The Biological and Clinical Significance of Glutaminase in Luminal Breast Cancer

Brendah Masisi
University of Nottingham University Park Campus: University of Nottingham
Rokaya El-Ansari
University of Nottingham
Lutfi Alfarsi
University of Nottingham
Madeleine Craze
University of Nottingham
Natasha Jewa
University of Nottingham
Andrew Oldfield
University of Nottingham
Hayley Cheung
University of Nottingham
Michael Toss
University of Nottingham
Emad Rakha
University of Nottingham
Andrew Green (andrew.green@nottingham.ac.uk)
University of Nottingham https://orcid.org/0000-0002-0488-5913

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The biological and clinical significance of glutaminase in luminal breast cancer

Brendah K. Masisi[1], Rokaya El Ansari[1], Lutfi Alfarsi[1], Madeleine L. Craze[1], Natasha Jewa, Andrew Oldfield[1], Hayley Cheung[1], Michael Toss[1], Emad A. Rakha[1, 2], Andrew R. Green[1]

[1]Nottingham Breast Cancer Research Centre, Division of Cancer and Stem Cells, School of Medicine, University of Nottingham Biodiscovery Institute, University Park, Nottingham NG7 2RD, UK. [2]Cellular Pathology, Nottingham University Hospitals NHS Trust, Hucknall Road, Nottingham NG5 1PB, UK.

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Abstract

Background: Glutamine metabolism has a key role in the regulation of uncontrolled tumour growth by modulating bioenergetics, redox homeostasis and serving as a precursor for biomass synthesis. Glutaminase is a key enzyme involved in glutaminolysis, a process which plays a crucial role in carcinogenesis and progression. This study aimed to evaluate the expression and prognostic significance of glutaminase in luminal breast cancer (BC).

Methods: The glutaminase protein isoforms (GLS and GLS2) were assessed at the genomic and transcriptomic levels, using METABRIC (n=1398) and GENE MINER datasets (n=4,712), and protein level using immunohistochemistry in large well characterised cohorts of luminal Oestrogen Receptor (ER)-positive and HER2-negative BC patients, including ductal carcinoma *in situ* (DCIS) (n=206) and invasive BC (IBC; n=717) cohorts. GLS and GLS2 expression was associated with clinicopathological features, patient outcome and other glutamine-metabolism related genes.

Results: In DCIS, GLS expression was an independent risk factor for shorter local recurrence-free interval ($p<0.0008$). In IBC high *GLS* and *GLS2* mRNA and protein expression significantly correlated with solute carriers with high glutamine affinity, SLC3A2 ($p\leq0.01$) SLC7A8 ($p\leq0.01$) and SLC7A5 ($p<0.001$), and glutamine related enzymes; GLUD1 ($p<0.001$) and ALDH18A1 ($p<0.001$). *GLS* and *GLS2* gene copy number gains were associated with poor patient outcome ($p=0.028$; $p=0.010$ respectively). High GLS2 protein was predictive of a longer disease-free survival ($p=0.006$).

Conclusion: GLS appears to play a role in the early non-invasive stage of BC and it could be used as a potential biomarker to predict DCIS progression to invasive disease. In IBC, both GLS and GLS2 play a key role in the biological function of luminal tumours. Further functional assessments are needed to explore the specific role played by each isoform in BC.

INTRODUCTION

Metabolic reprogramming has been recognised as a hallmark of cancer [1]. Malignant transformation and progression require alteration of signalling pathways related to cellular metabolism to meet the demand for both energy and biomass for proliferating malignant cells. Glutamine is the second most utilised amino acid after glucose for driving tumour cell proliferation and cell survival, with some breast cancers known to exhibit glutamine addiction [2]. Glutamine plays a role in the replenishment of biosynthetic intermediates to maintain a functioning tricarboxylic-acid (TCA) cycle and it allows for the synthesis of macromolecules and antioxidants for rapidly proliferating cells [3, 4]. Once in the cell, glutamine is catabolised to glutamate, an important metabolic intermediate that connects with a wide variety of biological processes through the mitochondrial enzyme glutaminase, which presents in two isoforms; kidney-type (GLS/KGA) and liver-type (GLS2/LGA) [5].

The prognostic and therapeutic significance of these 2 glutaminase isoforms remain an active area of research. GLS is the main isoform expressed in cancer cells and there is increasing evidence suggesting that GLS plays an important role in carcinogenesis and tumour progression in various solid cancers. Previous studies have established that high GLS correlates with higher rates of tumour growth and is associated with advanced tumour stage and poor patient outcome [6-8] . In contrast, GLS2 tends to have opposing functions as although studies are limited on GLS2, it is markedly increased in tumours that are more differentiated and less aggressive [9, 10]. High expression of GLS2 is associated with a significantly longer survival time in hepatocellular carcinoma (HCC) [11]. In breast cancer (BC), GLS is expressed at different levels in molecular subtypes and plays an important role in the aggressive subclass of luminal BC in addition to triple negative BC (TNBC) [12]. It has also been observed that patients with high *GLS* but not *GLS2* mRNA expression in high proliferative subsets of luminal BC, have the worst patient outcome compared with those classified as low proliferative ([13]. BC is a heterogeneous group of diseases with histological types and metabolic pathways together sustaining the initiation and progression [14, 15]. The progression from ductal carcinoma *in situ* (DCIS) into invasive disease is a complex multifactorial process that involves different mechanisms including metabolic pathways. There is need to explore the combined protein expression of GLS isoforms in the luminal oestrogen receptor positive (ER+) and human epidermal growth factor receptor 2 negative (HER2-) breast tumours which show higher glutamine metabolic activity in *in vitro* studies [16]. Previous study has confirmed glutamine dependency in TNBC. However, the prognostic significance of GLS isoforms in ER+/HER2-

subtype, in DCIS and IBC, remain to be validated. Therefore, we hypothesised that, both GLS and GLS2 play a role in the tumour progression and prognosis in those tumours. This study aimed to assess the expression levels and prognostic significance of GLS and GLS2 in ER+/HER2- patients in a well- characterised DCIS and BC cohorts.

MATERIALS AND METHODS

Study cohorts

Protein expression was conducted on two cohorts of ER+/HER2− BC comprising DCIS and IBC. (Supplementary Table 1A and 1B) summarises the clinicopathological parameters of the two study cohorts. Patients were presented and managed at Nottingham City Hospital, Nottingham, UK. Clinicopathological, treatment and outcome data were collected and are prospectively maintained.

The DCIS cohort included primary DCIS (n=206), without synchronous IBC as previously described [17, 18] . Clinicopathological data including age at diagnosis, method of diagnosis either screening or symptomatic presentation, tumour grade, size and comedo-type necrosis. Expression of ER, progesterone receptor (PR), HER2, and Ki67 were previously determined for this cohort [18]. Local recurrence-free interval (LRFI), defined as any event of ipsilateral local recurrence (either DCIS or IBC) occurred after 6 months from the primary treatment.

The IBC cohort includes well characterised series of tumours from patients (n=717) with long-term follow-up ([19]. Outcome data included recurrence-free interval (RFI) and BC specific survival (BCSS), defined as the time (in months) from the date of primary surgical treatment to the time of recurrence or death from BC, respectively.

