

Amino Acid Transporters in Breast Cancer: Promising Prognostic Markers and Emerging Targets Across Molecular Subtypes

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Abstract

Background: To maintain the necessary energy and cellular building blocks, tumour cells selectively upregulate expression of cell-surface nutrient transporters. One of the most prominent are amino acid transporters, which catalyse the uptake of vital amino acids, particularly glutamine. Glutamine promotes cell growth by supporting bioenergetic and biosynthetic metabolism, maintaining redox balance and activating the mTORC1 signalling pathway, which supports protein translation and prevents apoptosis in cancer cells. These metabolic alterations are mediated by mutations in oncogenes and/or tumour suppressors that result in sustained cancer cell growth and proliferation. Cellular interaction with a tumour microenvironment, at the metabolic level, can also promote cancer aggressiveness and metastasis. Breast cancer (BC) is a heterogeneous disease with various subtypes that differ in terms of biology and clinical behaviour. There is growing evidence that differences in amino acid metabolism exist between BC molecular subtypes. However, the expression of amino acid transporters and the subsequent prognostic implications in BC remain elusive, especially with regard to the BC molecular subtypes. It was therefore hypothesised that amino acid transporters involved in the glutamine pathway are overexpressed in BC and they have prognostic implications that vary between BC molecular subtypes. This study aimed to determine the prognostic significance of the eminent amino acid transporters and their downstream signal in different BC molecular subtypes and to investigate the usability of the key amino acid transporters as potential targets for therapeutic interventions in BC. Furthermore, this study sought to find whether upregulation of amino acid uptake in BC participates in shaping the tumour microenvironment through attracting specific immune cell subtypes, including the subsequent impact on patient outcome.

Methodologies: The solute carriers SLC1A5, SLC7A5, SLC3A2, SLC38A2, SLC7A11 and SLC7A8 and the downstream signal,p70S6K, were each assessed at the genomic level using METABRIC data (n=1980) and Breast Cancer Gene-Expression Miner dataset (n= 4904), and at the proteomic level using immunohistochemical analysis and tissue microarray (TMA) sections constructed from a large, well-characterised primary BC cohort (n=2770). Their expression was correlated with clinicopathological parameters, BC molecular subtypes, relevant biological markers and patient outcome. K-mean and PAM clustering

algorithms were applied to stratify the BC cohort into accredited "SLC clusters" based on the protein expression data of the key solute carriers (SLC1A5, SLC7A5 and SLC3A2). The clinicopathological relevance of the SLC clusters was assessed, including associations with patient outcome. In addition, SLC clusters were correlated with various immune cell markers (CD3, CD8, FOXP3, CD20, CD68, PD1 and PDL1) as components of tumour microenvironment. The co-expression of the two compartments was associated with patient outcome, considering the BC molecular subtypes. In a different approach, *in vitro* studies using different BC cell lines were conducted to investigate the effect of transient siRNA knockdown of *SLC1A5* and *SLC7A5* on cell proliferation, cell invasion and amino acid uptake. The effect of silencing the two solute carriers on PDL1 expression was also assessed.

Results: All biomarkers included in this study, apart from SLC7A8, showed a strong correlation with poor clinicopathological parameters and oestrogen receptor negative (ER-) status. The results of the survival analysis differed among BC molecular subtypes. While high SLC1A5, SLC7A5 and SLC3A2 expression shared the characteristics of being predictive of patient outcome only in the ER+ highly proliferative/luminal B subtype, SLC38A2 showed similar associations in triple negative (TN) tumours, and p70S6K and SLC7A8 were limited to the ER+ low proliferative/luminal A subtype. In multivariate analysis, all aforementioned biomarkers were independent factors for predicting patient survival, excluding SLC1A5, which was significant only in the univariate analysis, and SLC7A11, which did not show any significant associations with patient outcome.

A significant association was also observed with other relevant biological markers. The most prominent was the key regulator of tumour cell metabolism, MYC, and a group of glutamine metabolic enzymes, particularly those involved in the glutamine-proline regulatory axis.

Clustering analysis of SLC1A5, SLC7A5, and SLC3A2 protein expression resulted in classifying BC patients into three clusters; low SLCs (SLC1A5-/SLC7A5-/SLC3A2-), high SLC1A5 (SLC1A5+/SLC7A5-/SLC3A2-), and high SLCs (SLC1A5+/SLC7A5+/SLC3A2+). Each cluster had distinct correlations to known prognostic factors and patient outcome. Multivariate analysis showed that the SLC clusters were independent risk factors for shorter BC-specific survival only in the ER+ highly proliferative tumours. The high SLCs cluster showed a significant

association with different immune cell markers, primarily in the TN subtype. In addition, the co-occurrence of SLCs with immune markers had an additive effect on predicting patient outcome. Targeting *SLC7A5* but not *SLC1A5* significantly reduced the expression of PDL1 in the TN cell line.

In this context, while silencing *SLC1A5* or *SLC7A5* significantly reduced amino acid uptake in the respective BC cell lines, the impact on cell proliferation and cell invasion was significant only upon silencing *SLC7A5*.

Conclusion: Continued refinement in understanding the biological diversity of BC and linked development of classification strategies suitable for routine clinical use are essential to achieve a personalised approach to BC management. This thesis demonstrated that the amino acid transport system for glutamine has a crucial prognostic role in BC, which is variable among BC molecular subtypes. Solute carriers that showed the liability of being independent predictors for poor patient outcome can be potential targets for BC treatment, particularly in synergism. Furthermore, this study deduced that enhanced amino acid uptake by cancer cells can influence the composition of immune cell infiltrates, which has an additional effect on patient outcome. Co-targeting the key amino acid transporters and the pro-tumorigenic immune cells may be a novel approach for BC treatment, particularly in the TN subgroup. Further elaborated functional studies may provide new insights into the specific role played by the amino acid transporters in the aggressive BC molecular subtypes.

Publications

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1. **Glutamine Transporters SLC1A5 & SLC7A5 are Key Therapeutic Targets in Luminal B Breast Cancer.**
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2. **The Cooperative Expression of the Solute Carriers, SLC1A5, SLC7A5 and SLC3A2, Confers a Poor Prognosis in the Highly Proliferative ER+ Breast Cancer Subtype.**
   Rokaya El Ansari, Madeleine L. Craze, Daniele Soria, Maria Diez-Rodriguez, Christopher C. Nolan, Ian O. Ellis, Emad A. Rakha, Andrew R. Green

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3. **Enhanced glutamine uptake influences composition of immune cell infiltrates in breast cancer**
   Rokaya El Ansari, Madeleine L. Craze, Maryam Althobiti, Lutfi H. Alfarsi, Ian O. Ellis, Emad A. Rakha, Andrew R. Green

   Nottingham Breast Cancer Research Center meeting, 9 May 2019, Nottingham, United Kingdom.

4. **Enhanced glutamine uptake influences composition of immune cell infiltrates in breast cancer**
   Rokaya El Ansari, Madeleine L. Craze, Maryam Althobiti, Lutfi H. Alfarsi, Ian O. Ellis, Emad A. Rakha, Andrew R. Green

   Pathological Society meeting, 2-4 July 2019, Harrogate, United Kingdom.

Poster presentations

1. **Glutamine Transporter SLC7A5 is key therapeutic target in Luminal B breast cancer driven by MYC.**

   Rokaya El Ansari, Madeleine L. Craze, Islam Miligy, Maria Diez-Rodriguez, Christopher C. Nolan, Ian O. Ellis, Emad A. Rakha, Andrew R. Green.

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2. **Glutamine Transporter SLC7A5 is key therapeutic target in Luminal B breast cancer driven by MYC.**

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   Nottingham Breast Cancer Research Centre Launch Event, 25-26 March 2018, Nottingham, United Kingdom.

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4. **Prognostic and Predictive Value of Amino Acid Transporters SLC7A5 and SLC3A2 Co-expression in Breast Cancer**

   El Ansari, R.; Alfarsi, L.H.; Craze, M.L.; Diez-Rodriguez, M.; Nolan, C.C.; Ellis, I.O.; Rakha, E.A.; Green, A.R.

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   Midlands Academy of Medical Sciences Research Festival 2019, 27 March 2019, Birmingham, United Kingdom.

6. **Kinesin Family Member-18A (KIF18A) is a Predictive Biomarker of Poor Benefit from Endocrine Therapy in Early ER+ Breast Cancer**

   Alfarsi LH, **El ansari R**, Toss MS, Diez-Rodriguez M, Nolan CC, Ellis IO, Rakha EA, Green AR

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7. **Glutamine-Proline regulatory axis is associated with MYC expression in Luminal B breast cancer**

   Madeleine L. Craze, Hayley Cheung, Natasha Jewa, Nuno D.M. Coimbra, Daniele Soria, **Rokaya El Ansari**, Mohammed A. Aleskandarany, Kiu Wai Cheng, Maria Diez-Rodriguez, Christopher C. Nolan, Ian O. Ellis, Emad A. Rakha, Andrew R. Green.

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8. **Phosphatidylinositol 3-kinase is a potential regulator of glutaminolysis in luminal B breast cancer**

   Madeleine L. Craze, Natasha Jewa, Hayley Cheung, **Rokaya El-Ansari**, Maria Diez-Rodriguez, Christopher C. Nolan, Ian O. Ellis, Emad A. Rakha, Andrew R. Green.

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9. **GLUD1 Expression in Triple Negative Breast Cancer is Associated with Response to Chemotherapy**

   Madeleine L Craze, Mohammed A. Aleskandarany, Kiu Wai Cheng, **Rokaya El-Ansari**, Maria Diez-Rodriguez, Christopher C. Nolan, R. Douglas Macmillan, Elena Provenzano, Carlos Caldas, Ian O. Ellis, Emad A. Rakha and Andrew R. Green

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10. **SLC7A5/SLC3A2 co-expression predicts relapse in endocrine treated breast cancer**

    Lutfi Alfarsi, **Rokaya El Ansari**, Madeleine L. Craze, Maria Diez-Rodriguez, Christopher C. Nolan, Ian O. Ellis, Emad A. Rakha, Andrew R. Green

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11. SLC1A5 regulates cellular metabolism and survival in ER+ breast cancer and predicts resistance to endocrine therapy

Lutfi Alfarsi, **Rokaya El Ansari**, Madeleine L. Craze, Brendah Masisi, Holly Nicholls, Christopher C. Nolan, Ian O. Ellis, Emad. Rakha, Andrew R. Green

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12. Glutaminase Expression Predicts Recurrence in Breast Ductal Carcinoma in Situ

Madeleine L. Craze, Andrew Olfield, Michael S. Toss, Brendah K. Masisi, **Rokaya El Ansari**, Lutfi Alfarsi, Islam M. Miligy, Abdulbaqi Al-Kawaz, Holly Nicholls, Christopher C. Nolan, Ian O. Ellis, Emad A. Rakha, Andrew R. Green

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13. SLC1A5 interacts with TALDO1 to modulate metabolic pathway leading to endocrine resistance in oestrogen receptor positive breast cancer

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14. High SLC1A5 Expression Predicts Resistance to Endocrine Therapy in ER+ Breast Cancer via Interacting with Metabolic Pathway

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15. Glutaminase Expression Predicts Recurrence in Breast Ductal Carcinoma in Situ

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16. The Prognostic Significant of ALDH1A1 in Breast Cancer Molecular Subtypes

Maryam Althobiti, **Rokaya El Ansari**, Chitra Joseph, Ian O Ellis, Andrew R Green, Emad A Rakha

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17. Glutaminase 2 is associated with high glutaminolytic markers in Breast cancer

Brendah K. Masisi, **Rokaya El Ansari**, Lutfi Alfarsi, Madeleine L. Craze ,Christopher C. Nolan, Emad A. Rakha, Andrew R. Green

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18. TALDO1 interaction with SLC1A5 modulate metabolic pathway affecting response to endocrine therapy in luminal breast cancer

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Abbreviations

2HG	2 hydroxyglutarate
4EBP	Eukaryotic initiation factor 4E (eIF4E)-binding protein
AC-CoA	Acetyl coenzyme A
ADH	atypical ductal hyperplasia
AKT	Serine-threonine protein kinase
ALDH18A1	Aldehyde dehydrogenase 18 family, member A1
ALDH4A1	Aldehyde Dehydrogenase 4 Family Member A1
ALH	atypical lobular hyperplasia
AMPK	AMP- activated protein kinase
ASCO	American Society of Clinical Oncology
ASCT2	Alanine-Serine-Cysteine Transporter 2
ATF4	Activation transcription factor
BC	Breast cancer
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
BCSS	Breast Cancer Specific Survival
BRAF	B-Raf proto-oncogene, serine/threonine kinase
C/EBP	CCAAT-enhancer-binding protein
CAF	Cancer-associated fibroblasts
CK	cytokeratin
CMF	Methotrexate and 5-flurouracil
CN	Copy number
CNV	Copy number variation
CO_2	Carbon dioxide
CSCs	cancer stem cells
DAB	3'Diaminobenzidine
DCIS	ductal carcinoma *in situ*
DFS	disease free survival
DMEM	Dulbecco's Modified Eagle medium
DMFS	Distant Metastasis Free Survival
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DPX	Dibutylphthalate Polystyrene Xylene

ECL	Enhanced Chemiluminescence
ECM	Extra cellular matrix
eEF2K	Eukaryotic elongation factor 2 kinase
EGFR	Epidermal growth factor receptor
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
EPG	excellent prognostic group
ER	Oestrogen receptor
ERS	Endoplasmic Reticulum stress
FBS	Foetal bovine serum
FFPE	Formalin Fixed Paraffin Embedded
FIP200	FAK family-interacting protein of 200 kDa
FISH	Fluorescence *in situ* hybridisation
GAP	GTPase-activating protein
GC-MS	gas chromatography-mass spectrometry
G-CSF	Granulocyte colony-stimulating factor
GEP	gene expression profiling
Gln	Glutamine
GLS	Glutaminase
GLUD1	Glutamate dehydrogenase
GLUL	Glutamine synthetase
GLUT	glucose transporter
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GPG	good prognostic group
GPNA	Gamma-l-glutamyl-p-nitroanilide
GSH	Glutathione
H&E	Haematoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HEK	Human embryonic Kidney
HER2	Human epidermal growth factor receptor-2
HIF	Hypoxia-inducible factor
HR	Hormone receptors
HRP	Horseradish peroxidase

HSF1	heat shock factor 1
IARC	International Agency for Research on Cancer
IBC	invasive breast cancer
IDC	Invasive ductal carcinoma
IDH	isocitrate dehydrogenase
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
IMS	Industrial methylated spirit
IntClusts	integrative clusters
ITGB1	β1 integrins
KDM4C	Lysin demethylase4c
KRAS	KRAS proto-oncogene, GTPase
LAT1	L-Type Amino Acid Transporter 1
LAT2	L-Type Amino Acid Transporter 1
LCIS	lobular carcinoma *in situ*
LC-MS	liquid chromatography-mass spectrometry
LDHA	lactate dehydrogenase A
LeuRS	Leucyl-tRNA synthetase
LIMMA	Linear models of microarray data
LN	Lymph node
LVI	Lymphovascular invasion
MAPK	Mitogen-activated protein kinase
MaSCs	mammary stem cells
MeCP2	Methyl-CpG-binding protein 2
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
miRNA	micro RNA
MPG1	moderate prognostic group 1
MPG2	moderate prognostic group 2
MRS	magnetic resonance spectroscopy
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NADH	Nicotinamide adenine dinucleotide hydrogen

NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NMD	Nonsense-mediated mRNA decay
NMR	nuclear magnetic resonance
NPI	Nottingham Prognostic Index
NRF2	Erythroid 2-related factor 2
NST	no special type
O.D.	Optical density
OAA	oxaloacetate
OXPHOS	oxidative phosphorylation
p70S6K	p70 Ribosomal S6 Kinase-1
PAM	Partitioning around medoids
PAM50	Prediction Analysis of Microarray 50
PBS	Phosphate Buffer Saline
PD1	Programmed cell death 1
PDL1	Programmed death ligand 1
PET	Positron Emission Tomography
PgR	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-3 kinase
PP2A	Protein phosphatase 2A
PPG	poor prognostic group
PPP	pentose phosphate pathway
PRODH	Proline dehydrogenase 1
Pro-GLn	Proline-glutamine
PVDF	Polyvinylidene difluoride
PYCR1	Pyrroline-5-Carboxylate Reductase 1
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
Raptor	Regulatory associated protein of mTOR
Rb	Retinoblastoma
Rictor	Rapamycin insensitive companion of mTOR
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNF5	Ring Finger Protein 5
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute
RPS6KB1	Ribosomal Protein S6 Kinase B1
rRNA	ribosomal RNA
SCO2	cytochrome C oxidase-2
SDS	Sodium Dodecyl Phosphate
siRNA	Small double-stranded interfering RNA
SLC	Solute carriers
SLC1A5	Solute Carrier Family 1 Member 5
SLC38A2	Solute Carrier Family 38 Member 2
SLC3A2	Solute Carrier Family 3 Member 2
SLC7A11	Solute Carrier Family 7 Member 11
SLC7A5	Solute Carrier Family 7 Member 5
SLC7A8	Solute Carrier Family 7 Member 8
SNAT2	Sodium-coupled neutral amino acid transporter 2
TAM	Tumour-associated macrophages
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline-Tween
TCA	Tricarboxylic acid cycle
TDLU	terminal ductal lobular unit
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TMA	Tissue microarray
TME	Tumour microenvironment
TN	Triple negative
TNBC	Triple negative breast cancer
Treg	Regulatory T cells
TSC1	Tuberous sclerosis proteins 1
TSC2	Tuberous sclerosis proteins 2
UPR	Unfolded folded protein response
VEGFA	Vascular endothelial growth factor A
VEGFB	Vascular endothelial growth factor B
VPG	very poor prognostic group
W.B	Western blotting
xCT	Cystine-glutamate antiporter
α-KG	α-ketoglutarate

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Chapter 1. General Introduction

1.1 Breast cancer

1.1.1 Anatomy and histology of the normal breast

The female breast starts developing at 6 weeks of the prenatal period from remains of the ectodermal ridge (the milk line). In association with puberty, pregnancy, and lactation, the breast responds to steroid hormones and growth factor receptor signalling and undergoes dramatic changes in size, shape, and function [1,2]. The adult female breast has a complex glandular structure, where each mammary gland is composed of 15 to 20 large lobes. These lobes are comprised of several small lobules, each of which contains tens to hundreds of the milk secreting structures, acini. Ducts arises from the acini converge to form terminal ductules, that all assemble to create the functional unit of the breast, terminal ductal lobular unit (TDLU). This unit is finally drains into the lactiferous duct that leads to the nipple [3]. The ducts and ductules are lined by an inner layer of cuboidal or columnar epithelial cells and outer myoepithelial cells, which are all anchored on a basement membrane layer. A fibro-adipose connective tissue is identified in the interlobular and intralobular regions, with different cellular components, including fibroblasts, myofibroblasts, adipocytes, histiocytes and occasional lymphocytes [4,5].

1.1.2 Pathogenesis of breast cancer

Breast tissue can give rise to a variety of benign and malignant lesions. Ducts are the most common site of these lesions (70-80%), but it can occur in lobules (10-15%) or in the tissue in between [6]. Uncontrolled cell division or hyperplasia can develop into a typical ductal or lobular hyperplasia which potentially progress to pre-invasive lesions, namely ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS) [7,8]. *In situ* lesions have high potential to breach the myoepithelial layer and the basement membrane and ultimately give rise to invasive breast cancer (IBC). Indeed, malignant transformation is governed by highly sophisticated mechanisms that lead to dramatic cytological or architectural dysplastic changes and encourage cells to escape mechanisms that control their survival, proliferation and migration. These malignant transformations involve a collection of multistep processes that has been described as the hallmarks of cancer. These include: sustaining proliferative signalling, evading growth

suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [9]. Later, two additional hallmarks were added; reprogramming of energy metabolism and evading immune destruction (Figure 1-1) [10]. Genetic alterations; activated oncogenes and or inactivated tumour suppressor genes, are key drivers for the progressive transformation of normal cells to highly malignant derivatives. In addition, growing evidence indicates that epigenetic regulations and non-coding RNAs are also implicated in the development of breast neoplasia and contribute to metastatic events and heterogeneity of the disease [11]. Breast carcinogenesis is also linked to the existence of cancer stem cells (CSCs), which is thought to be responsible for tumour initiation and progression. There are two hypothesis which propose the origin of these cells. The first suggests that CSCs arise from normal mammary cell hierarchy while the other postulates the origination of these cells from the normal mammary stem cells (MaSCs) [12].

It is noteworthy to highlight that these mechanisms will produce a disease of various defined subtypes, with remarkably different phenotypic and genotypic features. It is of great importance to distinguish between these subtypes as they exhibit different prognosis and treatment implications [13].

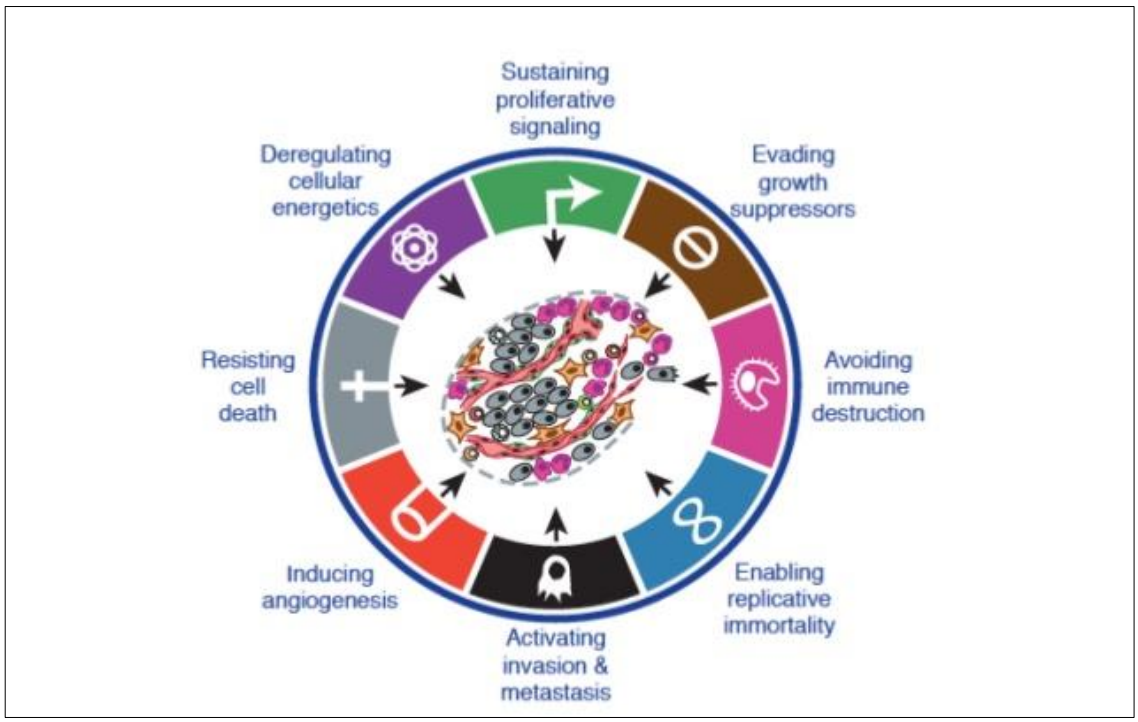


Figure 1-1. The hallmarks of cancer, including the previously identified six capabilities and the two emerging hallmarks, which together represent alterations that take place in cell physiology for the malignant transformation (adapted from Hanahan et al 2011).

1.1.3 Breast cancer incidence and mortality rates

Breast cancer (BC) is a major worldwide health issue and its incidence has increased in recent years. It is the most common female cancer in both developing and developed countries. Globally, it accounts for around 12% of the total cancer cases and 7% of the cancer related death. This is corresponding to about 2.1 million newly diagnosed BC female cases and 626,679 BC related death in 2018 [14]. Highest incidence rates are observed in developed countries such as Australia/ New Zealand, North Europe (including the UK), southern and western Europe and North America. This is attributed to a higher prevalence of known risk factors including menstruation, reproduction, nutrition and hormone supplements [14].

In the UK, there were over 55,000 new female cases diagnosed with BC in 2017 with 11,400 deaths, which is corresponding to about 31 cases every day (Cancerresearchuk.org).

1.1.4 Risk factors of developing breast cancer

Several factors have been associated with an increased risk for the development of BC which are introduced below:

1.1.4.1 Age

The risk of developing BC increases with age. Statistically, women aged more than 50 have increased risk to develop BC compared to younger patients. Noteworthy, BC in younger women is most likely to be of aggressive subtypes, such as triple negative and human epidermal growth factor receptor-2 (HER2)-enriched tumours, and is more likely to present in advanced stages that are associated with worse clinical outcome [15,16].

1.1.4.2 Family history

While less than 15% of BC patients have a family history of the disease, women who have close blood relatives with breast malignancies have a higher risk of developing the disease. The risk to develop BC almost doubles if she has a first-degree relative (mother, sister or daughter) affected with the disease. Furthermore, the risk increases almost three-fold if a woman has two first-degree relatives with BC [11,17].

1.1.4.3 Genetic predispositions

Overall, about 5-10% of BCs are linked to gene mutations, with the majority, about two thirds of hereditary BC, showing mutations in *BRCA1* or *BRCA2* DNA repair genes [17]. Carriers of these mutations are at higher risk to develop breast malignancies compared to the general population, with an estimated lifetime risk to develop the disease of more than 80% [18]. Mutated *BRCA1* and *BRCA2* BCs tend to affect both breasts with an early onset [19]. Although less common, mutations in other genes are also implicated in increasing the risk of developing BC. These include mutated *TP53*, *ATM*, *PTEN*, *CHEK2*, *CDH1*, *STK11* and *BALB2.* Individuals carrying mutations in these genes are also susceptible to other hereditary diseases and other types of cancers [20-27].

1.1.4.4 Exposure to oestrogen

Oestrogen is the primary female sex hormone that regulates many biological functions. Levels of endogenous oestrogen throughout a woman's lifetime have implications for the protection against or the development of BC. Early menarche age, in both pre- and post-menopausal women, is a risk for developing BC, with a two-fold increase in having hormone receptor positive tumours. Furthermore, the risk of having BC is higher in women with late menopausal age, where the risk increases 17% every five year delay in the menopause onset [28]. On the other hand, pregnancy and breastfeeding can reduce the exposure to oestrogen and decrease the risk of developing BC, through the protective effect of cellular differentiation of breast lobules [29].

Prolonged exposure to exogenous oestrogen from oral contraceptive medications or hormone replacement therapies are able to stimulate breast growth, therefore increasing BC risk. It is reported that menopausal hormone therapies containing both oestrogen and progestin (a synthetic progestogen) not only increase the risk of developing BC, but are also associated with delayed detection and diagnosis of the disease with a significant increase in the mortality rates [28].

1.1.4.5 Life style factors

Alcohol consumption has been associated with elevated risk of developing BC. According to the International Agency for Research on Cancer (IARC), risk of developing BC can increase by up to 10% in women who have daily intake of 10g

of alcohol [30]. In addition, there is an association between excessive alcohol intake and increase breast density in mammogram images [31]. Smoking is another factor which is related to BC. The risk of having the disease is higher in prolonged active smokers compared to women who are exposed to passive smoking. However, risk of BC is reduced if they have never smoked or been exposed to passive smoking [32]. Being overweight, especially in post-menopausal women is also associated with increased risk of having BC. Overweight or obese women, with Body Mass Index (BMI>25) have a 1.3 higher risk of developing BC compared to women within their normal weight range [33]. The relationship between obesity and BC can be assigned to the aromatase-driven oestrogen production in adipose tissue in addition to the state of insulin resistance and Hyperinsulinemia, which has been shown to be an independent risk of BC in postmenopausal women [34,35]. In contrast, regular physical activity is a factor which can significantly reduce the risk of BC development, in a dose dependent manner [36].

1.1.4.6 Breast pathology

Dense breast on mammogram has been shown to have 1.5 times more risk of developing BC compared to individuals with lower breast densities [37]. Certain non-proliferative lesions, such as simple cysts, fibrosis, mild hyperplasia and adenosis, may marginally affect BC risk [38,39]. A slight increase in BC risk is also associated with some proliferative lesions without atypia, such as fibroadenoma, papillomatosis and sclerosing adenosis. However, the risk in women with atypical proliferative lesions, including atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH), is 5 times that in women with non-proliferative lesions [11,40]. In addition, women with unilateral BC have an elevated risk to develop the same disease in the other breast or in another part in the same breast [11].

1.1.4.7 Prognostic and predictive factors in BC

Overall, prognostic factors are tumour characteristics that can predict patient clinical outcome; evaluating the risk of death, developing distant metastasis or disease recurrence at the time of primary diagnosis, while predictive factors include parameters that can provide information about the extent of response to certain treatments. Accurate prognostic and predictive parameters are vital for treatment decisions and improving BC patient outcome. The most commonly used

prognostic and predictive factors, which are routinely assessed in the pathologic reporting of BC, are presented in the following sub-sections.

1.1.4.8 Prognostic factors

1.1.4.8.1 Histological types

BC exhibits a wide scope of histological subtypes that have a distinct clinical course and outcome. Invasive ductal carcinoma (IDC) is the most common type of BC that consists of a heterogeneous group of tumours with various morphology. Some of them have special cytoarchitectural features and are classified as special types, such as, tubular, invasive cribriform, mucinous and medullary-like carcinomas, while the majority (~ 75%) are identified as IDC of no special type (NST) [13]. Invasive lobular carcinoma (ILC) is the second most common type which constitutes 5-15% of all breast carcinomas and it also has different histological variants [41]. From these types, mucinous, tubular, invasive cribriform and tubulolobular carcinomas show an excellent prognosis, with survival rates of more than 80% at 10 years. In contrast, solid lobular, IDC of NST and mixed ductal and lobular carcinomas exhibit poor prognostic features with survival rates of less than 50% at 10 years [42].

1.1.4.8.2 Tumour grade

Tumour grade is a powerful prognostic indicator that reflects the biological aggressiveness of the tumour through evaluating the degree of its similarity to the normal breast cells [43]. Grading system in BC consists of three distinctive categories, each of which has three different scores. The first is tubule formation (score 1: majority of tumour (> 75%), score 2: moderate degree (10-75%) and score 3: little or none (<10%)). The second component is nuclear pleomorphism (score 1: small, regular uniform cells, score 2: moderate increase in size and variability and score 3: marked variation). The final component is mitotic count (score 1: 1-9, score 2: 10-19 and score 3: ≥20). The mitotic count is assessed in ten high-power fields using light microscopy with a field diameter of 0.58mm. By summing the scores of the all components, a tumour grade is assigned; Grade 1 tumours, with a total score of 3-5, are well differentiated and have the best prognosis. Grade 2 tumours, with a total score of 6 or 7, are moderately differentiated with intermediate prognosis. Finally, Grade 3 tumours, with a total score of 8 or 9 are poorly differentiated and present the worst prognosis [43,44].

1.1.4.8.3 Tumour size

Tumour size is another important prognostic indicator that can predict BC behaviour and patient outcome. There is a direct proportion between the increased mortality among invasive BC patients and the size of their tumour, where patients with small tumour size have better survival compared to those with larger tumours [43,45].

1.1.4.8.4 Lymph node stage

The most significant prognostic indicator for patients with early-stage BC is the presence or absence of axillary lymph node (LN) involvement. The extent of nodal involvement is strongly correlated with patient outcome, in terms of disease free survival (DFS) and breast cancer specific survival (BCSS) [46,47]. There is a direct relationship between LN stage and patient survival, where the lower LN stage is associated with longer survival and vice versa. Furthermore, a positive association was observed between the number of involved axillary nodes and the risk for developing distant metastasis [48,49]. In this study, the pathological system used to assess staging is divided into three categories. 1) LN stage 1, if no LN is involved; 2) LN stage 2, if one, two, or three LNs are involved; and 3) LN stage 3, if more than three LNs are involved [49, 50]. The 5-year survival for patients with LN stage 1 is 83% compared with 73% for patients with LN stage 2 and 46% for patients with LN stage 3, from which the percentage declines to around 28% for patients with ≥13 positive LNs [50]. Although they act as independent prognostic factors, both tumour size and LN stage are positively associated, where nodal metastasis is observed in 30% of patients with tumour size <2cm while the percentage increased to 70% in patients who have breast tumours of >5cm in diameter [51].

1.1.4.8.5 Nottingham Prognostic Index

Using a combination of prognostic factors is more precise than using an individual one and this was the base of creating certain formulas that can enhance the prognostic power for BC patients, one of the most important is Nottingham Prognostic Index (NPI) [52]. NPI is classified into three prognostic groups; good-NPI, moderate-NPI, and poor-NPI, involving the three most powerful prognostic parameters; tumour grade, tumour size, and LN stage. Data from these parameters is included to calculate NPI, applying the following formula: NPI = [0.2 × tumour size (in centimetres)] + LN stage (1 to 3) + tumour grade (1 to 3).

The original NPI grouping assigns patients as good NPI group if their NPI is <3.4, while patients of moderate and poor NPI have NPI values of 3.4-5.4 and >5.4, respectively [53]. An improved NPI grouping system is also suggested by expanding the prognostic groups from three to six, which are: excellent prognostic group (EPG) if NPI is 2.08–2.4; good prognostic group (GPG) if NPI is 2.41–3.40; moderate prognostic group 1 (MPG1) if NPI is 3.41–4.40; moderate prognostic group 2 (MPG2) if NPI is 4.41–5.40; poor prognostic group (PPG) if NPI is 5.41–6.40; and very poor prognostic group (VPG) if NPI is >6.40 [54].

1.1.4.8.6 Lymphovascular invasion

Lymphovascular invasion (LVI) is defined as tumour emboli present within the lymphatic spaces and or blood vessels in the breast tissue surrounding the invasive cancer. The existence of LVI can identify patients who are at increased risk for node-positive disease and having metastatic events [55,56]. Furthermore, LVI shows a clear relationship with BC-related death and the development of local recurrence [57]. Although, LVI has been identified as an independent prognostic factor in patients with LN-negative and LN-positive BC [58,59] , its prognostic value in LN-positive patients is still controversial [56,60-62].

1.1.4.9 Predictive factors

Predictive factors or biomarkers are the indicators that can predict the response to a given treatment. These are also important in stratifying BC patients both to allow the accessibility to the optimum management and to avoid the adverse effects associated with over- or under-treatment [63].

1.1.4.9.1 Hormone receptors

Oestrogen receptor (ER) and progesterone receptor (PgR) are routinely assessed in clinical practice, by immunohistochemistry (IHC) on paraffin sections, in all BC cases [64]. According to the guidelines from the American Society of Clinical Oncology/College of American Pathologists, the recommended cut-off to determine ER and PgR positivity in invasive BC is set to 1% of nuclear staining of any intensity [65]. ER is considered as the most powerful indicator for predicting hormonal therapy after surgery [66]. However, the extended duration of the endocrine therapy regimen for ER+ patients is still debated [67]. Indeed, ER status provides limited prognostic information especially for younger, pre-menopausal, patients who are usually affected by aggressive BC phenotypes [68]. The co-

expression status of ER with PgR increases the predictive power of response to adjuvant hormonal therapy from 50%, in ER+/PgR- patients, to 80% in ER+/PgR+ patients [68]. Indeed, lack of PgR expression in ER+ tumours may be a surrogate marker of aberrant growth factor signalling that could contribute to resistance to endocrine treatment [69].

1.1.4.9.2 Human epidermal growth factor receptor 2

HER2 is a transmembrane tyrosine receptor kinase that encoded by the *ERBB2* gene. HER2 expression is usually evaluated by IHC. However, the assessment by Fluorescence *in situ* hybridisation (FISH) assay is required when evaluation by IHC is equivocal. HER2 gene amplification or protein overexpression is reported in approximately 13-20% of BC patients [70,71]. HER2 overexpression in invasive BC is associated with promoting cancer cells' proliferation, sustained angiogenesis, suppressing apoptosis, enhancing cellular motility and epithelial mesenchymal transition (EMT) [72,73]. These biological alterations render the HER2+ BC more aggressive with unfavourable behaviour. In addition, HER2 positivity is associated with high tumour grade, positive nodal metastasis and shorter patient survival [74,75]. Targeting HER2+ tumours with the monoclonal antibody, Trastuzumab (Herceptin), was previously established in BC with the best effect was observed in patients with early-stage disease [76]. The prediction ability of HER2 exceeds identifying BC patients who will respond to its targeted therapy, as it can also predict response to some systemic chemotherapies, such as, anthracycline and taxane based chemotherapeutic agents [77,78], in addition to its capability to predict resistance to endocrine therapy in ER+ BC patients [47].

1.1.5 Molecular classification of breast cancer

BC is a well-established heterogeneous disease at both phenotypic and genotypic levels. Microarray-based gene expression profiling (GEP) has conferred a new insight for a better understanding of the molecular heterogeneity of the disease. Based on the variations observed on their gene expression patterns, breast tumours have been classified into distinct subgroups with distinguishable clinical relevance. The best-characterised gene expression classification system is the Prediction Analysis of Microarray 50 (PAM50), which is a 50-gene classifier that groups breast tumours into five intrinsic subtypes. Perou et al, used this system to classify breast carcinomas into four different subtypes, namely, luminal, HER2-

enirched, basal-like and normal breast-like tumours [79]. Further clustering analysis has divided the luminal subtype into luminal A and luminal B tumours, giving rise to a total of five different molecular subtypes [80-82], which are characterised as follows:

Luminal A subtype: This subtype is ER+ and/or PgR+ and HER2- with low levels of the proliferation marker, Ki67. It is the commonest subtype and accounts for 50-60% of all BCs. Tumours belonging to this group are of low grade, slow growing and tends to have the best prognosis and longer survival compared to the other subtypes [83].

Luminal B subtype: This subtype represents 10-20% of all BCs and typified as ER+ and/or PgR+ and/or HER2+. Luminal B tumours have lower levels of PgR compared to luminal A subtype (79% vs 94%) [84]. In addition, they are more aggressive with higher histological grade, high expression of proliferative markers, worse prognosis and poorer patient outcome compared to luminal A tumours [81,83,85]. Changes in the expression levels of Ki67 are considered as an essential difference between the two luminal subtypes. Based on IHC assay, Cheang *et al*, has identified luminal A and luminal B tumours, as (ER+ and/or PgR+, HER2- and low ki67) and (ER+ and/or PgR+, HER2- and high Ki 67), respectively [86].

HER2-enirched subtype: This class comprises 15-20% of all BCs and characterised as, ER-, PgR- and HER2+ with high expression of proliferation genes and low expression of genes of luminal and basal-like groups. They grow faster than luminal subtypes and generally have worse prognosis [81]. While around 70% of clinical HER2+ BC are identified as HER2-enriched by microarray related methods, the remaining 30% are primarily luminal B subtype [82,87].

Triple negative/basal-like subtype: This category accounting for approximately 10-20% of all BCs. It is identified as ER-, PgR- and HER2-. Basal-like BCs differ from the non-basal triple-negative tumours by the positive expression of the basal markers; cytokeratin 5 and 6 (CK5/6) and/or epidermal growth factor receptor (EGFR) [88]. Generally, triple negative tumours usually have high histological grade and behave more aggressively than the other BC subtypes [89].

Normal breast-like subtype: This category has not been reproducibly defined. While it has been given the same definition as luminal A in some studies, others classified it as triple negative (ER-, PgR- and HER2-) tumours with lack of basal-like gene expression [11,82,90-92]. Indeed, normal breast-like tumours have been depicted as a technical artefact from high contamination with normal tissue [89]. Generally, these tumours are good prognostic but have worse prognosis compared to luminal A tumours [11].

In a different approach, a comprehensive genomic and transcriptomic profiling of 2,000 BC samples stratified BCs into 10 integrative clusters (IntClusts). Each cluster represents a distinct genomic landscape, molecular and clinical features and unique ER, PgR and HER2 expression [93,94] . Table 1-1 illustrates the molecular features of the ten clusters and their prognosis based on ten year BCSS.

Table 1-1. Molecular features, receptor status and prognosis of the ten integrative clusters.

IntClusts	Receptor status	Molecular feature	Prognosis
IntClust 1	ER+ (mainly luminal B subtype)	-Amplification of 17q23 locus -High prevalence of *GATA3* mutations	Intermediate
IntClust 2	ER+ (luminal A and B tumours)	-Amplification of 11q13/14	Worse
IntClust 3	ER+ (luminal A subtype)	-Low prevalence of *TP53* mutations -Highest frequency of *PIK3CA*, *CDH1* and *RUX1* mutations	Good
IntClust 4	ER+ and ER- tumours	-Deletion of the T-cell receptor loci on chromosome 7 and 14 (in 20% of cases)	Good
IntClust 5	HER2+ enriched	-HER2 amplification -High levels of TP53 mutations	Worse
IntClust 6	ER+ (luminal A and B tumours)	-Amplification of 8p12 locus -Low levels of PIK3CA mutations	Intermediate
IntClust 7	ER+ (luminal A)	-Specific 16p gain and 16q loss -Frequent amplification in 8q locus -High prevalence of MP3K1 and CTCF mutations	Good
IntClust 8	ER+ (luminal A)	-1q gain and 16q loss -High levels of PIK3CA, GATA7 and MPAP2K4 mutations	Good

Continued

IntClusts	Receptor status	Molecular feature	Prognosis
IntClust 9	ER+ (mainly luminal B)	-8q *cis*-acting mutations -20q amplification and *PPP2R2A* deletion -High levels of *TP53* mutations	Intermediate
IntClust 10	ER-, PgR- and HER2- (triple negative tumours)	-5q loss, 8q, 10p and 12p gain -Highest levels of *TP53* mutations	Worse

1.2 Altered amino acid metabolism in breast cancer; subtype dependencies and alternative adaptations

In addition to the heterogeneous pattern of different BC subtypes at the molecular level, they exhibit disparity in their metabolic profile and nutritional needs. One of the most prominent is alterations in their amino acid metabolism, particularly glutamine. The following sections highlight the metabolic modulations in BC, with emphasis on the role of altered glutamine metabolism and glutamine transporters in the different BC molecular subtypes.

1.2.1 Altered metabolic pathways in cancer

One of the hallmarks of cancer [10], altered metabolic profile, and its exploitation for anticancer therapy has recently attracted greater attention. Relentless proliferation of cancer cells is metabolically demanding, requiring ATP generation for the necessary energy and production of metabolic intermediates. The latter are the precursors for cellular 'building blocks', in the form of nucleotides for DNA/ RNA, amino acids for proteins and fatty acids for lipids. In addition to increasing the amount of metabolites the cells require, cancer cells actively alter the way nutrients are used. This adaptation process has been termed "metabolic transformation" [95].

In order to fuel the aberrant proliferation, cancer cells reprogram their glucose metabolism. Glycolysis is normally elevated under anaerobic conditions and is the major source of energy in malignant tumours, even with the presence of oxygen; a phenomenon known as aerobic glycolysis or the Warburg effect [96,97]. This phenomenon allows cancer cells to use carbon skeleton intermediates as precursors for the synthesis of macromolecules to sustain growth and proliferation

[98]. Furthermore, many cancers engage in *de novo* synthesis of fatty acids for ATP production and membrane biosynthesis [99-101]. Likewise amino acids, including glutamine, are important in supporting tumour growth and survival [102]. Although glutamine is a non-essential amino acid, certain cancer cell types are "addicted" to glutamine as they fail to grow or proliferate in its' absence.

Changes in metabolic pathways are controlled by activated oncogenes (e.g. *MYC*) and/or loss of tumour suppressors (e.g. *TP53*) which in turn, enhances the uptake of essential metabolites, via up-regulation of cellular transporters, supporting tumour replication and giving rise to aggressive cancer phenotypes [103,104]. Despite cellular transformation taking place in various cancer cell types, the metabolic requirements post transformation is generally comparable. However, as described above, BC is a heterogeneous disease with various subtypes that show heterogeneity in their metabolic reprogramming, including alterations in amino acid metabolism. Indeed, an initial link between tumour metabolites and different molecular BC subtypes has been established, where ER-negative tumours can be distinguished from those that are ER-positive depending on their metabolic signatures. Therefore, understanding the cellular metabolism in different subtypes might help in identifying potential novel therapeutic targets for BC therapy.

1.2.2 Mutational landscapes control glucose and glutamine metabolism in breast cancer

Glucose and glutamine serve as the primary nutrients to fuel cancer cell proliferation and, indeed, the rate of their utilisation far exceeds the consumption of other nutrients. It has been found that the metabolism of these two nutrients is co-regulated, as cancer cells which depend upon glycolysis must increase the rate of glutamine consumption to provide intermediates required for biosynthetic pathways. This reprogramming is controlled by mutations in oncogenes and/or tumour suppressors, or more directly by copy number aberrations of metabolic genes themselves [105].

The most frequent of these aberrations is *TP53* mutations, which occur in approximately 80% of basal-like and HER2+ tumours [106]. Wild-type P53 targets cytochrome C oxidase-2 (SCO2), which promotes aerobic respiration, and *TP53*-inducible glycolysis and apoptosis regulator (TIGAR), which inhibits glycolysis. Thus, loss of *TP53* function shifts metabolism towards aerobic glycolysis and

Warburg metabolism [107,108]. In addition, mutations in *PI3K* pathway, which occur commonly in the luminal subtypes of BC, not only stimulate glycolysis through the growth factor (PI3K/Akt/mTOR) signalling pathway, they also induce glycolysis through the activation and mobilisation of aldolase A from the actin cytoskeleton [108,109].

The oncogene *MYC* is amplified in approximately 15% of breast cancers, particularly in basal-like tumours, with a lower occurrence in HER2+ and luminal subtypes [110]. *MYC* is known to divert glucose metabolism towards aerobic glycolysis and lactate production. Simultaneously, it has a robust influence on glutamine metabolism as it facilitates glutamine uptake directly by binding to the promoters and inducing the expression of key glutamine transporters. It also promotes indirectly the expression of the glutamine metabolic enzyme, glutaminase (GLS), by repressing the expression of miR 23 a/b, miRNAs that repress the expression of this enzyme [111,112]. Glutamine uptake can also be regulated by the Retinoblastoma (Rb) family proteins via controlling the E2F-3 transcription factor, which associates with the promoter of the glutamine transporter, solute carrier family 1 member 5 (SLC1A5), and directly regulates its gene expression, where *Rb* knockout cells exhibit elevated SLC1A5 levels when compared to Rb wild-type cells [113].

1.2.3 Role of tumour microenvironment in metabolic reprogramming

Metabolic alterations are not only controlled by oncogenic signalling pathways, tumour microenvironment may also play essential roles in reprogramming tumour cell metabolism. The co-operation or "metabolic coupling" between cancer cell compartment and tumour microenvironment can promote malignant cell growth and give rise to cancer aggressiveness and metastasis. Tumour cells secrete high levels of reactive oxygen species (ROS) that cause a state of pseudohypoxia in the adjacent stromal cells [114]. Cancer cells can scavenge the stress caused by the ROS through activation of anti-oxidant pathways, such as the production of reduced glutathione [115], while the pseudo hypoxic state in the stromal compartment induces PI3K-AKT-mTOR and AMP- activated protein kinase (AMPK) pathways and results in favouring glycolysis over mitochondrial oxidative phosphorylation (OXPHOS), as a source of energy [116]. In addition, increase in

the level of ROS downregulates the stromal caveolin-1, up-regulates HIF1α and NFκB signalling, and increases the expression of the lactate transporter (MCT4), all of which promote glycolysis as well as acquiring the stromal cells a catabolic phenotype with enhanced macroautophagy. This catabolic state produces nutrient-rich environment, which include pyruvate, lactate, ketone bodies and glutamine, that fuels mitochondria and induces OXPHOS in cancer cells enabling the generation of adequate amount of ATP and building blocks required to support tumour growth and progression [116]. This metabolic coupling between the catabolic tumour microenvironment and the anabolic cancer cells is well known in BC and it is suggested that the ability to grow and develop metastasis in this tumour can be supported by the neighbour stromal cells through the increase in the mitochondrial metabolic activity [117]. On the other hand, in the same cancer this characteristic has been exploited in the treatment of ER+ subtype, where aromatase inhibitors target aromatase which are mainly expressed by the stromal compartment [118]. However, the tumour microenvironment can also induce chemotherapy resistance and in mammary carcinoma, it can cause tamoxifen resistance secondary to increased mitochondrial metabolism in cancer cells [119,120]. Resistance to cytotoxic chemotherapy, in addition, may occur as a result to a low pH in the tumour microenvironment, which is caused by lactate extrusion [121]. Hereby, being familiar with cancer-stromal symbiosis is important to understand the metabolic role of the adjacent non-malignant cells in promoting cancer progression and it should help improving the outcome of cancer patients.

1.2.4 Redox haemostasis is tightly linked to altered metabolic pathways in cancer

Oxidative stress is an observed hallmark of many tumours. Because rapidly growing cancer cells require high levels of energy to meet their metabolic demands, excessive amounts of ROS is generated in the cellular compartments. These oxidative compounds result in promoting cancer progression via modifying various signalling pathways, including activating oncogenes, loss of tumour suppressor genes and altering cancer cell metabolism [122,123]. However, extravagant increase in the concentration of ROS may induce oxidative damage-driven death. Thus, cancer cells should take protective measures to scavenge this stress, primarily through alterations in their metabolic pathways.

Indeed, reprogramming of glucose and glutamine pathways is considered as an essential determinant for redox haemostasis in cancer cells, as glycolytic intermediates can be shuttled to generate reducing equivalents, such as pentose phosphate pathway (PPP)-generated NADPH. The malate-aspartate shuttle is another mechanism that allows electrons, generated from glycolysis, to cross the inner mitochondrial membrane and thus restoring NADH imbalance [124,125]. Similarly, the consolidated up-regulation of glutaminolysis and the glutamate transporter, solute carrier family 7 member 11 (SLC7A11), reinforces the production of the anti-oxidant molecule, glutathione [115]. As anticipated, the capability to produce and metabolise ROS vary substantially among different BC subtypes, where the production of ROS and the subsequent scavenging activity was shown to be higher in the more aggressive ER- cell line, MDA-MB-231, compared to the ER+, MCF7, cells [126].

1.2.5 Importance of amino acid metabolism in sustaining tumour cell growth

Non-essential amino acids are synthesised endogenously by mammalian cells. However, they become essential in many tumour cells in order to meet the needs of rapid cell growth [127]. Amino acids, glutamine in particular, are important for tumour cell proliferation, survival and metabolism. The demand for glutamine in cancer cells was first noted by Harry Eagle, who concluded that cultured HeLa cells required 10 to 100-fold greater amounts of glutamine compared with other amino acids. Some cancer cells therefore rely on glutamine for their survival/progression and they display glutamine addiction, as it is a major source for metabolites that can facilitate cancer cell metabolism and sustain cancer growth in multiple ways (Figure 1-2). Firstly, glutamine provides the nitrogen required for the biosynthesis of nucleotides and non-essential amino acids [128]. In addition, glutamine can replenish the mitochondrial Tricarboxylic acid cycle (TCA) carbon pool being a source of α-ketoglutarate (α-KG) synthesis. Subsequently, α-KG can directly feed the TCA cycle or undergo reductive carboxylation, which supports citrate and fatty acid synthesis in stress conditions such as hypoxia [95,128].

Alternatively, glutamine can be metabolised by glutaminases to provide glutamate. The latter is a precursor for glutathione (GSH), which is used to reinforce the anti-oxidant response and maintain the redox balance within cancer

cells [95]. Conversion of glutamine to proline through the glutamine-proline regulatory axis also plays a special role in redox haemostasis and maintaining ATP levels [129]. A further role for glutamine is facilitating the import of essential amino acids, including leucine, which maintains activation of the mammalian target of rapamycin complex 1 (mTORC1), a master regulator of protein translation [130]. Additionally, in tumours that bear mutations in isocitrate dehydrogenase (IDH1/ IDH2), mitochondrial glutamine may be a source for 2 hydroxyglutarate (2HG) synthesis which competitively inhibits α-KG-dependent dioxygenase. If accumulated, 2HG causes aberrations in DNA and histone methylation that result in increased stem cell marker expression indicating a switch to a stem-like phenotype in cancer cells [131,132]. Interestingly, in endocrine resistant BC cells, glutamine in glucose-deprived conditions stimulates the unfolded protein response (UPR) pathway that, in turn, stimulates apoptosis in the majority of the cells whilst allowing survival of a small sub-population until an adequate energy supply becomes available to enable cell survival and proliferation [133]. Leucine is another essential amino acid, which is important for tumour cell growth. It is not only required for protein synthesis, it is also a potent activator of mTORC1 signalling pathway, which regulates cell growth, protein translation and prevents apoptosis in cancer cells [130]. In addition, about 40% of leucine is metabolised to acetyl-CoA which, in turn, involves in energy production and synthesis of other essential metabolites [134].

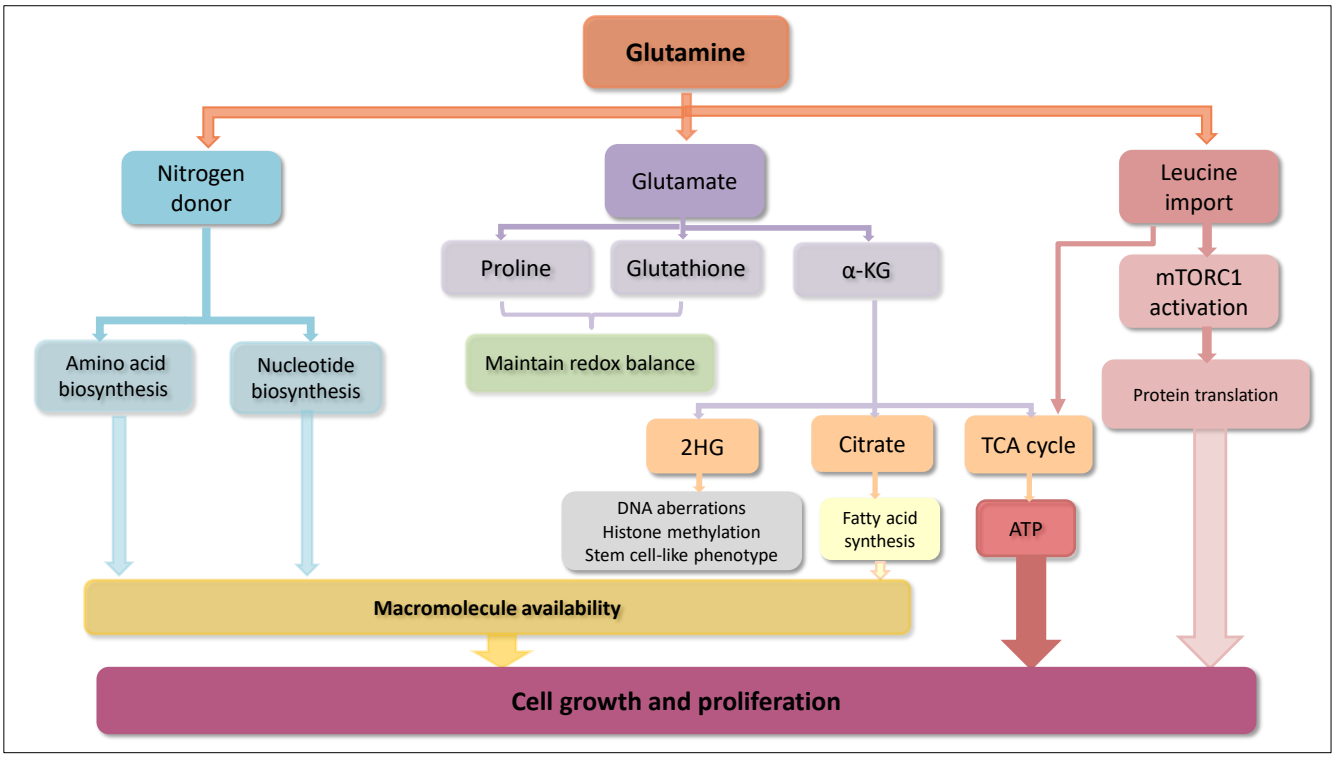


Figure 1-2. Role of glutamine in supporting tumour growth. Glutamine can act as a nitrogen donor and assist nucleotide biosynthesis and the synthesis of other amino acids. Additionally, Glutamine can be metabolised by glutaminases to provide glutamate. The latter, is a precursor for glutathione and proline, which neutralise the reactive oxygen species (ROS). Furthermore, glutamate can be converted to α-KG, which is used to replenish the TCA cycle. α-KG can also be a precursor for the oncometabolite (2HG), which causes methylation specific changes and increases the expression of stem cell markers. In stress conditions, such as hypoxia, α-KG may undergo reductive carboxylation giving rise to citrate, which supports fatty acid synthesis. Finally, glutamine efflux, through the glutamine transporter SLC7A5, is coupled by the influx of leucine, which then activates mTORC1. Alternatively, leucine can be metabolised to acetyl-CoA, which conveys the acetyl group to the TCA cycle to be oxidized for energy production. **α-KG**, α-Ketoglutarate; **mTORC1**, mammalian target of rapamycin1; **2HG**, 2 Hydroxyglutarate; **TCA**, Tricarboxylic acid cycle; **ATP**, Adenosine triphosphate.

1.2.6 Glutamine uptake and catabolism in breast cancer varies among different molecular classes

Glutamine dependent mechanisms can vary substantially among BC subtypes. Triple negative BC (TNBC) have increased expression of glutamine metabolic enzymes [135]. However, the highest levels of glutamine metabolism with increased glutaminase (GLS) and glutamate dehydrogenase (GLUD) expression is observed in HER2+ subtype, while luminal A tumours have the lowest level of these enzymes [136]. This is in concordance with higher levels of glutamate and lower levels of glutamine in TNBC and HER2+ subtypes compared with ER+

tumours suggesting an increase in glutamine consumption and glutaminolysis in the former subtypes [137,138]. Subsequently, glutamine deprivation can reduce the proliferation and progression of BC cells, especially among the most aggressive types of BC.

While glutamine uptake, by SLC1A5, is increased in TNBC and HER2+ subtypes, the ER+ tumours showed lower levels of this transporter. However, High expression of SLC1A5 was detected in the Aromatase inhibitor-resistant ER+ BC cells [136,139]. Similarly, higher expression of the glutamine/leucine antiporter, solute carrier family 7 member 5 (SLC7A5), was observed in TNBC and HER2+ subtypes compared with the ER+ tumours [140,141].

1.2.7 Glutamine transporters and their relevance to breast cancer

In general, all amino acids, including glutamine, require the aid of selective transporters to be able to cross the plasma membrane. Approximately 14 amino acid transporters accept glutamine as a substrate. However, none is solely selective for this amino acid, as they also transport other neutral, cationic and anionic amino acids. Furthermore, they function in either the influx or efflux of glutamine into/from cells. These transporters belong to four different gene families: solute carrier family (SLC) SLC1, SLC6, SLC7 and SLC38; namely, SLC1A5, SLC6A14, SLC6A19, SLC7A5-9 and SLC38A1-3, -5 and 7–8 [142]. Table 1-2 summarises the glutamine transporters along with their functions and relevance to breast cancer.

Table 1-2. Glutamine transporters, their functions and relevance to breast cancer

Transporter	Function	Observations in different BC subtypes
SLC1A5	Glutamine influx/efflux	• Expressed mainly in HER2+ and TNBC. • Inhibition or knockdown of SLC1A5 decreased tumour cell growth in basal-like cells (HCC1806) *in vitro* and *in vivo*, while the growth of MCF7 was unaffected [135].
SLC7A5	Glutamine influx/efflux	• Expressed mainly in HER2+ and TNBC. • Applying SLC7A5 inhibitor, BCH, or gene knockdown significantly inhibits MDA-MB-231 cell growth [141].
SLC6A14	Glutamine influx	• Upregulated in ER+ BC. • Treatment with SLC6A14 inhibitor, α-methyl-DL-tryptophan (α-MT), reduced ZR-75-1 cell growth, while it did not affect the growth of MDA-MB-231 [143].
SLC7A8	Glutamine efflux	• Upregulated in ER+ BC and it has the same amino acid substrate like SLC7A5. However, it exhibits relatively lower affinity [144].
SLC38A1	Glutamine influx	• SLC38A1 is upregulated and overexpressed in ER-cell lines • Knockdown of SLC38A1 reduce cell growth of the mouse BC cell line (4T1) [145].
SLC38A2	Glutamine influx	• Upregulated in the ER+ cell line and cause resistance to endocrine therapy [146].
SLC7A7 **SLC7A9** **SLC38A5**	Glutamine influx Glutamine efflux Glutamine influx/efflux	• Overexpressed in HER2+ BC cell lines [147].
SLC7A6 **SLC38A3** **SLC38A7** **SLC38A8**	Glutamine influx Glutamine influx/efflux Glutamine influx Glutamine influx	• Have yet to be studied in breast cancer

Within the field of cancer metabolism, SLC1A5 and SLC7A5 have been attracting particular attention due to their role in supporting tumour metabolism and potential as targets for anti-cancer therapy. As mentioned in the above section, SLC1A5 and SLC7A5 are both expressed in the aggressive BC subtypes, where they confer a poor clinical outcome. The two transporters co-operate at the functional level in promoting tumour growth, as SLC1A5 maintains the sodium-coupled influx of glutamine, which preserves the biosynthetic pathway of the rapidly growing cancer cells, whereas SLC7A5 mediates the efflux of this amino acid in exchange with the influx of the essential amino acid, leucine (Figure 1-3) [148].

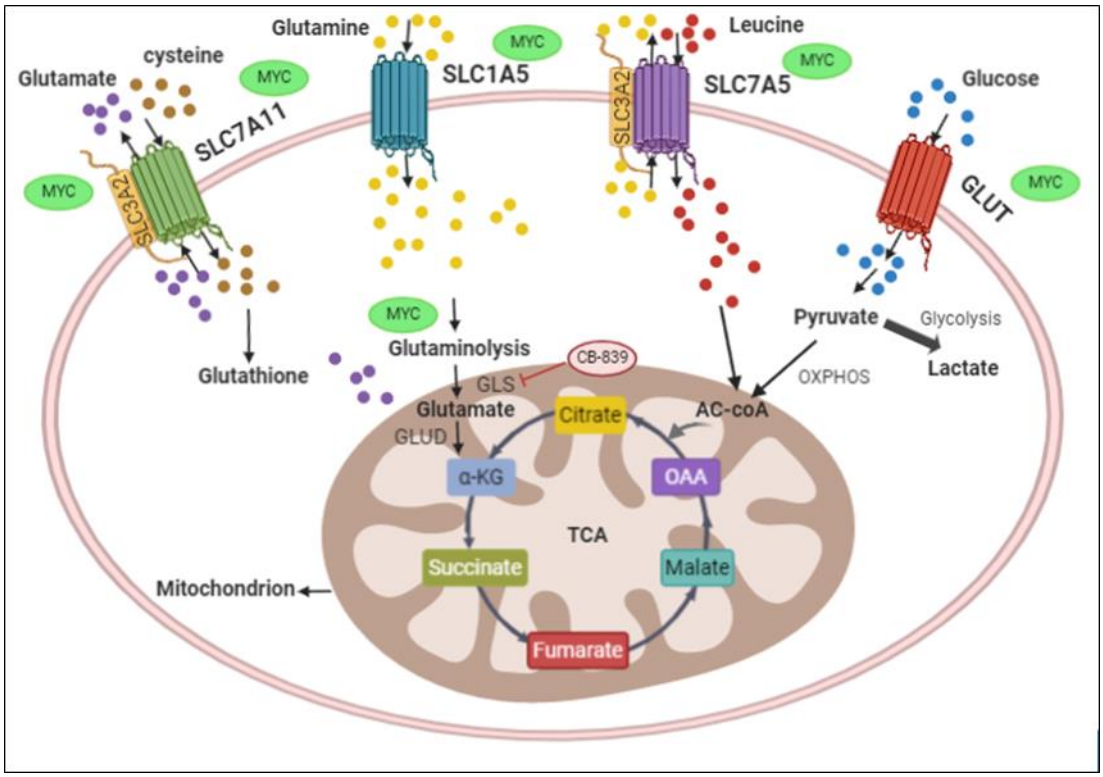


Figure 1-3. Glucose and amino acid transporters implicated in cancer cell growth. Glucose enters through glucose transporter (GLUT) and provides pyruvate, which mainly, in cancer cells, undergoes aerobic glycolysis. Glutamine influx mediated by glutamine transporters (e.g. SLC1A5) where it is metabolised by GLS to provide glutamate which can provide α-KG or exported through SLC7A11 with simultaneous entry of cysteine and subsequent formation of glutathione. Glutamine efflux coupled by influx of leucine, through SLC7A5, which can be converted to acetyl-CoA. All transporters involved in this figure, along with GLS, are regulated by MYC. SLC7A5 and SLC7A11 heterdimerise with SLC3A2 for their functional expression in the cell membrane. **GLUT**, Glucose transporter; **SLC1A5**, Solute carrier family 5 member1; **SLC7A5**, Solute carrier family 7 member 5; **SLC7A11**, Solute carrier family 7 member 11; SLC3A2, Solute carrier member 3 family 2; **TCA**. Tricarboxylic acid cycle; **AC-CoA**, Acetyl coenzyme A; **OAA**, Oxaloacetate; **α-KG**, α-Ketoglutarate, LDH; **GLS**, Glutaminase; **GLUD**, Glutamate dehydrogenase; **OXPHOS**, Oxidative phosphorylation.

Other glutamine transporters that have been investigated in BC include SLC6A14 and SLC7A8, which are upregulated in ER+ but not ER- breast tumours [143,144]. In addition, SLC7A7 may be considered as a PgR target gene, as a study carried out by Leo *et al*, showed a 7-fold expression change within the TNBC cell line, MDA-MB-231, after PgR-transfection [149]. Additionally, SLC7A7, along with SLC7A9 and SLC38A5, have been shown to be overexpressed in HER2+ BC cell lines [147]. Moreover, SLC38A1 is upregulated and overexpressed in different BC cell lines, particularly those that are ER-, compared with normal breast tissues [145]. SLC38A2 has been found to be upregulated by BRCA1, which is associated frequently with basal-like TNBC [150,151]. Upregulation of SLC38A2 by HIF1α, in the ER+, MCF7, cells was also established [146]. Other glutamine transporters have yet to be studied in BC. SLC7A11 is a glutamate/ cysteine antiporter that is closely related to transporters that reserve glutamine influx, as they provide the precursor required to operate its transport function. In BC, the expression levels of this transporter has been shown to be lower in the ER+ tumours compared to the TNBC subtype [152].

1.2.8 Metabolic biomarkers can be exploited to predict breast cancer progression and response to therapy

Various metabolic pathways of cancer cells harbour targets that can be exploited for treatment of malignant diseases, including BC. These agents can deactivate the essential pathways that result in lack of energy and the necessary molecules required for cancer cell growth and survival.

BC patients with ER+ and HER2+ subtypes respond well to endocrine therapies and anti-HER2 agents, respectively. However, some cancers exhibit resistance. Moreover, conventional chemotherapy remains the main strategy for treating the aggressive TNBC as targeted therapy is currently lacking for these tumours. Therefore, cellular metabolism in BC may be promising for developing targeted therapies with fewer complications which may also reverse drug resistance. Metabolic approaches including liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy can be applied to analyse different metabolites in tumour tissue and plasma in order to identify novel diagnostic and prognostic

biomarkers, as well as providing new anti-cancer agents especially in tumours that lack response to conventional treatment.

Differences in metabolic signatures between ER- and ER+ breast tumours may further guide therapy and predict disease outcome. Glutaminase inhibitor CB-839 for example, is currently being assessed in clinical trials in many solid tumours including in patients with TNBC [153]. Similarly, targeting glutamine transporters *in vitro* show a considerable inhibitory effect on the highly aggressive BC cells [135,141]. Interestingly, TNBC cells are particularly sensitive to the pharmacological inhibition of mTORC1 [154]. Likewise, a clinical trial showed that trastuzumab-refractory HER2+ patients can be actively treated with mTORC1 inhibitors combined with anti-HER2 agents [155]. Accordingly, blockade of glutamine transporters may afford a potential role in treating the most aggressive BC subtypes through impeding the import of leucine and the subsequent mTORC1 activation. Thus, designing specific inhibitors, which target this pathway, should be further investigated.

Targeting metabolic reprogramming can be potentially useful as a strategy to overcome resistance to therapy. It is been found that enhanced Warburg metabolism, via AKT/mTOR/HIF-1α modulations, is one of the mechanisms that cause tamoxifen resistance and impairing glycolysis can restore tamoxifen sensitivity in the endocrine-resistant BC cells [156]. Similarly, Zhao *et al* found that increased glycolysis in HER2+ cancer cells, through the upregulation of heat shock factor 1 (HSF1) and lactate dehydrogenase A (LDHA) can participate in Trastuzumab resistance, as a combination of glycolysis inhibitor and the anti-HER2+ drug inhibited the growth of Trastuzumab-resistant BC [157].

It is important to note that cancer cells exhibit metabolic plasticity which allows them to survive and grow under unfavourable and stressful environments, making this character a major issue facing metabolic targeting of cancer cells. Targeting a particular metabolic pathway may not be sufficient as a viable strategy for cancer treatment, as tumour cells could develop resistance through expressing other alternative pathways. For example, metastatic BC shows striking plasticity to adapt their metabolism and growth under different conditions.

To allow exposure to microenvironmental changes which give a better representation of *in vivo* systems, magnetic resonance spectroscopy (MRS) was

applied by Simões *et al*, in a high-density three-dimensional *in vitro* model. They observed that under glutamine starvation, metastatic BC cells (4T1) showed increased pyruvate carboxylase activity, which catalyses the carboxylation of pyruvate to form oxaloacetate (OAA), while glutamine supply can cause a decrease in the activity of this enzyme, as more glutamine is available to replenish the TCA cycle at the level of OAA. This indicates the presence of an alternative pathway into the TCA cycle driven by glutamine and independent of glucose [158]. Therefore, it can be suggested that the synergistic lethality targeting both glutamine and glucose pathways may prove to be more effective anti-cancer therapy of metastatic BC, taking into account the toxic effect of these on normal cells, otherwise this treatment regimen may be accompanied by the same types of toxicity caused by conventional chemotherapy.

1.3 Hypothesis

Amino acid transporters, which are involved in the glutamine pathway, are overexpressed in BC and they have prognostic implications that are vary among different molecular BC subtypes. These transporters co-operate to support proliferation of the aggressive BC subtypes and they can be potential molecular targets for BC treatment. Overexpression of the key members of the glutamine transport family has a role in shaping the tumour microenvironment via attracting specific subtypes of immune cells that has an additive prognostic function in BC.

1.4 Aims

1- To evaluate the expression of a range of amino acid transporters, alongside their downstream signal, at the mRNA and protein levels, in large well-characterised annotated cohorts of BC patients (chapters 3, 6 and 7).
2- To correlate the expression of the biomarkers of interest with clinicopathological variables and relevant biological markers (chapters 3, 6 and 7).
3- To determine the prognostic potential of the biomarkers of interest in the different molecular BC subtypes (chapters 3, 6 and 7).
4- To assess the co-localisation of the key glutamine transporters and assess their potential utility to stratify BC patients into accredited clusters based on their protein expression (chapter 4).

5- To investigate the impact of targeting the gene expression of the key glutamine transporters on BC cells' proliferation, invasion and transport activity (chapter 5).
6- To correlate the expression of the key glutamine transporters with various immune cell markers in addition to evaluating the prognostic potential of the co-occurrence of the two compartments in the different molecular BC subtypes and assessing the impact of targeting the key glutamine transports on the expression of the immune checkpoint ligand in BC cells (chapter 8).

Chapter 2. Material and methods

2.1 Patient cohorts

2.1.1 Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort

This study was previously conducted and comprised 1980 primary BC fresh-frozen samples from tumour banks in the UK and Canada. DNA and RNA were isolated and utilised for copy number/genotype analysis and transcriptional profiling respectively. The quality of extracted DNA was assessed by agarose gel electrophoresis, while RNA integrity was appraised using the Agilent 2100 Bioanalyser Nanochip (Agilent Technologies, Wokingham, UK). DNA probes were hybridised to Affymetrix SNP 6.0 arrays (Affymetrix, Santa Clara, CA) at AROS Applied Biotechnology (Aarhus, Denmark). Biotin-labelled cRNA was generated from total RNA, applying the Illumina Totalprep RNA amplification kit (Ambion, Warrington, UK), and hybridized onto Illumina Human HT-12 v3 platforms. The resulted gene expression raw data was pre-processed and normalised as described previously [93]. The linear models of microarray data (LIMMA) Bioconductor software package was utilised for the differential expression analysis. In this cohort, patients who were ER+ and/or lymph node (LN)-negative did not receive adjuvant chemotherapy, while ER- and LN-positive patients were offered the adjuvant treatment. None of the HER2+ patients was treated with anti-HER2 targeted therapy (trastuzumab). Table 2-1 summarises the clinicopathological parameters for the METABRIC cohort.

2.1.2 External validation

The online dataset, Breast Cancer Gene-Expression Miner v4.0 (bc-GenExMiner v4.0) (http://bcgenex.centregauducheau.fr) was utilised to further assess the mRNA expression of the all biomarkers included in this research. This dataset includes transcriptomic data of large pooled cohort of BC patients (n= 4904) and it is composed of three statistical mining modules; the prognostic module, which provides opportunity to assess the prognostic impact of candidate genes' expression in BC. This module was done by means of univariate cox regression proportional hazards model and Kaplan–Meier plot. The expression module, allows the evaluation of candidate gene expressions among the different prognostic parameters. The correlation module, offers the possibility to compute Pearson correlation coefficient between the candidate gene expressions and to provide lists

of genes that are most correlated with the gene of interest. Analysis can be conducted by splitting the cohort into sub-populations based on different clinicopathological variables, such as, nodal status, hormone receptor status and molecular subtypes [159].

2.1.3 Nottingham BC cohort

This is a well-characterised cohort of early stage primary operable invasive BC patients aged ≤ 70 years and presented between 1986 and 2006 at Nottingham City Hospital (n=2770). Patient management was uniform and based on tumour characteristics by NPI and hormone receptor status. Patients within the NPI excellent prognostic group (score ≤ 3.4) received no adjuvant therapy, but those patients with NPI > 3.4 received tamoxifen if ER-positive [± Goserelin (Zoladex) in premenopausal patients]. Whereas, ER-negative patients, who were fit enough to receive chemotherapy, were treated with cyclophosphamide, methotrexate and 5-flurouracil (CMF). None of the patients in this series received neoadjuvant therapy. In this cohort, clinical history, tumour characteristics and outcome data are prospectively maintained and regularly updated. Clinical details include, patient's age at diagnosis, menopausal status, tumour size, tumour grade, tumour type, NPI, nodal stage and lymphovascular invasion (LVI).

The outcome data recorded as follows: Breast Cancer Specific Survival (BCSS), which described the time, in months, from the date of the primary surgery to the date of BC-related death. Distant Metastasis Free Survival (DMFS), defined as the time in months from the primary surgery until developing distant-metastasis. Table 2-1 summarises the clinico-pathological characteristics for the Nottingham cohort.

Table 2-1. Frequency of clinicopathological parameters of the METABRIC and Nottingham BC series.

	METABRIC series	Nottingham series
Parameter	**Number (%)**	**Number (%)**
Age		
≥ 50 years	1426 (78.6)	1854 (67.0)
< 50 years	424 (21.4)	914 (33.0)
Tumour size		
≥ 2cm	1337 (68.2)	1233 (44.6)
<2cm	623 (31.8)	1534 (55.4)
Grade		
1	169 (9.0)	447 (16.2)
2	770 (40.7)	1024 (37.0)
3	952 (50.3)	1293 (46.8)
Tumour type		
Ductal (including mixed)	1545 (83.6)	2372 (85.8)
Lobular	148 (8.0)	216 (7.8)
Medullary-like	32 (1.7)	40 (1.4)
Miscellaneous	12 (0.6)	18 (0.7)
Special type	113 (6.1)	117 (4.3)
Vascular Invasion		
Definite	Not available	867 (31.4)
Negative/Probable		1890 (68.6)
Lymph node stage		
1	1035 (52.5)	1706 (61.8)
2	623 (31.5)	806 (29.2)
3	315 (16.0)	249 (9.0)
Nottingham prognostic index		
Good	680 (34.3)	895 (32.4)
Moderate	1101 (55.6)	1423 (51.5)
Poor	199 (10.1)	445 (16.1)

(Continued)

	METABRIC series	Nottingham series
Parameter	**Number (%)**	**Number (%)**
Follow-up Status		
Alive	1128 (71.6)	1887 (77.8)
Died from Breast Cancer	448 (28.4)	537 (22.2)
ER		
Negative	472 (23.8)	595 (21.6)
Positive	1508 (76.2)	2160 (78.4)
PgR		
Negative	938 (47.4)	1091 (40.4)
Positive	1042 (52.6)	1612 (59.6)
HER2		
Negative	1734 (87.5)	2353 (87.1)
Positive	246 (12.5)	350 (12.9)

2.1.4 Biomarker Data

A number of molecular biomarkers relevant to BC, previously studied in the Nottingham cohort, were used to determine associations with the amino acid transporters of interest. These include: HR (ER and PgR) [160], HER2 [161], tumour suppressor protein (P53) [160], cell proliferative marker (Ki-67) [162], the transcription factor (MYC) [163], proteins involved in PI3K/AKT/mTOR signalling pathway (PIK3CA, p-AKT, p-mTORC1) [164-166], proteins/enzymes involved in glutamine metabolism (GLS, GLUD1, ALDH4A1, ALDH18A1, PRODH, PYCR1) [167,168]. All biomarkers were assessed immunohistochemically on TMA excluding Ki-67, which was stained and assessed on full-face sections. The standard HercepTest guidelines (Dako) was followed to assess HER2 immunoreactivity. Briefly, a score of 0 and 1+ represented negative HER2 staining, while cases with a score of 3+ were deemed HER2 positive and cases with a score of 2+ were equivocal. Chromogenic *in situ* Hybridisation (CISH) was used to quantify HER2 gene amplification in equivocal cases using the HER2 FISH pharmDx™ plus HER2 CISH pharmDx™ kit (Dako) and was assessed according to the American Society of Clinical Oncology (ASCO) guidelines [169]. Antibody

clones, suppliers and cut-offs used to assess the high expression of the all biomarkers are listed in Table 2-2.

2.1.5 BC molecular subtypes in Nottingham cohort

BC molecular subtypes in the Nottingham cohort were determined, based on tumour immunohistochemical profiling and the Elston-Ellis mitotic score [170], which resulted in four subtypes defined as: ER+/HER2- Low Proliferation (mitotic score 1), ER+/HER2- High Proliferation (mitotic score 2 and 3), HER2+ class: HER2+ regardless of ER status and Triple Negative subtype: ER-, PgR- and HER2- [171].

Table 2-2. Antibody clones, suppliers and cut-offs of the biomarkers which were previously studied in the Nottingham cohort

Biomarker	Antibody clone	Supplier	Cut-off
ER	1D5	DAKO	≥1%
PgR	PgR 636	DAKO	≥1%
HER2	cerbB-2	DAKO	3+ and 2+ with amplification
P53	DO7	Novocastra	>10%
Ki-67	MIB-1	DAKO	≥10%
MYC	9E100	Abcam	>35 H-score
PIK3CA	HPA009985	Sigma-Aldrich	>100 H-score
p-AKT	RB-10369-P1	Neomarker	≥65 H-score
p-mTORC1	49F9	Cell signalling	>35 H-score
GLS	EP7212	Abcam	>120 H-score
GLUD1	Ab168352	Abcam	>215 H-score
ALDH4A1	HPA006401	Sigma-Aldrich	>200 H-score
ALDH18A1	HPA012604	Sigma-Aldrich	>130 H-score
PRODH	HPA020361	Sigma-Aldrich	>200 H-score
PYCR1	HPA047660	Sigma-Aldrich	>100 H-score

2.2 Western blotting (W.B)

Western blotting was used either to validate the specificity of antibodies involved in the immunostaining or to determine the knockdown efficiencies following siRNA transient transfection of interested genes. Western blotting was applied on a mixture of cell lysates, including human embryonic Kidney (HEK) 293T and whole

cell lysates of cervical/ breast cancer cell lines (HeLa, MCF7, T47D, MDA-MB-231, MDA-MB-175, SKBR3, ZR-75-1 and HCC1500).

2.2.1 Cell lines and preparation of cell lysates

HEK 293T lysate was purchased from (Origene Technologies, Rockville, MD, USA). Other cell lysates were prepared using cell lines obtained from American Type Culture Collection; Rockville, MD, USA. MCF7, T47D, MDA-MB-231, ZR-75-1 and HCC1500 cells were cultured in Roswell Park Memorial Institute (RPMI-1640) medium (GE Healthcare Life Science, AE25719279, South Logan, Utah) and SKBR3 cells were grown in McCoy's 5A medium (Sigma Life Science, SLCB4463, USA) while HeLa and MDA-MB-175 cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Sigma Life Sciences, RNBH6547, UK). All media were supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, UK). Cells were maintained in a 37°C humidified incubator with 5% carbon dioxide (CO_2).

Cells were grown until 80 to 90% confluent. Respective culture medium was discarded and cells were washed twice with sterile Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma Life Science, RNBH8631, UK), in order to remove the medium residue. Thereafter, cells were detached by adding trypsin (Sigma Life Science, 29259717, USA); a serine protease, which breaks down protein bounds to allow a gentle dissociation of cells from the tissue culture flasks, and incubated at 37°C for ≤ 5 minutes. Following this, cells were checked under the microscope to ensure they were detached and fresh media was added to neutralise the detached cells followed by centrifugation at 1000 rpm for 5 minutes. Cell pellets were re-suspended in fresh media and cells counted (the aim is a concentration of 1×10^7 cells/mL, which is roughly equalize 1µg of protein/1 µl). To count viable cells, a Neuber Haemocytometer was used; where a mixture was prepared by adding Trypan Blue (a substance that stains the non-viable cells in blue) to the cell suspension (with 1:1 ratio). 20µl of Trypan Blue/ cell mix was loaded under a coverslip on the haemocytometer and only viable cells were counted in the central (5x5) grid, using light microscopy. All counts were carried out in duplicate and the number of cells per ml was achieved by multiplying the average cell count by dilution factor (2) $x10^4$. After determining the cell count, the cell suspension was centrifuged at 1000 rpm for 5 minutes and cell pellet placed on ice. To lyse the cells, each $1x10^7$ cell pellet were re-suspended in 1 ml of RIPA lysis buffer (25mM

Tris, 150mM sodium chloride, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% Sodium Dodecyl Sulfate (SDS) (Thermo Scientific, #89901) and protease inhibitor (Roche, Complete Mini Protease Inhibitor Cocktail tablets, #04693124001). After 15 minutes incubation on ice, cell lysis was centrifuged at 4°C with 13000 rpm for 10 minutes. Finally, the supernatant was collected and stored in -20°C until use.

2.2.2 Sample preparation and gel electrophoresis

Samples, of approximately 30 µl volume, were prepared by adding 20 µg of protein lysates, 7.5 µl of NuPAGE/ Bolt LDS Sample Buffer (4x) (Novex Life technologies) and 3 µl of NuPAGE/ Bolt Reducing Agent (10x) (Novex Life technologies, NP0009). After mixing, samples were heated at 100°C for 5 minutes and placed on ice. Following that, samples were loaded onto NuPAGE/Bolt Bis-Tris protein gel (4-12%) (Novex Life Technologies, NW04120BOX) in 20x MOPS SDS running buffer (Novex Life Technologies). The PageRuler™ Plus Pre-stained Protein Ladder (Thermo Scientific) was used as a molecular weight standard and gels were run at 150 volt for 90 minutes or 160 volt for 60 minutes for NuPAGE or Bolt gels, respectively.

2.2.3 Protein transfer

After electrophoresis, proteins were transferred to Polyvinylidene difluoride (PVDF) membrane (Merck Millipore, R9DA13833, Ireland) and electro-blotting was performed at 25 Volt for 60 minutes in 20x NuPAGE/ Bolt transfer buffer (Novex Life Technologies), which was prepared by adding 1x NuPAGE/Bolt transfer buffer with 10% methanol and 0.1% NuPAGE/Bolt Antioxidant (Novex Life Technologies).

2.2.4 Blocking and antibody probing

The membrane was blocked in blocking buffer, using 5% milk powder (Marvel Original Dried Skimmed Milk, Premier Food Groups Ltd, UK) in Phosphate Buffer Saline (PBS) (Oxoid, Hampshire, UK) containing 0.1% Tween-20 (Sigma) for one hour at room temperature. After blocking, the membrane was incubated with primary antibodies at 4°C overnight, using the optimal dilutions detailed in Table 2-3. 1:5000 mouse monoclonal anti β-actin primary antibody (Sigma-Aldrich, A5441) was included as a positive control in all experiments. After washing with PBS + 0.1% Tween-20, 3 x 5 minutes each, the membrane was incubated for 60

minutes at room temperature with 1:15000 IRDye 680 RD donkey anti-mouse fluorescent secondary antibody (926-68072-LI-COR Biosciences)/ IRDye 800 CW donkey anti-rabbit fluorescent secondary antibody (926-32213-LI-COR Biosciences) followed by washing in PBS/Tween 3 x 5 minutes.

