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PRECLINICAL STUDY

Glutamate dehydrogenase (GLUD1) expression in breast cancer

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Abstract

Background Dysregulated cellular metabolism is one of the hallmarks of cancer with some tumours utilising the glutamine metabolism pathway for their sustained proliferation and survival. Glutamate dehydrogenase (GLUD1) is a key enzyme in glutaminolysis converting glutamate to α-ketoglutarate for entry into the TCA cycle. Breast cancer (BC) comprises a heterogeneous group of tumours in terms of molecular biology and clinical behaviour, and we have previously shown that altered glutamine metabolism varies substantially among the different molecular subtypes. We hypothesise that the prognostic value of GLUD1 expression will differ between the BC molecular subtypes and may act as a potential therapeutic target for BC tumours.

Methods GLUD1 was assessed at the DNA, mRNA ($n=1980$) and protein ($n=1300$) levels in large, well-characterised cohorts and correlated with clinicopathological parameters, molecular subtypes, patient outcome, and treatments.

Results There was a correlation between *GLUD1* mRNA and GLUD1 protein expression which were highly expressed in low grade luminal/ER + BC ($p<0.01$). GLUD1 mRNA and protein was associated with good patient outcome but not in any specific molecular subtypes. However, high GLUD1 protein expression was associated with a better outcome in triple negative (TN) patients treated with chemotherapy ($p=0.03$). High *GLUD1* mRNA was associated with the glutamine transporter, SLC1A5, and leucine transporter, SLC7A8 as well as mTOR ($p<0.0001$).

Conclusion We provide comprehensive data indicating GLUD1 plays an important role in luminal/ER + BC. GLUD1 expression predicts a better patient outcome and we show that it has the potential for predicting response to chemotherapy in TNBC patients.

Keywords GLUD1 · Breast cancer · Prognosis · Triple negative · Glutamine · Metabolism

Introduction

Dysregulated tumour metabolism is an important step in oncogenesis and is one of the revised hallmarks of cancer, whereby cancer cells are able to modify and re-programme their metabolism to most effectively provide the energy required for proliferation and survival [1].

The Warburg effect is one of the most well described metabolic changes in tumour cells where glycolysis is utilised to support increased energy requirements for the rapid growth of tumour cells, even in the presence of oxygen [2]. Besides glycolysis, glutaminolysis is also proving to be an essential metabolic pathway where the amino acid glutamine (Gln) is used to sustain proliferation and survival [3]. Indeed, many tumour cells undergo metabolic re-programming which makes them highly dependable upon this amino acid, and glutamine deprivation results in growth arrest and cell death [4].

There is abundant evidence surrounding the regulation of dysregulated tumour metabolism by oncogenes and/or tumour-suppressor genes such as c-Myc and p53,

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which are able to modulate the expression and activity of key transporters and enzymes involved in glutaminolysis [5–7].

BC heterogeneity is well documented and dysregulated metabolism can vary substantially among the different molecular subtypes of BC. Differences in metabolic profiles have been shown to discriminate between oestrogen receptor positive (ER+) and triple negative breast cancers (TNBC) [8], whereby TNBCs have an increased expression of metabolic enzymes involved in glutaminolysis and ER + BCs which have the lowest level of these enzymes [9, 10].

HER2 positive (HER2+) tumours have the highest levels of Gln metabolism with increased expression of glutaminase (GLS) and glutamate dehydrogenase (GLUD) [10]. This is in concordance with higher levels of glutamate and lower levels of Gln in TNBC and HER2+ tumours compared with ER+ tumours suggesting an increase in Gln consumption and glutaminolysis in these tumours [8, 11].

Oncometabolism is an attractive field for therapeutic intervention and the differences observed in metabolic signatures between ER−and and ER+ tumours may further guide therapy and predict disease outcome as well as allowing for the emergence of novel targets for therapeutic approaches to improve efficacy and reduce resistance.

We have previously reported the importance of the glutamine-proline regulatory axis in the highly proliferative luminal subgroup of BC and its regulation by MYC [12].

GLUD is a key enzyme in glutaminolysis converting glutamate to α-ketoglutarate (α-KG) for entry into the TCA cycle, reducing NAD(P)+ to NAD(P)H in the process. GLUD is activated by the direct binding of the essential amino acid leucine which stimulates the deamination of glutamate and hence the production of α-KG. Two different isoenzymes of GLUD exist, GLUD1 and GLUD2, both of which are upregulated in human cancers enabling the cancer cell to utilise this pathway for growth and proliferation [13]. In the cancer cell, GLUD1 is not only essential for sustaining the TCA cycle for rapid proliferation and growth, but also has a role in the activation of mTORC1 [14] and as a regulator of redox homeostasis, whereby the inhibition of GLUD1 results in imbalanced redox homeostasis and a reduction in cancer cell proliferation and growth [15].

In this study, we aimed to determine the role of GLUD1 in BC by assessing gene copy number, mRNA and protein expression in large well-characterised annotated cohorts of BC to determine its biological and clinical relevance within the different molecular subtypes.

Materials and methods

GLUD1 gene expression

GLUD1 gene expression was evaluated in a cohort of 1980 breast cancer samples using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort [16]. The METABRIC study provides data on genomic and transcriptomic profiling of breast cancer using the Affymetrix SNP 6.0 and Illumina HT-12 v3 platforms respectively. In addition, *TP53* mutational profiling was performed. Detailed description of the experimental assays and analytical methods used were described previously [17]. In this cohort, patients with ER+ and/or lymph node negative tumours did not receive adjuvant chemotherapy, whilst those with ER− and/or lymph node positive tumours received adjuvant chemotherapy. Dichotomisation of *GLUD1* mRNA expression was performed using X-tile (Version 3.6.1, Yale University, USA) based on prediction of breast cancer specific survival (BCSS) [18]. The BCSS is defined as the time (in months) from the date of primary surgery to the date of BC-related death. The relationship between gene copy number aberrations, both gains and losses, of *GLUD1* and *MYC* and *p53* mutations with *GLUD1* mRNA expression and patient outcome were also investigated.

To validate the data on the *GLUD1* mRNA expression, bc-GenExMiner v4.0 (Breast Cancer Gene-Expression Miner v4.0) online dataset (http://bcgenex.centregauduchea u.fr) was used as an external validation dataset [19].