Transcriptomic data

GLS and *GLS2* gene CN aberrations and gene expression were evaluated in a cohort of 1,398 ER+/HER2- BC cases in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort [20]. In addition, Breast Cancer Gene-Expression Miner v4.5 (bc-GenExMiner v4.5) incorporating TCGA and SCAN-B RNA sequencing data (n=4,712) was used. Correlation between *GLS* and *GLS2* mRNA expression with glutamine associated genes was also investigated. The selection of these genes was based on previous publications, in

which they are either regulatory genes or support the biological function of GLS or GLS2 in glutamine metabolism [13, 21-23].

Glutaminase protein expression

Prior to immunohistochemistry (IHC) staining, the specificity for rabbit monoclonal GLS [Clone EP7212, Abcam, UK] and GLS2 (Ab169954, Abcam Plc, Cambridge UK) primary antibodies were validated by western blotting (WB) using IBC (MCF7, ZR751, BT474, MDA-MB-231 and SKBR3) and DCIS (MCF10DCIS) cell lysates obtained from the American Type Culture Collection; Rockville, MD, USA as previously described [13]. GLS was used at dilution of 1:1000 overnight at 4ºC; while GLS2 was diluted at 1:1500 and incubated for 1 hour at room temperature. Mouse monoclonal anti-β-actin antibody (Sigma, Life Sciences) (1:5000) was included as a positive control. Donkey anti-rabbit and Donkey anti-mouse fluorescent secondary peroxidase-conjugated antibodies (1:15000 IRDye 680RD and IRDye 800CW, LI-COR Biosciences) were applied for 1hr at room temperature. Images were detected using the LI-COR Odyssey Fc machine with Image Studio 4.0 (LI-COR Biosciences) at wavelengths 700nm and 800nm. Specific bands were observed at the predicted size of 73 kDa and 65 kDa corresponding to KGA and GAC isoforms of GLS and 65 kDa and 31 kDa corresponding to LGA and GAB isoforms of GLS2 (Supplementary Figure 1).

Tissue microarray (TMA) was constructed from both cohorts as previously described [17, 24]. IHC was performed on 4μm TMA sections from both cohorts using the Novocastra Novolink TM Polymer Detection Systems Kit (Code: RE7280-K, Leica, Biosystems, UK) according to manufacturer instructions and as previously described [13]. Each antibody was used in a separate set of slides (non-dual staining). Heat-induced antigen epitope retrieval was performed in citrate buffer (pH 6.0) for 20 minutes using a microwave oven (Whirlpool JT359 Jet Chef 1000 W) for both antibodies. Tissues were incubated with either GLS antibody 1:50 (Clone EP7212, Abcam, UK) or GLS2 antibody 1:400 (Ab169954, Abcam Plc, Cambridge UK) diluted in Leica antibody diluent (RE AR9352, Leica, Biosysytems,UK) at 4ºC overnight and at room temperature for 1 hour respectively. Negative (omission of the primary antibody) and positive controls (human tonsil tissue) were included according to manufacturer's data sheet.

Scoring of GLS and GLS2 expression

Stained TMA slides were scanned using high resolution digital scanner (NanoZoomer; Hamamatsu Photonics, Welwyn Garden City, UK) at x20 magnification, and viewed using

Xplore viewing software (Philips Healthcare). Assessment of staining for GLS and GLS2 in DCIS and invasive BC was based on a semi-quantitative assessment using a modified histochemical score (H-score) which included an assessment of both the intensity of staining and the percentage of stained tumour cells. 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 of positive stained tumour cells for each intensity was estimated subjectively. The final H-score was calculated by multiplying the percentage of positive stained cells (0–100) by the intensity (0–3), producing a total range of 0–300 [25]. A pathologist blind scored 10% of the cases for inter-observer concordance. GLS and GLS2 protein expression were dichotomised into low and high expression using the median H-score as per previous publications [26, 27]. Breast cancer luminal subtypes were defined based on the IHC profile as: Luminal A: ER+/HER2-_ low proliferation (Ki67<10%), Luminal B: ER+/HER2- high proliferation (Ki67>10%). For GLS expression an H-score of 20 for DCIS and 100 for IBC were used. The median H-score for GLS2 expression was 103 and 90 in DCIS and IBC respectively.

STATISTICAL ANALYSIS

SPSS version 25(Chicago, IL, USA) was used to carry out statistical analyses. Continuous levels of *GLS* and *GLS2* mRNA and protein expressions were correlated with other parameters using the Pearson's correlation coefficient. Differences in mean between three or more groups were assessed using one-way analysis of variance (ANOVA) with the post-hoc Tukey multiple comparison test (for normalised data), while Mann Whitney and Kruskal-Wallis tests were applied for non-parametric data. Kaplan-Meier survival curves and log-rank test were used to investigate the association of glutaminase mRNA/protein expression with clinical outcome. Cox regression model was applied for the multivariate analysis against LRFI. A two-tailed p-value <0.05 for all the tests was considered significant.

RESULTS

Patterns of GLS and GLS2 protein expression

When present, GLS and GLS2 were predominantly in the cytoplasm of tumour cells of both DCIS and IBC; with intensity levels varying from low to high (Figure 1). GLS showed negative or faint staining in the adjacent apparently normal terminal duct lobular units (TDLUs), while GLS2 showed moderate expression. Occasional stained inflammatory cells and surrounding stromal fibroblasts were evident in a few cores (Figure 1). GLS expression was significantly higher in IBC than DCIS ($F=332.4$, $p<0.0001$) and GLS2 was higher in DCIS than IBC ($F=9.8$, $p=0.002$).

Glutaminase expression in ER+/HER2- DCIS

There was a significant positive linear correlation between GLS and GLS2 protein expression (Figure 2a; $r=0.202$, $p=0.009$). There was no difference in GLS expression between luminal subtypes (Figure 2h) but GLS2 expression was significantly higher in luminal B compared with luminal A tumours (Figure 2i, $p=0.004$). There were no associations between GLS or GLS2 with other clinical parameters including tumour size, DCIS grade (Figure 2b-g).

Glutaminase and outcome in ER+/HER2- DCIS

High GLS expression in DCIS was associated with shorter LRFI for all recurrences (Figure 6a; $p<0.0001$) whereas there was no association between GLS2 and DCIS outcome (Figure 6b $p=0.428$). When stratifying patients taking into account both GLS and GLS2 co-expression, DCIS with GLS+/GLS2- expression was associated with the shortest LRFI with GLS+/GLS2+ showing moderate outcome and those tumours without GLS expression, irrespective of GLS2 expression, having the best outcome (Figure 6c; $p=0.0001$). In multivariate Cox regression, GLS remained a predictor of shorter LRFI independent of tumour size, grade and comedo necrosis (Table 5; $p=0.0008$).

Glutaminase expression in ER+/HER2- invasive BC

In the METABRIC cohort consisting of ER+/HER2-ve tumours only, a total of 19/1398 IBC (1.4%) showed *GLS* copy number gain whereas 16 cases (1.1%) showed copy number loss. Regarding *GLS2*, copy number gain was observed in 50 cases (3.6%) and loss observed in only 7 cases (0.5%). There was a significant association between *GLS* and *GLS2* copy number variations (CNV) and their corresponding mRNA expression (Figure 3g, $p=0.006$ and figure 3h, $p=0.032$), respectively.

