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

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Abstract

Purpose: Breast cancer (BC) is a heterogeneous disease characterised by variant biology, metabolic activity, and patient outcome. Glutamine availability for growth and progression of BC is important in several BC subtypes. This study aimed to evaluate the biological and

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prognostic role of the combined expression of key glutamine transporters, SLC1A5, SLC7A5 and SLC3A2 in BC with emphasis on the intrinsic molecular subtypes.

Methods: SLC1A5, SLC7A5 and SLC3A2 were assessed at the protein level, using immunohistochemistry on tissue microarrays constructed from a large well characterised BC cohort (n=2,248). Patients were stratified into accredited clusters based on protein expression and correlated with clinicopathological parameters, molecular subtypes, and patient outcome.

Results: Clustering analysis of SLC1A5, SLC7A5 and SLC3A2 identified three clusters Low SLCs (SLC1A5-/SLC7A5-/SLC3A2-), High SLC1A5 (SLC1A5+/SLC7A5-/SLC3A2-) and High SLCs (SLC1A5+/SLC7A5+/SLC3A2+) which had distinct correlations to known prognostic factors and patient outcome (p<0.001). The key regulator of tumour cell metabolism, c-MYC, was significantly expressed in tumours in the High SLCs cluster (p<0.001). When different BC subtypes were considered, the association with the poor outcome was observed in the ER+ high proliferation/luminal B class only (p= 0.003). In multivariate analysis, SLC clusters were independent risk factor for shorter breast cancer specific survival (p= 0.001).

Conclusion: The co-operative expression of SLC1A5, SLC7A5 and SLC3A2 appears to play a role in the aggressive subclass of ER+ high proliferation/ luminal BC, driven by c-MYC, and therefore have the potential to act as therapeutic targets, particularly in synergism.

Key words: SLC1A5, SLC7A5, SLC3A2, clusters, breast cancer, prognosis.

Introduction

Altered metabolic profiles, one of the cancer hallmarks [1], has been substantiated for many years, and the significance of onco-metabolism and its exploitation for targeting cancers has recently attracted great attention. To maintain the unremitting proliferation, cancer cells reprogram their metabolism to provide nutrients required to support bioenergetics and biosynthetic pathways. 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 [2].

Glutamine is the second primary metabolite to fuel cancer cell proliferation after glucose, as it is characterised by its ability to replenish the carbon pool of the mitochondrial tricarboxylic acid cycle (TCA) via α-ketoglutarate (α-KG) synthesis, in addition to its role for maintaining the redox balance [3] Indeed, certain tumours may have glutamine-dependent cell growth or 'glutamine addiction' [4]. In BC, glutamine-dependent mechanisms can vary substantially between the molecular subtypes, as previous studies have revealed that the expression levels of glutamine metabolic enzymes, such as glutaminase (GLS), are mainly observed in Triple Negative (TN), HER2+ and luminal B subtypes compared with luminal A tumours [5-7]. Specific genetic alterations may drive cancer cells to resist harsh metabolic stress conditions and nutrient deprivation including the upregulation of the glutamine transporters, solute carrier family 1 member 5 (SLC1A5) and solute carrier family 7 member 5 (SLC7A5) by the oncogene c-MYC [8,9].

SLC7A5 is a sodium-independent amino acid transporter which imports large neutral amino acids such as leucine, isoleucine, tyrosine, and tryptophan, in exchange with intracellular glutamine [10]. It therefore functions in supplying amino acids to cancer cells as well as

maintaining intracellular leucine which is considered a master regulator of mTORC1 signalling pathway [11,12]. SLC7A5 requires a covalent association with the heavy chain of the membrane protein, solute carrier family 3 member 2 (SLC3A2), for its functional expression in plasma membrane [13]. We have previously described the potential utility of these two solute carriers as prognostic factors for the highly proliferative BC subtypes [14,15]. Additionally, other studies have revealed the prognostic value of the co-operative expression of SLC7A5 and SLC3A2 in transitional cell carcinoma [16], squamous cell carcinoma of the lung [17] and pulmonary adenocarcinoma [18].