GLUD1 protein expression

Immunohistochemistry was conducted using a large cohort of patients comprising a well-characterised consecutive series of early stage (TNM Stage I–III excluding T3 and T4 tumours) sporadic primary operable invasive BC. Patients (age ≤ 70 years) were enrolled into the Nottingham Tenovus Primary Breast Carcinoma Series, presented at Nottingham City Hospital between 1989 and 1998 ($n = 1300$) and managed in accordance to a uniform protocol. Patients' clinical history, tumour characteristics, information on therapy, and outcomes are prospectively maintained. Outcome data were collected on a prospective basis and included development and time to distant metastasis (DM) and BCSS. DM free survival (DMFS) is defined as the time (in months) from the date of primary surgery to the appearance of DM.

The clinicopathological parameters for Nottingham and METABRIC series of patients are summarised in Supplementary Table 1.

GLUD1 antibody validation

GLUD1 primary antibody specificity (Rabbit monoclonal, Ab168352, Abcam Plc, Cambridge UK) was determined using western blotting with MCF7 and MDA-MB-231 human BC cell lines (obtained from the American Type Culture Collection; Rockville, MD, USA). GLUD1 primary antibody was used at a 1:250 dilution and IRDye 800CW Donkey anti-Rabbit fluorescent secondary antibody (926-32213, LI-COR Biosciences) was used at a 1:15,000 dilution. Mouse monoclonal anti-β-actin primary antibody (1:5000, A5441, Sigma-Aldrich) with IRDye 680RD Donkey anti-Mouse fluorescent secondary antibody (1:15,000, 926-68072, LI-COR Biosciences) was used as a control. Samples were loaded at 10 µg alongside the protein ladder (26,619, Page Ruler Plus Prestained Protein Ladder, Thermo Scientific) to determine the correct molecular weight. Odyssey Fc with Image Studio 4.0 was used to visualise protein bands (LI-COR Biosciences) which showed a single specific band at the predicted molecular weight of 62 kDa (Fig. 1a).

Tissue arrays and Immunohistochemistry

Tissue microarrays (TMAs) were constructed as previously described [20]. Immunohistochemical staining for GLUD1 was performed on 4 µm thick sections using the Novolink polymer detection system (Leica Biosystems, RE7150-K), as per manufacturer's instructions. Heat mediated antigen retrieval was carried out using citrate buffer pH 6.0. GLUD1 primary antibody was used at a 1:100 dilution for 30 min at room temperature. Negative (omission of the primary antibody) and positive controls were included according to manufacturer's data sheet.

Assessment of GLUD1 protein expression

Stained TMA sections were assessed using high resolution digital images (NanoZoomer; Hamamatsu Photonics, Welwyn Garden City, UK) at x20 magnification. Assessment of staining for GLUD1 was based on a semi-quantitative assessment of immunoreactivity using a modified histochemical score (H-score) which includes an assessment of both the intensity of staining and the percentage of stained cells [21]. For the intensity, a score index of 0, 1, 2, and 3 corresponding to negative, weak, moderate, and strong staining was used and the percentage for each was estimated subjectively. A final score of 0 to 300 is the product of both the intensity and the percentage.

Scoring was carried out by KC and for inter-observer concordance, 10% of the cores were second scored by a pathologist (MA) blinded to previous scores, clinicopatholigcal variables and survival data.

Dichotomisation of GLUD1 protein expression was determined based on breast cancer specific survival (BCSS) using X-tile software [18].

Immunohistochemical staining and dichotomisation of the other biomarkers included in this study were as per previous publications [5]. ER and PR positivity was defined as $\geq 1\%$ staining. Immunoreactivity of HER2 was determined using standard HercepTest guidelines (Dako). Chromogenic in situ Hybridisation (CISH) was used to quantify HER2 gene amplification in borderline cases using the HER2 FISH pharmDx™ plus HER2 CISH pharmDx™ kit (Dako) and was assessed according to the American Society of Clinical Oncology guidelines. BC molecular subtypes were defined based on the immunohistochemical profile as: luminal A: ER+/HER2− low proliferation (Ki67 < 10%); luminal B: ER+/HER2− High Proliferation (Ki67 ≥ 10%); HER2-positive class: HER2+ regardless of ER status; and triple negative (TN): ER−,

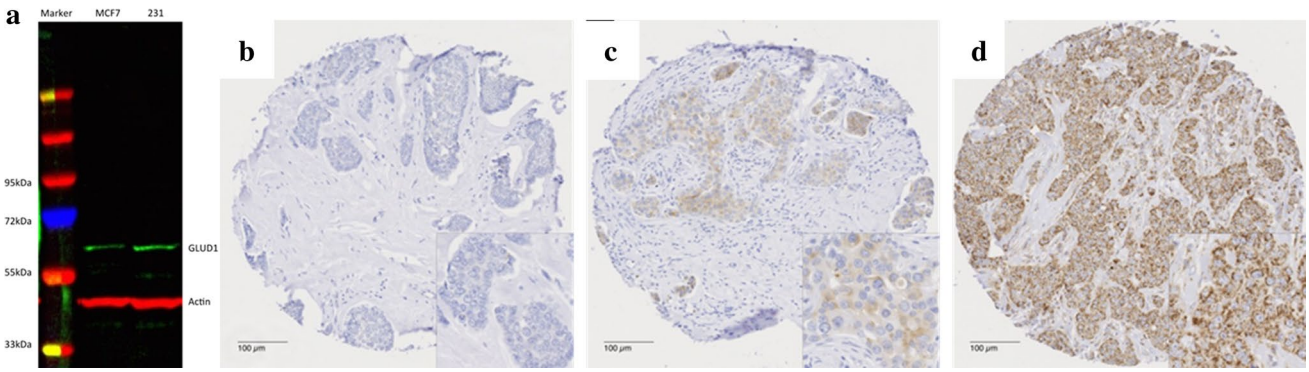

Fig. 1 GLUD1 Protein Expression in Breast Cancer. **a** Antibody validation of GLUD1 shows a single specific band is observed at the correct molecular weight of 62 kDa. Representative photomicrographs depicting: **b** negative GLUD1 expression, **c** low GLUD1 expression and **d** high GLUD1 expression in BC TMA using IHC

PgR−, and HER2−. Basal phenotype was defined as those tumours expressing cytokeratin (Ck) 5/6, and/or Ck14, and/or Ck17.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Univariate and multivariate analyses were performed by chi-squared test, Log rank and Cox regression analysis, respectively. One way ANOVA (Tukey) and Spearman's Correlation coefficient were used for continuous data. Survival curves were analysed by the method of Kaplan–Meier (Kaplan and Meier, 1958). A p-value < 0.05 was considered significant. This study complied with reporting recommendations for tumour marker prognostic studies (REMARK) criteria [22].

Ethics

This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer'. All samples from Nottingham used in this study were pseudo-anonymised and collected prior to 2006 and therefore under the Human Tissue Act informed patient consent was not needed. Release of data was also pseudo-anonymised as per Human Tissue Act regulations.