The correlation between GLS and GLS2 showed variable results in different cohorts and in the different molecular subtypes. At the protein level, there was a positive linear correlation between GLS and GLS2 protein in all ER+/HER2- BC cases (Figure 3b, p=0.011). When molecular classes were considered, GLS and GLS2 proteins were positively correlated in the high proliferation/luminal B tumours (Figure 4f; p=0.045), but not the low proliferation/luminal A tumours (Figure 4d; p=0.115). At the mRNA level, there was no correlation between *GLS* and *GLS2* mRNA expression in the ER+/HER2- invasive BC, luminal A or luminal B tumours (based on PAM50) in the METABRIC cohort (Figure 3a, c and e; all p>0.05). However, in the GeneMiner cohorts, there was a linear negative correlation between *GLS* and *GLS2* in all ER+ (p<0.00001) and luminal A (p=0.003) BC classes, but not the luminal B tumours (p=0.202; Supplementary Figure 2). 33

Association of glutaminase with clinicopathological parameters in invasive BC

High *GLS* and *GLS2* mRNA were significantly associated with lower tumour grade (Figure 4c, p=0.017, Figure 4d, p=0.026; respectively). There was no association between *GLS* or *GLS2* mRNA expression with tumour size (Figure 4a-b) or nodal stage (Figure 4e-f).

When comparing the levels of glutaminase mRNA expression in molecular subtypes, there was a significantly lower level of *GLS* in luminal B compared with luminal A tumours (Figure 4g, p<0.001). In contrast luminal B tumours showed higher *GLS2* expression than luminal A tumours (Figure 4h, p=0.03). Luminal B tumours were more likely to have *GLS* CNV, either gain or loss, compared with luminal A tumours (p=0.019). Similarly, *GLS2* copy number gains were primarily observed in luminal B tumours (p=0.00004).

Within the METABRIC Integrative clusters, high *GLS* mRNA expression was associated with cluster 4 (predominately luminal A) (Figure 4i; p<0.0001). In contrast, high *GLS2* was associated with cluster 6 (predominately luminal B) (Figure 4j; p<0.0001). *GLS2* copy number gain was associated with cluster 1 which are predominantly luminal B tumours p=0.000006). There were no other associations between CNV and Integrative clusters.

GLS and GLS2 protein were not associated with any of the key clinicopathological parameters: tumour size, tumour grade or nodal stage (Figure 5). There was a strong trend towards higher GLS protein expression in the high proliferative luminal tumours compared with the low proliferative tumours (Figure 5g; p=0.051). There was no significant difference between GLS2 protein expression and the luminal subtypes (Figure 5b).

Glutaminase and glutamine metabolism-related genes and proteins

There was a positive correlation between GLS and GLS2 with Glutamate Dehydrogenase (GLUD1), and the solute carriers (SLC38A2 and SLC7A8) at both the mRNA (all $p<0.05$; Tables 1 and 2) and protein levels ($p<0.01$; Tables 3 and 4). In addition, GLS mRNA and protein expression were positively correlated with ALDH4A1 ($p<0.001$; Tables 1 and 3) and GLS2 was correlated with the solute carrier (SLC7A11) at both mRNA and protein levels ($p<0.01$; Tables 2 and 4).

GLS and GLS2 protein, but not mRNA, was also positively correlated with c-Myc, SLC3A2 and enzymes involved in glutamine-proline regulatory axis (ALDH18A1 and PRODH) (all $p<0.001$; Tables 3 and 4). Additionally, there was a positive correlation between GLS and BRCA1 ($p<0.001$), p53 ($p<0.01$), PIK3CA ($p<0.001$), and the key glutamine solute carriers (SLC1A5 and SLC7A5) (all $p<0.001$; Table 3). GLS2 protein was positively correlated with ALDH4A1, SLC3A2, SLC7A11 (all $p<0.001$; Table 4).

With respect to the luminal subtypes, both luminal A (low proliferative) and B (high proliferative) tumours showed positive correlation between GLS mRNA and protein expression with GLUD1 and SLC38A2 ($p<0.01$; Tables 3 and 4). GLS protein expression, but not mRNA, was also similarly positively correlated with ALDH18A1, ALDH4A1, c-MYC, PRODH, SLC3A2, SLC7A11 and SLC7A5 in both luminal subtypes (all $p<=0.001$; Table 3). In addition, there was a positive correlation between GLS protein and BRCA1, p53, PIK3CA and SLC1A5 in low proliferative but not high proliferative luminal tumours (all $p<=0.001$; Table 3).

Both low and high proliferative luminal tumours showed positive correlation between GLS2 protein and ALDH18A1 ($p<0.001$), ALDH4A1 ($p<0.01$), ATF4 ($p=0.001$), PRODH ($p<0.001$), SLC38A2 ($p<=0.001$) and SLC7A11 ($p<0.001$; Table 4). Low proliferative, but not high proliferative, luminal tumours also showed a positive correlation between GLS2 protein and c-MYC ($p<0.001$), SLC1A5 ($p<0.05$) and SLC3A2 ($p<0.001$; Table 4).

Glutaminase and outcome

In the ER+/HER2- invasive breast cancer, copy number gain of *GLS* and *GLS2* were associated with poor patient survival ($p=0.028$; $p=0.010$; Figure 7a-b). There was no association between

GLS or GLS2 mRNA or protein expression with patient BCSS (Figure 7c-f). Likewise, there was no association with *GLS* or *GLS2* mRNA with either patient survival or disease free internal in Breast Cancer Gene-Expression Miner (Supplementary Figure 3). However, GLS2 protein (p=0.006), but not GLS, was predictive of a longer recurrence-free interval (Figure 7g-h) which remained independent of tumour size, grade and nodal stage (p=0.003, Table 6).

DISCUSSION

Glutamine metabolism is important in cancer cell proliferation and in promoting invasiveness [28]. It has been well established that glutamine synthesis is upregulated in most cancers including BC and consequently, glutaminase catalytic activity and levels are upregulated [5]. Several studies demonstrate that glutaminase contributes to cancer tumour growth in various human cancers such as prostate, lung and colorectal [7, 8]. Despite these findings, the role of glutaminase in the progression of DCIS into invasive disease stage remains poorly understood. In addition, studies on GLS2 expression in BC are limited. To provide an understanding on the prognostic significance of GLS and GLS2 in BC, the current study evaluated the transcriptomic and proteomic expression of GLS and GLS2 and the association with various clinicopathological parameters and linked each biomarker to patient outcome. To our knowledge, this is the first study to evaluate the role of both GLS and GLS2 in pre-invasive and invasive ER+/HER2- tumours. The ER+/luminal tumours are the most common type of BC accounting for about 55-80% of all BC types and have varied tumour biology, disease prognosis and recurrence [29].