2.2.5 Detection

Protein bands were visualised by fluorescent detection, using Odyssey Fc with Image Studio 4.0 (LI-COR Biosciences), at wavelengths of 600, 700 and 800 nm.

2.2.6 Western blotting using Enhanced Chemiluminescence (ECL)

ECL was used to visualise protein bands using MagicMark™ XP Western Protein Standard (Invitrogen) as a molecular weight standard and 1:1000 anti-rabbit or anti-mouse monoclonal horseradish peroxidase (HRP) conjugate (Dako, Carpinteria, CA, USA) was applied as a secondary antibody. For the detection, the following steps were carried out in the dark room, the membrane was incubated with ECL detection reagent (GE Healthcare, UK) for 1 minute and then removed from the solution and placed in a plastic pouch, making sure to remove air bubbles and excess reagent. Following that, the membrane pouch was placed into a cassette and a film sheet (Kodak Bio Max Film) was overlaid on the membrane pouch for 10 minutes. The film was soaked in developer (Sigma Aldrich, UK) for 1 minute, placed in fixing solution (Sigma Aldrich, UK) for 1 minute, then washed in water until the background was clear, and allowed to dry overnight.

2.3 TMA construction

TMA is a high-throughput technique, where representative tissue cores can be taken from more than 100 archival tissue blocks and embedded in a single recipient block. It allows for simultaneous high throughput assessment allowing time saving as well as uniformity of assay conditions across all tissue samples, leading to more reproducible and consistent results. TMAs, utilised in this research, were previously constructed following the next steps:

2.3.1 Preparation of donor blocks

Formalin Fixed Paraffin Embedded (FFPE) donor blocks, from BC patients, were retrieved from the Nottingham Health Science Biobank. Full-face sections were prepared from each donor block and were stained with haematoxylin and eosin

(H&E). Sections were assessed to determine the most appropriate invasive tumour area excluding areas with necrosis, haemorrhage and *in situ* lesions. The most representative area was marked on the slides and their corresponding tissue blocks.

2.3.2 Preparation of the recipient blocks

Blank paraffin blocks were used as a recipient block. Paraffin wax was melted at between 55-58°C, and placed into 5-10mm thick moulds. A histopathological tissue cassette was put on the surface of the melted paraffin, allowed to cool on ice, and the mould removed. The block was trimmed, using a microtome, to ensure a flat and regular surface.

2.3.3 Creation of TMA block

Donor and recipient blocks were loaded in an automated TMA machine, Grand Master® (3D HISTECH®, Budapest, Hungary). Tumours were arrayed using a single 0.6mm core needle sampled from the representative tumour areas. Each recipient block contained 150 tumour cores and three orientation (kidney, liver or tonsil) control tissue cores placed at the beginning of the first row and another two at the end of the last row to ensure correct orientation and precise core evaluation.

2.4 Immunohistochemistry (IHC)

IHC is a widely used technique in both diagnostic and research fields. It is based on the concept of detecting antigens (proteins) through the process of antigen-antibody binding in biological tissues. It allows the examination of differentially expressed proteins in tissues and understanding their distribution and cellular localisation. In this study, IHC was performed using Novolink polymer detection system (Leica Biosystems, RE7150-K). Staining was carried out on 4µm thick tissue sections.

2.4.1 Steps of IHC technique

2.4.1.1 Slide heating, Deparaffinisation and rehydration

Slides were placed on a 60°C hotplate for 10 minutes, to melt the paraffin and maintain firm fixation of the BC tissue on the slides. Slides then allowed to cool for 5 minutes and placed in an autostainer machine (Leica Biosystems), where

they were de-waxed, in two xylene baths for 5 minutes each, followed by three changes of industrial methylated spirit (IMS 100%, Thermo Fisher Scientific, M/4450/25) two minutes each. The slides were then rehydrated in water 3 x 5 minutes.

2.4.1.2 Antigen retrieval

Heat-induced antigen epitope retrieval was performed in citrate buffer (1M sodium citrate at pH6.0) for 20 minutes using a microwave oven (Whirlpool JT 359 Jet Chef 1000W) followed by cooling under running tap water for 5 minutes.

2.4.1.3 Applying Leica Max Polymer Detection kit

The following steps were accomplished manually either by using Sequenza trays, to stain full-face sections, or a flatbed for the TMA sections. For Sequenza staining, slides were loaded into Sequenza plates (Thermo Scientific) under the surface of tap water, making sure that they were free of air bubbles, and placed into Sequenza trays. Subsequently, slides were washed twice with Tris Buffered Saline (TBS, pH7.6). When flatbed staining was used, slides were placed onto a flatbed tray and a hydrophobic barrier pen was applied across the width of the slides to limit wetting area and ensuring reagents are confined to the TMA cores. Slides were washed two times with TBS-T (TBS pH 7.6, containing 0.1% Tween 20). The rest of the steps are the same in both ways, with one difference, which is the use of TBS for washing in Sequenza staining method, while TBS-T was used when the flatbed staining was applied. Endogenous peroxidase activity was blocked by applying peroxidase block (Novolink kit) for 5 minutes. Slides were rinsed with TBS/TBS-T for 2 x 5 minutes, followed by application of protein block (Novolink kit) for 5 minutes. After another TBS/TBS-T wash, sections were incubated with the primary antibody (Table 2-3), optimally diluted in Leica antibody diluent (Lieca, Biosysytems, UK, RE AR9352). Incubation times differed between 30 minutes or 1 hour at room temperature and overnight at 4°C (Table 2-3). Slides were washed with TBS/TBS-T for 2 x 5 minutes and incubated with Post Primary Block (Novolink kit) for 30 minutes followed by another washing step. Novolink polymer (Novolink kit) was added for 30 minutes and 3, 3'Diaminobenzidine (DAB) working solution (1:20 DAB chromogen in DAB substrate) (Novolink kit), for 5 minutes, was applied. Before and after adding the DAB, slides were washed twice

with TBS/TBS-T for 5 minutes. Slides were counterstained with Novolink haematoxylin for 6 minutes.

2.4.1.4 Dehydration and mounting

Slides were dehydrated through placing them in the autostainer machine, where they were immersed in IMS for 3 x 2 minutes, then cleared in xylene for 2 x 5 minutes. Finally, slides were mounted, with a glass coverslip, using Dibutylphthalate Polystyrene Xylene (DPX) (BDH, Leica Microsystems, Newcastle, UK), and left to dry overnight at room temperature.

Table 2-3. Details of the antibodies used in this research

				W.B		IHC		
Antibody	**Supplier**	**Species**	**Product code**	**Dilution**	**Molecular weight**	**Dilution**	**Incubation time**	**Positive control**
SLC1A5	Sigma-Aldrich	Rabbit polyclonal	HPA035240	1:250	80 KDa	1:200	30 minutes	BC tissue
SLC7A5	Abcam	Rabbit monoclonal	Ab208776	1:200	40 KDa	1:50	Overnight 4°C	Testis
SLC3A2	Sigma-Aldrich	Rabbit polyclonal	HPA017980	1:5000	80 KDa	1:1000	1 hour	Kidney
SLC38A2	Sigma-Aldrich	Rabbit polyclonal	HPA035180	1:1000	38 KDa	1:25	1hour	Tonsil
SLC7A8	Sigma-Aldrich	Rabbit polyclonal	HPA051950	1:200	58.4 KDa	1:100	1 hour	Kidney
SLC7A11	Abcam	Rabbit polyclonal	Ab37185	1:2000	55.5 KDa	1:50	1 hour	BC tissue
P70S6K	Sigma-Aldrich	Rabbit polyclonal	HPA010010	1:100	57 KDa	1:50	Overnight 4°C	BC tissue

2.4.2 Optimisation of primary antibody

All primary antibodies were optimised, using BC TMA sections, to ensure appropriate staining, including clear results with no or minimal background staining, and different staining intensities across the tested BC cores. Optimisation involved using different antibody dilutions, starting by the dilution recommended by the supplier's datasheet and adding other two or three dilutions above and below the recommended one. Optimisation also required changing other staining conditions such as incubation times.

2.4.3 Positive and negative controls

Positive and negative controls were included in all IHC experiments. Positive control tissues were selected based on antibodies' supplier recommendations or through choosing specific tissues, which showed high expression of the target

proteins, from Human Protein Atlas (http://www.proteinatlas.org/). BC tissues stained with rabbit polyclonal β2-microglobulin (Dako, Denmark), at a concentration of 1:2000, was also used as a positive control, in case specific positive control tissues were not available. Negative controls were included, where IHC staining was carried out on BC tissues with the omission of primary antibodies.

2.4.4 Assessment of IHC staining

Stained sections were scanned to produce high resolution digital images (0.45 μm/pixel), using NanoZoomer slide scanner (Hamamatsu Photonics, Welwyn Garden City, UK) at 20x magnification. Digital slides were viewed using Aperio ImageScope software (Aperio Technologies, Milton Keynes, UK) or Xplore 5.1.0 software (Philips, UK). TMA cores were evaluated semi-quantitatively using a modified histochemical score (H- score) [172], where the intensity of staining and the percentage of stained invasive BC cells were both considered. For the staining intensity, a score index of 0, 1, 2 and 3 is consistent with negative, weak, moderate and strong staining, respectively. The percentage of the positively stained tumour cells was estimated subjectively. The final H-score was calculated multiplying the percentage of positive cells (0- 100) by the intensity (0- 3), producing a total range of 0- 300. All non-representative cores were excluded from scoring, such as cores which contain < 15% tumour cells relative to the whole core area, cores which show only stroma or normal breast tissue and cores that were folded during processing or staining.

Prior to TMA staining, all biomarkers of interest were stained in twenty full-face BC tissue sections to assess the staining pattern and thus ensure that TMA cores are representative for the whole tumour. When co-localisation of two biomarkers was assessed, serial full-face sections were evaluated by estimating the percentage of positive immunostained cells and Caseviewer software (https://www.3dhistech.com/caseviewer) was utilised to view digital slides, where two corresponding slides, for both biomarkers, can be assessed simultaneously. All cases were scored without foreknowledge to patients' clinical or outcome data.

2.4.5 Setting up cut-off points

All biomarkers' expression data, at mRNA and protein levels, was dichotomised to identify a cut-off point which enables parsing the cohort into two categories;

expression levels > the identified cut-off point represent high expression group, while values ≤ the cut-off point reflect the low expression group. Dichotomisation was determined using x-tile bioinformatics software (version 3.6.1, Yale University, USA) [173]. This software works on splitting a single patient cohort into two sub-populations, training and validation sets. The optimally significant cut-off point, based on prediction of BCSS, is determined in a training group and validated in the second group.

2.5 Cell culture technique

2.5.1 Breast cancer cell lines

ZR-75-1, MCF7, HCC1500 and SKBR3 cells were chosen for further cell culture work, as they represent different BC subtypes (Table 2-4). A selection of these specific subtypes was based on the results obtained after studying the key targeted proteins (SLC1A5 and SLC7A5), at the IHC level, on different BC subtypes. Among the luminal cell lines, ZR-75-1 and HCC1500 cells were selected for SLC1A5 *in vitro* studies, as they express high levels of this protein, while the most representative luminal cell lines for SLC7A5 were ZR-75-1 and MCF7. All cells were obtained from American Type Culture Collection; Rockville, MD, USA. Cell growth was maintained as described in section 2.2.1. Characteristics of the cell lines, used in the *in vitro* work, were detailed in Table 2-4.

Table 2-4. Descriptions of breast cancer cell lines involved in the *in vitro* work.

Cell lines	Histological type	Receptors status	Subtype	Source	Reference
ZR-75-1	Human breast ductal carcinoma	ER+, PgR- and HER2-	Luminal B-like	Established in 1978 from ascites of a 63-year old Caucasian woman.	[174-176]
MCF7	Human breast adenocarcinoma	ER+, PgR+ and HER2-	Luminal A-like	Isolated from a pleural effusion of a 69-year old Caucasian woman in 1970.	[175,176]
HCC1500	Human breast ductal carcinoma	ER+, PgR+ and HER2-	Luminal A-like	Isolated in 1995 from mammary gland of a 32-year old black female.	[175,176]
SKBR3	Human breast adenocarcinoma	ER-, PgR- and HER2+	HER2+	Established in 1970 from pleural effusion of a 43-year-old Caucasian female.	[175-177]

2.5.2 Passaging of cell lines

The first steps of this procedure were identical to that described in section 2.2.1. After centrifugation and removing the residual trypsin, cells were re-suspended in fresh culture media and re-plated, in a desirable proportion, into new flasks. The flasks containing the cells were labelled with the cell name, passage number and date, and were then carefully placed in a 37°C humidified incubator with 5% CO_2. The passage number, for all cell lines, did not exceed 25.

2.5.3 Cryopreservation and thawing of cells

Cells of low passage numbers were stored in liquid nitrogen for future experiments. For cryopreservation, cells were trypsinised and re-suspended in freezing medium (complete growth medium containing 10% Dimethyl sulfoxide (DMSO, Sigma Life Science, SHBK2703, USA)). Following that, cell suspension was placed in pre-labelled cryovials and stored at -80°C overnight and transferred to liquid nitrogen for long-term storage. To commence a new cell culture experiment, stocks were thawed rapidly either at room temperature or in a 37°C pre-heated water bath. In order to extract the DMSO residue, cells were suspended in a fully supplemented culture medium and centrifuged at 1000 rpm for 5 minutes. Thereafter, cell pellets were re-suspended in a pre-warmed medium and cultured under standard conditions.

2.5.4 Mycoplasma detection

It is of a great importance to detect for mycoplasma infection, as these microorganisms are invisible by the naked eye and their contamination does not generate the turbidity typically associated with other microorganisms, such as fungi and bacteria. In addition, it can extensively alter phenotypic characteristics and affect the biology of the host cell lines causing inaccurate or inconsistent results.

In this study, mycoplasma testing was carried out on a routine basis using the MycoAlert Detection kit (R&D Systems, CUL001B, UK). This procedure involved using a 96-well hybridisation microplate that was washed twice in wash buffer (wash buffer concentrate diluted in RNase-free deionised water (1:10)). Following this, biotin-labelled capture oligonucleotide and digoxigenin-labelled detection probes, which are targeted to the ribosomal RNA (rRNA) of the most common

mycoplasma species, were diluted in the sample diluent and added, in designated wells, in the hybridization plate. Supernatant from targeted cells were diluted in cell lysis diluent (1:10) and were placed in duplicate, along with the positive control and the sample diluent (negative control) in designated wells. The plate was covered by plate sealer and incubated in a water bath at 65°C for 60 minutes. To allow higher binding efficiency, the hybridisation solution was transferred to streptavidin-coated 96 well microplate and incubated on an orbital shaker for 60 minutes at room temperature. After washing 4 times, anti-digoxigenin alkaline phosphatase conjugate was added to each well and incubated at room temperature for another 60 minutes, followed by rinsing 6 times with wash buffer. Substrate solution, prepared by reconstituting lyophilized substrate (NADPH) with substrate diluent, was added to each well and incubated, as before. Following that, amplifier solution was put in each well and incubated for 20 minutes at room temperature, to allow the development of colour commensurate with the amount of mycoplasma rRNA in the targeted samples. Finally, colour development was stopped by adding stop solution and colour intensities were measured at 490nm using a plate reader (TECAN Infinite F50). The results were calculated as follows, average optical density (O.D.) of the sample - average O.D. of the negative control. Calculated O.D. of <0.05 was considered as negative result while results of >0.1 were deemed positive for mycoplasma. All samples with values between 0.5 and 0.1, which treated as inconclusive results, were eligible for repeating the assay.

2.5.5 Transient siRNA knockdown of *SLC1A5* and *SLC7A5*

Small double-stranded interfering RNA (siRNA) is a type of RNA interference (RNAi), which results in gene silencing by a process of mRNA degradation. In this method, gene knockdown was accomplished by introducing siRNA into the cytoplasm, where it is incorporated into multi-protein complex, the RNA-induced silencing complex (RISC). Upon RISC activation, one of the siRNA strands is cleaved and detached from the complex, while the other works as a template located on the targeted mRNA. Once this occurs, the mRNA is cleaved and degraded by a ribonuclease [178] (Figure 2-1). In this study, siRNA was introduced to cells by lipofection, in order to obtain transient knockdown of the targeted gene.

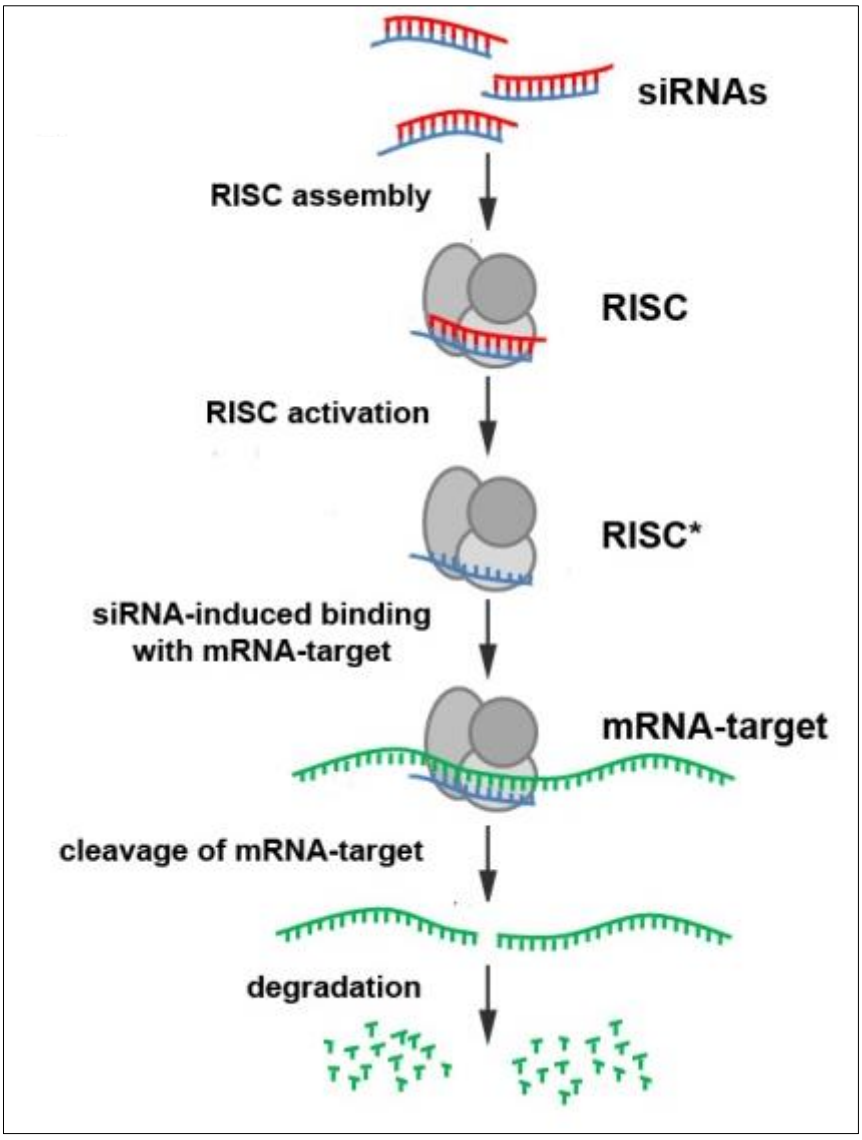


Figure 2-1. Scheme of mechanism of action of siRNA, modified from Petrova et al, 2013.

Silencer select siRNA constructs designed to *SLC1A5* (s12918) and *SLC7A5* (s15653) were purchased from ThermoFisher Scientific: anti-sense sequences were 5'-AAAGAGUAAACCCACAUCCtc-3' and 5'-UUGGGAUCUAGAUUGGACAca-3' (for *SLC1A5* and *SLC7A5* respectively). Transfection was carried out using lipofectamine RNAiMAX reagent (Thermofisher, 2121811, Lithuania). siRNAs were optimised alongside the negative, scrambled siRNA (ID: 4390843), control. This optimisation involved using different siRNA and lipofectamine concentrations, in order to achieve the maximum knockdown efficiency, which was determined by western blotting. In this study, a reverse transfection method was performed, where transfection mix (siRNA constructs and lipofectamine) was added in the

wells, after which cells and media are joined. This method characterised by being faster to perform compared to forward transfections, and it is the method of choice for high-throughput transfection.

2.5.5.1 siRNA transient transfection procedure

SLC1A5 transfection was performed by Dr. Madeleine Craze, while the *SLC7A5 in vitro* work was completed by the author. Transfection was carried out using 6-well plate and 24-well plate for *SLC1A5* and *SLC7A5* Knockdown respectively. For each well, 100 pmole/µl of *SLC1A5* siRNA constructs and 25 pmole/µl of *SLC7A5* siRNA constructs were added after being diluted in OptiMEM media (ThermoFisher, 2088880). 5 or 2µl lipofectamine, was added (for *SLC1A5* or *SLC7A5* studies respectively) to each well, and incubated for 15 minutes. Following that, $2x10^5$ cells were seeded per each well in the 6-well plate, while $5x10^4$ cells were seeded for the 24-well plate. Cells were then incubated at 37°C in a humidified, 5% CO_2 atmosphere, and either lysed at day 3, 5 and 7, for western blotting analysis or trypsinised to be used for functional assays, which will be detailed in the next sections.

2.5.5.2 Cell lysis for siRNA samples

Medium was aspirated from each well and the plate was placed on ice. Following that, wells were gently washed with cold DPBS followed by adding a previously prepared lysis buffer (prepared as mentioned in section 2.2.1) and incubating for 5 minutes. Cell lysis buffer was then collected and transferred to pre-chilled microfuge tubes, where they were incubated for 15 minutes and centrifuged at 4°C with 13000 rpm for 10 minutes. The supernatant was collected and stored in -20°C until used.

2.5.6 CellTiter 96® aqueous one solution cell proliferation assay (MTS assay)

MTS assay is a colourimetric method that is used to measure cell viability. The CellTiter 96® aqueous One Solution Reagent (Promega, US) contains tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). Metabolically active (viable) cells reduce this compound, by the action of dehydrogenase enzymes, to generate coloured

formazan, which is soluble in cell culture medium. This reaction is accompanied by production of reducing equivalents such as nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and nicotinamide adenine dinucleotide hydrogen (NADH). The amount of formazan formed is directly proportional to the number of viable cells.

MTS assay was performed to determine the effect of *SLC1A5* and *SLC7A5* knockdown on cell growth. 24 hours after transfection, control and transfected cells were seeded, in triplicate, at a density of 3000 cells per well in a 96-well plate and were allowed to adhere. Thereafter, 20µl of the proliferation reagent was added to each well, at 48 hours and 120 hours post transfection, and incubated for 1 hour under normal incubator conditions. The absorbance was recorded at 490nm using a microplate reader (TECAN Infinite F50). Each assay condition was done in triplicate, average taken and graphs plotted based on the percentage of cell growth, which was calculated as follows:

$$\text{Percentage of cell growth} = \frac{\text{O. D. (sample)} - \text{O. D. (blank) at 120 hours}}{\text{O. D. (sample)} - \text{O. D. (blank) at 48 hours}}$$

2.5.7 CytoSelect 24- well cell invasion assay

Cell invasion is defined as the ability of cells to penetrate through tissue barriers such as the basement membrane and the extra cellular matrix (ECM). The characteristic of invasion enables cancer cells to spread to secondary tissues, giving rise to metastasis. Thence, exploring new strategies to suppress cell invasion is extremely important in cancer studies.

In this study, cell invasion was assessed using Cytoselect 24-Well Cell Invasion Assay kit (Cell Biolabs Inc., CBA-110, USA) (Figure 2-2), according to manufacturer's instructions. This kit contains polycarbonate membrane inserts (8 µm pore size) in a 24-well plate. The upper surface of the insert is coated with a layer of dried basement membrane matrix solution. The basement membrane layer serves as a barrier to differentiate invasive cells from non-invasive cells. $1x10^6$, control or transfected, cells were cultivated, in duplicate, in the upper part of the insert in serum free medium, while the medium supplemented with 10% FBS was added in the lower chamber of the invasion plate. After 24 hours incubation, a cotton swab was used to remove the non-invasive cells from the upper part of the insert, whereas the invasive cells in the lower part were stained

with crystal violet and incubated for 10 minutes at room temperature. Following that, the stained inserts were gently washed in a beaker of water and allowed to air dry. The inserts were transferred to wells containing extraction solution and incubated for 10 minutes on an orbital shaker. Finally, 100 µl of each sample was transferred to a 96-well plate and absorbance was measured at 560 nm using a microplate reader (TECAN Infinite F50). Each assay condition was done in duplicate.

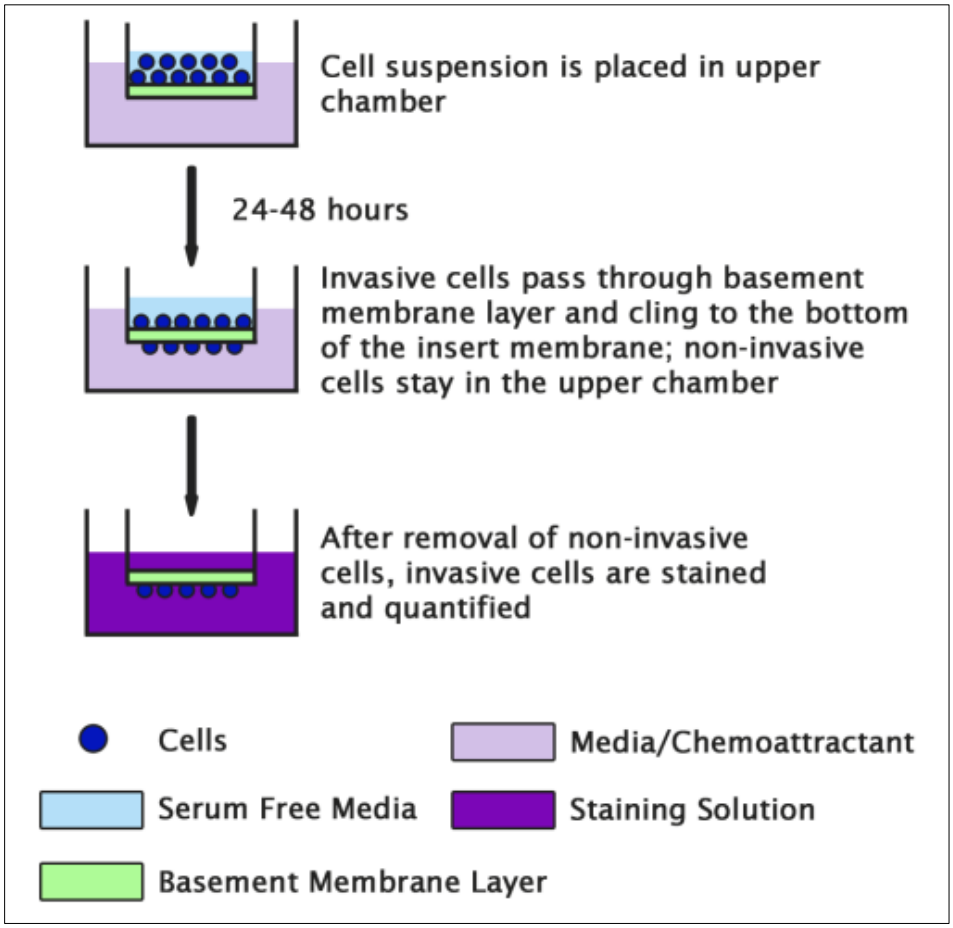


Figure 2-2. The schematic of CytoSelect cell invasion assay kit (Cell Biolabs, Inc).

2.5.8 Radioactive glutamine/leucine uptake assays

Radioactive glutamine/leucine uptake assays were performed to determine whether cells' ability to uptake glutamine or leucine were reduced after *SLC1A5* or *SLC7A5* knockdown, respectively.

2.5.8.1 Radioactive glutamine ([^{3}H]-L-Glutamine) uptake assay

This assay was carried out 120 hours after *SLC1A5* transfection in ZR-75-1 and HCC1500, control and transfected, cells. Firstly, medium was aspirated from the wells and replaced by glutamine-free medium 30 minutes before the procedure. Cells then were trypsinised, centrifuged and re-suspended in serum-free and glutamine-free medium. Cell suspension was transferred to pre-labelled microfuge tubes at a density of $5x10^4$ cells, followed by centrifugation at 6000x*g* for 5 minutes. After that, cell pellets were incubated in media, with or without L-[3,4-3H(N)]-Glutamine (0.3µCi/ml, Perkin Elmer, USA) for 30 minutes at 37°C in a humidified, 5% CO_2 atmosphere. This step was followed by washing twice with DPBS and spinning at 6000x*g* for 5 minutes. The cell pellet was re-suspended in serum-free and glutamine-free medium with 1% triton ™ X-100 (Sigma-Aldrich, UK), to avoid cell clumping. Thereafter, 50 µl from each sample was loaded, in triplicate, in Luma 96-well plate (PerkinElmer, USA) and left to air dry overnight. The next day, 20 µl of scintillation fluid (Microscint ™) (PerkinElmer, USA) was added to each well and radioactivity was measured, as raw scintillation counts, using a scintillation counter (PerkinElmer, USA). Each assay condition was done in triplicate.

2.5.8.2 Radioactive leucine ([^{14}C]-L-Leucine) uptake assay

This assay was performed on ZR-75-1, MCF7 and SKBR3, control and transfected, cells after 120 hours *SLC7A5* transfection. Steps of this procedure were similar to that described above with a few differences: cell pellet was incubated in Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich, UK) with or without Leucine, L-[4,5-3H(N)] (0.3µCi/ml, PerkinElmer, USA) for 5 minutes in standard incubator conditions.

2.6 Statistical analysis

The majority of statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software Version 24.0 (SPSS Inc., Chicago, IL, USA). GraphPad PRISM software Version 7.0 (GraphPad Prism Inc., San Diego, CA, USA) was used to plot graphs and analyse data generated from the cell culture work. For cluster analysis, two algorithms, partitioning around medoids (PAM) and K-means were used and the analysis was performed by Dr. Daniele Soria (Department of Computer Science, University of Westminster), applying the R

software. P-values were adjusted using Bonferroni Step-down (Holm) correction for multiple testing. This approach is an update of the Bonferroni correction and applied to reduce the chance of getting false positive results, which can be arisen from false discovery rate or type I error, when multiple analysis is performed on the same dependent variables. A p-value <0.05 was considered significant.

2.6.1 Univariate and correlation analysis

The chi-square test (x^2) was performed for inter-relationships between categorical variables, including associations with clinicopathological parameters and other biological markers. For the normalised continuous variables, differences between 3 or more groups were assessed using one way analysis of variance (ANOVA) with post-hoc Tukey multiple comparison test, while unpaired t-test was used to assess differences between two groups. To test correlation between two continuous normalised data, Pearson's correlation was performed.

2.6.2 Survival analysis

Survival analysis was assessed using Kaplan-Meier curves and the significance was analysed by the LogRank test. This was carried out with BC specific death, alive and censoring cases who lost to follow up or died of other causes. Cox's proportional hazard method was performed for multivariate analysis to test for confounders and prognostic independency of biomarkers. Thence, standard well-known prognostic factors were implied for this analysis, including tumour size, tumour grade and LN stage. The study endpoints were 10-year BCSS or DMFS.

2.6.3 Cluster analysis

Clustering is the partitioning of a set of objects into groups so that objects within a group (cluster) are similar and different to other objects set in other clusters. Therefore, cluster analysis aims to arrange unlabelled data into meaningful clusters with intra-cluster homogeneity and inter-cluster separation [179].

Prior to commencing the cluster analysis, validity indices (external validation criteria) were applied to determine the best number of clusters for the given data. All validity indices either depend on considering the data dispersion in a cluster and between clusters, or considering the scatter matrix of the data points and the one of the clusters centres [180]. Six validity indices were performed; Calinski

and Harabasz, Hartigan, Scott and Symons, Marriot, Trace W and Trace $W^{-1}B$ [181-186].

Two algorithms, PAM and K-means were applied in this study to cluster tumours based on protein expression (H-score) data of SLC1A5, SLC7A5 and SLC3A2. The K-means method pursues to split data into K clusters, where the sum of squares from points to the assigned cluster centres is minimised. The PAM (k-medoids) algorithm is a clustering method, which aims to minimise the distance between points labelled to be in a cluster and a point designated as the centre of that cluster. This can be achieved through selecting data points (medoids) as centres and allocating each point to its closest medoid [180].

2.7 Ethical approval

This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer' and the North West – Greater Manchester Central Research Ethics Committee under the title 'Nottingham Health Science Biobank (NHSB)' reference number 15/NW/0685.

Chapter 3. The prognostic significance of the key amino acid transporters in invasive breast cancer

3.1 Introduction

Amino acids are fundamental nutrients for all mammalian cells and any disruptions in intracellular amino acid contents can threaten their survival. Although the primary role of amino acids is proteogenic, they can support other biological functions, where they can be served as nitrogen donors for nucleotide synthesis and generation of other, non-essential, amino acids. The demand for these metabolites is increased in the fast growing cells, such as inflammatory, stem and cancer cells, which show a dramatic increase in their metabolic and biosynthetic needs [187,188]. Glutamine (Gln) is the most abundant amino acid in the human blood and is the second most essential nutrient used after glucose for driving tumour cell proliferation and survival [189,190]. Although it is non-essential, as it can be synthesised intracellularly, by glutamine synthetase (GLUL), it becomes "conditionally essential" to meet the demand of the highly proliferative tumour cells. This attributed to the plethora of biological functions that glutamine can provide to support cell survival and proliferation; beside its primary function, the carbon skeleton of glutamine can be involved in energy production in mitochondrial TCA cycle through α-ketoglutarate (α-KG) synthesis. α-KG can be converted to malate, which then used for production of NAPDH and pyruvate, which are in turn involved in anaerobic glycolysis and formation of anabolic intermediates [191,192]. Alternatively, glutamine can be catalysed by glutaminases to provide glutamate, the precursor of the anti-oxidant molecule, glutathione. Indeed, some cancer cells showed glutamine dependent cell growth and display the character of "glutamine addiction", as they fail to grow or proliferate in the absence of extracellular supply of this amino acid [102,193].

Essential amino acids, particularly leucine, are other examples of nutrients that cancer cells rely on for their growth and survival, as beside their function in protein synthesis, they considered as potent activators of mammalian target of rapamycin complex1 (mTORC1), which promote cellular growth through nutrient signalling pathways. Once activated, this complex enhance cellular anabolic programmes, such as mRNA translation, cell proliferation and repress apoptosis in cancer cells [41].

Thus, an intricate network of membrane transporters and enzymes is embroiled to maintain haemostasis of glutamine and other essential amino acids inside the

cells. To obtain the required amount of essential amino acids, cancer cells impose to increase the transcription of key amino acid transporters, such as SLC1A5 and SLC7A5.

3.1.1 SLC1A5/ Alanine-Serine-Cysteine Transporter 2 (ASCT2)

SLC1A5 is a cell surface sodium-dependent transporter that mediates the transport of neutral amino acids, including; alanine, serine, cysteine, threonine and glutamine, through the plasma membrane [142,194]. This transporter functions as an obligatory exchanger and is the only one in the SLC1 gene family that accepts glutamine as a substrate. The anti-port mode of SLC1A5 transporting system allows for regulation of amino acids pool in intracellular and extracellular spaces. SLC1A5 is normally expressed in lung, large intestine, T-cells, testis, kidney, brain, adipose tissue and skeletal muscles [195,196]. It has been also reported that SLC1A5 has a critical role in hematopoietic stem cells lineage specification, and targeting this transporter, with subsequent reduction of glutamine influx, inhibits the erythroid differentiation potential of progenitor cells [197].

The expression of this transporter increases dramatically in various cancers including, colorectal, melanoma, laryngeal squamous cell carcinoma, ovarian cancer, non-small cell lung cancer, breast and renal tumours [198-204,136]. This expression is induced by the up-regulation of MYC, where it selectively binds to the promotor regions of SLC1A5 leading to an increase in its expression levels [111]. A link with tumour suppressors is also seen, where down regulation of the tumour suppressor Retinoblastoma (Rb) can regulate glutamine uptake via controlling the E2F-3 transcription factor, which associates with the SLC1A5 promoter and directly up-regulates its transcription levels [113]. Further studies reveal that activation transcription factor (ATF4), a transcriptional master regulator of amino acid metabolism and stress responses, is implicated in the regulation of SLC1A5 expression in neuroblastoma and melanoma cells [205,206]. Mitogen-activated protein kinase (MAPK) and Phosphoinositide 3-kinase (PI3K) pathways are also involved in the regulation process, as they can maintain the stability of SLC1A5 in the plasma membrane of human enterocytes [207]. More recently, Edwards *et al* found that in HER2-positive BC cells, as well as in a mouse model, the molecular mechanism of activation of SLC1A5 expression can be

regulated through the tyrosine kinase receptor EphA2 [208]. Furthermore, SLC1A5 can also be regulated by micro RNA (miRNA-137); in an inversely correlated manner, as SLC1A5 can be activated when methylation of promoter region of miRNA-137, with consequent inhibition, takes place via methyl-CpG-binding protein 2 (MeCP2) and methyltransferases (DNMTs) [209].

3.1.2 SLC7A5/ L-Type Amino Acid Transporter 1 (LAT1)

SLC7A5 is a sodium-independent amino acid exchanger. It mediates uptake of large neutral amino acids such as leucine, histidine, phenylalanine, tryptophan and methionine by exchange with intracellular glutamine [210,211]. SLC7A5 has high affinity to its substrates, which renders it one of the key amino acid transporters that are able to cover cancer cells' needs for essential amino acids, in addition to its primary role in maintaining intracellular leucine, which activates the mTORC1 signalling pathway [212,213,130].

For functional expression on the plasma membrane, SLC7A5 must heterodimerise with the heavy chain of Solute Carrier Member 3 Family 2 (SLC3A2), also known as CD98hc or 4F2hc [211,130]. The heterodimeric complex formed when the two solute carriers covalently bind through di-sulfide bonds between two cysteine residues of the proteins [214,215]. Studies, on both murine and human isoforms, showed that SLC7A5 is the only component of the heterodimer which mediated the antiport of amino acids, whereas SLC3A2 does not demonstrate any intrinsic transport role [216]. However, it indirectly intervenes with amino acid transport, as it is the ancillary subunit of the heterodimer that assists trafficking of SLC7A5 to its eventual position in the plasma membrane [217-219]. SLC7A5 is normally expressed in placenta, brain, testis and T-cells [220-222]. Over-expression of SLC7A5 is described in variety of human cancers, including oral cancer, lung adenocarcinoma, TNBC, prostatic, gastric and pancreatic carcinomas [223-227,140]. It has been found that, SLC7A5 is co-expressed with SLC1A5 in many cancers [148], suggesting a functional coupling of these transporters, which certainly imputes to metabolic alterations occurring in tumour development and progression. In this setting, glutamine imported via SLC1A5 may supply the driving force for leucine uptake through SLC7A5 [228]. Like SLC1A5, the oncogene MYC regulates the expression of SLC7A5, as its' promoter has canonical binding sites for MYC and overexpression results in enhanced expression of SLC7A5

[193,112]. Recently, Zhao *et al* confirmed that lysin demethylase4c (KDM4C) directly induces mRNA expression of several amino acid transporters, including SLC7A5, via binding to their gene promoters. In response to amino acid deprivation, KDM4C indirectly activates this transporter, where it binds to the promoter of ATF4 and increases the production of its mRNA and protein [229]. Subsequently, ATF4-dependant transcripts encode for SLC7A5 and other key transporters to maintain homeostasis of intracellular amino acid pool [230]. A study on renal cell carcinoma showed that SLC7A5 expression is induced by the hypoxia-inducible factor (HIF2α) via binding to the SLC7A5 proximal promoter [231].

SLC7A5 can be exploited for tumour imaging via Positron Emission Tomography (PET), which allows the diagnosis of prostate, brain and breast cancers by tracing accumulation of the non-invasive SLC7A5 substrate, anti-1-amino-3-[18F]fluorocyclobutyl-1-carboxylic acid (anti-[18F]FACBC), which is delivered to cells through this transporter and enables a successful detection of tumour foci [232,233,141]. In terms of prognosis, SLC7A5 is included, along with p53, HTF9C, CEACAM5 and NDRG1, in the Mammostrat® test; which predicts early stage BC patients who are likely to develop recurrence during endocrine therapy [234]. Furthermore, SLC7A5 along with carcinoembryonic antigen-related adhesion molecules (CEACAM5 and CEACAM6), can predict poor response to neoadjuvant chemotherapy in locally advanced breast cancer thus providing a mechanism to avoid chemotherapy complications in patients who are improbable to respond to this treatment [235]. In lung cancer, SLC7A5 is also part of a five-antibody test, which differentiates squamous cell cancer from lung adenocarcinoma to identify patients competent for targeted therapy [236].

3.1.3 Solute Carrier Member 3 Family 2 (SLC3A2)

SLC3A2 is a trans-membrane protein which provides cells with the capacity of adjusting to their surroundings by mediating two distinct pathways. Primarily, it facilitates amino acid transport via acting as a chaperone that heterodimerises with a group of amino acid transporters (e.g. SLC7A5 and SLC7A11) for their functional expression in the plasma membrane [211,221]. SLC3A2 also binds to the cytoplasmic tail of β1 integrins (ITGB1) and regulates its function. Overexpression of SLC3A2 leads to amplification of integrin-dependent signals,

which involves extracellular matrix remodelling and increases cancer cell ability to respond to extracellular matrix rigidity resulting in promoting tumorigenesis and cancer growth [237,238]. Indeed the role of SLC3A2 in cancer biology goes beyond that, as it upregulates cellular anti-oxidant capacity when interacting with SLC7A11, the cysteine-glutamate exchanger which mediates the production of glutathione [239]. Furthermore, SLC3A2 assists glucose uptake and glycolysis through direct activation and stabilisation of glucose transporter 1 (GLUT1) independent of amino acid availability [240].

Noteworthy, SLC3A2 has an important role in the SLC classification even if this protein does not have a direct involvement in transport function. However, its expression is obligatory for the recruitment and hence the transport function of some SLC7 members. SLC3A2 is normally expressed in kidney, placenta, testis, pancreas and bone marrow. It has been found that expression of SLC3A2 associates with that of SLC7A5 in terms of localisation, as anticipated from the interaction between the two proteins. Nevertheless, SLC3A2 is also expressed in other tissues since it also works as an ancillary protein of other amino acid transporters in addition to its pleiotropic role in cell biology [221,241].

SLC3A2 is highly expressed in various human cancer types including gastric cancer, non-small cell lung cancer, osteosarcoma, head and neck squamous cell carcinoma, renal cell carcinoma, biliary tract cancer and TNBC [242-247,140]. Similar to SLC1A5 and SLC7A5, up-regulation of SLC3A2 is mediated by the oncogene MYC [193]. A relationship with protein kinases has also been revealed by a recent study, which shows SLC3A2 to promote tumour growth through regulation of PI3K/AKT signalling pathway in osteosarcoma [244]. Thus SLC3A2 could be implicated in mTORC1 activation by two major routes; either through interacting with the leucine importer, SLC7A5, or via regulating the protein kinases pathway.

To my knowledge there are no available molecules designed to inhibit SLC3A2 protein. nonetheless, gene knockdown of *SLC3A2* affects renal cancer cell growth *in vivo* [248] and efficiently decreases colony formation of osteosarcoma cells [244]. In addition, genetic deficiency of *SLC3A2* protects against Ras-driven skin carcinogenesis [249]. Since SLC3A2 is multi-functional protein, whether the effect of its deletion is due to its role in assisting amino acid transport or to its ability to

trigger intracellular signaling pathways that lead to cell growth stimulation and tumourigenesis is obscure. Furthermore, targeting SLC3A2 could be challenging since embryonic lethality in mice was observed after its deletion [250].

3.2 Hypothesis

SLC1A5, SLC7A5 and SLC3A2 are key solute carriers in BC and their expression and prognostic value will vary among the different BC molecular subtypes.

3.3 Aims

1. To assess SLC1A5, SLC7A5 and SLC3A2 gene copy number and mRNA expression, alongside protein expression in large well-characterised annotated cohorts of BC and to investigate their association with clinicopathological parameters and relevant biological markers.
2. To determine the prognostic potential of SLC1A5, SLC7A5 and SLC3A2, at mRNA and protein levels, in the different molecular BC subtypes.

3.4 Methods

3.4.1 Study cohorts

3.4.1.1 METABRIC cohort

METABRIC cohort was used to evaluate *SLC1A5*, *SLC7A5* and *SLC3A2* copy number and gene expression. This cohort comprised 1980 primary operable invasive BC patients. Clinical history, outcome data and tumour characteristics, including tumour size, tumour grade, LN stage and histological phenotypes, were available for this cohort. The intrinsic subtypes of BC (luminal A, luminal B, HER2-enriched, normal-like, and basal-like) were classified based on prediction analysis of microarray of 50 genes (PAM50). Integrating germline variants, somatic aberrations, and aberration with gene expression data augmented BC classification in this cohort by producing 10 integrative clusters. Each cluster represents a distinct genomic landscape, molecular and clinical features and unique ER, PR and HER2 expressions. The characteristics of the METABRIC cohort are detailed in section 2.1.1.

3.4.1.2 External validation cohort

For external validation of SLC1A5, SLC7A5 and SLC3A2 mRNA expression, Breast Cancer Gene-Expression Miner v4.0 online dataset was used. This dataset offers

the possibility to evaluate the clinicopathological and prognostic significance of candidate genes in large pooled cohort of BC patients, as detailed in section 2.1.2.

3.4.1.3 Nottingham BC cohort

SLC1A5, SLC7A5 and SLC3A2 protein expression was assessed using the Nottingham BC cohort. This is a well-characterised cohort of patients with early stage primary operable invasive BC. The clinicopathological profile of the Nottingham BC cohort includes patient's age, tumour size, tumour grade, nodal stage, NPI, LVI status and tumour histological types. The hormonal receptor expression profile and outcome data (BCSS and DMFS) were also recorded. The clinicopathological characteristics of this cohort are detailed in section 2.1.3.

The expression of SLC1A5, SLC7A5 and SLC3A2, at the mRNA and protein levels, was correlated with clinicopathological parameters, molecular subtypes, relevant biological markers and patient outcome.

3.4.2 Western blotting and immunohistochemistry

Western blotting was applied to validate the specificity of SLC1A5, SLC7A5 and SLC3A2 primary antibodies using whole cell lysates from MCF7, MDA-MB-231 and SKBR3 for SLC1A5, MDA-MB-175, T47D, MCF7 and HEK293T for SLC7A5 and MDA-MB-231 for SLC3A2. IHC staining was performed on TMA sections extracted from the Nottingham BC cohort, using the Novolink polymer detection system. The details of methodology, antibodies concentrations and incubation times are described in sections 2.2, 2.4 and table 2-3.

3.5 Results

3.5.1 Validation of SLC1A5, SLC7A5 and SLC3A2 antibodies

Western blotting analysis showed specific bands at the correct predicted size (80 kDa, 40 kDa and 80 kDa) corresponding to SLC1A5, SLC7A5 and SLC3A2 proteins, respectively. (Figure 3-1A-C).

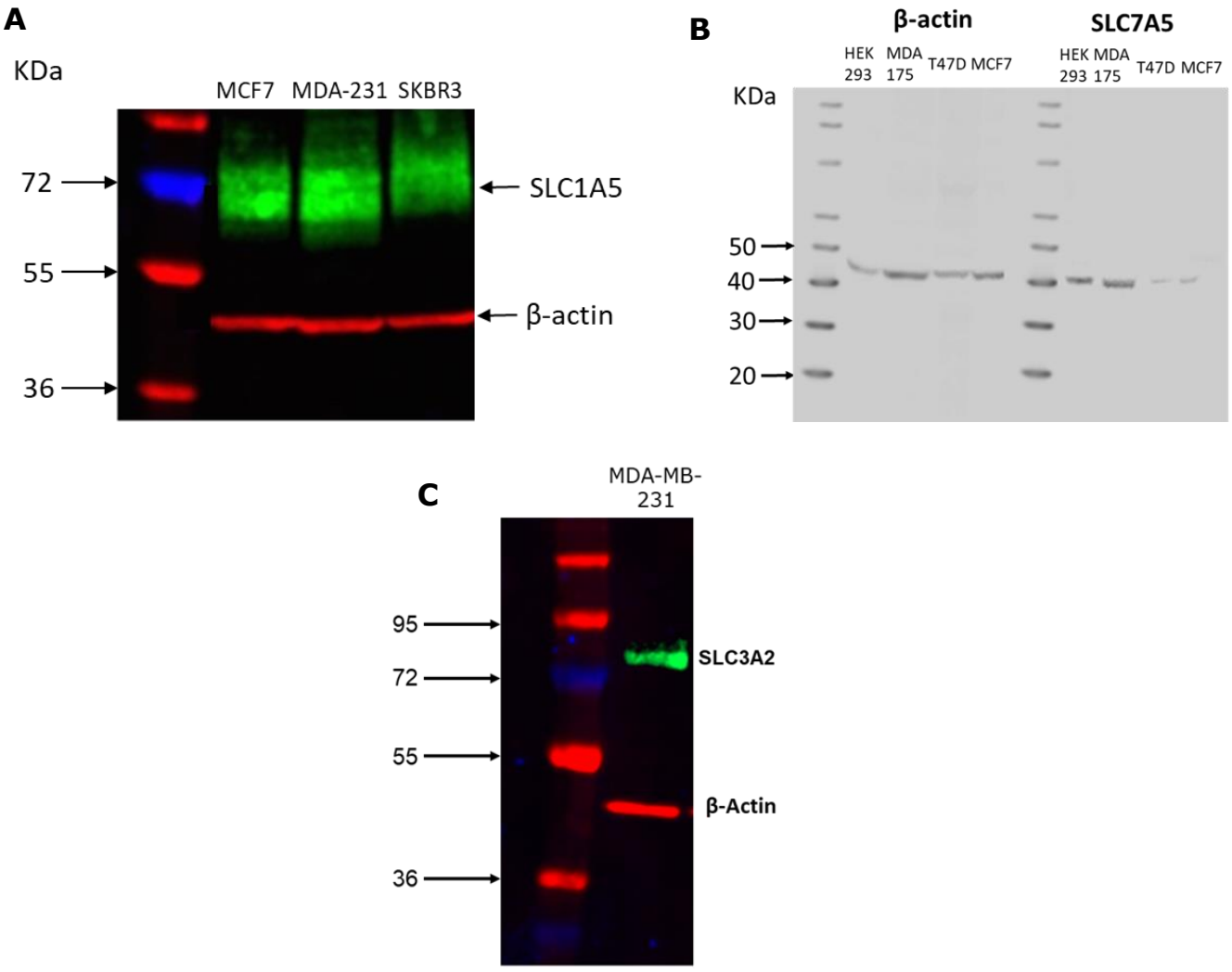


Figure 3-1. Western blot underlining antibody specificity of A) SLC1A5, B) SLC7A5 and C) SLC3A2. β-actin was included as a positive control.

3.5.2 Expression of SLC1A5, SLC7A5 and SLC3A2 in breast cancer

SLC1A5, SLC7A5 and SLC3A2 gene copy number and gene expression were evaluated using the METABRIC cohort. Data of *SLC1A5*, *SLC7A5* and *SLC3A2* mRNA expression was normally distributed and was dichotomised using cut-off points generated from X-tile software, based on prediction of BCSS. High *SLC1A5* mRNA expression (log2 intensity >7.9) was observed in 1,271/1,980 (64%) of the

METABRIC BC cases. In the same cohort, only 59/1,980 (3%) of cases showed a copy number (CN) gain of *SLC1A5* and 39/1,980 (2%) showed CN loss. Regarding *SLC7A5*, High mRNA expression (log2 intensity >8) was observed in 1,076/1,980 (54%) of the cases. A total of 49/1,980 (2.4%) cases showed a CN gain while 530/1,980 (26.7%) showed a CN loss. Frequency of *SLC3A2* mRNA was almost similar to that of *SLC7A5*, where, High *SLC3A2* expression (log2 intensity >6.6) was observed in almost half (52%) of the METABRIC cases. Amplification of *SLC3A2* was observed in 90 (4.5%) cases while 109 (5.5%) cases showed a CN loss. There was a positive association between copy number variations of *SLC1A5*, *SLC7A5* and *SLC3A2* and their corresponding mRNA expressions ($p \leq 0.001$, Figure 3-2A-C).

SLC1A5, SLC7A5 and SLC3A2 protein expressions were assessed using the Nottingham BC cohort. Protein expressions of the three solute carriers was initially evaluated in full-face BC tissue sections prior to TMA staining. This showed that the staining pattern was homogeneously distributed, thus validating the applicability of immunohistochemical detection in TMAs. SLC1A5, SLC7A5 and SLC3A2 proteins were expressed mainly in the membrane of invasive tumour cells, with intensity levels ranging from absent to high (Figure 3-3A-I). A total of 2,770, 2,664 and 2,500 cases were valid for assessing SLC1A5, SLC7A5 and SLC3A2 proteins, respectively. The distribution of protein expression data was left-skewed and did not display normal distribution and the cut-off point for SLC1A5, SLC7A5 and SLC3A2 positivity was set using X-tile software. Positive SLC1A5 protein (>40 H-score) was observed in 1,744/2,770 (63%) of BC cases while 459/2,664 (17%) of cases were positive for SLC7A5 protein (>15 H-score) and positive SLC3A2 expression (>15 H-score) was recognised in the half of the cohort (50%).

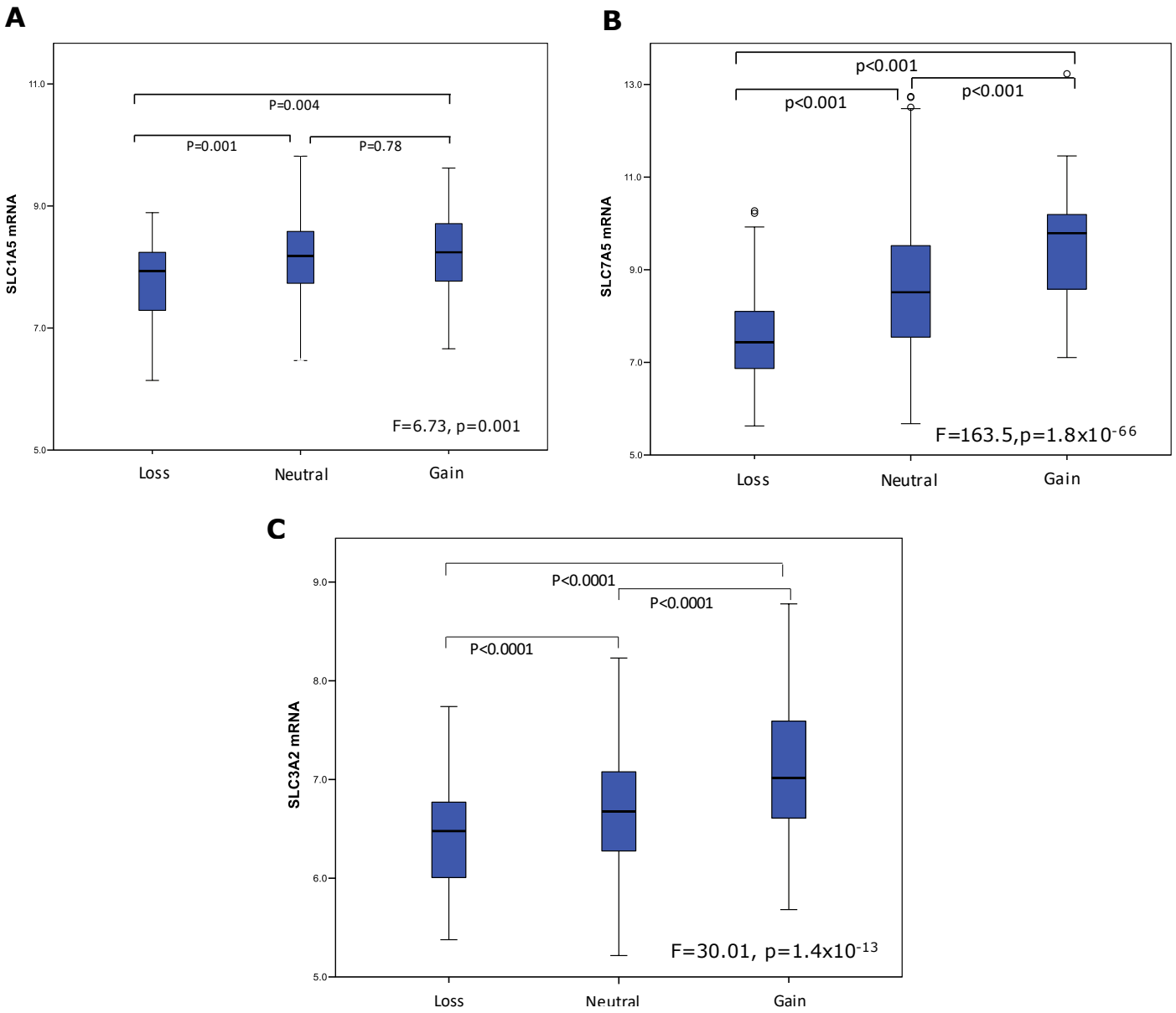


Figure 3-2. Relationship between Copy Number variations of A) SLC1A5, B) SLC7A5 and C) SLC3A2 and their corresponding mRNA expressions in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

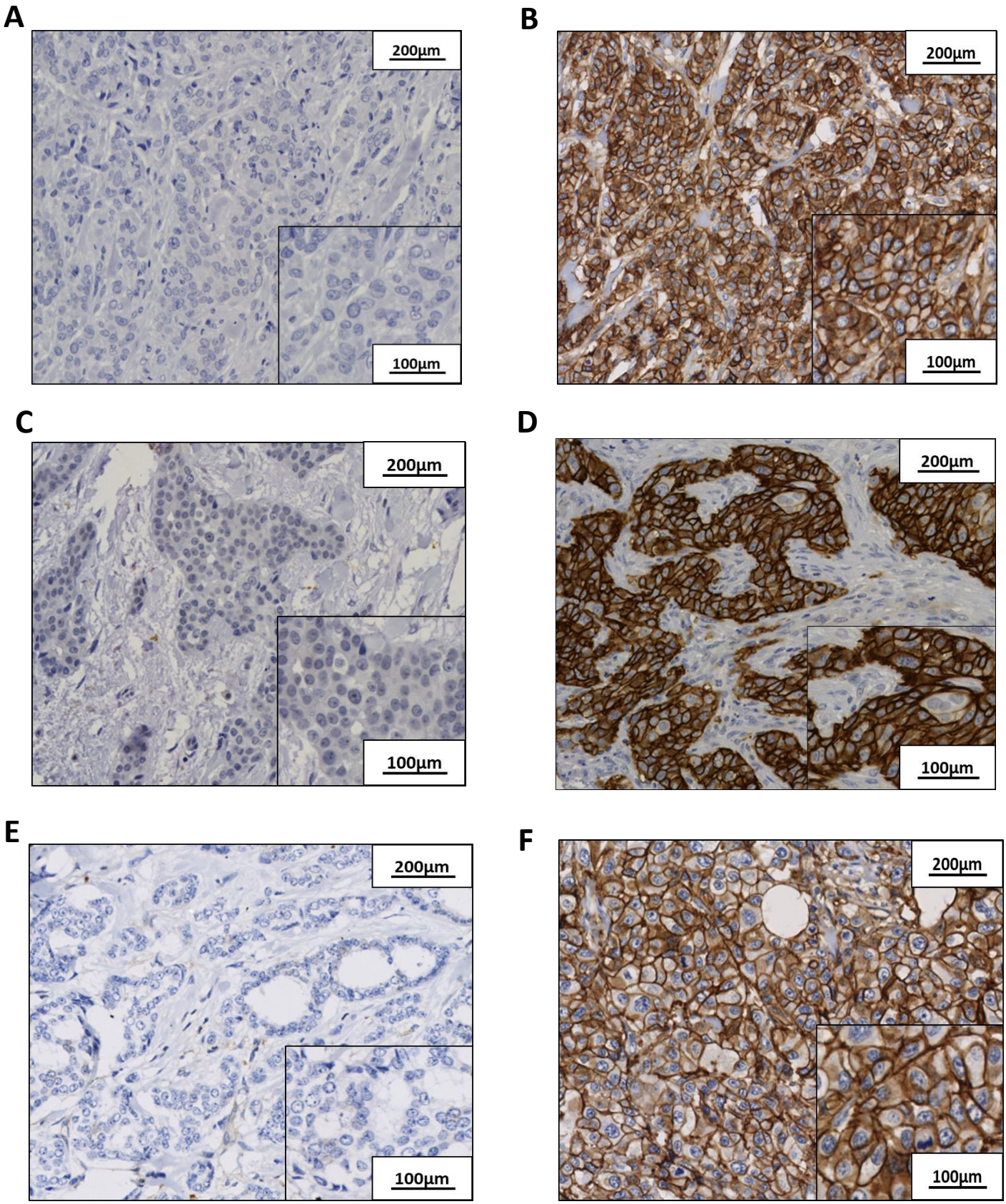


Figure 3-3. Protein expression of SLC1A5, SLC7A5 and SLC3A2 in invasive breast cancer. A) Negative SLC1A5 immunohistochemical (IHC) expression, B) Positive SLC1A5 IHC expression, C) Negative SLC7A5 IHC expression, D) Positive SLC7A5 IHC expression, E) Negative SLC3A2 IHC expression, F) Positive SLC3A2 IHC expression. Positive immunoreactivity is detected in the membrane of the invasive breast cancer cells. Magnification 10x. Scale bars=200µm. Inset, magnification 20x. Scale bars=100µm.

3.5.3 Association of SLC1A5, SLC7A5 and SLC3A2 with clinicopathological parameters

High mRNA expression of SLC1A5, SLC7A5 and SLC3A2 was significantly associated with poor prognostic parameters, including larger tumour size ($p \leq 0.02$, Figure 3-4, 3-5, 3-6 A), higher grade ($p < 0.001$, Figure 3-4, 3-5, 3-6B), positive nodal metastasis ($p<0.001$, Figure 3-4, 3-5, 3-6C) and poor prognostic group of NPI ($p<0.001$, Figure 3-4, 3-5, 3-6D). These associations were confirmed using the Breast Cancer Gene-Expression Miner, where available (Figures 3-7, 3-8, 3-9 A-C).

The associations between protein expression of the three solute carriers and clinicopathological variables are summarised in Tables 3-1, 3-2, 3-3. In the line with the mRNA, high expression of SLC1A5, SLC7A5 and SLC3A2 proteins was significantly associated with large tumour size ($p \leq 0.04$), high tumour grade, high pleomorphism, high mitotic count, less tubular formation and poor NPI (all $p<0.001$). A significant association was also observed between SLC1A5, SLC7A5 and SLC3A2 expression and histologic tumour type, where high proportions were observed within medullary-like carcinomas ($p<0.001$). There was a positive association between the expression of SLC1A5 and SLC7A5 proteins and the presence of lymphovascular invasion ($p<0.001$). Regarding associations with distant metastasis (DM), High SLC1A5 protein levels were associated with the development of DM in the bone and the liver (both $p \leq 0.001$), while SLC7A5 and SLC3A2 expressions were associated with developing secondary cancer to the brain (both $p<0.001$). No significant associations were observed with developing metastases in bone, lung or liver in patients who had high expression of SLC7A5 or SLC3A2 proteins ($p>0.05$).

Table 3-1. Clinicopathological associations of SLC1A5 expression in the Nottingham breast cancer cohort

	SLC1A5 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Age				
< 50	323 (35.3)	591 (64.7)	2.54 (0.11)	0.44
≥ 50	713 (38.5)	1141 (61.5)		
Tumour size				
< 2.0cm	670 (43.7)	864 (56.3)	57.82 ($2.8x10^{-14}$)	**<0.0001**
≥ 2.0cm	365 (29.6)	868 (70.4)		
Tumour Grade				
1	304 (68.0)	143 (32.0)	353.77 ($1.5x10^{-77}$)	**<0.0001**
2	459 (44.8)	565 (55.2)		
3	270 (20.9)	1023 (79.1)		
Mitosis				
1	609 (53.8)	524 (46.2)	280.62 ($1.1x10^{-61}$)	**<0.0001**
2	190 (36.2)	355 (63.8)		
3	200 (19.1)	847 (80.9)		
Pleomorphism				
1	33 (71.7)	13 (28.3)	252.04 ($1.8x10^{-55}$)	**<0.0001**
2	507 (56.0)	399 (44.0)		
3	459 (26.2)	1295 (73.8)		
Tubular formation				
1	124 (70.1)	53 (29.9)	123.69 ($1.3x10^{-27}$)	**<0.0001**
2	369 (42.4)	501 (57.6)		
3	507 (30.5)	1153 (69.5)		
Vascular Invasion				
Negative	780 (41.3)	1110 (58.7)	40.77 ($1.7x10^{-10}$)	**<0.0001**
Positive	248 (28.6)	619 (71.4)		
Lymph Node Stage				
1	696 (40.8)	1010 (59.2)	28.73 ($5.7x10^{-7}$)	**<0.0001**
2	275 (34.1)	531 (65.9)		
3	62 (24.9)	187 (75.1)		

(Continued)

	SLC1A5 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Nottingham Prognostic Index				
Good	505 (56.4)	390 (43.6)	219.55 ($2.1x10^{-48}$)	**<0.0001**
Moderate	438 (30.8)	985 (69.2)		
Poor	91 (20.4)	354 (79.6)		
IHC Subtypes				
ER+/HER2- Low Proliferation	704 (49.8)	710 (50.2)	231.2 ($7.5x10^{-50}$)	**<0.0001**
ER+/HER2- High Proliferation	99 (22.3)	344 (77.7)		
Triple Negative	77 (18.0)	351 (82.0)		
HER2+	61 (22.5)	210 (77.5)		
Histological type				
Ductal (including mixed)	836 (35.2)	1536 (64.8)	87.18 ($2.6x10^{-17}$)	**<0.0001**
Lobular	103 (47.7)	113 (52.3)		
Medullary	3 (7.5)	37 (92.5)		
Miscellaneous	9 (50.0)	9 (50.0)		
Special type	83 (70.9)	34 (29.1)		
Tubular	1 (33.3)	2 (66.7)		
Site of distant metastasis				
Brain				
No	973 (55.9)	767 (44.1)	1.73 (0.18)	0.54
Yes	45 (48.9)	47 (51.1)		
Lung				
No	929 (55.8)	737 (44.2)	0.282 (0.59)	1.18
Yes	89 (53.6)	77 (46.4)		
Bone				
No	848 (57.8)	620 (42.2)	14.45 (0.0001)	**0.0006**
Yes	170 (46.7)	194 (53.3)		
Liver				
No	903 (57.1)	679 (42.9)	10.73 (0.001)	**0.005**
Yes	115 (46.0)	135 (54.0)		

P values in bold means statistically significant

Table 3-2. Clinicopathological associations of SLC7A5 expression in Nottingham breast cancer cohort

	SLC7A5 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Age				
< 50	661 (76.5)	203 (23.5)	34.45 ($4.3x10^{-9}$)	**<0.0001**
≥ 50	1529 (85.7)	255 (14.3)		
Tumour size				
< 2.0cm	1280 (87.0)	191 (13.0)	43.14 ($5.07x10^{-11}$)	**<0.0001**
≥ 2.0cm	909 (77.3)	267 (22.7)		
Tumour Grade				
1	416 (98.8)	5 (1.2)	394.96 ($1.7x10^{-86}$)	**<0.0001**
2	942 (95.3)	46 (4.7)		
3	830 (67.2)	405 (32.8)		
Mitosis				
1	1075 (96.9)	34 (3.1)	401.08 ($8.0x10^{-88}$)	**<0.0001**
2	433 (87.3)	63 (12.7)		
3	634 (64.1)	355 (35.9)		
Pleomorphism				
1	44 (100.0)	0 (0.0)	224.86 ($1.4x10^{-49}$)	**<0.0001**
2	838 (97.8)	19 (2.2)		
3	1262 (74.5)	433 (25.5)		
Tubular formation				
1	164 (98.2)	3 (1.8)	100.84 ($1.2x10^{-22}$)	**<0.0001**
2	741 (90.6)	77 (9.4)		
3	1239 (76.9)	372 (23.1)		
Vascular Invasion				
Negative	1530 (84.6)	278 (15.4)	14.88 (0.0001)	**0.0007**
Positive	654 (78.5)	179 (21.5)		
Lymph Node Stage				
1	1376 (84.5)	253 (15.5)	12.74 (0.002)	**0.01**
2	625 (81.3)	144 (18.7)		
3	185 (75.8)	59 (24.2)		

(Continued)

	SLC7A5 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Nottingham Prognostic Index				
Good	829 (97.2)	24 (2.8)	206.69 ($1.3x10^{-45}$)	**<0.0001**
Moderate	1063 (78.1)	298 (21.9)		
Poor	294 (68.4)	136 (31.6)		
IHC Subtypes				
ER+/HER2- Low Proliferation	981 (97.8)	22 (2.2)	464.38 ($3.3x10^{-99}$)	**<0.0001**
ER+/HER2- High Proliferation	663 (84.1)	125 (15.9)		
Triple Negative	212 (51.7)	198 (48.3)		
HER2+	183 (70.7)	76 (29.3)		
Histological type				
Ductal (including mixed)	1836 (81.3)	421 (18.7)	101.71 ($2.2x10^{-20}$)	**<0.0001**
Lobular	220 (99.5)	1 (0.5)		
Medullary	16 (41.0)	23 (59.0)		
Miscellaneous	12 (75.0)	4 (25.0)		
Special type	101 (91.8)	9 (8.2)		
Site of distant metastasis				
Brain				
No	875 (83.8)	169 (16.2)	13.46 (0.0002)	**0.001**
Yes	40 (65.6)	21 (34.4)		
Lung				
No	839 (83.6)	164 (16.4)	5.43 (0.02)	0.08
Yes	76 (74.5)	26 (25.5)		
Bone				
No	752 (82.8)	151 (17.2)	0.005 (0.94)	1.88
Yes	190 (83.0)	39 (17.0)		
Liver				
No	790 (83.2)	159 (16.8)	0.914 (0.33)	0.99
Yes	125 (80.1)	31 (19.9)		

P values in bold means statistically significant

Table 3-3. Clinicopathological associations of SLC3A2 expression in Nottingham breast cancer cohort

	SLC3A2 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Age				
< 50	389 (48.3)	416 (51.7)	2.83 (0.09)	0.63
≥ 50	873 (51.9)	808 (48.1)		
Tumour size				
≥ 2.0 cm	526 (47.7)	576 (52.3)	7.49 (0.006)	**0.04**
<2.0 cm	736 (53.3)	646 (46.7)		
Tumour grade				
1	252 (62.7)	150 (37.3)	76.34 ($2.6x10^{-17}$)	**<0.0001**
2	543 (56.9)	412 (43.1)		
3	466 (41.4)	659 (58.6)		
Mitosis				
1	340 (84.6)	62 (15.4)	100.51 ($1.4x10^{-22}$)	**<0.0001**
2	759 (79.5)	196 (20.5)		
3	713 (63.4)	412 (36.6)		
Pleomorphism				
1	26 (59.1)	18 (40.9)	26.34 (0.000002)	**<0.0001**
2	467 (57.6)	344 (42.4)		
3	740 (46.8)	842 (53.2)		
Tubular formation				
1	106 (64.6)	58 (35.4)	22.86 (0.00001)	**0.0001**
2	406 (54.1)	345 (45.9)		
3	721 (47.4)	801 (52.6)		
Vascular Invasion				
Negative	882 (51.6)	826 (48.4)	1.59 (0.21)	1.05
Positive	377 (48.9)	394 (51.1)		
Lymph Node Stage				
1	794 (51.2)	756 (48.8)	0.292 (0.86)	2.25
2	350 (50.1)	349 (49.9)		
3	115 (50.2)	114 (49.8)		

(Continued)

	SLC3A2 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Nottingham Prognostic Index				
Good	481 (58.9)	337 (41.1)	36.37 ($8.0x10^{-7}$)	**<0.0001**
Moderate	611 (48.2)	657 (51.8)		
Poor	167 (42.5)	226 (57.5)		
IHC Subtypes				
ER+/HER2- Low Proliferation	748 (58.0)	542 (42.0)	66.58 ($2.3x10^{-14}$)	**<0.0001**
ER+/HER2- High Proliferation	156 (42.9)	208 (57.1)		
Triple Negative	153 (39.5)	234 (60.5)		
HER2+	94 (38.4)	151 (61.6)		
Histological type				
Ductal (including mixed)	1039 (49.5)	1060 (50.5)	29.73 (0.0002)	**0.001**
Lobular	141 (62.7)	84 (37.3)		
Medullary	9 (25.7)	26 (74.3)		
Miscellaneous	9 (50.0)	9 (50.0)		
Special type	59 (57.3)	44 (42.7)		
Site of distant metastasis				
Brain				
No	648 (72.6)	245 (27.4)	17.08 (0.00003)	**0.0003**
Yes	25 (46.3)	29 (53.7)		
Lung				
No	618 (71.7)	244 (28.3)	1.83 (0.17)	1.02
Yes	55 (64.7)	30 (35.3)		
Bone				
No	529 (70.9)	217 (29.1)	0.041 (0.83)	3.32
Yes	144 (71.6)	57 (28.4)		
Liver				
No	406 (50.3)	401 (49.7)	0.005 (0.94)	1.88
Yes	70 (50.0)	70 (50.0)		

P values in bold means statistically significant

3.5.4 SLC1A5, SLC7A5 and SLC3A2 expression in molecular BC subtypes

High expression of *SLC1A5*, *SLC7A5* and *SLC3A2* mRNA was significantly associated with negative expression of hormone receptors (ER- and PgR-). While the expression of the former two genes was also associated with HER2 positive tumours (all $p<0.001$, Table 3-4, 3-5), *SLC3A2* did not show a significant association ($p>0.05$, Table 3-6). However, protein expression of all solute carriers was associated with ER negative, PgR negative and HER2+ tumours ($p<0.001$, Table 3-4, 3-5, 3-6). In addition, they were highly expressed in malignancies that were TN compared to non-TN tumours ($p<0.001$, Table 3-4, 3-5, 3-6). Investigation of *SLC1A5* mRNA in the intrinsic (PAM50) subtypes [87] showed that high expression was observed in luminal B, HER2+ followed by basal-like tumours, while luminal A subtype displayed the lowest levels of *SLC1A5* ($p<0.001$, Figure 3-4F). Likewise, within the METABRIC Integrative Clusters, high *SLC1A5* mRNA expression was associated with clusters 1 and 5 which embrace Luminal B and ERBB2-amplified subgroups, respectively ($p<0.001$, Figure 3-4E). In the SCMGENE subtypes there was a higher expression of *SLC1A5* mRNA in the ER+/HER2- high proliferation class (Luminal B) compared with the ER+/HER2- low proliferation class (Luminal A) ($p<0.001$, Figure 3-4G). Expression levels were also high in the HER2+ and TN tumours compared with Luminal A (both $p<0.001$, Figure 3-4G) but were similar to that in the luminal B class.

When comparing the levels of *SLC7A5* mRNA expression in the intrinsic (PAM50) subtypes, high expression was observed in basal-like and HER2+ tumours. It is also noticeable that, within the luminal BC subtypes, luminal B showed higher expression of *SLC7A5* compared with luminal A tumours ($p<0.001$, Figure 3-5F). Similarly, within the METABRIC Integrative Clusters, high *SLC7A5* mRNA expression was associated with clusters 5 (ERBB2 amplified), 9 (Luminal B subgroup) and 10 (TN/basal-like) ($p<0.001$, Figure 3-5E). Furthermore, the results of the association with the SCMGENE subtypes were comparable to the above findings, where ER-/HER2- subtype showed the highest levels of *SLC7A5* mRNA expression, followed by HER2+ tumours. The expression levels within the ER+/HER2- group, was higher in the high proliferation class compared to the ER+/HER2- low proliferation class ($p<0.001$, Figure 3-5G).

When *SLC3A2* mRNA was assessed, high levels were observed in basal-like, luminal B and HER2+ tumours ($p < 0.001$, Figure 3-6F). In the METABRIC Integrative Clusters, high *SLC3A2* mRNA expression was associated with clusters 1 (luminal B subgroup), 5 (ERBB2-amplified) and 10 (TN/basal-like) ($p < 0.001$, Figure 3-6E). The association of *SLC3A2* mRNA with SCMGENE subtypes were comparable to the findings of *SLC7A5* ($p<0.001$, Figure 3-6G).

Associations of *SLC1A5*, *SLC7A5* and *SLC3A2* mRNA with ER- and PgR- tumours along with PAM 50 subtypes were confirmed using the Breast Cancer Gene-Expression Miner (Figure 3-7, 3-8, 3-9D-H).

Gene CN analysis showed that luminal B tumours had the highest proportion of *SLC1A5* CN loss ($p=0.02$, Table 3-7), whereas no significant association was observed between the *SLC1A5* gain and the BC intrinsic subtypes ($p>0.05$, Table 3-7). While basal-like tumours had a greater CN gain of *SLC7A5* ($p<0.001$, Table 3-8), CN loss was mainly observed in the Luminal A subtype ($p<0.001$, Table 3-8). In addition, *SLC3A2* CN gain was primarily observed in luminal B subtype and to lesser extent in HER2+ and TNBC ($p < 0.001$, Table 3-9).

SLC1A5, SLC7A5 and SLC3A2 protein expressions in the IHC defined molecular subtypes, in the Nottingham BC cohort, showed a significantly lower expression in the luminal A tumours compared to the other subtypes ($p<0.001$, Table 3-1, 3-2, 3-3).