SLC1A5 is a sodium-dependent transporter which regulates the transport of neutral amino acids; alanine, serine, cysteine, threonine, and glutamine. It has been suggested that glutamine influx by SLC1A5 triggers essential amino acid entry via SLC7A5 exchanger. These exchange mechanisms balance the cytoplasmic pool of the amino acids without expending energy and therefore give a strong advantage for cancer cell proliferation [19]. SLC1A5 is overexpressed in several human neoplasms [20-22,7]. In both esophageal squamous cell carcinoma [23] and laryngeal squamous cell carcinoma [24], co-expression of SLC7A5 and SLC1A5 is associated with a worse prognosis compared to expression of a single transporter, suggesting a potential functional coupling, at transportomic level, which supports tumour aggressiveness and progression.

Whilst approximately fourteen SLC members accept glutamine a substrate, SLC1A5 transporter and SLC7A5/SLC3A2 heterodimeric antiporter have a higher affinity for their substrates that confers them the potential to support the growth and survival of the aggressive tumour types.

In this study we sought to understand the prognostic impact of the co-operative expression of the transporters, SLC1A5, SLC7A5 and SLC3A2, in a large BC cohort. We stratified patients in to clusters based on assessment of SLC1A5, SLC7A5 and SLC3A2 protein expression, to

determine their clinicopathological and prognostic value with emphasis on the different molecular classes.

MATERIAL AND METHODS

Patient Cohort

This study evaluated a well-characterised cohort of early stage, primary operable, invasive BC patients aged ≤ 70 years. Patients (n=2,248) presented at Nottingham City Hospital between 1989 and 2006. Patient management was uniform and based on tumour characteristics by Nottingham Prognostic Index (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 case the patients were premenopausal]. Conversely, classical cyclophosphamide, methotrexate and 5-flurouracil (CMF) were used if the patients were ER negative and fit enough to receive chemotherapy. None of the patients in this study received neoadjuvant therapy. Clinical history, tumour characteristics, information on therapy and outcomes are prospectively maintained. Outcome data included development and time to distant metastasis (DM) and breast cancer specific survival (BCSS). The clinicopathological parameters for the BC series is summarized in (Supplementary Table 1).

Western Blotting

Primary antibody specificity of SLC1A5 (1:250; HPA035240, Sigma-Aldrich, UK), SLC7A5 (1:200; EPR17573, Abcam, UK) and SLC3A2 (1:2000; HPA017980, Sigma-Aldrich, UK) were validated using Western blotting in BC cell line lysates (American Type Culture Collection; Rockville, MD, USA) as previously described [15,14]. A single band for all biomarkers was visualized at the correct predicted size (75 KDa, 40 KDa and 80 KDa) respectively.

Tissue arrays and Immunohistochemistry

Tumour samples of 0.6mm cores were arrayed [25,14] and immunohistochemical (IHC) staining was performed on 4μm TMA sections using the Novolink polymer detection system (Leica Biosystems, RE7150-K), as previously described [5].

Stained TMA sections were scanned using high resolution digital images (NanoZoomer; Hamamatsu Photonics, Welwyn Garden City, UK), at x20 magnification. Evaluation of staining for SLC1A5, SLC7A5 and SLC3A2 was based on a semi-quantitative assessment of cores' digital images using a modified histochemical score (H-score) which includes an assessment of both the intensity and the percentage of stained cells [26]. Staining intensity was subjectively assessed as follows 0, negative; 1, weak; 2, medium; 3, strong and the percentage of the positively stained tumour cells was. 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.

Immunhistochemical staining and dichotomization of the other biomarkers included in this study were as per previous publication [27]. ER and PgR positivity was defined as ≥1% staining. Immunoreactivity of HER2 was scored using standard HercepTest guidelines (Dako). Chromogenic *in situ* Hybridization (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 [28]. BC molecular subtypes were defined, based on tumour IHC profile and the Elston-Ellis [29] mitotic score as: ER+/HER2- Low Proliferation (mitotic score 1), ER+/HER2- High Proliferation (mitotic score 2 and 3), HER2-positive class: HER2+ regardless of ER status, TN: ER-, PgR- and HER2- [30].

 Cluster analysis

Six cluster validity indices were calculated to determine the optimal number of clusters as previously described [32]. Two algorithms, partitioning around medoids (PAM) and K-means

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were used to cluster tumours into the optimal number of clusters based on the H-score of SLC1A5, SLC7A5 and SLC3A2 as previously described [31].

K-means clustering is based on the initial setting of the cluster assignments, and for this study, we used a fixed initialization obtained with hierarchical clustering. PAM is dependent on finding the k-representative objects (the so-called medoids) among the observations of the data set. K-clusters are constructed, after finding a set of k-mediods, through assigning each observation to its nearest medoid. Principle component analysis was applied to transform the original data space. Biplots were generated by plotting the points at their projected position on axes of the first and second principal components [32].