This study has revealed that high GLS expression is associated with shorter LRFI in DCIS. The results are consistent on multivariate analysis, proving GLS expression to be independent from other clinicopathological variables as a prognostic marker of shorter LFRI. In addition, when we assessed the co-expression of GLS and GLS2, those tumours expressing GLS and not GLS2 showed increase risk of recurrence. The findings highlighted the importance of GLS enzyme in tumour proliferation and invasiveness. With respect to invasive tumours increased expression of GLS2 protein predicted longer recurrence-free interval. This finding concurs with previous results that GLS2 has been linked to a role in suppressing tumour growth. It has been demonstrated that overexpression of GLS2 decreases HCC cell invasiveness by

counteracting the small GTPase Rac1[30]. Nevertheless, consistent with a previous study, copy number gain of GLS was associated with poor outcome in IBC [13].

Previous reports [5, 7] have shown that high proliferative tumours such as TNBC and luminal B have higher glutamine metabolism and show increased activity of glutaminase compared to low proliferating tumours. It is noteworthy that although GLS and GLS2 catalyse the conversion of glutamine to glutamate, the expression and regulation of the two isozymes is distinct. The former is the frequently upregulated isoform in most cancers. This study showed a strong trend towards higher GLS protein expression in the high proliferative ER+ tumours in invasive breast cancer and in DCIS. In addition, High GLS2 protein expression was associated with luminal B compared to Luminal A tumours in DCIS. When assessing the correlation between GLS and GLS2 between the two cohorts, we observed a positive correlation between high expression of GLS and GLS2 protein in pre-invasive tumours and high proliferative invasive tumours. This finding could suggest that in both pre-invasive stage and invasive stage, tumour cells might be overcoming the effect of GLS2 overexpression by overexpressing GLS. However, further mechanistic studies for this scenario are highly warranted to understand the underlying molecular mechanisms.

The relationship between GLS and GLS2 and other regulatory genes at both mRNA and protein expression was also investigated. A positive correlation between glutaminase isozymes and c-Myc in luminal types at protein level was observed. Evidence from various studies suggest that GLS is directly activated by c-Myc enabling sustained uncontrolled tumour cell proliferation. The level of GLS positively correlated to the level of c-Myc in both luminal tumours whereas GLS2 protein was confined to the low proliferative tumours. c-Myc is known as an important driver in maintaining a glutaminolytic phenotype particularly in ER- tumours and enhances GLS activity indirectly via suppressing the expression of miR-23a/b [21, 31]. Our data suggests that this regulation might also occur in the ER+/luminal subtype. In addition, the expression of GLS appears to be driven by alternative mechanisms in breast cancer. We observed a positive correlation between GLS with PI3KCa within the low proliferation subgroup. PI3KCa, a known oncogene, has a role in regulating cell proliferation and survival as well as an important role in regulating glucose and glutamine uptake and metabolism in different cancers. In lymphoma, PI3KCa increases the uptake of glucose and genes associated with glycolysis [32] whereas in colorectal cancer, cells become more dependent on glutamine due to mutations in PIK3Ca/p110α upregulating glutamine pyruvate transaminase 2, independent of Akt. In BC, PIK3Ca mutations tend to be associated with hormone receptor positive tumours and a study carried out by Lau *et al* have provided further evidence of the importance of PIK3Ca mutations

in metabolic re-programming, specifically increasing glutamine uptake and glutamate production by modulating pyruvate dehydrogenase activity [33]. *TP53* is well known tumour suppressor gene in cancer. It has been linked to regulating glutamine metabolism by mediating the *GLS2* gene and having tumour suppression effect on tumour cells [22]. Interestingly, in the current study a positive association between wild type *TP53* and GLS expression in low proliferating tumour was observed.

The association of both GLS and GLS2 with glutamine transporters and other enzymes involved in glutamine metabolism is not surprising. Our analysis demonstrated positive association between GLS and GLS2 with most of the glutamine metabolism related enzymes and solute carriers. Among these is GLUD1, which was associated with GLS and GLS2 at both mRNA and protein level in the low and high proliferative tumours. Craze and colleagues [34] have shown that there is a relationship between GLUD1 and luminal tumours compared to HER2+ tumours. In addition, Kim *et al.* has observed high expression of GLUD1 in luminal A and B tumours compared with TNBC. Furthermore, positive correlation with ALDH18A1 and PRODH was observed. Previously Craze *et al*. demonstrated that these enzymes were highly expressed in a subset of ER+ tumours that have high proliferation and were related to poor patient outcome[13].

The majority of solute carriers associated with a high affinity to glutamine were positively associated with either GLS or GLS2 at mRNA and/or protein levels including SLC3A2, SLC7A8, SLC7A5 and SLC7A11. Glutamine enters the cancer cells via SLC1A5, which then effluxes out of the cells via the SLC7A5/SLC3A2 complex coupled to the entry of leucine. SLC7A11 is a transporter for extracellular cysteine coupled to the efflux of intracellular glutamate [35]. In this study we show that high expression of GLS and GLS2 were associated with high expression of SLC7A5, SLC3A2 and SLC1A5. In their findings El Ansari *et al*, demonstrated that the combination of SLC1A5, SLC7A5 and SLC3A2, defined as high SLCs cluster, was associated with poor prognostic markers in highly proliferative ER positive tumours[23]. Our observation in this subset of BC is consistent with the previous studies. Although our findings did not show correlation with key clinicopathological features in invasive BC but mostly association with glutamine metabolism related genes, the findings may suggest that glutaminase isozymes expression in this subset of breast cancer are important in tumour biology rather than clinical outcome in invasive BC. Further investigation studies are needed to understand the underlying molecular mechanisms.

CONCLUSION

This study revealed that GLS may play an important role in DCIS progression as well as predicting recurrence in DCIS patients. This provides strong evidence of the potential use of GLS as a biomarker for invasive progression in DCIS and predict outcome of DCIS. This study also showed that GLS and GLS2 are associated with enhanced glutamine transport system in luminal BC. Further functional studies to decipher the role of GLS and its mechanism of action as a driver of DCIS to invasive BC and the specific role played by GLS and GLS2 in BC are warranted.

Abbreviations

ER +: Oestrogen receptor positive; HER2: Human epidermal growth factor receptor 2; IHC: Immunohistochemistry; TMAs: Tissue microarrays; DCIS: ductal carcinoma *in situ;* IBC: invasive breast cancer; LRFI: Local recurrence-free interval

Ethics approval and consent to participate

This study was approved by the Nottingham Research Ethics Committee 2 under the title "Development of a molecular genetic classification of breast cancer" (REC202313) and by North West – Greater Manchester Central Research Ethics Committee under the title "Nottingham Health Science Biobank (NHSB)" (15/NW/0685). All samples from Nottingham used in this study were pseudo-anonymised and stored in compliance with the UK Human Tissue Act.

Consent for publication

Not applicable

Availability of data and materials

The authors confirm that the datasets used and analysed during the current study are available from the corresponding author on reasonable request

Conflict of interest

The authors declare no conflict of interest.

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Authors' contributions

BM: Writing, methodology, data analysis and interpretation of the results, RE: data analysis, review and editing, LA and MC review, AO: Methodology, MT: Data analysis, review editing, ER: review and editing. AG: Conceived and designed the study, data analysis, and review and editing.