Table 3-4. Expression of SLC1A5 in breast cancer and the expression of other molecular biomarkers, at mRNA and protein levels

	SLC1A5							
	mRNA				Protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low n (%)**	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ER								
Negative	123 (26.1)	348 (73.9)	40.2 ($2.3x10^{-10}$)	**<0.0001**	107 (18.0)	488 (82.0)	120.0 ($6.2x10^{-28}$)	**<0.0001**
Positive	639 (42.4)	868 (57.6)			918 (42.5)	1242 (57.5)		
PgR								
Negative	298 (31.8)	640 (68.2)	34.4 ($4.6x10^{-9}$)	**<0.0001**	306 (28.0)	785 (72.0)	61.37 ($4.7x10^{-15}$)	**<0.0001**
Positive	464 (44.6)	576 (55.4)			691 (42.9)	921 (57.1)		
HER2								
Negative	710 (41.0)	1023 (59.0)	35.4 ($2.8x10^{-9}$)	**<0.0001**	929 (39.5)	1424 (60.5)	45.3 ($1.6x10^{-11}$)	**<0.0001**
Positive	52 (21.2)	193 (78.8)			73 (20.9)	277 (79.1)		
Triple Negative								
No	661 (39.8)	999 (60.2)	7.3 (0.007)	**0.008**	942 (40.8)	1367 (59.2)	83.1 ($7.7x10^{-20}$)	**<0.0001**
Yes	101 (31.8)	217 (68.2)			74 (17.5)	349 (82.5)		
TP53 mutation								
Wild-type	316 (44.1)	401 (55.9)	22.3 (0.000002)	**<0.0001**	N/A			
Mutation	19 (19.2)	80 (80.8)						
P53 protein								
Negative	N/A				344 (40.6)	503 (59.4)	32.7 ($1.1x10^{-8}$)	**<0.0001**
Positive					86 (23.5)	280 (76.5)		

N/A, not available; P values in bold means statistically significant

Table 3-5. Expression of SLC7A5 in breast cancer and the expression of other molecular biomarkers, at mRNA and protein levels

	SLC7A5							
	mRNA				**Protein**			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low n (%)**	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ER								
Negative	45 (9.6)	423 (90.4)	347.21		297 (52.1)	273 (47.9)	472.16	
Positive	859 (59.0)	596 (41.0)	(**$1.7x10^{-77}$**)	**<0.0001**	1882 (91.0)	185 (9.0)	**($1.08x10^{-104}$)**	**<0.0001**
PgR								
Negative	261 (28.6)	653 (71.4)	238.14		1303 (77.7)	374 (22.3)	79.07	
Positive	643 (63.7)	366 (36.3)	(**$9.9x10^{-54}$**)	**<0.0001**	784 (91.9)	69 (8.1)	**($5.9x10^{-19}$)**	**<0.0001**
HER2								
Negative	868 (51.6)	813 (48.4)	114.75		1904 (84.3)	354 (15.7)	30.63	
Positive	36 (14.9)	206 (85.1)	(**$8.9x10^{-27}$**)	**<0.0001**	183 (70.7)	76 (29.3)	**($3.1x10^{-8}$)**	**<0.0001**
Triple Negative								
No	456 (55.4)	367 (44.6)	101.67		1942 (89.0)	240 (11.0)	343.9	
Yes	16 (10.7)	134 (89.3)	(**$6.5x10^{-24}$**)	**<0.0001**	112 (51.7)	198 (48.3)	**($2.0x10^{-75}$)**	**<0.0001**
TP53 mutation								
Wild-type	374 (53.4)	327 (46.6)	42.41					
Mutation	19 (19.4)	79 (80.6)	(**$6.1x10^{-10}$**)	**<0.0001**		N/A		
P53 protein								
Negative					344 (40.6)	503 (59.4)	32.7	
Positive			N/A		86 (23.5)	280 (76.5)	(**$1.1x10^{-8}$**)	**<0.0001**

N/A, not available; P values in bold means statistically significant

Table 3-6. Expression of SLC3A2 in breast cancer and the expression of other molecular biomarkers, at mRNA and protein levels

	SLC3A2							
	mRNA				Protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low n (%)**	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ER								
Negative	182 (40.1)	272 (59.9)	6.13 (0.0003)	**0.0009**	208 (38.1)	338 (61.9)	44.97 ($1.9x10^{-11}$)	**<0.0001**
Positive	715 (50.9)	689 (49.1)			1051 (54.3)	883 (45.7)		
PgR								
Negative	390 (43.6)	504 (56.4)	14.94 (0.001)	**0.002**	708 (46.4)	818 (53.6)	28.5 ($9.3x10^{-8}$)	**<0.0001**
Positive	507 (52.6)	457 (47.4)			466 (58.0)	337 (42.0)		
HER2								
Negative	799 (49.2)	826 (50.8)	4.12 (0.04)	0.08	1090 (51.9)	1009 (48.1)	16.14 (0.00005)	**<0.0001**
Positive	98 (42.1)	135 (59.9)			94 (38.4)	151 (61.6)		
Triple Negative								
No	786 (50.7)	765 (49.3)	21.64 (0.000003)	**<0.0001**	1082 (53.0)	961 (47.0)	23.61 (0.000001)	**0.0001**
Yes	111 (36.2)	196 (63.8)			156 (39.6)	238 (60.4)		
TP53 mutation								
Wild-type	280 (42.1)	385 (57.9)	37.41 ($3.7x10^{-8}$)	**<0.0001**	N/A			
Mutation	27 (28.7)	67 (71.3)						
P53 protein								
Negative	N/A				499 (76.7)	152 (23.3)	40.29 ($2.1x10^{-10}$)	**<0.0001**
Positive					146 (55.5)	269 (29.4)		

N/A, not available; P values in bold means statistically significant

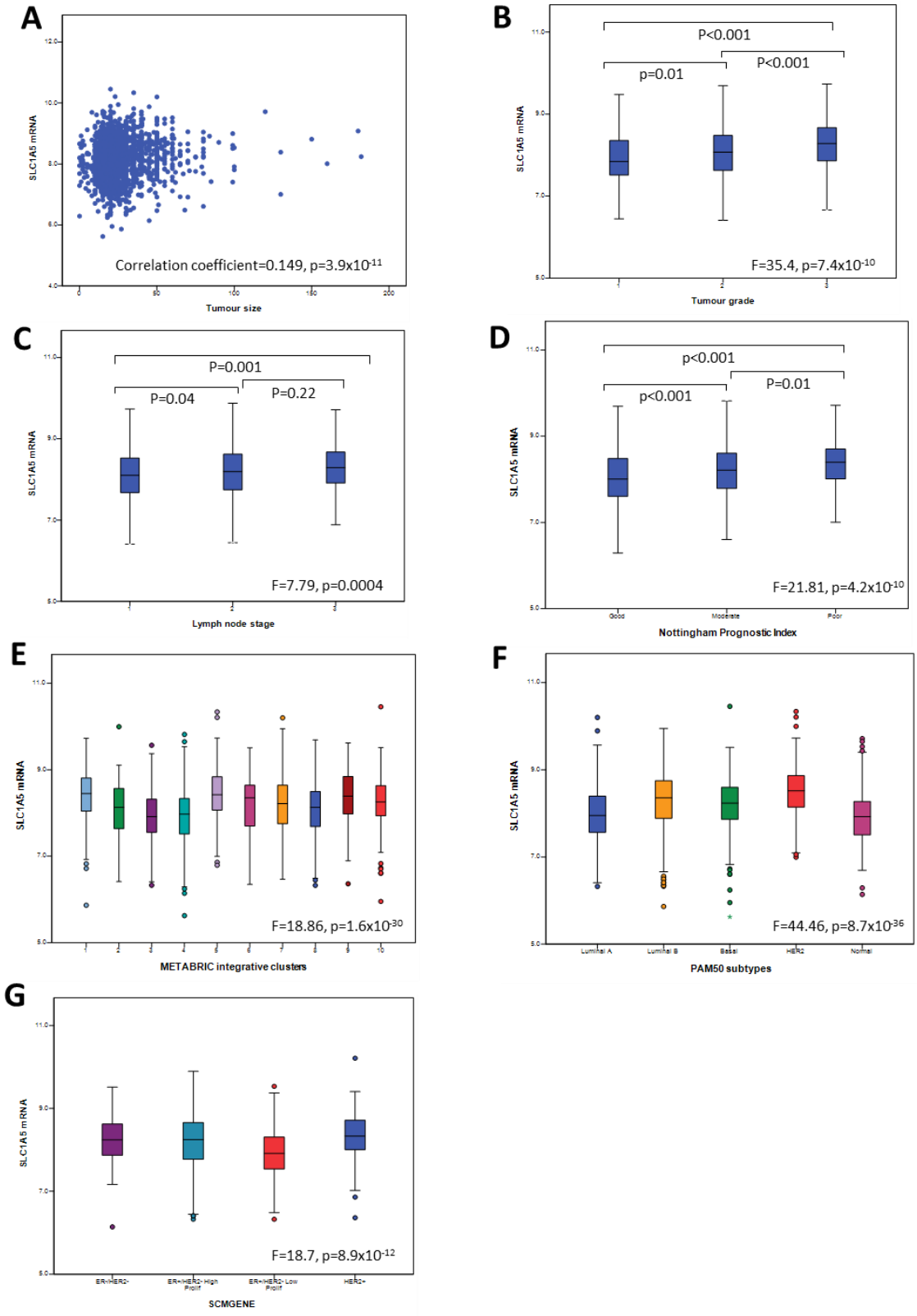


Figure 3-4. *SLC1A5* mRNA expression and its association with clinicopathological parameters and molecular subtypes: : A) *SLC1A5* and tumour size, B) *SLC1A5* and tumour grade, C) *SLC1A5* and lymph node stage, D) *SLC1A5* and NPI, E) *SLC1A5* and METABRIC Integrative Clusters, F) *SLC1A5* and PAM50 subtypes, G) *SLC1A5* and SMCGENE subtypes in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

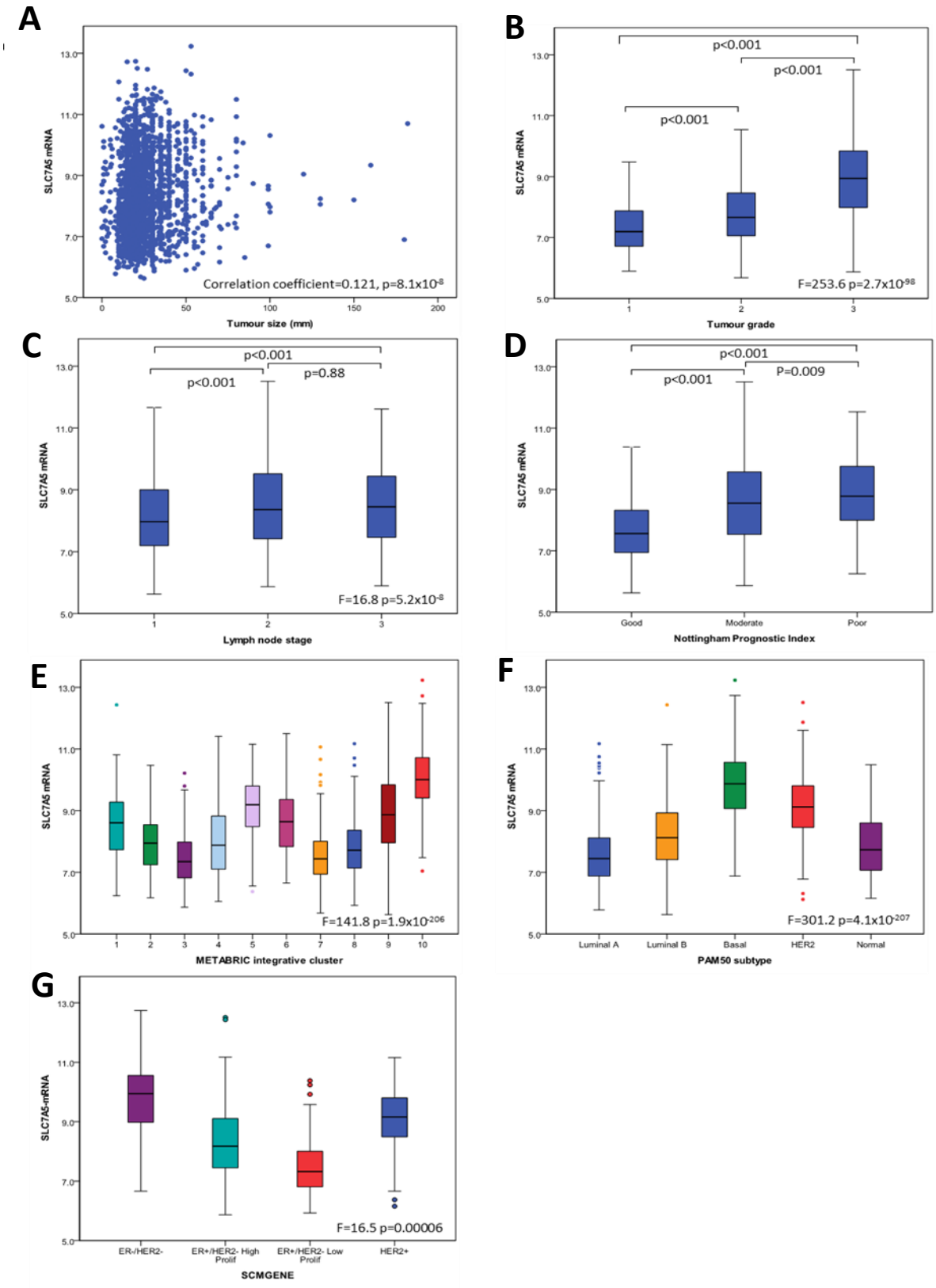


Figure 3-5. *SLC7A5* mRNA expression and its association with clinicopathological parameters and molecular subtypes: A) *SLC7A5* and tumour size, B) *SLC7A5* and tumour grade, C) *SLC7A5* and lymph node stage, D) *SLC7A5* and NPI, E) *SLC7A5* and METABRIC Integrative Clusters, F) *SLC7A5* and PAM50 subtypes, G) *SLC7A5* and SMCGENE subtypes in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

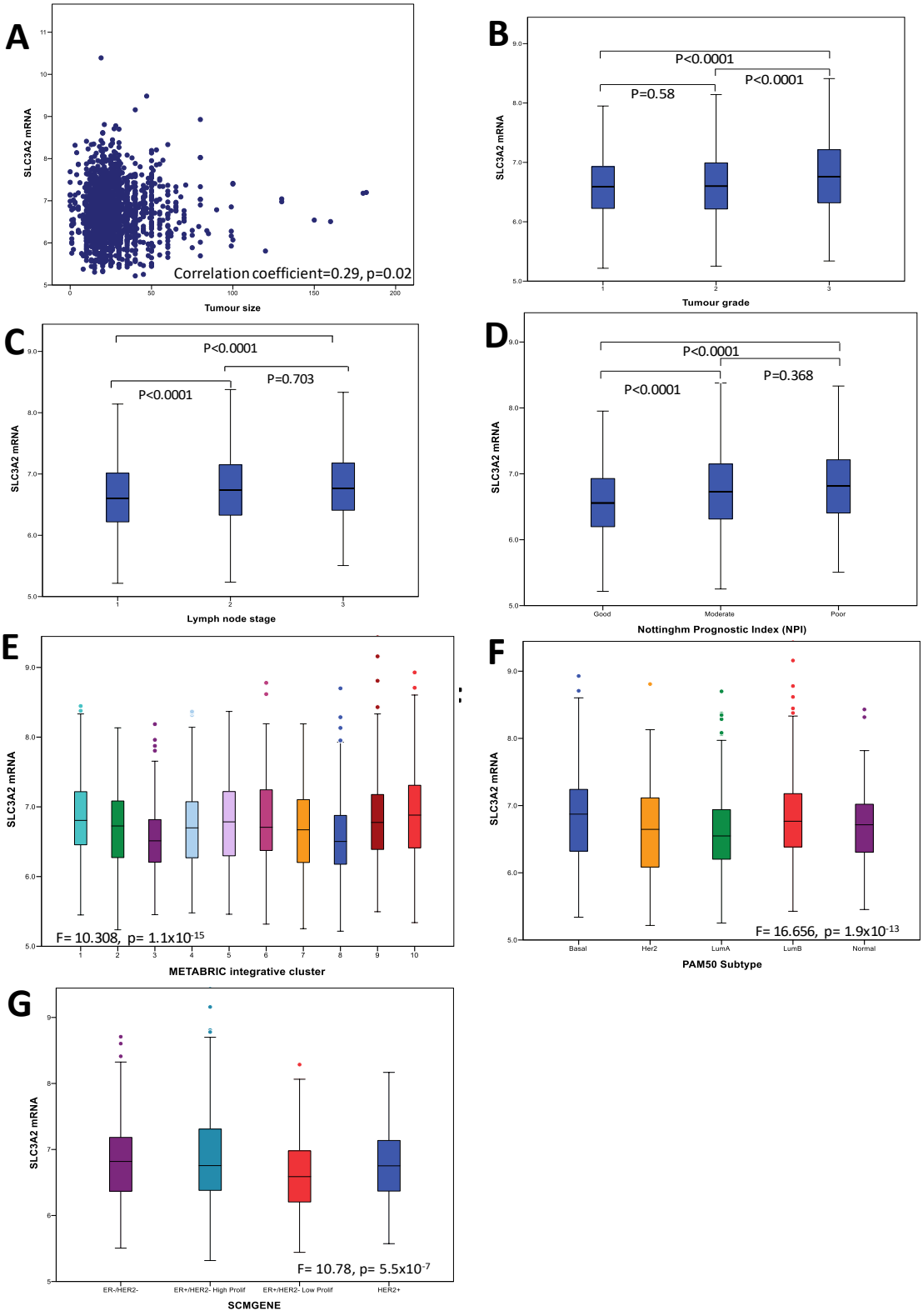


Figure 3-6. *SLC3A2* mRNA expression and its association with clinicopathological parameters and molecular subtypes: A) *SLC3A2* and tumour size, B) *SLC3A2* and tumour grade, C) *SLC3A2* and lymph node stage, D) *SLC3A2* and NPI, E) *SLC3A2* and METABRIC Integrative Clusters, F) *SLC3A2* and PAM50 subtypes, G) *SLC3A2* and SMCGENE subtypes in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

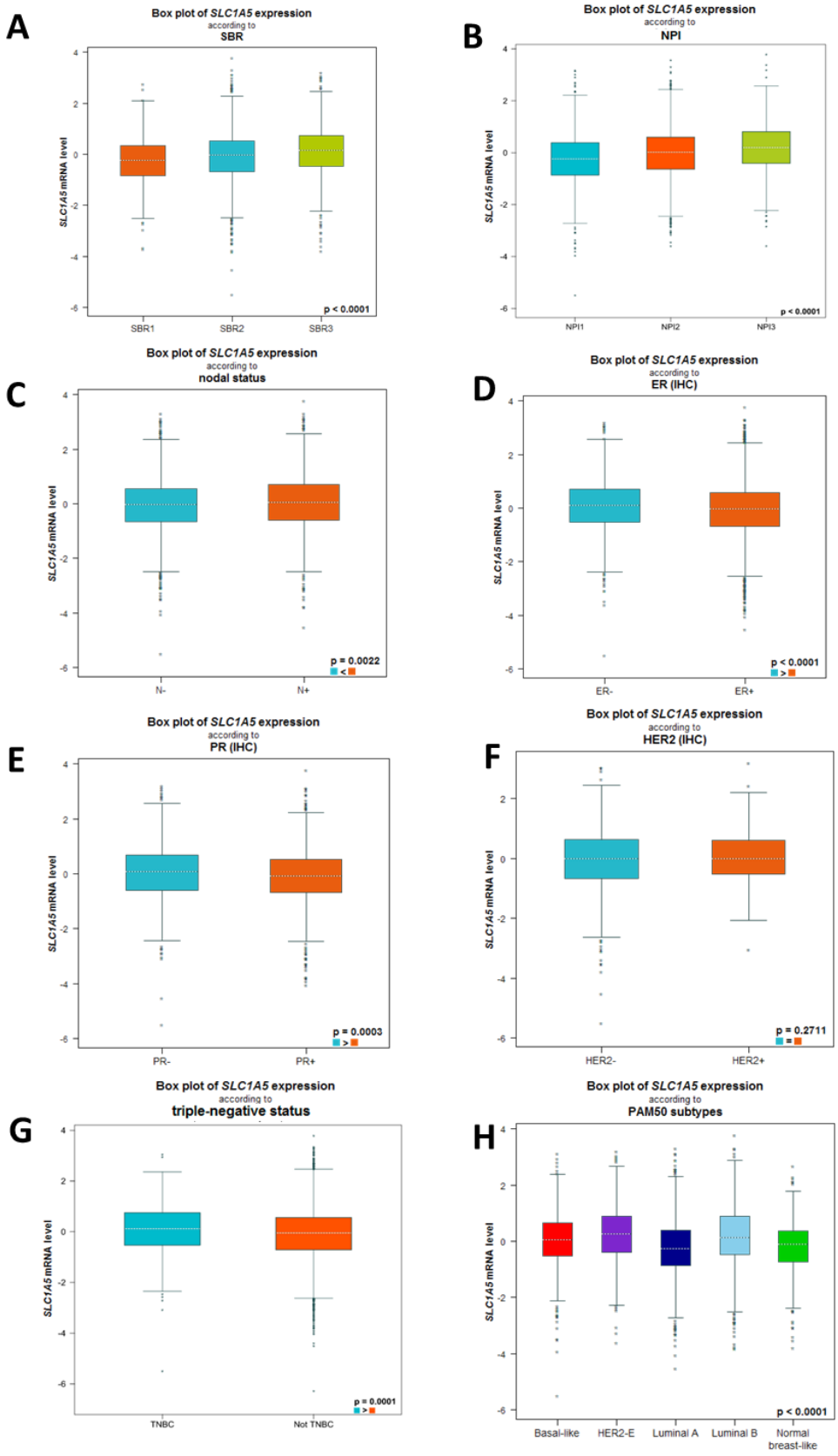


Figure 3-7. *SLC1A5* gene expression and its association, with: A) tumour grade, B) NPI, C) ER status, D) PR status, E) HER2 status, F) Triple Negative status, G) PAM50 subtypes, using Breast Cancer Gene-Expression Miner dataset.

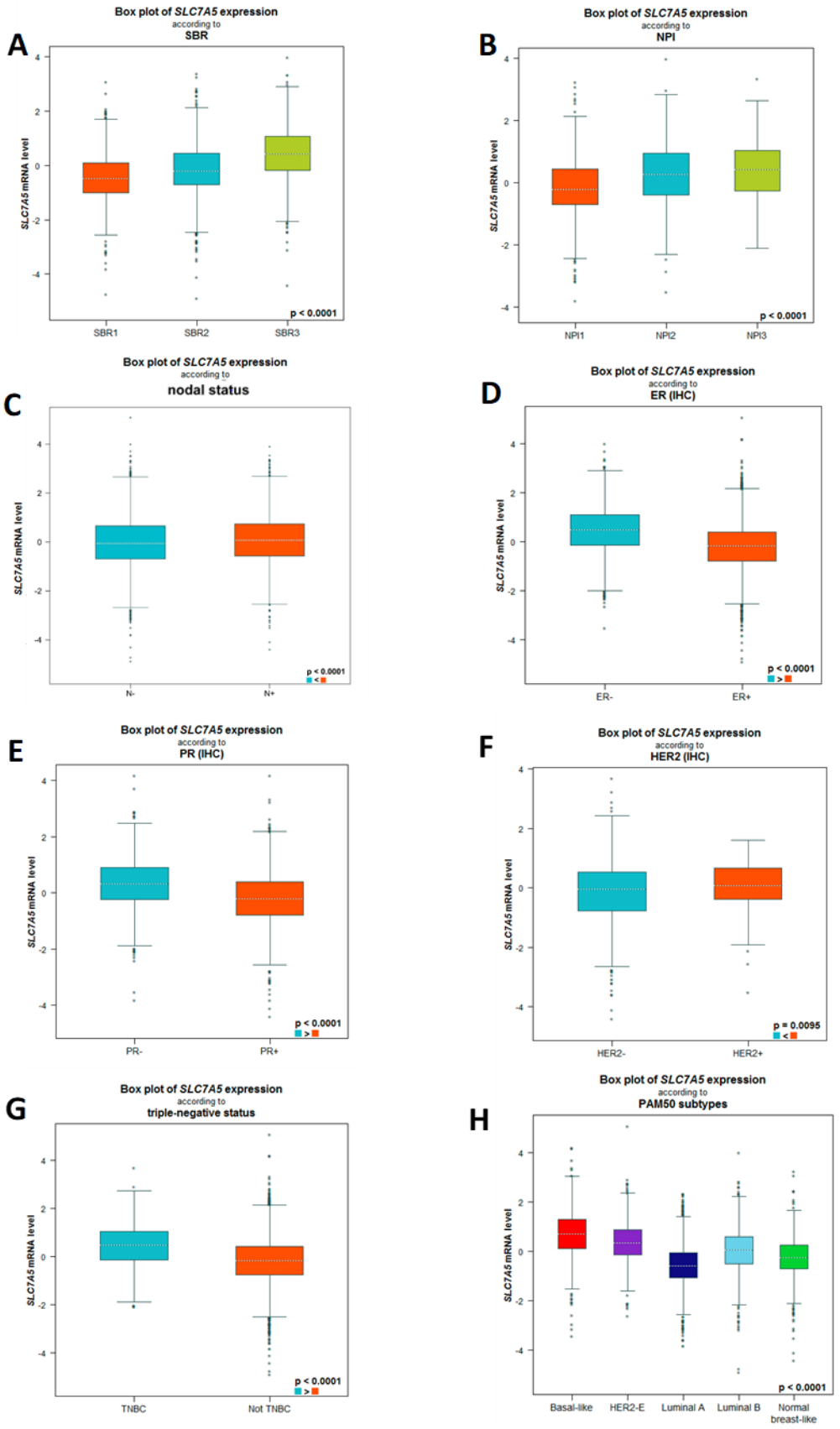


Figure 3-8. *SLC7A5* gene expression and its association, with: A) tumour grade, B) NPI, C) ER status, D) PR status, E) HER2 status, F) Triple Negative status, G) PAM50 subtypes, using Breast Cancer Gene-Expression Miner dataset.

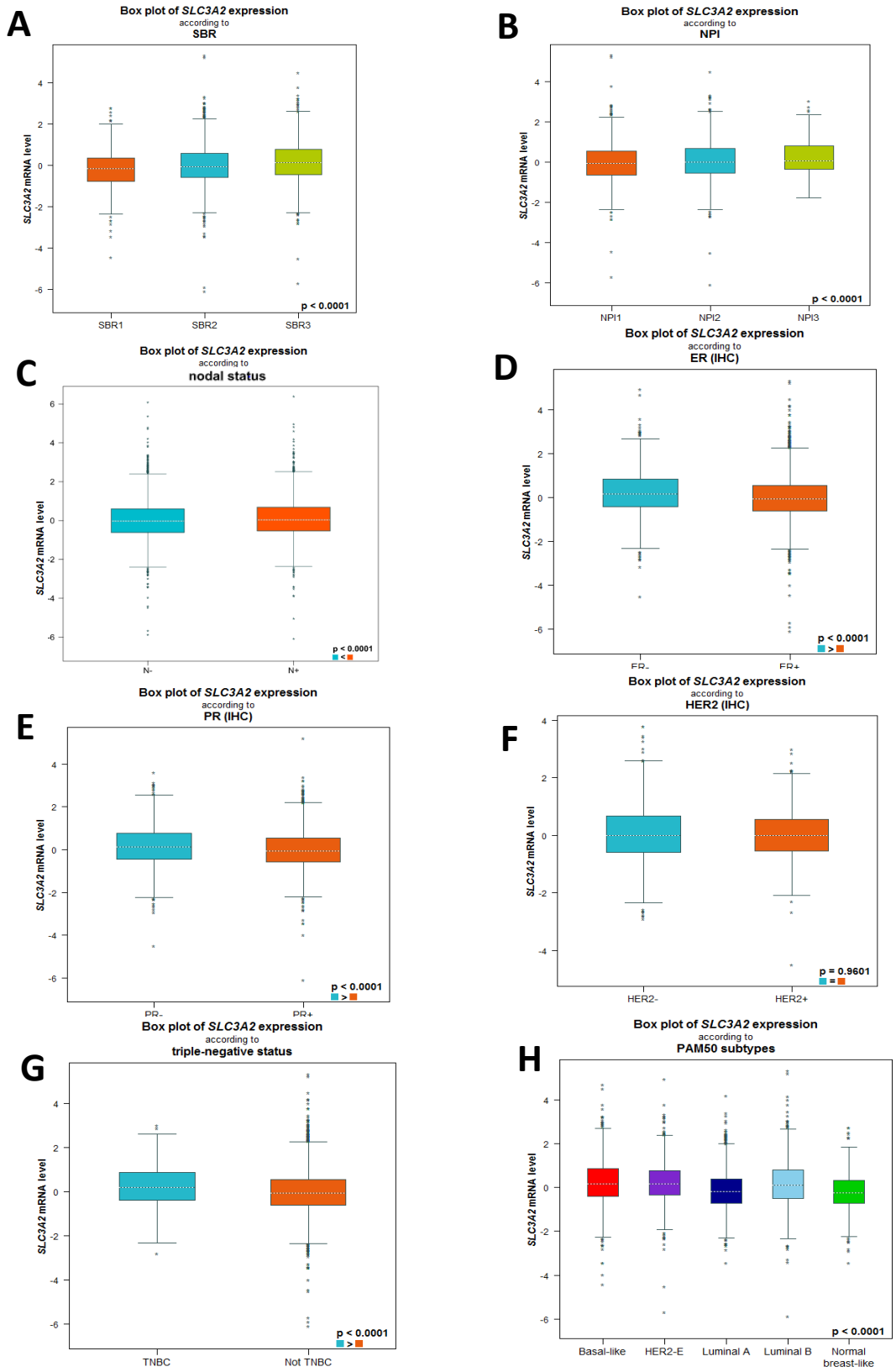


Figure 3-9. *SLC3A2* gene expression and its association, with: A) tumour grade, B) NPI, C) ER status, D) PR status, E) HER2 status, F) Triple Negative status, G) PAM50 subtypes, using Breast Cancer Gene-Expression Miner dataset.

Table 3-7. The association of *SLC1A5* copy number aberrations and PAM 50 subtypes in the METABRIC cohort.

	SLC1A5					
	Gain			**Loss**		
	No n (%)	**Yes n (%)**	**χ2 (p-value)**	**No n (%)**	**Yes n (%)**	**χ2 (p-value)**
PAM50 subtype						
Luminal A	702 (97.8)	16 (2.2)		711 (99.0)	7 (1.0)	
Luminal B	469 (96.1)	19 (3.9)	6.9	471 (96.5)	17 (3.5)	11.3
Basal	314 (95.4)	15 (4.6)	(0.139)	320 (97.3)	9 (2.7)	**(0.02)**
HER2	235 (97.9)	5 (2.1)		237 (98.8)	3 (1.3)	
Normal-like	195 (98.0)	4 (2.0)		196 (98.5)	3 (1.5)	

P value in bold means statistically significant

Table 3-8. The association of *SLC7A5* copy number aberrations and PAM 50 subtypes in the METABRIC cohort.

	SLC7A5					
	Gain			**Loss**		
	No n (%)	**Yes n (%)**	**χ2 (p-value)**	**No n (%)**	**Yes n (%)**	**χ2 (p-value)**
PAM50 subtype						
Luminal A	710 (36.9)	8 (16.3)		423 (29.3)	295 (55.8)	
Luminal B	477 (24.8)	11 (22.4)	40.515	312 (21.6)	176(33.3)	248.3
Basal	305 (15.8)	24 (49.0)	**($3.3x10^{-8}$)**	319 (22.1)	10 (1.9)	(**$1.4x10^{-52}$**)
HER2	235 (12.2)	5 (10.2)		219 (15.2)	21 (4.0)	
Normal-like	198 (10.3)	1 (2.0)		172 (11.9)	27(5.1)	

P values in bold means statistically significant

Table 3-9. Copy Number Aberrations of *SLC3A2* in breast cancer and their associations with MYC and molecular subtypes.

	SLC3A2					
	Gain			**Loss**		
	No n (%)	**Yes n (%)**	**χ2 (p-value)**	**No n (%)**	**Yes n (%)**	**χ2 (p-value)**
PAM50 subtype						
Luminal A	703 (97.9)	15 (2.1)		675 (94.0)	43 (6.0)	
Luminal B	445 (91.2)	43(8.8)	30.912	437 (89.5)	51 (10.5)	0.47
Basal	315 (95.7)	14 (4.3)	(**0.000003**)	326 (99.1)	3 (0.9)	(0.49)
HER2	229 (95.4)	11 (4.6)		232 (96.7)	8 (3.3)	
Normal-like	1884 (95.4)	7 (3.5)		195 (98.0)	4 (2.0)	

P value in bold means statistically significant

3.5.5 SLC1A5, SLC7A5 and SLC3A2 expression and other associated markers

The correlation between *SLC1A5*, *SLC7A5* and *SLC3A2* mRNA and the gene expression of other related biomarkers was investigated, using the METABRIC dataset (Table 3-10, 3-12, 3-14). The biomarkers were selected based on previous publications, being either regulatory genes or others that share the solute carriers' biological function, which primarily focused on glutamine transport and glutamine metabolism [111,113,205,207,208,230,231,167,168,251-254]. Association of SLC1A5, SLC7A5 and SLC3A2 protein expressions with other immunohistochemical biomarkers was also examined, using the Nottingham BC cohort, and shown in Table 3-11, 3-13, 3-15.

3.5.5.1 Associations with SLC1A5

There was a significant correlation between *SLC1A5* mRNA expression with the expression of all regulatory genes that were previously identified in the literature, including *MYC*, *Rb*, *ATF4*, *PIK3CA*, *EphA2* and genes involved in the *MAPK* pathway (*RAF1*, *BRAF* and *KRAS*) (all $p \leq 0.02$). While the majority of regulatory genes displayed a positive correlation with *SLC1A5*, an inverse relationship was observed with *MYC* and *Rb*, with the latter showing a consistent negative correlation across all BC subtypes ($p \leq 0.002$). However, the negative relationship between *MYC* and *SLC1A5* was only significant in luminal A tumours ($p=0.01$) and not in the other subtypes ($p>0.05$). *PIK3CA* was the only gene which showed a positive relationship in all BC subtypes, excluding HER2+ tumours ($p \leq 0.007$ and $p>0.05$) respectively.

Regarding associations with glutamine metabolic enzymes, *SLC1A5* expression was positively correlated with enzymes involved with conversion of glutamine to proline (GLS, PYCR1 and ALDH18A1) (all $p \leq 0.001$). A positive relationship was also observed with *GLUD1* enzyme, which catalyses glutamate to form α-KG ($p<0.001$). The significant association with the majority of enzymes was reserved within luminal A tumours ($p \leq 0.01$); the only subtype which also showed a positive association with glutamine synthetase enzyme (*GLUL*) ($p \leq 0.02$).

The majority of amino acid transporters were significantly associated with *SLC1A5* expression primarily in TNBC and to lesser extent luminal tumours (all $p \leq 0.02$). *SLC7A5* and *SLC38A2* were significantly expressed with *SLC1A5* in all BC subtypes

(p≤0.04). *TP53* mutations were also highly prevalent in breast tumours where there was high *SLC1A5* mRNA expression (p<0.001, Table 3-4).

SLC1A5 protein was significantly expressed in breast tumours with high Ki67 (p<0.001), and MYC (p=0.02). PIK3CA was also significantly expressed in breast tumours with high SLC1A5 expression (p=0.005). Interestingly, high expression of SLC1A5 protein was significantly associated with all enzymes involved in glutamine-proline regulatory axis, including enzymes which convert glutamine to proline (GLS, PYCR1 and ALDH18A1) (all p<0.001) and others which catalyse proline to glutamine (PRODH and ALDH4A1) (p≤0.03). Apart from SLC7A8, all other solute carriers included in this study (SLC7A5, SLC3A2, SLC7A11 and SLC38A2) were significantly expressed in breast tumours with high SLC1A5 expression (p≤0.002).

Table 3-10. Correlation of *SLC1A5* mRNA expression with the expression of other related genes in the METABRIC cohort.

	***SLC1A5* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)				**Adjusted p-value**					
Regulatory and other associated genes										
MYC	-0.126 ($1.6x10^{-8}$)	**<0.0001**	-0.179 (0.001)	**0.01**	-0.041 (0.438)	5.1	-0.263 (0.006)	0.13	-0.031 (0.709)	3.5
Rb	-0.23 ($2.4x10^{-25}$)	**<0.0001**	-0.154 (0.00003)	**0.0007**	-0.168 (0.0001)	**0.002**	-0.285 (0.000007)	**0.0002**	-0.305 ($1.7x10^{-8}$)	**<0.0001**
PIK3CA	0.196 ($1.5x10^{-18}$)	**<0.0001**	0.208 ($1.7x10^{-8}$)	**<0.0001**	0.134 (0.003)	**0.007**	0.126 (0.05)	0.56	0.236 (0.00001)	**0.0003**
AKT1	0.148 ($3.5x10^{-11}$)	**<0.0001**	0.230 (0.000008)	**0.0002**	0.006 (0.903)	2.7	0.189 (0.048)	0.48	0.204 (0.012)	0.16
RAF1	0.136 ($1.1x10^{-9}$)	**<0.0001**	0.103 (0.006)	0.11	0.098 (0.03)	0.63	0.134 (0.03)	0.45	0.112 (0.04)	0.60
BRAF	0.108 (0.000001)	**<0.0001**	0.08 (0.01)	0.12	-0.042 (0.357)	4.9	0.09 (0.162)	1.41	0.215 (0.00008)	**0.001**
KRAS	0.127 ($1.4x10^{-8}$)	**<0.0001**	0.07 (0.03)	0.27	0.119 (0.009)	0.19	-0.04 (0.49)	1.98	0.149 (0.007)	0.13
EPHA2	0.068 (0.002)	**0.02**	0.109 (0.003)	0.30	0.001 (0.987)	0.98	0.114 (0.07)	0.70	0.08 (0.13)	1.56
ATF4	0.273 ($4.3x10^{-35}$)	**<0.0001**	0.106 (0.042)	0.33	0.215 (0.00004)	**0.001**	0.445 (0.000001)	**<0.0001**	0.370 (0.000003)	**0.0001**

(Continued)

	***SLC1A5* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)					**Adjusted p-value**				
Enzymes involved in glutamine metabolism										
GLS	-0.087 (0.0001)	**0.001**	-0.111 (0.033)	0.30	-0.064 (0.219)	3.7	-0.183 (0.056)	0.60	0.076 (0.357)	1.58
ALDH4A1	0.042 (0.064)	0.30	-0.144 (0.006)	0.11	-0.037 (0.482)	5.2	0.220 (0.021)	0.34	0.055 (0.505)	3.04
PRODH	0.052 (0.021)	0.18	0.179 (0.001)	**0.01**	-0.014 (0.795)	4.7	0.222 (0.020)	0.36	0.071 (0.385)	2.10
PYCR1	0.335 ($4.7x10^{-53}$)	**<0.0001**	0.267 ($1.9x10^{-7}$)	**<0.0001**	0.245 (0.000002)	**0.0001**	0.445 (0.000001)	**<0.0001**	0.362 (0.000005)	**0.0001**
ALDH18A1	0.164 ($2.4x10^{-13}$)	**<0.0001**	0.227 (0.00001)	**0.0002**	0.057 (0.278)	4.3	0.278 (0.003)	**0.07**	0.217 (0.008)	0.14
GLUL	0.016 (0.478)	1.40	0.158 (0.002)	**0.02**	-0.065 (0.214)	3.5	-0.072 (0.454)	1.62	-0.067 (0.415)	2.52
GLUD1	0.134 ($2.5x10^{-9}$)	**<0.0001**	0.278 ($5.8x10^{-8}$)	**<0.0001**	0.087 (0.096)	0.80	0.116 (0.228)	1.45	0.076 (0.358)	2.00
Glutamine transporters										
SLC7A5	0.29 ($4.5x10^{-41}$)	**<0.0001**	0.170 (0.000005)	**0.0001**	0.150 (0.001)	**0.02**	0.208 (0.001)	**0.02**	0.25 (0.000002)	**<0.0001**
SLC3A2	-0.098 (0.00001)	**0.0001**	-0.188 (0.0003)	**0.006**	-0.077 (0.142)	2.6	-0.209 (0.028)	0.38	-0.129 (0.115)	1.43
SLC6A19	0.01 (0.47)	0.94	0.02 (0.44)	1.3	0.01 (0.74)	5.9	-0.11 (0.03)	0.46	0.04 (0.50)	3.42
SLC7A6	0.129 ($8.8x10^{-9}$)	**<0.0001**	0.03 (0.29)	1.4	0.01 (0.76)	5.3	0.137 (0.01)	0.20	0.18 (0.004)	0.08
SLC7A7	-0.01 (0.40)	1.60	-0.104 (0.005)	0.10	-0.04 (0.28)	4.2	-0.04 (0.39)	1.60	-0.21 (0.001)	**0.02**
SLC7A8	-0.07 (0.001)	**0.01**	0.06 (0.09)	0.54	0.007 (0.88)	3.2	-0.03 (0.62)	1.80	-0.20 (0.0002)	**0.003**
SLC7A9	0.057 (0.01)	0.08	0.06 (0.101)	0.70	-0.01 (0.72)	6.4	0.15 (0.006)	0.13	0.05 (0.38)	2.50
SLC38A1	0.066 (0.003)	**0.03**	-0.028 (0.596)	1.1	0.042 (0.427)	5.4	0.105 (0.275)	1.54	0.227 (0.005)	0.10
SLC38A2	0.18 ($1.5x10^{-16}$)	**<0.0001**	0.17 (0.000004)	**0.0001**	0.14 (0.002)	**0.02**	0.19 (0.002)	**0.02**	0.17 (0.002)	**0.04**
SLC38A3	0.069 (0.002)	**0.02**	0.053 (0.307)	1.2	-0.020 (0.704)	6.0	0.121 (0.206)	1.44	0.128 (0.119)	1.45
SLC38A5	0.04 (0.03)	0.18	-0.05 (0.11)	0.6	0.01 (0.79)	4.7	0.10 (0.06)	0.65	0.05 (0.42)	2.87
SLC38A7	0.241 ($1.2x10^{-27}$)	**<0.0001**	0.203 ($4.0x10^{-8}$)	**<0.0001**	0.145 (0.001)	**0.03**	0.168 (0.002)	0.05	0.31 (0.000001)	**<0.0001**
SLC38A8	0.01 (0.48)	0.48	0.01 (0.79)	0.79	0.005 (0.91)	1.8	-0.03 (0.47)	1.80	-0.01 (0.79)	3.85

P values in bold means statistically significant

Table 3-11. Association of SLC1A5 protein and other histochemical markers in the Nottingham cohort.

	SLC1A5 protein			
	Low, n (%)	**High, n (%)**	**χ2 (p-value)**	**Adjusted p-value**
c-MYC				
Negative	292 (35.4)	534 (64.6)	7.27	**0.02**
Positive	41 (24.6)	126 (75.4)	(0.007)	
Ki67				
Negative	184 (55.1)	150 (44.9)	89.8	**<0.0001**
Positive	184 (25.3)	544 (74.7)	($2.5x10^{-21}$)	
PIK3CA				
Negative	97 (43.7)	125 (56.3)	10.68	**0.005**
Positive	227 (31.7)	488 (68.3)	(0.001)	
GLUD1				
Negative	292 (33.7)	575 (66.3)	1.78	0.36
Positive	128 (37.8)	211 (62.2)	(0.18)	
GLS				
Negative	216 (39.2)	335 (60.8)	28.79	**<0.0001**
Positive	92 (22.8)	312 (77.2)	($8.0x10^{-8}$)	
PYCR1				
Negative	171 (39.7)	260 (60.3)	29.89	**<0.0001**
Positive	80 (21.7)	289 (78.3)	($4.5x10^{-8}$)	
ALDH4A1				
Negative	166 (38.2)	268 (61.8)	10.39	**0.006**
Positive	121 (27.9)	312 (72.1)	(0.001)	
ALDH18A1				
Negative	183 (40.1)	273 (59.9)	20.59	**<0.0001**
Positive	112 (25.8)	322 (74.2)	(0.000006)	
PRODH				
Negative	240 (33.8)	471 (66.2)	6.30	**0.03**
Positive	46 (24.2)	144 (75.8)	(0.01)	
SLC7A5				
Negative	825 (41.3)	1174 (58.7)	161.7	**<0.0001**
Positive	39 (9.0)	394 (91.0)	($4.7x10^{-37}$)	
SLC3A2				
Negative	423 (41.3)	601 (58.7)	32.40	**<0.0001**
Positive	308 (29.4)	741 (70.6)	($1.2x10^{-8}$)	
SLC38A2				
Negative	487 (34.7)	917 (65.3)	12.92	**0.002**
Positive	27 (19.6)	111 (80.4)	(0.0003)	
SLC7A8				
Negative	430 (33.4)	856 (66.6)	1.50	0.44
Positive	47 (28.7)	117 (71.3)	(0.22)	
SLC7A11				
Negative	430 (38.5)	686 (61.5)	27.8	**<0.0001**
Positive	197 (26.7)	541 (73.3)	($1.3x10^{-7}$)	

P values in bold means statistically significant

3.5.5.2 Associations with SLC7A5

There was a positive correlation between *SLC7A5* mRNA expression with the expression of regulatory genes such as *ATF4*, *HIF2a* and *MYC* ($p<0.001$). Interestingly, the correlation with *MYC* gene expression was only observed within luminal B tumours ($p<0.01$). High *SLC7A5* was also significantly associated with the enzyme involved in glutamine synthesis , *GLUL* ($p<0.001$) and enzymes involved with conversion of glutamine to proline, including *ALDH18A1* and *PYCR1*, which showed positive correlations in almost all BC subtypes ($p\leq0.02$). Finally, high *SLC7A5* mRNA expression was significantly correlated with its downstream signal, *mTOR*, and *VEGFA* ($p\leq0.001$).

The majority of glutamine transporters showed significant associations with *SLC7A5* expression, primarily in TN tumours and to lesser extent HER2+ and luminal subtypes. *SLC7A5* was significantly correlated with the key glutamine transporter, *SLC1A5*, in all BC subtypes ($p\leq0.02$). High proportion of *TP53* mutations were also observed in breast tumours where there was high *SLC7A5* mRNA expression ($p<0.001$, Table 3-5).

High SLC7A5 protein expression was detected in breast tumours which express high Ki67 and the upstream effector, MYC ($p<0.001$). All solute carriers included in this study, SLC1A5, SLC3A2, SLC38A2, SLC7A11, were significantly expressed in breast tumours with high SLC7A5 expression ($p<0.001$) with the exception of SLC7A8 ($p=0.05$). Although no significant association was observed with some glutamine-related enzymes; PRODH, ALDH4A1 and ALDH18A1 (all $p>0.05$), others; GLS, GLUD1 and PYCR1 were significantly associated with high SLC7A5 expression ($p\leq0.03$). With respect to downstream effectors, high SLC7A5 expression was positively associated with p70S6K while an inverse relationship was noticed with p-mTORC1 ($p<0.001$). However, PIK3CA (the growth factor which activates mTORC1 through PI3K/AKT/mTOR signalling pathway) showed a positive association with SLC7A5 protein ($p<0.001$). Moreover, SLC7A5 was highly expressed in breast cancers which express high p53 protein ($p<0.001$).

Table 3-12. Correlation of *SLC7A5* mRNA expression with the expression of other related genes in the METABRIC cohort.

	***SLC7A5* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)			**Adjusted p-value**						
Regulatory and other associated genes										
MYC	0.133 ($2.4x10^{-9}$)	**<0.0001**	0.012 (0.752)	4.14	0.155 (0.001)	**0.01**	0.066 (0.310)	4.65	0.103 (0.062)	0.43
HIF2A	-0.050 (0.028)	0.16	-0.023 (0.536)	4.89	0.215 (0.000002)	**<0.0001**	0.112 (0.083)	1.32	-0.282 ($1.8x10^{-7}$)	**<0.0001**
ATF4	0.159 ($1.0x10^{-12}$)	**<0.0001**	-0.029 (0.433)	5.10	0.057 (0.208)	2.08	0.143 (0.026)	0.46	0.108 (0.050)	0.45
KDM4C	0.004 (0.854)	1.69	-0.06 (0.07)	3.70	-0.02 (0.517)	3.10	0.02 (0.612)	5.93	-0.156 (0.01)	0.22
mTOR	0.085 (0.0001)	**0.001**	-0.005 (0.904)	1.82	0.088 (0.052)	0.72	-0.023 (0.723)	5.78	0.067 (0.226)	1.13
VEGFA	0.352 ($6.4x10^{-59}$)	**<0.0001**	0.166 (0.000008)	**0.0002**	0.260 ($5.3x10^{-9}$)	**<0.0001**	0.269 (0.00002)	**0.0005**	0.244 (0.000008)	**0.0002**
Enzymes involved in glutamine metabolism										
GLS	0.048 (0.032)	0.19	0.008 (0.829)	3.42	0.055 (0.222)	1.99	-0.006 (0.927)	4.63	-0.115 (0.038)	0.45
ALDH4A1	-0.053 (0.019)	0.13	0.018 (0.638)	4.51	0.063 (0.163)	1.79	-0.028 (0.663)	5.96	-0.134 (0.015)	0.22
PRODH	0.004 (0.858)	1.71	-0.034 (0.369)	4.76	-0.032 (0.483)	2.41	0.037 (0.573)	5.73	0.030 (0.582)	1.74
PYCR1	0.32 ($1.5x10^{-50}$)	**<0.0001**	0.143 (0.0001)	**0.001**	0.253 ($1.3x10^{-8}$)	**<0.0001**	0.210 (0.001)	**0.02**	0.303 ($1.9x10^{-8}$)	**<0.0001**
ALDH18A1	0.222 ($1.6x10^{-23}$)	**<0.0001**	0.151 (0.00004)	**0.0008**	0.144 (0.001)	**0.01**	0.168 (0.009)	0.18	0.356 ($2.9x10^{-11}$)	**<0.0001**
GLUL	-0.18 ($3.3x10^{-16}$)	**<0.0001**	0.134 (0.0003)	**0.005**	0.008 (0.863)	1.72	-0.001 (0.991)	1.98	-0.122 (0.028)	0.39
GLUD1	-0.38 ($4.3x10^{-69}$)	**<0.0001**	-0.161 (0.00001)	**0.0002**	-0.237 ($1.1x10^{-7}$)	**<0.0001**	-0.148 (0.022)	0.41	-0.112 (0.042)	0.42
Glutamine transporters										
SLC1A5	0.29 ($4.5x10^{-41}$)	**<0.0001**	0.170 (0.000005)	**0.0001**	0.150 (0.001)	**0.01**	0.208 (0.001)	**0.02**	0.25 (0.000002)	**<0.0001**
SLC3A2	0.17 ($1.1x10^{-14}$)	**<0.0001**	0.067 (0.072)	3.78	0.193 (0.00001)	**0.0002**	0.184 (0.004)	0.08	0.158 (0.004)	0.06
SLC6A19	0.004 (0.869)	1.73	0.041 (0.273)	4.42	-0.008 (0.859)	2.57	0.047 (0.473)	5.67	-0.103 (0.061)	0.48
SLC7A6	0.362 ($2.7x10^{-62}$)	**<0.0001**	0.254 ($5.3x10^{-12}$)	**<0.0001**	0.33 ($2.0x10^{-14}$)	**<0.0001**	0.284 (0.000008)	**0.0002**	0.071 (0.201)	1.20
SLC7A7	0.19 ($4.6x10^{-19}$)	**<0.0001**	0.007 (0.857)	2.71	0.085 (0.061)	0.79	0.041 (0.530)	5.83	-0.22 (0.00005)	**0.0001**

(Continued)

	***SLC7A5* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)				**Adjusted p-value**					
SLC7A8	-0.47 ($1.3x10^{-112}$)	**<0.0001**	-0.155 (0.00003)	**0.0005**	-0.11 (0.02)	0.35	-0.18 (0.004)	0.08	-0.45 ($3.3x10^{-18}$)	**<0.0001**
SLC7A9	-0.068 (0.002)	0.01	0.025 (0.510)	4.82	0.044 (0.333)	2.33	-0.123 (0.056)	0.95	0.283 ($1.8x10^{-7}$)	**<0.0001**
SLC38A1	-0.10 (0.000003)	**<0.0001**	-0.041 (0.270)	3.54	0.039 (0.391)	2.34	0.053 (0.413)	5.36	0.113 (0.041)	0.45
SLC38A2	-0.06 (0.007)	0.06	-0.06 (0.06)	1.05	-0.12 (0.002)	**0.02**	-0.10 (0.04)	0.90	-0.03 (0.56)	1.72
SLC38A3	0.18 ($8.3x10^{-17}$)	**<0.0001**	0.140 (0.0001)	**0.002**	0.046 (0.311)	2.48	0.003 (0.958)	2.87	0.196 (0.0003)	**0.005**
SLC38A5	0.011 (0.627)	2.57	-0.069 (0.066)	1.08	-0.077 (0.090)	1.08	-0.017 (0.793)	5.55	-0.017 (0.757)	1.51
SLC38A7	0.306 ($3.8x10^{-44}$)	**<0.0001**	0.270 ($2.0x10^{-13}$)	**<0.0001**	0.32 ($1.2x10^{-13}$)	**<0.0001**	0.064 (0.324)	4.53	0.177 (0.001)	**0.01**
SLC38A8	0.023 (0.312)	1.56	-0.019 (0.612)	4.46	0.011 (0.801)	3.20	-0.006 (0.930)	3.72	-0.039 (0.482)	1.92

P values in bold means statistically significant

Table 3-13. Association between SLC7A5 protein expression and other histochemical biomarkers in the Nottingham cohort.

	SLC7A5 protein			
	Low, n (%)	**High, n (%)**	**χ^2 (p-value)**	**Adjusted p-value**
c-MYC				
Negative	638 (83.9)	122 (16.1)	23.4	**0.0001**
Positive	91 (66.4)	46 (33.6)	(0.000001)	
Ki67				
Negative	268 (90.2)	29 (9.8)	18.6	**0.0001**
Positive	477 (78.6)	130 (21.4)	(0.00001)	
PIK3CA				
Negative	183 (92.0)	16 (8.0)	15.96	**0.0003**
Positive	509 (79.7)	130 (20.3)	(0.00006)	
GLUD1				
Negative	581 (79.7)	184 (20.3)	5.05	**0.03**
Positive	227 (86.0)	37 (14.0)	(0.02)	
GLS				
Negative	467 (90.7)	48 (9.3)	87.12	**<0.0001**
Positive	245 (65.3)	130 (34.7)	($1.01x10^{-20}$)	
PYCR1				
Negative	408 (89.1)	50 (10.9)	59.8	**<0.0001**
Positive	250 (67.2)	122 (32.8)	($1.04x10^{-14}$)	

(Continued)

	SLC7A5 Protein			
	Low, n (%)	**High, n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ALDH4A1				
Negative	371 (81.2)	86 (18.8)	0.55	0.90
Positive	370 (79.2)	97 (20.8)	(0.45)	
ALDH18A1				
Negative	405 (82.2)	88 (17.8)	0.95	0.96
Positive	360 (79.6)	92 (20.4)	(0.32)	
PRODH				
Negative	517 (80.3)	127 (19.7)	0.37	1.08
Positive	125 (78.1)	35 (21.9)	(0.54)	
SLC1A5				
Negative	825 (41.3)	1174 (58.7)	161.74	**<0.0001**
Positive	39 (9.0)	394 (91.0)	($4.7x10^{-37}$)	
SLC3A2				
Negative	926 (87.1)	137(12.9)	42.61	**<0.0001**
Positive	833 (76.2)	260 (23.8)	($6.6x10^{-11}$)	
SLC38A2				
Negative	1158 (80.9)	273 (19.1)	15.56	**0.0003**
Positive	98 (67.1)	48 (32.9)	(0.00008)	
SLC7A8				
Negative	1040 (88.1)	140 (11.9)	4.66	0.05
Positive	253 (92.7)	20 (7.3)	(0.03)	
SLC7A11				
Negative	942 (62.0)	577 (38.0)	13.22	**0.0006**
Positive	189 (51.6)	177 (48.4)	(0.0002)	
p-mTORC1				
Negative	364 (76.6)	11 (23.4)	17.41	**0.00006**
Positive	320 (87.9)	44 (12.1)	(0.00003)	
p70S6K				
Negative	709 (81.1)	165 (18.9)	26.13	**<0.0001**
Positive	139 (65.0)	75 (35.0)	($3.1x10^{-7}$)	

P values in bold means statistically significant

3.5.5.3 Associations with SLC3A2

There was a relationship between *SLC3A2* and its upstream effector, *MYC*, which showed significant correlation across all BC subtypes ($p < 0.001$). High *SLC3A2* mRNA expression was significantly associated with its heterodimers, the glutamine exchanger, *SLC7A5,* and the glutamate transporter, *SLC7A11* ($p<0.001$ and $p = 0.002$, respectively). A positive correlation was also observed with *ITGB1* and *VEGFB* (all $p<0.001$). Nevertheless, the majority of other glutamine transporters were negatively correlated with *SLC3A2* expression. A similar association was also observed with glutaminase (GLS) enzyme, which mediates the conversion of glutamine to glutamate ($p < 0.001$). High *SLC3A2* mRNA expression was

associated with those tumours where there were *TP53* mutations ($p < 0.001$, Table 3-6).

SLC3A2 protein was significantly expressed with high Ki67 and MYC proteins (all $p < 0.001$). SLC7A5, SLC1A5, SLC38A2, SLC7A11, GLS and PIK3CA were all significantly expressed in breast tumours with high SLC3A2 expression (all $p \leq 0.04$). No significant association was observed with SLC7A8 ($p > 0.05$). While the low expression of SLC3A2 was associated with high levels of p-mTORC1 ($p < 0.001$), a positive relationship was observed with p70S6K ($p = 0.01$). Moreover, high SLC3A2 protein was positively associated with high p53 protein expression ($p < 0.001$, Table 3-6).

Table 3-14. Correlation of *SLC3A2* mRNA expression with the expression of other related genes in the METABRIC cohort.

	***SLC3A2* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)				**Adjusted p-value**					
Regulatory and other associated genes										
MYC	0.390 (4.7×10^{-73})	**<0.0001**	0.388 (3.1×10^{-27})	**<0.0001**	0.348 (2.4×10^{-15})	**<0.0001**	0.502 (1.04×10^{-16})	**<0.0001**	0.301 (2.5×10^{-8})	**<0.0001**
mTOR	-0.272 (5.2×10^{-35})	**<0.0001**	-0.326 (2.7×10^{-19})	**<0.0001**	-0.128 (0.005)	0.07	-0.221 (0.001)	**0.01**	-0.349 (7.0×10^{-11})	**<0.0001**
VEGFB	0.149 (2.8×10^{-11})	**<0.0001**	0.129 (0.001)	**0.01**	0.213 (0.000002)	**<0.0001**	0.134 (0.03)	0.27	0.310 (9.2×10^{-9})	**<0.0001**
ITGB1	0.459 (1.3×10^{-103})	**<0.0001**	0.458 (1.7×10^{-38})	**<0.0001**	0.314 (1.3×10^{-12})	**<0.0001**	0.676 (1.9×10^{-33})	**<0.0001**	0.443 (3.2×10^{-17})	**<0.0001**
PIK3CA	-0.24 (2.7×10^{-28})	**<0.0001**	-0.28 (2.06×10^{-14})	**<0.0001**	-0.349 (2.1×10^{-15})	**<0.0001**	-0.23 (0.00002)	**<0.0001**	-0.30 (0.000002)	**<0.0001**
AKT	-0.27 (1.7×10^{-34})	**<0.0001**	-0.29 (1.02×10^{-15})	**<0.0001**	-0.12 (0.005)	0.08	-0.29 (5.9×10^{-8})	**<0.0001**	-0.25 (0.00004)	**<0.0001**
Glutamine metabolism										
GLS	-0.127 (1.4×10^{-8})	**<0.0001**	-0.150 (0.00005)	**0.0006**	-0.041 (0.365)	3.228	0.160 (0.01)	0.10	0.164 (0.003)	**0.03**
Glutamine/Glutamate transporters										
SLC7A5	0.172 (1.1×10^{-14})	**<0.0001**	0.067 (0.072)	0.49	0.193 (0.00001)	**0.0002**	0.184 (0.004)	**0.04**	0.158 (0.004)	**0.04**
SLC7A11	0.13 (1.1×10^{-9})	**<0.0001**	0.11 (0.002)	**0.02**	0.14 (0.001)	**0.02**	0.05 (0.40)	1.72	0.17 (0.002)	**0.03**
SLC1A5	-0.098 (0.00001)	**0.0001**	-0.188 (0.0003)	**0.004**	-0.054 (0.14)	2.36	-0.209 (0.02)	0.20	-0.129 (0.11)	0.67

(Continued)

	***SLC3A2* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)					**Adjusted p-value**				
SLC6A19	0.041 (0.07)	0.28	0.073 (0.051)	0.40	0.002 (0.967)	2.90	0.022 (0.732)	1.46	0.123 (0.025)	0.14
SLC7A6	-0.211 ($2.1x10^{-21}$)	**<0.0001**	-0.285 ($7.1x10^{-15}$)	**<0.0001**	-0.097 (0.03)	0.39	-0.339 ($7.0x10^{-8}$)	**<0.0001**	-0.320 ($2.9x10^{-9}$)	**<0.0001**
SLC7A7	0.191 ($1.1x10^{-17}$)	**<0.0001**	0.196 ($1.2x10^{-7}$)	**<0.0001**	0.081 (0.075)	0.84	0.235 (0.0002)	**0.003**	0.137 (0.01)	0.09
SLC7A8	-0.08 (0.0003)	**0.003**	0.01 (0.61)	1.98	0.08 (0.06)	0.71	-0.21 (0.001)	**0.01**	-0.05 (0.35)	0.92
SLC7A9	-0.155 ($3.9x10^{-12}$)	**<0.0001**	-0.186 ($4.8x10^{-7}$)	**<0.0001**	-0.037 (0.410)	3.28	-0.197 (0.002)	**0.02**	-0.103 (0.063)	0.36
SLC38A1	-0.101 (0.000006)	**0.0001**	-0.080 (0.03)	0.27	0.001 (0.978)	1.95	-0.037 (0.568)	2.27	-0.222 (0.00004)	**0.0006**
SLC38A2	-0.223 ($7.7x10^{-24}$)	**<0.0001**	-0.231 ($3.9x10^{-10}$)	**<0.0001**	-0.188 (0.00002)	**0.0003**	-0.283 (0.000009)	**0.0002**	-0.199 (0.0002)	**0.002**
SLC38A3	-0.064 (0.004)	**0.02**	-0.114 (0.002)	**0.02**	-0.033 (0.464)	3.24	-0.113 (0.08)	0.64	-0.082 (0.139)	0.69
SLC38A5	-0.016 (0.472)	0.94	-0.016 (0.673)	2.02	0.004 (0.933)	3.73	-0.101 (0.118)	0.71	-0.059 (0.290)	0.87
SLC38A7	-0.297 ($1.4x10^{-41}$)	**<0.0001**	-0.354 ($1.2x10^{-22}$**)**	<0.0001	-0.143 (0.002)	**0.03**	-0.578 ($8.9x10^{-23}$)	**<0.0001**	-0.318 ($3.7x10^{-9}$)	**<0.0001**
SLC38A8	0.034 (0.134)	0.40	0.37 (0.322)	1.93	-0.011 (0.812)	0.40	-0.041 (0.526)	2.63	0.034 (0.538)	0.11

P values in bold means statistically significant

Table 3-15. Association between SLC3A2 protein expression and other histochemical biomarkers in the Nottingham cohort

SLC3A2 protein				
	Low, n (%)	**High, n (%)**	**χ2 (p-value)**	**Adjusted p-value**
c-MYC				
Negative	341 (51.7)	318 (48.3)	20.73	**<0.0001**
Positive	35 (29.2)	85 (70.8)	(0.000005)	
Ki67				
Negative	158 (61.7)	98 (38.3)	18.28	**0.0001**
Positive	238 (45.4)	286 (54.6)	(0.00001)	
GLS				
Negative	274 (58.7)	193 (41.3)	62.18	**<0.0001**
Positive	101 (30.4)	231 (69.6)	($3.1x10^{-15}$)	
PIK3CA				
Negative	109 (61.1)	68 (38.4)	13.67	**0.008**
Positive	248 (45.6)	296 (54.4)	(0.0002)	

(Continued)

	SLC3A2 protein			
	Low, n (%)	**High, n (%)**	**χ2 (p-value)**	**Adjusted p-value**
SLC1A5				
Negative	423 (57.9)	308 (42.1)	32.40	**<0.0001**
Positive	601 (44.8)	741 (55.2)	($1.2x10^{-8}$)	
SLC7A5				
Negative	926 (87.1)	137 (12.9)	42.61	**<0.0001**
Positive	833 (76.2)	260 (23.8)	($6.6x10^{-11}$)	
SLC38A2				
Negative	977 (73.5)	352 (26.5)	5.89	**0.04**
Positive	84 (63.6)	48 (36.4)	(0.01)	
SLC7A8				
Negative	863 (73.1)	317 (26.9)	0.78	1.14
Positive	106 (69.7)	46 (30.3)	(0.37)	
SLC7A11				
Negative	778 (74.9)	261 (25.1)	9.25	**0.01**
Positive	474 (68.2)	221 (31.8)	(0.002)	
P-mTORC1				
Negative	182 (42.6)	245 (57.4)	19.35	**0.0001**
Positive	183(59.0)	127 (41.0)	(0.00001)	
P70S6K				
Negative	419 (82.3)	90 (17.7)	9.72	**0.01**
Positive	362 (74.2)	126 (25.8)	(0.002)	

P values in bold means statistically significant

3.5.6 Association of SLC1A5, SLC7A5 and SLC3A2 and patient outcome

Survival analysis of both mRNA and protein revealed that higher SLC1A5 and SLC7A5 expression was associated with shorter BCSS (all $p<0.001$, Figure 3-10, 3-11, 3-12, 3-13A). High SLC3A2 protein expression, but not mRNA, was also associated with poor patient outcome ($p < 0.001$, Figure 3-15A) and ($p>0.05$, Figure 3-14A), respectively.

When the analysis was carried out in the intrinsic molecular classes, none of the subtypes showed significant association between mRNA expression of the three solute carriers and patient outcome (all $p>0.05$, Figure 3-10, 3-12, 3-14B-E). However, high expression of SLC1A5 protein was only predictive of shorter BCSS in the ER+ high-proliferation subtype ($p = 0.004$, Figure 3-11C). There was no association between SLC1A5 protein and outcome in the ER+ low-proliferation, TN and HER2+ tumours (all $p>0.05$, Figure 3-11B, 3-11D, 3-11E). In addition, high expression of SLC7A5 protein was predictive of shorter BCSS in the ER+ high

proliferation (p=0.007, Figure 3-13C) and HER2+ tumours (p=0.03, Figure 3-13E) while no significant association was observed in the other subtypes (all p>0.05, Figure 3-13B, D). Regarding SLC3A2 protein, high expression was predictive of shorter BCSS in the ER+ high-proliferation class (p = 0.009, Figure 3-15C), and TN tumours (p = 0.03, Figure 3-15D) but not in the ER+ low-proliferation and HER2+ tumours (all p>0.05, Figure 3-15B, 3-15E).

Multivariate Cox regression analysis showed that *SLC1A5* mRNA predicted poor survival independent of the three most powerful BC prognostic variables; tumour size, tumour grade, and lymph node stage (P= 0.005, Table 3-16A) but not in any specific subtype (all p>0.05, Table 3-16B). Nonetheless, no significant association was observed when the same analysis was carried out involving SLC1A5 protein (all p>0.05 Table 3-16A, C). Similarly, high expression of *SLC7A5* mRNA was an independent poor prognostic factor regardless of the same variables (P=0.006, Table 3-17A) but not in any specific subtype (all p>0.05, Table 3-17B). However, SLC7A5 protein showed significant association in the ER+ high proliferation tumours (p=0.02, Table 3-17C) while no significant association was observed in the other subtypes (all p>0.05, Table 3-17C). Finally, multivariate analysis of SLC3A2 protein showed that it was a predictor of shorter BCSS independent of the same confounding variables (p<0.001, Table 3-18A). The same significant result was remained in the ER+ high-proliferation and TN tumours (all p=0.01, Table 3-18B) when different subtypes were considered.

Regarding associations with DM, protein expression of all solute carriers was associated with shorter DMFS (p<0.001; Figure 3-16, 3-17, 3-18A) within the ER+ high proliferation tumours (p=0.002, Figure 3-16, 3-17, 3-18C) but not with the ER+ low proliferation, HER2+ and TN subtypes (all p>0.05, Figure 3-16, 3-17, 3-18B-E). The relationship between high mRNA expression of SLC1A5, SLC7A5 and SLC3A2 and poor patient outcome in ER+ disease, but not ER- disease, was confirmed using Breast Cancer Gene-Expression Miner (Figure 3-19, 3-20, 3-21A-C).

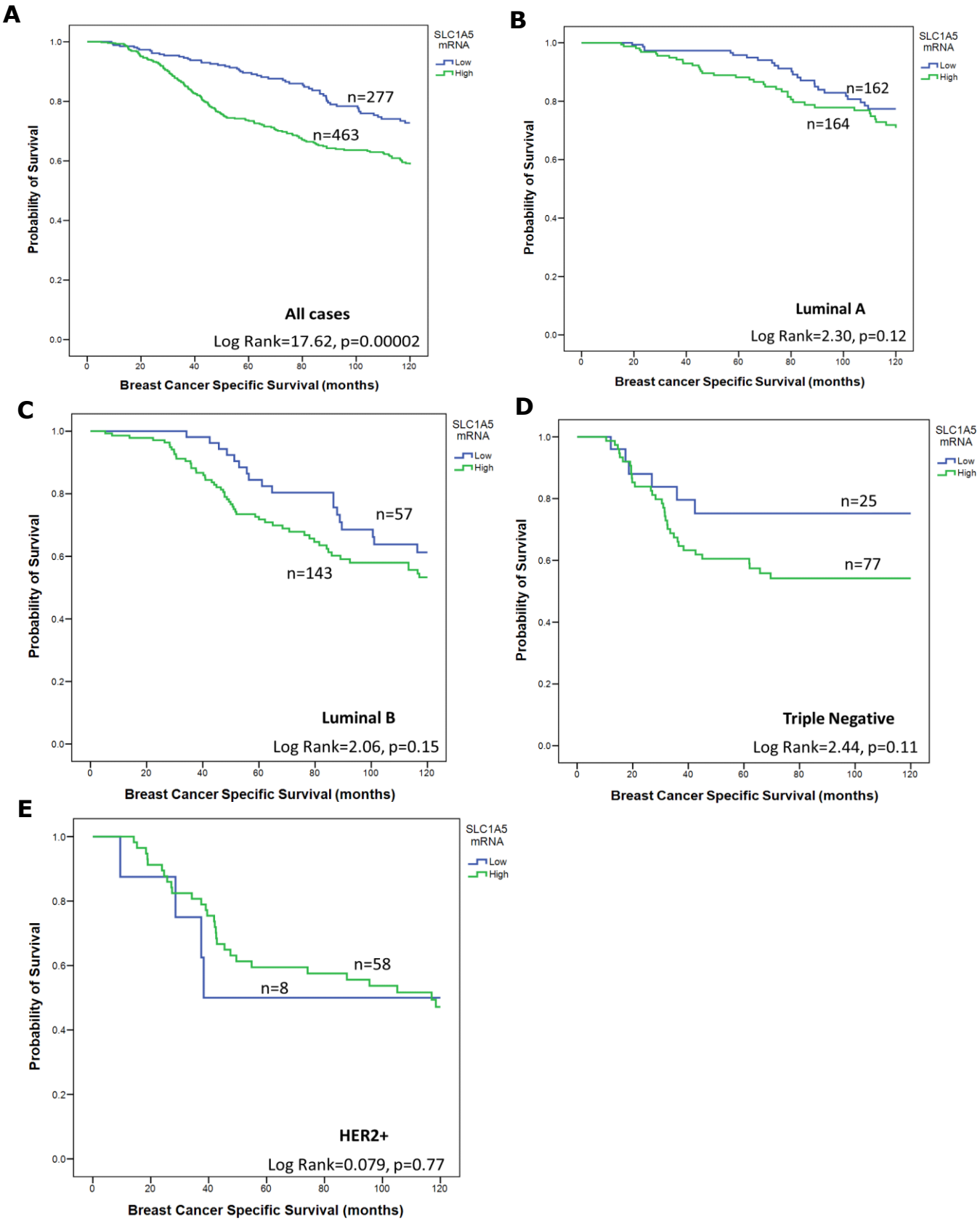


Figure 3-10. SLC1A5 mRNA and breast cancer patient outcome. A) *SLC1A5* vs BCSS in all cases, B) *SLC1A5* vs BCSS in Luminal A tumours, C) *SLC1A5* vs BCSS in Luminal B tumours, D) *SLC1A5* vs BCSS in Triple Negative tumours, E) *SLC1A5* vs BCSS in HER2+ tumours.

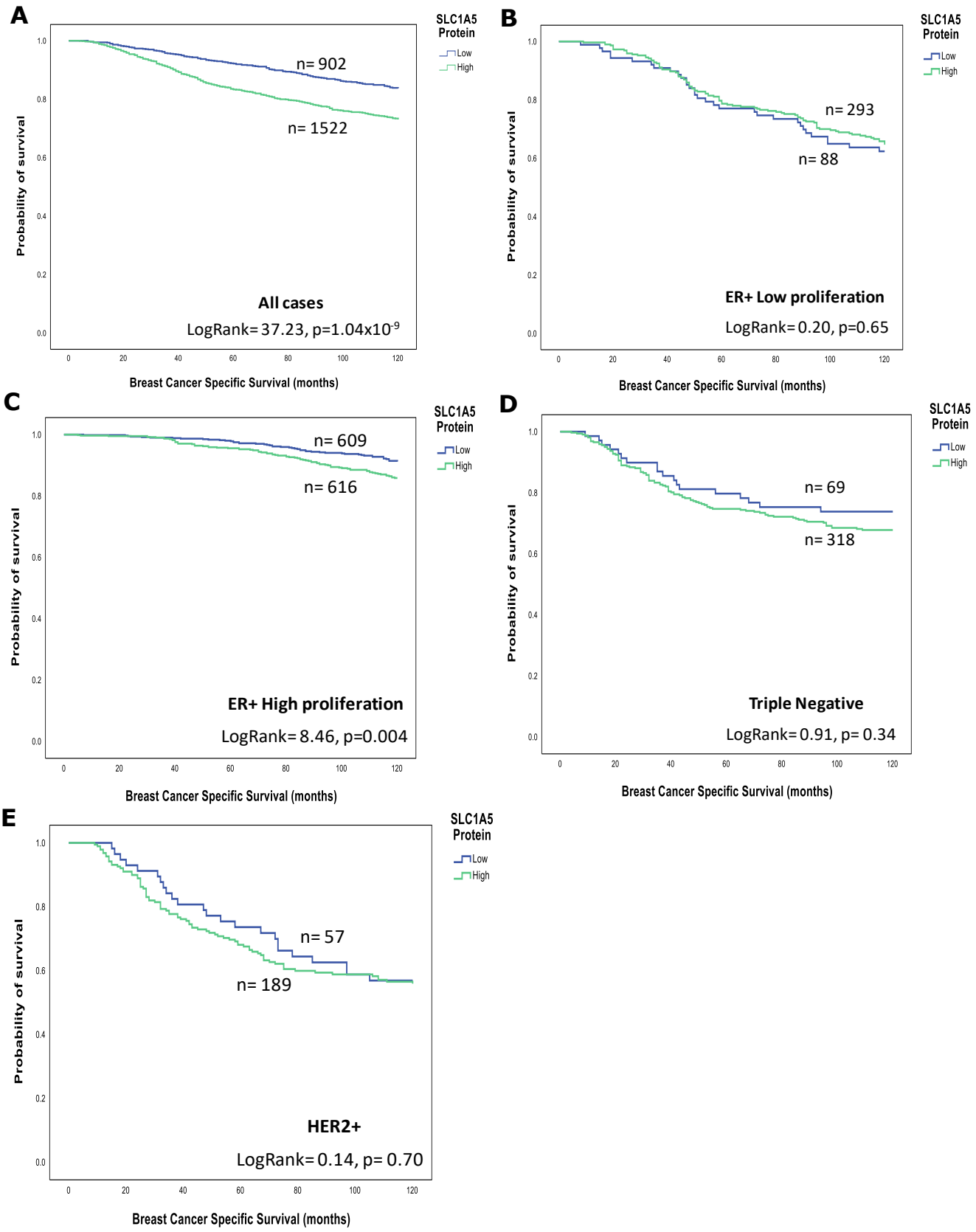


Figure 3-11. SLC1A5 protein expression and breast cancer patient outcome. A) SLC1A5 vs BCSS in all cases , B) SLC1A5 vs BCSS in ER+- Low Proliferation tumours, C) SLC1A5 vs BCSS in ER+- High Proliferation tumours, D) SLC1A5 vs BCSS in Triple Negative tumours E) SLC1A5 vs BCSS in HER2+ tumours.

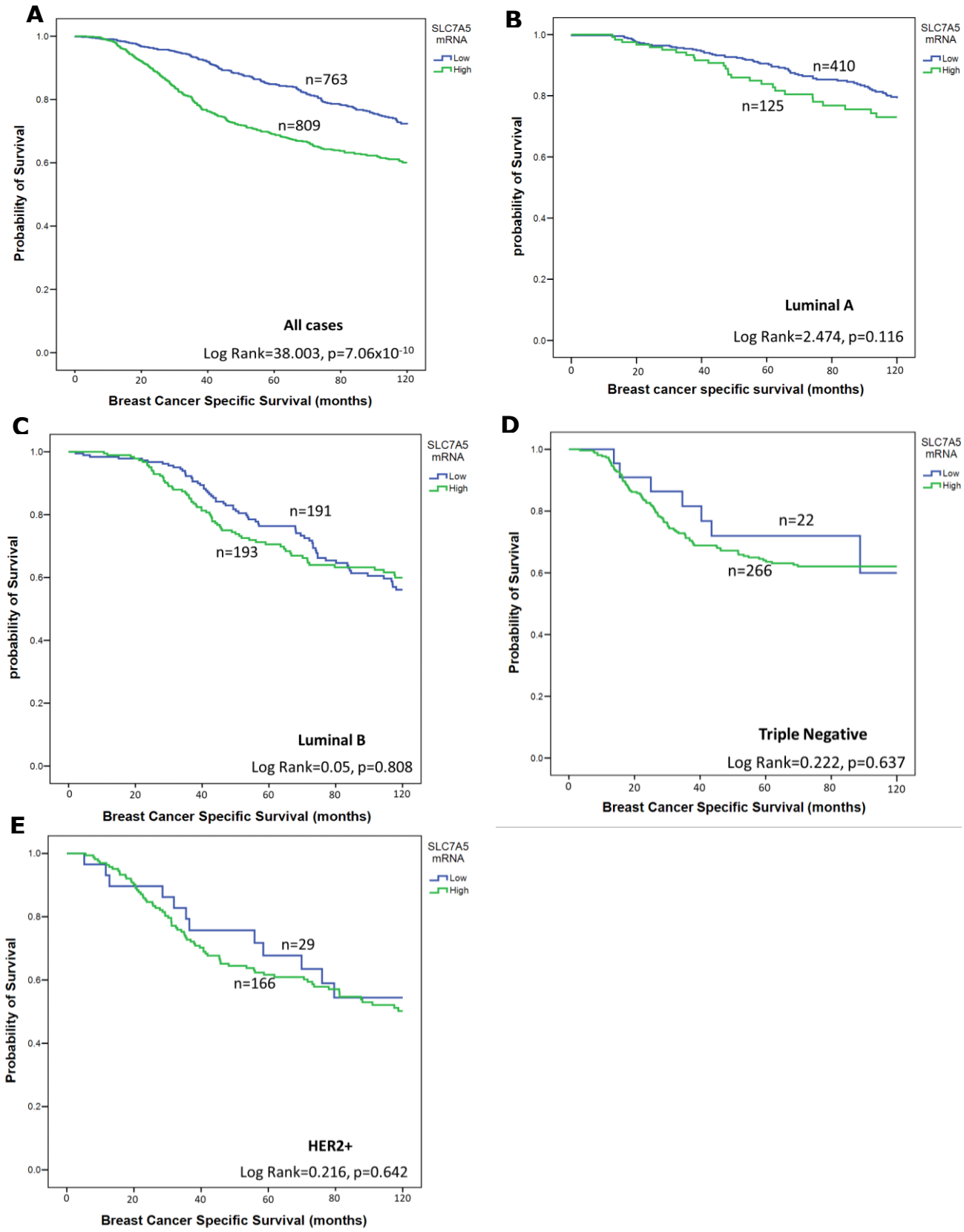


Figure 3-12. SLC7A5 mRNA and breast cancer patient outcome. A) *SLC7A5* vs BCSS in all cases, B) *SLC7A5* vs BCSS in Luminal A tumours, C) *SLC7A5* vs BCSS in Luminal B tumours, D) *SLC7A5* vs BCSS in Triple Negative tumours, E) *SLC7A5* vs BCSS in HER2+ tumours.

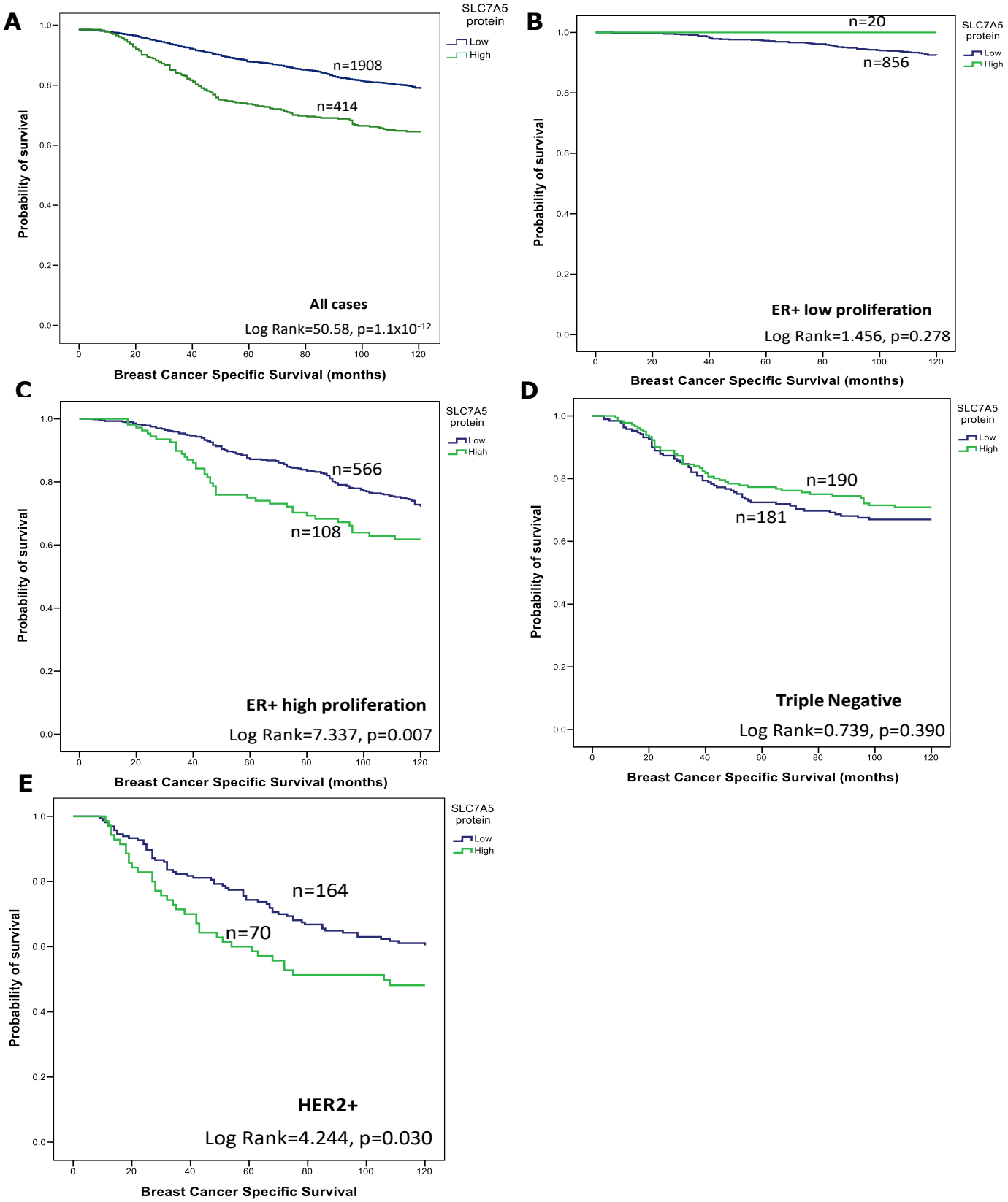


Figure 3-13. SLC7A5 protein expression and breast cancer patient outcome. A) SLC7A5 vs BCSS in all cases , B) SLC7A5 vs BCSS in ER+- Low Proliferation tumours, C) SLC7A5 vs BCSS in ER+- High Proliferation tumours, D) SLC7A5 vs BCSS in Triple Negative tumours E) SLC7A5 vs BCSS in HER2+ tumours.

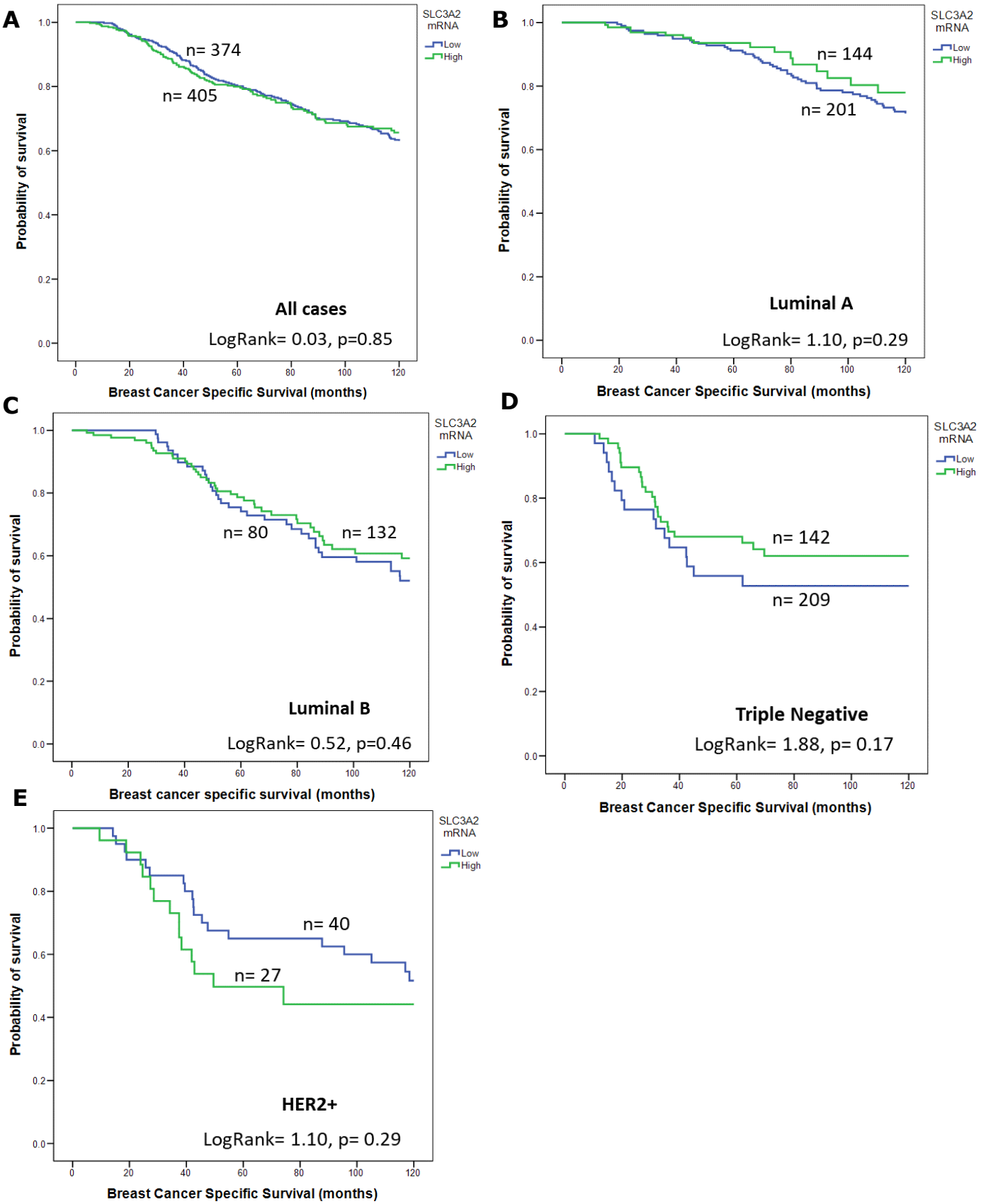


Figure 3-14. SLC3A2 mRNA and breast cancer patient outcome. A) *SLC3A2* vs BCSS in all cases, B) *SLC3A2*vs BCSS in Luminal A tumours, C) *SLC3A2* vs BCSS in Luminal B tumours, D) *SLC3A2* vs BCSS in Triple Negative tumours, E) *SLC3A2* vs BCSS in HER2+ tumours.