Statistical analysis

Statistical analysis was performed using SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA). The Chi-square test was performed for inter-relationships between categorical variables. Survival curves were analyzed by Kaplan-Meier with Log Rank test. This was performed with the BC specific death, those who died of other causes, alive and lost to follow-up were censored. Cox's proportional hazard method was performed for multivariate analysis to identify the independent prognostic factors. P-values were adjusted using Bonferroni correction for multiple testing. A p-value <0.05 was considered significant. The study endpoints were 5-year BCSS or distant metastasis free survival (DMFS). This study complied with reporting recommendations for tumour marker prognostic studies (REMARK) criteria [33].

Results

Solute carrier protein expression in breast cancer

Expression of the three solute carriers was predominantly in the membrane of the invasive BC cells, with intensity levels varying from absent to high (Figures 1A-C).

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, acceptable classification was applied by clustering the tumours into three groups. An appropriate separation between clusters was attained as indicated from the biplots (Supplementary Figure 1A-B). The final three clusters were obtained from the overlap of the two clustering algorithms. The three clusters were characterised as follows: Low SLCs (SLC1A5$^-$/SLC7A5$^-$/SLC3A2$^-$), High SLC1A5 (SLC1A5$^+$/SLC7A5$^-$/SLC3A2$^-$) and High SLCs (SLC1A5$^+$/SLC7A5$^+$/SLC3A2$^+$) (Figure 2).

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 larger tumour size, higher histologic grade, poor NPI and lymph node stage 3 (p<0.001), whereas tumours in the Low SLCs cluster were associated with good prognostic factors (Table 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 2). Tumours with HER2 positivity were more frequent in High SLCs and High SLC1A5 clusters compared with Low SLCs (p<0.001, Table 2).

The prevalence of the three clusters in different BC subtypes was varied, where the Low SLCs cluster was primarily observed in 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 1).

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 3A). Within the different molecular subtypes, the High SLCs cluster was predictive of shorter BCSS but only in ER+ high proliferation/luminal B tumours (p=0.003, Figure 3B). There was no association between the SLC clusters and outcome in ER+ low proliferation/luminal A, HER2+ or TN tumours. (Figure 3C-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 4A). Similarly, the result is only observed in the ER+ high proliferation/luminal B Class (p=0.01, Figure 4B). There was no association with the SLC clusters in predicting DMFS in ER+ low proliferation/luminal A, HER2+ or TN subtypes (Figure 4C-E). In multivariate Cox regression analysis, the SLCs clusters remained a predictor of shorter BCSS independent of tumour size, histologic grade and lymph node stage in unselected tumours (p=0.001, Table 3). The result remained significant in the ER+ high proliferation /luminal B tumours only (p=0.01, Supplementary Table 2).

SLC clusters and other molecular biomarkers

The expression of c-MYC protein was primarily observed in the High SLCs cluster (Table 2, p<0.001). This observation remained significant only in ER+ High proliferation/luminal B tumours (p=0.01, data not shown). Furthermore, patients in High SLCs cluster with c-MYC positive tumours showed shorter BCSS compared to those tumours that did not express c-MYC (Supplementary Figure 2A, B). Low PIK3CA expression was predominant in low SLCs cluster, while High SLC1A5 and High SLCs clusters were mainly associated with positive PIK3CA expression (Table 2, p<0.001). The association with poor outcome was only observed in patients with PIK3CA positive expression and not with the cases who did not express this protein (Supplementary Figure 3A, B). The proliferation marker, Ki67, was also significantly expressed in High SLC1A5 and High SLCs clusters (Table 2, p<0.001) and patients belonging to these clusters showed shorter BCSS when accompanied with the positive expression of Ki67 (Supplementary Figure 4A, B). Conversely, mTORC1 was mainly observed in Low SLCs cluster (Table 2, P<0.001) and the association with poor outcome showed no difference between mTORC+ and mTORC- tumours (Supplementary Figure 5A, B).