Results

GLUD1 in breast cancer

A total of 26/1980 (1.3%) of cases showed a copy number gain of *GLUD1* and 58/1980 (2.9%) showed a copy number loss. The distribution of *GLUD1* mRNA expression in the METABRIC cohort showed high expression (Log2 intensity > 10.0 units in 909/1980 (46%)). Loss of *GLUD1* was correlated with lower *GLUD1* mRNA expression and gain of *GLUD1* was associated with higher *GLUD1* mRNA expression (both $p < 0.001$, Fig. 2a).

GLUD1 protein expression was observed in the cytoplasm of breast tumour cells ranging from absent to high (Fig. 1b, c, d), with high protein expression (> 215 H-score) being observed in 367/1300 (28%) of tumours. There was a significant correlation between *GLUD1* mRNA expression and GLUD1 protein expression (correlation coefficient = 0.161, $p = 0.021$).

GLUD1 is associated with clinicopathological parameters of good prognosis

Higher *GLUD1* mRNA expression was significantly associated with lower tumour grade (Fig. 3a; Table 2, $p < 0.001$) and lymph node stage (Fig. 3b; Table 2, $p < 0.001$). The association between *GLUD1* mRNA and tumour grade, but not nodal stage, was confirmed using the Breast Cancer Gene-Expression Miner v4.0 (Supplementary Fig. 1a). There was also a significant association between high *GLUD1* mRNA and lobular and special type tumours ($p < 0.001$, Fig. 3j; Table 2). A similar association was observed between high GLUD1 protein expression and lower tumour grade ($p = 0.047$, Table 2).

High expression of *GLUD1* mRNA and GLUD1 protein were significantly associated with ER+, and PgR+ tumours (all $p < 0.001$; Table 2; Fig. 3c, d). Additionally, high *GLUD1* mRNA, but not GLUD1 protein, expression was significantly expressed in HER2 negative tumours ($p < 0.001$; Table 2; Fig. 3e). High *GLUD1* mRNA and GLUD1 protein was associated with non-TN tumours ($p < 0.001$; Table 2; Fig. 3f).

In addition, loss of GLUD1 copy number was associated with high grade tumours ($p = 0.001$), ER− ($p = 0.008$), HER2− ($p = 0.012$), and TN ($p < 0.001$) tumours (Table 1).

GLUD1 is differentially expressed within the molecular subtypes of BC

When comparing the levels of *GLUD1* mRNA expression in the intrinsic (PAM50) molecular subtypes, high expression was observed in luminal A and B classes (Fig. 3g, $p < 0.001$). Association of *GLUD1* mRNA with these classes was confirmed using the Breast Cancer Gene-Expression Miner v4.0 (Supplementary Fig. 1f). Similarly within the METABRIC Integrative Clusters, high *GLUD1* mRNA expression was associated with clusters 2 (luminal A and luminal B tumours) and 7 (luminal A tumours) (Fig. 3h, $p < 0.001$), and low levels of *GLUD1* mRNA expression was observed in cluster 10 (TN tumours). In the SCMGENE subtypes there was a higher expression of *GLUD1* mRNA in ER+/HER2− tumours compared with HER2 + and ER−/HER2− tumours ($p < 0.001$; Fig. 3i). ER+/HER2− low proliferation tumours had a significantly higher expression of *GLUD1* mRNA than ER+/HER2− high proliferation tumours ($p = 0.02$).

Similarly, expression of GLUD1 protein in BC subtypes showed a significantly higher expression in ER+/HER2− tumours compared with the other subtypes ($p < 0.001$, Table 2).

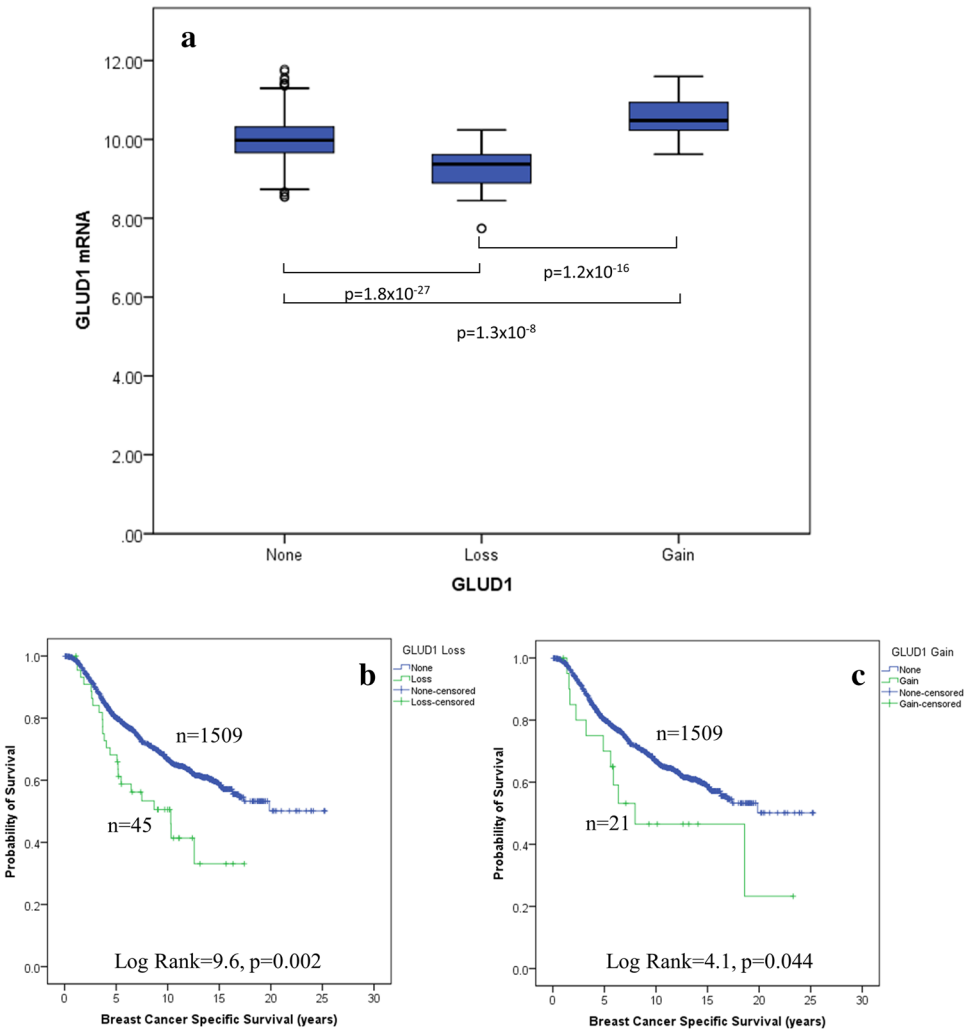

Fig. 2 GLUD1 copy number aberrations and relationship with **a** mRNA expression, **b** loss vs BCSS, **c** amplification vs BCSS

Both luminal B and basal subtypes showed a greater copy number loss of GLUD1 ($p < 0.001$) with a similar trend for GLUD1 copy number gain (Table 1).