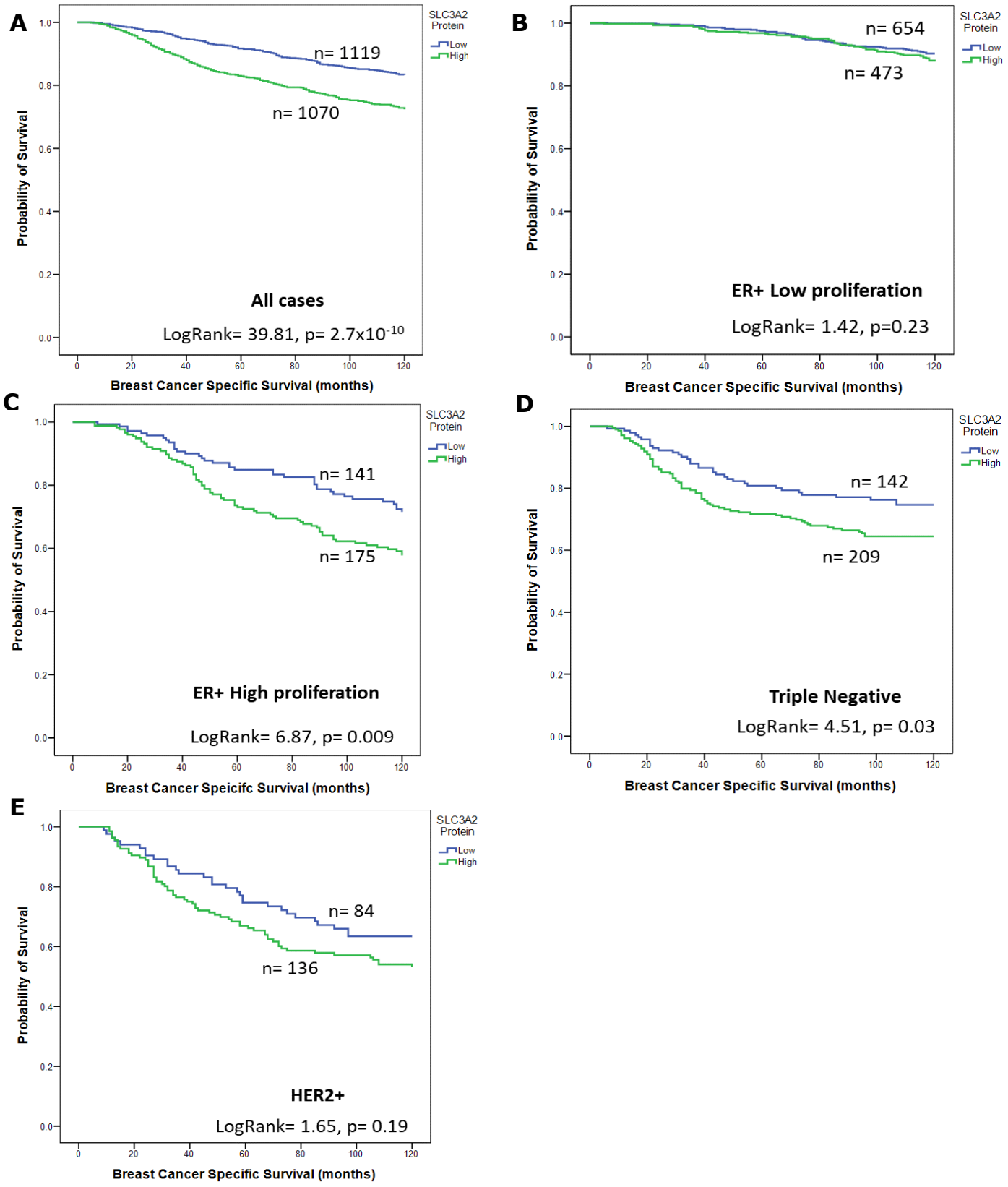


Figure 3-15: SLC3A2protein expression and breast cancer patient outcome. A) SLC3A2 vs BCSS in all cases , B) SLC3A2 vs BCSS in ER+- Low Proliferation tumours, C) SLC3A2 vs BCSS in ER+- High Proliferation tumours, D) SLC3A2 vs BCSS in Triple Negative tumours E) SLC3A2 vs BCSS in HER2+ tumours.

Table 3-16A. Multivariate analysis of prognostic variables and SLC1A5 mRNA and protein expressions in all cases.

	***SLC1A5* mRNA**		**SLC1A5 protein**	
Parameter	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC1A5	1.55 (1.14-2.12)	**0.005**	1.09 (0.89-1.34)	0.36
Nodal stage	1.72 (1.43-2.05)	$2.7x10^{-9}$	1.89 (1.68-2.12)	$1.4x10^{-27}$
Size	1.45 (1.02-2.06)	0.03	1.34 (1.12-1.61)	0.001
Grade	1.41 (1.10-1.82)	0.007	2.41 (2.03-2.85)	$2.5x10^{-24}$

P value in bold means high SLC1A5 expression significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 3-16B. Multivariate analysis of prognostic variables and *SLC1A5* mRNA expression in different BC subtypes.

	***SLC1A5* mRNA**							
	Luminal A		**Luminal B**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC1A5	1.35 (0.80-2.27)	0.25	1.32 (0.77-2.28)	0.30	1.58 (0.65-3.82)	0.31	0.89 (0.27-2.99)	0.86
Nodal stage	1.23 (0.86-1.76)	0.24	1.96 (1.44-2.67)	0.00001	1.91 (1.19-3.07)	0.007	2.24 (1.36-3.68)	0.001
Size	1.93 (1.03-3.61)	0.03	1.53 (0.77-3.05)	0.22	0.72 (0.35-1.49)	0.38	1.92 (0.58-6.35)	0.28
Grade	1.23 (0.79-1.91)	0.35	1.003 (0.65-1.53)	0.98	2.36 (0.31-17.6)	0.40	0.79 (0.35-1.79)	0.58

Table 3-16C. Multivariate analysis of prognostic variables and SLC1A5 protein expression in different IHC subtypes.

SLC1A5 Protein								
	ER-low proliferation		ER-High proliferation		Triple Negative		HER2+	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC1A5	0.83 (0.56-1.25)	0.38	1.27 (0.88-1.82)	0.19	1.09 (0.65-1.81)	0.73	1.00 (0.63-1.59)	0.98
Nodal stage	1.71 (1.37-2.14)	0.000002	1.76 (1.36-2.27)	0.00001	2.08 (1.64-2.65)	$1.5x10^{-9}$	1.75 (1.35-2.28)	0.00002
Size	1.28 (0.88-1.87)	0.19	1.48 (1.02-2.15)	0.03	1.34 (0.90-1.98)	0.13	1.66 (1.08-2.54)	0.01
Grade	3.20 (0.78-13.1)	0.11	1.86 (1.38-2.51)	0.00004	1.54 (0.77-3.05)	0.21	1.33 (0.82-2.17)	0.24

Table 3-17A. Multivariate analysis of prognostic variables and *SLC7A5* mRNA and protein expressions in all cases.

	***SLC7A5* mRNA**		**SLC7A5 protein**	
Parameter	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC7A5	1.121 (1.039-1.209)	**0.003**	1.001 (1.000-1.003)	0.063
Nodal stage	1.877 (1.656-2.129)	$8.9x10^{-23}$	2.060 (1.813-2.341)	$1.7x10^{-28}$
Size	1.550 (1.219-1.971)	0.0003	1.365 (1.111-1.678)	0.003
Grade	1.331 (1.108-1.599)	0.002	2.454 (2.023-2.977)	$1.8x10^{-20}$

P value in bold means high SLC7A5 expression significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 3-17B. Multivariate analysis of prognostic variables and *SLC7A5* mRNA expression in different BC subtypes.

***SLC7A5* mRNA**								
	Luminal A		**Luminal B**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC7A5	1.048 (064-1.69)	0.84	0.934 (0.65-1.33)	0.70	0.941 (0.40-2.21)	0.88	1.207 (0.64-2.25)	0.55
Nodal stage	1.344 (1.005-1.79)	0.04	2.125 (1.68-2.67)	$1.4x10^{-10}$	1.847 (1.40-2.43)	0.00001	2.060 (1.55-2.73)	$5.2x10^{-7}$
Size	2.502 (1.45-4.31)	0.001	1.467 (0.91-2.34)	0.11	1.288 (0.80-2.07)	0.29	1.24 (0.72-2.13)	0.42
Grade	1.289 (0.91-1.82)	0.15	0.989 (0.72-1.35)	0.94	1.571 (0.68-3.59)	0.28	0.803 (0.51-1.25)	0.33

Table 3-17C. Multivariate analysis of prognostic variables and SLC7A5 protein expression in different IHC subtypes.

SLC7A5 Protein								
	ER-low proliferation		**ER-High proliferation**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC7A5	0.959 (0.84-1.08)	0.51	1.004 (1.001-1.006)	**0.006**	0.999 (0.99-1.002)	0.69	1.002 (0.99-1.005)	0.39
Nodal stage	1.703 (1.12-2.57)	0.01	1.756 (1.42-2.16)	$1.04x10^{-7}$	2.427 (1.86-3.15)	$2.7x10^{-11}$	2.103 (1.57-2.80)	$4.4x10^{-7}$
Size	2.192 (1.19-4.01)	0.01	1.169 (0.83-1.63)	0.358	1.568 (1.01-2.42)	0.04	1.480 (0.92-2.36)	0.10
Grade	2.161 (1.04-4.45)	0.03	1.756 (1.15-2.67)	0.009	1.479 (0.73-2.99)	0.27	1.426 (0.84-2.41)	0.18

P value in bold means high SLC7A5 expression significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 3-18A. Multivariate analysis of prognostic variables and SLC3A2 protein expression in all cases.

	SLC3A2 protein	
Parameter	**Hazard ratio (95% CI)**	**p-value**
SLC3A2	1.62 (1.34-1.96)	**$5.4x10^{-7}$**
Nodal stage	1.96 (1.74-2.22)	$3.0x10^{-27}$
Size	1.42 (1.16-1.73)	0.0004
Grade	2.48 (2.08-2.97)	$2.1x10^{-23}$

P value in bold means high SLC3A2 expression significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 3-18B. Multivariate analysis of prognostic variables and SLC3A2 protein expression in different IHC subtypes.

	SLC3A2 protein							
	ER-low proliferation		**ER-High proliferation**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC3A2	1.24 (0.86-1.80)	0.24	1.67 (1.13-2.48)	**0.01**	1.69 (1.11-2.55)	**0.01**	1.56 (1.01-2.43)	0.05
Nodal stage	1.92 (1.47-2.51)	0.000001	1.53 (1.19-1.97)	0.001	2.17 (1.67-2.81)	$4.2x10^{-9}$	2.03 (1.55-2.65)	$1.9x10^{-7}$
Size	1.63 (1.09-2.45)	0.01	1.33 (0.87-2.02)	0.17	1.51 (0.99-2.29)	0.05	1.43 (0.92-2.21)	0.10
Grade	1.81 (1.31-2.50)	0.0003	4.71 (1.19-1.97)	0.12	1.46 (0.73-2.90)	0.27	1.41 (0.84-2.35)	0.19

P value in bold means high SLC3A2 expression significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

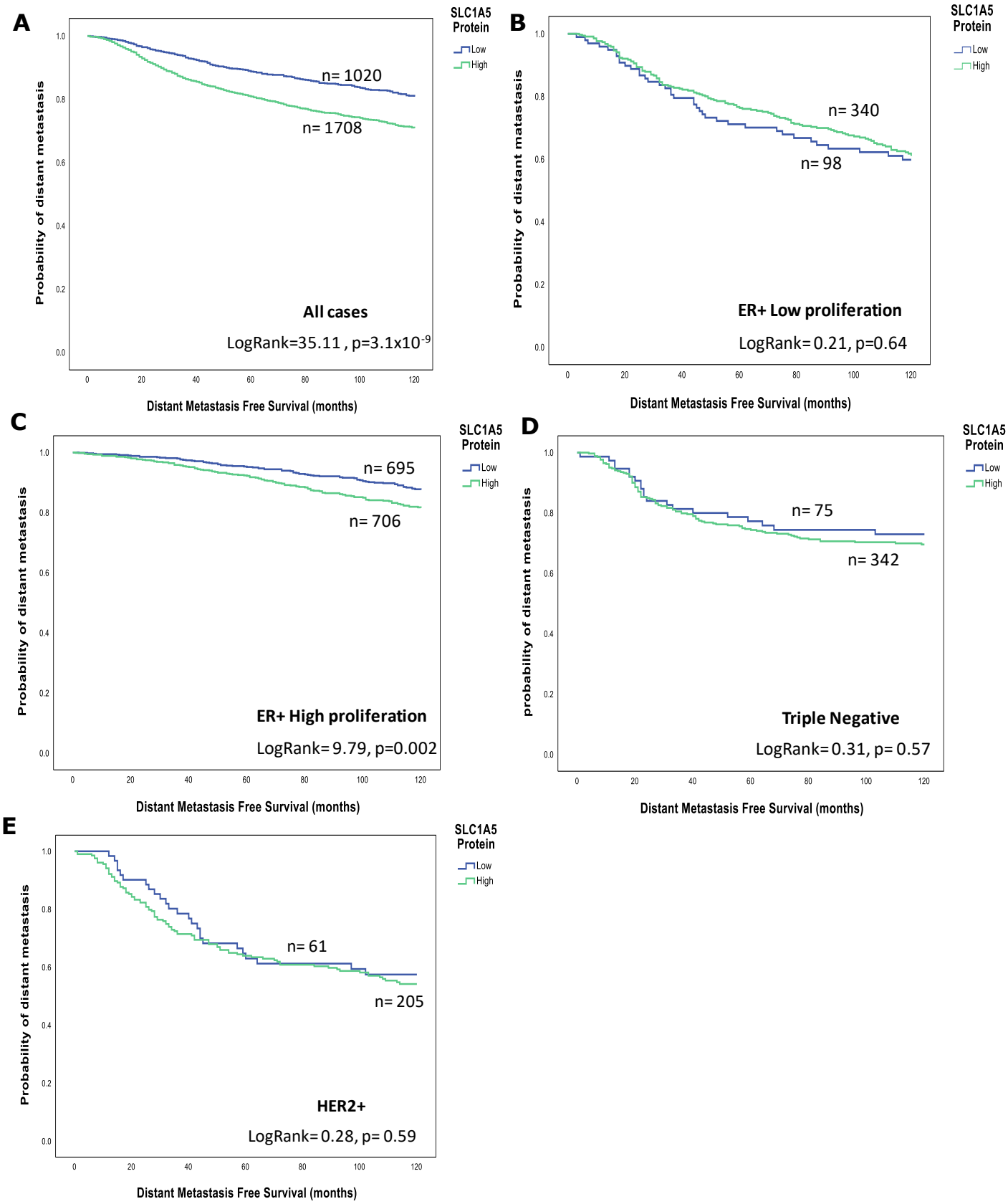


Figure 3-16. SLC1A5 protein expression and distant metastasis. A) SLC1A5 Vs DMFS in all cases, B) SLC1A5 Vs DMFS in ER+- Low Proliferation tumours, C) SLC1A5 Vs DMFS in ER+- High Proliferation tumours, D) SLC1A5 Vs DMFS in Triple negative tumours, E) SLC1A5 Vs DMFS in HER2+ tumours.

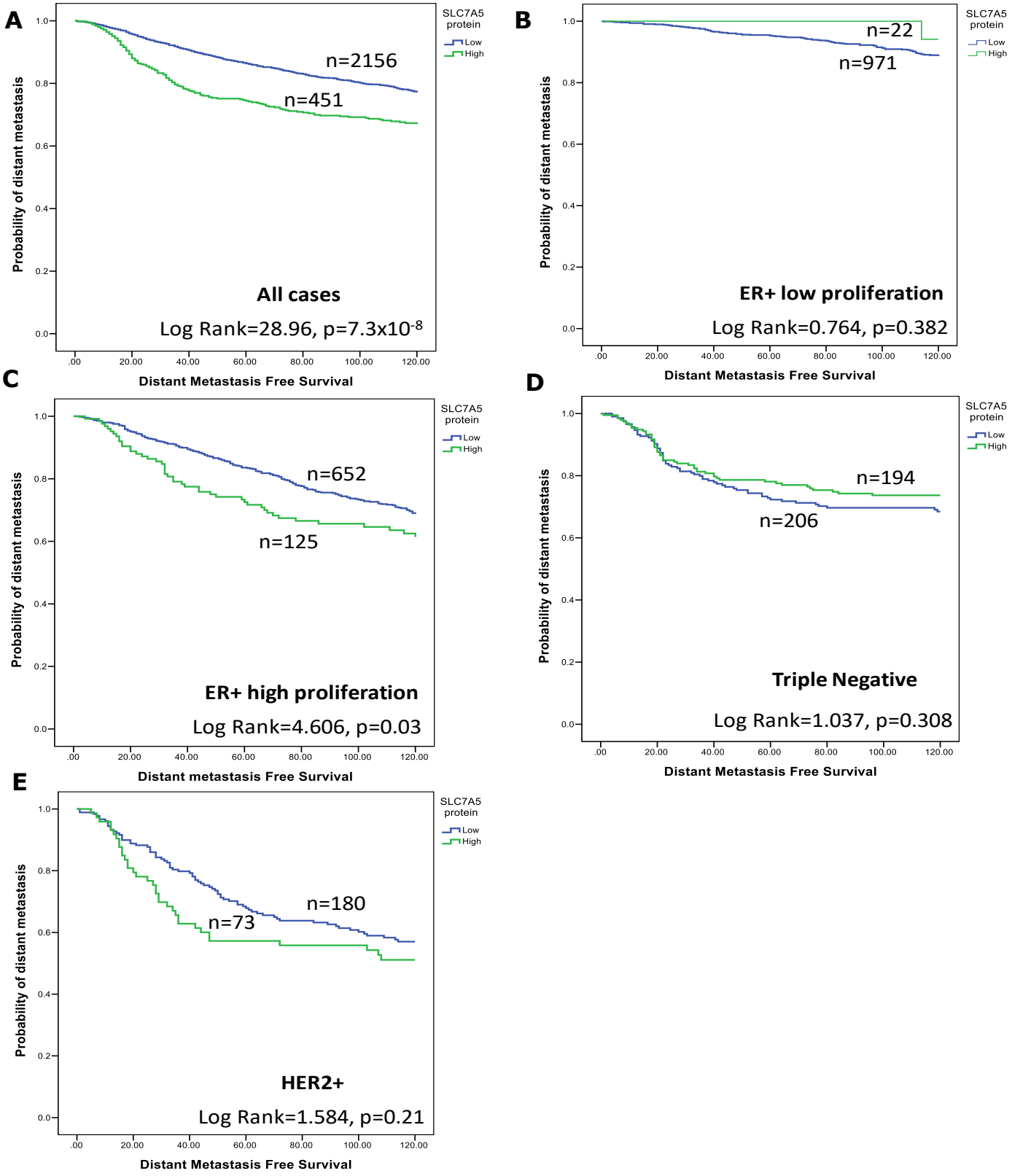


Figure 3-17. SLC7A5 protein expression and distant metastasis. A) SLC7A5 Vs DMFS in all cases, B) SLC7A5 Vs DMFS in ER+- Low Proliferation tumours, C) SLC7A5 Vs DMFS in ER+- High Proliferation tumours, D) SLC7A5 Vs DMFS in Triple negative tumours, E) SLC7A5 Vs DMFS in HER2+ tumours.

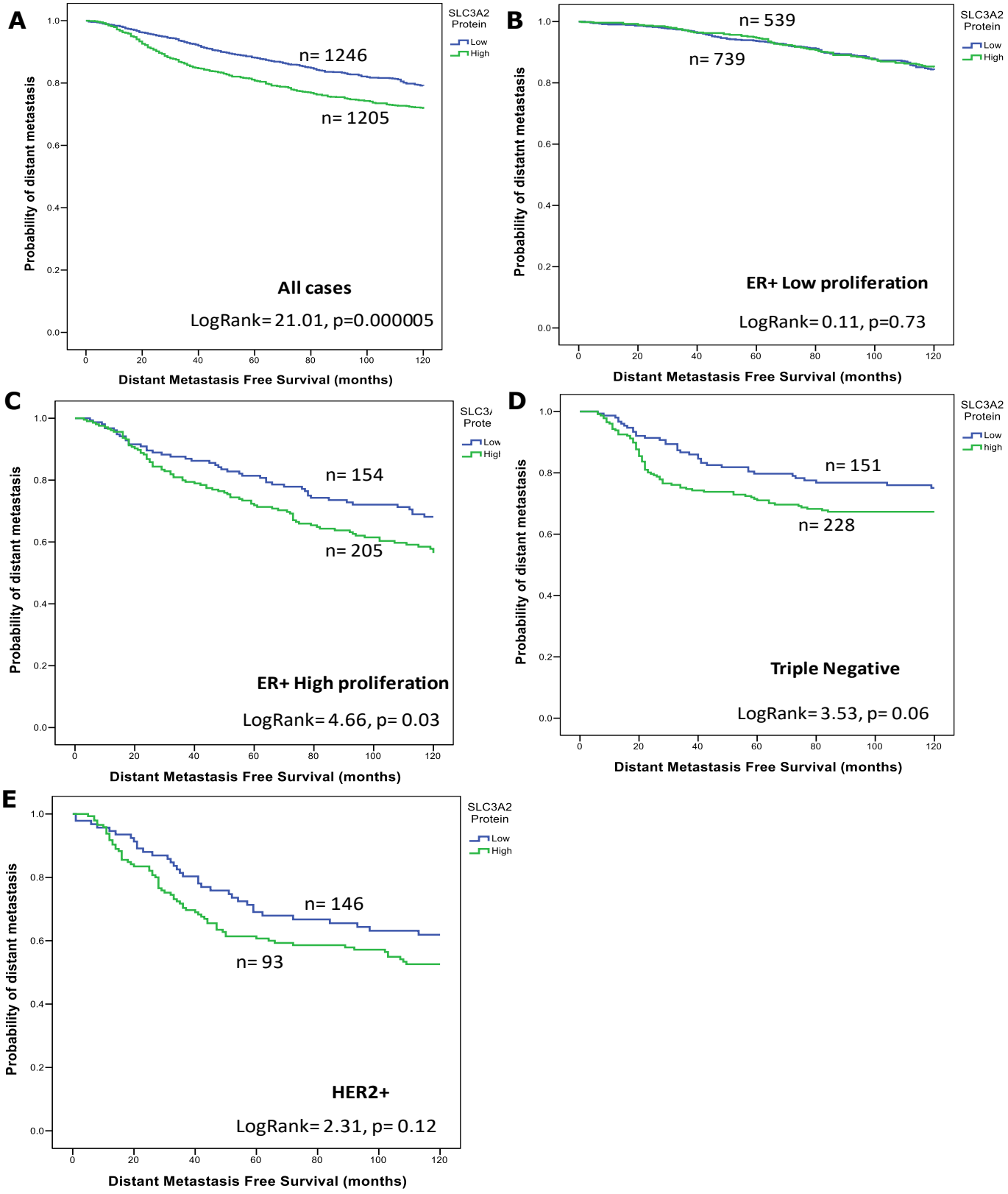


Figure 3-18. SLC3A2 protein expression and distant metastasis. A) SLC3A2 Vs DMFS in all cases, B) SLC3A2 Vs DMFS in ER+- Low Proliferation tumours, C) SLC3A2 Vs DMFS in ER+- High Proliferation tumours, D) SLC3A2 Vs DMFS in Triple negative tumours, E) SLC3A2 Vs DMFS in HER2+ tumours.

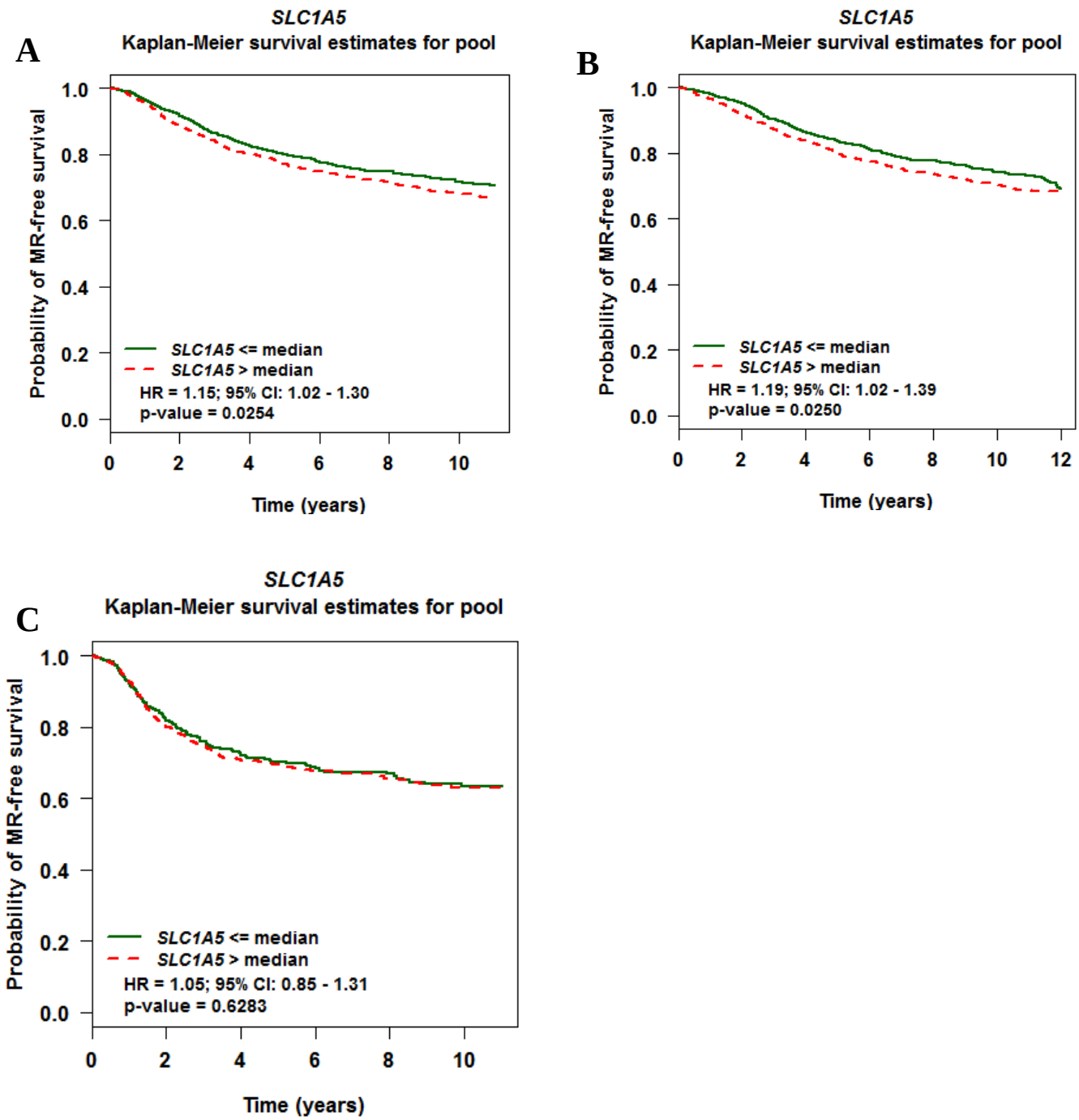


Figure 3-19. SLC1A5 mRNA and breast cancer patient outcome using Breast Cancer Gene-Expression Miner: A) Unselected cases, B) ER+ disease, C) ER- disease.

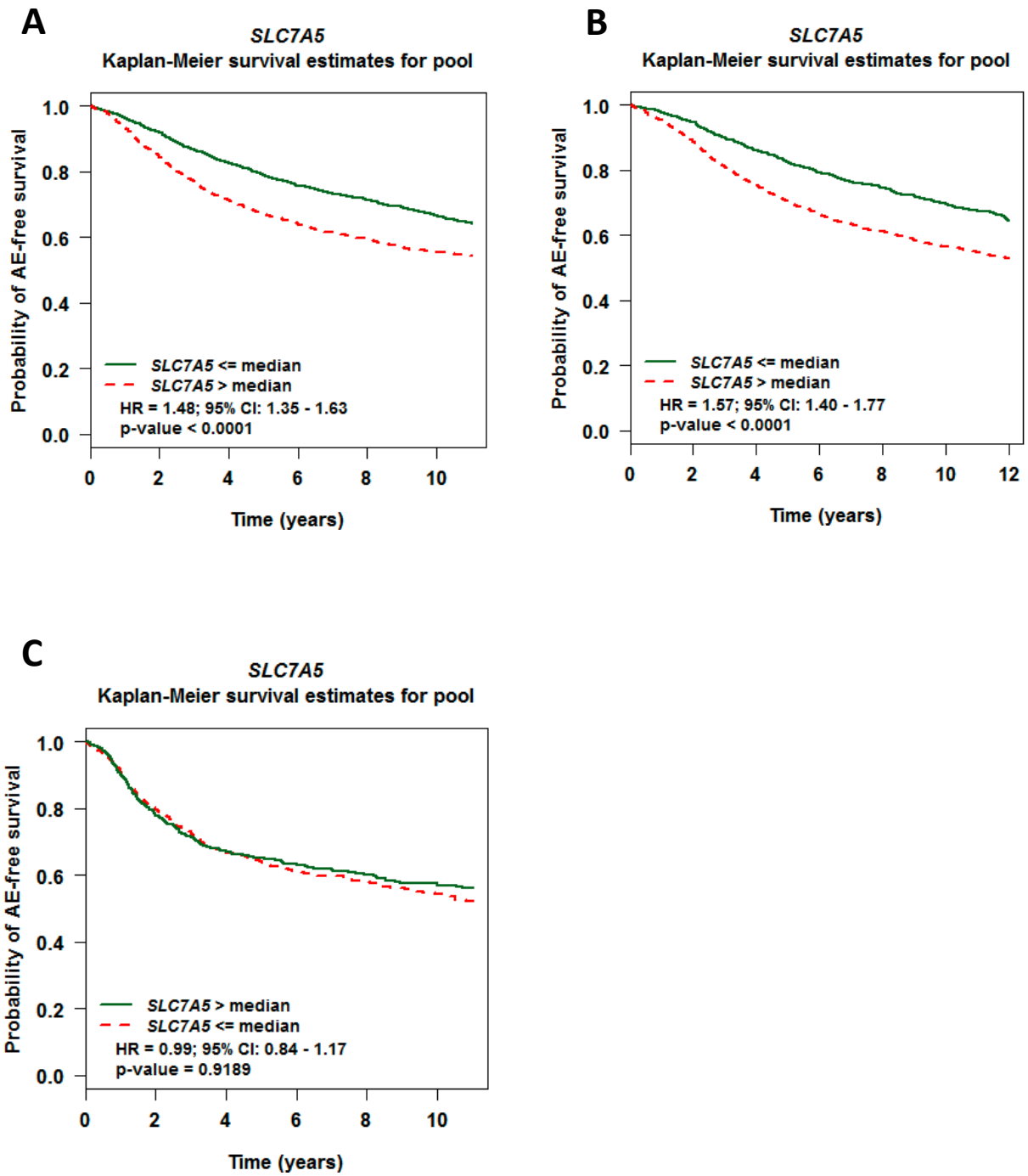


Figure 3-20. SLC7A5mRNA and breast cancer patient outcome using Breast Cancer Gene-Expression Miner: A) Unselected cases, B) ER+ disease, C) ER- disease.

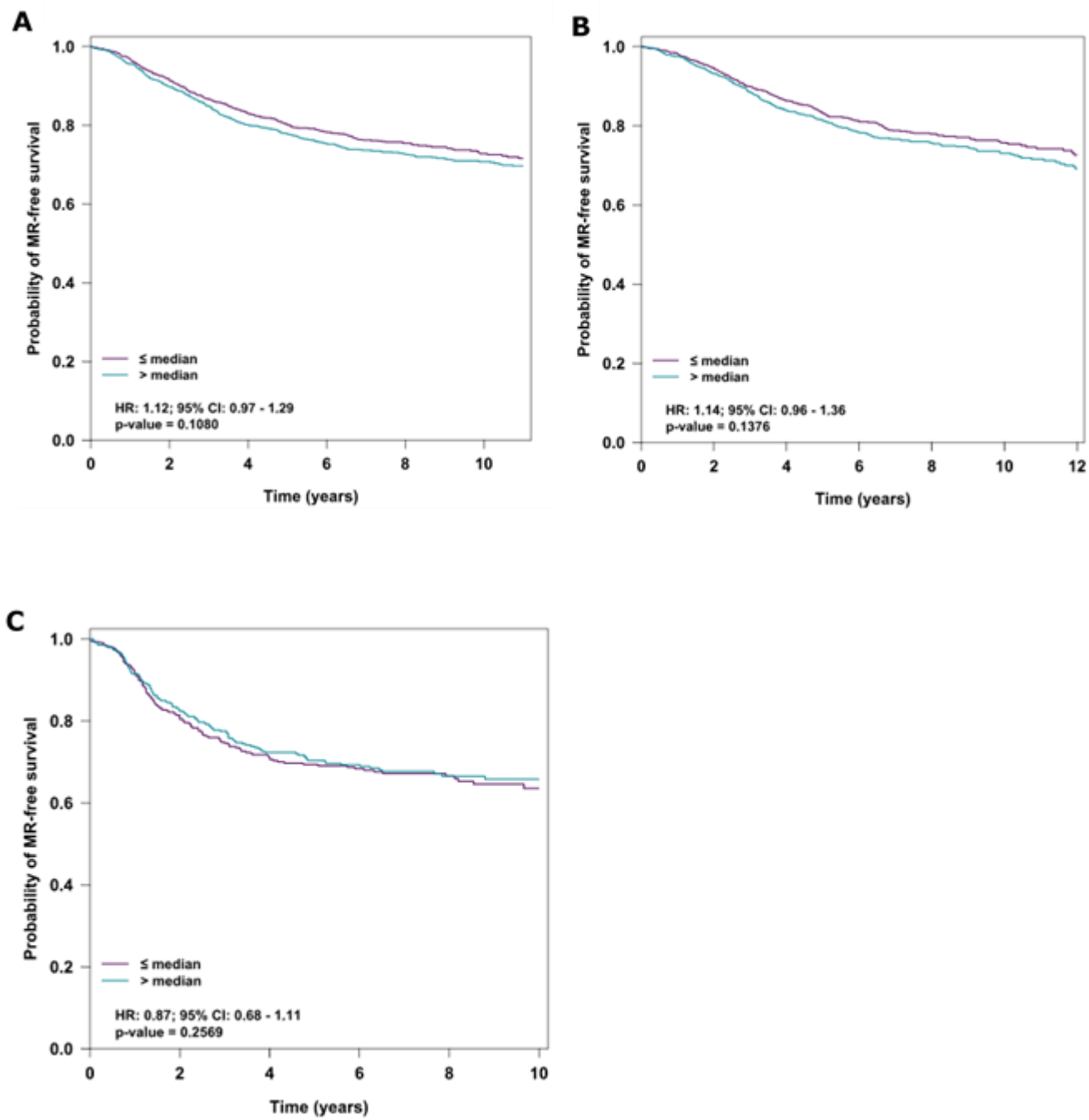


Figure 3-21. SLC3A2 mRNA and breast cancer patient outcome using Breast Cancer Gene-Expression Miner: A) Unselected cases, B) ER+ disease, C) ER- disease.

3.6 Discussion

Altered metabolic pathways in cancer cells is essential to satisfy the demands of necessary energy and cellular building blocks. Many studies have emphasised the role of amino acid transporters in patronising the growth and proliferation of cancer cells through supplying the required amino acids for protein synthesis as well as activation of mTORC1 complex. Furthermore, it has been reported that tumour cells are reliant on glutamine metabolism and become "addicted" to this amino acid for sustained proliferation and survival. However, studies that address the prognostic significance of the key amino acid transporters (SLC1A5, SLC7A5 alongside SLC3A2) in BC and their potential influence on different subtypes remain limited particularly in luminal BC. Therefore, this study investigated large cohorts of BC cases in order to unravel the potential role of SLC1A5, SLC7A5 and SLC3A2 mRNA and protein expressions in BC and its molecular subtypes.

3.6.1 SLC1A5

Consistent with previous studies demonstrating the poor prognostic impact of SLC1A5 expression in many cancers [198,200,255], the results showed that SLC1A5 mRNA and protein are over-expressed in tumours with poor prognostic variables including larger tumour size, higher tumour grade and positive nodal metastasis. With respect to BC molecular classes, high expression of SLC1A5, at both mRNA and protein levels, was associated with ER+ highly proliferative/luminal B, HER2+ and TN tumours while it was less frequent in the ER+ low proliferative/luminal A subtype. This is in agreement with another study which revealed lowest levels of SLC1A5 within the luminal A tumours [136]. Furthermore, our results showed that SLC1A5 over-expressing cases had shorter BCSS and DMFS. Moreover, higher proportions of cases suffered from bone or liver metastatic recurrence tended to overexpress SLC1A5 than those who developed metastatic recurrence elsewhere. ER+ highly proliferative tumours was the only subtype which showed association with patient outcome, probably due to their heavier energy and amino acid requirements for excessive proliferation.

A previous study in mouse embryonic fibroblasts reported that dysfunction of the tumour suppressor *Rb* and *Rb*-controlled transcription factor *E2F-3* altered glutamine uptake through the direct regulation of *SLC1A5* mRNA and protein expression [113]. In the line, results of this study indicated that loss of *Rb*

activates *SLC1A5* transcription, which was observed in the all BC molecular subtypes. In addition, *PIK3CA* seemingly has the potential to influence glutamine uptake through the upregulation of *SLC1A5*, particularly in luminal and TN tumours, but not the HER2+ subtype. Cancer cells evolve adaptive mechanisms to respond to amino acid deprivation via activation of amino acid response signal transduction pathway, which is mediated by *ATF4*-dependent transcription [256]. In this study, direct correlation between *ATF4* and *SLC1A5* mRNA expression was observed in the more aggressive and highly proliferative BC subtypes; luminal B, HER2+ and TN tumours, which implicates the ability of these subtypes to sustain growth and proliferation despite the challenges faced due to fluctuation in dietary nutrients.

Previous studies have raised awareness and revealed the importance of the Proline-Glutamine (Pro-Gln) regulatory axis in BC [167]. Acquisition of glutamine via SLC1A5 undoubtedly is important, but proline metabolism may be an alternative glutamine source. This study showed that luminal A is the only subtype which has significant association with *PRODH*, which involved in the conversion of proline to glutamine. Furthermore, this subtype was the only that correlates with *GLUL*, indicating that luminal A could depend on intracellular glutamine synthesis rather than uptake through SLC1A5. In this context, the association with transporters which mediate glutamine influx was primarily observed in TN and luminal B subtypes and to lesser extent in luminal A tumours. This can explain the findings of Geldermalsen *et al*, who showed that the TN HCC1806 cell growth was affected by glutamine deprivation when SLC1A5 is blocked with gamma-l-glutamyl-p-nitroanilide (GPNA) while minimal effect was observed in the luminal-A like MCF7 cells [135]. The correlation between *SLC1A5* and *GLUD1*, which is involved in the conversion of glutamine to α-KG for the TCA cycle and subsequent gluconeogenesis, in luminal A tumours, suggests that glutamine is utilised for this process. Regarding SLC7A5, which mediates glutamine efflux, it seems to be functionally coupled with SLC1A5 to allow the potential transport of glutamine in and out of tumour cells. The co-expression of SLC1A5 and SLC7A5 in all BC subtypes suggests that they have a key role in glutamine transport.

The effect of blocking SLC1A5 on tumour growth in basal-like TNBC but not in luminal A tumours was previously demonstrated [135]. However, the consequences of blocking the action of SLC1A5 in luminal B tumours remain

undetermined, this study suggests that SLC1A5 could potentially be used as a target in luminal B tumours to reduce glutamine uptake and thus cell proliferation and growth.

3.6.2 SLC7A5

SLC7A5 is widely expressed in many human cancers and various cancer cell lines [257]. The current study involved large BC cohorts to affirm the significant association between high SLC7A5 mRNA and protein expressions and the poor clinico-pathological variables such as larger tumour size, positive nodal involvement and higher tumour grade. As per another study, high expression of SLC7A5 was observed predominantly in TNBC and HER2+ tumours [140]. Nevertheless, in these subtypes the significant association between SLC7A5 protein expression and patient outcome was only observed in the HER2+ tumours. Noteworthy, within ER+ subtypes, SLC7A5 expression was lower in ER+ tumours that have low proliferation /luminal A subtype compared to the highly proliferative ER+ /luminal B tumours, and it was associated with shorter BCSS and DMFS in the latter class only. Furthermore, among all BC subtypes, SLC7A5 protein expression was an independent predictor for short BCSS in the ER+ high proliferation tumours only. The role of SLC7A5 in metastasis is well established in many cancers [258]. In addition, Kaira *et al* demonstrated that SLC7A5 is co-expressed with vascular endothelial growth factor (VEGF) in primary and metastatic sites of many cancers, which could be implicated in the metastatic process [254]. In the current study, the most significant positive correlation between mRNA expression of SLC7A5 and VEGFA was observed in the luminal B tumours.

Previous studies have detailed about the regulation of the amino acid transporter, *SLC7A5*, by other genes including *ATF4*, *MYC* and *HIF2a* and this study confirmed the positive correlation with these genes. However, for the last two genes, the correlation remained significant only in luminal B tumours, when different BC subtypes were examined. The positive associations with *SLC7A5* expression, which is confined to luminal B tumours, might underscore the poor prognostic impact of SLC7A5 on this specific BC subtype.

Intracellular leucine that is taken up via SLC7A5, is sequestered by Sestrin2 causing an increase of free GATOR2 with consequent activation of mTOR signalling

[259,260]. This study also showed a positive correlation between *SLC7A5* and *mTOR* at the mRNA level. However, the protein levels of SLC7A5 showed discrepant results, where high SLC7A5 expression was associated with lower levels of the mTORC1 phosphorylated at ser (2448), which was included in this study. In line with that, a previous study confirmed that phosphorylation of mTORC1 at ser (2448), which is stimulated by growth factors, was mutually exclusive with mTORC1 phosphorylated at thr (2446), which is regulated by amino acids [261]. Alternatively, this can be explained based on Figueiredo *et al* findings which demonstrated that p-mTORC1 at Ser (2448) is an inadequate measure for mTORC1 kinase activity, as it is targeted by the downstream effectors in a negative feedback loop mechanism, and the substitutional should be the downstream and effector proteins, such as p70S6K [262]. Our results showed that the latter protein is highly expressed in tumours with high SLC7A5 expression.

In this study, the association with other glutamine transporters was variable across different BC subtypes. However, the positive correlation with SLC1A5 was consistent among all of them, suggesting a functional coupling between the key glutamine transporters which allows for cellular influx and efflux of glutamine, as SLC1A5 mediates glutamine uptake, while SLC7A5 uses intracellular glutamine concentrations to adjust essential amino acid cytoplasmic pool for metabolic requirements and activation of mTORC1 [148].

SLC7A5 appeared to be co-expressed with the enzymes involved in the (Pro-Gln) regulatory axis. In particular, SLC7A5 was associated with the enzymes which mediate glutamate degradation to form the amino acid proline, which has been shown to play a role in assisting tumour growth by different mechanisms [129].

3.6.3 SLC3A2

SLC proteins are related to tumourigenesis and drug resistance in cancer cells [263] and SLC3A2 is characterised by its diverse effect to promote cancer cell growth and survival. Besides its role in regulating the function of amino acid transporter system, it modulates integrin-induced signal transduction, which promotes malignant tumour cells' behaviour, including cell spreading and migration [248].

The present study revealed the significant association between the high SLC3A2 mRNA and protein expressions and the poor prognostic clinicopathological

parameters. Furthermore, high SLC3A2 expression was significantly associated with proliferation. This supports the results of previous studies, which reported that these, SLC3A2 and Ki67, are significantly correlated in non-small-cell lung cancer and hypo-pharyngeal squamous cell carcinoma [264,265] confirming that SLC3A2 is most likely critical for proliferation in cancer cells.

Similar to SLC1A5 and SLC7A5, SLC3A2 showed lower expression in ER+ tumours that have low proliferation compared with the highly proliferative ER+ tumours, and it was associated with poor patient outcome in the latter class only. This is doubtlessly attributable to the role that SLC3A2 provides for the more aggressive luminal tumours, in particular supplying the amino acids necessary for growth and proliferation.

SLC3A2 mRNA and protein were also highly expressed in TNBC and HER2+ tumours, in concordance with Furuya *et al*. [140] However, the significant association between SLC3A2 protein expression and patient outcome was only restricted to TNBC. In this regard, it has been shown that over-expression of SLC3A2 is actively involved in the proliferation of vascular smooth muscle cells and is necessary for efficient angiogenesis [266,267]. In this study, ER+ high-proliferation and TN tumours showed the most significant positive correlation between the mRNA expression of SLC3A2 and vascular endothelial growth factor B (VEGFB), which maintains the continuity of angiogenesis and thus implicated for the metastatic process [268].

Generally, the association with patient outcome was observed at the protein, but not the mRNA, level. This can be attributed to the post-translational modification, N-glycosylation, of the SLC3A2 protein, which is required to make this protein functioning as it is renowned that the glycosylated SLC3A2 (~80 kDa) is necessary to form the heterodimeric complex, which further assists the amino-acid transport function.

Regarding association with other related biomarkers, a positive relationship was observed, at both mRNA and protein levels, between SLC3A2 and its regulator, MYC, and this correlation was observed in all BC subtypes. The heavy chain of SLC3A2 forms a disulphide bond with the light chain of a group of amino-acid transporters mediating their functions in the plasma membrane. This study revealed the depth of the positive correlation between SLC3A2 and SLC7A5, which

remained significant in almost all subtypes, apart from luminal A tumours. This study further investigated the association of SLC3A2 expression with other glutamine transporters, which provide the substrate, glutamine, required to operate the SLC3A2-SLC7A5 heterodimeric complex and GLS, which converts glutamine to glutamate, the substrate needed for SC3A2-SLC7A11 function. Although the mRNA showed a negative correlation with most glutamine transporters and GLS, the high SLC3A2 protein expression was associated with the higher levels of SLC1A5, SLC7A5 and GLS, indicating a system of functional coupling between these biomarkers at the protein level.

The role of the tumour microenvironment is well known with respect to disease development and progression, and SLC3A2 appears to have a role in this, as the SLC3A2 heavy chain binds to the cytoplasmic tail of integrin β1, which in turn mediates extracellular matrix remodelling that controls cell spreading, survival and growth [269,270]. The SLC3A2 interaction with integrin is well studied in renal cancer cell [248] and the current study also confirmed the positive correlation between gene expressions in all BC subtypes.

A recent study reported that SLC3A2 influences osteosarcoma growth through the PI3K/AKt signalling pathway [244] and this could be the case in BC as demonstrated by our finding that high levels of SLC3A2 protein is positively associated with PIK3CA expression at the mRNA and protein levels. Therefore, it appears that all functions of SLC3A2 in BC were associated with poor prognosis and would not be in favour of patients with aggressive subtypes.

Altogether, this study revealed that over expression of SLC1A5, SLC7A5 and SLC3A2, at both mRNA and protein levels, is linked to ER negative status and poor clinicopathological parameters, including larger tumour size, higher tumour grade and positive nodal metastasis. The three solute carriers share the character of being predictive of shorter BCSS and DMFS only in the ER+ highly proliferative/luminal B tumours but not in the other subtypes. Additionally, SLC1A5, SLC7A5 and SLC3A2 were significantly associated with the oncogene, MYC, and other glutamine related enzymes and transporters. Therefore, these solute carriers could be utilised as potential prognostic markers. Functional assessment is necessary to reveal the specific role played by these solute carriers

in the aggressive forms of BC and to explore their potentiality as targets for BC treatment.

Chapter 4. The combined expression of solute carriers is associated with a poor prognosis in highly proliferative ER+ breast cancer

4.1 Introduction

Cancer cells, including BC, require a constant supply of nutrients to support their growth and proliferation. To do so, they need to elevate the expression levels of nutrient transporters in the plasma membrane, a process which has a strong relationship with tumorigenesis and drug resistance [263]. Amino acids considered one of the most important nutrients for survival and progression of cancer. However, amino acid homeostasis is a complex and intricate network, in which more than one transporter or enzyme should be integrated to act in favour of tumour progression. Among the many glutamine transporters identified in human physiology [142], at least two transporters have emerged as playing a critical role in the control of cancer growth and aggressiveness, considering their increased level of expression and correlation with poor disease prognosis. These include the bidirectional essential amino acid transporter complex, SLC7A5/SLC3A2, and the potentially functionally coupled SLC1A5. It has been suggested that glutamine influx by SLC1A5 triggers essential amino acid entry via SLC7A5-SLC3A2 exchanger. These exchange mechanisms balance the cytoplasmic pool of the amino acids without expending energy and therefore give a strong advantage for cancer cell growth and proliferation [193].

Although a large number of biomarkers have been suggested to be prognostic markers in breast cancer, other studies have demonstrated that combination of prognostic indicators is superior to recruiting single markers [167,271,272]. Therefore, applying cluster analysis is not only competent for identifying biologically relevant groups of biomarkers, but it can also allow more detailed prognostication and potentially provide optimal treatment strategies with the objective of improving patient outcome and quality of life.

In the previous chapter, it has been demonstrated that the solute carriers SLC1A5, SLC7A5 and SLC3A2 have an important prognostic role in BC, as they were associated with poor prognosis particularly in the ER+ high proliferative BC subtype [273,274]. Since these key transporters act complementary to each other, at the functional level, it is important to highlight the prognostic significance of the co-operative expression of the three solute carriers, taking into account their influence on molecular BC subtypes.

4.2 Hypothesis

The solute carriers SLC1A5, SLC7A5 and SLC3A2 are co-localised in BC, they are functionally coupled, in order to equilibrate the cytoplasmic amino acid pool, and their co-expression is associated with a poor prognosis, with differential effects in the molecular BC subtypes.

4.3 Aims

1. To determine whether SLC1A5, SLC7A5 and SLC3A2 are co-localised.
2. To stratify BC patients in to accredited clusters based on assessment of SLC1A5, SLC7A5, and SLC3A2 protein expression in a large BC cohort.
3. To assess the co-operative expression of the solute carriers, SLC1A5, SLC7A5, and SLC3A2 in the generated clusters and evaluate their association with clinicopathological parameters and relevant biological markers.
4. To determine their prognostic potential with emphasis on the different BC molecular subtypes.

4.4 Methods

4.4.1 Study cohort

The Nottingham BC cohort was used in this study. This cohort is a well-characterised series of early-stage, primary operable, invasive BC patients (n = 2248). Clinical history, tumour characteristics and outcomes are prospectively maintained and regularly updated. Outcome data included development and time to distant metastasis and BC-specific death. The clinicopathological characteristics of this cohort are detailed in section 2.1.3.

4.4.2 Immunohistochemistry and cluster analysis

To assess co-localisation of SLC1A5, SLC7A5 and SLC3A2, cases showing protein expression data of the three solute carriers were identified from the previous chapter. IHC and assessment of staining in invasive BC cells was carried out as described in section 2.4.4 using serial whole tissue sections. Additionally, SLC1A5, SLC7A5, and SLC3A2 were assessed at the protein level, as described in the previous chapter, using IHC on TMA constructed from the Nottingham BC cohort.

Based on the H-score of SLC1A5, SLC7A5 and SLC3A2, patients were stratified into accredited clusters, applying k-mean and PAM clustering algorithms, as detailed in section 2.6.3, then correlated with clinicopathological parameters, molecular subtypes, and patient outcome.

4.5 Results

4.5.1 Co-localisation of SLC1A5, SLC7A5 and SLC3A2 in BC

Regardless of the intensity of the staining, membranous immunoreactivity of SLC1A5, SLC7A5 and SLC3A2 was homogenous and comparable in most cases (Figure 4-1A-C). There was a positive correlation between the percentage of membranous expression of the three proteins (multiple correlation coefficient=0.67, p=0.001), confirming that they are co-localised in the invasive breast tumour cells.

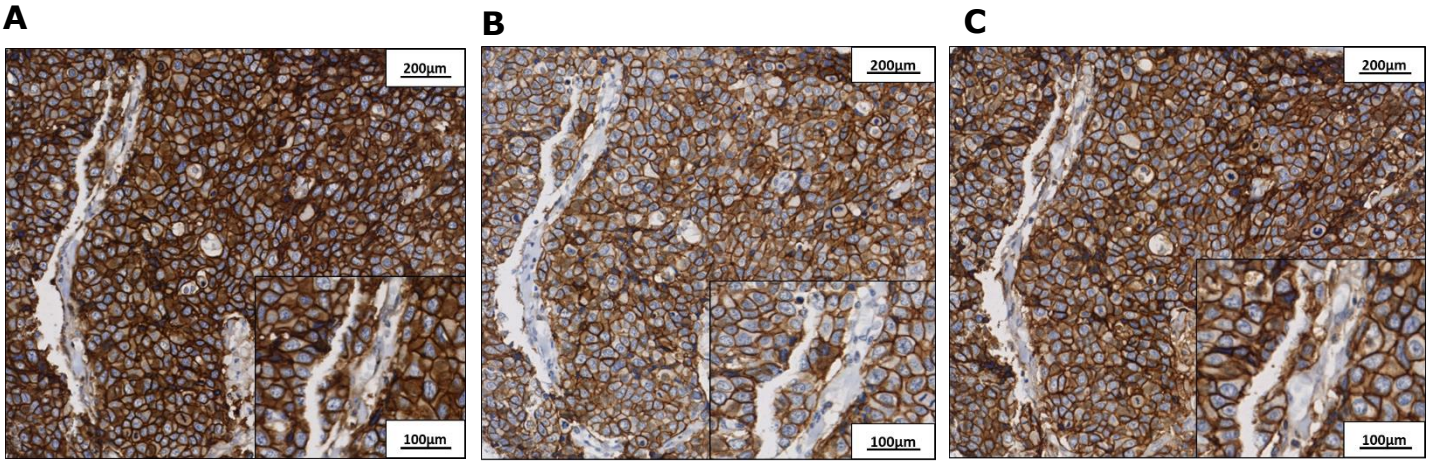


Figure 4-1. Representative images of immunohistochemical staining to evaluate co-localisation of SLC1A5, SLC7A5 and SLC3A2 in serial full face invasive breast cancer sections. A) SLC1A5, B) SLC7A5 and C) SLC3A2. Positive immunoreactivity is detected in the membrane of the invasive breast cancer cells. Magnification 10x. Scale bars=200µm. Inset, magnification 20x. Scale bars=100µm.

4.5.2 Clustering of SLCs expression in breast cancer

To explore the prognostic value of the co-expression of SLC1A5, SLC7A5, and SLC3A2 in invasive BC, unsupervised clustering analysis was applied. Based on several indices that were previously identified in section 2.6.3, acceptable classification was applied by clustering the tumours into three clusters. An appropriate separation between clusters was attained as indicated from the biplots (Figure 4-2). The final three clusters were obtained from the overlap of the two clustering algorithms. Starting from the three clusters of K-means and PAM, those

cases that were classified in the same group by the two different methods were considered. By doing so, 1722 cases were clustered into one of the common groups, while 526 remained unclassified (NC). The three clusters were characterised as follows: low SLCs (SLC1A5−/SLC7A5−/SLC3A2−), high SLC1A5 (SLC1A5+/SLC7A5−/SLC3A2−), and high SLCs (SLC1A5+/SLC7A5+/SLC3A2+) (Figure 4-3).

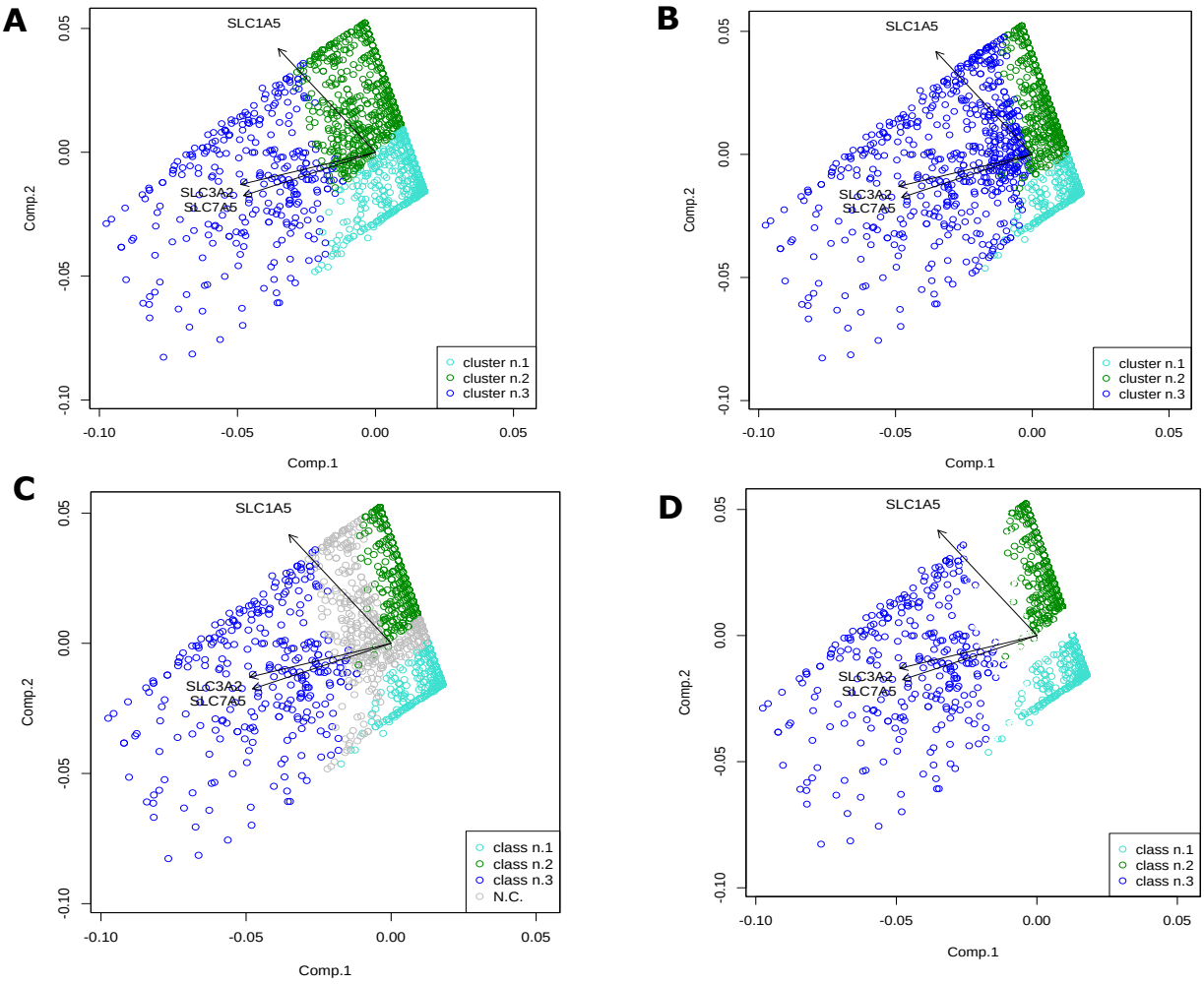


Figure 4-2. Biplots of clusters projected on the first and second principle component axes: A) K-means clustering, B) PAM clustering, C) Final clusters including the unclassified cases, D) Final (interesting) clusters; N.C., Not Classified.

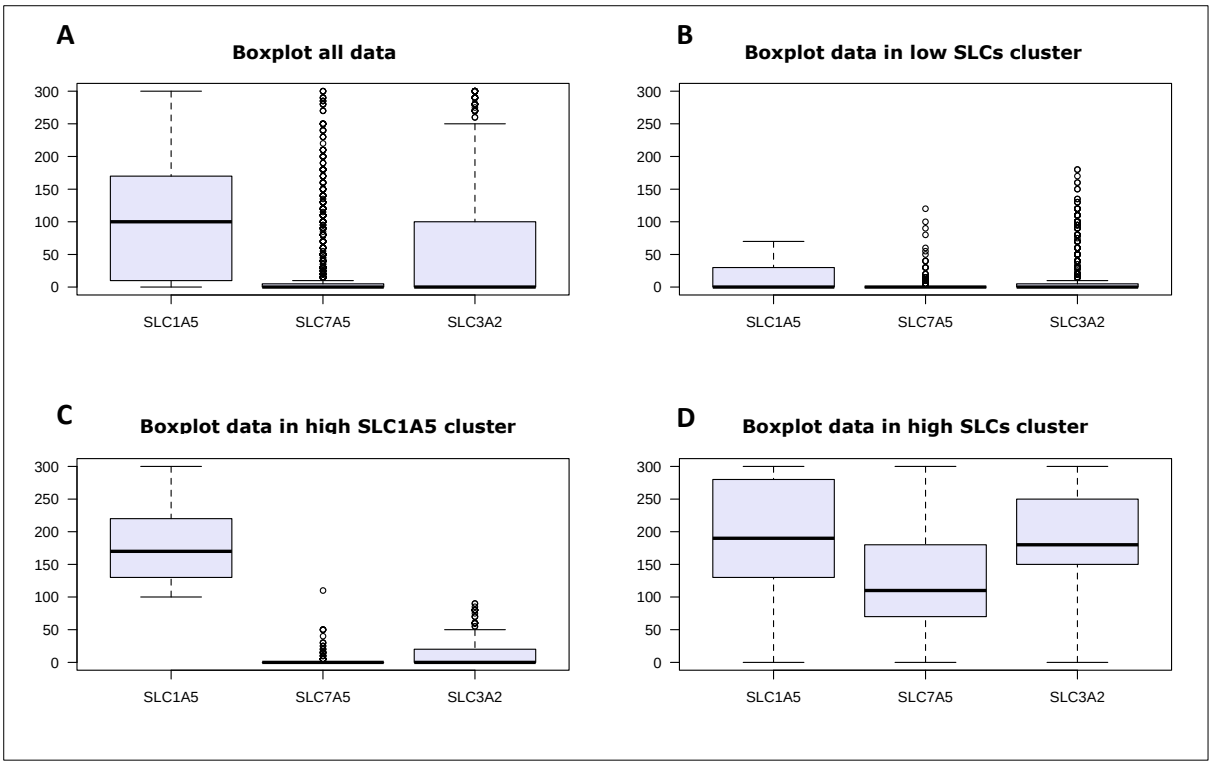


Figure 4-3. Cluster analysis of SLC1A5, SLC7A5 and SLC3A2 in breast cancer. Boxplots showed the distribution of the SLCs protein expression in A) all data, B) Low SLCs cluster, C) High SLC1A5 cluster, D) High SLCs cluster.

4.5.3 SLC clusters correlate with the clinicopathological features and breast cancer subtypes

BCs in the high SLCs and high SLC1A5 clusters were significantly associated with poor prognostic parameters including younger age, larger tumour size, higher histologic grade, high pleomorphism, high mitotic count, less tubular formation, poor NPI, and lymph node stage 3 (all $p < 0.001$, Table 4-1), whereas tumours in the Low SLCs cluster were associated with good prognostic factors ($p<0.001$, Table 4-1). When association with hormone receptor status was examined, both low SLCs and high SLC1A5 clusters showed a significant association with hormone receptor (ER and PgR)-positive tumours, while the high SLCs cluster included mainly tumours with hormone receptor-negative status ($p < 0.001$, Table 4-2). Tumours with HER2 positivity were more frequent in high SLCs and high SLC1A5 clusters compared with low SLCs ($p < 0.001$, Table 4-2). The prevalence of the three clusters in different BC subtypes was varied, where the low SLCs cluster was primarily observed in the ER+ low proliferation/luminal A tumours and the high SLC1A5 cluster was mainly found in the ER+ high proliferation/luminal B and

HER2+ subtypes. In contrast, the poor prognostic TN class was strongly represented in patients having tumours with high SLCs ($p < 0.001$, Table 4-1).

4.5.4 SLC clusters and other molecular biomarkers

The expression of MYC protein was primarily observed in the high SLCs cluster ($p<0.001$, Table 4-2). In addition, high SLC1A5 and high SLCs clusters were mainly associated with positive PIK3CA expression ($p < 0.001$, Table 4-2). The proliferation marker, Ki67, was also significantly expressed in high SLC1A5 and high SLCs clusters ($p < 0.001$, Table 4-2). Conversely, mTORC1 was mainly observed in the low SLCs cluster ($p < 0.001$, Table 4-2) while p70S6K was significantly expressed in the high SLCs and high SLC1A5 clusters ($p<0.001$, Table 4-2).

Table 4-1. Clinicopathological associations of the SLC clusters in breast cancer.

Parameter	Low SLCs n (%)	High SLC1A5 n (%)	High SLCs n (%)	χ2 (p-value)	Adjusted p-value
Age					
<50	267 (47.5)	160 (28.5)	135 (24.0)	27.86 ($8.8x10^{-7}$)	**<0.0001**
≥50	643 (55.8)	349 (30.3)	160 (13.9)		
Tumour size					
<2cm	583 (60.9)	262 (27.4)	112 (11.7)	67.5 ($2.2x10^{-15}$)	**<0.0001**
≥2cm	326 (43.1)	247 (32.7)	183 (24.2)		
Tumour Grade					
1	256 (83.7)	48 (15.7)	2 (0.7)	422.5 ($3.7x10^{-90}$)	**<0.0001**
2	421 (64.5)	205 (31.4)	27 (4.1)		
3	232 (30.8)	256 (34.0)	266 (35.3)		
Mitosis					
1	555 (71.9)	199 (25.8)	18 (2.3)	430.33 ($7.7x10^{-92}$)	**<0.0001**
2	165 (54.8)	105 (34.9)	31 (10.3)		
3	163 (27.0)	196 (32.5)	244 (40.5)		
Pleomorphism					
1	30 (93.8)	2 (6.3)	0 (0.0)	265.49 ($3.5x10^{-56}$)	**<0.0001**
2	438 (76.2)	124 (21.6)	13 (2.3)		
3	417 (38.9)	374 (34.9)	280 (26.1)		

(Continued)

Parameter	Low SLCs n (%)	High SLC1A5 n (%)	High SLCs n (%)	χ2 (p-value)	Adjusted p-value
Tubular formation					
1	96 (80.0)	22 (18.3)	2 (1.7)	131.79 ($1.6x10^{-27}$)	**<0.0001**
2	342 (63.1)	162 (29.9)	38 (7.0)		
3	447 (44.0)	316 (31.1)	253 (24.9)		
Vascular invasion					
Negative	217 (41.7)	189 (36.3)	115 (22.1)	39.12 ($3.1x10^{-9}$)	**<0.0001**
Positive	689 (58.0)	319 (26.9)	180 (15.2)		
Lymph Node Stage					
1	613 (57.0)	286 (26.6)	176 (16.4)	21.8 (0.0002)	**0.0004**
2	236 (48.2)	165 (33.7)	89 (18.2)		
3	60 (40.5)	58 (39.2)	30 (20.3)		
Nottingham Prognostic Index					
Good	443 (75.5)	132 (22.5)	12 (2.0)	241.5 ($1.06x10^{-47}$)	**<0.0001**
Moderate	381 (44.3)	279 (32.4)	201 (23.3)		
Poor	84 (31.8)	98 (37.1)	82 (31.1)		
IHC Subtypes					
ER+/HER2- Low Proliferation	639 (68.9)	259 (27.9)	30 (3.2)	504.4 ($9.4x10^{-106}$)	**<0.0001**
ER+/HER2- High Proliferation	88 (34.0)	112 (43.2)	59 (22.8)		
Triple Negative	59 (22.2)	54 (20.3)	153 (57.5)		
HER2+	55 (39.3)	51 (36.4)	34 (24.3)		

P values in bold means statistically significant

Table 4-2. Associations of the SLC clusters with the expression of other molecular biomarkers in breast cancer.

Parameter	Low SLCs n (%)	High SLC1A5 n (%)	High SLCs n (%)	χ2 (p-value)	Adjusted p-value
ER					
Negative	80 (23.3)	77 (22.4)	186 (54.2)	418.6 ($1.2x10^{-91}$)	**<0.0001**
Positive	825 (60.4)	432 (31.6)	109 (8.0)		
PgR					
Negative	521 (50.6)	253 (24.6)	255 (24.8)	114.8 ($1.3x10^{-25}$)	**<0.0001**
Positive	358 (57.2)	237 (37.9)	31 (5.0)		
HER2					
Negative	809 (54.3)	433 (29.1)	247 (16.6)	12.2 (0.002)	**0.004**
Positive	55 (39.3)	51 (36.4)	34 (24.3)		
Triple Negative					
No	839 (59.1)	446 (31.4)	134 (9.4)	369.1 ($7.1x10^{-81}$)	**<0.0001**
Yes	59 (22.1)	55 (20.6)	153 (57.3)		
MYC					
Negative	270 (59.7)	103 (22.8)	79 (17.5)	44.6 ($2.0x10^{-10}$)	**<0.0001**
Positive	34 (38.6)	10 (11.4)	44 (50.0)		
PIK3CA					
Negative	94 (76.4)	14 (11.4)	15 (12.2)	24.8 (0.000004)	**<0.0001**
Positive	182 (50.7)	81 (22.6)	96 (26.7)		
Ki67					
Negative	142 (78.5)	22 (12.2)	17 (9.4)	50.5 ($1.08x10^{-11}$)	**<0.0001**
Positive	159 (46.5)	83 (24.3)	100 (29.2)		
p-mTORC1					
Negative	141 (48.3)	66 (22.6)	85 (29.1)	21.1 (0.00002)	**<0.0001**
Positive	143 (66.5)	43 (20.0)	29 (13.5)		
P70S6K					
Negative	318 (53.3)	190 (31.8)	89 (14.9)	40.62 ($1.5x10^{-9}$)	**<0.0001**
Positive	43 (27.4)	63 (40.1)	51 (32.5)		

P values in bold means statistically significant

4.5.5 High SLCs is associated with poor patient outcome in breast cancer

Clusters of the three solute carriers were significantly associated with patient outcome, where patients with tumours showing high SLCs had the worst survival compared to tumours with high SLC1A5, which had moderate outcome and low SLCs which showed the best survival ($p < 0.001$, Figure 4-4A). Within the different molecular subtypes, the high SLCs cluster was predictive of shorter BCSS but only in the ER+ high proliferative/luminal B tumours ($p = 0.01$, Figure 4-4C). There was no association between the SLC clusters and outcome in the ER+ low proliferative/luminal A, HER2+, or TN tumours (all $p>0.05$, Figure 4-4B, D, E).

There was a similar observation regarding the association of SLC clusters with DMFS, where patients in the high SLCs cluster had significantly shorter DMFS compared with the other clusters ($p < 0.001$, Figure 4-5A). Similarly, the result was only observed in the ER+ high proliferative/luminal B class ($p = 0.01$, Figure 4-5C). There was no association with the SLC clusters in predicting DMFS in the other subtypes (all $P>0.05$, Figure 4-5B, D, E). In multivariate Cox regression analysis, the SLC clusters remained predictor of shorter BCSS independent of tumour size, histologic grade, and lymph node stage in unselected tumours ($p = 0.001$, Table 4-3A). The result remained significant in the ER+ high proliferative/luminal B tumours only ($p = 0.01$, Table 4-3B).

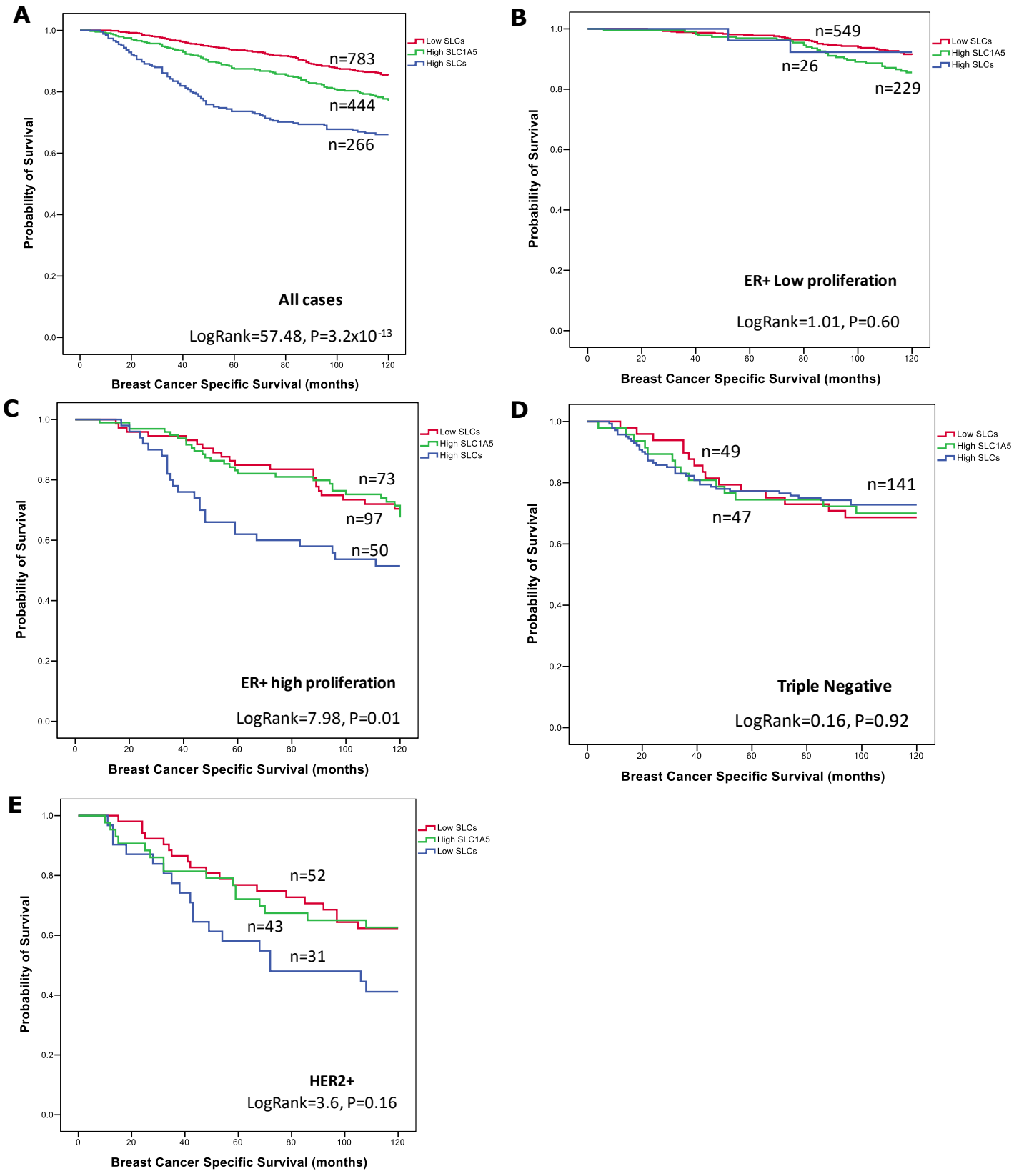


Figure 4-4. SLC clusters and breast cancer patient outcome in A) All cases, B) ER+- low proliferation tumours, C) ER+- high proliferation tumours, D) Triple negative tumours, E) HER2+ tumours.

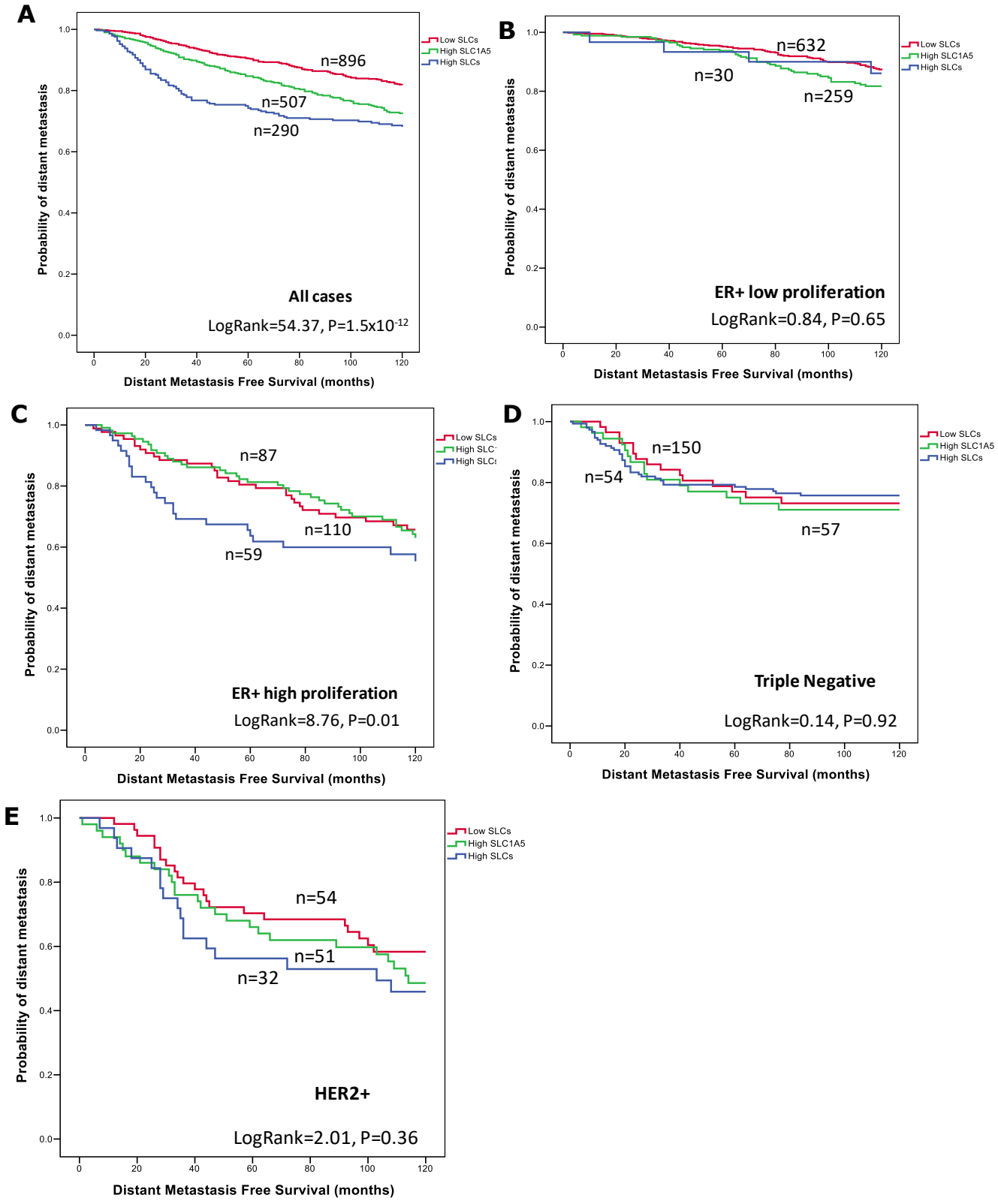


Figure 4-5. SLC clusters vs Distant metastasis free survival in A) all cases, B) ER+- low proliferation tumours, C) ER+- high proliferation tumours, D) Triple negative tumours, E) HER2+ tumours.

Table 4-3A. Multivariate analysis of prognostic variables and the SLC clusters in relation to BCSS.

Variable	Hazard ratio (95% CI)	p-value
SLC clusters	1.4 (1.1-1.7)	**0.001**
Tumour Size	1.3 (0.9-1.8)	0.13
Nodal stage	2.1 (1.7 -2.5)	$1.9x10^{-13}$
Grade	4.2 (2.8-6.2)	$1.5x10^{-12}$

P value in bold means SLC clusters significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 4-3B. Multivariate analysis of prognostic variables and SLC clusters, in relation to BCSS, in different BC subtypes.

	ER-low proliferation		ER-High proliferation		Triple Negative		HER2+	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
Clusters	1.11 (0.49-2.45)	0.80	1.38 (1.01-1.90)	**0.01**	0.97 (0.70-1.36)	0.89	1.40 (0.92-2.12)	0.11
Size	0.67 (0.24-1.90)	0.45	1.57 (0.81-3.06)	0.18	1.31 (0.73-2.35)	0.35	1.53 (0.73-3.21)	0.25
Grade	1.95 (0.86-4.42)	0.11	1.51 (0.47-4.77)	0.48	2.31 (0.75-7.04)	0.14	1.28 (0.59-2.76)	0.52
Nodal Stage	3.82 (1.93-7.54)	0.0001	1.66 (1.14-2.41)	0.007	2.43 (1.72-3.44)	$5.2x10^{-7}$	1.88 (1.18-2.98)	0.007

P value in bold means SLC clusters significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

4.6 Discussion

This study has revealed differential expression levels of three solute carriers, SLC1A5, SLC7A5 and SLC3A2, across a large BC cohort which can be used to stratify the patients into three clusters, where the low SLCs cluster did not show expression or had low expression of transporter proteins, high SLC1A5 cluster showed high expression of SLC1A5 only, and the high SLCs cluster included tumours with high expression of all solute carriers. The prognostic value of the three clusters was varied, where patients with tumours in the high SLC1A5 and high SLCs clusters were associated with poor clinicopathological parameters. In contrast, tumours with good prognostic characteristics were predominantly in the low SLCs cluster. It was further established that patients with tumours in the high SLCs cluster had the poorest outcome compared to those cases in the low SLCs or high SLC1A5 clusters. This is anticipated, as the high SLCs cluster embraces the overexpression of the three solute carriers together, where SLC1A5 mediates uptake of glutamine, while the SLC7A5-SLC3A2 heterodimeric complex uses the intracellular glutamine concentrations to adjust the essential amino acid cytoplasmic pool for metabolic demands and the subsequent activation of mTORC1 signalling pathway, suggesting a system of functional coupling between these transporters to confer the more aggressive character to BC cells. It is also known that the SLC7A5-SLC3A2 complex is a thyroid hormone transporter, and recently, Søgaard *et al* have identified an association between hyperthyroidism and increasing BC risk [275].

With respect to the luminal BC subtypes, the three solute carriers were highly expressed in a subset of the highly proliferative ER+/luminal B tumours and were related with poor patient outcome and shorter DMFS. We noted that the single expression of SLC1A5, the driver of high SLC1A5 cluster, was not enough to predict the shorter BCSS in this subtype. To do so, it requires the concurrent expression of SLC7A5 and SLC3A2. Furthermore, we and others have previously demonstrated that SLC7A5 and SLC3A2 are significantly correlated with the proliferative marker Ki67 in thymic carcinomas, tongue cancer, neuroendocrine carcinoma of the lung, non-small cell lung cancer, and BC [276,277,264,255,140,274,273]. In this study, Ki67 was mainly expressed in tumours of high SLC1A5 and high SLCs clusters, indicating that the studied solute

carriers can support cancer cell proliferation, the main characteristic which distinguishes luminal B from luminal A subtypes.

The BC subtypes, TN and HER2+, were also commonly represented in cases demonstrating the high SLCs cluster, in concordance with Kim *et al* [136] and Furuya *et al* [140], who studied the expression levels of SLC1A5 and SLC7A5/SLC3A2, respectively, within the BC molecular subtypes. However, neither the TN nor the HER2+ subtype showed a significant association between the SLC clusters and patient outcome.

The oncogene MYC has a robust influence in tumour cell metabolism through enhancing the uptake and utilisation of the necessary nutrients via upregulation of cellular transporters and enzymes supporting tumour growth and giving rise to aggressive cancer phenotypes [111,251]. In addition, SLC7A5 can control MYC expression and constitute a positive feedback loop mechanism to encourage essential amino acid transport and tumorigenesis [278,279]. The current study revealed that MYC can regulate the concurrent expression of the three studied solute carriers.

Mutations in PIK3CA are known to have a relation to metabolic alterations in cancer cells and a recent work carried out by our group has shown that PIK3CA is the driver for GLS expression, in the luminal B tumours (data unpublished). In this study, we showed that PIK3CA is primarily over-expressed in the high SLC1A5 and high SLCs clusters. Previous research has detailed the role of amino acids in activating mTORC1, which in turn controls cell proliferation and prevents apoptosis in cancer cells [280]. This study, confirms the direct relationship between the high expression of the three solute carriers and the positive expression of the mTORC1 downstream signal, p70S6K.

With the increasing number of treatment strategies available for cancer patients, it is critical to find effective approaches which can support personalisation of care and allow tailored treatment plan for BC patients, taking into consideration its molecular subtypes. Although the consequences of blocking the action of SLC1A5, SLC7A5 and SLC3A2 in ER+ high proliferative tumours remain undetermined, our data suggests that the synergistic lethality targeting the three solute carriers may prove to be more effective in BC treatment.

Elaboration in understanding the biological diversity of BC is essential, with the linked development of classification strategies suitable for clinical use. This study revealed that clustering analysis of SLC1A5, SLC7A5, and SLC3A2 allow stratifying BC patients into three accredited clusters (high SLCs, high SLC1A5 and low SLCs), which had distinct correlations to known prognostic factors and patient outcome. High SLCs cluster was associated with the poor prognostic characteristics and poor patient outcome particularly in the ER+ high proliferative/luminal B subtype. The key regulator of tumour cell metabolism, MYC, was significantly expressed in the high SLC cluster. Further functional assessment is warranted to reveal the specific role played by solute carrier co-expression in the highly proliferative ER+ BC.

Chapter 5. SLC1A5 and SLC7A5 are key therapeutic targets in the highly proliferative breast cancer subtypes; insights from *in vitro* studies

5.1 Introduction

To sustain their persistent proliferation, cancer cells require a constant supply of essential metabolites that uphold cellular biosynthetic and bioenergetic pathways. Amino acids are fundamental metabolites for all rapidly growing cells, as they are the building blocks of proteins required for biomass production. Beside their role in replenishing TCA cycle, amino acids also mediate the synthesis of essential metabolic intermediates such as nucleotides, lipids, glucosamine, polyamines and glutathione [281,282]. Moreover, amino acids, particularly leucine, act as signalling molecules to activate the master regulator of growth and cell metabolism, mTORC1 [283,212].

Most importantly, the need for acquiring amino acids is even more in the highly proliferative and more aggressive tumour types, which are able to grow in challenging environments such as hypoxia, and nutrient deprivation [284]. In order to encounter stressful situations, tumour cells need to evolve an increased ability to express nutrient transporters in the cell membrane; a process which is controlled by different transcriptional pathways including, MYC oncogenic transcription and nutrient starvation responses [251,257]. Overexpression of various amino acid solute carriers is implicated in tumourigenesis and indeed is now considered as a hallmark of cancer metabolism [128].