Discussion

BC is a heterogeneous disease with various molecular subtypes differing in terms of tumour biology and patient outcome [34]. In addition, different subtypes exhibit disparity in their metabolic pathways, where ER-negative tumours can be distinguished from the ER-positive based on their metabolic signatures. ER+/luminal tumours which comprise the majority of BC cases (approximately 75%) remain heterogeneous in terms of mortality rates and disease prognosis [35,36]. There is thus a clear need to understand the biology of BC which then can help in exploring methods of prognostic and predictive stratification as well as identifying potential novel therapeutic targets.

Currently, the prognostic stratification of invasive BC is mainly based on the known clinicopathological parameters, such as tumour grade, stage, and hormone receptor status. Although a large number of biomarkers have been suggested to be prognostic markers in this tumour, other studies have demonstrated that combination of prognostic indicators is superior to recruiting single markers [5,37,38]. Therefore, applying cluster analysis is not only competent for identifying biologically relevant groups of biomarkers, it can also allow more detailed prognostication and potentially provide optimal treatment strategies with the objective of improving patient outcome and quality of life.

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 [39]. We have recently demonstrated that the solute carriers SLC7A5 and SLC3A2 are associated with poor prognosis particularly in the ER+ high proliferation subtype of BC [14,15] and the co-expression of SLC7A5 and SLC3A2 in TNBC has been previously established [40]. However, studies that address the prognostic significance of the co-operative

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expression of those two proteins alongside the glutamine transporter SLC1A5 in BC, and their influence in the other molecular subtypes, remain limited.

This study has revealed differential expression levels of the three solute carriers 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 of any transporter proteins, High SLC1A5 cluster was derived by the high expression of SLC1A5 only and the High SLCs cluster included tumours with high expression of all three transporters. 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, including larger tumour size, higher grade and poor NPI. 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 breast cancer risk [41]. It is noteworthy, that SLC7A5 and SLC3A2 can be expressed independently. Furthermore, the single expression of SLC3A2, but not SLC7A5, can predict the poor patient outcome (data not shown). This is can be attributed to the extra role played by SLC3A2 in favoring cancer growth, as it can modulates integrin-

induced signal transduction which derives malignant tumour cells' behavior including cell spreading and migration [42].

With respect to the ER+ 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. This is most likely due to their heavier energy and nutrient requirements for cell survival and proliferation. Furthermore, we and others have previously demonstrated that SLC7A5 and SLC3A2 are significantly correlated with the proliferative marker Ki67 in thymic carcinomas [43], tongue cancer [44], neuroendocrine carcinoma of the lung [45], non-small cell lung cancer [46] and BC [14,15,40]. In this study, Ki 67 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 [6] and Furuya et al [40], 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 utilization of the necessary nutrients via up−regulation of cellular transporters and enzymes supporting tumour growth and giving rise to aggressive cancer phenotypes [47,9]. Previous studies have showed that MYC controls the expression of SLC1A5 and SLC7A5 via binding to specific promoters and enhancing protein transcription [48,8]. Upon glutamine deprivation, Activation Factor 4 (ATF4), in co-ordination with MYC, activates the expression

of both transporters directly to maintain intracellular levels of amino acids [49,50,7]. In addition, SLC7A5 can control MYC expression and constitute a positive feedback loop mechanism to encourage essential amino acid transport and tumorigenesis [51,52]. The current study revealed that MYC not only regulates the concurrent expression of the three studied solute carriers, it also conferred poorer outcome, in patients with tumours in the High SLCs cluster, compared to lack of its expression, concluding that this cluster might play a role in endocrine therapy resistance as previous work by our group showing that MYC overexpression confers resistance to endocrine therapy [27].

Mutations in PIK3CAis known to have a relation to metabolic alterations in cancer cells. Hao et al, have demonstrated that PIK3CA mutations in colorectal cancer increase glutamine dependency by upregulating glutamate pyruvate transaminase 2 (GPT2), which enhances glutamine conversion to αkG to replenish TCA and assist ATP production through an ATF4-dependent mechanism [53]. Recent work carried out by our group has shown that PIK3CA is the driver for glutaminase 1 expression, which converts glutamine to glutamate, 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 and is therefore a potential driver of shorter BCSS in these clusters.

Previous research has detailed the role of amino acids in activating mTORC1, which in turn controls cell proliferation and prevent apoptosis in cancer cells [2]. This study however showed that mTORC1 was mainly expressed in Low SLCs cluster and its expression did not make a difference in patient outcome compared to its absence. This can be explained by a previous study, which reported that phosphorylation of mTORC1 at ser (2448), which included in this study, is stimulated by growth factors and it was mutually exclusive with mTORC1 phosphorylated at thr (2446), which is regulated by amino acids [54]. In addition, Figueiredo et al concluded that mTORC1 phosphorylated at Ser (2448) is an inadequate

measure for mTORC1 kinase activity and the alternative should be the downstream and effector proteins, such as p70S6K [55].