GLUD1 showed significant molecular association with other significant biomarkers

We investigated correlation of *GLUD1* mRNA expression with associated glutaminolytic genes using the METABRIC dataset which are summarised in Table 5. The genes were selected based on previous publications, being either regulatory genes or others that share or support the biological function of GLUD1. There was a positive correlation between *GLUD1* with several amino acid transporters including *SLC7A5* ($p < 0.001$) and genes involved in the glutamine-proline regulatory axis including *ALDH18A1* ($p < 0.001$). There was an inverse relationship between *GLUD1* and *MYC*, and the MYC regulated gene *NDRG2*. At the protein level, high GLUD1 was associated with low c-MYC expression ($p < 0.001$), ALDH18A1 ($p = 0.007$), and SLC7A5 ($p = 0.025$) (Table 3) but not any other amino acid transporters or enzymes involved in glutaminolysis (Table 3).

High GLUD1 protein expression was significantly expressed in breast tumours that were negative for, PI3K ($p < 0.001$) and the cell cycle regulator Cyclin E ($p = 0.006$, Table 4). High expression of GLUD1 was positively associated with high levels of Bcl-2 ($p = 0.022$) and pAKTs437 ($p < 0.001$; Table 4). There was no association between GLUD1 and Ki67.

When investigating the subtypes of BC, the negative relationship between *MYC* and *GLUD1* was only observed in luminal A ($p = 0.001$) and luminal B ($p = 0.015$) subtypes. In ER+ tumours, high *GLUD1* mRNA expression was specifically associated with those enzymes involved with the Pro-Gln regulatory axis. The majority of amino acid transporters were significantly associated with *GLUD1* expression in ER+ tumours and to a lesser extent TN and Basal tumours. *SLC1A5, SLC38A1, SLC7A5*, and *SLC7A8* were significantly expressed with *GLUD1* in all subtypes.

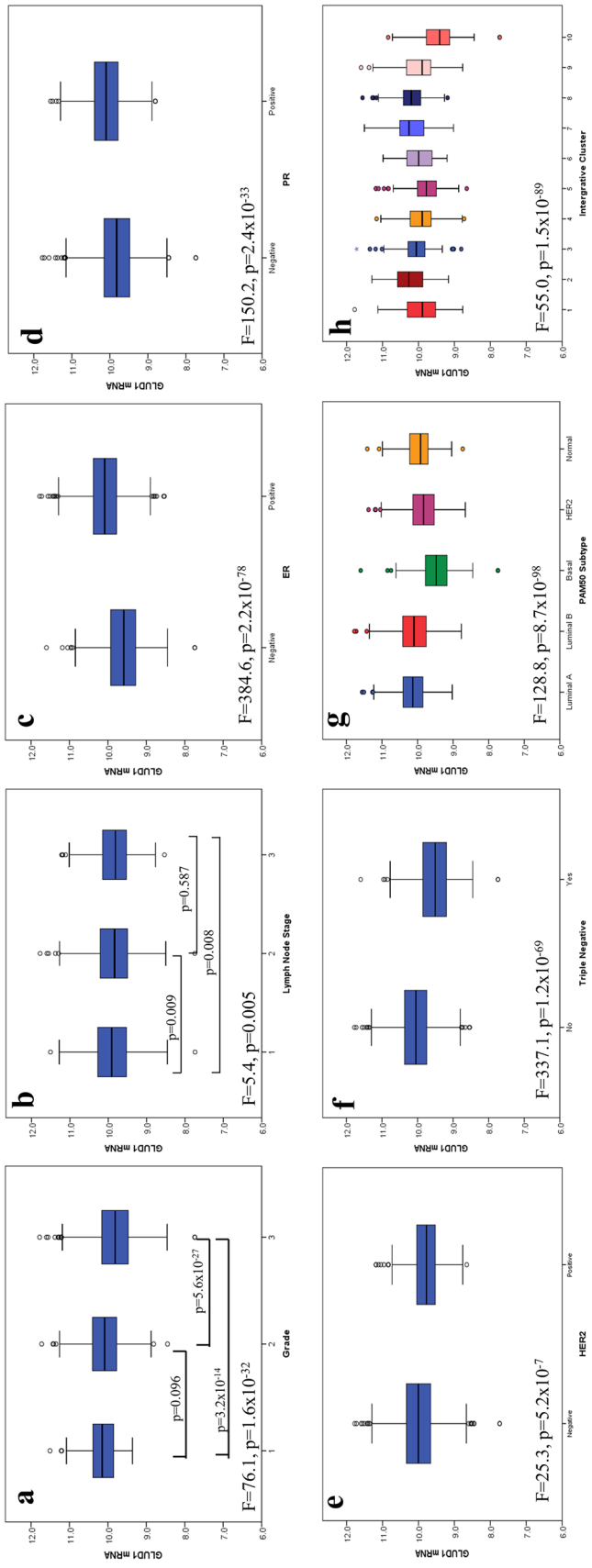

Fig. 3 *GLUD1* expression and its association with clinicopathological parameters and molecular subtypes: *GLUD1* and **a** tumour grade, **b** lymph node stage, **c** ER status, **d** PR status, **e** HER2 status, **f** triple negative status, **g** PAM50 subtypes, **h** METABRIC integrative clusters, **i** SMCGENE subtypes, **j** histological type (*IDC* invasive ductal carcinoma, *IDC + ILC* invasive ductal carcinoma and invasive lobular carcinoma, *IDC-TUB* tubular, *IDC-MUC* mucinous, *IDC-MED* medullary, *ILC* invasive lobular carincoma)

Table 1 Copy number aberrations (CNA) of GLUD1 in breast cancer and their associations with MYC CNA and BC molecular subtypes