The transporters for glutamine and essential amino acids, SLC1A5 and SLC7A5, respectively, have emerged as major pro-tumoral transporters that showed increased expression levels in various aggressive cancer types [223,264,198,201]. Therefore, targeting these solute carriers has attracted attention in the field of drug discovery for advancing the treatment of cancers in which these proteins are involved. Detailed information on SLC1A5 and SLC7A5 biology and function has been illustrated in chapter 3.

Furthermore, in chapter 3, it has been demonstrated that SLC1A5 and SLC7A5 are associated with poor patient outcome. Both solute carriers showed significant results within the ER+ highly proliferative/luminal B subtype but not with the low proliferative/luminal A tumours. Furthermore, SLC7A5 also exhibited correlation with poor patient prognosis within the aggressive HER2+ tumours [273].

Blockade of SLC1A5 decreases the growth of various cancer cell lines such as lung cancer [201], prostate cancer [285] acute myeloid leukaemia [286] and TNBC

[135]. Furthermore, it is noted that mice with deleted SLC1A5 are viable and fertile with no obvious pathological phenotype, suggesting that there could be few side effects to this as a therapeutic approach [193]. Inhibition of SLC1A5 can be achieved via the chemotherapeutic reagent paclitaxel, which mediates degradation of this transporter by the ubiquitin ligase RNF5 that plays a critical role in Endoplasmic Reticulum stress (ERS) and unfolded folded protein response (UPR) [287]. SLC1A5 can also physically associates in a molecular complex with epidermal growth factor receptor (EGFR) and is co-targeted by the EGFR inhibitor "cetuximab", as this agent can induce co-internalisation of the SLC1A5-EGFR complex via endocytosis resulting in downregulation of SLC1A5 in an EGFR expression dependant manner [288]. Inhibition of SLC1A5 by the small molecule inhibitor, GPNA, was also reported. However, recent studies have demonstrated that it has limited selectivity to this solute carrier as it is also able to inhibit other sodium-dependent and independent amino acid transporters [289,290]. Most recently, Schulte *et al* have used the novel SLC1A5 selective inhibitor, V-9302, which results in reducing cancer cell growth and proliferation and increased oxidative stress *in vitro* and in murine models *in vivo* [291].

Indeed, the interest around SLC7A5 is continuously increased, as this transporter is implicated in many human cancers. However, therapeutic targeting of SLC7A5 could be challenging as deletion of SLC7A5 in mice is embryonically lethal [292], reflecting its physiological role in transporting essential amino acids into the brain across the blood brain barrier and that of thyroid hormones (T3 and T4) through the placental membrane [220,293]. It has been found that targeting SLC7A5 with the inhibitor of system L amino acid transporters, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) or the gene knockdown significantly reduces proliferation of many human cancer cells, including bladder cancer, oral epidermoid carcinoma, osteogenic sarcoma, colon adenocarcinoma and human BC cell lines [294,295,219,296]. However, this inhibitor is not selective for SLC7A5 as it attenuates leucine uptake through inhibiting all four members of system L amino acid transporters. Nevertheless, recent studies have reported the application of the selective inhibitor, JPH203, which successfully reduced the growth and survival of various cancer types [297-299].

The PIK3CA/AKT/mTOR signalling is often enhanced in human cancers, and thought to be attractive target for the anti-cancer therapy. Indeed, recent

advances in therapeutics designed to target this pathway have resulted in the release of many anti-cancer compounds currently undergoing Phase I/II trials [300,301]. The amino acid, leucine, is a master regulator of this critical cell growth pathway, as it allows the activation of the mTORC1/Regulator complex. Furthermore, it has been thought that mTORC1 depends on the availability of leucine, arginine and glutamine to begin the translation process [228,302,303,212]. Thus, blocking the entrance of these amino acids, through targeting the key transporters, could be successful approach to cease mTORC1-dependent cancer growth.

It is known that SLC7A5 requires the engagement of SLC3A2 to form the functioning heterodimeric complex. A study carried out by Cormerais *et al* revealed that targeting SLC3A2 alone results in a remarkable reduction in the amino acid transport. However, they confirmed that SLC7A5 is the key growth-limiting step of the heterodimer and it is the member of the complex which has the most prevalent pro-tumoural action in cancer cells [219]. Furthermore, it has been found that the pro-tumoural role in teratocarcinoma and promoting growth in T and B cells are mediated by the integrins-SLC3A2 signalling rather than that of SLC7A5 activity [270,304]. To be certain and focus on the amino acid transport function, herein we investigated the consequences of targeting SLC7A5 member of the complex along with SLC1A5, using the relevant BC cell lines.

5.2 Hypothesis

Targeting SLC1A5 and SLC7A5 will inhibit BC cell proliferation, invasion and reduce amino acid uptake in the more aggressive highly proliferative BC subtypes thereby acting as potential molecular targets for BC treatment.

5.3 Aims

1. To investigate the impact of transient siRNA knockdown of *SLC1A5* and *SLC7A5* on BC cell proliferation using MTS assay.
2. To determine the consequences of genetic disruption of *SLC1A5* and *SLC7A5* on BC cell invasion using the Cytoselect 24-Well Cell Invasion Assay.
3. To assess the cellular glutamine and leucine uptake after targeting the key glutamine transporters, *SLC1A5* and *SLC7A5*, respectively.

5.4 Methods

BC cell lines used in the *in vitro* work were selected based on the results obtained from the IHC study of SLC1A5 and SLC7A5 (Chapter 3). SLC1A5 has a prognostic impact in the luminal B, but not luminal A subtype. In order to compare between these two subtypes, luminal B-like (defined as ER+, PgR-, and HER2-) and luminal A-like cells (defined as ER+, PgR+, HER2-) [176,175] were selected as models for *in vitro* studies. Relative protein expression, in the all available luminal cells, was assessed using western blotting and quantification was carried out using LI-COR Odyssey machine. Cells that expressed highest levels of SLC1A5 were selected for the knockdown and other assays. In addition for SLC7A5, SKBR3 (HER2+) cells were assessed. Characteristics of the cell lines and conditions of maintaining cell growth are detailed in Chapter 2.

Respective cells were transfected by using the reverse transfection method with siRNAs designed to *SLC1A5* and *SLC7A5*. Cell proliferation assays were carried out using the CellTiter 96® aqueous one solution cell proliferation assay (MTS assay) to determine the effect of *SLC1A5* and *SLC7A5* knockdown on cell growth while the invasive behaviour of the cells was assayed using the Cytoselect 24-Well Cell Invasion Assay according to the manufacturer's instructions. Finally, radioactive glutamine/leucine uptake assays were performed to determine whether cells' ability to uptake glutamine or leucine were reduced after *SLC1A5* or *SLC7A5* knockdown, respectively. The detailed material and methods about the knockdown and assays used in this study are in section 2.5.1.

5.5 Results

5.5.1 Expression of SLC1A5 and SLC7A5 in luminal cell lines.

SLC1A5 and SLC7A5 protein expression was assessed in different luminal A-like (MCF7, T47D and HCC1500) and luminal B-like (ZR-75-1 and MDA-MB-175) cell lines (Figure 5-1A, C). The expression of SLC1A5 was variable among the cell lines, with the highest level being observed in ZR-75-1 cells ($p<0.01$, Figure 5-1B). Within the luminal A-like cells, HCC1500 showed the highest level of SLC1A5 compared to the other two luminal A models. Therefore, ZR-75-1 and HCC1500 cells were selected for *SLC1A5* knockdown. Similarly luminal cells varied in their SLC7A5 expression, where ZR-75-1 and MCF7 showed higher expression levels

compared to the other cell line models ($p<0.01$, Figure 5-1D), which rendered them suitable candidates for SLC7A5 knockdown.

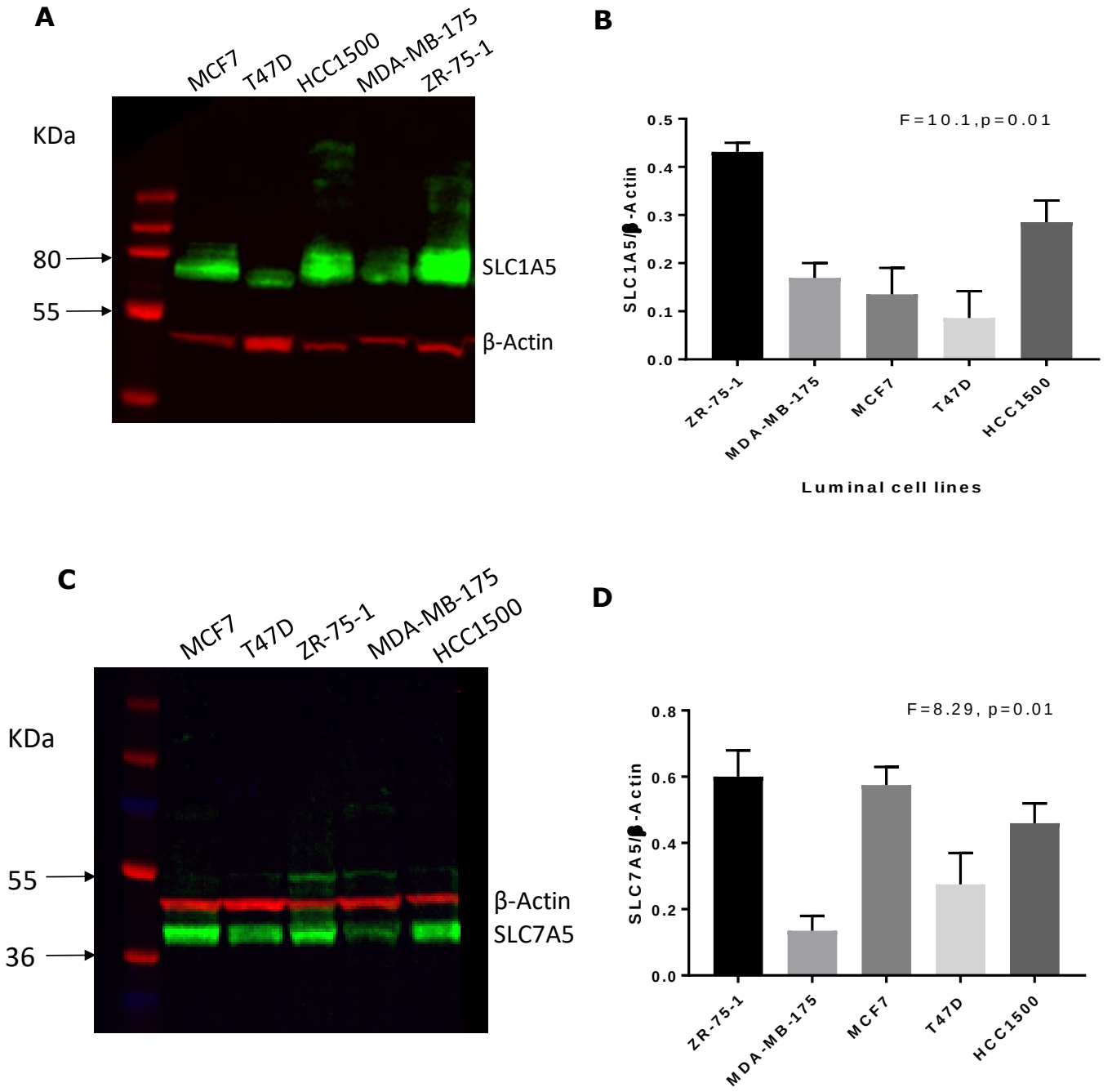


Figure 5-1. Expression levels of SLC1A5 and SLC7A5 proteins in luminal breast cancer cell lines. A) SLC1A5 expression using western blotting, B) Quantification of SLC1A5 protein in different luminal cell lines, C) SLC7A5 expression using western blotting, D) Quantification of SLC7A5 protein in different luminal cell lines. Each bar represents mean ± S.E. of two independent experiments. β-actin was included as a loading control.

5.5.2 Evaluation of siRNA knockdown efficiency

Western blot methodology is the most efficient way to monitor knockdown protein levels by siRNA [305,306]. To determine the knockdown efficiency of *SLC1A5* and *SLC7A5*, western blotting was performed in cell lysates prepared from transfected and control cells. At day 5 (120 hours) post transfection, the expression of SLC1A5 and SLC7A5 proteins were not detectable in the transfected cells, indicating efficient knockdown which was consistent among all transfected cells (Figure 5-2).

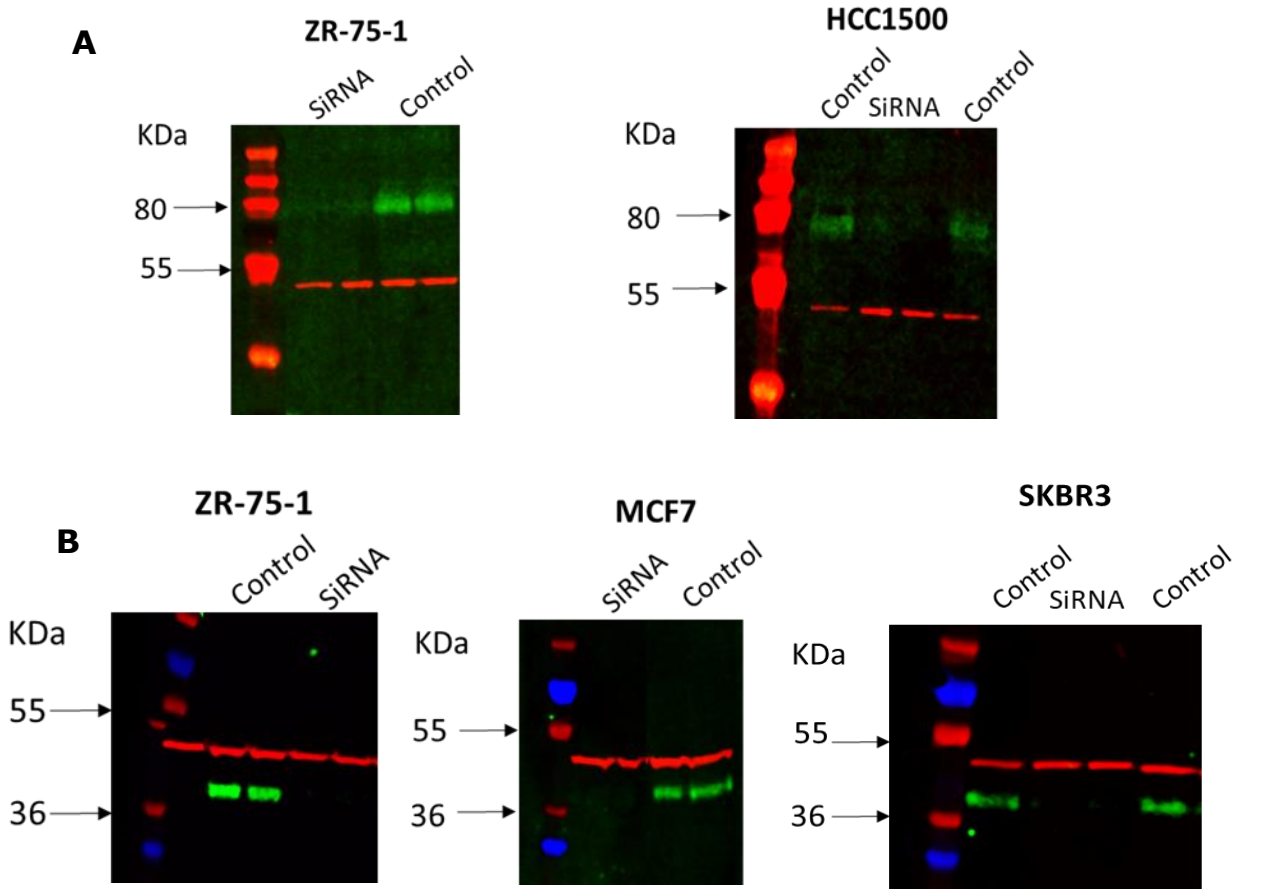


Figure 5-2. The efficiency of SLC1A5 and SLC7A5 knockdown in BC cell lines. A) SLC1A5 expression in ZR-75-1 and HCC1500 transfected with siRNA targeted to *SLC1A5* and control cells. B) SLC7A5 expression in ZR-75-1, MCF7 and SKBR3 transfected with siRNA targeted to *SLC7A5* and control cells. β-actin was included as a loading control.

5.5.3 Knockdown of *SLC1A5* and *SLC7A5* decreases cell proliferation in a variable degree among the selected BC cell lines.

MTS cell viability assay was performed to determine whether knockdown of *SLC1A5* or *SLC7A5* reduces cell proliferation in the transfected cells and whether the effect differs among the selected luminal cell lines. Transfected and control cells were assayed 24 and 120 hours after transfection, with siRNA targeted to *SLC1A5* or *SLC7A5*, and cell viability was represented as the percentage of cell growth, as detailed in section 2.5.6.

Regarding *SLC1A5* knockdown, a greater effect in cell proliferation was observed between the ZR-75-1, transfected and control cells, compared to the difference observed in HCC1500 cells. However, no significant effect on cell growth was observed in either cell line (ZR-75-1 $p=0.19$ and HCC1500 $p=0.51$, Figure 5-3A, B). Nevertheless, upon silencing *SLC7A5*, the percentage of cell growth in both ZR-75-1 and SKBR3 control cells was significantly higher than that of the transfected cells ($p=0.01$ and $p=0.02$, respectively, Figure 5-3C, E). There was no significant difference in the percentage of cell viability between MCF7 control and transfected cells ($p=0.46$, Figure 5-3D).

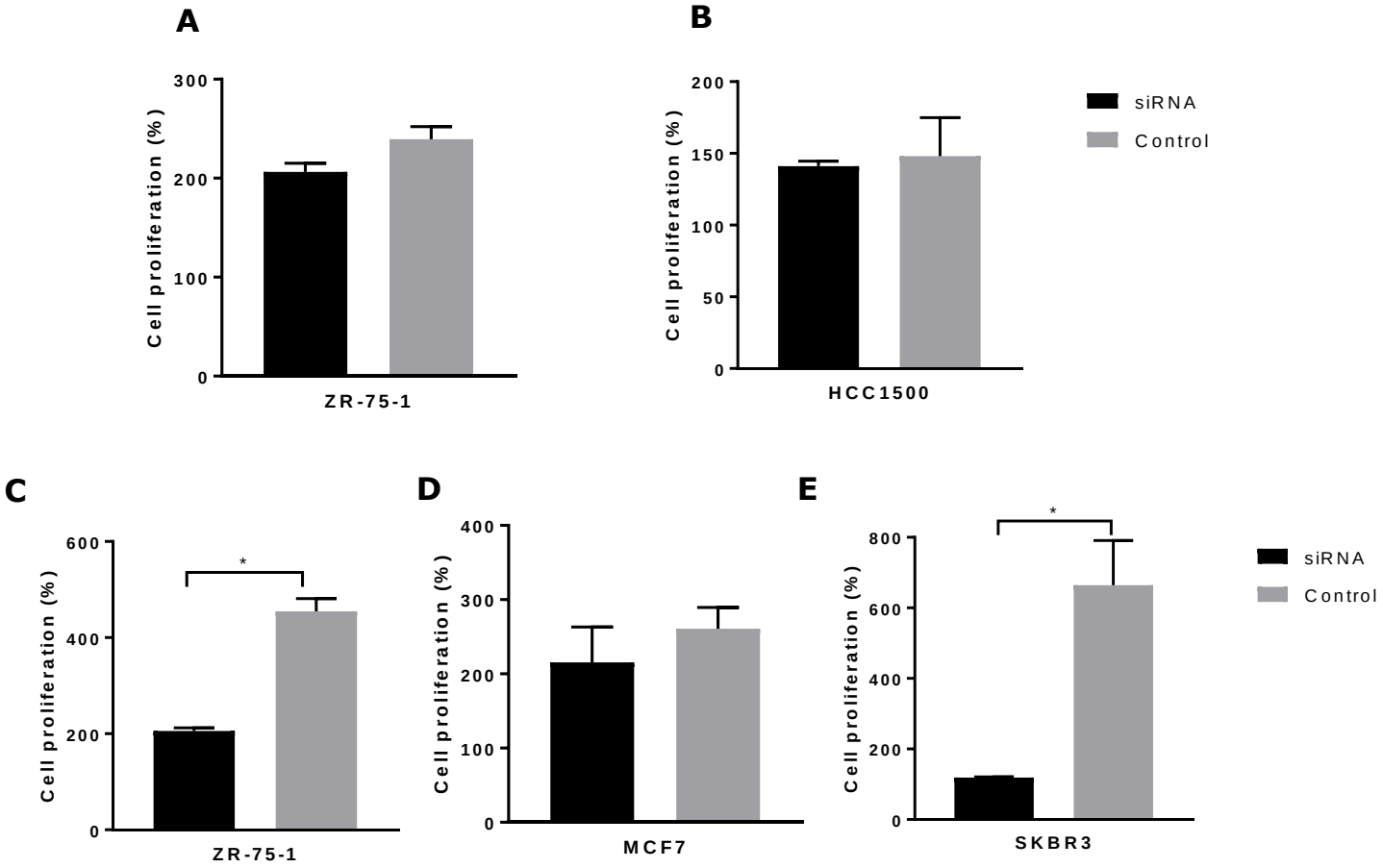


Figure 5-3. Impact of SLC1A5 and SLC7A5 knockdown on cell proliferation. The effect of SLC1A5 knockdown on cell proliferation in A) ZR-75-1 and B) HCC1500 lines compared to negative control cells. The effect of SLC7A5 knockdown on cell proliferation in C) ZR-75-1, D) MCF7 and E) SKBR3 lines compared to negative control cells. Each bar represents mean ± S.E. of at least three independent experiments.

5.5.4 SLC7A5 but not SLC1A5 regulates cell invasion in the luminal B-like cell line

The functions of SLC1A5 and SLC7A5 on the invasive phenotype of the selected cell lines was investigated using a cell invasion assay. As shown in Figure 5-4, both, ZR-75-1 and HCC1500, cell lines, did not show a significant difference in cell invasion between the *SLC1A5* transfected and control cells (p=0.32 and p=0.22, respectively). However, the invasiveness of ZR-75-1 cells was significantly decreased in *SLC7A5* knockdown cells compared to the negative control (p=0.03). No significant difference was observed between the transfected and control cells within the MCF7 and SKBR3 cells, upon *SLC7A5* silencing (p=0.74 and p=0.29, respectively).

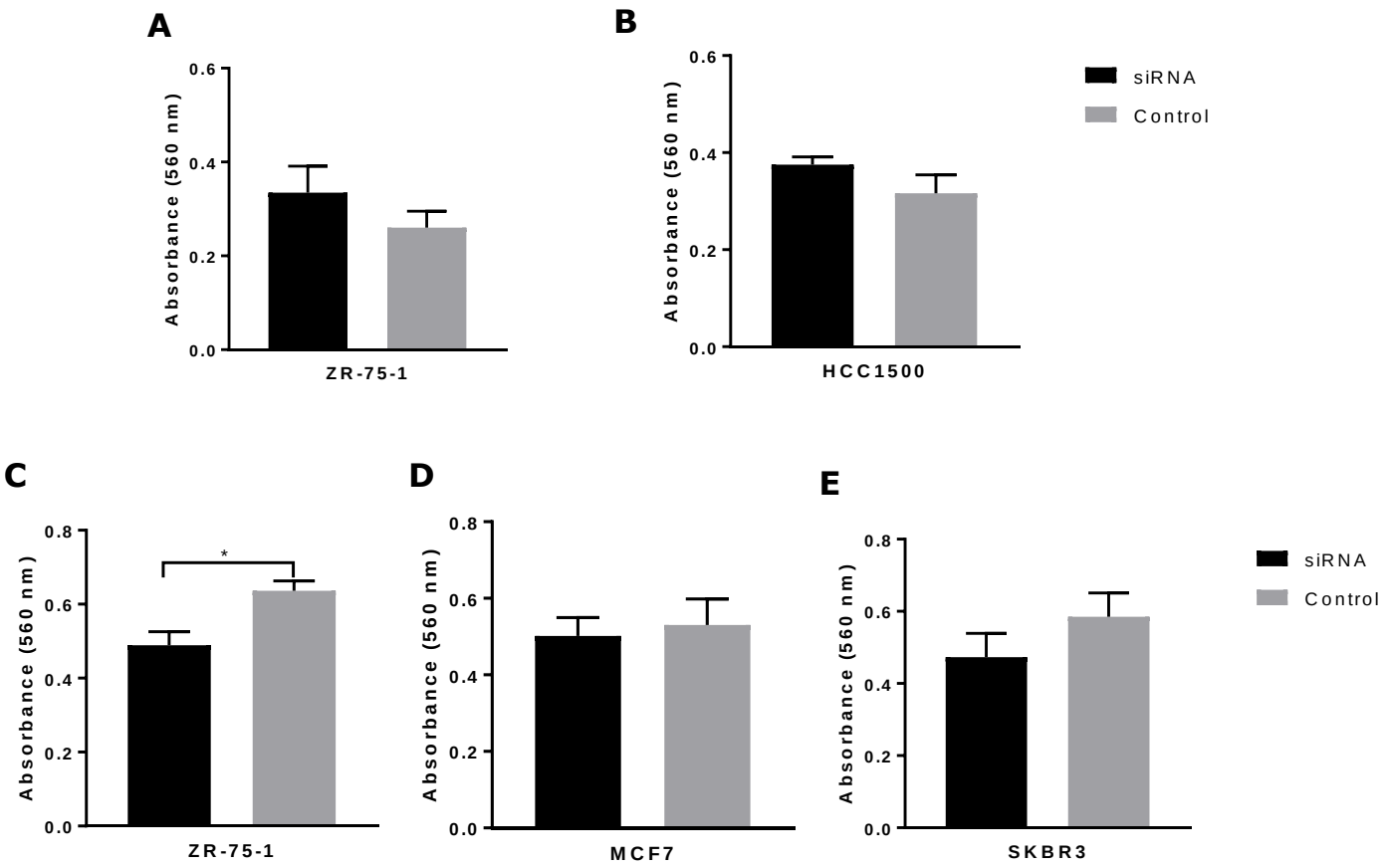


Figure 5-4. Effect of SLC1A5 and SLC7A5 knockdown on cell invasion. The effect of SLC1A5 knockdown on cell invasion in A) ZR-75-1 and B) HCC1500 lines compared to negative control cells. The effect of *SLC7A5* knockdown on cell invasion in C) ZR-75-1, D) MCF7 and E) SKBR3 lines compared to negative control cells. Each bar represents mean ± S.E. of two independent experiments.

5.5.5 SLC1A5 and SLC7A5 mediates the amino acid uptake in BC cells

In order to examine the effect of *SLC1A5* and *SLC7A5* knockdown in amino acid transport, [^{3}H]-L-Glutamine and [^{14}C]-L-Leucine uptake assays, respectively, were performed in the respective cell lines transfected, with siRNA targeted to *SLC1A5* or *SLC7A5,* and negative control cells. Upon *SLC1A5* knockdown, [^{3}H]-L-Glutamine uptake was significantly decreased in ZR-75-1 and HCC1500 transfected cells compared to the control cells ($p=0.01$ and $p=0.02$, respectively, Figure 5-5A, B). Furthermore, knockdown of *SLC7A5* in ZR-75-1 and SKBR3 cells significantly attenuated [^{14}C]-L-Leucine uptake in transfected cells compared to the control cells ($p=0.007$ and $p=0.008$, respectively, Figure 5-5C, E). However, no significant difference in [^{14}C]-L-Leucine uptake was observed between the MCF7 transfected and control cells ($p=0.32$, Figure 5-5D).

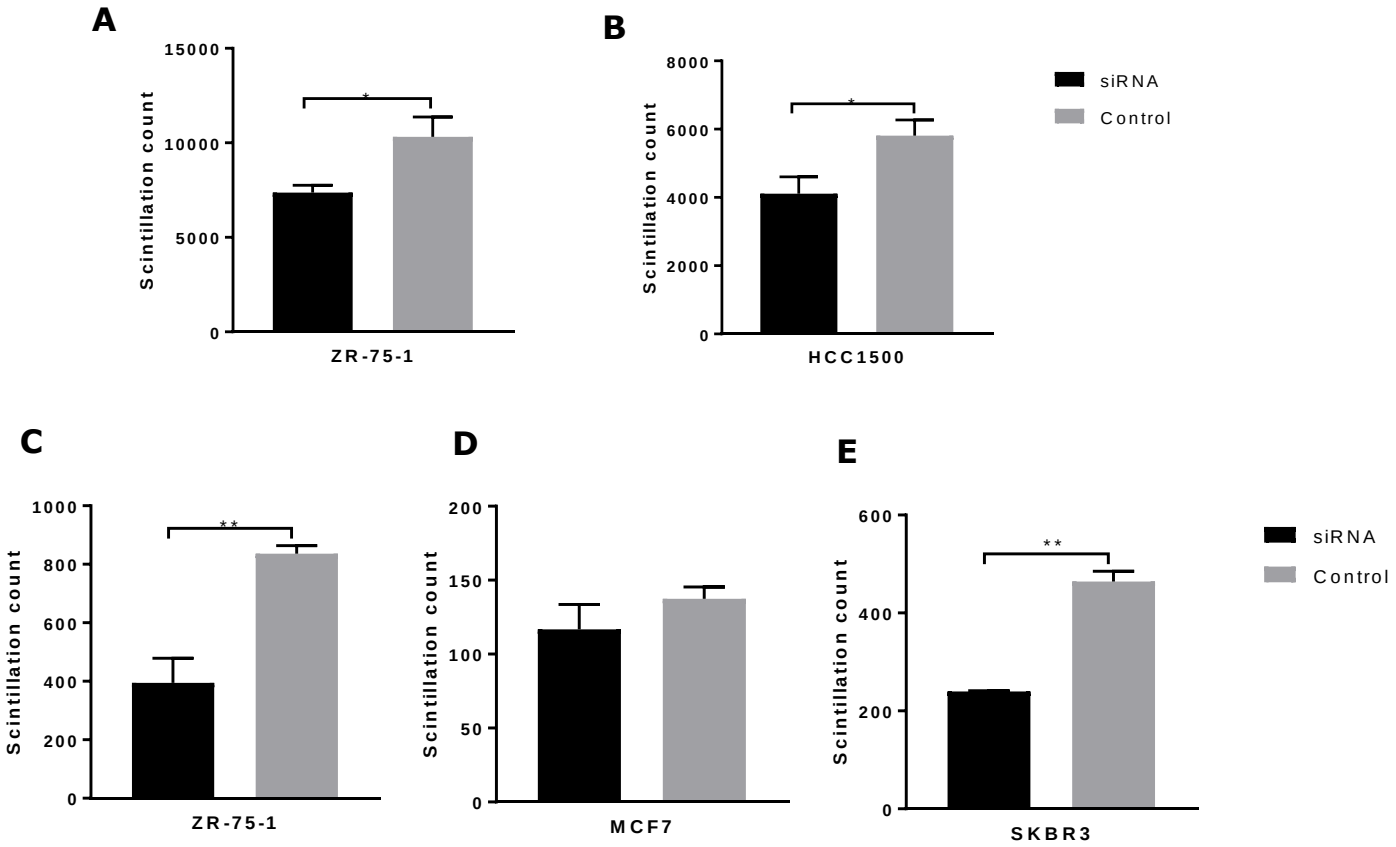


Figure 5-5. Impact of SLC1A5 and SLC7A5 knockdown on amino acid uptake. Effect of *SLC1A5* knockdown in glutamine transport activity in A) ZR-75-1 and B) HCC1500 transfected and control cells. Effect of *SLC7A5* knockdown in leucine uptake in A) ZR-75-1, B) MCF7 and C) SKBR3 transfected and control cells. Each bar represents mean ± S.E. of three independent experiments.

5.6 Discussion

Breast cancer is a heterogeneous disease with different subtypes that vary in terms of histology, molecular profiles, response to therapy and clinical behaviour [79]. In addition, different subtypes display variance in their metabolic pathways and nutritional needs. The most common form of BC is ER+/luminal tumours [94,307] and those that belong to this class are also variable in terms of disease prognosis, recurrence and mortality rates [94].

Currently, anti-oestrogen drugs such as tamoxifen and the HER2-targeting antibody, trastuzumab, are the only molecularly targeted drugs for BC treatment. Although these treatments improve overall survival and reduce risk for relapse, a subset of patients still develop drug resistance and die as a result of the disease [308,309]. Therefore, identification of new molecular therapeutic targets is urgently needed, especially for the highly proliferative BC subtypes. In this study, different BC cell lines were used to determine whether SLC1A5 or SLC7A5 could be potentially utilised as novel molecular targets for treatment of the more aggressive luminal BC subtype and also the HER2+ subtype.

It is known that the demand for amino acids increased in the highly proliferating cells and to satisfy their hunger, they upregulate the required amino acid transporters [310]. Increased expression of SLC1A5 and SLC7A5 is well documented in various types of human cancer and it has been reported that these proteins could be useful as prognostic markers in malignant tumour tissues, including BC [277,311,312,255,200,313,136,273]. In chapter 3, it was shown that SLC1A5 and SLC7A5 are highly expressed in the more aggressive BC subtypes compared to the low proliferative/luminal A tumours. In addition, the significant association with poor survival was restricted to the highly proliferative tumours, indicating that blocking these solute carriers can be valuable in treating these BC subtypes.

Although the inhibitors of SLC1A5 and SLC7A5 (GPNA and BCH, respectively) were widely used to study the function of these transporters, they are known to be inhibitors for other members of the amino acid transport system. In addition, the recently identified SLC1A5 and SLC7A5 selective inhibitors (V-9302 and JPH203, respectively) were not commercially available when this study was performed. Therefore, we investigated the impact of blocking SLC1A5 and SLC7A5 in two

luminal (luminal B-like and luminal A-like) and HER2+ cell lines using transient siRNA knockdown.

Since SLC7A5 recognises essential neutral amino acids with bulky side chains including leucine, targeting SLC7A5-mediated amino acid-transport could have a significant impact on protein synthesis and cell proliferation. In this regard, this thesis and others have demonstrated that SLC7A5 significantly correlated with the proliferative marker Ki-67 [273,255,277,276,140] and the expression of other proliferative related genes [314]. This study showed that silencing SLC7A5 impaired leucine uptake and proliferation of the luminal B-like and HER2+ cells but not the luminal A subtype, indicating that SLC7A5, with the subsequent import of essential amino acids, is implicated in supporting cell proliferation in the highly proliferative subtypes of BC. In agreement with this notion, another study reported that knockdown of SLC7A5 expression using shRNA reduced leucine uptake and proliferation of prostate cancer cells [315]. The anti-proliferative effect of blocking SLC7A5, with the following suppression in leucine uptake, can be attributed to the subsequent inhibition of mTOR/p70S6K signalling pathway. On the other hand, the lack of effect of SLC7A5-knockdown on cell proliferation in luminal A cells could be caused by the dependence of this subtype to another members of system L transporters, particularly SLC7A8, as recently it has been determined that this is highly expressed in luminal A tumours [316]. In the line with that, other studies also have shown that targeting SLC7A5 did not affect cell proliferation in human cholangiocarcinoma and ovarian cancer cells [317,318].

In chapter 3, it was shown that SLC7A5 was associated with development of distant metastasis especially in the ER+ high proliferative BC subtype. It has also been documented that this protein is expressed in the primary and metastatic sites of many cancers [254], which renders it implicated in the metastatic process. Tumour cell invasion considered as the first event of the multi-step metastatic cascade [319] and this study showed that silencing *SLC7A5* by siRNA caused significant reduction in ZR-75-1 cell invasion. However, no effect was observed in the luminal A-like and HER2+ cell lines, indicating that SLC7A5 could be driving the tumorigenesis and aggressiveness in the more aggressive luminal B subtype. The effect observed in the latter subtype is consistent with other studies, which confirmed that targeting SLC7A5 suppressed cell invasion of cholangiocarcinoma lines and mouse trophoblast cells [318,320].

With respect to SLC1A5, neither the luminal B-like nor the luminal A-like cells showed any significant effect in their proliferation when *SLC1A5* was targeted by RNA interference although significant reduction in glutamine uptake was observed in the two cell lines. Previously published work supports this fact where it was found that the single expression of SLC1A5 was not enough to predict the poor patient survival in the ER+ highly proliferative BC subtype. Instead, it requires the combinational expression with the SLC7A5-SLC3A2 heterodimeric complex [321]. In addition, it has been shown, in chapter 3, that SLC1A5 fails to predict patient outcome independent of the most powerful prognostic factors, indicating that targeting SLC1A5 alone may not benefit patients whose their BC belongs to the highly proliferative ER+ tumours. Data presented here, however, suggests that SLC1A5 may be a target for combination therapy with anti-SLC7A5 agents to combat the more aggressive luminal subtype.

In this regard, most recent work carried out by Cormerias *et al*, confirmed that SLC1A5 works independent of SLC7A5 in promoting cancer cell growth, where knocking down SLC1A5 significantly reduced glutamine import in colon and lung adenocarcinoma cells whereas no impact on leucine uptake was observed in both cell lines [322]. This data suggests that in some cancers, SLC7A5 might co-operate with other transporters that mediate the net influx of glutamine.

It is clearly observed in this study that the knockdown of *SLC1A5* significantly reduced glutamine import in both, ZR-75-1 and HCC1500, cell lines compared to the control cells. However, this reduction in glutamine was not sufficient to cause a significant decrease in cell proliferation or invasion in both cell lines. This can be attributed to the compensation by the upregulation of other amino acid transporters as a response to blocking the action of SLC1A5.

Nevertheless, a study in the TNBC cell lines showed that SLC1A5 is not only highly expressed in this subtype, but it also promotes cell proliferation *in vitro* and *in vivo*. In addition, they found that knockdown of SLC1A5 significantly attenuated glutamine uptake, reduced cell growth and arrested cell cycle in G0/G1 stage, in the same BC subtype [135]. Furthermore, a recent study showed that blocking SLC1A5, with the selective inhibitor, efficiently reduces TNBC cell growth and proliferation [291], Wang *et al* also supports the above findings, as they revealed that SLC1A5 promotes prostate cancer proliferation and metastasis *in vivo*,

suggesting that this solute carrier could be a potential therapeutic target in TNBC subtype and prostate cancer [285]. The anti-proliferative effect of inhibiting SLC1A5 in gastric cancer cells was also examined *in vitro* and *in vivo*, along with suppressing the ability of the same cells to migrate and invade [323].

Altogether, these statements indicate a differential reliance on SLC1A5 and SLC7A5 activity across BC cell lines, where only TNBC may require SLC1A5-mediated glutamine uptake for cell growth and proliferation while SLC7A5 may have a great value for the proliferation and invasiveness of luminal B and HER2+ subtypes. This study revealed that although silencing *SLC1A5* or *SLC7A5* significantly reduced amino acid uptake in the respective BC cell lines, the effect on cell proliferation and invasion of the luminal B-like cells was only detected upon targeting *SLC7A5* but not *SLC1A5*. Further experiments are needed to confirm the effect of blocking these transporters using selective inhibitors, as well as exploring whether there is any synergistic effect from co-targeting both solute carriers in different BC cell lines.

Chapter 6. Prognostic significance of the mTORC1 substrate (p70S6k) in breast cancer: as a downstream effector of amino acid transporters

6.1 Introduction

Mammalian target of rapamycin (mTOR) is a member of the phosphoinositide-3-kinase related kinase (PIKK) family that has catalytic activity as a protein serine-threonine kinase. Within the cell, mTOR binds to other proteins to form two major complexes, mTORC1 and mTORC2 [257]. mTORC1 is characterised by its association with regulatory associated protein of mTOR(Raptor), whereas rapamycin insensitive companion of mTOR (Rictor) is involved in the mTORC2 structure [324]. Both, Raptor and Rictor, are scaffold proteins that guide mTOR activity towards specific substrates, and thus, obtaining specificity in determining cellular functions [325-327]. mTORC1 is considered as a master regulator of protein synthesis, energy metabolism, autophagy, cell growth and proliferation [328-330] which renders it highly responsive to signals from growth factors, nutrients and energy stress [331,326]. On the other hand, mTORC2 acts in controlling other cellular functions such as regulating aspects of cell survival and the organization of actin cytoskeleton [332,333].

Currently, mTORC1 is receiving a greater attention in the field of cancer research due to its role in supporting various cellular functions in addition to its ability to respond to the anti-proliferative agent, rapamycin [334]. This study is focussed on mTORC1 as a downstream effector of amino acid transporters as amino acids considered one of its potent regulators.

It is well known that growth factor signalling transduction triggers PI3K-Akt Pathway with the subsequent activation of mTORC1. Akt can indirectly activate mTORC1 through phosphorylating Tuberous sclerosis proteins 2 (TSC2). In situations where this protein is inactive, it forms a heterodimeric complex with TSC1, which acts as a tumour suppressor that inactivates mTORC1. However, once phosphorylated, TSC2 dissociates from TSC1 and acts as a GTPase-activating protein (GAP) [335,336], which converts Rheb-GDP to its active form, Rheb-GTP. The latter binds to mTORC1 and activates its kinase activity [337]. In addition to Akt, MAPK pathway, is also implicated in inhibiting TSC2 therefore activating mTORC1 [338]. Amino acids, especially leucine, are other essential extracellular stimuli that activate mTORC1 in mammalian cells [339].

In response to amino acid availability, the Rag GTPases, members of the Ras GTPase super family, mediate mTORC1 activation [340-342]. The mechanism is

started by interaction of RagA or B with RagC or D to form a heterodimer that is anchored to the surface of the lysosome [343-345]. In the status of amino acid deprivation, RagA/B is GDP-loaded and RagC/D is GTP-loaded, and the resulting Rag heterodimer is unable to bind and activate mTORC1. However, the presence of sufficient amino acids stimulates the conversion of GDP by GTP in RagA/B and the exchange of GTP to GDP in Rag C/D, allowing the binding of the Rag heterodimer to mTORC1 therefore recruiting it to the lysosome, where it is directly activated through interacting with the Rheb-GTP molecule [342].

The presence of glutamine and subsequent glutaminolysis, along with arginine and leucine are thought to be implicated in the mTORC1 activation [346,347]. Interestingly, the influx of leucine through system L transporters, has an additional effect in the activation process [348], as it has been found that intracellular leucine can be detected by leucyl-tRNA synthetase (LeuRS), a member of aminoacyl-tRNA synthetase family, which catalyses the ATP-dependent ligation of L-leucine to leucyl-tRNA during protein synthesis [349]. LeuRS activates the Rag GTPase complex where it acts as a GAP, making RagD in a GDP-bound form, which is required for mTORC1 activation. In addition, LeuRS may also activate mTORC1 signalling via binding to Raptor on the surface of lysosome [349,213]. Furthermore, another study has revealed that leucine is involved in the activation of mTORC1 signalling via stimulating glutaminolysis [346], where it directly binds and activates glutamate dehydrogenase, with subsequent conversion of glutamate to α-KG [350]. The latter is sufficient to stimulate recruitment of mTORC1 to the lysosome through activating RagB [346].

Previous *in vitro* studies suggest that there is cross-talk between the more prominent mTORC1 regulators, nutrients and growth factor signals, where amino acids sufficiency is essential for the insulin-induced activation of mTORC1 [339,351]. However, mTOR activation by nutrient sensing involves phosphorylation at Thr2446 site, while growth factors stimulate Ser2448 phosphorylation of mTOR and both isoforms are mutually exclusive and therefore act as a switch that merges the counteracting signals of growth factors and nutrient status [261].

The two best-characterised downstream effectors of mTORC1 are p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF4E)-binding

protein (4EBP). Phosphorylation of these substrates, upon mTORC1 activation, stimulates protein translation process. While both substrates mediate translation initiation, p70S6K also assists translation elongation through phosphorylation and subsequent suppression of eukaryotic elongation factor 2 kinase (eEF2K) activity [338] (Figure 6-1). In addition, p70S6K regulates protein translation through direct phosphorylation of ribosomal protein S6, a subunit of the 40S protein complex, which involved in the translation of mRNAs that contain an oligopyrimidine tract at their transcriptional start site [352].

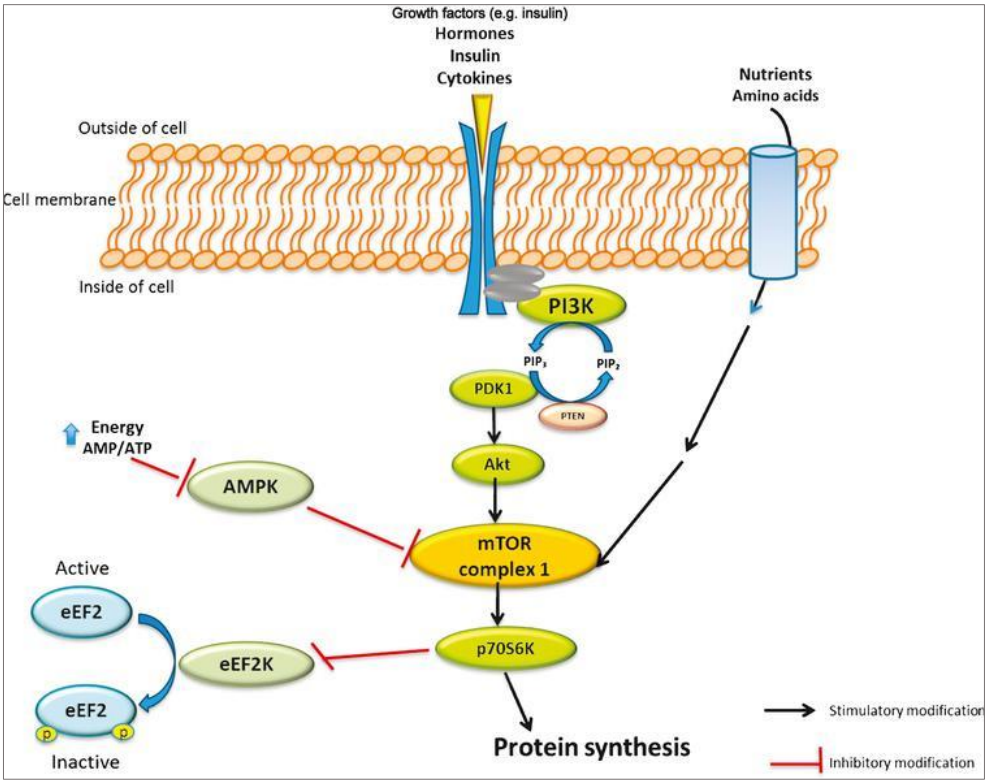


Figure 6-1. Schematic view of mammalian target of rapamycin complex 1 (mTORC1) signalling pathway, its upstream signals including, growth factors, insulin and amino acids, and the downstream effector, p70S6K, which triggers a cascade of responses that subsequently assist protein synthesis (adapted from Farnaz et al 2015).

Figueiredo *et al* have found that activation of p70S6K targets Ser2448 in a negative feedback mechanism, inferring that p-mTORC1 at Ser2448 is an inadequate measure for mTORC1 kinase activity and the substitutional should be the downstream effector proteins such as p706SK [262].

p70S6K is encoded by the Ribosomal Protein S6 Kinase B1 (*RPS6KB1*) gene and is frequently activated in various human cancer types, including pancreatic cancer, small cell lung cancer, cervical and oesophageal cancers [353-355]. Studies in BC

have revealed that expression levels of p70S6K are twice in the highly malignant MCF10CA1a cells compared to the benign MCF10A cells [356] and cases with tumours having increased p70S6K levels had poor outcome and increased metastatic events [357].

6.2 Hypothesis

p70S6K is a downstream effector of amino acid transporters that is over-expressed in BC and it has prognostic value that is vary among different molecular BC subtypes.

6.3 Aims

1. To assess *RPS6KB1* gene copy number and mRNA expression, alongside p70S6K protein expression in large well-characterised annotated cohorts of BC and to evaluate their association with clinicopathological parameters, amino acid transporters and glutaminolytic enzymes.
2. To determine the prognostic potential of *RPS6KB1* mRNA and p70S6K protein in the different molecular BC subtypes.

6.4 Methods

6.4.1 Study cohorts

6.4.1.1 METABRIC cohort

RPS6KB1 gene copy number and gene expression were evaluated using the METABRIC cohort. This cohort comprised 1980 primary operable invasive BC patients. Clinical history, outcome data and tumour characteristics, including tumour size, tumour grade, LN stage and histological phenotypes, were available for this cohort. The intrinsic subtypes of BC (luminal A, luminal B, HER2-enriched, normal-like, and basal-like) were classified based on prediction analysis of microarray of 50 genes (PAM50). Integrating germline variants, somatic aberrations, and aberration with gene expression data augmented BC classification in this cohort by producing 10 integrative clusters. Each cluster represents a distinct genomic landscape, molecular and clinical features and unique ER, PR and HER2 expressions. The characteristics of the METABRIC cohort are detailed in section 2.1.1.

6.4.1.2 External validation cohort

For external validation of *RPS6KB1* mRNA expression, Breast Cancer Gene-Expression Miner v4.0 online dataset was used. This dataset offers the possibility to evaluate the clinicopathological and prognostic significance of candidate genes in large pooled cohort of BC patients, as detailed in section 2.1.2.

6.4.1.3 Nottingham BC cohort

Protein expression of p70S6K was assessed using IHC and TMAs extracted from the Nottingham BC cohort (n= 1338). This is a well-characterised cohort of patients with early stage primary operable invasive BC. The clinicopathological profile of the study samples includes patient's age, tumour size, tumour grade, nodal stage, NPI, LVI status and tumour histological types. The hormonal receptor expression profiles and outcome data (BCSS and DMFS) were also recorded. The clinicopathological characteristics of this cohort are detailed in section 2.1.3.

The expression of *RPS6KB1*/p70S6K, at the mRNA and protein levels, was correlated with clinicopathological parameters, molecular subtypes, relevant biological markers and patient outcome.

6.4.2 Western blotting and immunohistochemistry

Western blotting was applied to validate the specificity of p70S6K primary antibody using whole cell lysates from MCF7, MDA-MB-175, and SKBR3 cells. IHC staining was performed on TMA sections using the Novolink polymer detection system. The details of methodology, antibodies concentrations and incubation times are described in sections 2.2, 2.4 and table 2-3.

6.5 Results

6.5.1 Validation of p70S6K antibody

The specificity of the p70S6K antibody was evaluated using western blotting which revealed a single specific band at the right size (57 KDa) for the p70S6K protein (Figure 6-2).

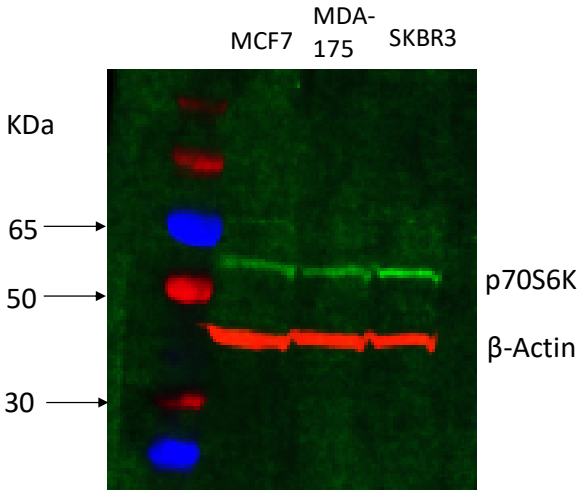


Figure 6-2. Western blot underlining p70S6K antibody specificity. β-actin was included as a positive control.

6.5.2 *RPS6KB1*/P70S6K expression in breast cancer

RPS6KB1 gene copy number and gene expression were evaluated using the METABRIC cohort. Dichotomisation of *RPS6KB1* normalised data was set using the x-tile software. High *RPS6KB1* mRNA expression (log2 intensity >8.6) was observed in 792/1,980 (40%) of the METABRIC BC cases. A total of 365/1,980 (18.4%) cases showed a CN gain of *RPS6KB1* while only 40/1,980 (2.0%) cases showed a CN loss. A significant association was observed between *RPS6KB1* CNV and *RPS6KB1* mRNA expression ($p<0.001$, Figure 6-3).

p70S6K protein expression was assessed using the Nottingham BC cohort. Staining of p70S6K in full-face BC tissue sections demonstrated a homogenous pattern of p70S6K immunoreactivity. A total of 1336 cases were valid for assessment. p70S6K was expressed predominantly in the cytoplasm of invasive tumour cells, with intensity levels varying from absent to high (Figure 6-4A, B). The distribution of this expression was unimodal and left-skewed and the x-tile software was used to identify the cut-off point for p70S6K positivity. Positive

p70S6K protein expression (>100 H-score) was observed in 286/2664 (21%) of BC cases.

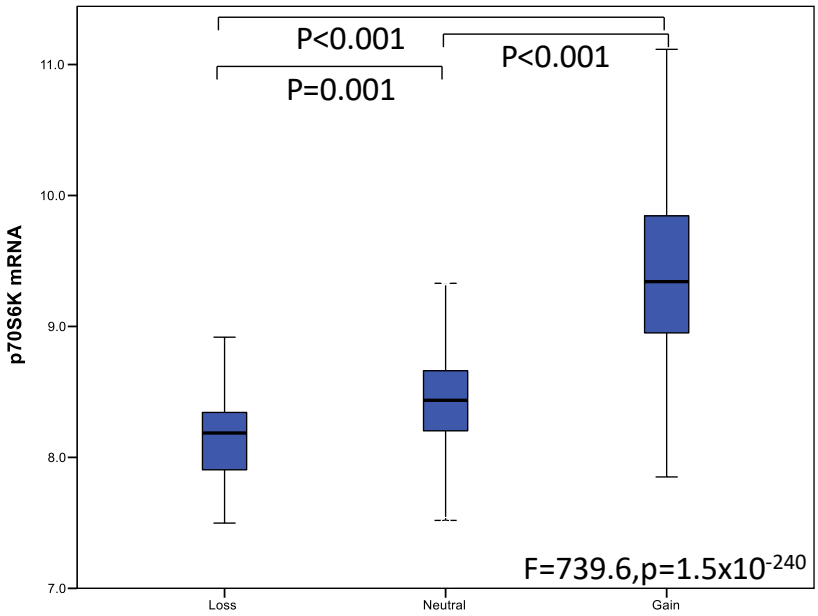


Figure 6-3. RPS6KB1 Copy Number Variation and relationship with mRNA expression in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

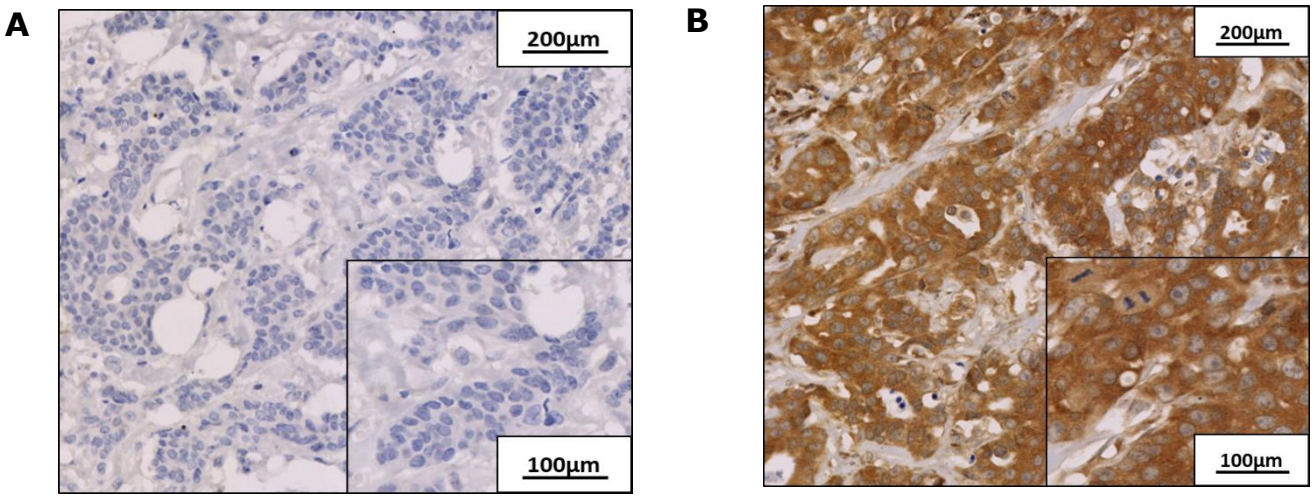


Figure 6-4. Protein expression of p70S6K in invasive breast cancer. A) Negative immunohistochemical (IHC) expression, B) Positive IHC expression. Positive immunoreactivity is detected in the cytoplasm of the invasive breast cancer cells. Magnification 10x. Scale bars=200µm. Inset, magnification 20x. Scale bars=100µm.

6.5.3 Association of *RPS6KB1*/p706SK with clinicopathological parameters

High *RPS6KB1* mRNA expression was significantly associated with poor prognostic parameters, including larger tumour size (p=0.02, Figure 6-5A), higher grade

(p=0.002, Figure 6-5B) and poor NPI (p=0.02, Figure 6-5C). However, no significant association was observed with lymph node stage (p>0.05, Figure 6-5D). Breast Cancer Gene-Expression Miner dataset was used to confirm these correlations, where possible (Figure 6-6A-C).

The associations between p70S6K protein expression and clinicopathological variables are summarised in Table 6-1. High expression of p70S6K was significantly associated with younger patient age, large tumour size, high tumour grade, high pleomorphism, high mitotic count, less tubular formation and poor NPI (all p≤0.007). In addition, there was a significant association between high p70S6K expression and medullary tumour type (p=0.04). There was no significant association between p70S6K and the presence of lymphovascular invasion or lymph node stage (p>0.05).

Table 6-1. Clinicopathological associations of p70S6K expression in breast cancer

	p70S6K protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Age				
< 50	336 (73.0)	124 (27.0)	13.81 (0.001)	**0.005**
≥ 50	712 (81.5)	162 (18.5)		
Tumour size				
< 2.0cm	608 (82.2)	132 (17.8)	14.31 (0.001)	**0.006**
≥ 2.0cm	439 (74.0)	154 (26.0)		
Tumour Grade				
1	165 (90.7)	17 (9.3)		
2	404 (85.6)	68 (14.4)	55.65 ($8.2x10^{-13}$)	**<0.0001**
3	482 (70.6)	201 (29.4)		
Mitosis				
1	461 (88.7)	59 (11.3)		
2	205 (83.7)	40 (16.3)	80.64 ($3.1x10^{-18}$)	**<0.0001**
3	373 (66.8)	185 (33.2)		
Pleomorphism				
1	14 (93.3)	1 (6.7)		
2	323 (88.7)	41 (11.3)	34.25 ($3.6x10^{-8}$)	**<0.0001**
3	701 (74.3)	242 (25.7)		

(Continued)

	p70S6K protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Tubular formation				
1	67 (93.1)	5 (6.9)	14.89 (0.001)	**0.007**
2	332 (81.6)	75 (18.4)		
3	640 (75.8)	204 (24.2)		
Vascular Invasion				
Negative	700 (78.5)	192 (21.5)	1.37 (0.50)	1.17
Positive	347 (78.7)	94 (21.3)		
Lymph Node Stage				
1	652 (79.8)	165 (20.2)	1.86 (0.39)	1.00
2	299 (76.9)	90 (23.1)		
3	99 (76.2)	31 (23.8)		
Nottingham Prognostic Index				
Good	352 (88.9)	44 (11.1)	39.21 ($1.5x10^{-8}$)	**<0.0001**
Moderate	524 (75.0)	175 (25.0)		
Poor	169 (71.6)	67 (28.4)		
IHC Subtypes				
ER+/HER2- Low Proliferation	579 (88.3)	77 (11.7)	79.17 ($2.6x10^{-16}$)	**<0.0001**
ER+/HER2- High Proliferation	181 (74.2)	63 (25.8)		
Triple Negative	147 (66.8)	73 (33.2)		
HER2+	91 (63.2)	53 (36.8)		
Histological type				
Ductal (including mixed)	915 (78.1)	257 (21.9)	16.03 (0.01)	**0.04**
Lobular	75 (88.2)	10 (11.8)		
Medullary	11 (52.4)	10 (47.6)		
Miscellaneous	7 (77.8)	2 (22.2)		
Special type	38 (84.4)	7 (15.6)		
Tubular	1 (100.0)	0 (0.0)		

P values in bold means statistically significant

6.5.4 *RPS6KB1*/p70S6K expression in molecular BC subtypes

High expression of *RPS6KB1* mRNA was significantly associated with ER positive status (p<0.001, Table 6-2) and HER2+ tumours (p<0.001, Table 6-2). These results were in agreement with the results of the Breast Cancer Gene-Expression Miner (Figure 6-6D-G). However, p70S6K protein expression was associated with negative hormone receptor status and HER2+ tumours and it was highly expressed in TN malignancies compared to non-TN tumours (p<0.001, Table 6-2). When comparing the levels of *RPS6KB1* mRNA expression in the intrinsic (PAM50) subtypes, high expression was observed in luminal B and HER2+ tumours. (p<0.001, Figure 6-5F). Similarly, within the METABRIC Integrative Clusters, high *RPS6KB1* mRNA expression was predominantly associated with cluster 1 (luminal B subgroup) and to lesser extent in clusters 6 (luminal A/B tumours) and 9 (luminal B subgroup) (p<0.001, Figure 6-5E). Furthermore, the results of association with the SCMGENE subtypes showed that the ER+/HER2- high proliferative subtype and HER2+ tumours had the highest levels of *RPS6KB1* mRNA expression (p<0.001, Figure 6-5G). Similar results were obtained when the association of *RPS6KB1* mRNA with PAM50 subtypes was assessed using the Breast Cancer Gene-Expression Miner (Figure 6-6H). Gene CN analysis showed that luminal B and HER2+ tumours more commonly had a CN gain of *RPS6KB1* (p<0.001, Table 6-3), while no significant association was observed between *RPS6KB1* CN loss and the PAM50 subtypes (p>0.05, Table 6-3). Regarding p70S6K protein, the lowest expression was observed in the ER+/ low proliferation tumours compared to the other IHC defined molecular subtypes (p<0.001, Table 6-1).

Table 6-2. Association of *RPS6KB1*/p70S6K expression and hormone/ HER2 receptor status in breast cancer

	***RPS6KB1* mRNA**				**p70S6K Protein**			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low n (%)**	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ER								
Negative	342 (72.2)	132 (27.8)	38.34 ($5.9x10^{-10}$)	**<0.0001**	196 (65.1)	105 (34.9)	42.05 ($8.8x10^{-11}$)	**<0.0001**
Positive	846 (56.2)	660 (43.8)			855 (82.5)	181 (17.5)		
PgR								
Negative	568 (60.4)	372 (39.6)	0.135 (0.71)	1.42	392 (72.1)	152 (27.9)	23.09 (0.000002)	**<0.0001**
Positive	620 (59.6)	420 (40.4)			648 (83.1)	132 (16.9)		
HER2								
Negative	1077 (62.1)	656 (37.9)	26.67 ($2.4x10^{-7}$)	**<0.0001**	924 (81.3)	212 (18.7)	30.21 ($2.7x10^{-7}$)	**<0.0001**
Positive	111 (44.9)	136 (55.1)			117 (63.6)	67 (36.4)		
Triple Negative								
No	925 (55.7)	735 (44.3)	78.29 ($8.8x10^{-19}$)	**<0.0001**	901 (81.0)	211 (19.0)	22.85 (0.000002)	**<0.0001**
Yes	263 (82.2)	57 (17.8)			145 (66.5)	73 (33.5)		

P values in bold means statistically significant

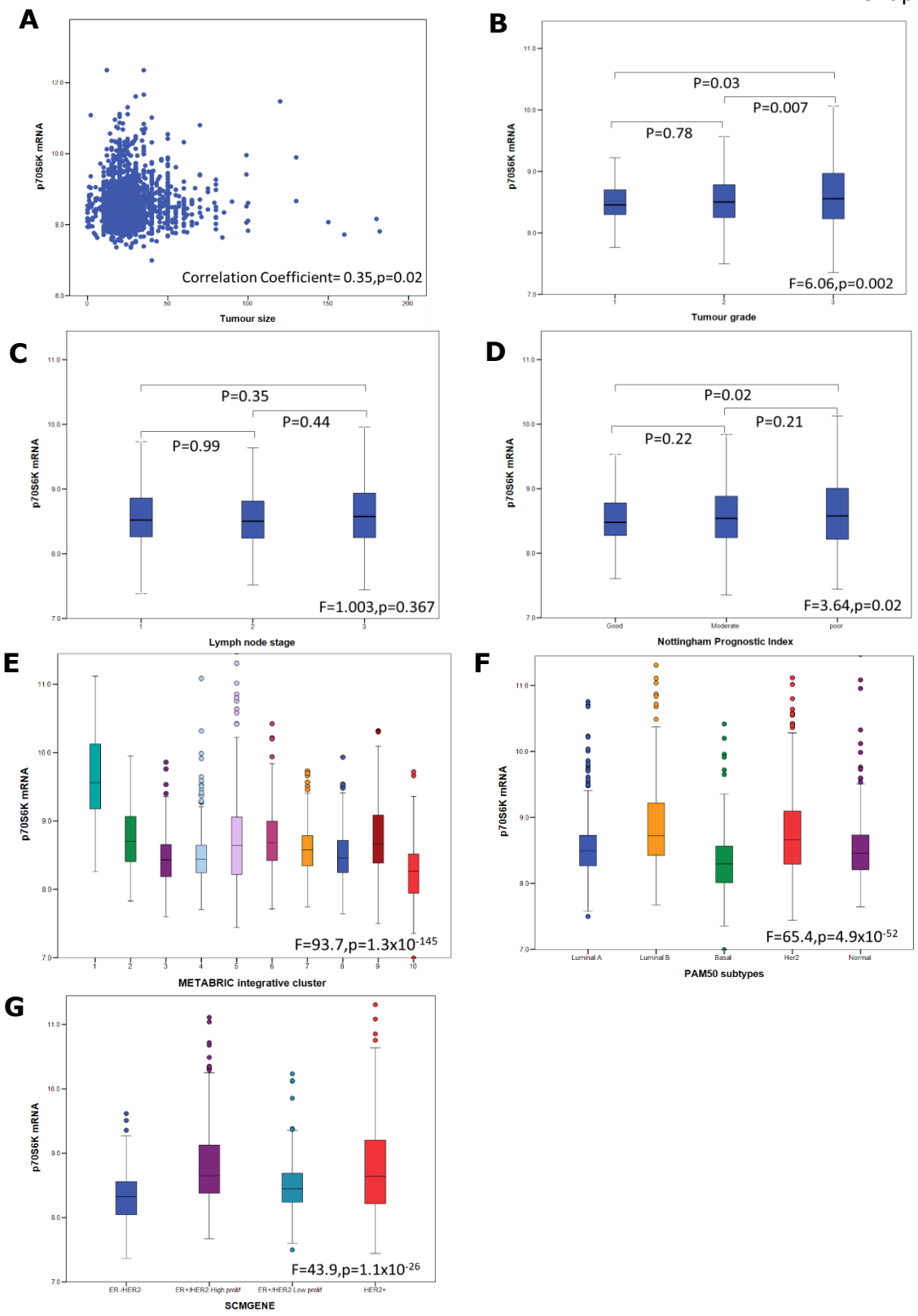


Figure 6-5. *RPS6KB1* mRNA expression and its association with clinicopathological parameters and molecular subtypes: A) *RPS6KB1* and tumour size, B) *RPS6KB1* and tumour grade, C) *RPS6KB1* and lymph node stage, D) *RPS6KB1* and NPI, E) *RPS6KB1* and METABRIC Integrative Clusters, F) *RPS6KB1* and PAM50 subtypes, G) *RPS6KB1* and SMCGENE subtypes in the METABRIC cohort using one-way ANOVA with post-hoc tukey test.

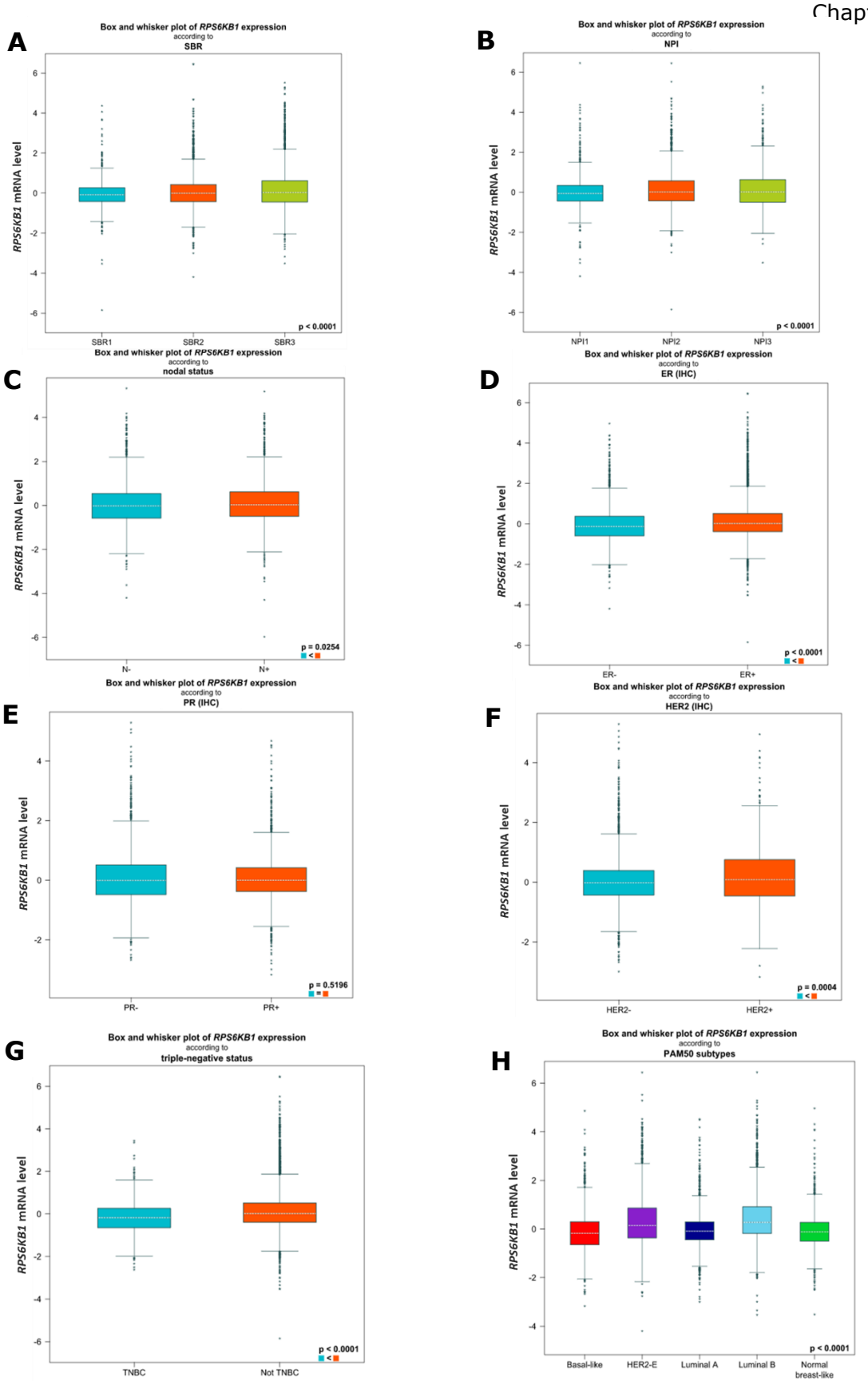


Figure 6-6. *RPS6KB1* gene expression and its association, with: A) tumour grade, B) NPI, C) ER status, D) PR status, E) HER2 status, F) Triple Negative status, G) PAM50 subtypes, using Breast Cancer Gene-Expression Miner dataset.

Table 6-3. The association of *RPS6KB1* copy number aberrations and PAM 50 subtypes in the METABRIC cohort.

	RPS6KB1					
	Gain			**Loss**		
	No	**Yes**	**χ2 (p-value)**	**No**	**Yes**	**χ2 (p-value)**
PAM50 subtype						
Luminal A	658 (91.6)	60 (8.4)		702 (97.8)	16 (2.2)	
Luminal B	322 (66.0)	166 (34.0)	172.45	477 (97.7)	11 (2.3)	2.53
Basal	289 (87.8)	40 (12.2)	(**$3.1x10^{-36}$**)	321 (97.6)	8 (2.4)	(0.63)
HER2	163 (67.9)	77 (32.1)		238 (99.2)	2 (0.8)	
Normal-like	177 (88.9)	22 (11.1)		196 (98.5)	3 (1.5)	

P values in bold means statistically significant

6.5.5 *RPS6KB1*/p70S6K expression and other associated markers

The correlation between *RPS6KB1* mRNA and the gene expression of other related biomarkers was investigated, using METABRIC dataset (Table 6-4). This comprises correlations with *RPS6KB1* upstream signals, including amino acid transporters that are involved in this study. Both upstream signals, *AKT* and *PIK3CA*, showed a positive correlation with *RPS6KB1* ($p<0.001$). Interestingly, the correlation with *PIK3CA* gene expression was only observed within luminal tumours ($p<0.001$). High *RPS6KB1* mRNA was also significantly observed with enzymes involved in glutaminolysis, *GLS* and *GLUD1*, but only in the luminal A subtype ($p\leq0.007$). There was a positive correlation with the gene expression of glutamine transporters, *SLC1A5* and *SLC38A2*, in almost all BC subtypes ($p\leq0.01$) while the positive association with *SLC7A5* was only noticed in the luminal B tumours ($p=0.04$). Furthermore, there was a positive correlation between *RPS6KB1* mRNA and the amino acid transporter, *SLC7A8* ($p<0.001$).

In the Nottingham BC cohort, high p70S6K protein expression was detected in breast tumours which express high PIK3CA, GLS, SLC1A5, SLC7A5, SLC3A2 and SLC38A2 (all $p\leq0.02$, Table 6-5). No significant association was observed with SLC7A8, SLC7A11 and the upstream effector p-mTORC1 (all $p>0.05$, Table 6-5). However, the latter showed a negative relationship trend with p70S6K expression.

Table 6-4. Correlation of *RPS6KB1* mRNA expression with the expression of other related genes in the METABRIC cohort.

	***RPS6KB1* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)				**Adjusted p-value**					
Regulatory and other associated genes										
mTOR	-0.01 (0.58)	1.16	0.06 (0.11)	0.66	-0.01 (0.80)	3.20	-0.001 (0.98)	0.50	-0.001 (0.98)	1.96
AKT	0.08 (0.0001)	**0.0006**	-0.05 (0.15)	0.75	-0.001 (0.98)	1.96	0.08 (0.20)	1.00	0.10 (0.05)	0.40
PIK3CA	0.16 ($7.2x10^{-14}$)	**<0.0001**	0.17 (0.000005)	**0.0001**	0.23 ($9.3x10^{-8}$)	**<0.0001**	0.14 (0.02)	0.16	0.07 (0.17)	0.85
Enzymes involved in glutaminolysis										
GLS	-0.04 (0.05)	0.25	0.14 (0.00006)	**0.0005**	0.001 (0.97)	2.91	-0.09 (0.13)	0.91	0.02 (0.71)	2.13
GLUD	0.13 ($1.8x10^{-9}$)	**<0.0001**	0.12 (0.001)	**0.007**	-0.03 (0.50)	3.00	0.09 (0.15)	0.90	0.08 (0.11)	0.66
Amino acid transporters										
SLC1A5	0.18 ($5.1x10^{-17}$)	**<0.0001**	0.14 (0.00006)	**0.0005**	0.17 (0.00007)	**0.0008**	0.21 (0.001)	**0.01**	0.05 (0.33)	1.32
SLC7A5	-0.01 (0.43)	1.72	0.02 (0.48)	1.44	0.11 (0.01)	**0.04**	0.04 (0.50)	1.00	-0.09 (0.09)	0.63
SLC3A2	-0.15 ($3.5x10^{-12}$)	**<0.0001**	-0.21 ($7.1x10^{-9}$)	**<0.0001**	-0.05 (0.22)	1.54	-0.25 (0.00008)	**0.0009**	-0.24 (0.000006)	**0.0001**
SLC7A8	0.10 (0.000004)	**<0.0001**	0.04 (0.21)	0.84	-0.10 (0.02)	0.16	0.08 (0.20)	0.80	0.13 (0.01)	0.09
SLC38A2	0.22 ($5.9x10^{-24}$)	**<0.0001**	0.29 ($1.8x10^{-15}$)	**<0.0001**	0.16 (0.0003)	**0.003**	0.17 (0.005)	**0.04**	0.22 (0.00002)	**0.0002**
SLC7A11	-0.01 (0.58)	1.74	0.00 (0.99)	1.98	-0.01 (0.71)	3.55	-0.07 (0.27)	0.81	-0.13 (0.01)	0.10

P values in bold means statistically significant

Table 6-5. Association between p70S6K protein expression and other biomarkers

	p70S6K protein			
	Low, n (%)	**High, n (%)**	**χ2 (p-value)**	**Adjusted p-value**
p-mTORC1				
Negative	150 (70.8)	62 (29.2)	1.66	0.76
Positive	101 (77.1)	30 (22.9)	(0.19)	
PIK3CA				
Negative	143 (81.7)	32 (18.3)	13.44	**0.001**
Positive	118 (64.5)	65 (35.5)	(0.0002)	
GLS				
Negative	133 (76.9)	40 (23.1)	8.19	**0.02**
Positive	98 (62.4)	59 (37.6)	(0.004)	
GLUD				
Negative	192 (69.3)	85 (30.7)	0.37	1.62
Positive	69 (72.6)	26 (27.4)	(0.54)	
SLC1A5				
Negative	308 (86.0)	50 (14.0)	21.96	**<0.0001**
Positive	551 (73.5)	199 (26.5)	(0.000003)	
SLC7A5				
Negative	709 (81.1)	165 (18.9)	26.13	**<0.0001**
Positive	139 (65.0)	75 (35.0)	($3.1x10^{-7}$)	
SLC3A2				
Negative	417 (81.9)	92 (18.1)	8.74	**0.02**
Positive	362 (74.2)	126 (25.8)	(0.003)	
SLC38A2				
Negative	611 (79.6)	157 (20.4)	8.57	**0.02**
Positive	53 (65.4)	28 (34.6)	(0.003)	
SLC7A8				
Negative	562 (79.0)	149 (21.0)	0.01	1.84
Positive	66 (78.6)	18 (21.4)	(0.92)	
SLC7A11				
Negative	71 (84.5)	13 (15.5)	1.70	0.95
Positive	607 (78.4)	167 (21.6)	(0.19)	

P values in bold means statistically significant

6.5.6 *RPS6KB1*/p70S6K expression and patient outcome

Both high *RPS6KB1* mRNA and p70S6K protein expression were associated with poor BCSS ($p<0.006$ and $p<0.01$, Figure 6-7A and 6-8A, respectively). When the analysis was carried out within the BC subtypes, the significant association with poor survival was only observed in the ER+ low proliferation/luminal A subtype ($p=0.001$ and $p=0.01$, Figure 6-7B and 6-8B, respectively). There was no significant association between *RPS6KB1* mRNA or p70S6K protein and BCSS in the ER+ high proliferative/luminal B ($p>0.05$, Figure 6-7C and 6-8C, respectively), TNBC ($p>0.05$, Figure 6-7D and 6-8D, respectively) or HER2+ tumours ($p>0.05$,

Figure 6-7E and 6-8E, respectively). Multivariate Cox regression analysis showed that high expression of *RPS6KB1*mRNA was an independent poor prognostic factor for BCSS, regardless of the other variables; tumour size, tumour grade, and lymph node stage (P=0.01, Table 6-6A). This result remained significant only in luminal A tumours (p<0.001, Table 6-6B). Similarly, p70S6K protein was a predictor of shorter BCSS independent of the same prognostic variables, in the ER+ low proliferative tumours (p=0.02, Table 6-6C) whilst other subtypes did not show any significant associations (all p>0.05, Table 6-6C).

High p70S6K protein expression showed a trend toward significance with shorter DMFS (p=0.05; Figure 6-9A) within the ER+ low proliferative tumours (p=0.05, Figure 6-9B) but not with any other BC subtypes (all p>0.05, Figure 6-9C-E). Breast Cancer Gene-Expression Miner was used to confirm the association between high *RPS6KB1* mRNA expression and poor outcome in ER+, but not ER- tumours (Figure 6-10A-C).

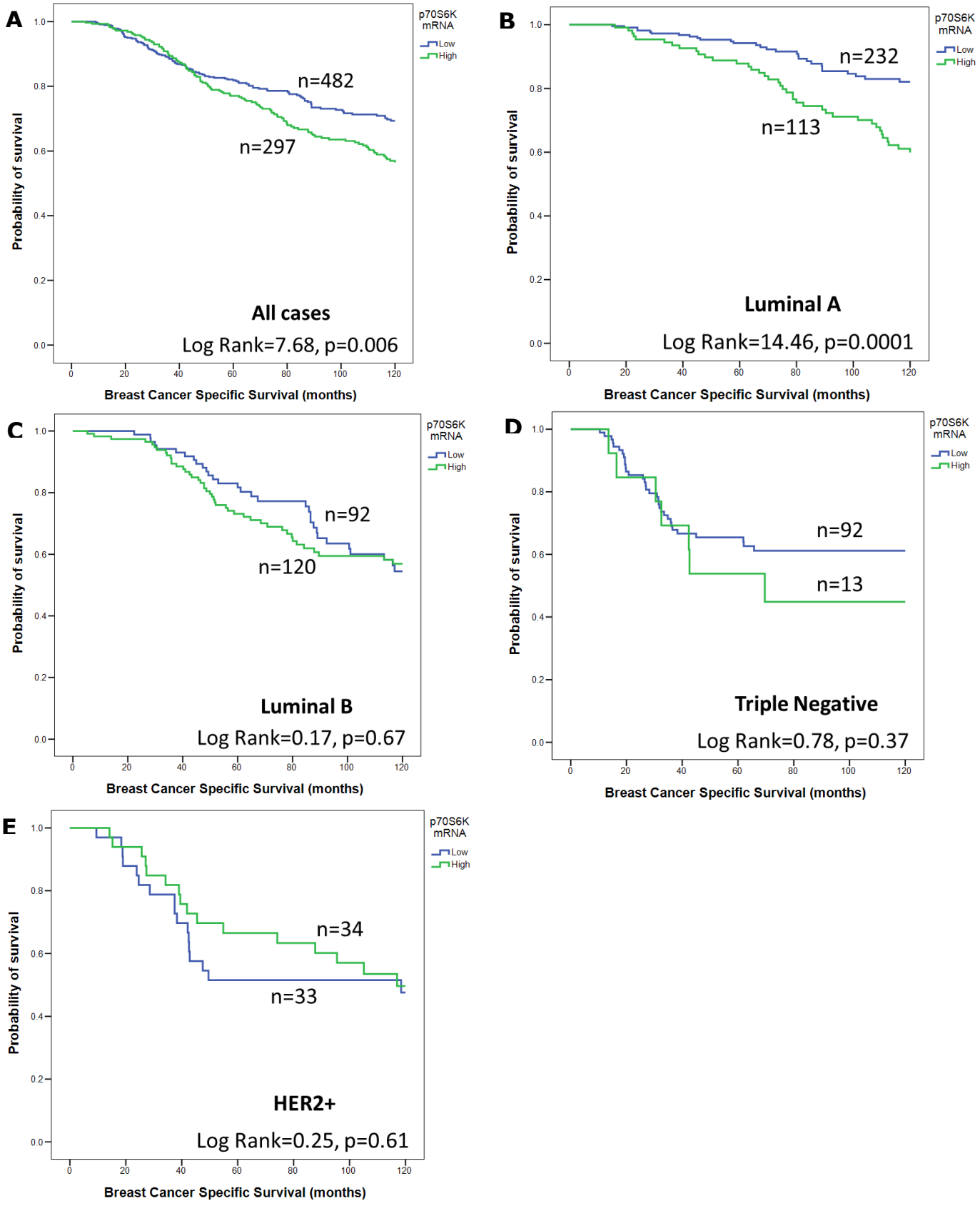


Figure 6-7. RPS6KB1 mRNA and breast cancer patient outcome. A) *RPS6KB1* vs BCSS in all cases, B) *RPS6KB1* vs BCSS in Luminal A tumours, C) *RPS6KB1* vs BCSS in Luminal B tumours, D) *RPS6KB1* vs BCSS in Triple Negative tumours, E) *RPS6KB1* vs BCSS in HER2+ tumours.