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Figures

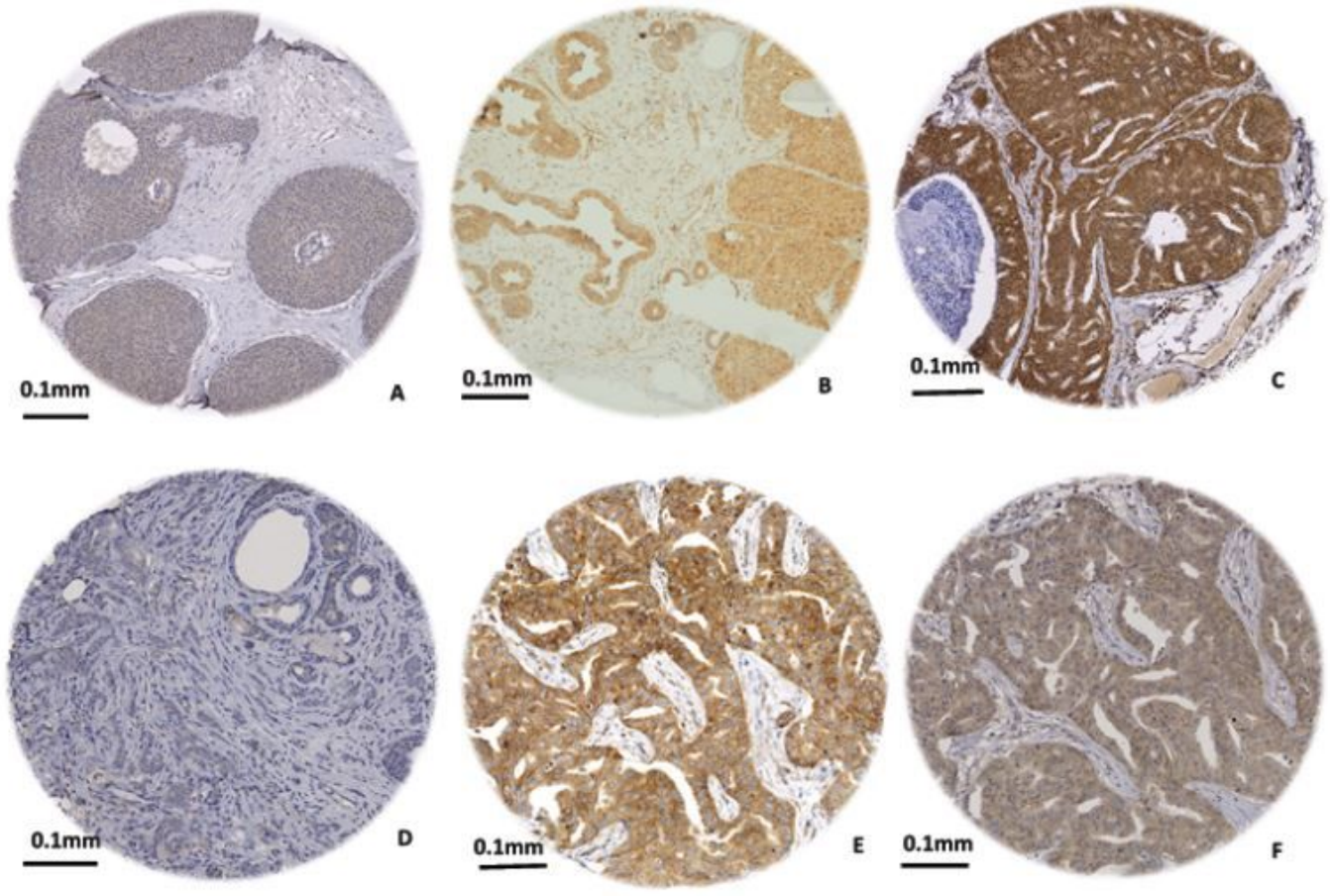

Figure 1

GLS and GLS2 protein expression in ER+/HER2- DCIS and invasive breast cancer. Representative TMA images (x20 magnification) depicting (A) negative immunostaining, positive GLS (B) and GLS2 (C) immunostaining in DCIS cases. (D) Negative immunostaining, positive GLS (E) and GLS2 (F) expression in invasive breast tumours.

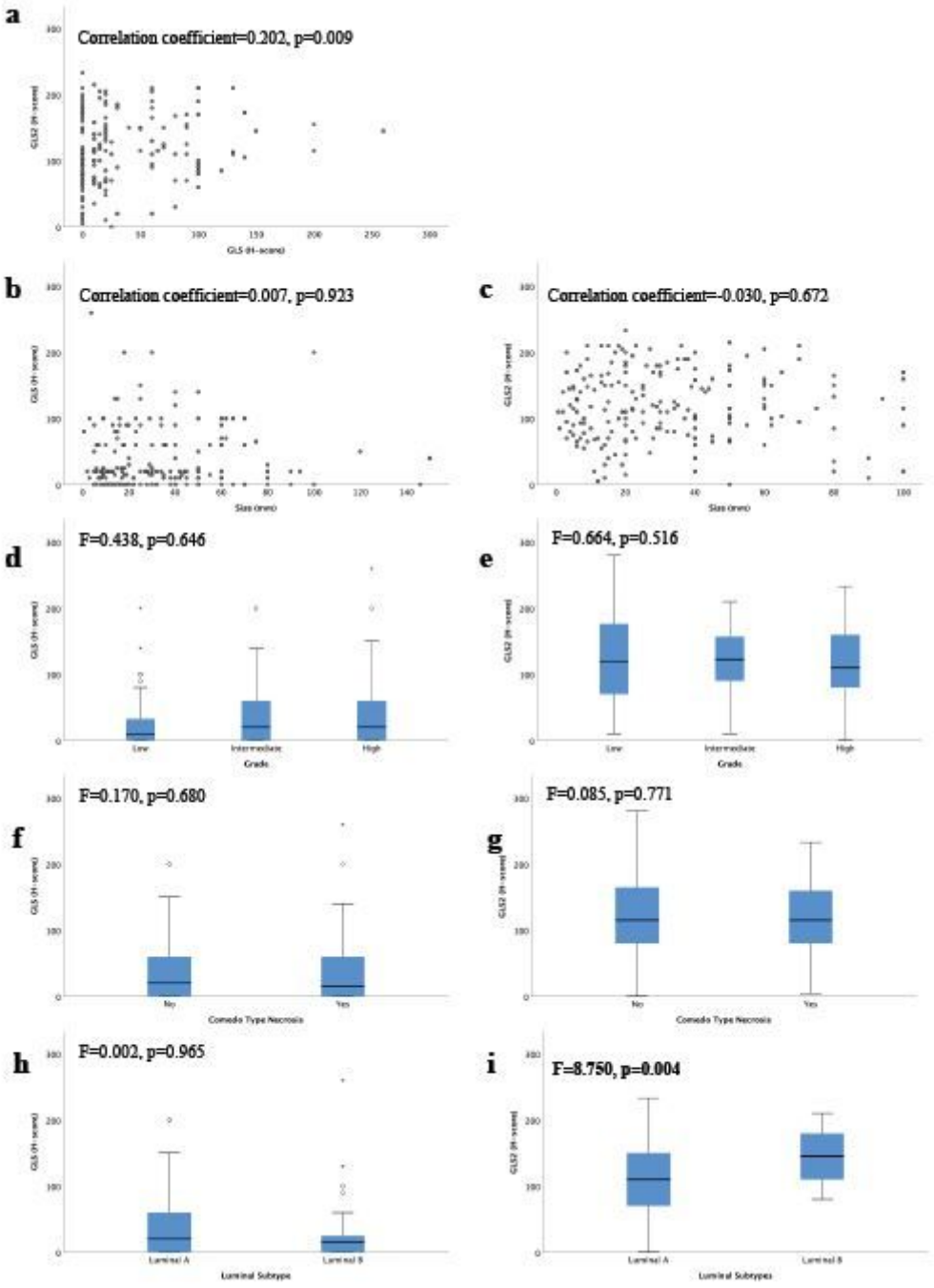

Figure 2

Glutaminase protein expression and its association with clinicopathological parameters and molecular subtypes in ER+/HER2- DCIS: a GLS and GLS2, GLS and b tumour size, d tumour grade, f comedo type necrosis, h luminal subtypes; GLS2 and c tumour size, e tumour grade, g comedo type necrosis, i luminal subtypes.