	GLUD1 copy number			
	No	Loss	Gain	χ^2 (p-value)
Grade				
1	169 (99.4)	0	1 (0.6)	18.0 (**0.001**)
2	745 (96.8)	22 (2.9)	3 (0.4)	
3	896 (94.1)	36 (3.8)	20 (2.1)	
Lymph node stage				
1	995 (96.1)	26 (2.5)	14 (1.4)	3.3 (0.507)
2	593 (95.3)	23 (3.7)	6 (1.0)	
3	301 (95.3)	9 (2.8)	6 (1.9)	
Oestrogen receptor				
Negative	442 (93.2)	22 (4.6)	10 (2.1)	9.7 (**0.008**)
Positive	1454 (96.5)	36 (2.4)	16 (1.1)	
Progesterone receptor				
Negative	892 (94.9)	34 (3.6)	14 (1.5)	3.5 (0.178)
Positive	1004 (96.5)	24 (2.3)	12 (1.2)	
HER2				
Negative	1656 (95.6)	57 (3.3)	20 (1.2)	8.9 (**0.012**)
Positive	240 (97.2)	1 (0.4)	6 (2.4)	
Triple negative				
No	1063 (96.6)	37 (2.2)	20 (1.2)	18.8 (**0.00008**)
Yes	293 (91.6)	21 (6.6)	6 (1.9)	
PAM50 subtype				
Luminal A	703 (97.9)	10 (1.4)	5 (0.7)	42.1 (**0.000001**)
Luminal B	458 (93.9)	20 (4.1)	10 (2.0)	
Basal	301 (91.5)	23 (7.0)	5 (1.5)	
HER2	231 (96.3)	3 (1.3)	6 (2.5)	
Normal	197 (99.0)	2 (1.0)	0	
SCMGENE				
ER+/HER2− low proliferation	361 (98.1)	4 (1.1)	3 (0.8)	15.0 (**0.021**)
ER+/HER2− high proliferation	352 (95.7)	12 (3.3)	4 (1.1)	
ER−/HER2−	140 (92.7)	9 (6.0)	2 (1.3)	
HER2+	108 (98.2)	0	2 (1.8)	

Bold values indicate statistically significant

Correlations between *GLUD1* expression and *mTOR* were observed in the luminal A and Basal molecular subtypes, along with *VEGFA*, whereas *VEGFB* was only seen in ER+/HER2− tumours (Table 5). A similar pattern of co-expression of *GLUD1* and other genes was observed using the Breast Cancer Gene-Expression Miner (Supplementary Table 2) particularly in ER+ tumours with genes involved in the Pro-Gln regulatory axis.

GLUD1 expression is associated with patient outcome and response to chemotherapy

High *GLUD1* mRNA ($p < 0.001$) and GLUD1 protein ($p = 0.006$) expression were both associated with a good BCSS (Fig. 4a, b). However, when investigating associations with outcome within the molecular subtypes, high expression of *GLUD1* was only predictive of longer BCSS in luminal A tumours at the mRNA level ($p = 0.027$, data not shown). There was no association between *GLUD1* mRNA or protein and outcome in the other molecular subtypes.

The relationship between high *GLUD1* mRNA expression and good patient outcome in ER+ disease, but not ER− disease, was confirmed using Breast Cancer Gene-Expression Miner (Supplementary Fig. 2). CN loss or gain of *GLUD1* was associated with poor patient survival ($p = 0.002$; $p = 0.044$) (Fig. 2b, c).

In multivariate Cox regression analysis, GLUD1 protein, but not *GLUD1* mRNA, was an independent predictor of BCSS in all cases ($p = 0.005$) (Table 6).

Table 2 Clinicopathological associations of *GLUD1* mRNA and GLUD1 protein expression in breast cancer

	GLUD1					
	mRNA			protein		
	Low, *n* (%)	High, *n* (%)	χ^2 (*p*-value)	Low, *n* (%)	High, *n* (%)	χ^2 (*p*-value)
Tumour size (cm)						
< 2.0	344 (55.3)	278 (44.7)	0.52 (0.473)	443 (69.5)	194 (30.5)	3.85 (0.050)
≥ 2.0	713 (53.6)	618 (46.4)		503 (74.4)	173 (25.6)	
Grade						
1	59 (34.7)	111 (65.3)	114.3 (**1.49×10^{-25}**)	140 (67.6)	67 (32.4)	6.107 (**0.047**)
2	340 (44.2)	430 (55.8)		300 (39.8)	130 (30.2)	
3	632 (66.4)	320 (33.6)		505 (75.0)	168 (25.0)	
Histological type						
Ductal (including mixed)	910 (55.7)	724 (44.3)	**42.9 (2.7×10^{-9})**	806 (72.3)	309 (27.7)	5.74 (0.220)
Lobular	60 (40.80)	87 (59.2)		76 (69.1)	34 (30.9)	
Medullary-like	27 (84.4)	5 (15.6)		27 (87.1)	4 (12.9)	
Special type	38 (33.6)	75 (66.4)		37 (64.91)	20 (35.1)	
Lymph node stage						
1	515 (49.8)	520 (50.2)	18.5 (**0.0001**)	585 (73.8)	208 (26.2)	4.53 (0.104)
2	354 (56.9)	268 (43.1)		282 (68.3)	131 (31.7)	
3	197 (62.3)	119 (37.7)		78 (64.9)	26 (25.0)	
Oestrogen receptor						
Negative	385 (81.2)	89 (18.8)	184.7 (**4.5×10^{-42}**)	275 (82.3)	59 (17.7)	23.50 (**0.000001**)
Positive	686 (45.6)	820 (54.4)		662 (68.5)	304 (31.5)	
Progesterone receptor						
Negative	611 (65.0)	329 (35.0)	85.8 (**1.6×10^{-32}**)	390 (76.0)	123 (24.0)	6.56 (**0.010**)
Positive	460 (44.2)	580 (55.8)		518 (69.4)	228 (30.6)	
HER2						
Negative	892 (51.5)	841 (48.5)	38.4 (**5.8×10^{-10}**)	776 (71.0)	313 (29.0)	3.07 (0.080)
Positive	179 (72.5)	68 (27.5)		128 (77.6)	37 (22.4)	
Triple negative						
No	801 (48.3)	859 (51.7)	141.0 (**1.6×10^{-32}**)	724 (69.2)	322 (30.8)	26.47 (**2.7×10^{-7}**)
Yes	270 (54.1)	50 (15.6)		197 (86.0)	32 (14.0)	
SCMGENE/IHC molecular subtypes						
ER+/HER2− high proliferation	179 (48.6)	189 (51.4)		285 (67.4)	138 (32.6)	
ER+/HER2− low proliferation	153 (41.6)	215 (58.4)	106.9 (**5.1×10^{-23}**)	198 (68.8)	90 (31.3)	27.7 (**0.000004**)
Triple negative	130 (86.1)	21 (13.9)		200 (84.7)	36 (15.3)	
HER2+	81 (73.6)	29 (26.4)		128 (77.6)	37 (22.4)	

Bold values indicate statistically significant

When considering TNBC patients treated with chemotherapy, patients with high GLUD1 protein expression had a significantly better outcome (Fig. 5c, $p = 0.027$) whilst those patients who did not receive chemotherapy had no survival advantage (Fig. 5b, $p = 0.570$).