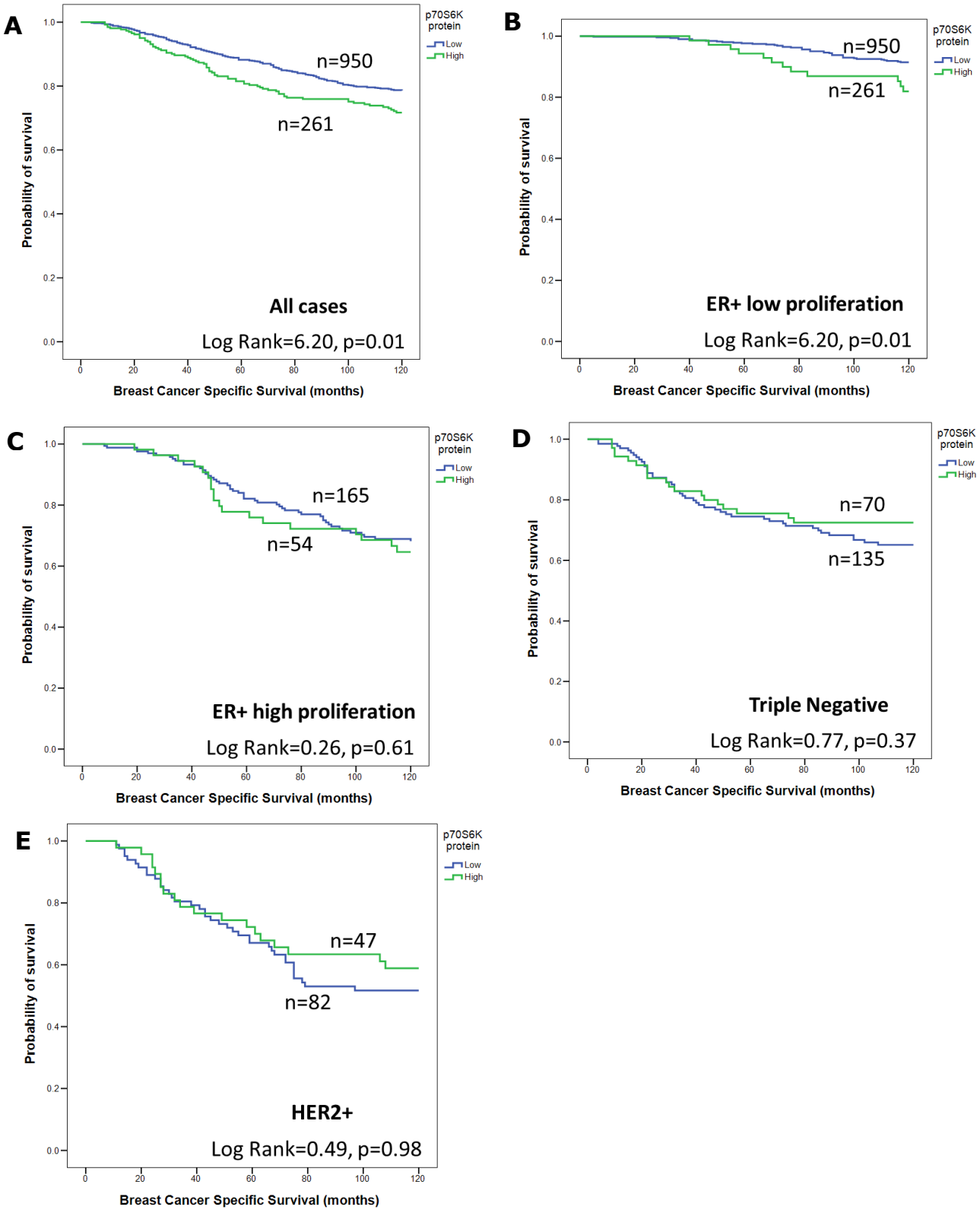


Figure 6-8. p70S6K protein expression and breast cancer patient outcome. A) p70S6K vs BCSS in all cases , B) p70S6K vs BCSS in ER+- Low Proliferation tumours, C) p70S6K vs BCSS in ER+- High Proliferation tumours, D) p70S6K vs BCSS in Triple Negative tumours E) SLC7A5 vs BCSS in HER2+ tumours.

Table 6-6A. Multivariate analysis of prognostic variables and p70S6K mRNA and protein expressions in all cases.

	***RPS6KB1* mRNA**		**p70S6K protein**	
Parameter	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
***RPS6KB1*/ p70S6K**	1.37 (1.06-1.78)	**0.01**	1.08 (0.82-1.42)	0.55
Nodal Stage	1.76 (1.48-2.09)	$1.4x10^{-10}$	2.04 (1.74-2.39)	$8.2x10^{-19}$
Size	1.47 (1.05-2.06)	0.02	2.66 (2.07-3.42)	$2.7x10^{-14}$
Grade	1.42 (1.11-1.81)	0.004	1.38 (1.07-1.79)	0.01

P value in bold means high expression of *RPS6KB1* significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 6-6B. Multivariate analysis of prognostic variables and p70S6K mRNA expression in different BC subtypes.

	***RPS6KB1* mRNA**							
	Luminal A		**Luminal B**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
RPS6KB1	2.48 (1.51-4.06)	**0.0003**	1.16 (0.73-1.85)	0.51	1.32 (0.57-3.05)	0.50	1.05 (0.51-2.20)	0.88
Nodal Stage	1.26 (0.89-1.77)	0.17	1.94 (1.43-2.63)	0.00001	1.84 (1.16-2.92)	0.009	2.31 (1.40-3.79)	0.001
Size	2.09 (1.15-3.82)	0.01	1.50 (0.77-2.93)	0.22	0.75 (0.37-1.54)	0.44	1.88 (0.61-5.74)	0.26
Grade	1.09 (0.72-1.64)	0.67	0.94 (0.62-1.44)	0.80	3.05 (0.41-22.5)	0.27	0.77 (0.34-1.72)	0.53

P value in bold means high expression of *RPS6KB1* significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 6-6C. Multivariate analysis of prognostic variables and p70S6K protein expression in different IHC subtypes.

	p70S6K protein							
	ER-low proliferation		ER-High proliferation		Triple Negative		HER2+	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
p70S6K	2.06 (1.08-3.92)	**0.02**	1.12 (0.65-1.91)	0.67	0.92 (0.53-1.59)	0.92	0.97 (0.54-1.73)	0.93
Nodal Stage	1.46 (0.98-2.17)	0.06	1.88 (1.39-2.55)	0.00004	2.58 (1.87-3.55)	$6.2x10^{-9}$	2.36 (1.64-3.39)	0.000003
Size	1.32 (0.74-2.37)	0.34	1.42 (0.83-2.44)	0.20	1.62 (0.94-2.78)	0.07	1.13 (0.63-2.03)	0.66
Grade	2.07 (1.30-3.30)	0.002	1.71 (0.41-7.05)	0.45	1.69 (0.63-4.52)	0.29	1.60 (0.80-3.17)	0.17

P value in bold means high expression of p70S6K significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

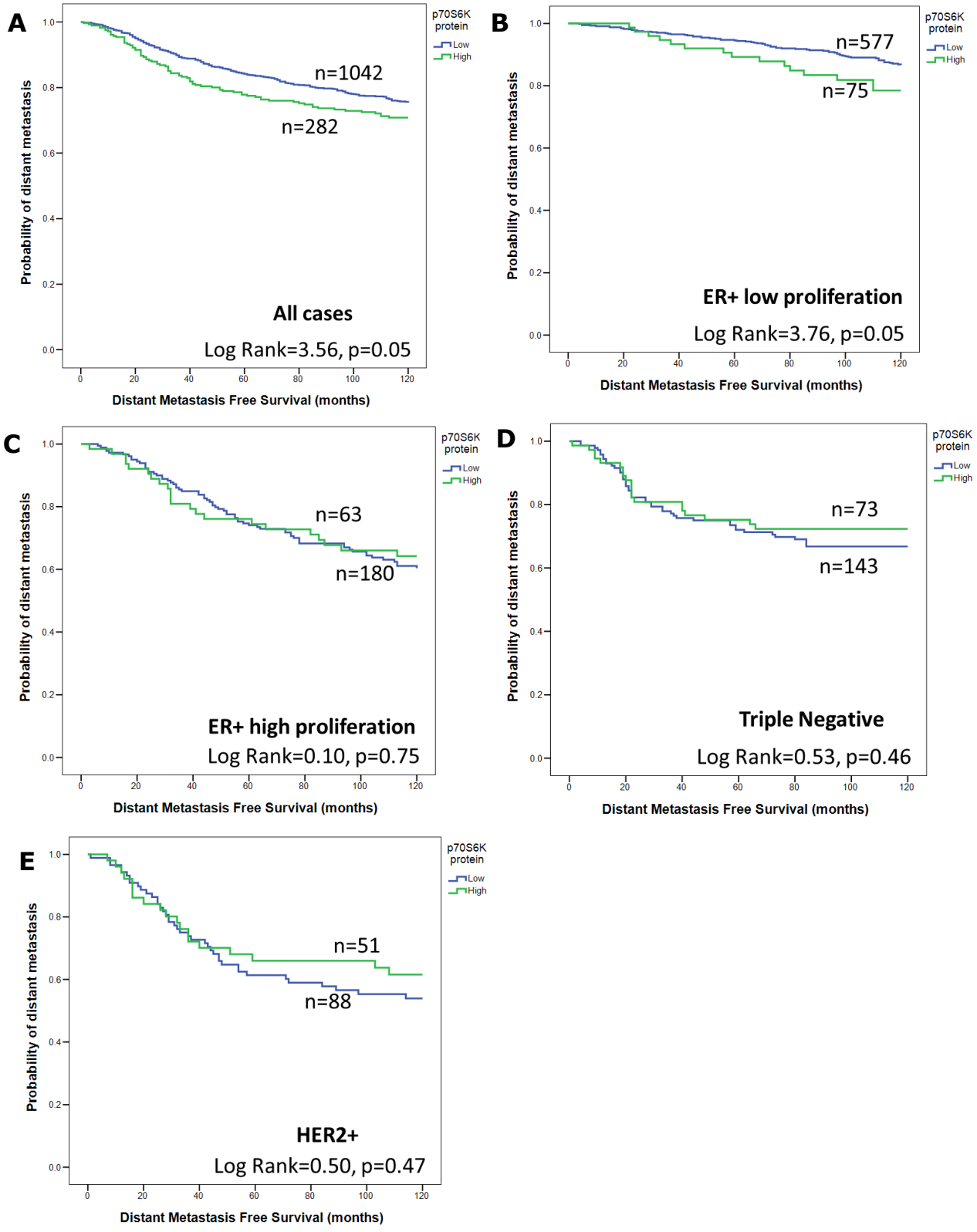


Figure 6-9. p70S6K protein expression and distant metastasis. A) p70S6K Vs DMFS in all cases, B) p70S6K Vs DMFS in ER+- Low Proliferation tumours, C) p70S6K Vs DMFS in ER+- High Proliferation tumours, D) p70S6K Vs DMFS in Triple Negative tumours, E) p70S6K Vs DMFS in HER2+ tumours.

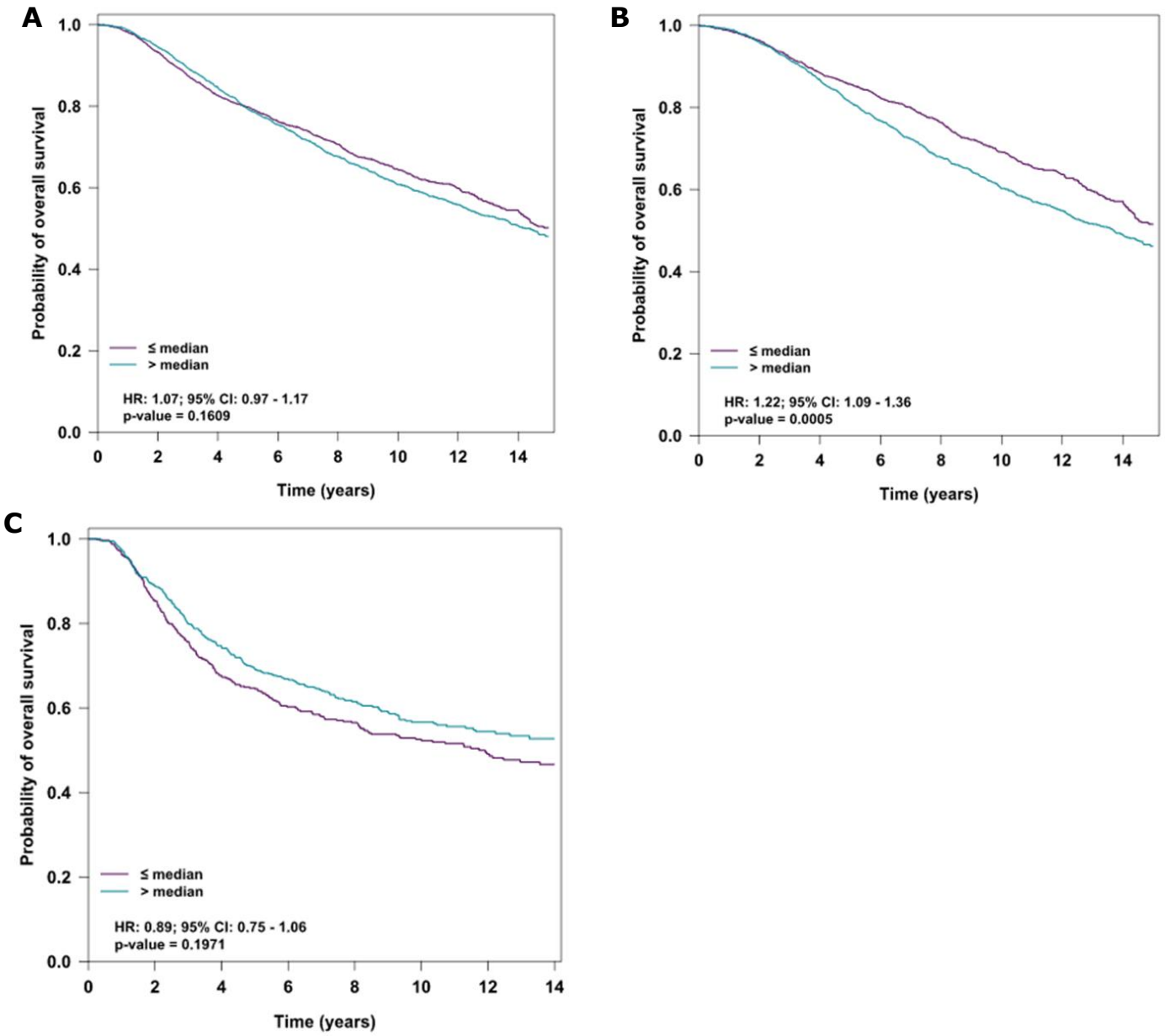


Figure 6-10. RPS6KB1 mRNA and breast cancer patient outcome using Breast Cancer Gene-Expression Miner: A) Unselected cases, B) ER+ disease, C) ER- disease.

6.6 Discussion

Stimulation of cells by growth factors, nutrients and hormones, commences a coordinated set of events which support cell survival, growth and proliferation. p70S6K is the most prominent downstream effector of the extracellular growth stimulation and many studies have emphasised the role of amino acids in triggering mTORC1/p706SK signalling [348,280]. Nevertheless, studies that address the prognostic significance of p70S6K in BC and its potential influence in different BC subtypes remain limited particularly in luminal BC. Therefore, this study investigated large cohorts of BC in order to unravel the potential role of p70S6K in BC and its molecular subtypes, focusing on the involvement of amino acid transporters.

The results of this chapter showed that *RPS6KB1* mRNA and p70S6K protein are over-expressed in tumours with poor prognostic variables including larger tumour size, higher tumour grade and poor NPI. With respect to BC molecular classes, *RPS6KB1* mRNA and p70S6K protein, were highly expressed in HER2+ tumours. This is perhaps anticipated, since both *RPS6KB1* and *HER2* genes are located on the same chromosomal region, 17q23.1 and 17q12, respectively, which is frequently amplified in BC and leads to p70S6K and HER2 overexpression. Furthermore, HER2 is a member of epidermal growth factor (EGF) receptor family that is known to activate PI3K-mTOR-p70S6K signalling in BC [357].

While *RPS6KB1* mRNA was associated with the ER+ status, the p70S6K protein was highly prevalent in the ER- and TN tumours. This can be attributed to the upstream activators, PIK3CA/mTORC1 and or amino acids/amino acid transporters that are more frequent in ER- BC [165,358,273] and may lead to p70S6K phosphorylation and subsequent activation in this specific subtype.

Furthermore, transcriptomic and proteomic analysis of p70S6K were consistent with the finding that the lowest levels of p70S6K was observed in the ER+ low proliferative/luminal A tumours. It is noteworthy that *PIK3CA* mutations are more frequent in the luminal A subtype and they result in decreased p70S6K expression and subsequent favourable prognosis [359,360]. However, the results here showed that the association with shorter BCSS was only observed in the ER+ low proliferative/luminal A tumours and this association was independent of the other confounding variables. Indicating that, there could be mechanisms, other than the

PIK3CA mutation, that control the oncogenic effect of p70S6K in luminal A tumours. On the other hand, another study has showed that PIK3CA can predict poor survival in the luminal-like tumours but not in the other subtypes [165], suggesting that the activation of p70S6K by the wild type PIK3CA can be linked to the results observed in the luminal A tumours, especially that this study showed a positive correlation between *p70S6K* and *PIK3CA* particularly in the luminal tumours.

It has been shown that mTORC1 activates p70S6K via phosphorylation both directly and through inhibition of protein phosphatase 2A (PP2A), which selectively dephosphorylates p70S6K [361,362]. This study however showed a trend toward a negative association between p70S6K and p-mTORC1. This observation is consistent with a recent study which demonstrated that p70S6K targeted p-mTORC1 phosphorylated at Ser2448 in a negative feedback loop mechanism, providing an evidence not to use Ser2448 as a proxy of mTORC1 kinase activity, alternatively, p70S6K should be used for the preferential analysis of mTORC1 activation [262].

mTORC1 is known to be activated by amino acids. Glutamine with subsequent glutaminolysis is demonstrated to be involved in activating mTORC1. While the association with amino acid transporters that mediate glutamine influx (*SLC1A5* and *SLC38A2*) was observed in almost all of the subtypes, enzymes engaged in glutaminolysis showed a significant correlation in luminal A tumours only, indicating that mTORC1/p70S6K activation by glutaminolysis could be specific to luminal A subtype.

Interestingly, the association with *SLC7A5*, which mediates leucine uptake, was only observed in luminal B tumours. In the line with that, Chapters 3 and 5 have shown that SLC7A5 is a predictor of poor survival in the luminal B subtype and targeting SLC7A5 significantly reduced proliferation and invasion in the luminal B-like cell line (ZR-75-1), indicating that the effect of SLC7A5 on this subtype may be partly mediated through the activation of P70S6K.

Previous studies showed that elevated levels of p70S6K was associated with poor clinical outcome in patients with early stage oesophageal squamous cell carcinoma and osteosarcoma [363,364]. In addition, p70S6K was linked to malignant transformation, invasion and metastasis in patients with gastric carcinoma [365].

Consistent with these studies, our results revealed that overexpression of *RPS6KB1*/p70S6K in BC, at both mRNA and protein levels, is linked to poor clinicopathological parameters, including larger tumour size, higher tumour grade and poor NPI. It was also associated with poor patient outcome, especially in the ER+ low proliferative/ luminal A tumours thus it could act as a potential prognostic marker. Additionally, *RPS6KB1*/p70S6K was significantly associated with the upstream signal, PIK3CA, and the key glutamine transporters. These findings lay a foundation for the necessity of making further assessment on p70S6K as a potential target for BC treatment.

Chapter 7. The prognostic significance of SLC38A2, SLC7A11 and SLC7A8 in invasive breast cancer

7.1 Introduction

Many cancer cells alter their metabolism to provide energy and cellular building blocks required for their rapid proliferation. One common mechanism used by cancer cells for metabolic reprogramming is to increase uptake of nutrients crucial for bioenergetic and biosynthetic pathways, including amino acids such as glutamine [128,366]. To allow this, cancer cells upregulate various transporters that mediate uptake of amino acids. Indeed, the urgent need for important amino acids might require the expression of more than one transporter which all share the same function. For example, SLC1A5 is a key glutamine transporter and it has been previously studied in many cancers [198-201,136]. Solute Carrier Member 38 Family 2 (SLC38A2) also considered one of the major routes for glutamine uptake and thus could co-express with the SLC1A5 to boost its function.

Metabolic reprogramming in cancer cells also increases oxidative stress which subsequently leads to cell death. To encounter this, cancer cells upregulate their antioxidant capabilities through different mechanisms such as increasing biosynthesis of antioxidant molecules including glutathione [367]. Amino acids are one of the important molecules that assist the generation of these antioxidants through supplying carbon and cofactors (e.g. NADPH). SLC7A11 is the cystine/glutamate antiporter which plays a critical role in maintaining redox balance [368]. As this transporter requires glutamate, it is hypothesised that functional coupling could occur with the key transporters that provide glutamine to be converted to glutamate. This suggests that cancer cells not only require the expression of transporters that share the same functions, they also demand the existence of other transporters which support further processes, so all work in synergy in favour of cancer cells survival and progression.

Another example of the transporters that share the same substrate selectivity are SLC7A5 and Solute Carrier Family 7 Member 8 (SLC7A8). Although there is a general thought that SLC7A5 is over expressed in many cancers and it is associated with poor prognosis, other studies revealed that the expression levels of SLC7A8 are low in cancer cells [318,226].

This chapter will focus on other amino acid transporters that are functionally related to SLC1A5 and SLC7A5 (i.e. SLC38A2, SLC7A11 and SLC7A8) as their prognostic significance in breast cancer remains unknown.

7.1.1 SLC38A2/ sodium-coupled neutral amino acid transporter 2 (SNAT2)

SLC38A2 is a sodium-dependent transporter that belongs to system A amino acid transporters. It mediates the transport of neutral amino acids including glutamine, serine, proline and alanine [369]. Although this transporter has a narrow substrate selectivity, it represents one of the major routes of glutamine entry into cells under physiologic conditions [142]. SLC38A2 is expressed ubiquitously in all nucleated mammalian cells [370]. In neurones, this transporter assists the continuity of glutamate/GABA-glutamine cycle that takes place between neurons and astrocytes, where this transporter takes up the glutamine from extracellular spaces and utilises it as a source of glutamate, which is then released into the synaptic cleft during neuronal activation [371]. In the liver, hepatocytes take up glutamine through SLC38A2, which is subsequently involved in ammonia metabolism and urea synthesis [372]. SLC38A2 is also critically involved in the transfer of amino acids from the mother to the developing foetus across the placenta, indicating the crucial role played by this transporter in foetal development [373].

Indeed, there is some evidence that SLC38A2 might be involved in tumourigenesis, as the gene encoding *SLC38A2* is a transcriptional target for the tumour suppressor p53, thus the wild-type p53 repress the expression of this transporter [374]. This indicates that the expression of SLC38A2 is increased in p53-mutant cancers; therefore providing cancer cells with glutamine. In addition, SLC38A2 appears to have a role in supporting cancer cell growth and proliferation and it is over-expressed in various cancer cell types, including cervical cancer, osteosarcoma and breast cancer [375,289,146].

Among the SLC38 family, SLC38A2 is the primary isoform induced when mammalian cells are deprived of amino acids and the rate of its transcription is enhanced following amino acid depletion [376]. It has been found that ATF4 and CCAAT-enhancer-binding protein (C/EBP) are implicated in the regulation of SLC38A2 transcription in amino acid deprived conditions [370]. Furthermore, under hypoxic environment, HIF1α increased the expression of SLC38A2 in the ER+ breast cancer cells and this expression is linked to developing resistance to endocrine therapy [146]. Altogether, SLC38A2 seems to be a marker of stressful

conditions, such as hypoxia and amino acid depletion, which are usually associated with the more aggressive and highly proliferative cancers.

SLC38A2, like SLC1A5, is also regulated by proteasomal degradation through the chemotherapeutic agent, paclitaxel, which induces endoplasmic reticulum stress and activates the ubiquitin ligase RNF5 with subsequent ubiquitination and degradation of SLC38A2 [287]. Hindering glutamine uptake into cancer cells might underlain the therapeutic efficacy of this agent in cancer treatment.

Interestingly, the inhibition of SLC38A2 not only showed a decrease in its substrates, it also reduces the essential amino acid (leucine), which may confirm the extent of co-operation between this transporter and other system L transporters, such as SLC7A5, especially in stressful conditions [375]. In this regard, Bröer *et al* reported that SLC38A2 is one of the system A amino acid transporters that mediates the net influx of glutamine with subsequent glutaminolysis while SLC1A5 is an obligatory exchanger for small neutral amino acids and does not mediate net uptake of glutamine unless other amino acids are available for release [289]. This indicates that these two transporters could act as a switch, in providing glutamine in cancer cells, based on amino acid availability.

7.1.2 SLC7A11/ xCT

SLC7A11 is a sodium independent cysteine/glutamate antiporter, which takes up extracellular cystine in exchange for intracellular glutamate at 1:1 ratio [377]. This transporter plays a crucial role in maintaining redox homeostasis and protecting cancer cells from apoptosis, where the amino acid cystine is reduced to cysteine; the rate-limiting precursor in the synthesis of the antioxidant peptide (glutathione) [377]. SLC7A11, like SLC7A5, is expressed in the plasma membrane in the form of a heterodimer complex with the chaperone protein (SLC3A2). The latter does not demonstrate any intrinsic transport activity, but its expression is necessary for the recruitment and therefore the transport function of SLC7A11 [221].

SLC7A11 is normally expressed in brain, pancreas, fibroblasts and immune cells [377]. Over-expression of SLC7A11 is demonstrated in many cancers including tongue cancer, liver carcinoma, oesophageal and laryngeal squamous cell carcinomas [255,378-380]. Interestingly, the expression of this transporter is induced directly by the oncogene c-Myc, the same inducer of the previously

studied SLCs (SLC1A5, SLC7A5 and SLC3A2) [381]. In addition, another study showed the role of proto-oncogene Ets-1 in increasing SLC7A11 expression and activity [382].

Previous studies demonstrate that SLC7A11 expression and activity is induced in response to various stress conditions such as amino acid starvation, especially cysteine, glucose starvation and oxidative stress [383,384]. Stress-induced SLC7A11 expression is regulated by the master regulator of anti-oxidant response, erythroid 2-related factor 2 (NRF2) and ATF4 through binding to SLC7A11 promotor [385]. The tumour suppressor, P53, is another transcription factor that inhibits SLC7A11 expression and induces cell cycle arrest and cell death [386].

SLC7A11 expression is also regulated by posttranscriptional mechanisms including micro RNAs such as miR-26b and miR-27a, which directly degrade *SLC7A11* mRNA reducing its stability. These microRNAs are downregulated in many cancer cells and its overexpression reduces cell proliferation and invasion of different cancer cells, most likely via inhibiting SLC7A11 expression [387,388]

Posttranscriptional control of SLC7A11 expression also includes the regulation by nonsense-mediated mRNA decay (NMD), which acts on targeting mRNAs, that often encode proteins involved in stress responses, with premature stop codons. However, under different cellular stresses, the activity of NMD is inhibited resulting in *SLC7A11* mRNA stabilisation and enhancing its protein levels [389].

Posttranslational modifications are other mechanisms that regulate SLC7A11 protein activity. Besides SLC3A2, CD44 also acts to maintain SLC7A11 protein stability and assists its localisation in the plasma membrane [390]. Phosphorylation of SLC7A11 protein is a further regulatory mechanism, which supresses SLC7A11 protein activity and function [391].

As discussed earlier, intracellular glutamate is necessary for cystine entry through SLC7A11. This confirms the importance of intracellular glutamine, which converts into glutamate via glutaminase and ultimately drives SLC7A11-mediated cystine entry. Hence, SLC7A11 requires the functional coupling with other glutamine transporters. SLC38A2 could be one candidate, as it is capable of mediating the influx of extracellular glutamine in stressful conditions. Furthermore, intracellular cystine undergoes cystine/cysteine cycle where intra cellular cystine is reduced to cysteine for glutathione synthesis while the rest is exported outside the cell and

oxidised to cystine [392]. The obligatory exchanger, SLC1A5, could be implicated in cysteine efflux, which is coupled by glutamine entry, thus it operates like the factory that supplies SLC7A11 with its substrates. This suggests that SLC1A5 could be another potential associate for SLC7A11 function [193].

7.1.3 SLC7A8/ L-Type Amino Acid Transporter 1 (LAT2)

SLC7A8 is a sodium-independent amino acid exchanger (antiporter), which transports small and large neutral amino acids such as alanine, serine, threonine, cysteine, phenylalanine, tyrosine, leucine and glutamine [6]. Similar to SLC7A5 and SLC7A11, this solute carrier also requires heterodimerisation with the heavy chain of SLC3A2 for its proper localisation in the plasma membrane [211,393]. Although SLC7A8 shares the same substrate selectivity with SLC7A5, the former has a narrower tissue expression pattern and displays lower affinity for its substrates, especially glutamine and serine [142].

SLC7A8 plays an important role in the growth and proliferation of normal cells [394]. More specifically, it is highly expressed in the absorptive cells of the intestine and kidneys where it presents in the basolateral membrane, therefore involved in the efflux of amino acids from the cells into the bloodstream [142]. It is also expressed in the ciliary and lens epithelia and its deletion results in a significant reduction in lens essential amino acid levels and development of cataracts [395]. In addition, this transporter, alongside SLC7A5, is considered among the carriers that transport thyroid hormones across the blood brain barrier [396].

SLC7A5 has been extensively studied in a variety of cancers, including results presented in this thesis, and it is regulated by various oncogenes, such as MYC [397,398,227,140]. However, there is limited information whether SLC7A8 plays an equal role in cancer. Janpipatkul *et al* showed high levels of SLC7A5 in cholangiocarcinoma cells while SLC7A8 expression was barely detectible in the same cell line [318]. Another study confirmed that human oral epidermoid carcinoma cells and human bladder cancer cells, which co-express SLC7A5 and SLC3A2 in the plasma membrane did not express SLC7A8 [226], suggesting that the higher affinity transporter is the predominant in the cancer cells. This also raises doubts about whether SLC7A8 has any role to play in cancer cells. Furthermore, knockdown of *SLC7A5* did not show any changes in the SLC7A8

mRNA expression, indicating that there are no compensatory responses by the latter after silencing *SLC7A5* [318]. It has previously been described above the potential utility of SLC7A5 as a poor prognostic factor for the highly proliferative BC subtypes [273]. However, the prognostic significance of SLC7A8 in BC is still obscure.

Information about genes that regulate SLC7A8 expression is scarce. However, previous studies have revealed that hormone receptors could be implicated in the regulation process. It has been found that SLC7A8 is upregulated in ER+ BC and it is controlled by oestrogen [399,144]. On the other hand, Luo *et al* identified SLC7A8 as a novel progesterone target gene in uterine leiomyoma cells [400].

7.2 Hypothesis

The amino acid transporters, SLC38A2, SLC7A11 and SLC7A8, are over-expressed in BC and they have prognostic implications that will vary among the different molecular BC subtypes.

7.3 Aims

1. To assess the expression of SLC38A2, SLC7A11 and SLC7A8, at the mRNA and protein levels, using large well-characterised annotated cohorts of BC and to evaluate their association with clinicopathological parameters and a range of relevant biological markers.
2. To evaluate the prognostic potential of SLC38A2, SLC7A11 and SLC7A8, at mRNA and protein levels, in the different molecular BC subtypes.

7.4 Methods

7.4.1 Study cohorts

7.4.1.1 METABRIC cohort

METABRIC cohort was utilised to assess gene copy number and expression of *SLC38A2*, *SLC7A11* and *SLC7A8*. This cohort comprised 1980 primary operable invasive BC patients. Clinical history, outcome data and tumour characteristics, including tumour size, tumour grade, LN stage and histological phenotypes, were available for this cohort. The intrinsic subtypes of BC (luminal A, luminal B, HER2-enriched, normal-like, and basal-like) were classified based on prediction analysis of microarray of 50 genes (PAM50). Integrating germline variants, somatic

aberrations, and aberration with gene expression data augmented BC classification in this cohort by producing 10 integrative clusters. Each cluster represents a distinct genomic landscape, molecular and clinical features and unique ER, PR and HER2 expressions. The characteristics of the METABRIC cohort are detailed in section 2.1.1.

7.4.1.2 External validation cohort

For external validation of *SLC38A2*, *SLC7A11* and *SLC7A8* mRNA expression, Breast Cancer Gene-Expression Miner v4.0 online dataset was used. This dataset offers the possibility to evaluate the clinicopathological and prognostic significance of candidate genes in large pooled cohort of BC patients, as detailed in section 2.1.2.

7.4.1.3 Nottingham BC cohort

Protein expression of SLC38A2, SLC7A11 and SLC7A8 was assessed using the Nottingham BC cohort. This is a well-characterised cohort of patients with early stage primary operable invasive BC. The clinicopathological profile of the study samples includes patient's age, tumour size, tumour grade, nodal stage, NPI, LVI status and tumour histological types. The hormonal receptor expression profiles and outcome data (BCSS and DMFS) were also recorded. The clinicopathological characteristics of this cohort are detailed in section 2.1.3.

The expression of SLC38A2, SLC7A11 and SLC7A8, at the mRNA and protein levels, was correlated with clinicopathological parameters, molecular subtypes, relevant biological markers and patient outcome.

7.4.2 Western blotting and immunohistochemistry

Western blotting was applied to validate the specificity of SLC38A2, SLC7A11 and SLC7A8 primary antibodies using whole cell lysates from HeLa, MCF7, ZR-75-1 and SKBR3 cells. IHC staining was performed on TMA sections using the Novolink polymer detection system. The details of methodology, antibodies concentrations and incubation times are described in sections 2.2, 2.4 and table 2-3.

7.5 Results

7.5.1 Validation of SLC38A2, SLC7A11 and SLC7A8 antibodies

Western blotting analysis showed specific bands at the correct predicted size (36 kDa for SLC38A2 protein, 55 kDa and 135kDa for SLC7A11 protein isoforms and 58 kDa for SLC7A8 protein) (Figure 7-1A-C).

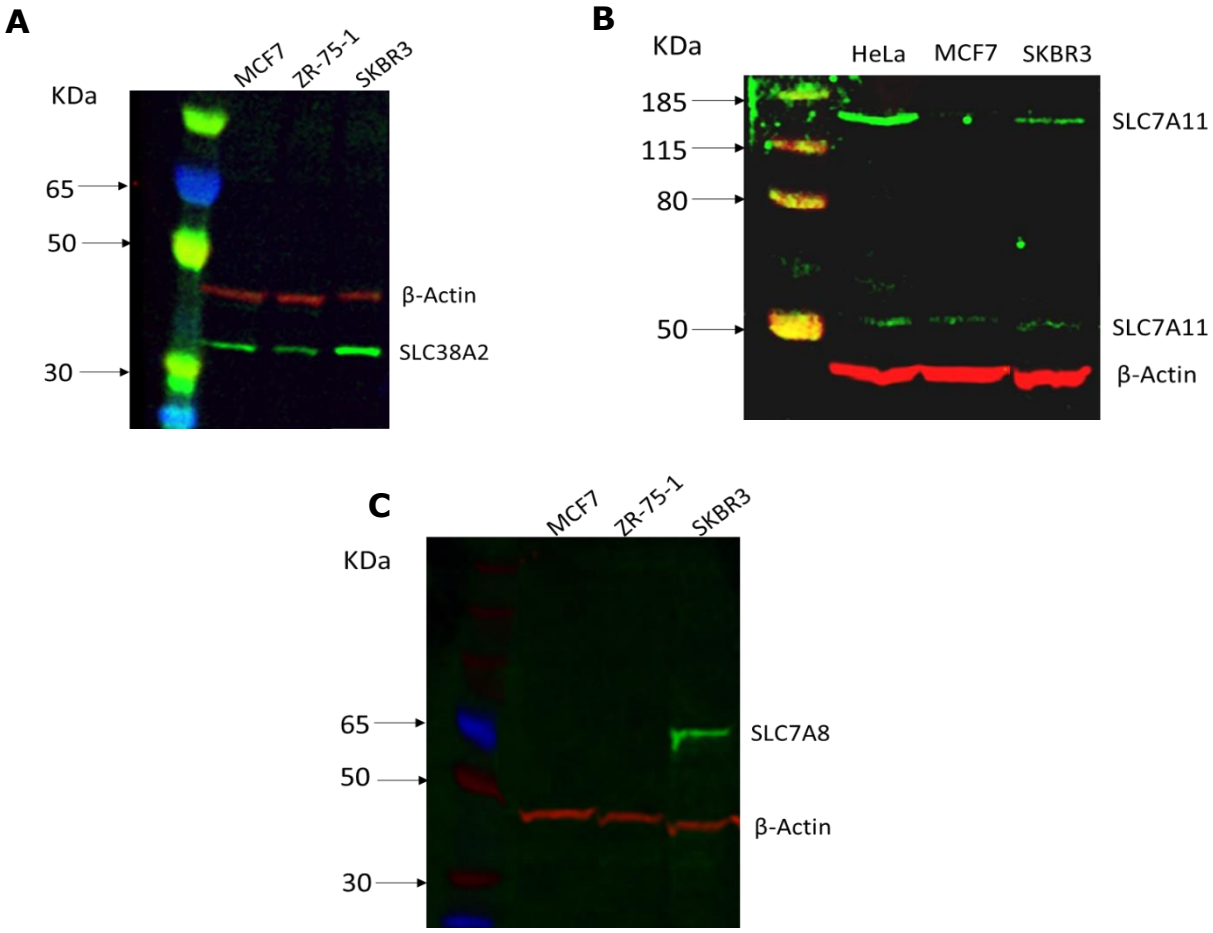


Figure 7-1. Western blot underlining antibody specificity of A) SLC38A2, B) SLC7A11 and C) SLC7A8. β-actin was included as a positive control.

7.5.2 Expression of SLC38A2, SLC7A11 and SLC7A8 in breast cancer

SLC38A2, SLC7A11 and SLC7A8 gene copy number and gene expressions were evaluated using the METABRIC cohort. Dichotomisation of *SLC38A2*, *SLC7A11* and *SLC7A8* mRNA normalised data was set using X-tile software, based on prediction of BCSS. High *SLC38A2* mRNA expression (log2 intensity >8.5) was observed in 988/1,980 (50%) of the METABRIC BC cases. A total of 63/1,980 (3.2%) cases showed a CN gain of *SLC38A2* while only 14/1,980 (0.7%) cases showed a CN loss. Regarding *SLC7A11*, high mRNA expression (log2 intensity >5.5) was observed in 239/1,980 (12%) of the cases. Only 23/1980 (1.2%) cases showed *SLC7A11* CN gain while the gene loss was observed in 66/1980 (3.3%) of the cases. High *SLC7A8* mRNA expression was observed in almost two-thirds (67%) of the cases. *SLC7A8* was amplified in 90/1,980 (4.5%) cases, whereas 45/1,980 (2.3%) cases showed a CN loss. A positive association was observed between CNV of *SLC38A2* and *SLC7A8* and their corresponding mRNA expressions ($p<0.001$, Figure 7-2A and C). However, no significant association was detected between *SLC7A11* CNV and mRNA expression ($p>0.05$, Figure 7-2B).

SLC38A2, SLC7A11 and SLC7A8 protein expressions were assessed using the Nottingham BC cohort. Staining of SLC38A2, SLC7A11 and SLC7A8 in full-face BC tissue sections demonstrated a homogenous pattern of their immunoreactivity. A total of 1,685, 1,981 and 1,562 cases were valid for assessing SLC38A2, SLC7A11 and SLC7A8 proteins, respectively. Protein expression of the three solute carriers was mainly observed in the cytoplasm of the invasive breast tumour cells, with intensity levels varying from absent to high (Figure 7-3A-F). The distribution of SLC38A2, SLC7A11 and SLC7A8 expression was unimodal and left-skewed and the x-tile software was used to identify the cut-off points for their positivity. High SLC38A2 protein (>130 H-score) was observed in 153/1,685 (9%) of BC cases while 784/1,981 (40%) of cases were high for SLC7A11 protein (>100 H-score) and 177/1,562 (11%) were high for SLC7A8 protein (>20 H-score).

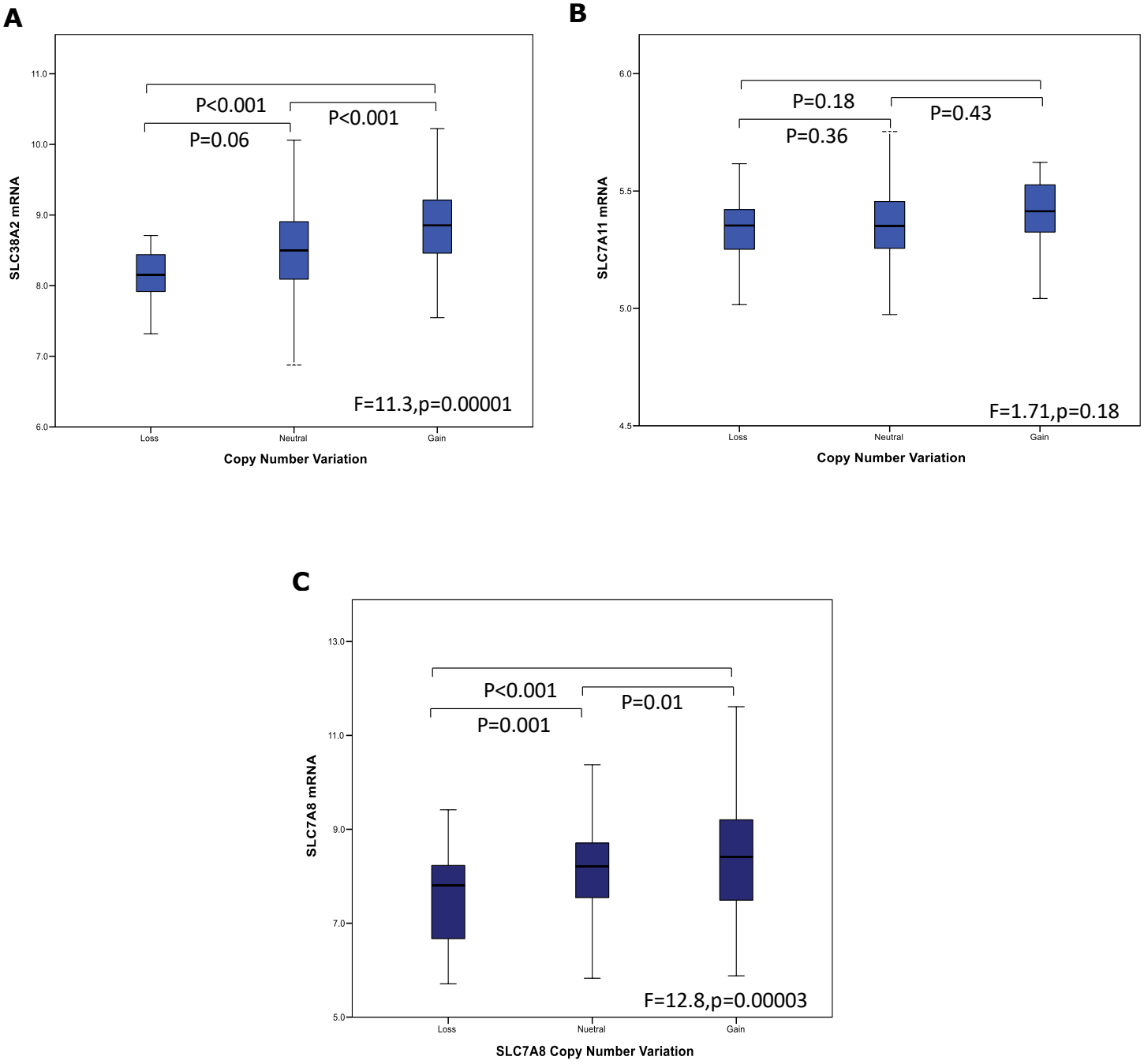


Figure 7-2. Relationship between Copy Number variations of A) SLC38A2, B) SLC7A11 and C) SLC7A8 and their corresponding mRNA expressions in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

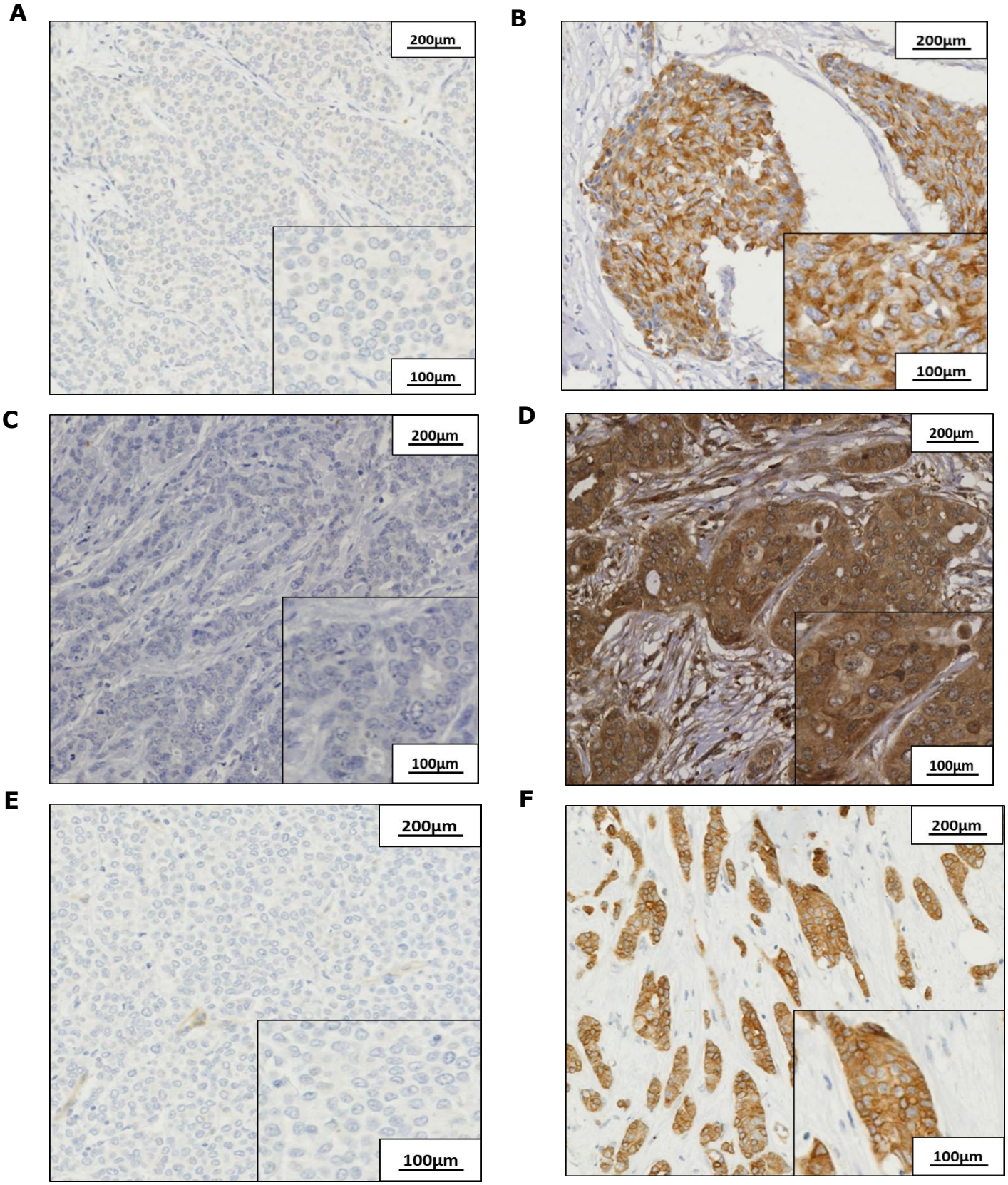


Figure 7-3. Protein expression of SLC38A2, SLC7A11 and SLC7A8 in invasive breast cancer. A) Negative SLC38A2 immunohistochemical (IHC) expression, B) Positive SLC38A2 IHC expression, C) Negative SLC7A11 IHC expression, D) Positive SLC7A11 IHC expression, E) Negative SLC7A8 IHC expression, F) Positive SLC7A8 IHC expression. Positive immunoreactivity is detected in the cytoplasm of the invasive breast cancer cells. Magnification 10x. Scale bars=200µm. Inset, magnification 20x. Scale bars=100µm.

7.5.3 Association of SLC38A2, SLC7A11 and SLC7A8 with clinicopathological parameters

SLC38A2 and SLC7A11 were primarily associated with clinicopathological features of poor prognosis. Whilst high *SLC38A2* mRNA expression did not show any significant associations with clinicopathological parameters; tumour size, grade, lymph node stage or NPI in the METABRIC dataset (all p>0.05, Figure 7-4 A-D) or Breast Cancer Gene-Expression Miner (Figure 7-7A-C), SLC38A2 protein was significantly associated with high tumour grade (p<0.001, Table 7-1), high mitotic count (p<0.001, Table 7-1) and poor NPI (p=0.03, Table7-1). There was no association with tumour size, nodal stage, the presence of lymphovascular invasion or development of metastasis in distal organs (all p>0.05, Table 7-1). With regards to *SLC7A11*, high mRNA levels were associated with high tumour grade (p=0.01, Figure 7-5B) but not with tumour size, nodal stage and NPI (all p>0.05, Figure 7-5A, C, D). Nevertheless, analysis of *SLC7A11* in Breast Cancer Gene-Expression Miner v4.0 dataset showed significant associations with these parameters, where available (Figure 7-8A-C). At the protein level (Table 7-2), SLC7A11 was significantly associated with higher tumour grade (p=0.02), high mitotic count (p=0.02) and more tubule formation (p=0.007) whereas it was not associated with tumour size, NPI, lymph node stage and lymphovascular invasion (all p>0.05).

SLC7A8 was a marker of favourable prognosis, as high *SLC7A8* mRNA expression was significantly associated with smaller tumour size (P=0.007, Figure 7-6A), lower tumour grade (P<0.001, Figure 7-6B), and good NPI (P<0.001, Figure 7-6D). These associations were confirmed using the Breast Cancer Gene-Expression Miner (Figure 7-9A-C). Similar associations were observed with SLC7A8 protein expression. Table 7-3 summarises the observed findings between high SLC7A8 protein and good prognostic factors, including small tumour size (P=0.03), low tumour grade (P<0.001), low mitotic count (p=0.002), low pleomorphism (p=0.005), more tubule formation (p=0.01) and good NPI (P<0.001). SLC7A8 was not associated with nodal stage and the presence of lymphovascular invasion (all p>0.05) nor with the development of distant metastasis to brain, lung, bone and liver (all p>0.05, Table 7-1, 7-3).

Table 7-1. Clinicopathological associations of SLC38A2 expression in Nottingham breast cancer cohort

	SLC38A2 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Age				
< 50	508 (88.8)	64 (11.2)	4.63 (0.03)	0.09
≥ 50	1023 (92.0)	89 (8.0)		
Tumour size				
< 2.0cm	853 (91.8)	76 (8.2)	2.07 (0.14)	0.26
≥ 2.0cm	677 (89.8)	77 (10.2)		
Tumour Grade				
1	217 (93.9)	14 (6.1)		
2	578 (94.0)	37 (6.0)	19.37 (0.00006)	**0.0003**
3	734 (87.8)	102 (12.2)		
Mitosis				
1	601 (93.9)	39 (6.1)		
2	334 (94.4)	20 (5.6)	31.5 ($1.4x10^{-7}$)	**<0.0001**
3	561 (85.9)	92 (14.1)		
Pleomorphism				
1	21 (87.5)	3 (12.5)		
2	460 (92.9)	35 (7.1)	3.89 (0.14)	0.30
3	1016 (90.0)	113 (10.0)		
Tubular formation				
1	87 (93.5)	6 (6.5)		
2	481 (93.0)	36 (7.0)	6.03 (0.04)	0.10
3	930 (89.5)	109 (10.5)		
Vascular Invasion				
Negative	1024 (90.5)	108 (9.5)	0.78 (0.37)	0.51
Positive	503 (91.8)	45 (8.2)		
Lymph Node Stage				
1	925 (90.9)	93 (9.1)		
2	463 (91.0)	46 (9.0)	0.006 (0.99)	1.98
3	138 (90.8)	14 (9.2)		

(Continued)

	SLC38A2 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Nottingham Prognostic Index				
Good	477 (94.1)	30 (5.9)	8.98 (0.01)	**0.03**
Moderate	792 (89.6)	92 (10.4)		
Poor	257 (89.2)	31 (10.8)		
IHC Subtypes				
ER+/HER2- Low Proliferation	788 (94.1)	49 (5.9)	24.96 (0.00001)	**0.0001**
ER+/HER2- High Proliferation	249 (88.3)	33 (11.7)		
Triple Negative	226 (85.0)	40 (15.0)		
HER2+	170 (89.5)	20 (10.5)		
Histological type				
Ductal (including mixed)	1347 (90.8)	136 (9.2)	2.35 (0.79)	1.58
Lobular	99 (89.2)	12 (10.8)		
Medullary	25 (96.2)	1 (3.8)		
Miscellaneous	7 (100.0)	0 (0.0)		
Special type	50 (92.6)	4 (7.4)		
Tubular	2 (100.0)	0 (0.0)		
Site of distant metastasis				
Brain				
No	573 (92.6)	46 (7.4)	1.49 (0.22)	0.36
Yes	34 (87.2)	5 (12.8)		
Lung				
No	547 (92.6)	44 (7.4)	0.75 (0.38)	0.55
Yes	60 (89.6)	7 (10.4)		
Bone				
No	473 (91.8)	42 (8.2)	0.54 (0.46)	0.57
Yes	134 (93.7)	9 (6.3)		
Liver				
No	519 (92.0)	45 (8.0)	0.28 (0.59)	0.68
Yes	88 (93.6)	6 (6.4)		

P values in bold means statistically significant

Table 7-2. Clinicopathological associations of SLC7A11expression in Nottingham breast cancer cohort

	SLC7A11 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Age				
< 50	111 (17.2)	535 (82.8)	0.91 (0.33)	0.44
≥ 50	177 (15.4)	970 (84.6)		
Tumour size				
< 2.0cm	644 (60.5)	420 (39.5)	0.008 (0.92)	1.00
≥ 2.0cm	552 (60.3)	363 (39.7)		
Tumour Grade				
1	156 (56.3)	121 (43.7)	8.66 (0.01)	**0.02**
2	473 (64.5)	260 (35.5)		
3	566 (58.5)	402 (41.5)		
Mitosis				
1	492 (63.0)	289 (37.0)	8.45 (0.01)	**0.02**
2	248 (62.8)	147 (37.2)		
3	432 (56.3)	335 (43.7)		
Pleomorphism				
1	19 (70.4)	8 (29.6)	16.56 (0.0002)	**0.002**
2	400 (66.7)	200 (33.3)		
3	754 (57.2)	564 (42.8)		
Tubular formation				
1	55 (49.1)	57 (50.9)	12.57 (0.002)	**0.007**
2	346 (56.9)	262 (43.1)		
3	772 (63.0)	453 (37.0)		
Vascular Invasion				
Negative	802 (61.4)	505 (38.6)	1.32 (0.24)	0.33
Positive	392 (58.7)	276 (41.3)		
Lymph Node Stage				
1	744 (62.4)	449 (37.6)	4.89 (0.08)	0.13
2	342 (57.3)	255 (42.7)		
3	108 (57.8)	79 (42.2)		

(Continued)

	SLC7A11 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Nottingham Prognostic Index				
Good	365 (62.4)	220 (37.6)	1.71 (0.42)	0.50
Moderate	628 (60.0)	419 (40.0)		
Poor	201 (58.3)	144 (41.7)		
IHC Subtypes				
ER+/HER2- Low Proliferation	634 (63.2)	369 (36.8)	12.97 (0.01)	**0.04**
ER+/HER2- High Proliferation	201 (60.0)	134 (40.0)		
Triple Negative	159 (52.6)	143 (47.4)		
HER2+	118 (56.5)	91 (43.5)		
Histological type				
Ductal (including mixed)	1027 (59.4)	702 (40.6)	21.09 (0.001)	**0.005**
Lobular	118 (76.1)	37 (23.9)		
Medullary	13 (54.2)	11 (45.8)		
Miscellaneous	7 (77.8)	2 (22.2)		
Special type	30 (50.0)	30 (50.0)		
Tubular	1 (50.0)	1 (50.0)		

P values in bold means statistically significant

Table 7-3. Clinicopathological associations of SLC7A8 expression in Nottingham breast cancer cohort

	SLC7A8 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Age				
<50	469 (90.2)	51 (9.8)	1.18 (0.27)	0.77
≥ 50	911 (88.4)	120 (11.6)		
Tumour size				
< 2.0cm	756 (87.1)	112 (12.9)	7.03 (0.008)	**0.02**
≥ 2.0cm	623 (91.3)	59 (8.7)		
Tumour Grade				
1	182 (82.7)	38 (17.3)	20.62 (0.00003)	**0.0004**
2	491 (86.7)	75 (13.3)		
3	704 (92.4)	58 (7.6)		
Mitosis				
1	526 (84.8)	94 (15.2)	18.18 (0.0001)	**0.002**
2	300 (92.3)	25 (7.7)		
3	527 (91.5)	49 (8.5)		
Pleomorphism				
1	19 (82.6)	4 (17.4)	14.92 (0.001)	**0.005**
2	384 (84.4)	71 (15.6)		
3	950 (91.0)	94 (9.0)		
Tubular formation				
1	74 (83.1)	15 (16.9)	11.28 (0.004)	**0.01**
2	408 (85.9)	67 (14.1)		
3	871 (90.9)	87 (9.1)		
Vascular Invasion				
Negative	924 (89.1)	113 (10.9)	0.07 (0.77)	1.30
Positive	452 (88.6)	58 (11.4)		
Lymph Node Stage				
1	840 (87.9)	116 (12.1)	4.84 (0.08)	0.18
2	400 (89.7)	46 (10.3)		
3	136 (93.8)	9 (6.2)		

(Continued)

	SLC7A8 protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted P value**
Nottingham Prognostic Index				
Good	397 (83.8)	77 (16.2)	20.09 (0.00004)	**0.0005**
Moderate	746 (90.6)	77 (9.4)		
Poor	234 (93.2)	17 (6.8)		
IHC Subtypes				
ER+/HER2- Low Proliferation	706 (87.7)	99 (12.3)	5.67 (0.12)	0.36
ER+/HER2- High Proliferation	230 (90.9)	23 (9.1)		
Triple Negative	207 (88.5)	27 (11.5)		
HER2+	145 (93.5)	10 (6.5)		
Histological type				
Ductal (including mixed)	1217 (89.6)	142 (10.4)	15.93 (0.01)	**0.02**
Lobular	88 (87.1)	13 (12.9)		
Medullary	23 (92.0)	2 (8.0)		
Miscellaneous	7 (77.8)	2 (22.2)		
Special type	41 (78.8)	11 (21.2)		
Tubular	2 (100.0)	0 (0.0)		
Site of distant metastasis				
Brain				
No	349 (86.0)	57 (14.0)	0.24 (0.62)	0.82
Yes	25 (89.3)	3 (10.7)		
Lung				
No	336 (86.4)	53 (13.6)	0.12 (0.72)	0.83
Yes	38 (84.4)	7 (15.6)		
Bone				
No	286 (84.1)	54 (15.9)	5.57 (0.01)	0.05
Yes	88 (93.6)	6 (6.4)		
Liver				
No	308 (84.6)	56 (15.4)	4.60 (0.03)	0.13
Yes	66 (94.3)	4 (5.7)		

P values in bold means statistically significant

7.5.4 SLC38A2, SLC7A11 and SLC7A8 expression in molecular BC subtypes

While *SLC7A11* mRNA expression did not show significant associations with hormone receptors and HER2 status, *SLC38A2* was highly expressed in HER2+ tumours ($p>0.05$ and $p<0.001$, Table 7-5, 7-4, respectively). In contrast, high expression of *SLC7A8* mRNA was significantly associated with hormone receptor positive (ER+ and PgR+) and HER2- BC (all $P<0.001$, Table 7-6). In addition, *SLC7A8* mRNA was highly expressed in non-triple negative compared with TN tumours ($P<0.001$, Table 7-6).

Analysis of *SLC38A2* mRNA in intrinsic (PAM50) subtypes revealed that the high expression was observed in HER2+ tumours followed by luminal B subtype ($p<0.001$, Figure 7-4F). Similarly, within the METABRIC integrative clusters, high *SLC38A2* expression was associated with clusters 5 (ERBB2-amplified) and 2 (luminal tumours) ($p<0.001$, Figure 7-4E). In the SCMGENE subtypes, *SLC38A2* was predominantly expressed in HER2+ and ER+ high proliferation tumours ($p<0.001$, Figure 7-4G).

When *SLC7A11* mRNA was assessed in PAM50 subtypes, high levels were observed in basal-like tumours ($p<0.001$, Figure 7-5F). Likewise, within the METABRIC integrative clusters, high *SLC7A11* expression was associated with cluster 10 which embraces triple negative tumours ($p=0.02$, Figure 7-5E). There was no significant association between *SLC7A11* and SCMGENE subtypes ($p>0.05$, Figure 7-5G).

Regarding the association of *SLC7A8* with PAM50 subtypes, high mRNA expression was observed primarily in luminal A and B tumours ($P<0.001$, Figure 7-6F). In the METABRIC Integrative Clusters, high *SLC7A8* mRNA expression was associated with clusters 7 and 8 which encompass ER+ tumours predominately of the luminal A intrinsic subtype ($P<0.001$, Figure 7-6E). Furthermore, the result of the association with the SCMGENE subtypes was comparable to the above findings, where the ER+/HER2- subtype showed the highest levels of *SLC7A8* mRNA expression ($p<0.001$, Figure 7-6G). Associations of *SLC38A2*, *SLC7A11* and *SLC7A8* with the BC molecular subtypes were confirmed using Breast Cancer Gene-Expression Miner v4.0 (Figure 7-7, 7-8, 7-9H).

Gene CN analysis showed that HER2+ tumours had the highest proportion of *SLC38A2* CN gain ($p<0.001$, Table 7-7), while no significant association was

observed between *SLC7A11* CN status and BC intrinsic subtypes (p>0.05, Table7-8). However, *SLC7A8* gene was mainly amplified in the luminal B subtype (p<0.001, Table 7-9).

In the Nottingham BC cohort, SLC38A2 and SLC7A11 proteins were significantly associated with ER- tumours and they were highly expressed in malignancies that were TN compared to non-TN tumours (all p≤0.008, Table 7-4, 7-5), respectively. Furthermore, both transporters were observed in TNBC compared to the other IHC defined molecular subtypes (p<0.001 and p=0.04, Table 7-1 and 7-2), respectively. On the other hand, high SLC7A8 protein expression was associated with HER2- tumours (P=0.01, Table 7-6) and although the result of the association with the defined IHC subtypes were not nominally significant, it showed higher SLC7A8 expression in the ER+ low proliferation tumours compared with the other BC subtypes (p>0.05, Table 7-3).

Table 7-4. Expression of SLC38A2 in breast cancer and the expression of other molecular biomarkers, at mRNA and protein levels

	SLC38A2							
	mRNA				Protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low n (%)**	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ER								
Negative	235 (49.6)	239 (50.4)	0.06 (0.79)	1.58	309 (84.0)	59 (16.0)	27.35 ($1.6x10^{-7}$)	**<0.0001**
Positive	757 (50.3)	749 (49.7)			1219 (92.8)	94 (7.2)		
PgR								
Negative	484 (51.5)	456 (48.5)	1.38 (0.24)	0.96	613 (88.7)	78 (11.3)	7.67 (0.006)	**0.02**
Positive	508 (48.8)	532 (51.2)			896 (92.7)	71 (7.3)		
HER2								
Negative	902 (52.0)	831 (48.0)	21.07 (0.00004)	**0.0002**	1301 (91.4)	122 (8.6)	2.99 (0.08)	0.16
Positive	90 (36.4)	157 (63.6)			212 (88.0)	29 (12.0)		
Triple Negative								
No	810 (48.8)	850 (51.2)	7.00 (0.008)	**0.02**	1299 (92.1)	111 (7.9)	14.35 (0.0001)	**0.0005**
Yes	182 (56.9)	138 (43.1)			224 (84.8)	40 (15.2)		
TP53 mutation								
Wild-type	363 (50.3)	358 (49.7)	1.33 (0.51)	1.53		N/A		
Mutation	44 (44.4)	55 (55.6)						
P53 protein								
Negative			N/A		424 (93.6)	29 (6.4)	3.02 (0.08)	0.24
Positive					163 (89.6)	19 (10.4)		

N/A, not available; P values in bold means statistically significant

Table 7-5. Expression of SLC7A11 in breast cancer and the expression of other molecular biomarkers, at mRNA and protein levels

	SLC7A11							
	mRNA				Protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low n (%)**	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ER								
Negative	376 (81.2)	87 (18.8)	3.57 (0.05)	0.16	222 (52.5)	201 (47.5)	14.01 (0.0001)	**0.0006**
Positive	1259 (84.9)	224 (15.1)			971 (62.5)	582 (37.5)		
PgR								
Negative	760 (82.3)	164 (17.7)	4.09 (0.04)	0.15	461 (58.2)	331 (41.8)	2.73 (0.09)	0.27
Positive	875 (85.6)	147 (14.4)			716 (61.9)	440 (38.1)		
HER2								
Negative	1425 (83.5)	282 (16.5)	3.00 (0.08)	0.24	1022 (61.0)	654 (39.0)	0.94 (0.33)	0.66
Positive	210 (87.9)	29 (12.1)			158 (57.9)	115 (42.1)		
Triple Negative								
No	1386 (84.9)	246 (15.1)	6.21 (0.01)	0.05	1031 (61.9)	635 (38.1)	9.69 (0.002)	**0.008**
Yes	249 (79.3)	65 (20.7)			157 (52.3)	143 (47.7)		
TP53 mutation								
Wild-type	609 (85.3)	105 (14.7)	2.09 (0.35)	0.42		N/A		
Mutation	77 (80.2)	19 (19.8)						
P53 protein								
Negative			N/A		770 (62.7)	458 (37.3)	9.57 (0.002)	**0.01**
Positive					350 (55.3)	283 (44.7)		

N/A, not available; P values in bold means statistically significant

Table 7-6. Expression of SLC7A8 in breast cancer and the expression of other molecular biomarkers, at mRNA and protein levels

	SLC7A8							
	mRNA				protein			
	Low n (%)	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low n (%)**	**High n (%)**	**χ2 (p-value)**	**Adjusted p-value**
ER								
Negative	365 (77.2)	108 (22.8)	553.2 ($2.5x10^{-122}$)	**<0.0001**	296 (89.4)	35 (10.6)	0.09 (0.76)	1.52
Positive	283 (18.9)	1215 (81.1)			1083 (88.8)	136 (11.2)		
PgR								
Negative	453 (48.3)	484 (51.7)	193.6 ($5.0x10^{-44}$)	**<0.0001**	559 (91.0)	55 (9.0)	3.53 (0.06)	0.30
Positive	195 (18.9)	839 (81.1)			807 (88.0)	110 (12.0)		
HER2								
Negative	519 (30.1)	1206 (69.9)	48.7 ($2.9x10^{-12}$)	**<0.0001**	1174 (88.3)	155 (11.7)	8.82 (0.003)	**0.01**
Positive	129 (52.4)	117 (47.6)			198 (95.2)	10 (4.8)		
Triple Negative								
No	388 (23.5)	1263 (76.5)	405.07 ($4.3x10^{-90}$)	**<0.0001**	1169 (89.4)	139 (10.6)	0.56 (0.45)	1.80
Yes	260 (81.3)	60 (18.8)			207 (87.7)	29 (12.3)		
***TP53* mutations**								
Wild-type	204 (28.5)	512 (71.5)	38.36 ($4.6x10^{-9}$)	**<0.0001**		N/A		
Mutation	59 (59.6)	40 (40.4)						
P53 protein								
Negative			N/A		860 (89.0)	106 (11.0)	0.24 (0.62)	1.86
Positive					435 (89.9)	49 (10.1)		

N/A, not available; P values in bold means statistically significant

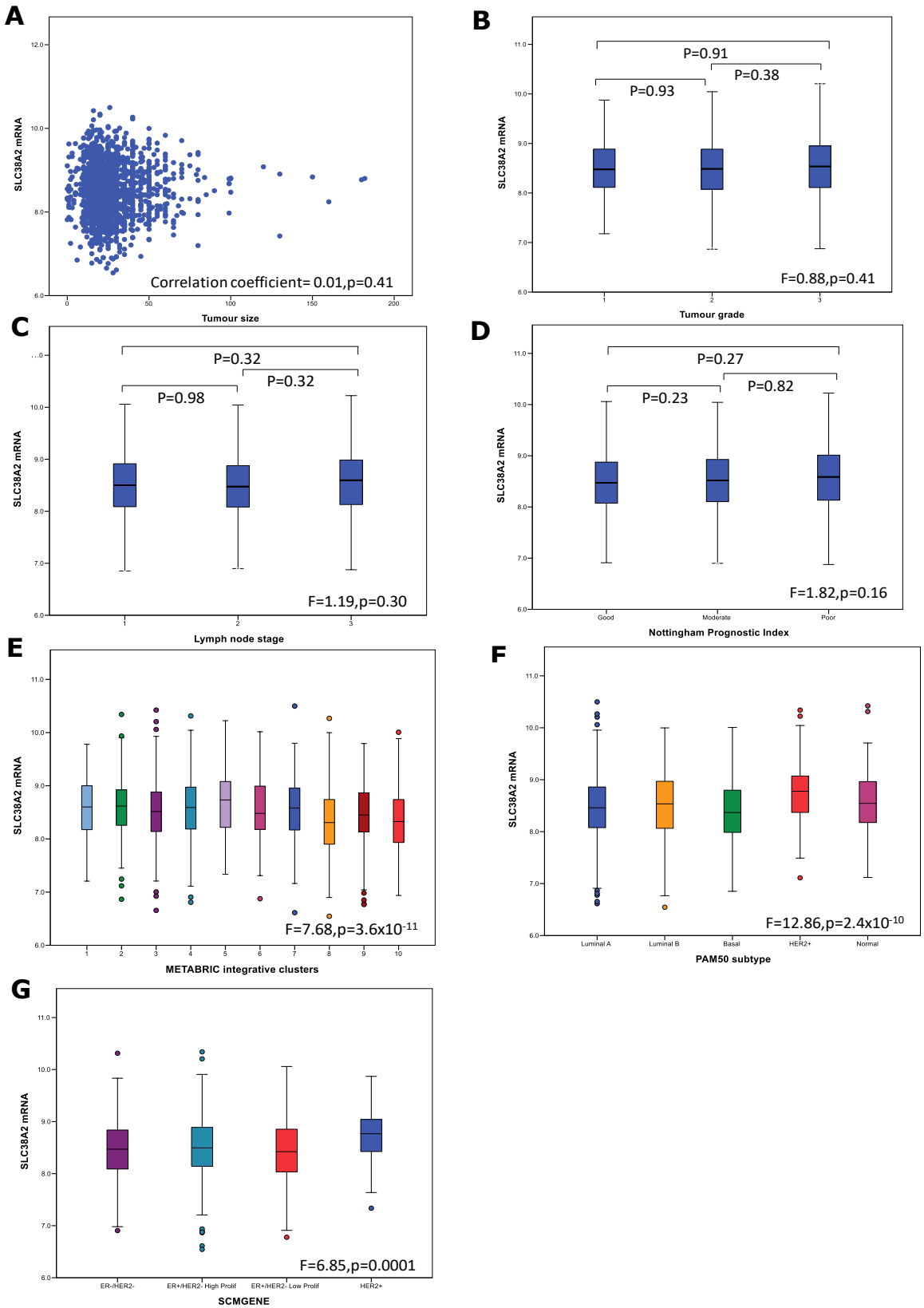


Figure 7-4. *SLC38A2* mRNA expression and its association with clinicopathological parameters and molecular subtypes: A) *SLC38A2* and tumour size, B) *SLC38A2* and tumour grade, C) *SLC38A2* and lymph node stage, D) *SLC38A2* and NPI, E) *SLC38A2* and METABRIC Integrative Clusters, F) *SLC38A2* and PAM50 subtypes, G) *SLC38A2* and SMCGENE subtypes in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

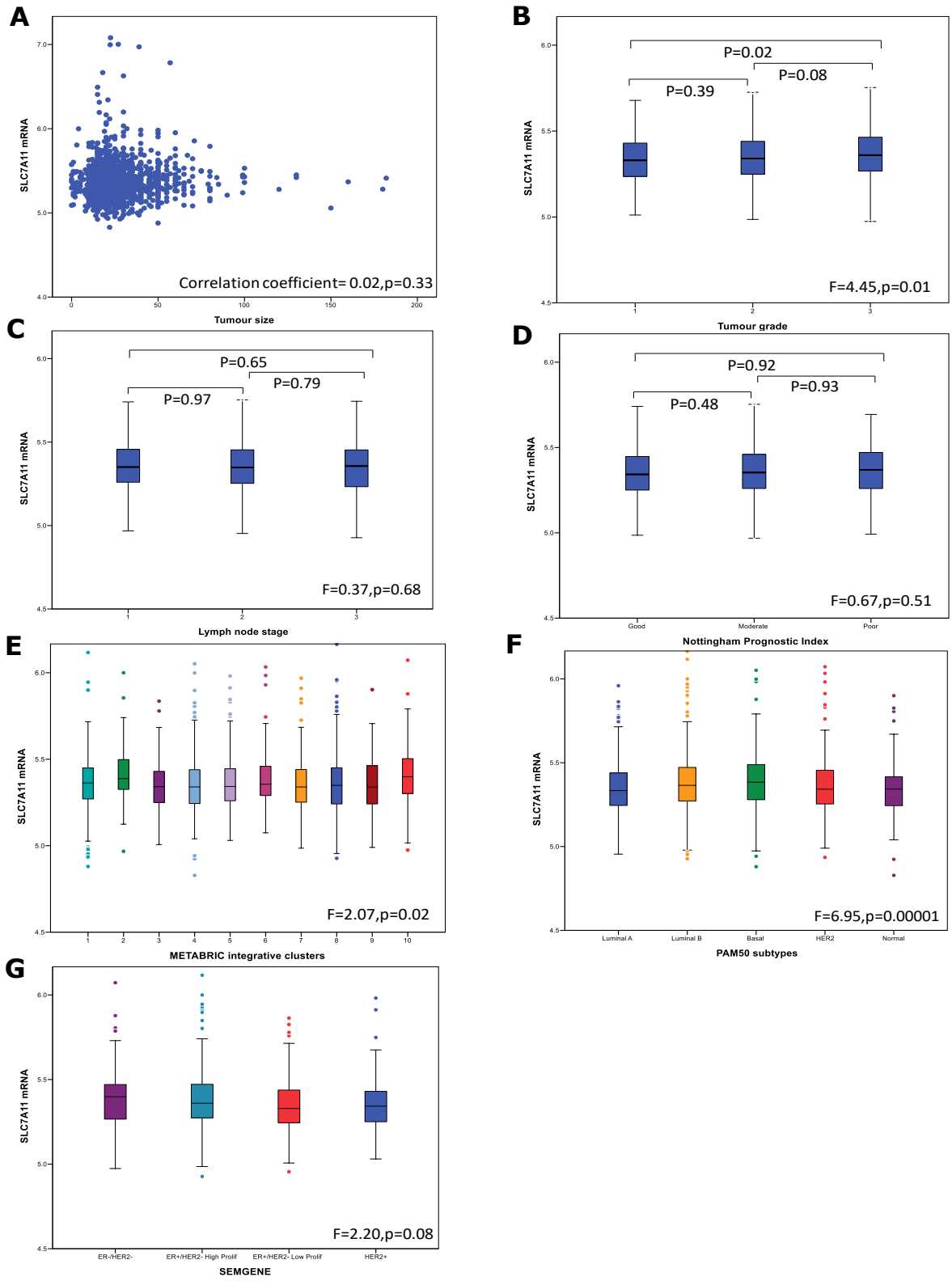


Figure 7-5. *SLC7A11* mRNA expression and its association with clinicopathological parameters and molecular subtypes: A) *SLC7A11* and tumour size, B) *SLC7A11* and tumour grade, C) *SLC7A11* and lymph node stage, D) *SLC7A11* and NPI, E) *SLC7A11* and METABRIC Integrative Clusters, F) *SLC7A11* and PAM50 subtypes, G) *SLC7A11* and SMCGENE subtypes in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

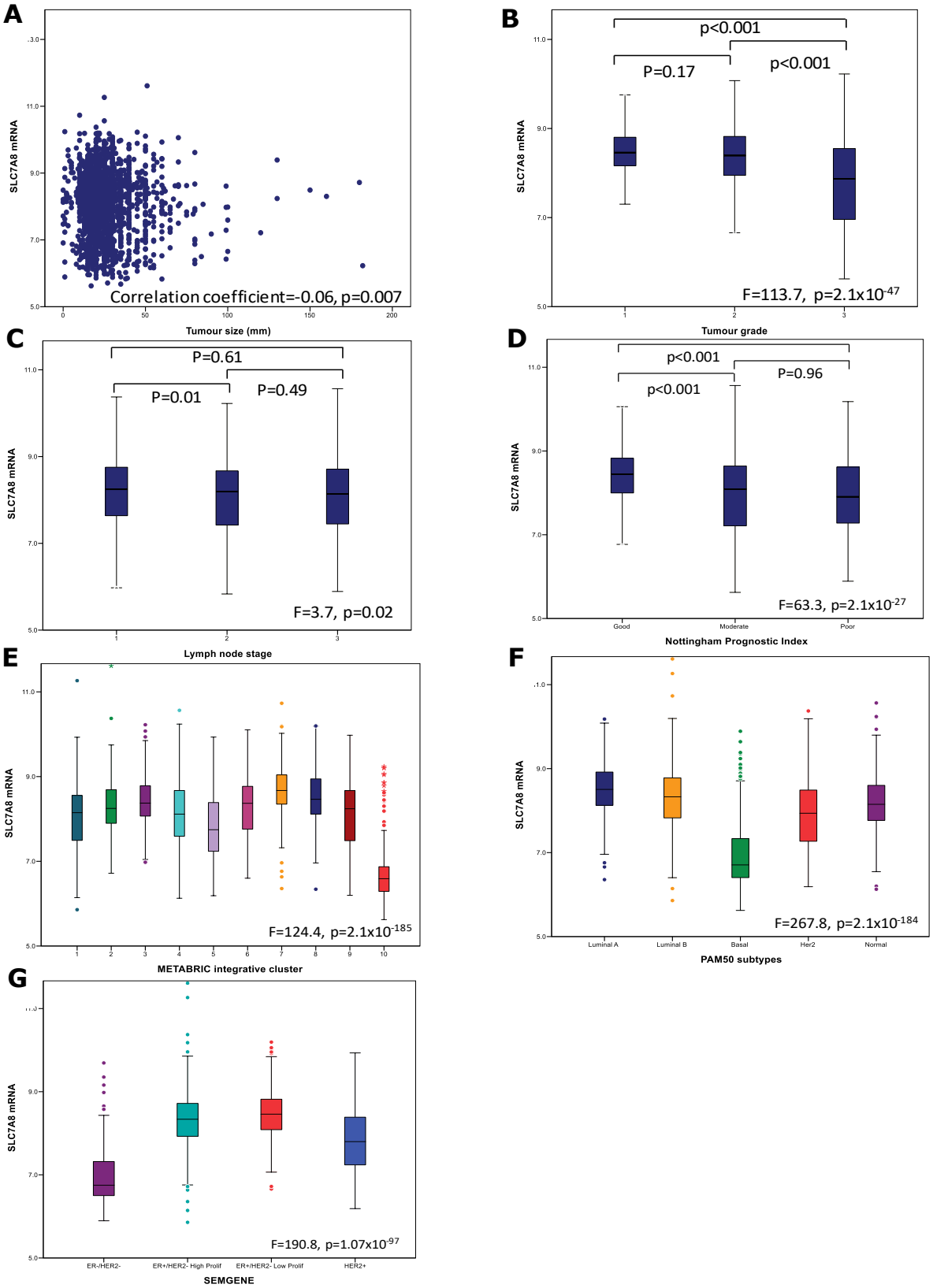


Figure 7-6. *SLC7A8* mRNA expression and its association with clinicopathological parameters and molecular subtypes: A) *SLC7A8* and tumour size, B) *SLC7A8* and tumour grade, C) *SLC7A8* and lymph node stage, D) *SLC7A8* and NPI, E) *SLC7A8* and METABRIC Integrative Clusters, F) *SLC7A8* and PAM50 subtypes, G) *SLC7A8* and SMCGENE subtypes in the METABRIC cohort using One-way ANOVA with post-hoc tukey test.

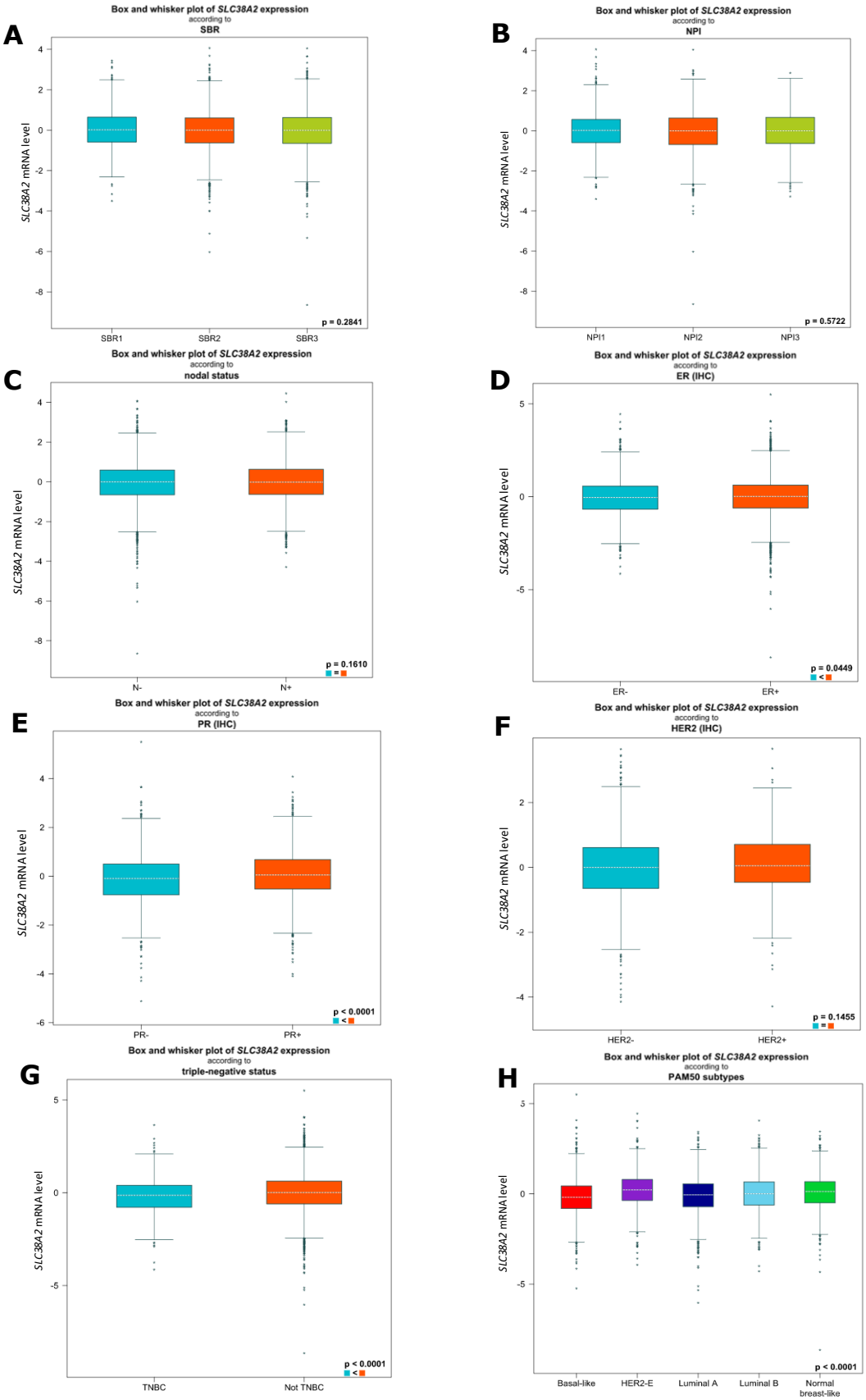


Figure 7-7. *SLC38A2*gene expression and its association, with: A) tumour grade, B) NPI, C) ER status, D) PR status, E) HER2 status, F) Triple Negative status, G) PAM50 subtypes, using Breast Cancer Gene-Expression Miner dataset.