With the increasing number of treatment strategies available for cancer patients it is critical to find effective approaches which can support personalization of care and allow tailored treatment plan for BC patients, taking into consideration its molecular subtypes. Inhibition of SLC1A5, via the inhibitor l-γ-glutamyl-p-nitroanilide (GPNA), decreases the growth of various cancer cell lines including TN BC [56,7,57]. Blocking SLC7A5, using 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), efficiently decreased colony formation of MDA-MB-231 TNBC cells [58]. In addition, the selective SLC7A5 inhibitor (JPH203/KYT-0353) is undergoing Phase I clinical trials (UMIN000016546) as a novel adjuvant treatment approach for solid tumours including BC [59,60]. Although the consequences of blocking the action of these solute carriers in ER+ high proliferation tumours remain undetermined, our data suggest that the synergistic lethality targeting the three solute carriers may prove to be more effective in BC treatment.

Conclusion

This study reveals that the co-operative expression of SLC1A5, SLC7A5 and SLC3A2 was associated with the poor prognostic characteristics and poor patient outcome particularly in the ER+ high proliferation/luminal B subtype. Therefore, elaboration in understanding the biological diversity of BC is essential, with the linked development of classification strategies suitable for clinical use. Further functional assessment is essential to reveal the specific role played by solute carrier co-expression in the highly proliferative BC subclass of ER+ disease.

Table 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
Tumour size					
<2cm	583 (60.9)	262 (27.4)	112 (11.7)	67.5 (2.2×10^{-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 (3.7×10^{-90})	**<0.0001**
2	421 (64.5)	205 (31.4)	27 (4.1)		
3	232 (30.8)	256 (34.0)	266 (35.3)		
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 (1.06×10^{-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)		
ER+/HER2- High Proliferation	88 (34.0)	112 (43.2)	59 (22.8)		
Triple Negative	59 (22.2)	54 (20.3)	153 (57.5)	504.4 (9.4×10^{-106})	**<0.0001**
HER2+	55 (39.3)	51 (36.4)	34 (24.3)		

Table 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 (%)	χ^2 (p-value)	Adjusted p-value
ER					
Negative	80 (23.3)	77 (22.4)	186 (54.2)	418.6	
Positive	825 (60.4)	432 (31.6)	109 (8.0)	(1.2×10^{-91})	**<0.0001**
PR					
Negative	521 (50.6)	253 (24.6)	255 (24.8)	114.8	
Positive	358 (57.2)	237 (37.9)	31 (5.0)	(1.3×10^{-25})	**<0.0001**
HER2					
Negative	809 (54.3)	433 (29.1)	247 (16.6)	12.2	
Positive	55 (39.3)	51 (36.4)	34 (24.3)	(0.002)	**0.004**
Triple Negative					
No	839 (59.1)	446 (31.4)	134 (9.4)	369.1	
Yes	59 (22.1)	55 (20.6)	153 (57.3)	(7.1×10^{-81})	**<0.0001**
MYC					
Negative	270 (59.7)	103 (22.8)	79 (17.5)	44.6	
Positive	34 (38.6)	10 (11.4)	44 (50.0)	(2.0×10^{-10})	**<0.0001**
PIK3CA					
Negative	94 (76.4)	14 (11.4)	15 (12.2)	24.8	
Positive	182 (50.7)	81 (22.6)	96 (26.7)	(0.000004)	**<0.0001**
Ki67					
Negative	142 (78.5)	22 (12.2)	17 (9.4)	50.5	
Positive	159 (46.5)	83 (24.3)	100 (29.2)	(1.08×10^{-11})	**<0.0001**
mTORC1					
Negative	141 (48.3)	66 (22.6)	85 (29.1)	21.1	
Positive	143 (66.5)	43 (20.0)	29 (13.5)	(0.00002)	**<0.0001**