Discussion

Glutaminolysis is proving to be an essential metabolic pathway where the amino acid Gln is used to sustain proliferation and survival. In BC, dysregulated metabolism can vary substantially among the different molecular subtypes, with differences in metabolic profiles being able to discriminate between ER+ and TNBC.

Studies that address the prognostic significance of the enzyme GLUD1 in BC and its potential influence on Gln metabolism remains limited.

We therefore investigated GLUD1 at the genomic, transcriptomic, and proteomic level utilising a large number of breast tumours in order to better understand the potential role of this enzyme in BC and its molecular subtypes.

In this study, we have shown for the first time that high GLUD1 expression at the mRNA and protein level

Table 3 Correlation of GLUD1 protein expression with other glutamine-related proteins

	GLUD1		
	Low, *n* (%)	High, *n* (%)	χ^2 (*p*-value)
PRODH			
Negative	546 (74.5)	187 (25.5)	0.892 (0.345)
Positive	138 (71.1)	56 (28.9)	
ALDH18A1			
Negative	370 (76.6)	113 (23.4)	7.362 (**0.007**)
Positive	307 (68.7)	140 (31.1)	
ALDH4A1			
Negative	337 (74.9)	113 (25.1)	0.597 (0.440)
Positive	329 (72.6)	124 (27.4)	
PYCR1			
Negative	195 (69.9)	84 (30.1)	1.234 (0.267)
Positive	352 (73.6)	126 (26.4)	
GLS			
Negative	634 (72.2)	244 (27.8)	0.304 (0.581)
Positive	91 (74.6)	31 (25.4)	
SLC7A11			
Negative	411 (73.7)	147 (26.3)	1.060 (0.303)
Positive	122 (77.7)	35 (22.3)	
SLC7A5			
Negative	581 (71.9)	227 (28.1)	5.053 (**0.025**)
Positive	148 (80.0)	37 (20.0)	
SLC3A2			
Negative	434 (72.0)	169 (28.0)	1.262 (0.261)
Positive	196 (75.7)	63 (24.3)	
SLC1A5			
Negative	342 (70.2)	145 (29.8)	1.101 (0.294)
Positive	506 (73.0)	187 (27.0)	

Bold values indicate statistically significant

Table 4 Expression of GLUD1 in breast cancer and the expression of other molecular biomarkers

	GLUD1		
	Low, *n* (%)	High, *n* (%)	χ^2 (*p*-value)
c-MYC			
Negative	415 (48.7)	437 (51.3)	19.077 (<**0.001**)
Positive	111 (67.3)	54 (32.7)	
BCL2			
Negative	211 (55.8)	167 (44.2)	5.235 (**0.022**)
Positive	376 (48.6)	397 (51.4)	
Cyclin E			
Negative	169 (45.6)	202 (54.4)	7.695 (**0.006**)
Positive	54 (62.1)	33 (37.9)	
Ki67			
Negative	173 (50.9)	167 (49.1)	0.521 (0.47)
Positive	403 (53.2)	354 (46.8)	
pAKTs437			
Negative	135 (61.6)	84 (38.4)	15.381 (<**0.001**)
Positive	290 (46.3)	337 (53.7)	
PI3K			
Negative	346 (47.3)	385 (52.7)	12.555 (<**0.001**)
Positive	484 (50.5)	474 (49.5)	

Bold values indicate statistically significant

is associated with tumours of good prognosis; specifically tumours of lower grade and hormone receptor positivity (ER+/PR+).

Kim et al., 2013, investigated the expression of glutamine metabolism-related proteins, including GLUD1, in a smaller study within the different molecular subtypes of breast cancer observing high expression of tumoural GLUD1 protein in HER2+ and luminal A/B subtypes compared with TNBC [10]. In our study, we confirm the relationship with GLUD1 and luminal tumours but we found no correlation between GLUD1 expression and HER2+ tumours.

We did observe a CN loss of GLUD1 which was significantly associated with ER−, HER2−, and TN tumours as well as a greater CN loss being observed in Basal-like tumours.

Several studies have been carried out looking at quantitative proteomics to generate profiles to identify functional differences between the BC molecular subtypes [23, 24]. These studies revealed noticeable differences in energy metabolism networks between the subtypes of BC, suggesting the use and synthesis of metabolites to support growth and survival differs within each of the subtypes. For example, a significant increase in energy metabolism was observed in ER/PR 'luminal-like' tumours compared to a down regulation of this pathway in HER2+ tumours and KEGG and STRING analysis show higher expression of GLUD1 and glutamate-ammonia ligase/glutamine synthetase (GLUL) with lower expression of GLS within the ER+/PR+ tumours compared to the HER2+ and TNBC [24].

Another study using computational modelling and metabolic phenotypic analysis (MPA), showed considerable metabolic differences between ER+ and ER− tumours, with 73% of metabolic processes having significantly different MPA scores. This study identified an increase in glutamine uptake to be typical of ER− tumours and an increase in glutamine production and secretion to be typical of ER+ tumours showing glutamine biosynthesis and secretion is significantly higher in ER+ whereas serine metabolism and glutamine uptake were significantly higher in ER− tumours [23]. These observation support the outcome of our study where we have shown the metabolic biosynthesis involving GLUD1 to be associated with the luminal subtypes of BC.

Table 5 Correlation of *GLUD1* mRNA expression with other glutamine-related genes