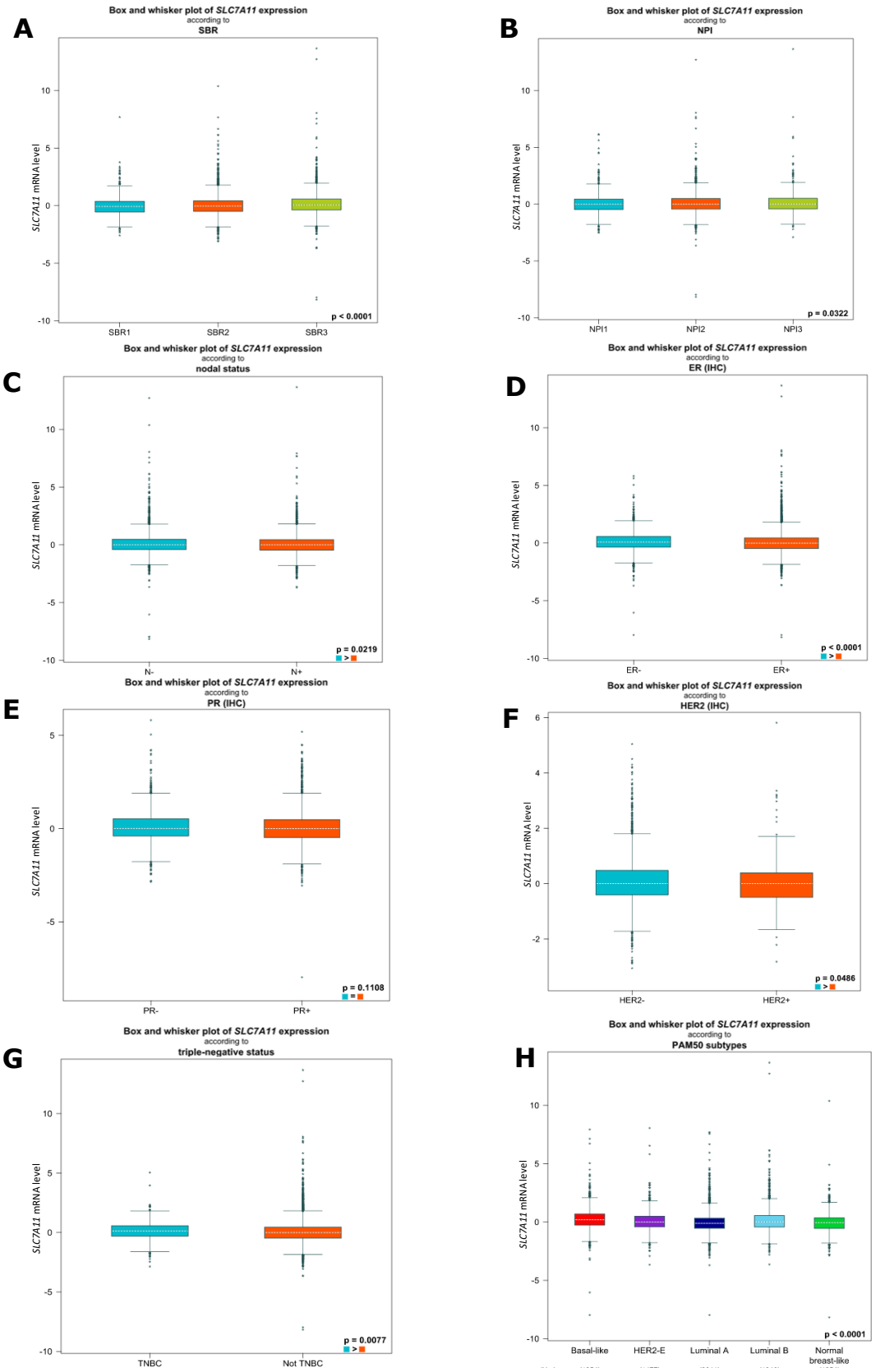


Figure 7-8. *SLC7A11*gene expression and its association, with: A) tumour grade, B) NPI, C) ER status, D) PR status, E) HER2 status, F) Triple Negative status, G) PAM50 subtypes, using Breast Cancer Gene-Expression Miner dataset.

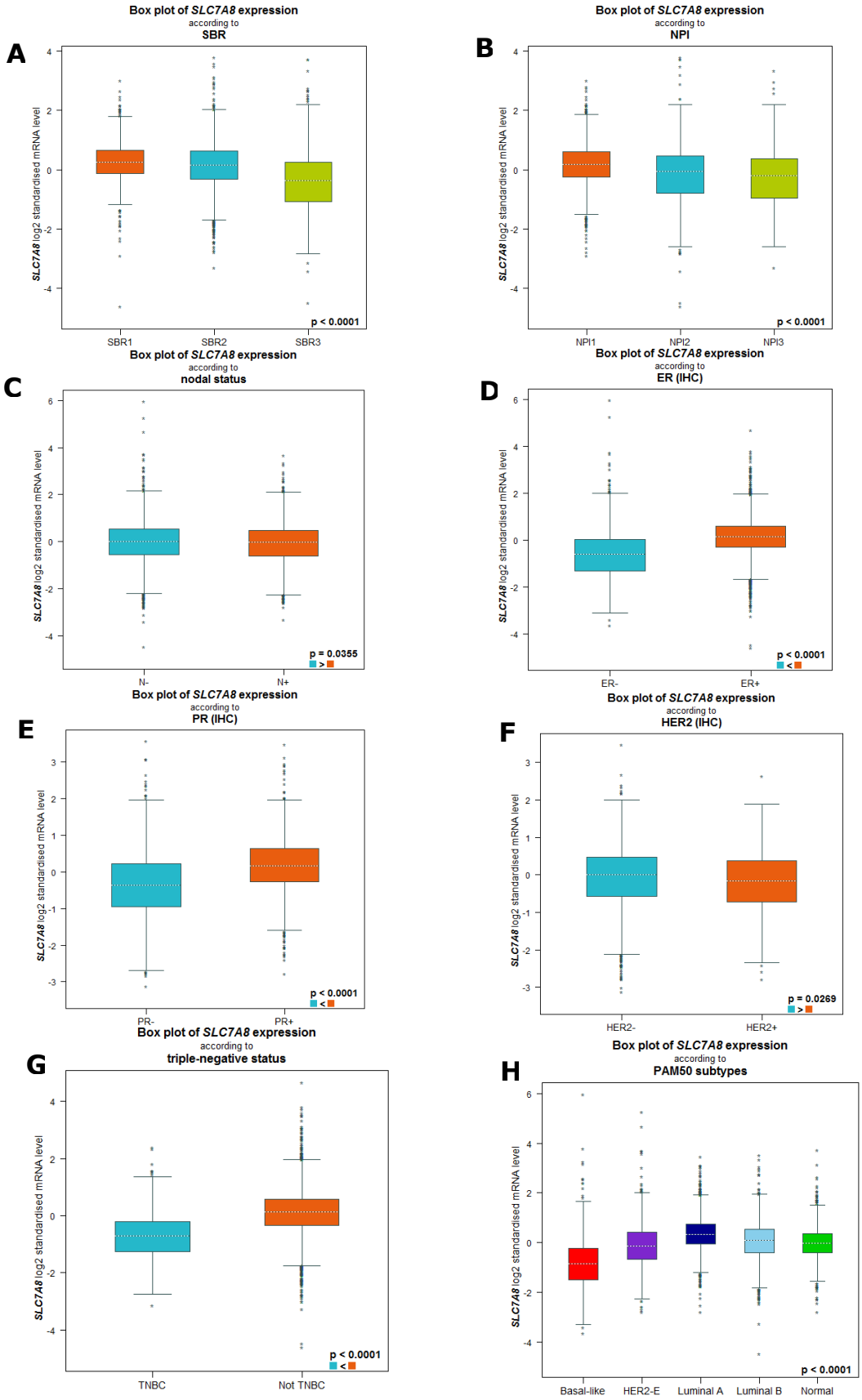


Figure 7-9. *SLC7A8* gene expression and its association, with: A) tumour grade, B) NPI, C) ER status, D) PR status, E) HER2 status, F) Triple Negative status, G) PAM50 subtypes, using Breast Cancer Gene-Expression Miner dataset.

Table 7-7. The association of SLC38A2 copy number aberrations and PAM50 subtypes in the METABRIC cohort.

	SLC38A2					
	Gain			Loss		
	No	Yes	χ2 (p-value)	No	Yes	χ2 (p-value)
PAM50 subtype						
Luminal A	703 (97.9)	15 (2.1)		718 (100.0)	0 (0.0)	
Luminal B	462 (94.7)	26 (5.3)	20.52	485 (99.4)	3 (0.6)	32.27
Basal	323 (98.2)	6 (1.8)	(**0.0003**)	319 (97.0)	10 (3.0)	(**0.000002**)
HER2	226 (94.2)	14 (5.8)		239 (99.6)	1 (0.4)	
Normal-like	197 (99.0)	2 (1.0)		199 (100.0)	0 (0.0)	

P values in bold means statistically significant

Table 7-8. The association of SLC7A11 copy number aberrations and PAM 50 subtypes in the METABRIC cohort.

	SLC7A11					
	Gain			Loss		
	No	Yes	χ2 (p-value)	No	Yes	χ2 (p-value)
PAM50 subtype						
Luminal A	712 (99.2)	6 (0.8)		696 (96.9)	22 (3.1)	
Luminal B	481 (98.6)	7 (1.4)	5.09	470 (96.3)	18 (3.7)	8.21
Basal	323 (98.2)	6 (1.8)	(0.27)	311 (94.5)	18 (5.5)	(0.08)
HER2	236 (98.3)	4 (1.7)		235 (97.9)	5 (2.1)	
Normal-like	199 (100.0)	0 (0.0)		196 (98.5)	3 (1.5)	

Table 7-9. The association of SLC7A8 copy number aberrations and PAM 50 subtypes in the METABRIC cohort.

	SLC7A8					
	Gain			Loss		
	No	Yes	χ2 (p-value)	No	Yes	χ2 (p-value)
PAM50 subtype						
Luminal A	700 (97.5)	18 (2.5)		705 (98.2)	13 (1.8)	
Luminal B	448 (91.8)	40 (8.2)	26.94	477 (97.7)	11 (2.3)	7.68
Basal	314 (95.4)	15 (4.6)	(**0.00002**)	315 (95.7)	14 (4.3)	(0.10)
HER2	226 (94.2)	14 (5.8)		237 (98.8)	3 (1.3)	
Normal-like	196 (98.5)	3 (1.5)		195 (98.0)	4 (2.0)	

P values in bold means statistically significant

7.5.5 SLC38A2, SLC7A11and SLC7A8 expression and other associated markers

The METABRIC dataset was used to investigate the correlation between *SLC38A2*, *SLC7A11* and *SLC7A8* mRNA and the gene expression of other related biomarkers (Table 7-10, 7-12, 7-14). The biomarkers were selected based on other publications, being either regulatory genes or others that share the solute carriers' biological function [142,251,370,146,287,385,390,188]. Association of SLC38A2, SLC7A11 and SLC7A8 protein expressions with other immunohistochemical biomarkers was also examined, using the Nottingham BC cohort (Table 7-11, 7-13, 7-15).

7.5.5.1 Associations with SLC38A2

There was a significant correlation between *SLC38A2* mRNA expression with the expression of all regulatory genes that were previously identified in the literature. While *ATF4* and *HIF1α* displayed a positive relationship with *SLC38A2*, a negative correlation was observed with *C/EBP* and *RNF5* (all $p<0.001$). The associations with *ATF4* and *RNF5* were detected in the all BC subtypes (all $p<0.001$). The majority of glutamine metabolic enzymes were positively correlated with *SLC38A2*. This included enzymes involved in glutamine-proline regulatory axis; *GLS*, *ALDH18A1*, *ALDH4A1, PRODH* in addition to *GLUD1* and *GLUL* enzymes (all $p<0.001$). These associations were primarily seen within the luminal A and luminal B tumours. Regarding the associations with the other glutamine transporters, a positive relationship was observed with *SLC1A5*, *SLC7A8*, *SLC38A1*, *SLC38A5* and *SLC38A7* transporters (all $p\leq0.003$), primarily within the luminal tumours. However, the association with *SLC1A5* was observed in all BC subtypes ($p\leq0.04$).

Similar to mRNA, SLC38A2 protein was significantly associated with enzymes involved in glutamine-proline regulatory axis and GLUD1 (all $p\leq0.01$). Furthermore, all solute carriers included in this study (SLC1A5, SLC7A5, SLC3A2, SLC7A8 and SLC7A11), along with Ki67, were significantly expressed in breast tumours with high SLC38A2 protein (all $p\leq0.04$).

Table 7-10. Correlation of *SLC38A2* mRNA expression with the expression of other related genes in the METABRIC cohort.

	***SLC38A2* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)					**Adjusted p-value**				
Regulatory genes										
ATF4	0.46 ($3.1x10^{-105}$)	**<0.0001**	0.47 ($5.7x10^{-42}$)	**<0.0001**	0.52 ($7.4x10^{-36}$)	**<0.0001**	0.43 ($1.1x10^{-12}$)	**<0.0001**	0.41 ($8.3x10^{-15}$)	**<0.0001**
CEBPB	-0.11 (0.000001)	**<0.0001**	-0.10 (0.004)	0.05	-0.14 (0.001)	**0.01**	-0.06 (0.29)	1.95	-0.13 (0.01)	0.11
HIF1a	0.12 ($3.6x10^{-8}$)	**<0.0001**	0.08 (0.02)	0.22	0.21 (0.000003)	**0.0001**	-0.003 (0.96)	4.16	0.17 (0.001)	**0.02**
RNF5	-0.31 ($1.1x10^{-46}$)	**<0.0001**	-0.36 ($8.3x10^{-24}$)	**<0.0001**	-0.24 ($8.1x10^{-8}$)	**<0.0001**	-0.32 ($2.2x10^{-7}$)	**<0.0001**	-0.25 (0.000004)	**0.0001**
Enzymes involved in glutamine metabolism										
GLS	0.15 ($2.9x10^{-12}$)	**<0.0001**	0.24 ($2.9x10^{-11}$)	**<0.0001**	0.13 (0.002)	**0.02**	0.04 (0.47)	2.80	0.11 (0.04)	0.40
ALDH4A1	0.17 ($4.0x10^{-15}$)	**<0.0001**	0.17 (0.000003)	**0.0001**	0.12 (0.007)	0.08	0.03 (0.58)	3.08	0.15 (0.005)	0.08
PRODH	0.09 (0.00001)	**0.0001**	0.08 (0.03)	0.30	0.06 (0.15)	0.90	-0.02 (0.68)	3.80	0.09 (0.10)	0.70
PYCR1	0.001 (0.97)	1.94	0.01 (0.70)	2.65	-0.01 (0.75)	2.15	-0.02 (0.65)	3.40	-0.09 (0.08)	0.64
ALDH18A1	-0.12 ($5.9x10^{-8}$)	**<0.0001**	-0.08 (0.02)	0.24	-0.22 ($6.6x10^{-7}$)	**<0.0001**	-0.10 (0.09)	1.05	-0.19 (0.0003)	**0.006**
GLUL	-0.11 ($3.7x10^{-7}$)	**<0.0001**	-0.19 ($1.3x10^{-7}$)	**<0.0001**	-0.16 (0.0003)	**0.005**	-0.06 (0.28)	1.92	0.09 (0.10)	0.72
GLUD1	0.15 ($3x10^{-12}$)	**<0.0001**	0.21 ($1.6x10^{-8}$)	**<0.0001**	0.15 (0.001)	**0.01**	0.11 (0.07)	0.72	0.14 (0.007)	0.09
Glutamine transporters										
SLC1A5	0.18 ($1.5x10^{-16}$)	**<0.0001**	0.17 (0.000004)	**0.0001**	0.14 (0.002)	**0.02**	0.19 (0.002)	**0.04**	0.17 (0.002)	**0.04**
SLC6A19	-0.04 (0.06)	0.14	-0.05 (0.16)	1.12	0.02 (0.53)	1.95	-0.09 (0.16)	1.95	-0.13 (0.01)	0.13
SLC7A5	-0.06 (0.007)	0.06	-0.06 (0.06)	0.54	-0.12 (0.002)	**0.03**	-0.10 (0.04)	0.64	-0.03 (0.56)	1.12
SLC7A6	0.02 (0.27)	0.36	0.02 (0.59)	2.36	-0.11 (0.01)	0.11	0.09 (0.15)	1.92	0.12 (0.02)	0.22
SLC7A7	-0.005 (0.80)	1.40	-0.05 (0.14)	1.12	0.03 (0.43)	1.50	-0.04 (0.52)	2.90	0.07 (0.18)	0.84
SLC7A8	0.08 (0.0003)	**0.003**	0.12 (0.001)	**0.01**	0.02 (0.65)	2.12	-0.003 (0.96)	4.16	0.13 (0.01)	0.14

(Continued)

	***SLC38A2* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)					**Adjusted p-value**				
SLC7A9	-0.06 (0.008)	0.06	-0.02 (0.47)	1.98	-0.10 (0.02)	0.20	-0.10 (0.11)	1.54	-0.05 (0.33)	0.99
SLC38A1	0.25 ($2.8x10^{-30}$)	**<0.0001**	0.27 ($4.5x10^{-14}$)	**<0.0001**	0.31 ($2.2x10^{-12}$)	**<0.0001**	0.20 (0.002)	**0.04**	0.13 (0.01)	0.15
SLC38A3	-0.02 (0.35)	1.35	0.00 (0.99)	2.82	-0.07 (0.11)	0.77	0.13 (0.04)	0.68	-0.06 (0.21)	0.90
SLC38A5	0.15 ($3.5x10^{-12}$)	**<0.0001**	0.12 (0.001)	**0.01**	0.17 (0.00009)	**0.001**	0.17 (0.006)	0.12	0.16 (0.003)	0.05
SLC38A7	0.15 ($5.2x10^{-12}$)	**<0.0001**	0.15 (0.00001)	**0.0002**	0.07 (0.09)	0.72	0.16 (0.01)	0.19	0.155 (0.005)	0.08
SLC38A8	0.05 (0.02)	0.14	0.02 (0.53)	2.10	0.08 (0.06)	0.54	-0.003 (0.95)	4.06	0.10 (0.05)	0.45

P values in bold means statistically significant

Table 7-11. Association between SLC38A2 protein expression and other histochemical biomarkers in the Nottingham cohort

	SLC38A2 protein			
	Low, n (%)	**High, n (%)**	**χ2 (p-value)**	**Adjusted p-value**
Ki67				
Negative	164 (96.5)	6 (3.5)	11.97	**0.008**
Positive	340 (90.9)	34 (9.1)	(0.001)	
GLS				
Negative	330 (95.7)	15 (4.3)	17.02	**0.0004**
Positive	204 (86.1)	33 (13.9)	(0.00003)	
GLUD1				
Negative	298 (95.8)	13 (4.2)	10.61	**0.008**
Positive	371 (88.9)	34 (11.1)	(0.001)	
PYCR1				
Negative	292 (95.1)	15 (4.9)	9.72	**0.01**
Positive	208 (87.8)	29 (12.2)	(0.002)	
ALDH4A1				
Negative	282 (95.6)	13 (4.4)	9.06	**0.01**
Positive	267 (89.0)	33 (11.0)	(0.003)	
ALDH18A1				
Negative	309 (95.4)	15 (4.6)	10.51	**0.009**
Positive	247 (88.2)	33 (11.8)	(0.001)	
PRODH				
Negative	387 (93.3)	28 (6.7)	5.35	0.06
Positive	80 (86.0)	13 (14.0)	(0.02)	
SLC1A5				
Negative	487 (94.7)	27 (5.3)	12.92	**0.003**
Positive	917 (89.2)	111 (10.8)	(0.0003)	

(Continued)

	SLC38A2 protein			
	Low, n (%)	**High, n (%)**	**χ^2 (p-value)**	**Adjusted p-value**
SLC7A5				
Negative	1158 (92.2)	98 (7.8)	15.56	**0.001**
Positive	273 (85.0)	48 (15.0)	(0.00008)	
SLC3A2				
Negative	977 (92.1)	84 (7.9)	5.89	**0.04**
Positive	352 (88.0)	48 (12.0)	(0.01)	
SLC7A8				
Negative	997 (91.9)	88 (8.1)	13.53	**0.002**
Positive	117 (82.4)	25 (17.6)	(0.0002)	
SLC7A11				
Negative	768 (92.4)	63 (7.6)	5.81	**0.04**
Positive	473 (88.6)	61 (11.4)	(0.01)	

P values in bold means statistically significant

7.5.5.2 Associations with SLC7A11

There was a positive correlation between *SLC7A11* mRNA expression and the expression of the regulatory gene, *ATF4* (p=0.004). However, no significant association was observed with *MYC*, *ETS-1* or *NRF2* (all p>0.05). High *SLC7A11* was also correlated with the ancillary genes, *SLC3A2* and *CD44* (all p≤0.001). While the former was associated with almost all subtypes (p≤0.03), correlation with *CD44* was only observed within luminal B tumours (p=0.03). No significant association was observed with enzymes involved in glutamine metabolism/synthesis (*GLS*, *GLUD1* and *GLUL*) (all p>0.05). Nevertheless, a positive association was observed with *SLC1A5* and *SLC7A5* (all p≤0.001). There was no significant association with *SLC7A8*, *SLC38A2*, glutamate transporters (S*LC1A3, SLC1A6 and SLC1A7*) and cystine/glutamate antiporter (*SLC1A1*) apart from *SLC1A2* (p=0.04) which losts its significance when different BC subtypes were examined.

At the protein level, SLC7A11 was significantly expressed with breast tumours that express high MYC, Ki67 and GLS (all p≤0.01). There was no significant association between SLC7A11 and GLUD1 (p>0.05). Nonetheless, a positive association was observed with SLC7A11 and all solute carriers included in this study (all p≤0.04). Moreover, SLC7A11 was highly expressed in breast cancers which express high p53 protein (p=0.01, Table 7-5).

Table 7-12. Correlation of *SLC7A11* mRNA expression with the expression of other related genes in the METABRIC cohort.

	***SLC7A11* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)				**Adjusted p-value**					
Regulatory and other associated genes										
MYC	0.02 (0.38)	2.48	0.02 (0.43)	2.64	0.02 (0.60)	1.64	0.01 (0.78)	5.20	-0.04 (0.38)	3.44
ETS-1	-0.05 (0.01)	0.12	-0.08 (0.02)	0.32	-0.06 (0.17)	1.30	-0.08 (0.17)	2.76	0.02 (0.59)	3.72
NRF2	-0.02 (0.25)	1.75	-0.02 (0.44)	2.73	-0.05 (0.20)	1.40	-0.02 (0.70)	4.80	0.06 (0.26)	2.68
ATF4	0.08 (0.0003)	**0.004**	0.07 (0.05)	0.75	0.06 (0.17)	1.36	0.06 (0.29)	2.88	0.08 (0.13)	1.96
CD44	0.07 (0.001)	**0.01**	0.007 (0.85)	3.87	0.13 (0.002)	**0.03**	0.19 (0.003)	0.05	0.06 (0.25)	2.08
Enzymes involved in glutamine metabolism										
GLS	0.01 (0.48)	2.50	-0.02 (0.58)	3.30	0.06 (0.13)	1.26	0.06 (0.32)	2.89	0.04 (0.43)	3.50
GLUL	0.03 (0.14)	1.44	0.02 (0.47)	2.73	0.01 (0.82)	3.00	0.04 (0.52)	4.64	0.15 (0.006)	0.10
GLUD1	0.02 (0.31)	1.75	0.01 (0.64)	3.52	0.11 (0.01)	0.15	0.02 (0.69)	4.76	-0.06 (0.25)	2.43
Glutamine transporters included in this study										
SLC3A2	0.13 ($1.1x10^{-9}$)	**<0.0001**	0.11 (0.002)	**0.03**	0.14 (0.001)	**0.01**	0.05 (0.40)	4.26	0.17 (0.002)	**0.03**
SLC1A5	0.13 ($2.1x10^{-9}$)	**<0.0001**	0.15 (0.00004)	**0.0008**	0.13 (0.003)	**0.04**	-0.02 (0.71)	4.84	0.11 (0.05)	0.85
SLC7A5	0.08 (0.00008)	**0.001**	0.03 (0.37)	2.64	0.08 (0.06)	0.77	.09 (0.16)	4.97	0.05 (0.36)	3.42
SLC7A8	-0.007 (0.75)	2.90	-0.04 (0.22)	2.32	0.05 (0.21)	1.53	0.04 (0.44)	4.42	0.05 (0.32)	2.95
SLC38A2	-0.03 (0.15)	1.50	-0.04 (0.21)	1.70	-0.02 (0.60)	1.64	-0.01 (0.83)	5.60	0.02 (0.67)	3.75
Cystine/glutamate transporters										
SLC1A1	0.06 (0.006)	0.07	0.09 (0.01)	0.17	0.10 (0.02)	0.28	0.006 (0.92)	6.10	0.01 (0.81)	3.80
SLC1A2	0.06 (0.003)	**0.04**	0.04 (0.21)	1.92	0.08 (0.07)	0.78	0.06 (0.34)	3.32	0.03 (0.53)	3.52

(Continued)

	***SLC7A11* mRNA**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)				**Adjusted p-value**					
Glutamate transporters										
SLC1A3	-0.02 (0.35)	2.10	-0.02 (0.55)	2.80	-0.11 (0.01)	0.16	-0.06 (0.34)	3.90	0.05 (0.32)	3.38
SLC1A6	0.02 (0.35)	2.45	0.02 (0.56)	3.29	0.08 (0.07)	0.84	-0.02 (0.71)	4.97	-0.03 (0.58)	3.60
SLC1A7	-0.02 (0.35)	2.45	-0.04 (0.24)	2.44	-0.01 (0.73)	2.40	0.004 (0.95)	6.21	-0.01 (0.98)	3.84

P values in bold means statistically significant

Table 7-13. Association between SLC7A11 protein expression and other histochemical biomarkers in the Nottingham cohort

	SLC7A11 protein			
	Low, n (%)	**High, n (%)**	**χ^2 (p-value)**	**Adjusted p-value**
c-MYC				
Negative	328 (58.7)	231 (41.3)	21.28	**<0.0001**
Positive	22 (30.1)	51 (69.9)	(0.000004)	
Ki67				
Negative	122 (64.6)	67 (35.4)	9.67	**0.01**
Positive	219 (51.0)	210 (49.0)	(0.002)	
GLS				
Negative	252 (63.8)	143 (36.2)	27.11	**<0.0001**
Positive	123 (43.6)	159 (56.4)	($1.9x10^{-7}$)	
GLUD1				
Negative	212 (57.3)	158 (42.7)	0.73	0.78
Positive	185 (54.1)	157 (45.9)	(0.39)	
SLC1A5				
Negative	430 (68.6)	197 (31.4)	27.80	**<0.0001**
Positive	686 (55.9)	541 (44.1)	($1.3x10^{-7}$)	
SLC7A5				
Negative	942 (62.0)	577 (38.0)	13.22	**0.001**
Positive	189 (51.6)	177 (48.4)	(0.0002)	
SLC3A2				
Negative	778 (62.1)	474 (37.9)	9.25	**0.01**
Positive	261 (54.1)	221 (45.9)	(0.002)	
SLC38A2				
Negative	765 (61.9)	470 (38.1)	6.25	**0.04**
Positive	62 (50.4)	61 (49.6)	(0.01)	
SLC7A8				
Negative	744 (64.5)	410 (35.5)	11.20	**0.005**
Positive	70 (50.0)	70 (50.0)	(0.001)	

P values in bold means statistically significant

7.5.5.3 Associations with SLC7A8

High *SLC7A8* mRNA expression was significantly associated with enzymes involved in glutamine metabolism; glutaminases (*GLS* and *GLS2*) (all P<0.001), which both mediate the conversion of glutamine to glutamate. While the correlation with *GLS* was negative, it was positive with *GLS2*. There was also a positive association with *GLUL*, *GLUD1* and the enzymes which mediate conversion of glutamine to proline, namely, *ALDH4A1* and *PRODH* (all P<0.001). In contrast, some glutamine transporters were negatively correlated with SLC7A8 expression (all P≤ 0.009), including *SLC1A5*, *SLC7A5*, *SLC7A6*, *SLC7A7*, and *SLC38A3*, while others showed positive associations, including *SLC7A9*, *SLC38A1*, *SLC38A2*, and *SLC38A7* (all P≤0.003). The associations between *SLC7A8* and glutamine metabolic enzymes and transporters were primarily observed within luminal A tumours and to lesser extent in luminal B, HER2+ and TN subtypes. High *SLC7A8* mRNA expression was associated with tumours which showed wild-type *TP53* expression (P<0.001, Table 7-6).

SLC7A8 protein was significantly expressed with high GLS and GLS2 enzymes (P<0.001 and P=0.03), respectively. High PRODH, ALDH18A1, and ALDH4A1 were expressed in breast tumours with high SLC7A8 expression (all P≤0.02). There was no significant associations with SLC1A5, SLC7A5, SLC3A2 and p70S6K proteins (all p>0.05).

Table 7-14. Correlation of *SLC7A8* expression with the expression of other related genes in the METABRIC cohort.

	***SLC7A8* mRNA expression**									
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)					**Adjusted p-value**				
Glutamine metabolism										
GLS	-0.10 (0.000009)	**0.0001**	0.07 (0.05)	0.40	0.05 (0.19)	1.89	0.01 (0.85)	3.84	-0.09 (0.08)	1.52
GLS2	0.25 ($1.1x10^{-30}$)	**<0.0001**	0.07 (0.04)	0.36	0.01 (0.69)	2.69	0.28 (0.000006)	**0.0001**	-0.03 (0.53)	1.06
ALDH4A1	0.22 ($3.1x10^{-23}$)	**<0.0001**	0.11 (0.004)	0.05	0.07 (0.09)	1.08	0.13 (0.03)	0.75	0.37 ($3.1x10^{-12}$)	**<0.0001**
PRODH	0.10 (0.000003)	**0.0001**	0.16 (0.00001)	**0.0002**	0.12 (0.005)	0.09	-0.03 (0.56)	4.96	0.13 (0.01)	0.19
PYCR1	-0.05 (0.01)	0.08	0.09 (0.01)	0.11	0.10 (0.02)	0.32	-0.06 (0.33)	4.90	-0.11 (0.05)	0.55

(Continued)

***SLC7A8* mRNA expression**										
	All cases (n=1,980)		**Luminal A** (n=368)		**Luminal B** (n=367)		**HER2+** (n=110)		**Triple negative** (n=150)	
	Correlation Coefficient (p-value)					**Adjusted p-value**				
ALDH18A1	-0.004 (0.86)	1.72	0.11 (0.003)	**0.04**	0.11 (0.01)	0.34	0.09 (0.16)	3.12	-0.15 (0.004)	0.15
GLUL	0.21 ($3.8x10^{-21}$)	**<0.0001**	-0.11 (0.002)	**0.03**	-0.02 (0.58)	3.25	0.01 (0.83)	4.25	0.38 ($3x10^{-13}$)	**<0.0001**
GLUD1	0.42 ($1.7x10^{-84}$)	**<0.0001**	0.26 ($2.9x10^{-13}$)	**<0.0001**	0.15 (0.001)	**0.02**	0.27 (0.00001)	**0.0002**	0.11 (0.05)	0.70
Glutamine/Glutamate transporters										
SLC1A5	-0.07 (0.001)	**0.009**	0.06 (0.09)	0.54	0.007 (0.88)	1.76	-0.03 (0.62)	4.34	-0.20 (0.0002)	**0.003**
SLC3A2	-0.08 (0.0003)	**0.003**	0.01 (0.61)	1.83	0.08 (0.06)	1.17	-0.21 (0.001)	**0.01**	-0.05 (0.35)	2.04
SLC6A19	-0.007 (0.74)	2.96	-0.002 (0.95)	1.90	-0.04 (0.37)	3.22	0.03 (0.62)	4.98	0.03 (0.51)	1.59
SLC7A5	-0.47 ($1.3x10^{-112}$)	**<0.0001**	-0.155 (0.00003)	**0.0005**	-0.11 (0.02)	0.60	-0.18 (0.004)	0.48	-0.45 ($3.3x10^{-18}$)	**<0.0001**
SLC7A6	-0.12 ($2.6x10^{-8}$)	**<0.0001**	0.07 (0.05)	0.35	0.07 (0.09)	1.54	-0.001 (0.98)	1.98	0.10 (0.07)	0.72
SLC7A7	-0.25 ($5.9x10^{-31}$)	**<0.0001**	-0.21 ($2.3x10^{-8}$)	**<0.0001**	-0.14 (0.001)	**0.02**	-0.33 ($7.8x10^{-8}$)	**<0.0001**	0.20 (0.0002)	**0.005**
SLC7A9	0.21 ($1.2x10^{-20}$)	**<0.0001**	1.33 (0.0003)	**0.005**	0.15 (0.001)	**0.02**	0.35 ($1.7x10^{-8}$)	**<0.0001**	-0.06 (0.25)	1.92
SLC38A1	0.25 ($1.3x10^{-29}$)	**<0.0001**	0.27 ($8.4x10^{-14}$)	**<0.0001**	0.22 ($7.5x10^{-7}$)	**<0.0001**	0.12 (0.05)	0.84	-0.21 (0.00007)	**0.001**
SLC38A2	0.08 (0.0003)	**0.003**	0.12 (0.001)	**0.01**	0.02 (0.65)	2.76	-0.003 (0.96)	2.94	0.13 (0.01)	0.13
SLC38A3	-0.14 ($3x10^{-11}$)	**<0.0001**	0.05 (0.13)	0.65	0.09 (0.04)	0.84	0.19 (0.002)	0.06	-0.19 (0.0003)	0.06
SLC38A5	0.02 (0.28)	1.68	0.10 (0.006)	0.07	0.03 (0.46)	3.00	0.11 (0.06)	2.08	0.13 (0.01)	0.60
SLC38A7	0.08 (0.0001)	**0.001**	0.22 ($5.9x10^{-10}$)	**<0.0001**	0.14 (0.002)	**0.03**	0.31 ($7.1x10^{-7}$)	**<0.0001**	0.26 ($9.6x10^{-7}$)	**<0.0001**
SLC38A8	-0.05 (0.02)	0.14	-0.07 (0.03)	0.30	-0.06 (0.14)	1.90	-0.07 (0.26)	3.63	0.07 (0.19)	1.75
SLC7A11	-0.007 (0.75)	2.25	-0.04 (0.22)	0.88	0.05 (0.21)	2.96	0.04 (0.44)	5.04	0.05 (0.32)	1.75

P values in bold means statistically significant

Table 7-15. Association between SLC7A8 protein expression and other histochemical biomarkers in the Nottingham cohort

	SLC7A8 protein			
	Low, n (%)	**High, n (%)**	**χ2 (p-value)**	**Adjusted p-value**
GLS				
Negative	226 (92.6)	18 (7.4)	18.45	**0.0001**
Positive	118 (77.6)	34 (22.4)	(0.00001)	
GLS2				
Negative	171 (89.5)	20 (10.5)	7.41	**0.03**
Positive	155 (79.5)	40 (20.5)	(0.006)	
PRODH				
Negative	255 (89.8)	29 (10.2)	10.93	**0.007**
Positive	46 (74.2)	16 (25.8)	(0.001)	
ALDH4A1				
Negative	185 (92.0)	16 (8.0)	11.19	**0.008**
Positive	152 (80.4)	37 (19.6)	(0.001)	
ALDH18A1				
Negative	190 (91.8)	17 (8.2)	8.17	**0.02**
Positive	157 (82.2)	34 (17.8)	(0.004)	
SLC1A5				
Negative	430 (90.1)	47 (9.9)	1.50	0.81
Positive	856 (88.0)	117 (12.0)	(0.22)	
SLC7A5				
Negative	1040 (88.1)	140 (11.9)	4.66	0.09
Positive	253 (92.7)	20 (7.3)	(0.03)	
SLC38A2				
Negative	997 (89.5)	117 (10.5)	13.53	**0.002**
Positive	88 (77.9)	25 (22.1)	(0.0002)	
SLC7A11				
Negative	744 (91.4)	70 (8.6)	11.20	**0.009**
Positive	410 (85.4)	70 (14.6)	(0.001)	
SLC3A2				
Negative	863 (89.1)	106 (10.9)	0.78	0.54
Positive	317 (87.3)	46 (12.7)	(0.37)	
P70S6K				
Negative	265 (89.5)	66 (10.5)	0.10	1.48
Positive	148 (88.6)	19 (11.4)	(0.74)	

P values in bold means statistically significant

7.5.6 Association of SLC38A2, SLC7A11 and SLC7A8 and patient outcome

High SLC38A2 protein expression, but not mRNA, was associated with shorter BCSS (p=0.004, Figure 7-11A) and (p>0.05, Figure 7-10A) respectively. When investigating within the molecular classes, no significant associations was observed in any of the BC subtypes at the mRNA level (all p>0.05, Figure 7-10B-E). However, high expression of SLC38A2 protein was predictive of shorter BCSS only within TN tumours (p=0.01, Figure 7-11D). There was no association between SLC38A2 protein and outcome in ER+ and HER2+ subtypes (all p>0.05, Figure 7-11B, C, E). High SLC38A2 protein expression was also associated with shorter DMFS (p=0.01, Figure 7-16A) and this was observed in TN tumours (p=0.01, Figure 7-16D) but not with the other subtypes (all p>0.05 Figure 7-16B, C, E). In multivariate Cox regression analysis, SLC38A2 protein was a predictor of shorter BCSS independent of tumour size, tumour grade and lymph node stage in TN tumours only (p=0.004, Table 7-16B).

Survival analysis of *SLC7A11* mRNA showed a significant association with shorter BCSS (p=0.02, Figure 7-12A). No significant associations were observed when the analysis was carried out within the molecular BC subtypes (all p>0.05, Figure 7-12B-E). However, multivariate analysis showed that *SLC7A11* mRNA was a predictive of poor survival independent of the same confounding variables (p=0.008, Table 7-17A) and the result remained significant within the luminal A tumours (p=0.02, Table 7-17B), when different subtypes were considered. SLC7A11 protein was not associated with patient outcome, in terms of BCSS and DMFS either in the whole cohort (all p>0.05, Figure 7-13A, 7-17A) or when the different subtypes were examined (all p>0.05, Figure 7-13B-E, Figure 7-17B-E).

Regarding SLC7A8, high expression of both mRNA and protein was associated with longer BCSS (all P≤0.001, Figure 7-14A, 7-15A). While *SLC7A8* mRNA expression was not predictive for BCSS in any specific molecular class (all p>0.05, Figure 7-14 B-E), high expression of SLC7A8 protein was predictive of good survival in only the ER + low proliferation tumours (P=0.01, Figure 7-15B). There was no association between SLC7A8 protein and outcome in the ER+ high proliferation, HER2+ or TN subtypes (all p>0.05, Figure 7-15C-E). Multivariate Cox regression analysis showed that *SLC7A8* mRNA and SLC7A8 protein were predictors of longer

BCSS independent of tumour size, grade, and lymph node stage (P=0.01 and P=0.03, Table 7-18A) respectively. Likewise, high SLC7A8 protein expression was associated with longer DMFS (P<0.001, Figure 7-18A) within the ER+ low proliferation class (P=0.003, Figure 7-18B) but not with the other subtypes (all p>0.05, Figure 7-18C-E).

The relationships between high SLC38A2, SLC7A11 and SLC7A8 mRNA expression and patient outcome were verified using Breast Cancer Gene-Expression Miner (Figure 7-19, 7-20, 7-21 A-C).

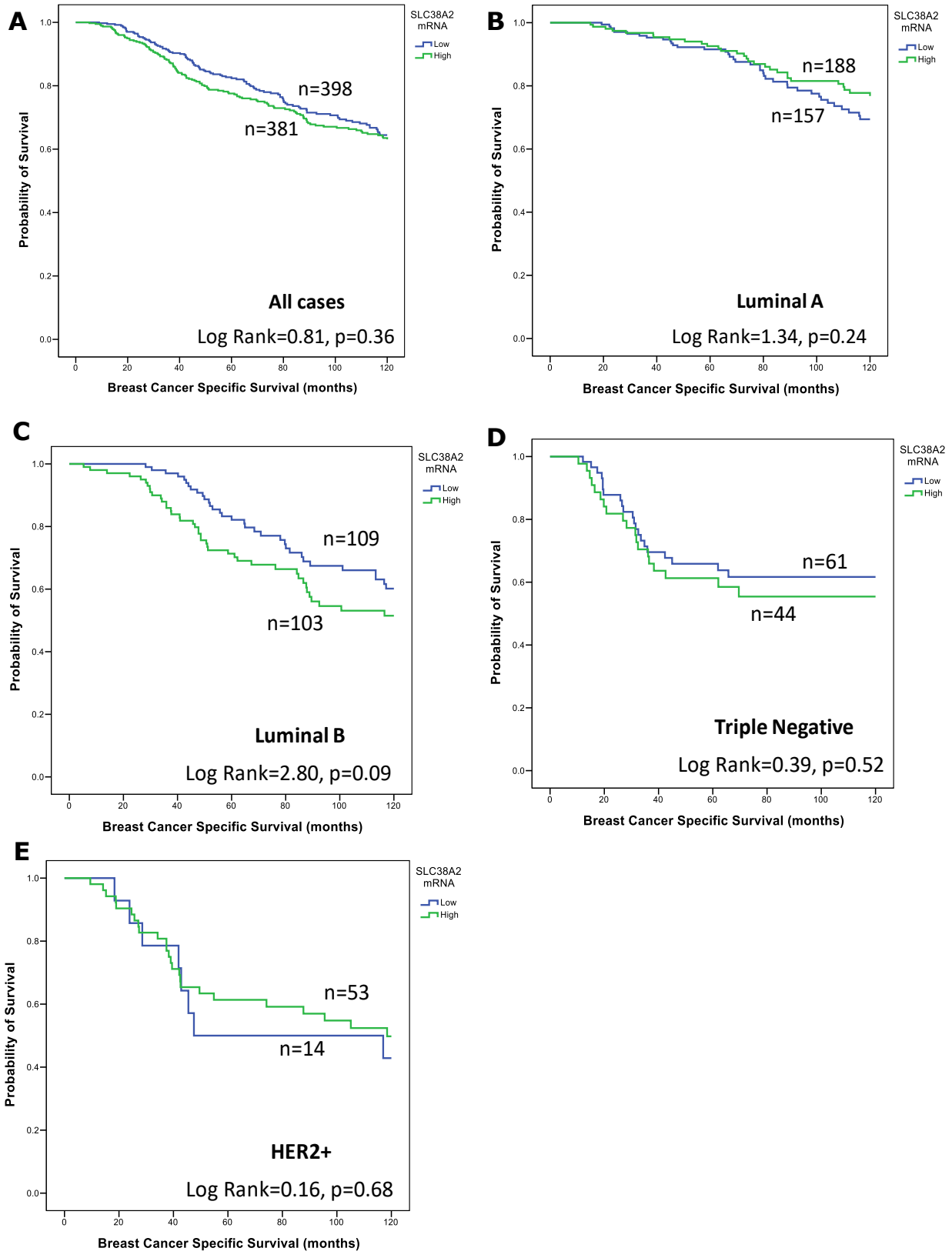


Figure 7-10. SLC38A2 mRNA and breast cancer patient outcome. A) *SLC38A2*vs BCSS in all cases, B) *SLC38A2*vs BCSS in Luminal A tumours, C) *SLC38A2* vs BCSS in Luminal B tumours, D) *SLC38A2* vs BCSS in Triple Negative tumours, E) *SLC38A2* vs BCSS in HER2+ tumours.

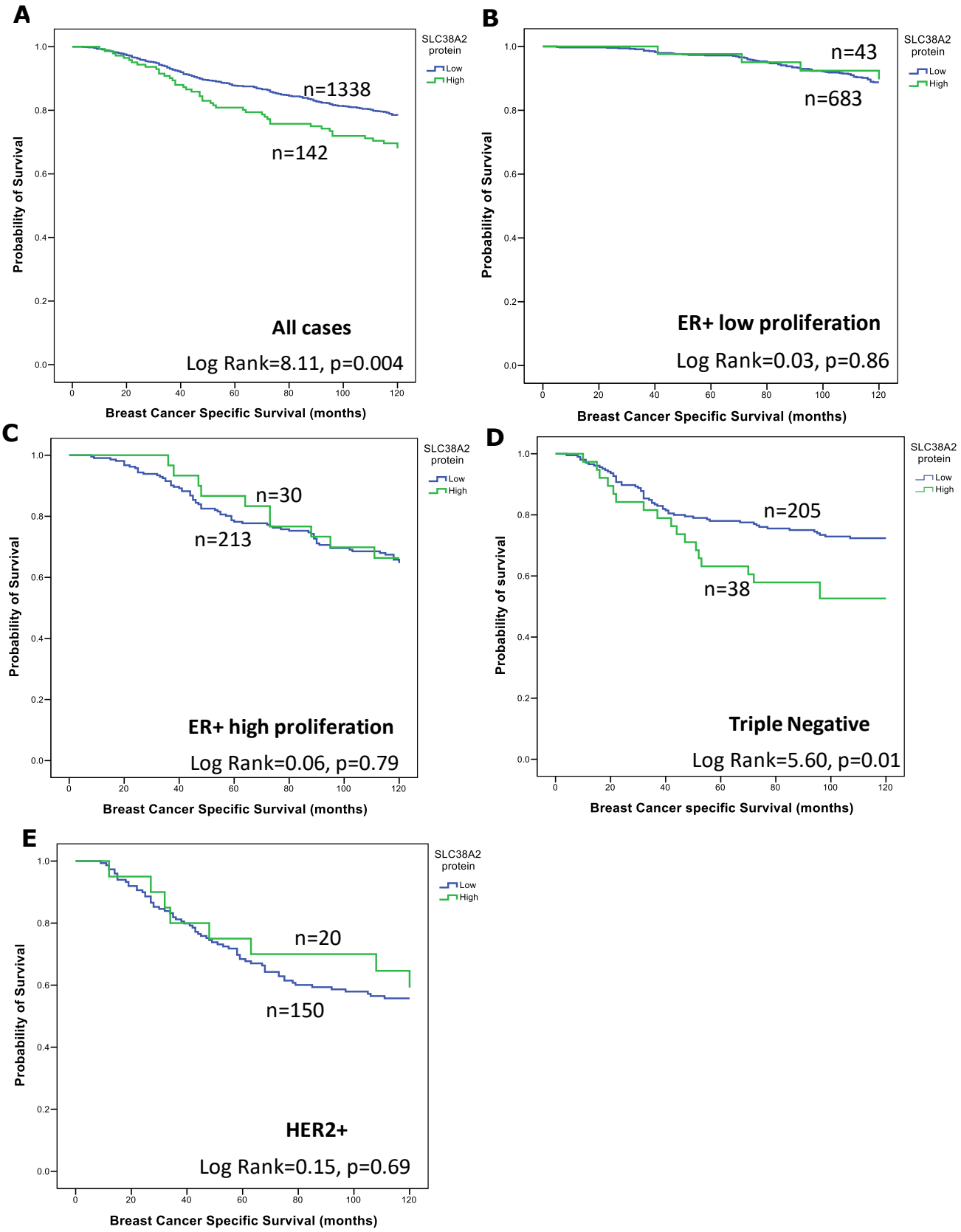


Figure 7-11. SLC38A2 protein expression and breast cancer patient outcome. A) SLC38A2 vs BCSS in all cases , B) SLC38A2 vs BCSS in ER+- Low Proliferation tumours, C) SLC38A2 vs BCSS in ER+- High Proliferation tumours, D) SLC38A2 vs BCSS in Triple Negative tumours E) SLC38A2 vs BCSS in HER2+ tumours.

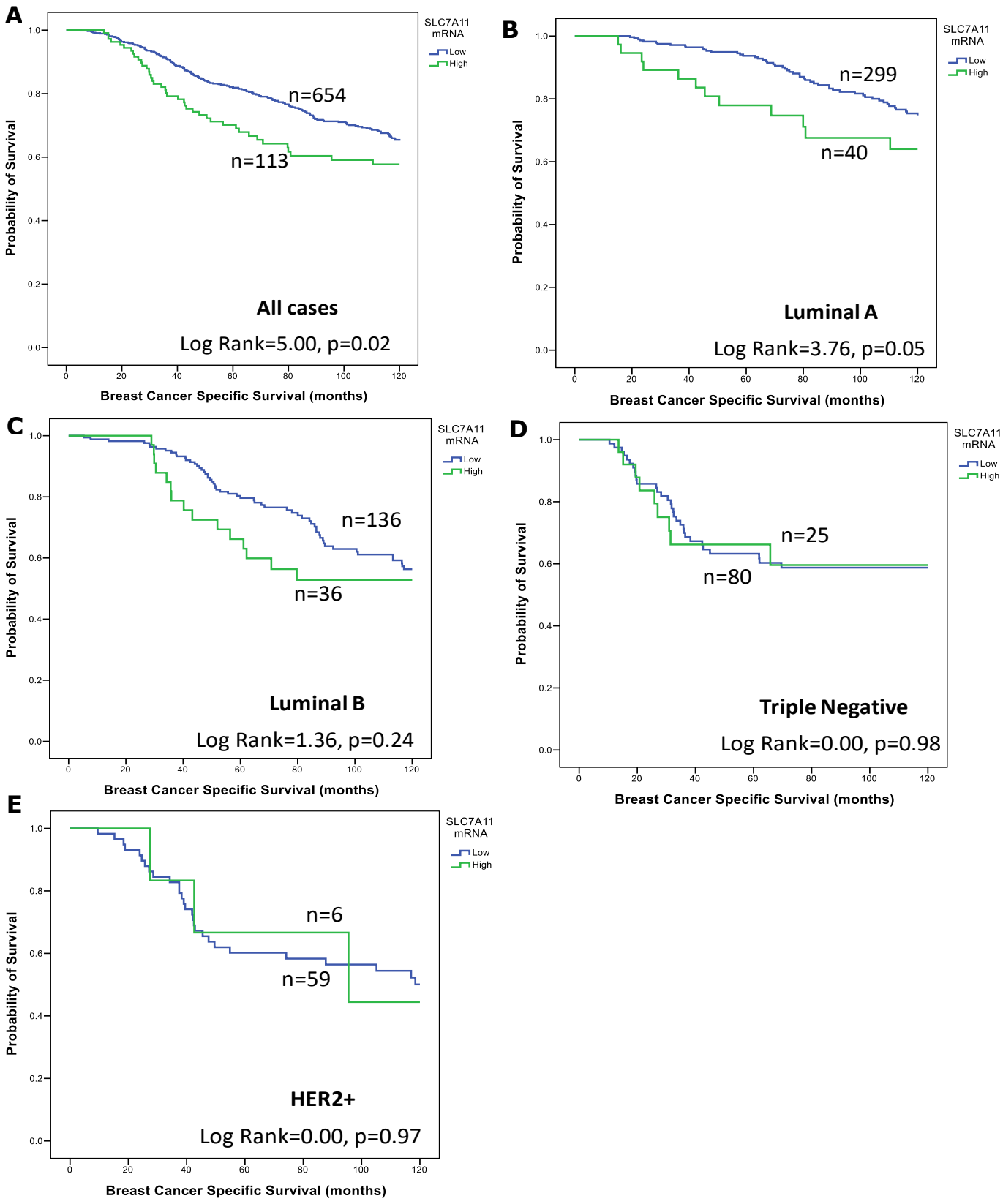


Figure 7-12. SLC7A11 mRNA and breast cancer patient outcome. A) *SLC7A11*vs BCSS in all cases, B) *SLC7A11*vs BCSS in Luminal A tumours, C) *SLC7A11* vs BCSS in Luminal B tumours, D) *SLC7A11* vs BCSS in Triple Negative tumours, E) *SLC7A11* vs BCSS in HER2+ tumours.

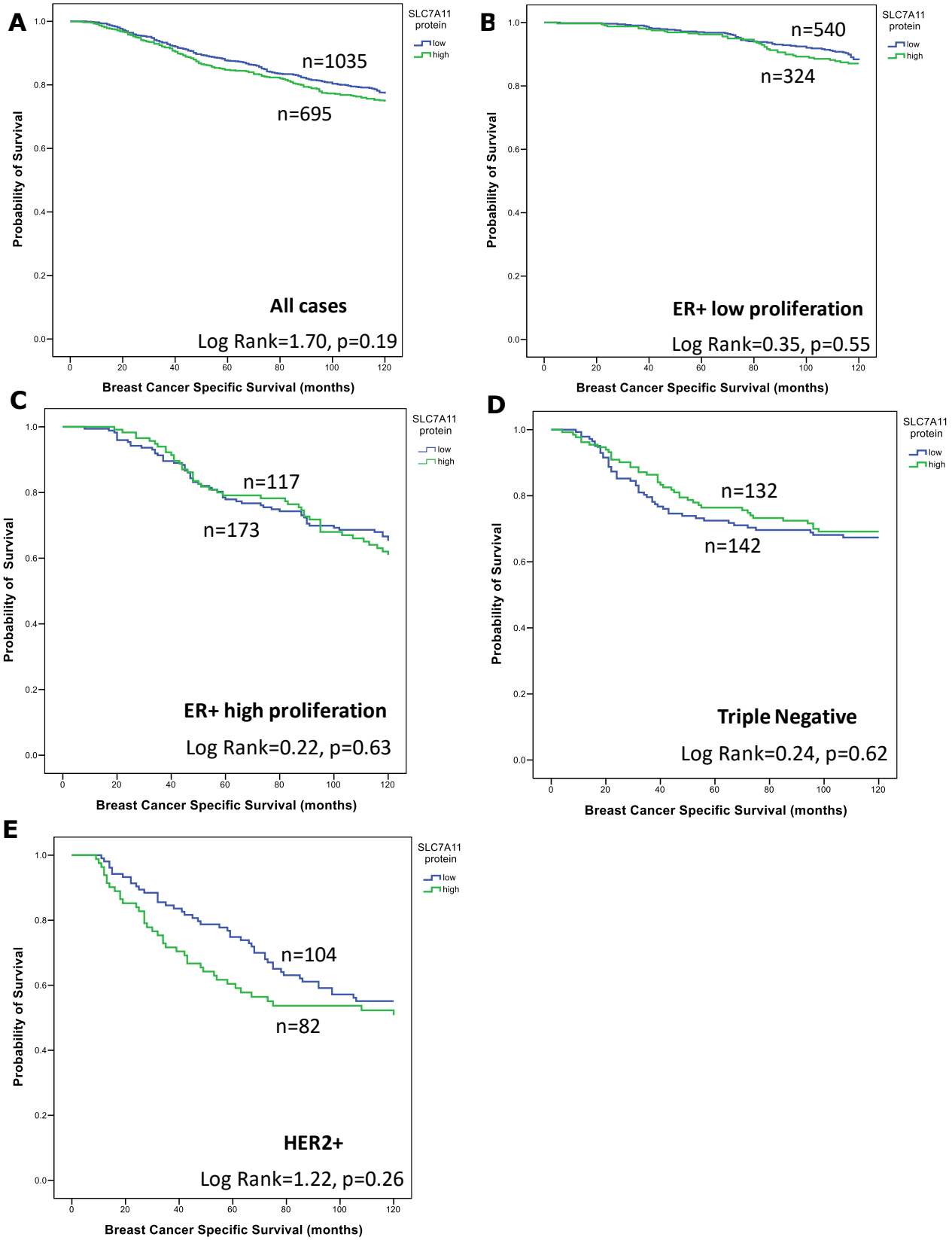


Figure 7-13. SLC7A11 protein expression and breast cancer patient outcome. A) SLC7A11 vs BCSS in all cases , B) SLC7A11 vs BCSS in ER+- Low Proliferation tumours, C) SLC7A11 vs BCSS in ER+- High Proliferation tumours, D) SLC7A11 vs BCSS in Triple Negative tumours E) SLC7A11 vs BCSS in HER2+ tumours.

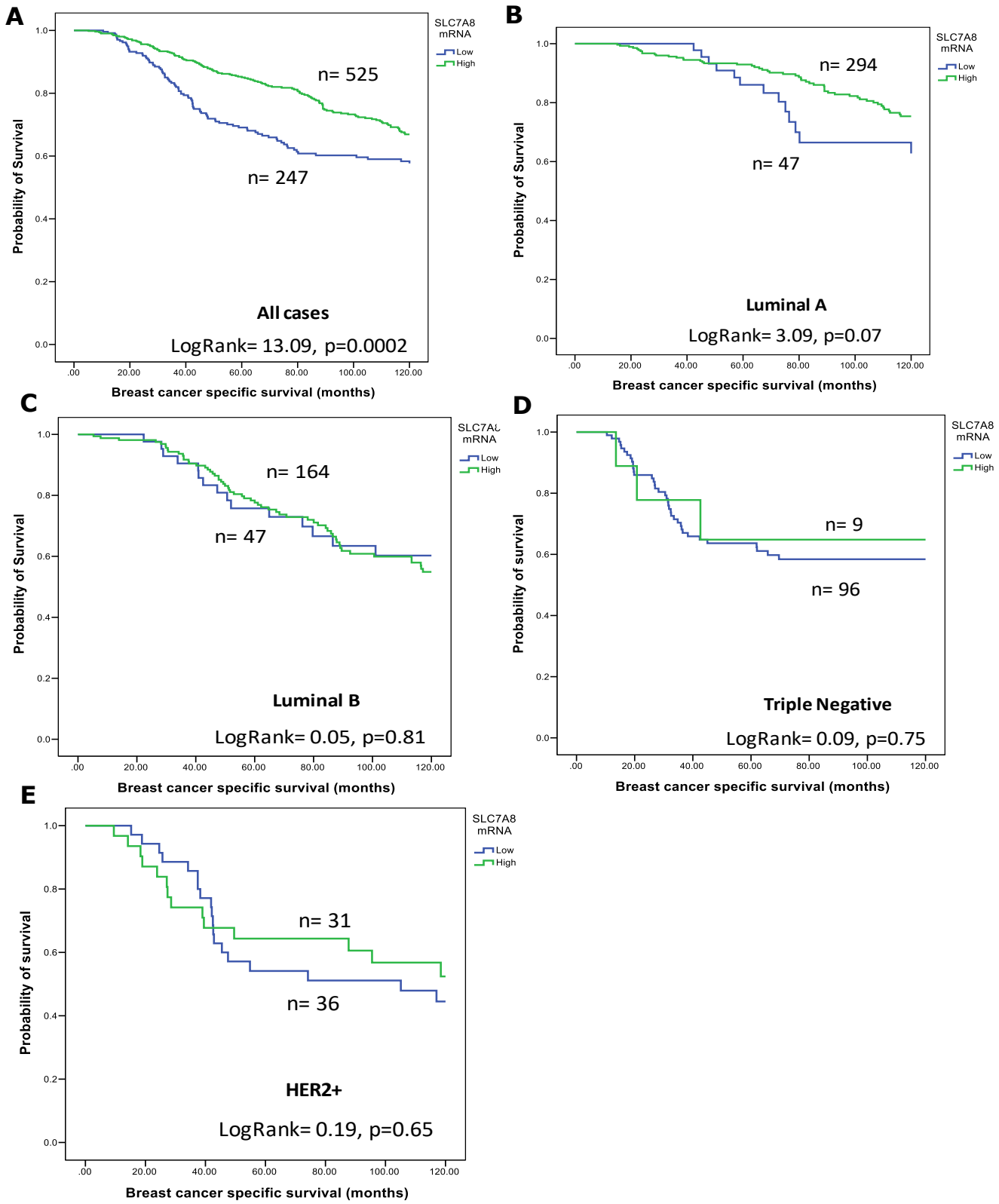


Figure 7-14. SLC7A8 mRNA and breast cancer patient outcome. A) *SLC7A8*vs BCSS in all cases, B) *SLC7A81*vs BCSS in Luminal A tumours, C) *SLC7A8* vs BCSS in Luminal B tumours, D) *SLC7A8* vs BCSS in Triple Negative tumours, E) *SLC7A8* vs BCSS in HER2+ tumours.

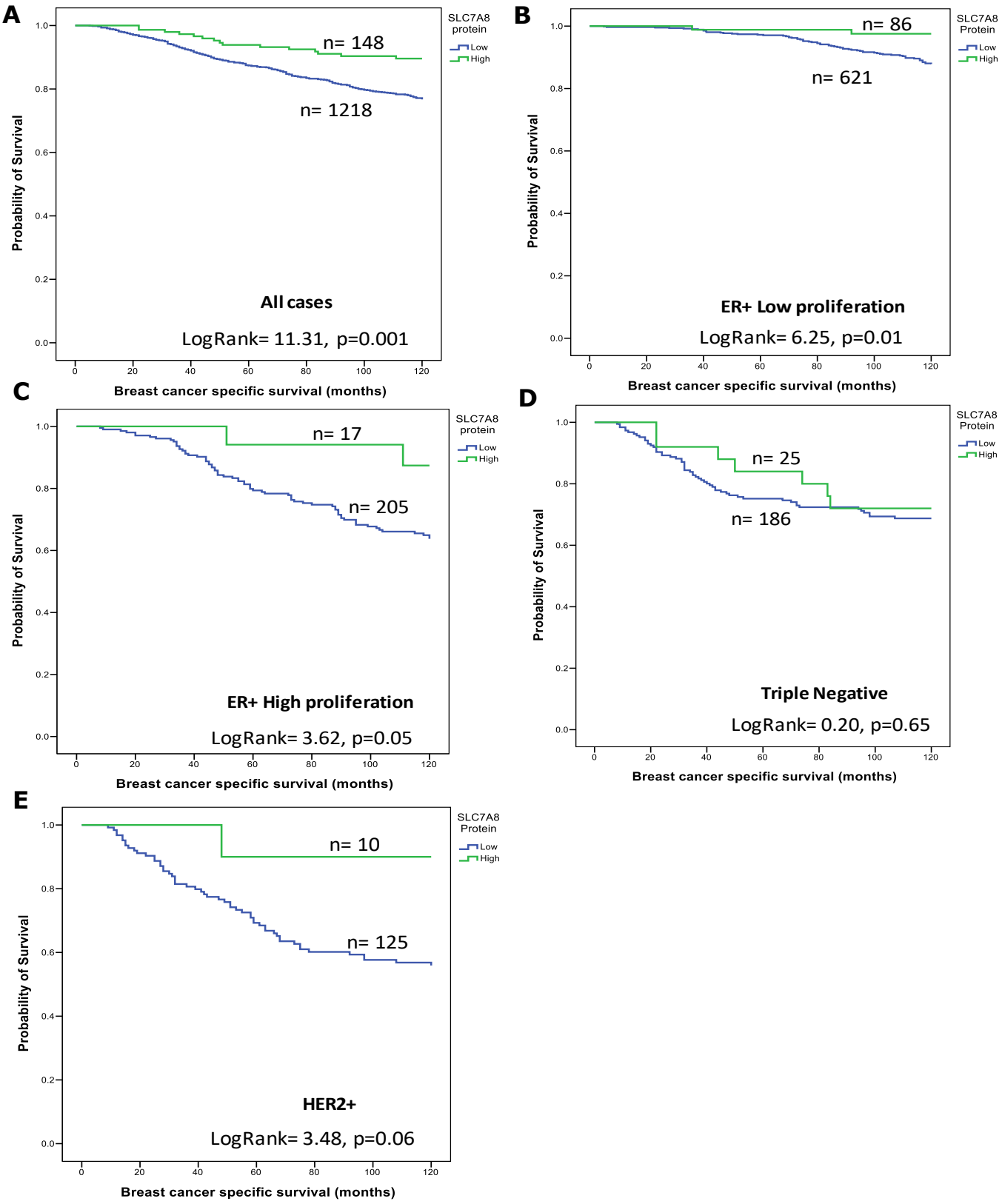


Figure 7-15. SLC7A8 protein expression and breast cancer patient outcome. A) SLC7A8 vs BCSS in all cases , B) SLC7A8 vs BCSS in ER+- Low Proliferation tumours, C) SLC7A8 vs BCSS in ER+- High Proliferation tumours, D) SLC7A8 vs BCSS in Triple Negative tumours E) SLC7A8 vs BCSS in HER2+ tumours.

Table 7-16A. Multivariate analysis of prognostic variables and SLC38A2 protein expressions in all cases.

	SLC38A2 protein	
Parameter	**Hazard ratio (95% CI)**	**p-value**
SLC38A2	1.36 (0.99-1.87)	0.05
stage	1.91 (1.64-2.22)	$3.8x10^{-17}$
Size	1.48 (1.16-1.87)	0.001
Grade	2.38 (1.91-2.97)	$7.2x10^{-15}$

Table 7-16B. Multivariate analysis of prognostic variables and SLC38A2 protein expression in different IHC subtypes.

	SLC38A2 protein							
	ER-low proliferation		**ER-High proliferation**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC38A2	0.87 (0.31-2.39)	0.79	1.01 (0.52-1.96)	0.97	2.19 (1.28-3.76)	**0.004**	0.91 (0.43-1.91)	0.81
Nodal stage	1.81 (1.28-2.55)	0.001	1.42 (1.06-1.90)	0.01	2.11 (1.54-2.91)	0.000003	2.06 (1.51-2.83)	0.000006
Size	1.67 (1.02-2.74)	0.04	1.53 (0.94-2.51)	0.08	1.29 (0.79-2.13)	0.30	1.68 (1.01-2.77)	004
Grade	1.72 (1.16-2.54)	0.007	3.73 (0.51-6.89)	0.19	1.20 (0.52-2.79)	0.66	1.36 (0.78-2.37)	0.27

P value in bold means high expression of SLC38A2 significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 7-17A. Multivariate analysis of prognostic variables and *SLC7A11* mRNA expressions in all cases.

	***SLC7A11* mRNA**	
Parameter	**Hazard ratio (95% CI)**	**p-value**
SLC7A11	1.57 (1.12-2.21)	**0.008**
Nodal stage	1.71 (1.43-2.04)	$2.1x10^{-9}$
Size	1.51 (1.07-2.14)	0.01
Grade	1.45 (1.14-1.86)	0.003

P value in bold means high expression of *SLC7A11* significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 7-17B. Multivariate analysis of prognostic variables and SLC7A11 mRNA expression in different BC molecular subtypes.

	***SLC7A11* mRNA**							
	Luminal A		**Luminal B**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC7A11	2.08 (1.11-3.84)	**0.02**	1.29 (0.73-2.30)	0.37	1.11 (0.52-2.36)	0.77	1.26 (0.36-4.38)	0.71
Nodal stage	1.31 (0.93-1.84)	0.12	1.82 (1.34-2.48)	0.0001	1.89 (1.19-3.01)	0.007	2.19 (1.31-3.65)	0.003
Size	2.03 (1.11-3.73)	0.02	1.56 (0.80-3.04)	0.19	0.77 (0.37-1.58)	0.48	2.47 (0.72-8.46)	0.14
Grade	1.23 (0.81-1.87)	0.33	0.96 (0.62-1.49)	0.88	2.95 (0.39-21.84)	0.28	0.71 (0.32-1.58)	0.41

P value in bold means high expression of *SLC7A11* significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 7-18A. Multivariate analysis of prognostic variables and SLC7A8 mRNA and protein expressions in all cases.

	***SLC7A8* mRNA**		**SLC7A8 protein**	
Parameter	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC7A8	0.70 (0.52-0.94)	**0.01**	0.57 (0.33-0.96)	**0.03**
Nodal stage	2.00 (1.56-2.55)	$2.4x10^{-8}$	1.89 (1.62-2.22)	$8.7x10^{-16}$
Size	1.48 (0.99-2.20)	0.05	1.39 (1.08-1.78)	0.009
Grade	1.41 (1.08-1.82)	0.01	2.49 (1.97-3.16)	$2.8x10^{-14}$

P value in bold means high expression of SLC7A8 significantly predicts survival after adjusting with tumour grade, tumour size and lymph node stage

Table 7-18B. Multivariate analysis of prognostic variables and *SLC7A8* mRNA expression in different BC molecular subtypes.

	***SLC7A8* mRNA**							
	Luminal A		**Luminal B**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC7A8	0.80 (0.43-1.49)	0.49	1.01 (0.63-1.61)	0.94	0.77 (0.36-1.66)	0.51	0.75 (0.43-1.29)	0.30
Nodal stage	1.57 (0.99-2.48)	0.05	2.10 (1.50-2.96)	0.00001	2.36 (1.53-3.64)	0.00009	2.34 (1.48-3.72)	0.0002
Size	1.94 (1.02-3.70)	0.04	1.37 (0.76-2.48)	0.28	0.96 (0.54-1.73)	0.91	1.23 (0.57-2.62)	0.58
Grade	1.24 (0.85-1.82)	0.26	1.07 (0.73-1.58)	0.70	1.92 (0.70-5.27)	0.20	0.79 (0.45-1.39)	0.42

Table 17-18C. Multivariate analysis of prognostic variables and SLC7A8 protein expression in different IHC subtypes.

SLC7A8 protein								
	ER-low proliferation		**ER-High proliferation**		**Triple Negative**		**HER2+**	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
SLC7A8	0.25 (0.06-1.03)	0.05	0.34 (0.08-1.42)	0.14	0.87 (0.39-1.95)	0.87	0.22 (0.03-1.66)	0.14
Nodal stage	1.61 (1.14-2.26)	0.006	1.43 (1.06-1.94)	0.01	2.17 (1.55-3.05)	0.000007	2.27 (1.56-3.32)	0.00002
Size	1.50 (0.91-2.48)	0.11	1.30 (0.78-2.17)	0.30	1.63 (0.93-2.85)	0.08	1.44 (0.82-2.52)	0.19
Grade	1.89 (1.27-2.82)	0.002	1.35 (0.77-2.35)	0.28	1.63 (0.57-4.63)	0.35	1.36 (0.70-2.65)	0.36

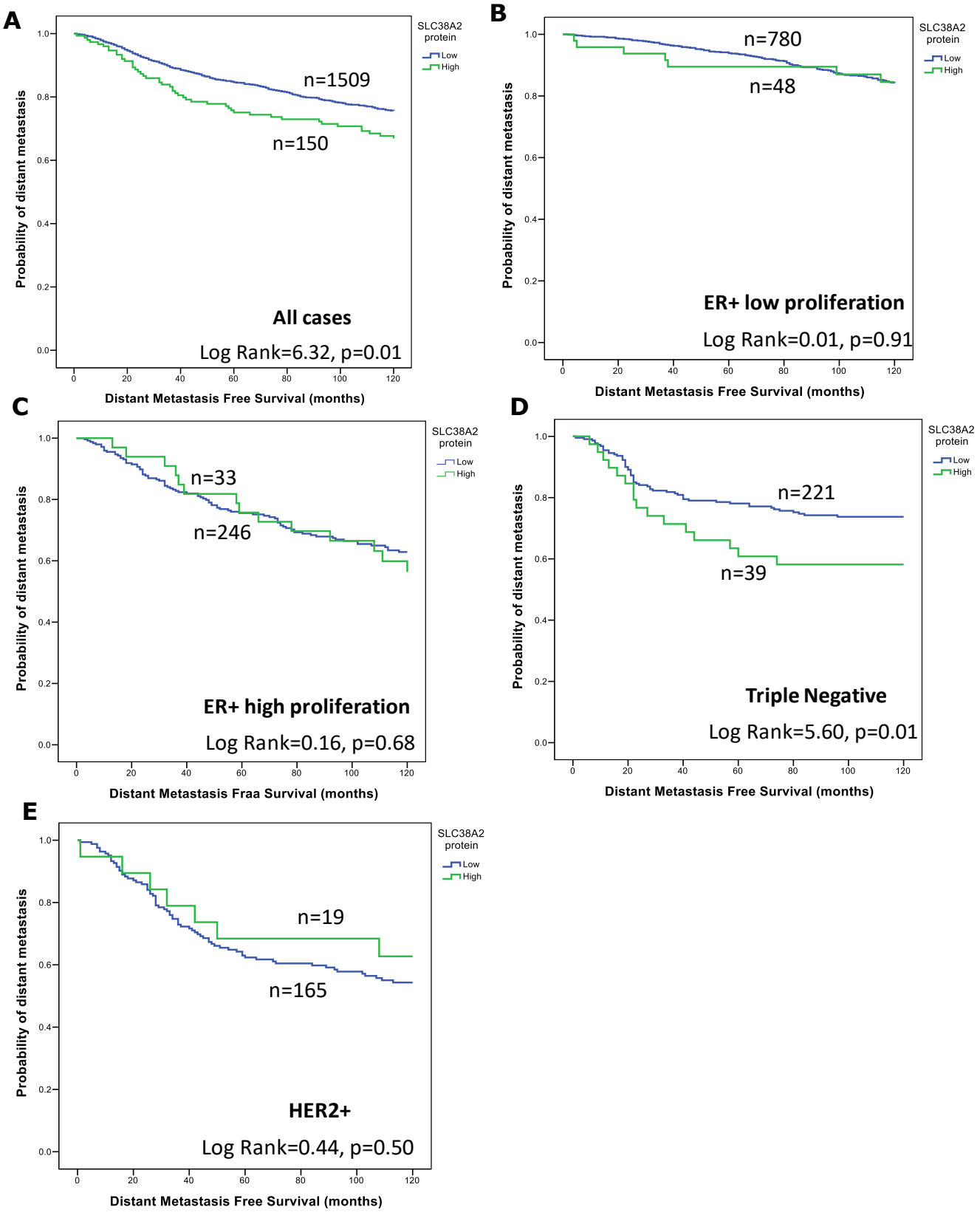


Figure 7-16. SLC38A2 protein expression and distant metastasis. A) SLC38A2 Vs DMFS in all cases, B) SLC38A2 Vs DMFS in ER+- Low Proliferation tumours, C) SLC38A2 Vs DMFS in ER+- High Proliferation tumours, D) SLC38A2 Vs DMFS in Triple negative tumours, E) SLC38A2 Vs DMFS in HER2+ tumours.

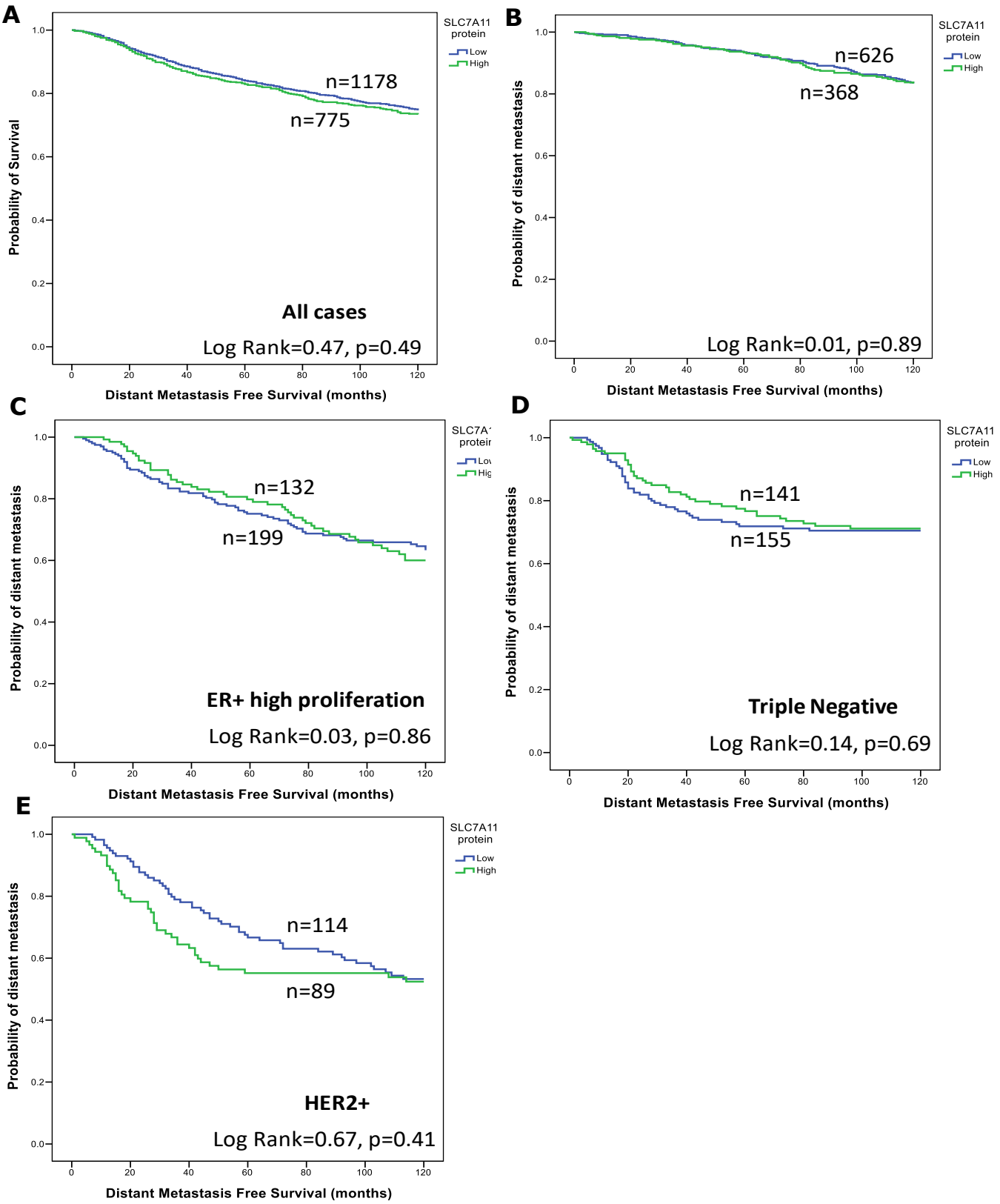


Figure 7-17. SLC7A11 protein expression and distant metastasis. A) SLC7A11 Vs DMFS in all cases, B) SLC7A11 Vs DMFS in ER+- Low Proliferation tumours, C) SLC7A11 Vs DMFS in ER+- High Proliferation tumours, D) SLC7A11 Vs DMFS in Triple negative tumours, E) SLC7A11 Vs DMFS in HER2+ tumours.

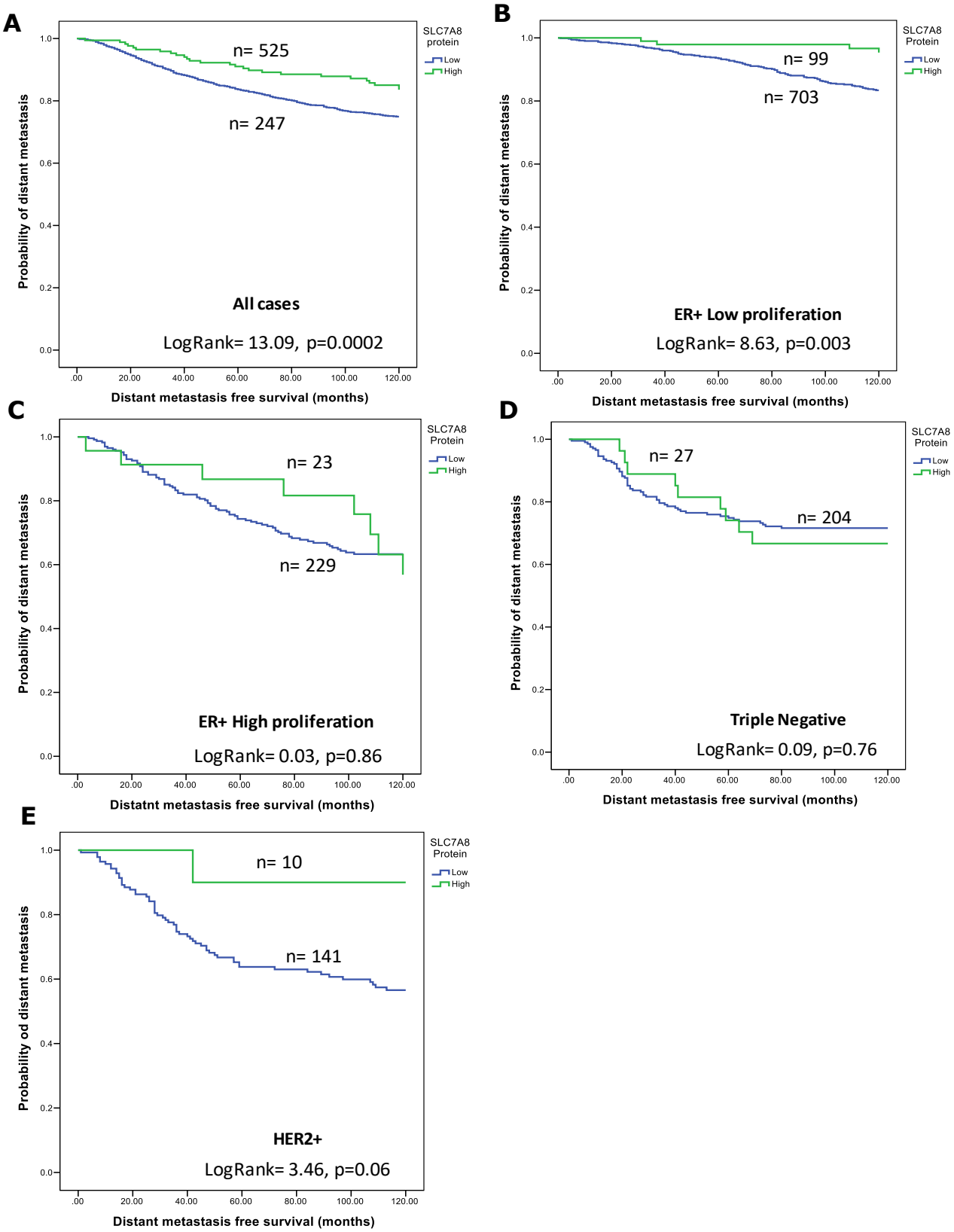


Figure 7-18. SLC7A8 protein expression and distant metastasis. A) SLC7A8 Vs DMFS in all cases, B) SLC7A8 Vs DMFS in ER+- Low Proliferation tumours, C) SLC7A8 Vs DMFS in ER+- High Proliferation tumours, D) SLC7A8 Vs DMFS in Triple negative tumours, E) SLC7A8 Vs DMFS in HER2+ tumours.

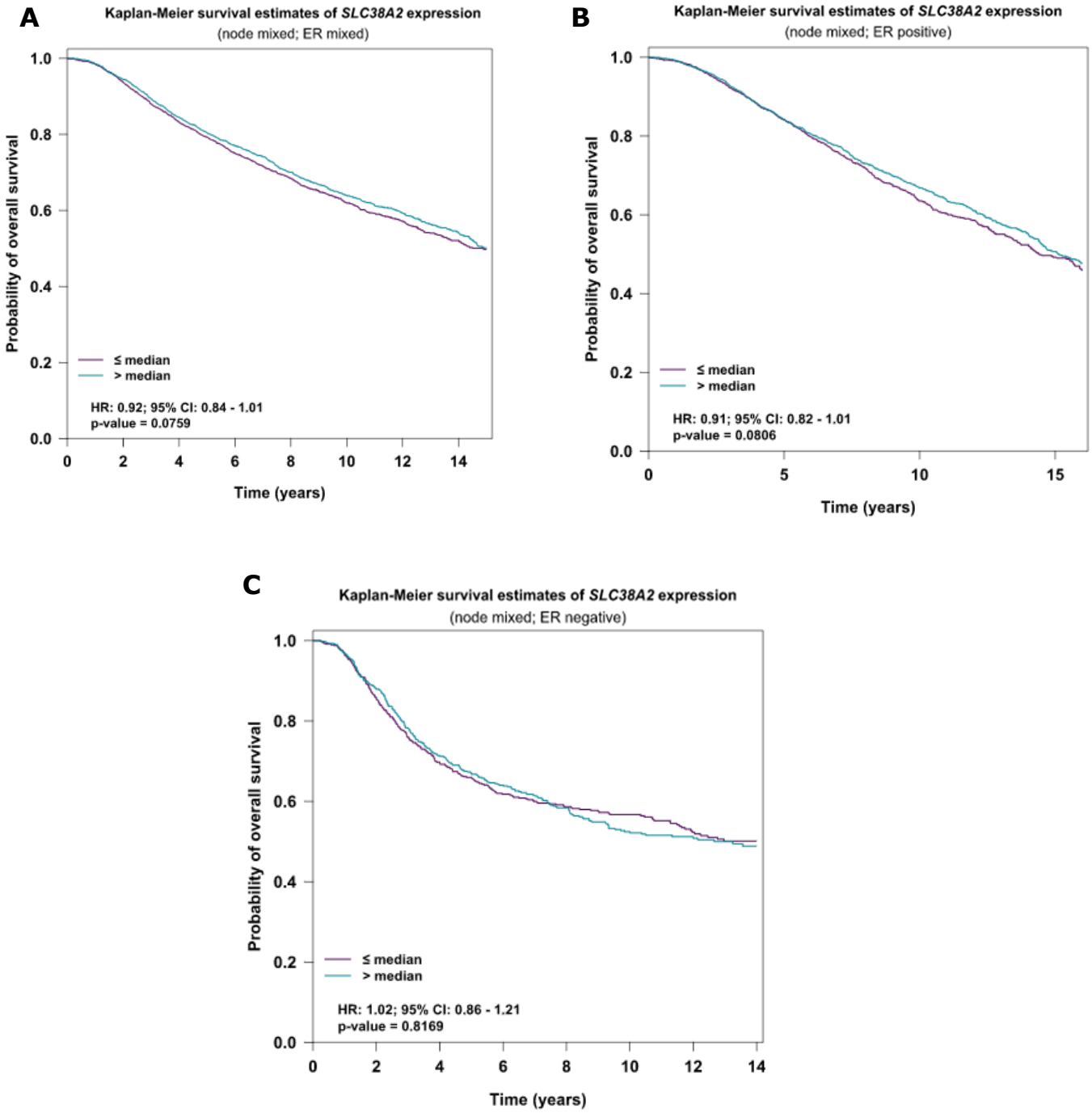


Figure 7-19. SLC38A2 mRNA and breast cancer patient outcome using Breast Cancer Gene-Expression Miner: A) Unselected cases, B) ER+ disease, C) ER- disease.