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

Variable	Multivariate		Univariate	
	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
SLC clusters	1.4 (1.1-1.7)	**0.001**	2.1 (1.8-2.6)	**5.9×10^{-17}**
Tumour Size	1.3 (0.9-1.8)	0.13	2.8 (2.3-3.5)	**1.1×10^{-25}**
Lymph node stage	2.1 (1.7 -2.5)	**1.9×10^{-13}**	2.4 (2.2-2.8)	**2.2×10^{-50}**
Grade	4.2 (2.8-6.2)	**1.5×10^{-12}**	4.3 (3.5-5.4)	**2.5×10^{-42}**

Figure legends

Figure 1. SLCs protein expression in invasive breast cancer cores. A) Represent Low SLCs cluster; from left to right, negative immunohistochemical (IHC) expression in SLC1A5, SLC7A5 and SLC3A2. B) Represent High SLC1A5 cluster; form left to right, positive IHC expression in SLC1A5 and negative IHC expression in SLC7A5 and SLC3A2. C) Represent High SLCs cluster; from left to right, positive IHC expression in SLC1A5, SLC7A5 and SLC3A2.

Figure 2. 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.

Figure 3. SLC clusters and breast cancer patient outcome in A) All cases, B) ER+- High Proliferation tumours, C) ER+- Low Proliferation tumours, D) HER2+ tumours, E) Triple negative tumours.

Figure 4. SLC clusters vs DMFS in A) all cases, B) ER+- High Proliferation tumours, C) ER+- Low Proliferation tumours, D) HER2+ tumours, E) Triple negative tumours.

Supplementary Figure 1. 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.

Supplementary Figure 2. SLC clusters and breast cancer patient outcome in A) MYC- tumours, B) MYC+ tumours.

Supplementary Figure 3. SLC clusters and breast cancer patient outcome in A) PIK3CA- tumours, B) PIK3CA+ tumours.

Supplementary Figure 4. SLC clusters and breast cancer patient outcome in A) Ki67- tumours, B) Ki67+ tumours.

Supplementary Figure 5. SLC clusters and breast cancer patient outcome in A) mTORC1-

tumours, B) mTORC1+ tumours.

Compliance with ethical standards

Conflicts of interest

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

Research involving human participants and/or animals

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. Release of data was also pseudo-anonymised as per the UK Human Tissue Act regulations. This article does not contain any studies with animals performed by any of the authors.

Informed consent

All tissue samples from Nottingham used in this study were pseudo-anonymised and collected prior to 1st September 2006; therefore under the UK Human Tissue Act informed patient consent was not needed.