	GLUD1 mRNA				
	All cases ($n=1980$)	Luminal A ($n=368$)	Luminal B ($n=367$)	HER2+ ($n=110$)	Basal ($n=150$)
	Correlation coefficient (p-value)				
MYC	**−0.204 (3.9×10^{-20})**	**−0.127 (0.001)**	**−0.110 (0.015)**	−0.111 (0.085)	0.015 (0.785)
NDRG2	**−0.072 (0.001)**	−0.007 (0.850)	0.002 (0.970)	−0.060 (0.352)	−0.040 (0.475)
GLS	0.039 (0.087)	**0.151 (0.000049)**	**0.173 (0.000122)**	0.029 (0.656)	**0.117 (0.033)**
ALDH4A1	**0.159 (1.1×10^{-12})**	**0.078 (0.037)**	−0.040 (0.377)	0.120 (0.063)	**0.232 (0.000022)**
PRODH	0.046 (0.463)	0.131 (0.163)	0.041 (0.727)	0.212 (0.383)	0.040 (0.858)
PYCR1	**−0.070 (0.002)**	−0.002 (0.954)	−0.020 (0.662)	0.051 (0.432)	**−0.123 (0.026)**
ALDH18A1	**0.110 (9.8×10^{-7})**	**0.201 (5.6×10^{-8})**	**0.158 (0.000458)**	**0.278 (0.000013)**	0.091 (0.100)
GLUL	**0.272 (0.000011)**	0.166 (0.077)	**0.402 (0.000385)**	−0.256 (0.290)	0.081 (0.713)
SLC1A5	**0.130 (9.8×10^{-7})**	**0.255 (3.6×10^{-12})**	**0.192 (0.000019)**	**0.202 (0.002)**	**0.128 (0.020)**
SLC38A1	**0.334 (1.4×10^{-17})**	**0.297 (4.8×10^{-16})**	**0.183 (0.000048)**	**0.313 (7.7×10^{-7})**	**0.108 (0.049)**
SLC38A2	**0.154 (6.1×10^{-12})**	**0.207 (2.1×10^{-8})**	**0.154 (0.001)**	0.082 (0.207)	**0.188 (0.001)**
SLC38A3	−0.029 (0.200)	0.048 (0.186)	**0.154 (0.001)**	0.077 (0.235)	**−0.117 (0.034)**
SLC38A5	**−0.125 (2.3×10^{-8})**	−0.073 (0.050)	**−0.161 (0.000349)**	−0.073 (0.261)	−0.683 (0.135)
SLC38A7	−0.025 (0.283)	0.072 (0.053)	0.072 (0.114)	0.053 (0.415)	0.059 (0.283)
SLC38A8	**−0.047 (0.035)**	−0.041 (0.275)	−0.046 (0.314)	−0.119 (0.066)	−0.066 (0.232)
SLC7A11	−0.027 (0.230)	−0.013 (0.721)	0.074 (0.100)	0.017 (0.798)	−0.044 (0.421)
SLC7A5	**−0.380 (4.4×10^{-69})**	**−0.161 (0.000014)**	**−0.237 (1.1×10^{-7})**	**−0.148 (0.022)**	**−0.112 (0.042)**
SLC7A6	**−0.205 (3.7×10^{-20})**	**−0.127 (0.001)**	**−0.152 (0.001)**	−0.52 (0.421)	**0.162 (0.003)**
SLC7A7	**−0.363 (1.4×10^{-62})**	**−0.361 (1.4×10^{-23})**	**−0.316 (8.1×10^{-13})**	**−0.214 (0.001)**	0.009 (0.867)
SLC7A8	**0.409 (1.4×10^{-17})**	**0.275 (6.0×10^{-14})**	**0.171 (0.000144)**	**0.245 (0.000130)**	**0.128 (0.020)**
SLC6A19	−0.009 (0.700)	−0.041 (0.273)	0.036 (0.503)	0.030 (0.643)	−0.107 (0.053)
MTOR	**0.053 (0.018)**	**0.135 (0.000293)**	−0.017 (0.701)	0.053 (0.413)	**0.200 (0.000264)**
PIK3AP1	**−0.200 (2.1×10^{-19})**	−0.060 (0.106)	**−0.131 (0.004)**	**−0.157 (0.015)**	0.027 (0.631)
VEGFA	**−0.118 (1.4×10^{-7})**	**0.097 (0.010)**	0.003 (0.952)	−0.083 (0.202)	**−0.283 (1.7×10^{-7})**
VEGFB	**−0.080 (0.000390)**	**−0.199 (7.3×10^{-8})**	**−0.167 (0.000204)**	−0.068 (0.293)	−0.090 (0.103)
AKT1	**0.089 (0.000075)**	0.033 (0.376)	0.027 (0.555)	0.064 (0.327)	−0.046 (0.403)
ATF4	**−0.070 (0.002)**	0.004 (0.915)	0.060 (0.184)	0.022 (0.737)	−0.068 (0.217)
BRCA1	**0.086 (0.000121)**	**0.102 (0.006)**	**0.109 (0.016)**	0.092 (0.137)	0.069 (0.213)

Bold values indicate statistically significant

The association of GLUD1 at the mRNA and protein level with key amino acid transporters and enzymes involved in glutaminolysis is not unsurprising. GLUD1 is the key enzyme in the second deamination step of glutaminolysis, where it is activated by the amino acid leucine to deaminate glutamate to α-KG for incorporation into the TCA cycle, which is a crucial anaplerotic step in proliferating cells. Leucine is known to activate mammalian target of rapamycin complex 1 (mTORC1) which has many functions in the cancer cell including regulation of protein translation, prevention of apoptosis, and cancer cell proliferation [25].

Several studies have shown the importance of glutaminolysis in the mTOR signalling pathway which, when active, mTORC1 is known to have a major role in promoting cancer cell growth and proliferation. mTORC1, one of the two multi-protein complexes, is regulated by several upstream signals including growth factors and nutrients such as amino acids and glucose. The amino acid leucine is thought to be a key activator of mTORC1 by stimulating the GTP state of the RagA/B complex which in turn recruits mTORC1 to the lysosome where it is activated by the lysosome-associated Rheb [26, 27].

Glutamine has also been implicated as an important amino acid for mTORC1 signalling as it can indirectly stimulate this pathway by increasing the uptake of leucine [28].

Recent findings also suggest leucine stimulates mTORC1 indirectly through glutaminolysis. Duran et al. demonstrated that glutamine, in combination with leucine, increased the GTP charge of exongenously expressed RagB, promoting mTORC1 activation and enhancing glutaminolysis and α-KG production, suggesting glutaminoloysis and α-KG