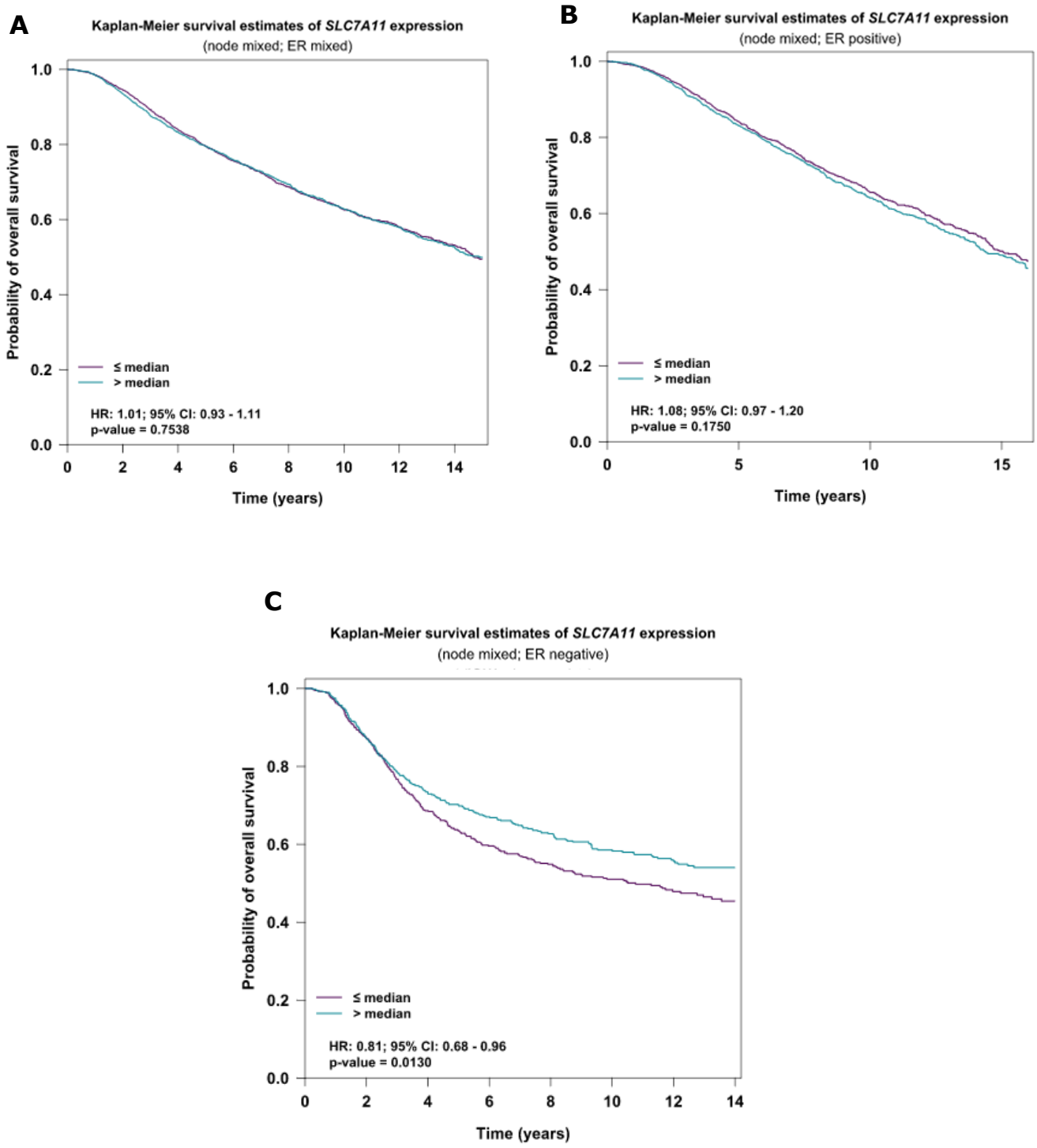


Figure 7-20. SLC7A11 mRNA and breast cancer patient outcome using Breast Cancer Gene-Expression Miner: A) Unselected cases, B) ER+ disease, C) ER- disease.

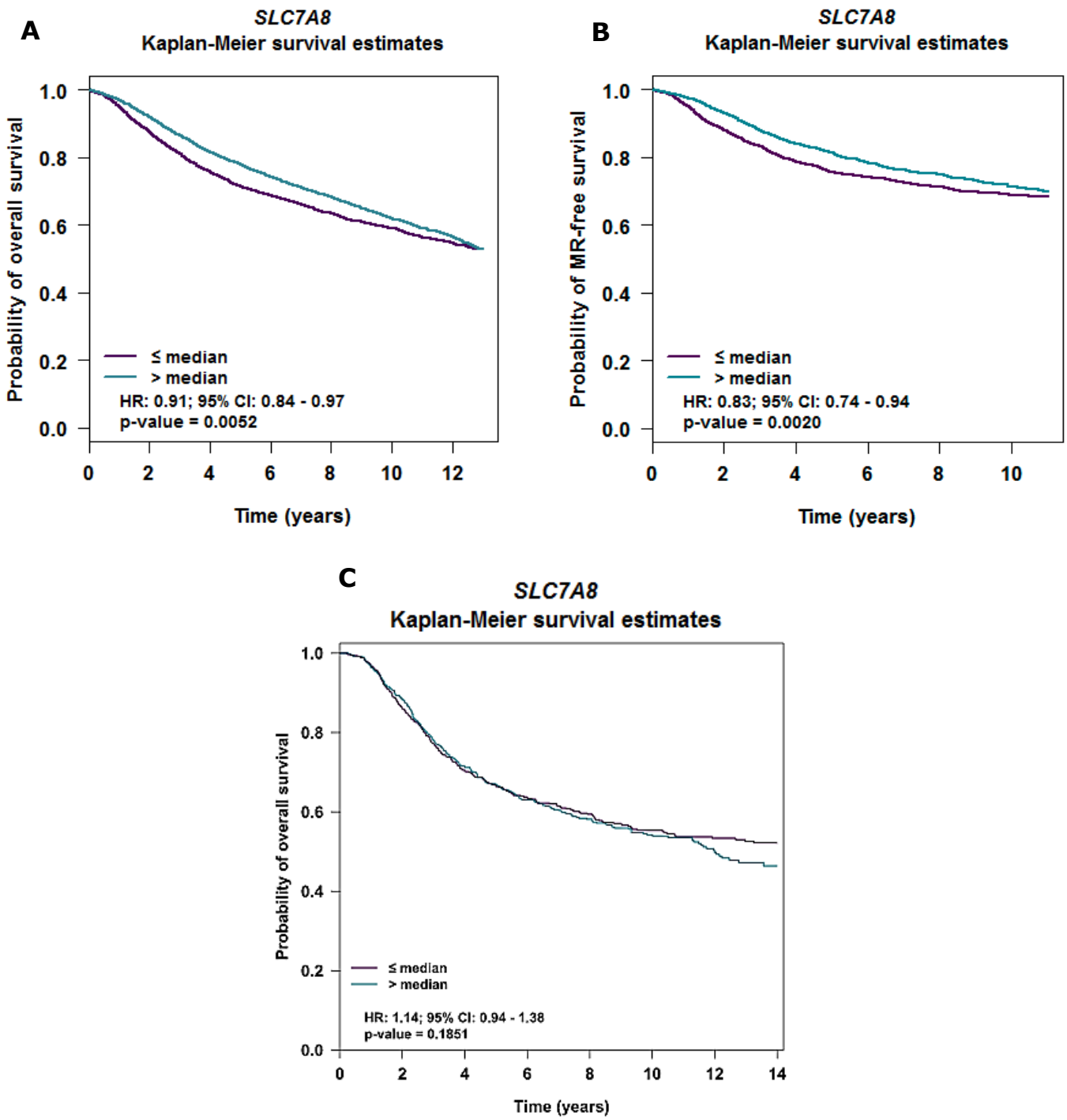


Figure 7-21. SLC7A8 mRNA and breast cancer patient outcome using Breast Cancer Gene-Expression Miner: A) Unselected cases, B) ER+ disease, C) ER- disease.

7.6 Discussion

SLC proteins are related to tumourigenesis in cancer cells [263]. As tumour initiates, the metabolic activity of cancer cells rises, resulting in an increase in the oxidative stress and subsequent activation of signalling pathways that support cell proliferation and survival [401]. Therefore, cancer cells require the expression of SLCs that can provide important molecules, such as glutamine, under extreme conditions, including hypoxia, nutrient deprivation and oxidative stress. Furthermore, some SLCs, such as SLC7A11, protect cellular damage through providing amino acids that assist in the formation of the antioxidant molecule, glutathione. However, some less aggressive cancers that have lower metabolic activity may express lower levels of these transporters or replace that via expressing solute carriers that have low affinity to their substrates, such SLC7A8.

Studies that address the clinical significance of the solute carriers (SLC38A2, SLC7A11 and SLC7A8) in patients with BC and their potential influence on different subtypes remain elusive. Therefore, we hypothesise that SLC38A2, SLC7A11 and SLC7A8, are over-expressed in BC and the consequences of these expressions will vary among the different molecular BC subtypes. To prove this hypothesis, large cohorts of BC cases were investigated in order to decipher the potential role of SLC38A2, SLC7A11 and SLC7A8 mRNA and protein expressions in BC and its molecular subtypes.

7.6.1 SLC38A2

SLC38A2 protein expression, but not mRNA, was significantly associated with poor prognostic parameters, including high tumour grade, high mitotic count and poor NPI. Furthermore, high SLC38A2 expression was significantly associated with the higher expression of the proliferative marker (Ki67), indicating that this solute carrier is critical for proliferation in BC cells. In agreement with this finding, other studies found that upregulation of *SLC38A2 in vivo* increased the expression of Ki67 and proliferation of MCF7 xenografts. Additionally, inhibition of SLC38A2 significantly reduced cell proliferation of the same cell line *in vitro* [375,146].

Regarding BC molecular classes, the highest proportion of *SLC38A2* CN gain was observed in HER2+ tumours. Similarly, high *SLC38A2* mRNA was observed mainly in HER2+ subtype. However, SLC38A2 protein was highly expressed in TN tumours. Furthermore, the results showed that SLC38A2 over-expressing TN

cases had shorter BCSS and DMFS. The discrepant findings between the mRNA and protein can be attributed to the tight control of SLC38A2 protein at posttranslational level. The N-glycosylation is an example of posttranslational modifications that occur in a variety of membrane transporters and they play a role in their function, such as membrane targeting, substrate binding, and stability [402-404]. A recent study found that SLC38A2 contains 3 N-glycosylation sites, which are linked to its expression and function [405].

On the other hand, ubiquitination is another posttranslational mechanism which mediates SLC38A2 protein degradation, as it has been found that the ubiquitin ligase RNF5 targets SLC38A2 and resulted in decreased glutamine uptake and proliferation of BC cells [287]. In addition, targeting RNF5 in MDA-MB-231 TNBC cells not only promoted tumourigenesis but also decreased responsiveness to chemotherapy [287]. In the line with that, this study showed a negative correlation between *RNF5* and *SLC38A2*. The ubiquitination process could take place under nutrient replete-conditions as SLC38A2 is known as a key transporter when amino acids are deficient. Indeed, upon amino acid deprivation, the General Control Non-derepressible-2 (GCN2) kinase is activated and phosphorylates the translation initiation factor eIF2α (eukaryotic initiation factor 2α), which in turn, decreases global protein synthesis. However, it stimulates the translation of a selected number of mRNAs, including that coding for ATF4 [406]. To encounter amino acid stress, ATF4 increased transcription of a subset of target genes, including SLC38A2 [370]. In this study, direct correlation between *ATF4* and *SLC38A2* mRNA expression was observed in all BC subtypes.

A recent study revealed that *SLC38A2* induction in MCF7 cells was stimulated by *ER*, but it became predominantly a *HIF1α*–dependent gene under hypoxic conditions [146]. The latter could be the regulator in breast cancers that lack ER expression, as it was positively correlated with *SLC38A2* in TNBC. This association was also observed within the luminal B subtype which is relevant to the clinical behaviour of TNBC and shows a prognosis similar to that of the basal-like tumours [407]. Furthermore, being aggressive and highly proliferative, these subtypes are more prone to face hypoxic environments with subsequent upregulation of *HIF1α*.

The current study showed significant associations between SLC38A2 and glutamine metabolic enzymes, at both mRNA and protein levels, including

enzymes involved in the glutamine-proline regulatory axis, GLS and GLUD1. This indicates that glutamine uptake by SLC38A2 is sequentially converted to glutamate which then supports other cellular pathways such as the formation of proline. The latter is important to face cellular stress conditions, as it plays a special role in ROS signalling and redox balance, in addition to its ability to interconvert to other conditionally-essential amino acids under various nutritionally stressed situations [408].

The association with other glutamine transporters was variable across BC subtypes and SLC1A5 was the only transporter which showed associations with SLC38A2 in the all subtypes. Furthermore, SLC38A2 protein was associated with all solute carriers included in this study, suggesting that they all cooperate to support cancer cells to resist various stressful situations. With regard to this, Menchini *et al* explained how SLC38A2 interacts with the key transporters, SLC1A5 and SLC7A5, where the last two are obligatory exchangers and act on amino acid uptake against their concentration gradient in exchange with other amino acid substrates. As a unidirectional transporter, SLC38A2 is able to generate high intra-cellular concentration of amino acid substrates which in turn power the action of SLC1A5 and SLC7A5 by exchanging with their substrates [409].

It has been found that the co-expression of SLC38A2 and SLC7A5 leads to a substantial increase in intracellular leucine levels compared to the expression of SLC7A5 alone [410]. Furthermore, targeting SLC38A2 in rat myocytes resulted in a decrease in intracellular concentrations of both glutamine and leucine, which subsequently repressed mTORC1 signalling [411]. This indicates that SLC38A2 may indirectly control amino acid sensing through SLC7A5 and mTORC1 [412].

7.6.2 SLC7A11

Oxidative stress is a harmful phenomenon that leads to cellular damage through the oxidative modification of DNAs, proteins and lipids and therefore plays a crucial role in many diseases. In addition, oxidative stress often affects cancer cells due to their relatively high metabolic rate [413]. SLC7A11-mediated cystine transport for GSH synthesis plays a key role in the prevention of oxidative stress signalling, thereby, is strongly associated with cancer cell proliferation and tumour growth [377].

SLC7A11 is overexpressed in various tumour tissues and cancer cell lines and its expression is associated with poor prognosis and shortened overall survival [378-380,414,152,415]. In agreement with these studies, the results of this chapter demonstrated that overexpression of SLC7A11 is associated with features of undesirable prognosis, including high tumour grade and high mitotic count. Furthermore, high SLC7A11 expression was significantly associated with proliferation. This supports the results of previous studies, which reported the significant correlation between SLC7A11 and Ki67 labelling index in human oesophageal and laryngeal squamous cell carcinomas [379,380], confirming that SLC7A11 is most likely critical for proliferation in cancer cells.

In addition, current results showed that ER- tumours and TNBC subtype have the highest levels of SLC7A11 mRNA and protein. In the line with that, other studies revealed that SLC7A11 expression and activity is higher in the TN cell line, MDA-MB-231 compared to MCF7 cells [152,416]. This can be assigned to the increased metabolic activity of TNBC which causes elevated ROS levels with subsequent upregulation of antioxidant mechanisms, such as SLC7A11.

The Kaplan-Meier plots showed that BC patients with high *SLC7A11* mRNA expression, but not protein, have shorter BCSS. However, this association was lost when the analysis was carried out among BC molecular subtypes. The inconsistent results of mRNA and protein can possibly be attributed to the destitution of SLC7A11 protein to the assistance of other chaperone proteins such as, SLC3A2 and CD44, for its stability and cell surface localisation [221,390]. In this study, a positive correlation was observed between *CD44*, *SLC3A2* and *SLC7A11*. Additionally, the association between the last two was also confirmed at the protein level.

A positive association was also observed with the oncogene MYC which is known to directly induce SLC7A11 expression [381]. Interestingly, MYC also regulates the expression of SLC1A5, SLC7A5, SLC3A2 and GLS [381,193,111]. Most likely, SLC1A5 provides glutamine which is required to operate the SLC7A5-SLC3A2 heterodimeric complex, GLS catalyses glutamine to provide glutamate, the substrate needed for SLC7A11-SLC3A2 function. Furthermore, the high SLC7A11 protein expression was associated with the higher levels of the all aforementioned

proteins, which confirms the depth of the relationship between these proteins to perform their function.

SLC7A11 protein was also significantly associated with the glutamine transporter, SLC38A2. The latter is a key transporter under nutrient deprived conditions, which could be the provider of glutamine-derived glutamate in such situations. In addition, this study did not show correlations with other glutamate transporters, indicating that SLC7A11 depends on intra-cellular glutamine as a source of glutamate rather than the extracellular supply.

P53 is a well-known tumour suppressor gene that responds to various stress signals through modulating other cellular processes, including cell cycle arrest and apoptosis. Jiang *et al* revealed that p53 also regulates a non-apoptotic form of cell death, named ‘‘ferroptosis’’ through suppressing the expression of SLC7A11 with subsequent inhibition of cystine uptake [386]. In the present study, although the association with *TP53* status was not significant, mutations of this gene was highly prevalent in breast tumours where there was high *SLC7A11* mRNA expression. In addition, SLC7A11 protein was highly expressed in breast cancers which expressed high mutant p53 protein.

Studies on various cancer cell lines showed that targeting SLC7A11 by gene knockdown or using the pharmacological inhibitor, sulfasalazine, significantly inhibited cell growth and proliferation through inducing cell cycle arrest [379,380,417-419]. In addition, blocking the action of SLC7A11 completely inhibited cell growth and proliferation in TNBC cell line but not the ER+ cells [152,416], indicating that SLC7A11 could be used as a target of cancer therapy, particularly in TNBC.

7.6.3 SLC7A8

Unlike SLC7A5, SLC7A8 lacks studies that illustrate its prognostic role in human cancer. Data from Oncomine revealed a significant upregulation of SLC7A8 in several cancers, including breast, colorectal, head and neck, leukaemia, lymphoma, and melanoma [257]. However, this has only been validated at the mRNA level in a subset of breast tumours [144] and melanoma cell lines, which showed however more than five times increase in SLC7A5 expression compared to SLC7A8 [199]. In the current study, large BC cohorts were used to reveal the significant associations between the high SLC7A8 expression, at mRNA and protein

levels, and good prognostic clinicopathological parameters, including small tumour size, low tumour grade and good NPI.

With respect to BC subtypes, the highest levels of SLC7A8 mRNA were observed in the ER+ tumours, particularly luminal A subtype, while it was lower in the TNBC. These results were compatible with other studies which showed that SLC7A8 mRNA was expressed in the ER+, MCF7, cell line but not in the ER-, MDA-MB-231 cells [296]. Furthermore, this study showed that SLC7A8 was associated with better patient outcome and longer DMFS in the ER+ low proliferative tumours only. Thakkar *et al* also found that upregulation of SLC7A8 alongside GATA3 and MLPH significantly associated with longer relapse free survival in ER+ lymph node positive breast tumours [144]. These results may suggest that ER+ low proliferative tumours settle for the lower affinity transporter, SLC7A8, to satisfy their nutritional needs as they exhibit lower metabolic activity compared to the aggressive forms of BC.

The relationship between hormone receptor positivity and SLC7A8 indicates that hormone receptors have a possible role in stimulating SLC7A8 expression. It has been shown that 17β oestradiol, in ER+ BC cells, regulates leucine uptake through SLC7A5 and SLC7A8 while no effect was observed in ER- BC cells [399]. It has been further reported that SLC7A8 has oestrogen-dependent expression, in ER+ BC cells, and the existence of inhibitors of oestrogen signalling pathway (ICI182780 and tamoxifen) eliminates the oestrogen-induced upregulation of SLC7A8 [144]. Another study also revealed that progesterone significantly upregulates SLC7A8 mRNA and protein expressions, in uterine leiomyoma tissues, and knockdown of *SLC7A8* markedly increased leiomyoma cell proliferation [400].

It has been found that P53 plays a role in controlling metabolic pathways such as regulating glutamine metabolism through inducing GLS2 expression [420]. Interestingly, this study showed a positive association between wild-type *TP53* and *SLC7A8*. This suggests that SLC7A8 may contribute to P53-dependent tumour suppression, which resulted in the presence of favourable prognosis and patient outcome in tumours expressing high SLC7A8. In addition, *SLC7A8* was upregulated alongside two tumour suppressor genes, *CEACAM1* and *BMP2*, in human colon cancer cells after exposure to anti-tumour agent [421].

It has previously been shown that SLC7A5 is highly expressed in the aggressive BC subtypes and it was predictive of poor prognosis and poor patient outcome [273] while analysing SLC7A8 in the same cohort has resulted in opposite findings. Furthermore, this study showed that expression of *SLC7A8* mRNA was mutually exclusive with *SLC7A5*. In addition, a trend toward a negative association was observed between the two biomarkers at the protein level. It is noteworthy that although SLC7A5 and SLC7A8 have similar substrate selectivity and function, the latter has narrower tissue expression pattern and exhibits lower affinity to its substrates [142]. There is also evidence which suggests that the role of SLC7A8 is limited to equilibration of amino acids distribution across the cell membrane while SLC7A5 mediates the actual net of amino acid flux [422], which is required for further mTORC1 activation and cellular proliferation.

Furthermore, it seems that both solute carriers have contrasting effect in tumourigenesis and they could be a subject of different regulatory mechanisms, as SLC7A5 expression is induced by MYC while hormone receptors appear to control SLC7A8 expression [399,400,193].

It is noteworthy that different transcriptional pathways such as MYC oncogenic transcription, hormone receptors and nutrient starvation responses regulate the expression of amino acid transporters in human cancers [251,257]. This also applies to SLC7A5 and SLC7A8, as both belong to the system L transport family but their expression is controlled by different pathways. Furthermore, both solute carriers mediate the uptake of large neutral amino acids, with the latter showing decreased transporting capacity. However, SLC7A8 can also accept smaller neutral amino acids, such as glycine, alanine, serine, cysteine, and glutamine [423], which are known substrates of the key system A amino acid transporter, SLC1A5 that is highly expressed in TNBC [142,135]. These statements indicate that SLC7A8 could be a key transporter in luminal A tumours through determining the actual net flux of not only the large but also the small neutral amino acids.

This study further investigated the association of SLC7A8 expression with other solute carriers which involved in amino acid transport. While the majority of these transporters were negatively associated with SLC7A8, others were positively correlated, including SLC38A7 which showed consistent correlation in all BC subtypes. Positive associations with several glutamine metabolic enzymes were

also detected, among these, GLS2 and GLUD1, which are associated with tumours of good prognosis and favourable patient outcome [168,424]. Some variability in the expression of investigated markers across molecular subtypes was observed. For example, luminal A tumours were the main class which showed associations between SLC7A8 and markers required for glutamine transport and metabolism. This could be attributed to the increased SLC7A8 expression and function in this particular BC subtype.

In this chapter, all solute carriers included can be potential prognostic factors in BC. While SLC38A2 and SLC7A11 are linked to poor prognosis, SLC7A8 appears to have tumour suppressive characteristics. Despite some discrepancies between the findings of mRNA and protein, SLC38A2 and SLC7A11 showing features of being poor prognostic indicators, including the associations with high tumour grade and ER negative status. In addition, SLC38A2 was independent predictor for shorter BCSS, which was limited to TN tumours when different BC subtypes were considered. However, SLC7A8 was an indicator for good prognosis. More specifically, high expression of SLC7A8, mRNA and protein, was associated with good prognostic parameters, ER positive status and better patient outcome. In addition, ER+ low proliferative/luminal A tumours was the only subtype which showed significant associations with longer BCSS and DMFS. Further functional studies to decipher the specific role of these solute carriers in different BC subtypes are warranted.

Chapter 8. Enhanced amino acid uptake influences composition of immune cell infiltrates in breast cancer

8.1 Introduction

Altered metabolic pathways are readily accepted as part of the revised hallmarks of cancer where cancer cells adapt their metabolism in order to resist the unfavourable, nutrient-deprived conditions and to respond to the increased energy demands required by their unremitting proliferation [10]. Many cancer cells are highly reliant on amino acids for their growth, not only because they are precursors for nucleotide and protein synthesis, but also because they activate mammalian target of rapamycin complex1 (mTORC1) through nutrient-signalling pathways, which in turn regulates protein translation and cell growth [280,252]

The role of the tumour microenvironment (TME) is well known with respect to disease development and progression. One of the important components of the TME are immune cells, including the regulatory T cells (Treg) and tumour-associated macrophages (TAM), which gain pro-tumoural functions stimulating tumour growth, progression, invasion and metastasis. Conversely, other immune cells such as CD8 + and CD20 + lymphocytes are responsible for anti-tumoural responses by activating host defence mechanisms preventing immune evasion [425,426] (Figure 8-1). Immune evasion is another emerging hallmark of cancer which is a strategy used by tumours to evade a host's immune response in an attempt to maximise their ability to continue surviving and growing [10]. Tumour immune evasion includes several mechanisms such as progressive formation of an immune-suppressive environment within the tumour and the selection of tumour variants resistant to immune effectors (immunoediting) [426].

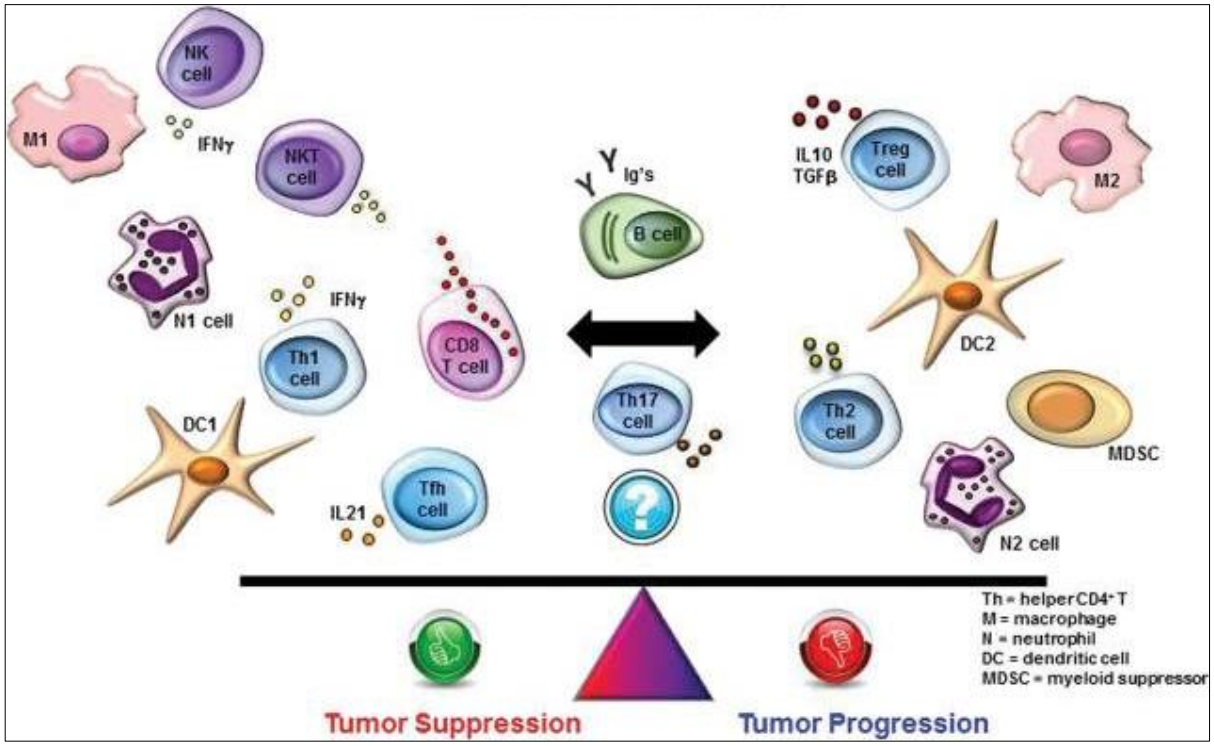


Figure 8-1. Tumour infiltrating lymphocytes play a crucial role in shaping tumour microenvironment via having either an immune mediate anti-tumoural activity, such as CD8 and Th1cells, or pro-tumoural activity, such as M2 macrophages and regulatory (Treg) cells. (Adapted from Salgado et al 2015).

Composition of the inflammatory cell infiltrates in BC also correlates with clinical outcome, where an abundant infiltration of pro-tumorigenic cells is associated with poor outcome, while TME enriched in cells with anti-tumorigenic functions has a favourable effect on patient survival [427-429]. The immune checkpoint, Programmed cell death 1 (PD1), and it's activating ligand, programmed death ligand 1 (PDL1) act in attenuating the anticancer immune response and promoting T-reg cell development and function. PD1 is expressed at the cell surface of activated immune cells while PDL1 is expressed by tumour cells of several cancer types, with evidence of an association with aggressive tumour behaviour and poor prognosis [430-434]. Indeed, monoclonal antibodies have been developed to target PD1/PDL1 pathway for cancer immunotherapy and the use of these molecules in clinical trials have shown impressive response rates in patients with various cancer types [435].

Previous studies show that the cellular contents of the TME change in parallel with tumour growth/progression. For example, the accompanied alterations in glucose

metabolism in cancer cells are tightly linked to the composition of the surrounding immune cells [436,437].

It has been found that the composition of immune infiltrates in tumours can be shaped by the glucose availability in the TME, where tumour cells can deplete glucose from the TME cells including the anti-cancer immune cells and thus block their effector function [438]. In ovarian cancer, glucose deprivation in CD8+ T cells can cause endoplasmic reticulum stress and activate unfolded protein response via the IRE1α–XBP1 pathway, which limits mitochondrial respiration and cytokine production [439].

In addition, lactate production downstream of tumour cell glycolysis diminishes the function of anti-tumoural T-cells [440,441] through supressing FAK family-interacting protein of 200 kDa (FIP200) with subsequent apoptosis [442] . However, the function of Treg cells remains intact, as they can resist low glucose, high lactate-rich environment through inhibiting Myc-driven glycolysis and activating oxidative phosphorylation [440].

On the other hand, restricting glycolysis in cancer tissues may directly allow greater immune cell activation owing to greater glucose availability for effector cells that rely on this substrate. It has been reported that restricting glycolysis in two mouse models of TNBC inhibited the production of granulocyte–macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) by tumour cells, thereby diminishing recruitment of the pro-tumoural cells resulted in reduced tumour growth, metastasis, and prolonged survival in these models [443].

Amino acids, such as glutamine and leucine, are other metabolites that play a crucial role for immune cells' proliferation and function, as they are essential for protein and nucleotide synthesis in addition to their role in supporting other biosynthetic and bioenergetic pathways [444,445]. It has been found that naïve T cell differentiation and activation depends on rapid glutamine influx through SLC1A5 [446]. In addition, SLC7A5-mediated leucine uptake contributes to pro-inflammatory cytokine production in activated human macrophages and targeting SLC7A5 significantly inhibits cell proliferation and activation of T-cells [447,222]. SLC3A2 is also involved in lymphocyte maturation and immune cell regulation, not only because it assists SLC7A5 function but it also supports lymphocyte clonal

expansion by amplifying integrin signals that enable proliferation and prevent apoptosis [304].

In Chapter 3, the potential utility of SLC1A5, SLC7A5 and SLC3A2 as prognostic factors in certain BC subtypes was described. Further, in Chapter 4, BC patients were stratified into three accredited clusters based on the protein expression of these three solute carriers. However, their impact on immune cells structure in the BC TME is still obscure.

8.2 Hypothesis

Reprogramming of amino acid metabolism, which is represented by increase glutamine and leucine uptake, in BC can determine the existence of specific subtypes of immune cells, in addition to their supportive role in estimating the clinical outcome.

8.3 Aims

1. To evaluate the association between the expression of SLCs and various immune cell markers, which include (pan T-cell CD3, cytotoxic T-cell CD8, B-cell CD20, Histiocytic cell marker CD68 and T-reg FOXP3 in addition to the immune checkpoint marker PD1 and its ligand PDL1, in the different molecular BC subtypes.
2. To assess the prognostic potential of co-occurrence of SLCs with different immune cell infiltrates in BC.
3. To investigate the impact of targeting *SLC1A5* and or *SLC7A5* on PDL1 expression *in vitro*, using a TNBC cell line.

8.4 Methods

8.4.1 Study cohort

Nottingham BC cohort was used in this study. This cohort is a well-characterised series of early-stage, primary operable, invasive BC patients. Clinical history, tumour characteristics and outcomes are prospectively maintained and regularly updated. Outcome data included development and time to distant metastasis and BC-specific death. The clinicopathological characteristics for this cohort are detailed in section 2.1.3.

8.4.2 Immunohistochemistry

Protein expression of SLC1A5, SLC7A5 and SLC3A2 was assessed using IHC and TMAs extracted from the Nottingham BC cohort, as described in Chapters 2 and 3. Clustering analysis of SLC1A5, SLC7A5 and SLC3A2 protein expression was previously performed as described in Chapter 4 and [321]. The three clusters were characterised as follows: low SLCs (SLC1A5–/SLC7A5–/SLC3A2–), high SLC1A5 (SLC1A5+/SLC7A5–/SLC3A2–) and high SLCs (SLC1A5+/SLC7A5+/SLC3A2+).

The IHC staining and assessment of the immune cells markers, PD1, PDL1, CD3, CD8, CD68, CD20 and FOXP3 was previously performed [427-429]. The total number of each immune cell type was counted in each tumour core by using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). PD1 and PDL1 were evaluated based on a semi quantitative assessment by using H-score as detailed in section 2.4.4.

8.4.3 siRNA transfection of *SLC1A5* and *SLC7A5*

The impact of *SLC1A5* and/or *SLC7A5* knockdown on PDL1 expression was investigated in MDA-MB-231 cells, which were transfected, using reverse transfection method, with siRNA designed to *SLC1A5* or *SLC7A5*. The expression of PDL1 was detected in the transfected and negative control cells, using western blotting. Conditions of maintaining cell growth and the detailed material and methods about the knockdown experiments are described in section 2.5.

8.5 Results

8.5.1 Expression of SLC1A5, SLC7A5, SLC3A2 and immune cell markers in BC

Expression of the three solute carriers was predominantly in the membrane of the invasive BC cells, with intensity levels varying from absent to high, as described in Chapter 3. Interestingly, high expression of the solute carriers was also observed in lymphocytic infiltrates, which were in the stroma adjacent to the tumour cells (Figure 8-2A-C). Immunohistochemical expression of immune cell markers in invasive BC cores is illustrated in (Figure 8-3A-).

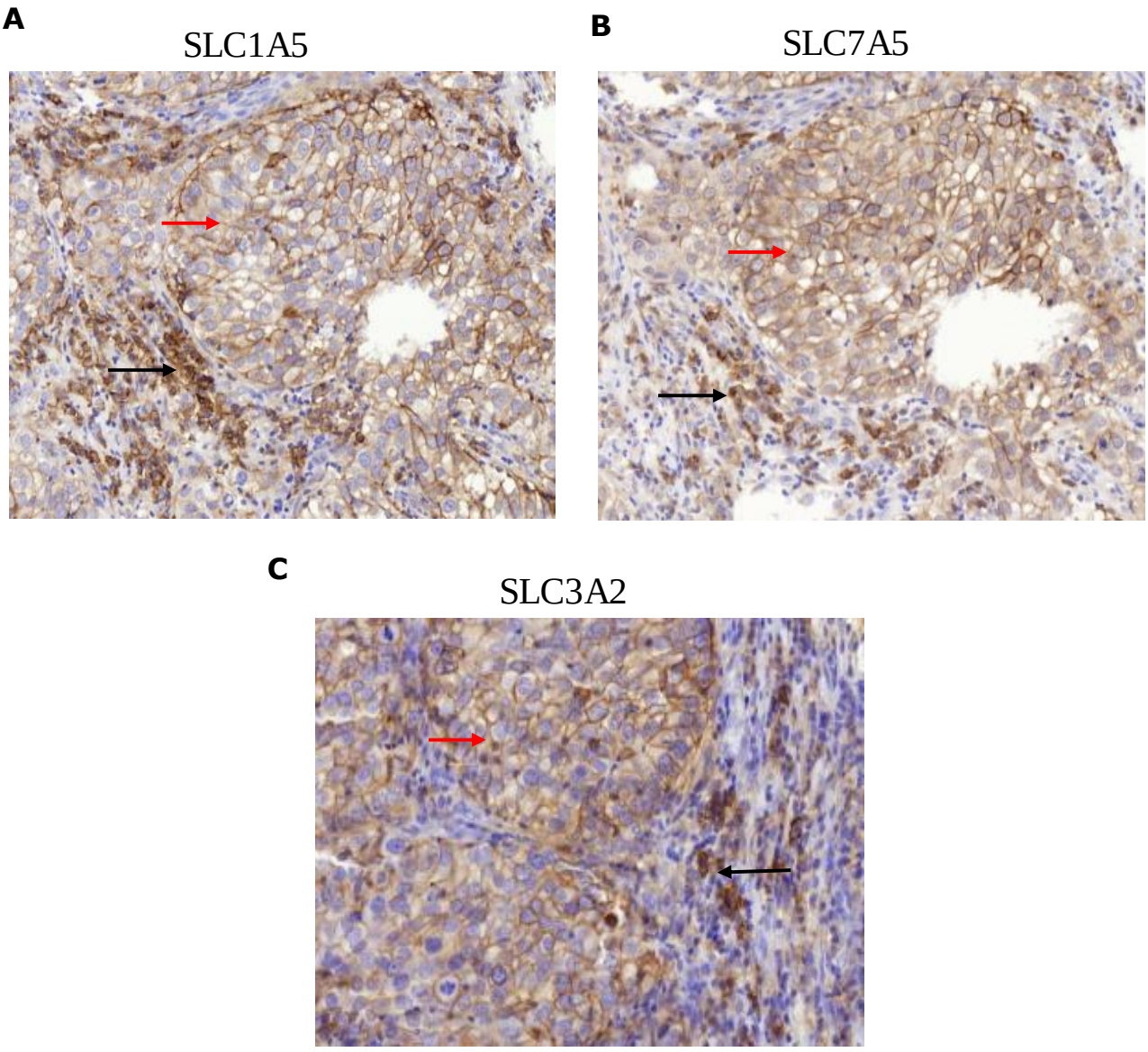


Figure 8-2. The expression of solute carriers of A) SLC1A5, B) SLC7A5 and C) SLC3A2 was predominant in the cell membrane of the breast cancer cells (red arrows) and the adjacent immune cell infiltrates (black arrows).

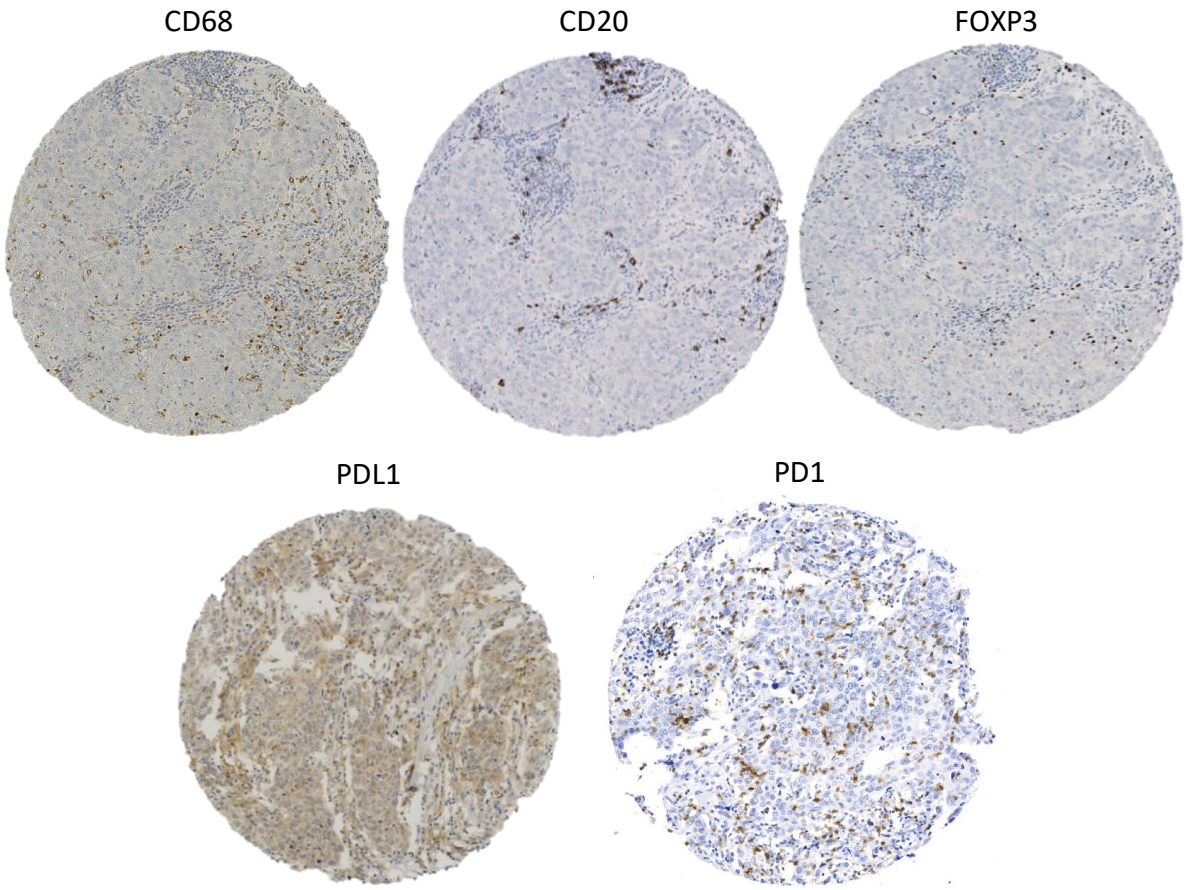


Figure 8-3. Immunohistochemical expression of CD68, CD20, FOXP3 and PD1 in lymphocytic infiltrates of the invasive BC cores. Immunohistochemical expression of PDL1 in invasive BC tumour cells.

8.5.2 Association of SLCs with immune cell infiltrates

Tumour-infiltrating FOXP3 + lymphocytes and CD68 + macrophages were both predominant in tumours with high SLCs and to lesser extent in those tumours with high SLC1A5 expression ($p<0.001$, Table 8-1). CD20 + lymphocytes were mainly observed in tumours with high SLCs expression ($p<0.001$, Table 8-1). While PDL1 was highly expressed in tumours with high SLCs and high SLC1A5 expression, PD1 + cells were mainly expressed with tumours with high SLCs expression (all $p<0.001$, Table 8-1). In contrast, there was no significant association between the SLC clusters and CD3 + or CD8 + T lymphocytes ($p=0.84$ and $p=0.24$, Table1-8), respectively.

Table 8-1. Association of SLCs with different subtypes of immune cell markers

Immune cell marker	Low SLCs n (%)	High SLC1A5 n (%)	High SLCs n (%)	χ2 (p-value)	Adjusted p-value
CD3					
Negative	36 (56.3)	21 (32.8)	7 (10.9)	1.7 (0.42)	0.84
Positive	268 (54.1)	142 (28.7)	85 (17.2)		
CD8					
Negative	71 (57.7)	35 (28.5)	17 (13.8)	4.9 (0.08)	0.24
Positive	236 (48.9)	140 (29.0)	107 (22.2)		
FOXP3					
Negative	148 (63.8)	67 (28.9)	17 (7.3)	37.3 ($7.9x10^{-9}$)	**<0.0001**
Positive	188 (44.9)	122 (29.1)	109 (26.0)		
CD68					
Negative	139 (67.1)	47 (22.7)	21 (10.1)	35.0 ($2.5x10^{-8}$)	**<0.0001**
Positive	165 (42.7)	124 (32.1)	97 (25.1)		
CD20					
Negative	255 (55.4)	134 (29.1)	71 (15.4)	17.1 (0.0001)	**0.0004**
Positive	81 (44.5)	47 (25.8)	54 (29.7)		
PD1					
Negative	203 (52.1)	111 (28.5)	76 (19.5)	42.5 ($6.0x10^{-10}$)	**<0.0001**
Positive	134 (36.1)	85 (22.9)	152 (41.0)		
PDL1					
Negative	81 (67.5)	28 (23.3)	11 (9.2)	16.8 (0.0002)	**0.0004**
Positive	287 (48.2)	178 (29.9)	130 (21.8)		

P values in bold means statistically significant

8.5.3 Association between SLC clusters and immune cell infiltrates varied among BC molecular subtypes

CD68 + cells were significantly associated with high SLCs expression in both ER + high proliferative and TN subtypes (p=0.02 and p=0.03, Table 8-2, 8-3), respectively. In contrast, FOXP3 + cells were only associated within TN tumours showing high SLCs expression (p=0.02, Table 8-3). CD20 + cells were only observed in ER + high proliferative tumours with high SLCs (p=0.04, Table 8-2). PD1 and PDL1 were also significantly expressed in HER2 + tumours with high SLCs expression (p=0.03 and p=0.04, Table 8-3), respectively. However, within TN tumours, only PDL1 expression was associated with high SLCs expression (p=0.04, Table 8-3). There were no significant association between the SLC clusters and immune cell infiltrates in ER + low proliferative tumours (Table 8-2). In addition, no significant association was observed between the SLC clusters and CD3 + or CD8 + T lymphocytes in any of the BC subtypes (all p>0.05, Table 8-2, 8-3).

Table 8-2. Association of SLCs with immune cell markers in ER+ low proliferative/luminal A and ER+ high proliferative/luminal B breast cancer subtypes

	ER+ low proliferative/luminal A					ER+ high proliferative/luminal B				
Immune cell marker	**Low SLCs n (%)**	**High SLC1A5 n (%)**	**High SLCs n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low SLCs n (%)**	**High SLC1A5 n (%)**	**High SLCs n (%)**	**χ2 (p-value)**	**Adjusted p-value**
CD3										
Negative	10 (66.7)	4 (26.7)	1 (6.7)	1.41	3.92	10 (41.7)	12 (50.0)	2 (8.3)	1.31	4.08
Positive	95 (79.8)	20 (16.8)	4 (3.4)	(0.49)		93 (52.5)	67 (37.9)	17 (9.6)	(0.51)	
CD8										
Negative	22 (75.9)	5 (17.2)	2 (6.9)	0.22	6.23	20 (47.6)	16 (38.1)	6 (14.3)	0.56	5.25
Positive	82 (78.8)	17 (16.3)	5 (4.8)	(0.89)		84 (50.6)	65 (39.2)	17 (10.2)	(0.75)	
FOXP3										
Negative	61 (80.3)	12 (15.8)	3 (3.9)	0.94	3.72	47 (55.3)	34 (40.0)	4 (4.7)	3.76	0.90
Positive	50 (73.5)	14 (20.6)	4 (5.9)	(0.62)		69 (51.1)	49 (36.3)	17 (12.6)	(0.15)	
CD68										
Negative	55 (85.9)	7 (10.9)	2 (3.1)	4.75	0.45	50 (66.7)	21 (28.0)	4 (5.3)	10.6	**0.02**
Positive	47 (70.1)	15 (22.4)	5 (7.5)	(0.09)		56 (43.8)	54 (42.2)	18 (14.1)	(0.005)	
CD20										
Negative	92 (79.3)	19 (16.4)	5 (4.3)	0.56	3.00	83 (50.3)	69 (41.8)	13 (7.9)	8.03	**0.04**
Positive	20 (74.1)	5 (18.5)	2 (7.4)	(0.75)		32 (60.4)	12 (22.6)	9 (17.0)	(0.01)	
PD1										
Negative	85 (79.4)	19 (17.8)	3 (2.8)	3.01	0.66	64 (50.8)	53 (42.1)	9 (7.1)	6.02	
Positive	42 (71.2)	12 (20.3)	5 (8.5)	(0.22)		54 (50.5)	35 (32.7)	18 (16.8)	(0.04)	0.12
PDL1										
Negative	24 (82.8)	4 (13.8)	1 (3.4)	0.60	1.46	19 (70.4)	6 (22.2)	2 (7.4)	3.64	0.30
Positive	99 (76.2)	24 (18.5)	7 (5.4)	(0.73)		109 (0.9)	80 (37.4)	25 (11.7)	(0.15)	

P values in bold means statistically significant

Table 8-3. Association of SLCs with immune cell markers in HER2+ and Triple Negative breast cancer subtypes

	HER2+					Triple Negative				
Immune cell marker	**Low SLCs n (%)**	**High SLC1A5 n (%)**	**High SLCs n (%)**	**χ2 (p-value)**	**Adjusted p-value**	**Low SLCs n (%)**	**High SLC1A5 n (%)**	**High SLCs n (%)**	**χ2 (p-value)**	**Adjusted p-value**
CD3										
Negative	3 (75.0)	1 (25.0)	0 (0.0)	3.02	1.76	3 (30.0)	4 (40.0)	3 (30.0)	4.68	0.72
Positive	22 (33.8)	26 (40.0)	17 (26.2)	(0.22)		16 (25.4)	9 (14.3)	38 (60.3)	(0.09)	
CD8										
Negative	5 (62.5)	3 (37.5)	0 (0.0)	4.37	0.77	6 (30.0)	7 (35.0)	7 (35.0)	7.72	0.14
Positive	23 (31.1)	30 (40.5)	21 (28.4)	(0.11)		18 (21.2)	11 (12.9)	56 (65.9)	(0.02)	
FOXP3										
Negative	6 (46.2)	6 (46.2)	1 (7.7)	2.47	1.74	9 (42.9)	6 (28.6)	6 (28.6)	9.86	**0.02**
Positive	22 (30.6)	31 (43.1)	19 (26.4)	(0.29)		18 (19.8)	13 (14.3)	60 (65.9)	(0.007)	
CD68										
Negative	4 (26.7)	4 (26.7)	7 (46.7)	5.18	0.35	3 (20.0)	7 (46.7)	5 (33.3)	10.1	**0.03**
Positive	24 (35.3)	31 (45.6)	13 (19.1)	(0.07)		18 (21.4)	11 (13.1)	55 (65.5)	(0.007)	
CD20										
Negative	20 (42.6)	17 (36.2)	10 (21.3)	3.05	0.84	13 (21.7)	13 (21.7)	34 (56.7)	1.55	1.84
Positive	9 (24.3)	18 (48.6)	10 (27.0)	(0.21)		12 (25.0)	6 (12.5)	30 (62.5)	(0.46)	
PD1										
Negative	14 (37.8)	20 (54.1)	3 (8.1)	8.57	**0.03**	11 (29.7)	7 (18.9)	19 (51.4)	1.35	1.50
Positive	16 (29.1)	20 (36.4)	19 (34.5)	(0.01)		19 (20.4)	18 (19.4)	56 (60.2)	(0.50)	
PDL1										
Negative	8 (47.1)	9 (52.9)	0 (0.0)	7.40	**0.04**	7 (50.0)	3 (21.4)	4 (28.6)	7.48	**0.04**
Positive	19 (26.0)	32 (43.8)	22 (30.1)	(0.02)		21 (19.4)	20 (18.5)	67 (62.0)	(0.02)	

P values in bold means statistically significant

8.5.4 The co-occurrence of SLCs and immune cell infiltrates correlates with patient outcome

Variable associations with patient outcome were observed when investigating the co-occurrence of the SLC clusters with the immune cell markers. The coexistence of high SLCs with FOXP3 + T lymphocytes was predictive of a shorter BCSS ($p<0.001$, Figure 8-4A). Similar associations were observed when CD68 + macrophages, PD1 + cells and PDL1 expression were considered ($p<0.001$, Figure 8-4B-D). However, patients with tumours showing both high SLCs and CD20 + lymphocytes, showed better BCSS ($p<0.001$, Figure 8-4E).

There was a comparable observation regarding the association of SLCs and immune cell markers with DMFS, where high SLCs expression accompanied by the presence of FOXP3 +, CD68 + and PD1 + cells or PDL1 expression showed significantly shorter DMFS (all $p<0.001$, Figure 8-5A-D). In contrast, the presence of CD20 + lymphocytes and high SLCs conferred a longer DMFS ($p=0.002$, Figure 8-5E).

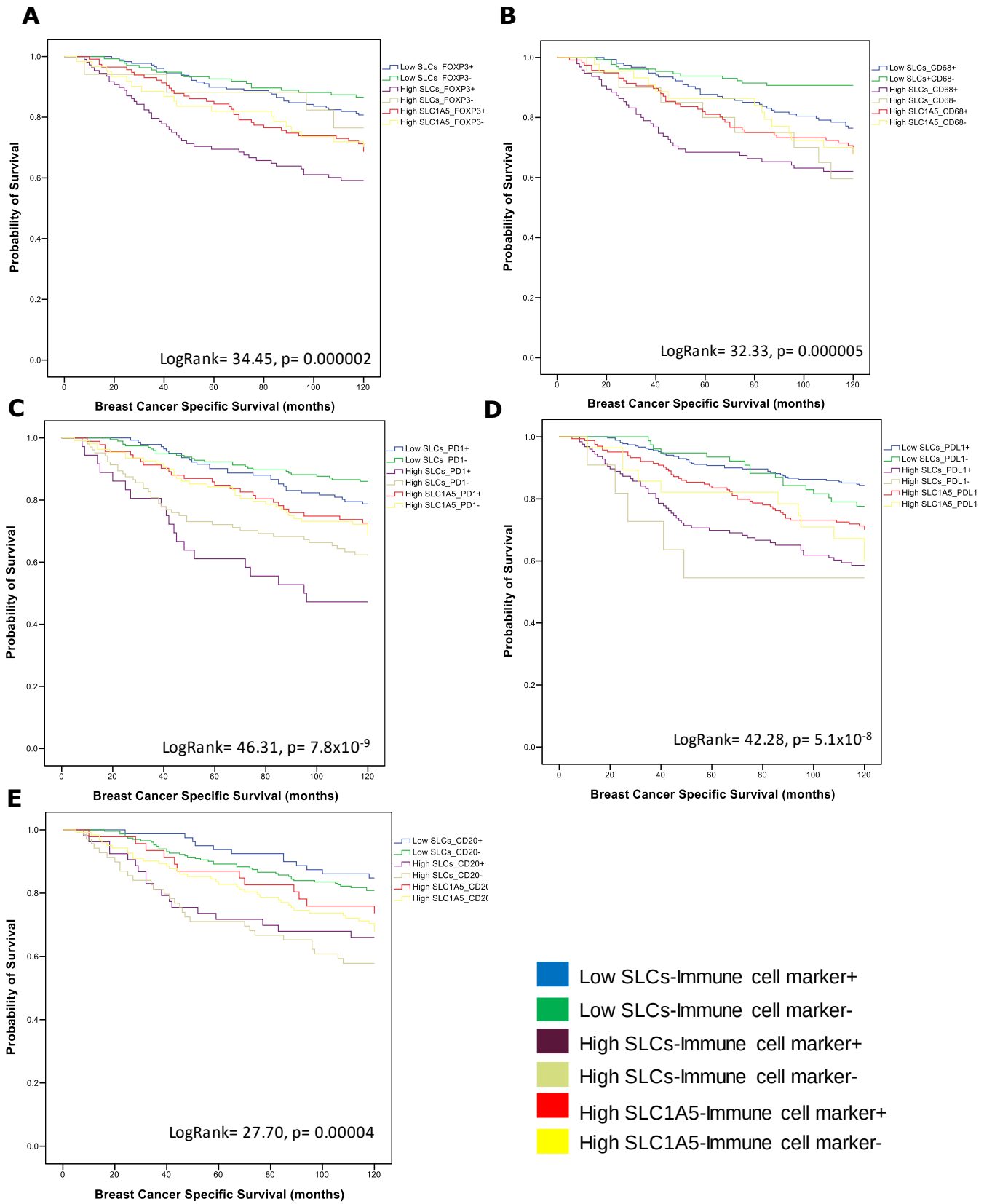


Figure 8-4. Breast cancer specific survival in SLCs and immune markers co-expression. A) SLCs and FOXP3. B) SLCs and CD68. C) SLCs and PD1. D) SLCs and PDL1. E) SLCs and CD20.

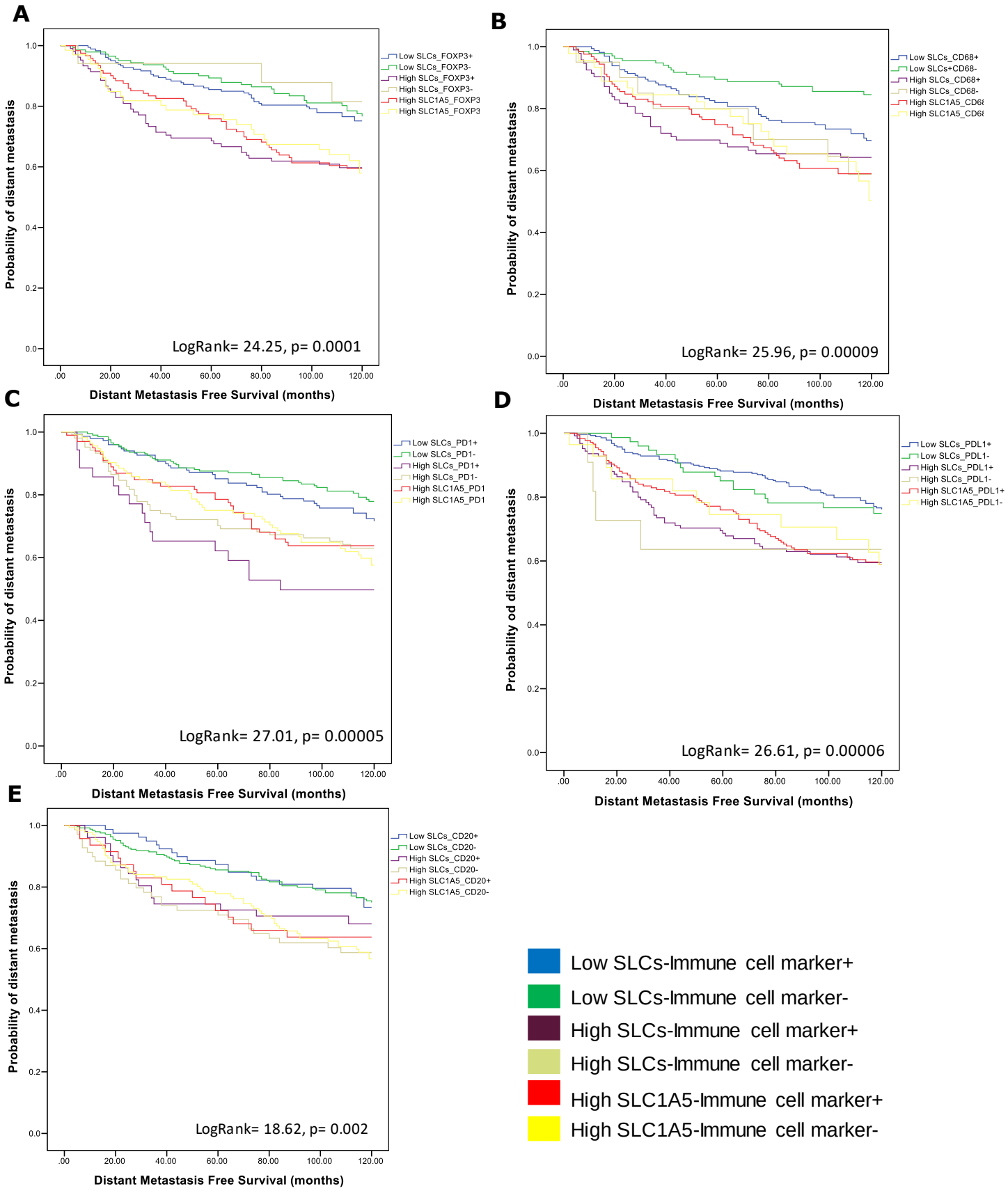


Figure 8-5. Distant metastasis Free Survival in SLCs and immune markers co-expression. A) SLCs-FOXP3. B) SLCs-CD68. C) SLCs-PD1. D) SLCs-PDL1. E) SLCs-CD20.

8.5.5 SLC7A5 plays a role in PDL1 expression in TNBC

The association between high SLCs and immune cell markers, including PDL1, was predominantly observed in TNBC subtype. Thus, the knockdown study was carried out using the TNBC cells, MDA-MB-231.

Knockdown efficiency of *SLC1A5* and *SLC7A5* in MDA-MB-231 transfected cells was determined by western blotting, at day 5 post transfection. Figure 8-6A, B showed an efficient knockdown of *SLC1A5* and *SLC7A5* in the transfected cells compared to the control cells.

siRNA knockdown of *SLC1A5* or *SLC7A5* in MDA-MB-231 reduced the protein expression of PDL1. However, this observation was only significant upon targeting *SLC7A5* ($p=0.01$, Figure 8-6E) but not *SLC1A5* ($p=0.13$, Figure 8-6D). Significant reduction of PDL1 protein expression was also observed in cells transfected with both *SLC1A5* and *SLC7A5* siRNAs ($p=0.02$, Figure 8-6F).

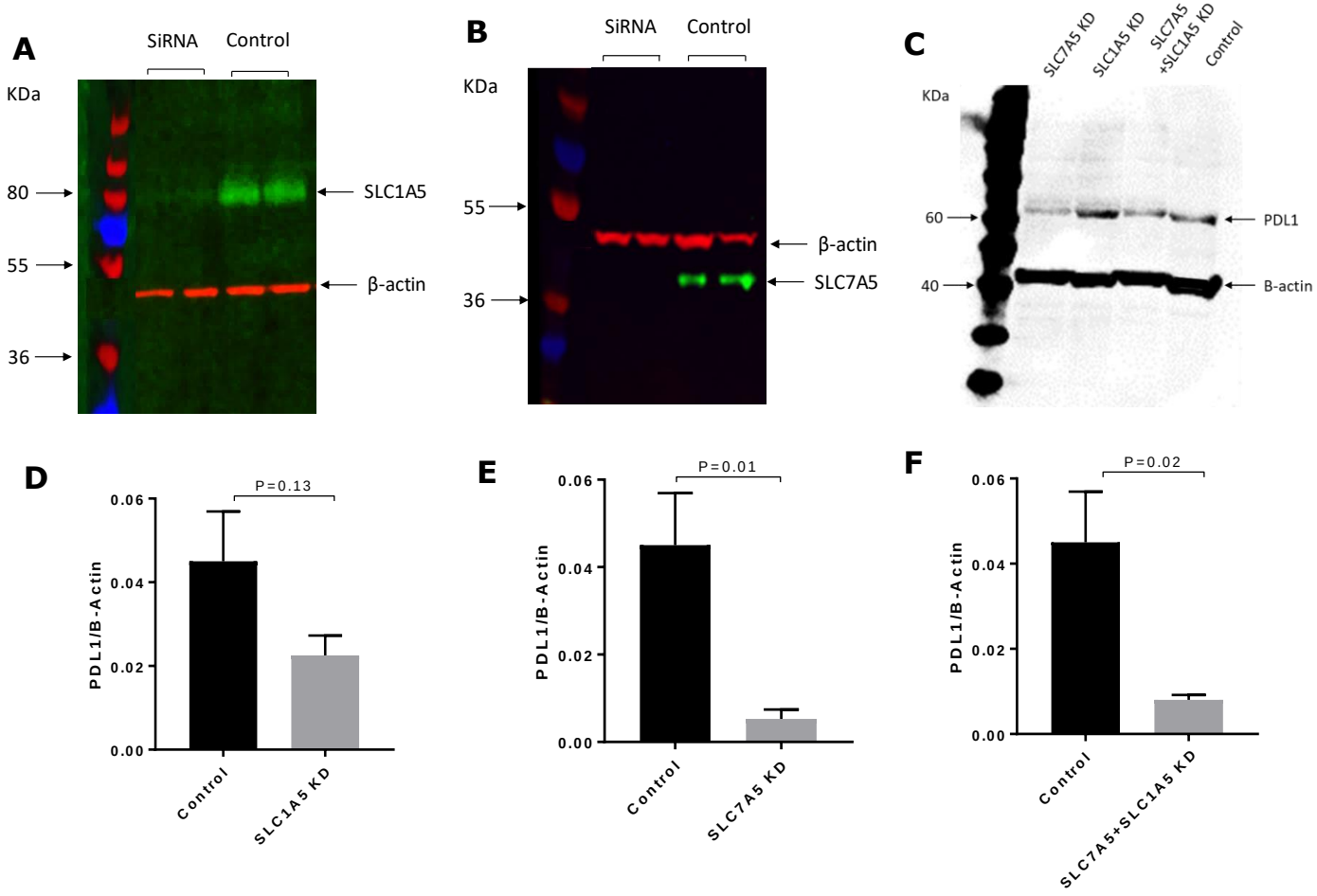


Figure 8-6. The effect of SLC1A5 and SLC7A5 knockdown on PDL1 expression. Knockdown of *SLC1A5* and *SLC7A5* in MDA-MB-231 cell line. A) *SLC1A5* expression in MDA-MB-231 transfected with *SLC1A5* siRNA and control cells. B) *SLC7A5* expression in MDA-MB-231 transfected with *SLC7A5* siRNA and control cells. C) Western blot analysis of PDL1 protein expression in MDA-MB-231 cells transfected with *SLC1A5* and/or *SLC7A5* siRNA. The bar graph summarises the expression levels of PDL1 protein, using β-actin as normalised control, upon D) *SLC1A5* siRNA transfection. E) *SLC7A5* siRNA transfection. F) *SLC1A5* and *SLC7A5* siRNA transfection. Data represent the mean and error bars of three independent experiments.

8.6 Discussion

Cancer cells undergo metabolic changes in order to satisfy the demands of necessary energy and cellular building blocks. One of the most prominent changes is the increase in the consumption of glutamine and other essential amino acids such as leucine. To achieve this, tumour cells upregulate the key amino acid transporters (SLC1A5 and the SLC7A5–SLC3A2 heterodimeric complex) at their cell surface. In Chapter 4, it was demonstrated that the combined expression of the three solute carriers (SLC1A5, SLC7A5 and SLC3A2) is associated with poor prognosis and short BCSS, particularly in the highly proliferative BC subtypes [321].

Besides metabolic reprogramming, immune evasion is also considered as an emerging hallmark of cancer [10]. The role of immune cells in tumour evasion is increasingly intricate, as many tumours not only escape recognition by the adaptive immune response but also sometimes cooperate with the pro-tumorigenic immune cells to become invasive and more aggressive. Furthermore, there is a link between the two mentioned hallmarks, as changes in the tumour cell metabolism can influence the component and function of the inflammatory infiltrates [436,448]. This study showed that altered amino acid metabolism, which was detected by the overexpression of the key solute carriers (SLC1A5, SLC7A5 and SLC3A2) was significantly associated with the existence of specific subtypes of immune cells, namely CD68 + macrophages, FOXP3 + regulatory T cells (Tregs), CD20 + B lymphocytes and PD1 + lymphocytes along with its tumour-expressing ligand (PDL1). However, no association was observed between the SLCs and CD3 + or CD8 + T lymphocytes.

A recent study on the same BC cohort has showed that the main component of the inflammatory infiltrates is the pan-T-lymphocyte population (CD3 + cells) along with CD8 + cells being more frequent than FOXP3 + cells. The CD68 + macrophages were more frequent while CD20 + B-lymphocytes were the least [449]. In this study, however, changes in the metabolic activity of the cancer cells, which is reflected by an increase in amino acid transport, attract specific components of immune cells, which were restricted to CD68 +, FOXP3 + and CD20 + along with PD1 + cells. This indicates that in these circumstances, the

antigen- presenting cells (APC), CD68 + cells are recognised only by specific subpopulations of T- and B- lymphocytes.

When different BC subtypes were examined, a significant association was observed in ER + highly proliferative/luminal B, TN and HER2 + tumours, but not the ER + low proliferative/luminal A subtype. Both luminal B and TN tumours showed associations with CD68 + macrophages. These two subtypes are aggressive, highly proliferative and exhibit high metabolic activity. Consequently, aggressive cancer cells secrete high levels of ROS in their microenvironment [119]. The latter can cause a state of pseudohypoxia in the adjacent stromal compartment with concomitant upregulation of HIF1α, which is known to induce the pro-tumorigenic CD68 + macrophages [450,451]. The same scenario can be applied when amino acids, particularly leucine, activate mTORC1 that upregulates HIF1α [452]. Previous studies have shown that PD1/PDL1 are mainly expressed in HER2 + and TN subtypes [434,453,454]. This study further shows that the expression of PD1 and/or PDL1 is mainly associated with high SLCs expression but restricted to HER2 + and TN tumours.

In this study, high expression of the solute carriers in the stromal lymphocytes was observed. This is expected as amino acid transporters are not only necessary for cancer cells, but they are also important for optimal lymphocyte proliferation and differentiation [446,455,279,456]. In addition, macrophages may require glutamine as it is the main precursor for arginine [457]. The latter can be catalysed by Arginase 1 to support cell proliferation and tissue remodelling [458].

Indeed, the upregulation of amino acid transporters in the cancer cells and their neighbouring immune cells might indicate that both cell types are substantially comparable in their requirements of amino acids, which can be obtained from the TME, to support their survival and proliferation. Furthermore, TME might be a source of stromal glutamine, as metabolic stress in TME triggers genomic instability, which subsequently acquires the non-malignant cancer-associated fibroblasts (CAF) a catabolic phenotype with enhanced macroautophagy. This catabolic state produces a nutrient-rich environment, with increased amounts of pyruvate, lactate, ketone bodies and glutamine [116]. This phenomenon also substantiates the cancer-stromal symbiosis that subsequently supports cancer growth and progression.

We and others revealed that high expression of glutamine solute carriers is associated with poor patient outcome [274,273,204]. Similarly, the presence of FOXP3 + and CD68 + cells also correlates with shorter survival [449]. However, CD20 + cells tend to be associated with better survival [449]. In this study, we showed that the co-occurrence of SLCs with FOXP3 + or CD68 + cells can predict shorter survival compared with the presence of one without the other, whereas the combination of the SLCs with CD20 + cells predicts better patient outcome.

The association between PD1/PDL1 and survival in BC is controversial [434,459-461]. This study, however, showed that the co-expression of SLCs with PD1 or PDL1 was associated with shorter DMFS and BCSS, indicating that patients with BC show an increase in their amino acid metabolic activity that might influence poor outcome in PD1/PDL1 + tumours.

This study showed that targeting SLC7A5 by transient siRNA significantly reduced the expression of PDL1 in TN cells. This can be attributed to the role played by this protein in activating the mTORC1 pathway, through importing essential amino acids, such as leucine. This might take place in parallel with the activation of mTORC1 through the AKT–mTOR signalling pathway, which is previously identified as a tight regulator of PDL1 expression in several cancers, including TNBC [453,462,463].

Although clinical trials with monoclonal antibodies targeting PD1/PDL1 interaction have shown promising results, with durable responses, in several human cancers, not all patients respond to this targeted therapy [464-466]. Therefore, it is critical to find effective approaches that could allow personalisation of treatment of PD1/PDL1 + tumours. This study not only provides clinical evidence that SLCs in BC could aid the personalisation of anti-PD1/PDL1 inhibition therapies, but it also emphasises that targeting the amino acid transporter, SLC7A5, along with the anti-PDL1 immunotherapy could be considered as a novel approach to synergistically enhance the therapeutic effect.

Altogether, this study revealed that there are associations between the two cancer hallmarks, metabolic reprogramming and immune evasion. More specifically, enhanced amino acid uptake in cancer cells can influence the existence of specific subtypes of inflammatory infiltrates, which act either with or against the aggressiveness and progression of the BC cells. The association with the pro-

tumorigenic immune cells was predominantly observed within TN tumours and was associated with poor patient outcome. Therefore, targeting both the pro-tumorigenic inflammatory cells and their nutrient suppliers can be a new approach that will counteract the highly proliferative and aggressive BC subtypes.

Chapter 9. General discussion and conclusion

9.1 General discussion

Breast cancer is the most common cancer in women and is the leading cause of cancer related death in females worldwide. Therefore, continuous development of disease prevention, diagnosis, prognosis and treatment options is of great importance to improve patient outcome and quality of life. However, this task is challenging, as BC is a heterogeneous disease with various subtypes differing in terms of morphology, molecular profiles, response to therapy and clinical behaviour [79]. In addition, different subtypes exhibit disparity in their metabolic pathways and their nutritional needs. The most common form of BC (~ 75%) is the ER+/luminal tumours [94,307], and those that belong to this class are also variable in terms of recurrence, mortality rates, metabolic requirements and disease prognosis [94]. Therefore, understanding the biology of BC and exploring the metabolic pathways could help to identify potential novel therapeutic targets to increase personalisation of care.

The most challenges facing management decision of BC patients is the determination of disease prognosis. Indicators that evaluate the risk of death, metastasis or relapse of the disease during treatment is still largely insufficient. Therefore, identification of new factors and combinations of biomarkers not only will improve prognostication of BC patients but also will indicate the potential value of additional treatment strategies and potential targets.

Indeed, classifying BC into biologically distinct clusters can confer more reliable and detailed prognostication for BC patients. Studies of gene expression profiling using clustered analysis have provided new insights into the classification of BC [80,81]. However, the use of these in routine clinical practice remains evasive or prohibitively expensive. Alternatively, Green *et al* have successfully utilised IHC on FFPE as a robust approach to classify BC, with results that are similar to the established genotypic biological classes of BC [467].

Cancer cells must alter their metabolism in order to satisfy the demands of necessary energy and cellular building blocks. It is widely known that amino acid transport systems play a principle role in sustaining the proliferation of cancer cells by supplying the required amino acids for protein synthesis and by activation of nutrient signalling through the mTORC1 pathway [252,280]. Amino acid metabolism can vary substantially among BC subtypes, where TN tumours display

increased activity of amino acid consumption and metabolism compared with ER+ tumours [137,138]. Furthermore, within the luminal tumours, the ER+ highly proliferative/luminal B subtype are more rapacious for nutrients, to sustain their excessive proliferation, compared to the less aggressive luminal A tumours.

Amino acid transporters are poorly studied as prognostic markers in BC especially with regard to BC molecular subtypes. Therefore, the hypothesis of this thesis stated that amino acid transporters can be reliable prognostic markers in BC and their prognostic implications will vary among the different molecular BC subtypes. To verify this hypothesis, the expression levels of the key amino acid transporters (SLC1A5, SLC7A5 and SLC3A2) were examined, at the mRNA and protein levels, against the clinicopathological and biological features of large cohorts of BC patients, alongside assessing their association with the patient outcome. In addition, the use of these biomarkers as prognostic indicators was investigated while in clusters. The consequences of targeting *SLC1A5* and *SLC7A5* in BC cell lines with emphasis on the luminal subtypes was further investigated.

Finally, the prognostic implications of the downstream signal, p70S6K, alongside other amino acid transporters (SLC38A2, SLC7A11 and SLC7A8), that are functionally coupled or share the same biological function with the key glutamine transporters, were also investigated and presented in this thesis.

9.2 Cardinal findings of the study

To my knowledge, this is the largest study which has revealed for the first time that SLC1A5, SLC7A5 and SLC3A2 are key solute carriers in the ER+ highly proliferative/luminal B BC subtype. Consistent with other studies that demonstrate the poor prognostic impact of these solute carriers in many human cancers [204,203,198,254,225,243], the results showed that the elevated expression of SLC1A5, SLC7A5 and SLC3A2, at both mRNA and protein levels, are associated with poor prognostic parameters and ER negative status. Within the luminal subtype, luminal B tumours showed highest proportions of these biomarkers compared to luminal A. In addition, SLC1A5, SLC7A5 and SLC3A2 proteins are associated with poor patient outcome.

Interestingly, when the survival analysis was carried out within the BC molecular subtypes, all three solute carriers share the character of being predictive of shorter

BCSS and DMFS only in the ER+ highly proliferative/luminal B subtype but not in the other subtypes. Which indicates the substantial need of these amino acid transporters for its survival, proliferation and invasiveness. However, on multivariate analysis, only SLC7A5 and SLC3A2 were independent prognostic factors for shorter BCSS.

Noteworthy, when the association with patient outcome was carried out at the level of SLCs clusters, it seems that the ER+ highly proliferative BC was not reliant on SLC1A5 alone, instead it required the concurrent expression of SLC7A5-SLC3A2 heterodimeric complex, the functional combination which not only will provides glutamine, but also adjusts the essential amino acid cytoplasmic pool for subsequent activation of mTORC1 signalling pathway. This statement might explain the inability of SLC1A5 as an independent prognostic factor for BCSS.

To confirm the above findings, functional analysis was carried out in Chapter 5 and showed that silencing *SLC7A5*, but not *SLC1A5*, significantly reduced cell proliferation and invasion of the luminal B-like BC cell line but not the luminal A-like cells.

The functional collaboration between amino acid transporters may exceed those described above. Pinilla *et al* demonstrated the potential coupling of SLC7A5 with SLC38A2. This is anticipated, as the latter is one of the major routes of glutamine entry especially, under nutrient deprived conditions [375]. SLC7A11 is another candidate that requires the expression of transporters that mediate glutamine entry, such as SLC1A5 and SLC38A2, to ultimately provide the antioxidant molecule, glutathione. Indeed, many studies have demonstrated the upregulation of SLC7A11 and SLC38A2 in various cancers [146,289,378,379]. In this study, we not only showed that all these transporters are overexpressed in BC, but we also found that they were significantly expressed with each other, confirming the state of symbiosis which takes place between these transporters in BC.

Furthermore, this study revealed the prognostic significance of SLC38A2 and SLC7A11 in BC. Despite some discrepancies between the findings of mRNA and protein, both transporters showing features of being poor prognostic indicators, including the associations with high tumour grade and ER negative status. In addition, SLC38A2 was independent predictor for shorter BCSS, which was limited to TN tumours when different BC subtypes were considered. However, SLC7A11

was not associated with patient outcome and this was ascribed to the necessity of this transporter to other proteins that reserve its optimal stability and functional localisation in the cell surface [221,390].

SLC7A8 is another amino acid transporter that is functionally related to the key transporter, SLC7A5, as they both share the same substrates. However, among all transporters included in this study, only SLC7A8 was an indicator for good prognosis. More specifically, high expression of SLC7A8, mRNA and protein, was associated with good prognostic parameters, ER positive status and better patient outcome. In addition, ER+ low proliferative/luminal A tumours was the only subtype which showed significant associations with longer BCSS and DMFS. The above findings along with the positive relationship that was observed between wild type *TP53* and *SLC7A8* give a suggestion that the latter could have tumour suppressive characteristics in BC. Further functional assessment is warranted to reveal the specific role of SLC7A8 in the ER+ low proliferative tumours.

Remarkably, some level of heterogeneity was observed among all these solute carriers, while some of them showed an impact on the ER+ high proliferative tumours others affect the survival of ER+ low proliferative or TN subtypes. Furthermore, while the majority are poor prognostic factors, SLC7A8 was an opposite indicator. Additionally, some transporters may function in normal situations whereas others were the key transporters in stressful situations, such as nutrient deprived conditions and oxidative stress. Despite these differences, this study has identified promising prognostic biomarkers of clinicopathological importance and strong associations with BC patient outcome.

This study further highlights the associations with regulatory effectors. The most prominent, is the oncogene, MYC, which is known to stimulate the expression of SLC1A5, SLC7A5, SLC3A2 and SLC7A11 [381,111]. Here, the associations between these proteins was confirmed, the observation which further confirms the extensive collaboration of these solute carriers at the functional level.

Associations with glutamine metabolic enzymes was also assessed and showed the extent of harmony between these enzymes and the studied amino acid transporters. In particular, it was shown that glutamine import may have several fates; with potential conversion to glutamate, α-KG, proline or targeted by antiporters to assist the influx of other essential amino acids, such as leucine. All

these destinies will ultimately support cellular bioenergetics and biosynthetic pathways in addition to protecting cells from the damage caused by ROS.

TME plays essential roles in promoting malignant cell growth, proliferation and metastasis. More importantly, metabolic coupling established by stromal cells and tumour cells is an example of the symbiotic relationship between the two compartments. This process is well described in several tumour types such as breast, prostate and colon cancers [468-470]. Immune cells are one of the important components of the TME that have diverse roles in cancer progression. Previous studies describe a potential link between cancer and stromal immune cells, where altered metabolic behaviour in cancer cells may influence the composition of the surrounding immune cells [436,437]. In this study, for the first time, evidence has been provided which indicates that enhanced amino acid uptake in BC can attract specific subtypes of immune cell infiltrates; namely CD20+ B-cells, CD68 + macrophages, FOXP3+ and PD1 + lymphocytes. It was further found that the association with the pro-tumorigenic immune cells was predominantly observed within TN tumours and targeting *SLC7A5* significantly reduced the expression of PDL1 in the TNBC cell line.

Altogether, this thesis showed how the functional collaboration of amino acid transporters can support the cornerstones of tumour metabolic ecology; assisting bioenergy, maintaining redox balance and reacting with microenvironment, which in turn promote BC cell aggressiveness and progression.

9.3 Final conclusions

The present study confirmed the hypothesis that the amino acid transport system has an important prognostic role in BC, which is variable among different BC subtypes. It was further identified that amino acid transporters can be potentially used for risk stratification and treatment selection of BC patients. Amino acid transporters included in this study were co-expressed, giving the evidence of potential collaboration to support BC progression and aggression and targeting these transporters may be a new form of synergistic lethality that can prove to be effective in treatment of BC patients. It appears evident that targeting *SLC7A5* and *SLC1A5* significantly reduced amino acid uptake in the luminal BC cells and the former plays an important role in the proliferation and invasiveness of luminal B and HER2+ BC subtypes.

The findings of this thesis further strengthen the link between the two cancer hallmarks, metabolic reprogramming and immune evasion, where it was shown that enhanced amino acid uptake can attract specific subtypes of inflammatory infiltrates and induce the expression of the immune checkpoint legend, PDL1. The use of molecules that target the pro-tumourigenic immune cells and the key amino acid transporters can be another novel approach which can provide longer lives to patients with aggressive forms of BC. Additional functional studies are highly recommended for further validation.

9.4 Strengths of the study

1- The samples utilised in this study were primarily from BC patients instead of cell lines or xenografts.
2- The utilisation of two large well-characterised BC cohorts, at mRNA and protein levels, with the availability of clinicopathological data and relevant biomarkers' data.
3- The use of BC- Gene Expression Miner software as a validation cohort for the transcriptomic data.
4- The identification of reliable prognostic markers, with promising significance in different BC molecular subtypes.
5- Using western blotting to verify the specificity of the investigated markers.
6- The inclusion of controls with each IHC experiment to ensure genuine staining.
7- Clustering analysis was included and allows the stratification of BC patients into accredited clusters, based on the protein IHC data.
8- The inclusion of *in vitro* work with different functional assays and optimum selection of BC cell lines based on the current findings of the IHC study.
9- Several published articles were generated from this study.

9.5 Limitations of the study

1- IHC H-scoring is a semi-quantitative subjective method dependent on the subjectivity of the scorers although standard methods were considered to minimise this limitation.
2- Despite the fact that expression patterns for the IHC-stained proteins were proved to be homogenous in full-face sections, it would have been ideal to

include more than one core per case to enhance the assessment of these proteins.

3- The TMA blocks used in this study were historic, and some TMA cores were missing or the tumour burden was marginal.
4- Limitation of the number of cell lines used for the *in vitro* work to reflect different BC subtypes.
5- Specific inhibitors were not commercially available, which was an impediment for including them in this study.

9.6 Future work

1- Investigation of other candidates of glutamine transporters to explore their prognostic significance in different BC subtypes
2- Applying cluster analysis to the whole portrait of glutamine transporters that are relevant to BC.
3- Designing functional studies for other amino acid transporters including the use of BC cell lines based on results obtained from molecular subtyping of BC.
4- Applying the selective inhibitors in the *in vitro* work and including other functional assays.
5- Applying three-dimensional (3D) cell culture to reliably reflect the *in vivo* cellular responses and more adequately represent the actual microenvironment, which allows exploring the metabolic coupling between the tumour cells and the stromal cells.

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