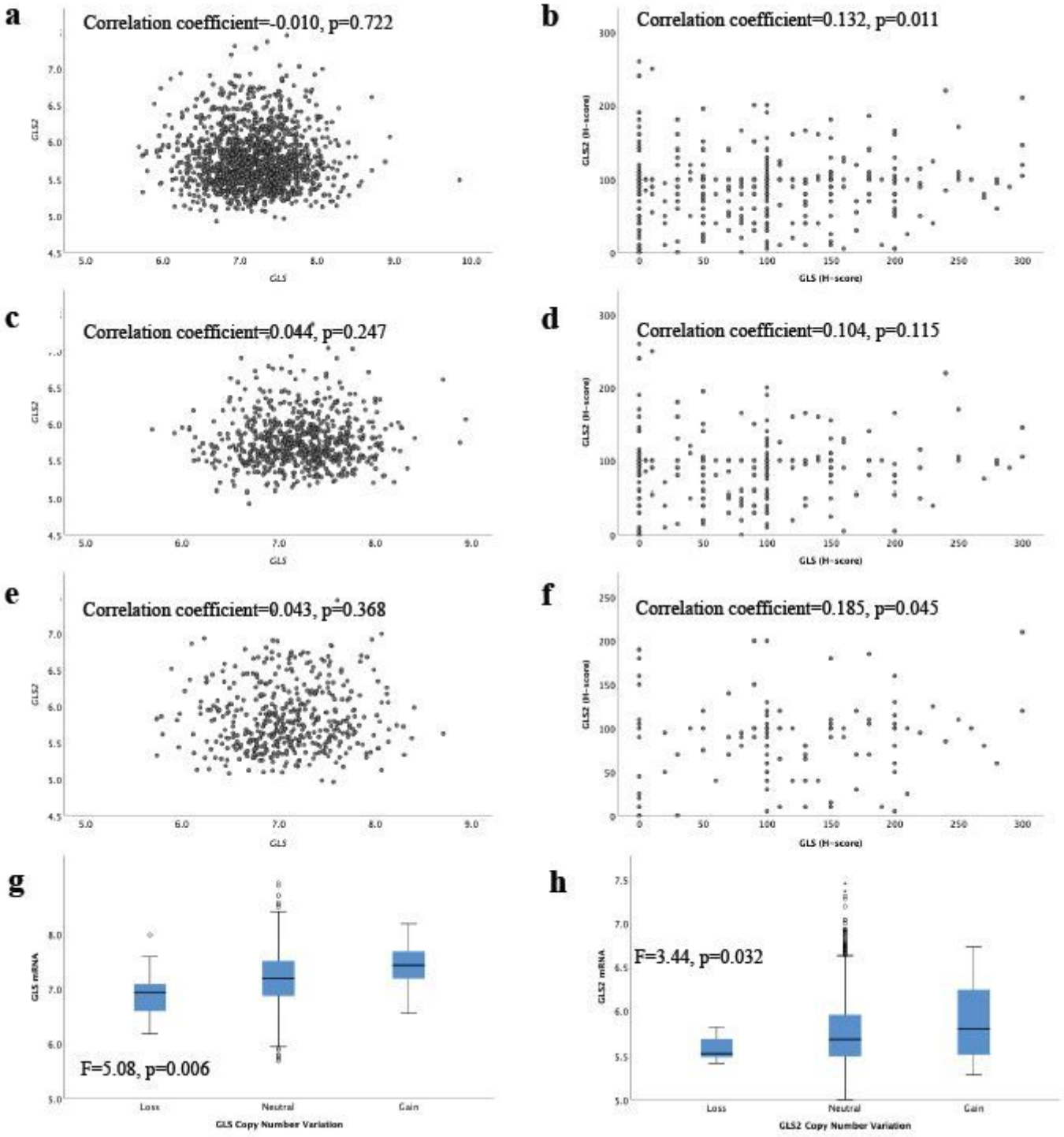

Figure 3

Correlation between glutaminase mRNA and protein expression in ER+/HER2- invasive breast cancer: GLS and GLS2 mRNA in a all tumours, c luminal A tumours, e luminal B tumours; GLS and GLS2 protein in b all tumours, d low proliferation tumours, f high proliferation tumours. Copy number gain and relationship with mRNA expression for g GLS, h GLS2.