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	SLC1A5	SLC7A5	SLC3A2
A Low SLCs			
B High SLC1A5			
C High SLCs			
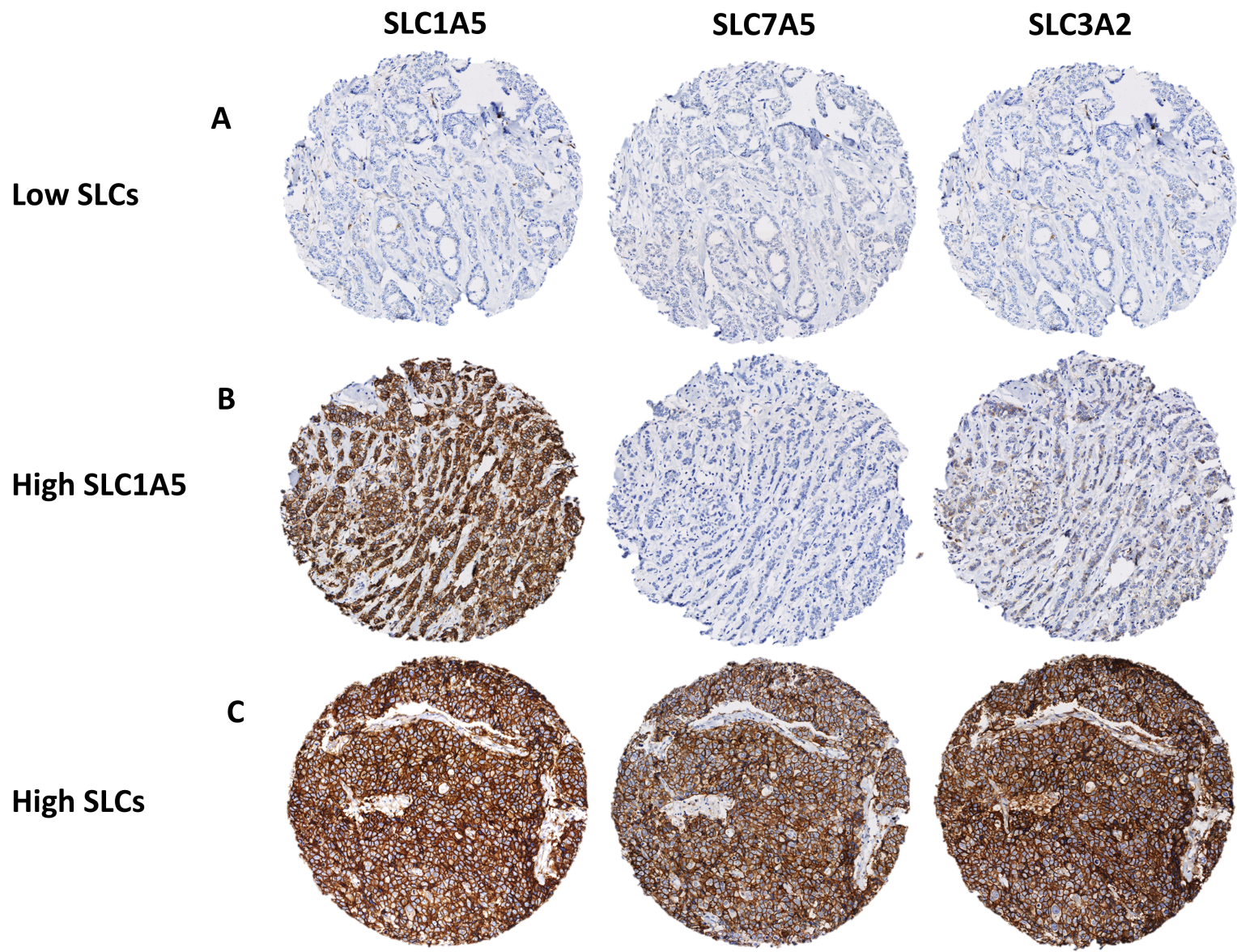
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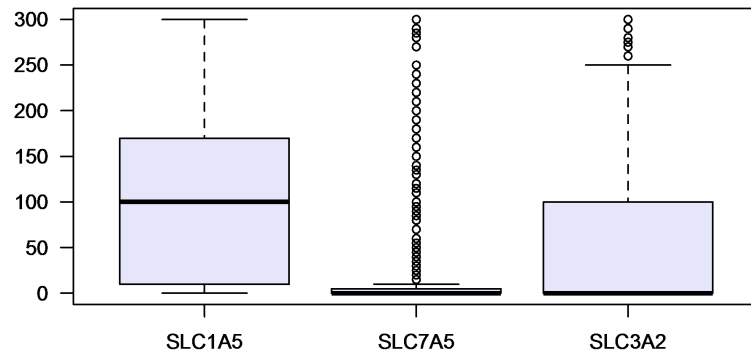
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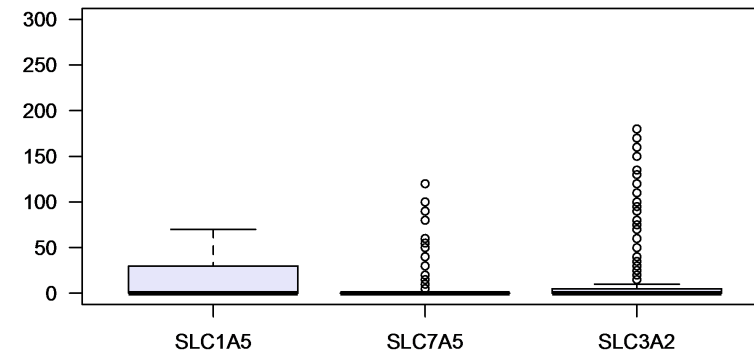