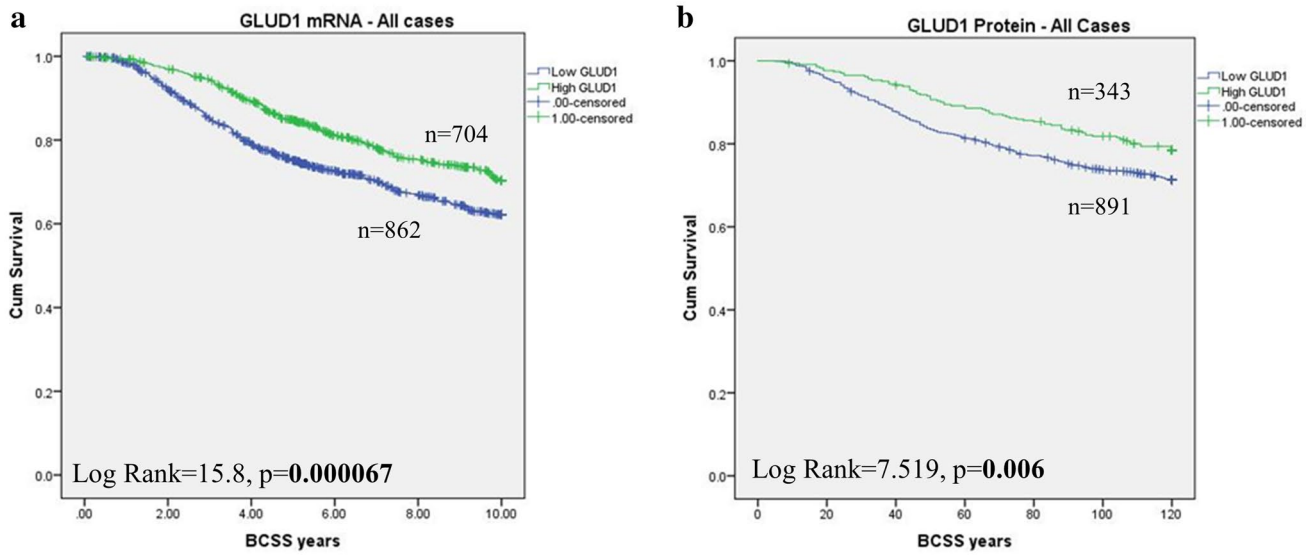

Fig. 4 GLUD1 and Breast Cancer Specific Survival (BCSS) at 10 years: **a** *GLUD1* vs BCSS in all cases, **b** GLUD1 vs BCSS in all cases

Table 6 GLUD1 and patient outcome

Parameter	mRNA		Protein	
	Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
All cases				
GLUD1	0.84	0.072	0.69	0.005
LN stage	1.86	**3.2×10^{-24}**	1.89	2.5×10^{-15}
Size	1.64	**0.00002**	1.27	0.051
Grade	1.26	**0.006**	2.41	6.6×10^{-14}
ER	0.77	**0.013**	0.91	0.454

Bold values indicate statistically significant

production may be key events for leucine to activate Rag-mTORC1 signalling [14]. As it is known that leucine directly binds and regulates GLUD1, enhancing the conversion of glutamate into α-KG, it was proposed that mTORC1 senses the fluctuations of glutamine and leucine together by sensing leucine-dependent production of α-KG. However, as leucine isn't the only activator of GLUD1, several mechanisms could be at play [29].

Several of the amino acid transporter molecules are involved in transporting leucine into the cell in exchange for glutamine, including SLC7A5 and SLC7A8.

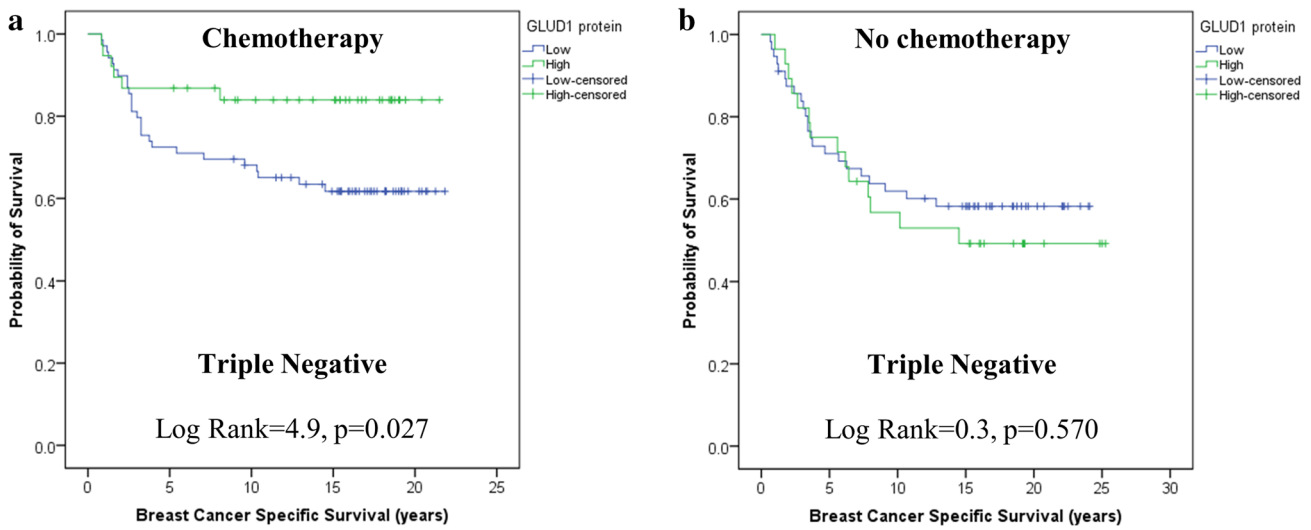

Fig. 5 GLUD1 protein expression and breast cancer patient outcome in triple negative tumours according to adjuvant treatment: **a** protein in patients treated with chemotherapy, **b** protein in patients not treated with chemotherapy. Green = high protein expression; blue = low protein expression

In this study we observed high GLUD1 expression at the mRNA and the protein level to have an inverse correlation with the solute carrier SLC7A5 and a positive correlation with SLC7A8 at the mRNA level. SLC7A5 is highly associated with TNBC and SLC7A8 with ER+ tumours, suggesting within luminal/ER+ tumours, SLC7A8 has a higher affinity for leucine transport which activates GLUD1 for its energy metabolism.

In this study, we have shown that GLUD1 is highly expressed in ER+ luminal tumours and are related with good overall patient outcome. We have also shown high GLUD1 is associated with the transporters of glutamine and leucine and mTOR, suggesting this is the metabolic pathway utilised by these tumours for cell survival and proliferation.

In addition, we observed good patient outcome within TNBC patients who had high GLUD1 protein expression and received chemotherapy, compared with patients who did not receive chemotherapy. Further validation studies are required to confirm whether GLUD1 is able to predict response to chemotherapy within the poor prognostic TN patients.

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Author Contributions MLC and ARG conceived and designed study. MLC, RE, MAA, KWC, LA, BM, MDR, CCN, IOE, EAR, ARG carried out experiments and collected data. MLC, KWC, ARG analysed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Compliance with ethical standards

Conflict of interest The authors confirm that they do not have any conflict of interests to declare.

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'. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All tissue samples from Nottingham used in this study were pseudo-anonymised and collected prior to 1st September 2006; therefore under the Human Tissue Act informed patient consent was not needed. Release of data was also pseudo-anonymised as per Human Tissue Act regulations. This article does not contain any studies with animals performed by any of the authors.

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