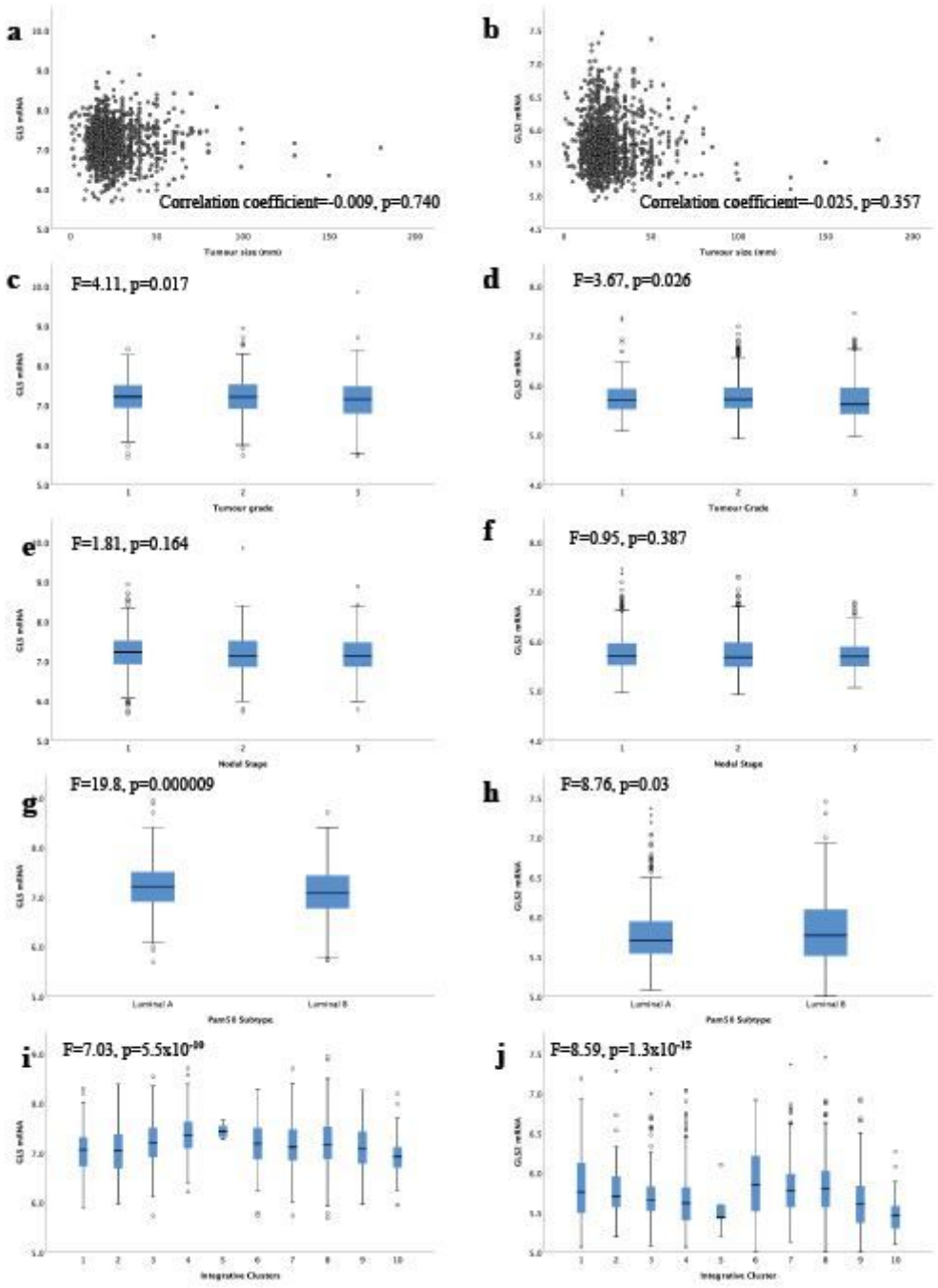

Figure 4

Glutaminase mRNA expression and its association with clinicopathological parameters: GLS and a tumour size, c tumour grade, e lymph node stage, g luminal subtypes, i METABRIC integrative clusters; GLS2 and b tumour size, d tumour grade, f lymph node stage, h luminal subtypes, j METABRIC integrative clusters.

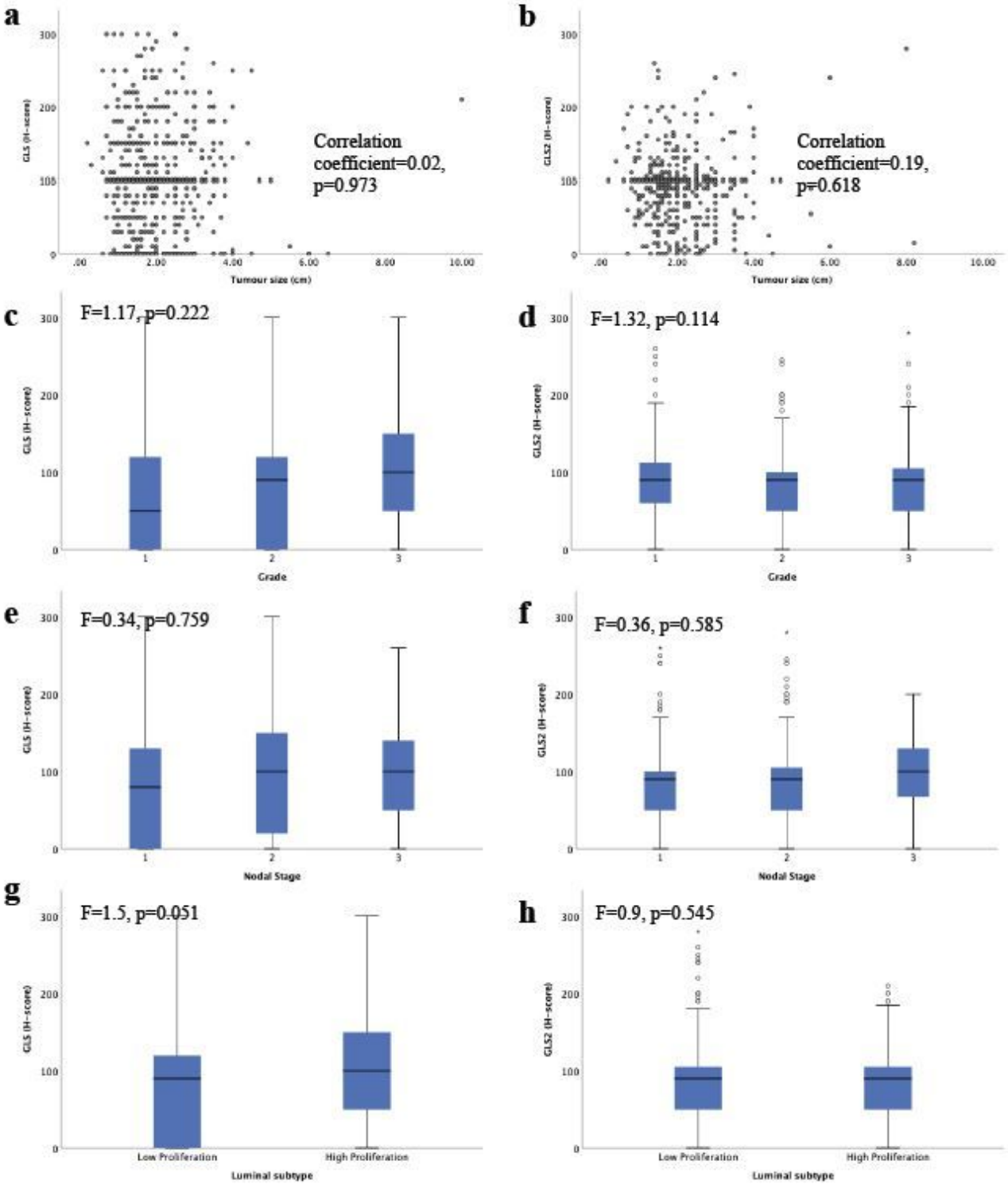

Figure 5

Glutaminase protein expression and its association with clinicopathological parameters and molecular subtypes in ER+/HER2- invasive breast cancer: GLS and a tumour size, c, tumour grade, e lymph node stage, g luminal subtypes; GLS2 and b tumour size, d tumour grade, f lymph node stage, h luminal subtypes.

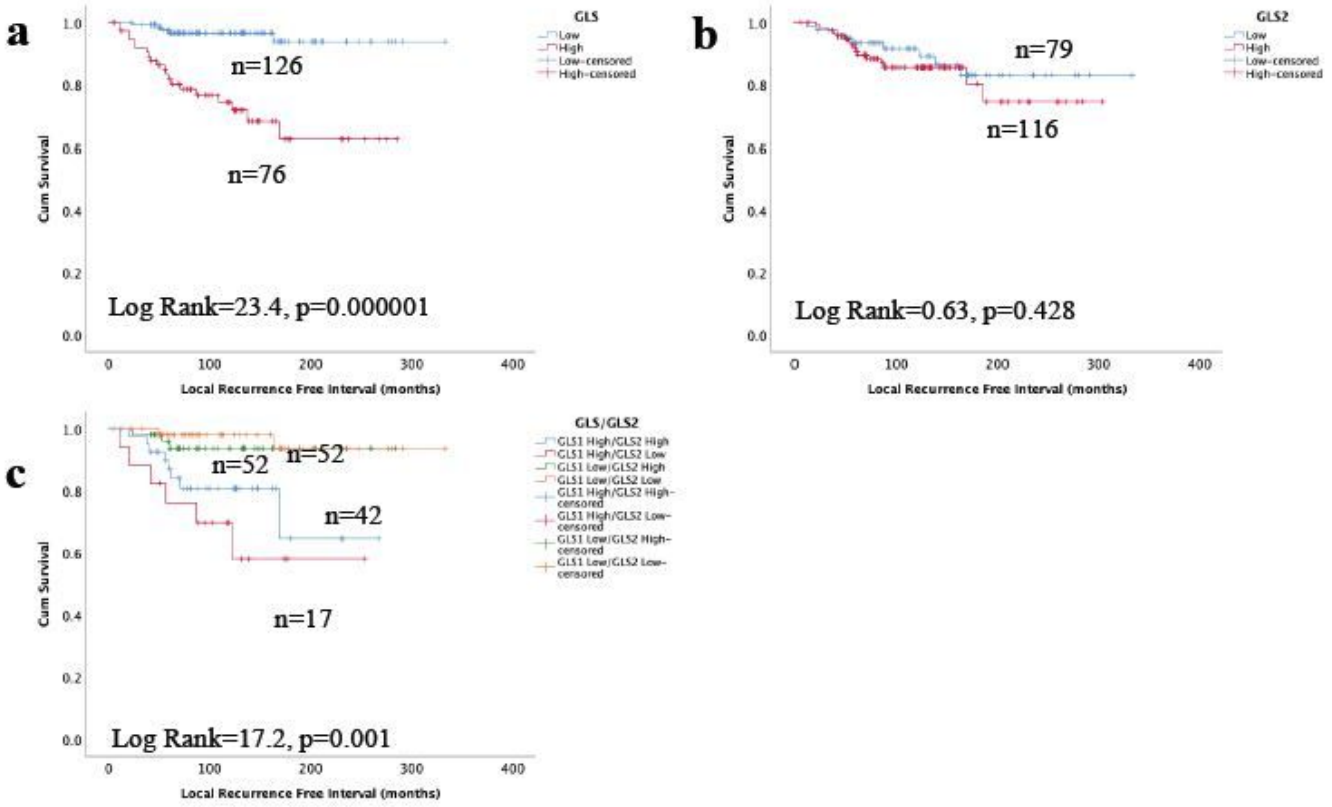

Figure 6

Association of GLS and GLS2 protein expression with tumour recurrence in ER+/HER2- DCIS: a GLS, b GLS2 and, c combined expression of GLS and GLS2.

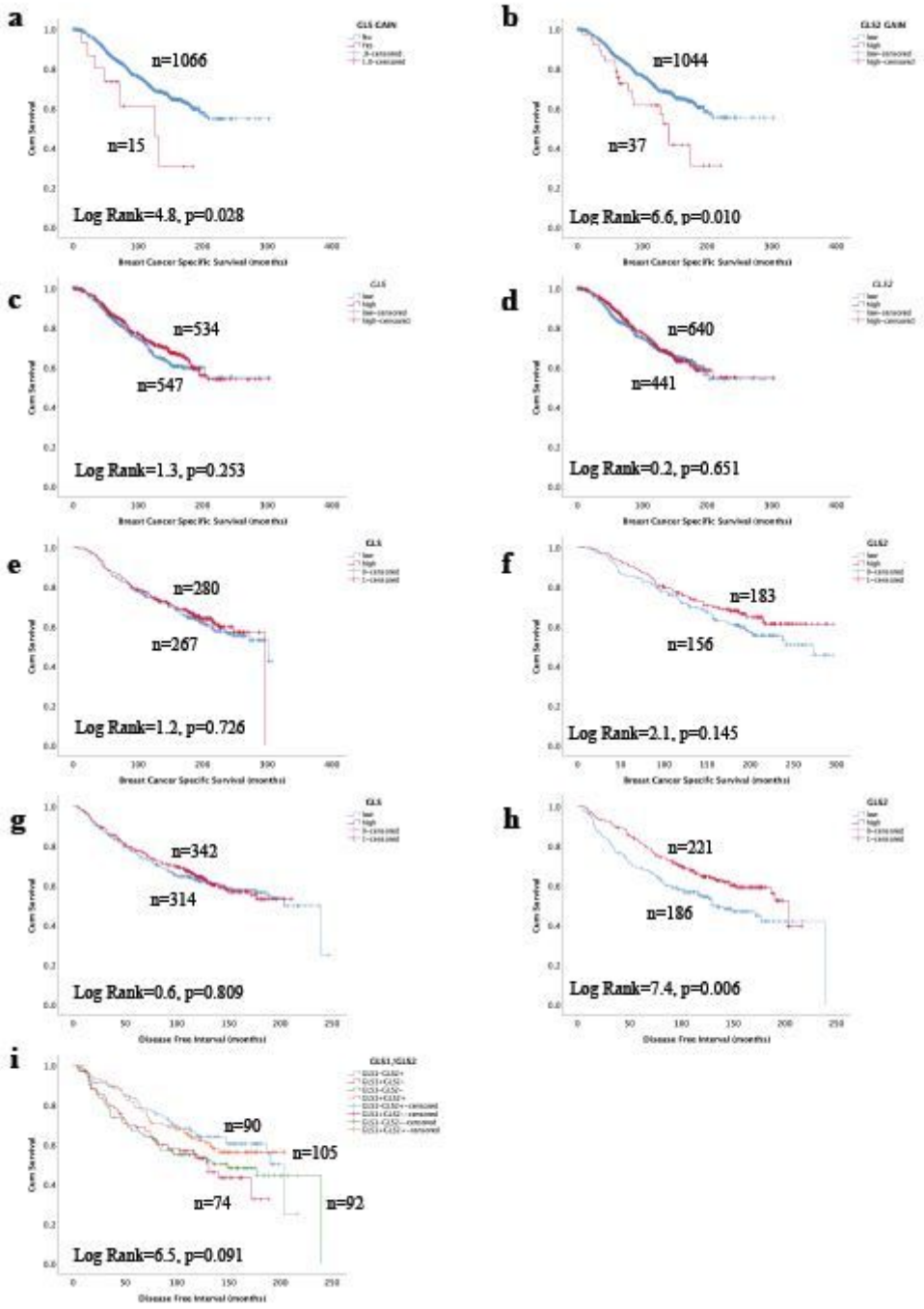

Figure 7

Association of glutaminase expression with patient outcome in ER+/HER2- invasive breast cancer in the METABRIC and Nottingham series: breast cancer specific survival of a GLS copy number gain, b GLS2 copy number gain, c GLS mRNA, d GLS2 mRNA, e GLS protein and f GLS2 protein, disease free interval of g GLS protein, h GLS2 protein, i combined expression of GLS and GLS2.

Supplementary Files

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