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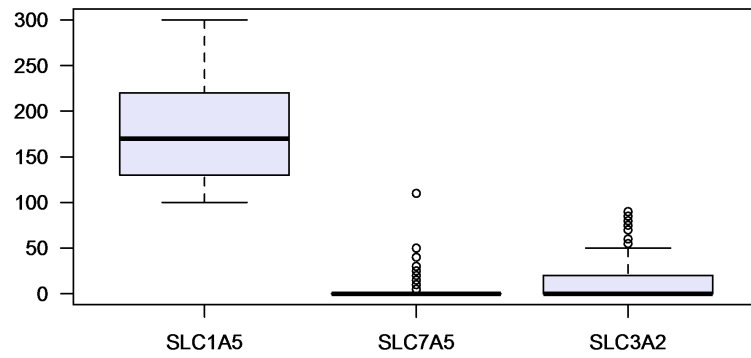
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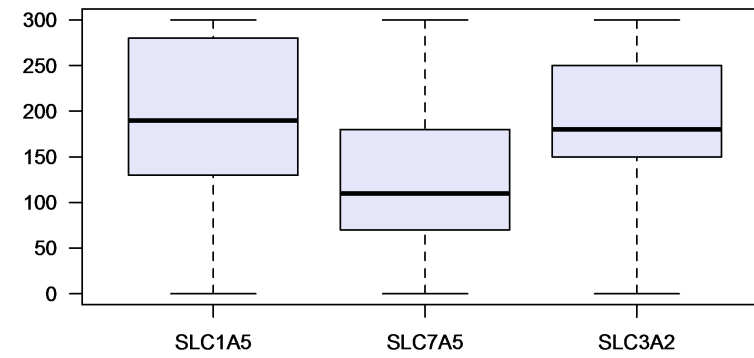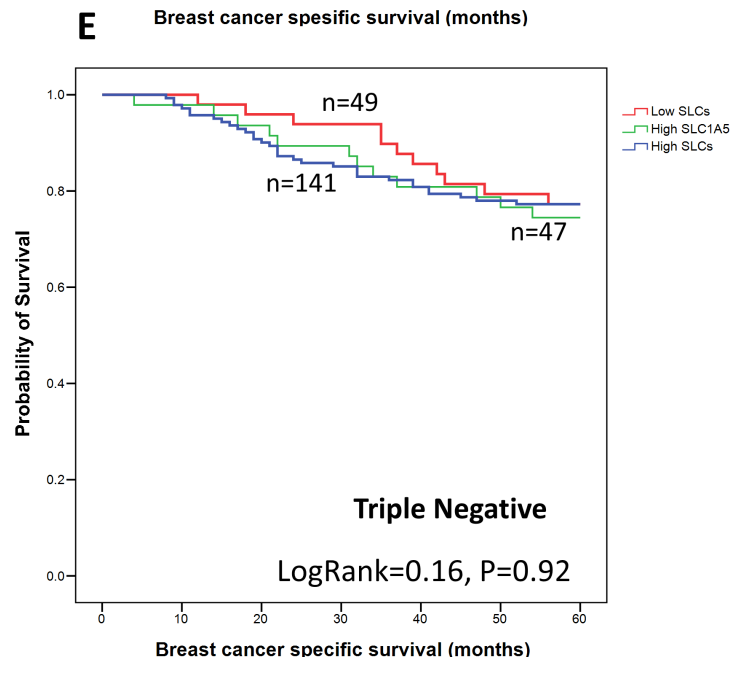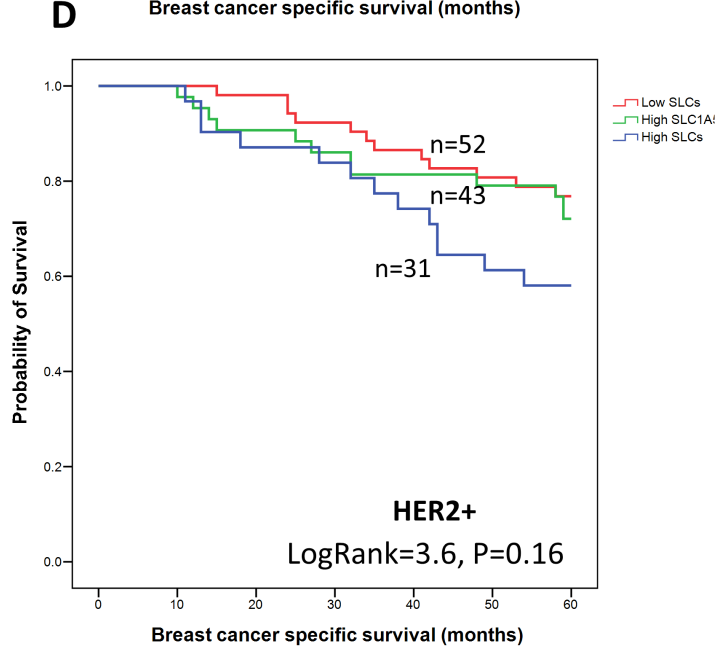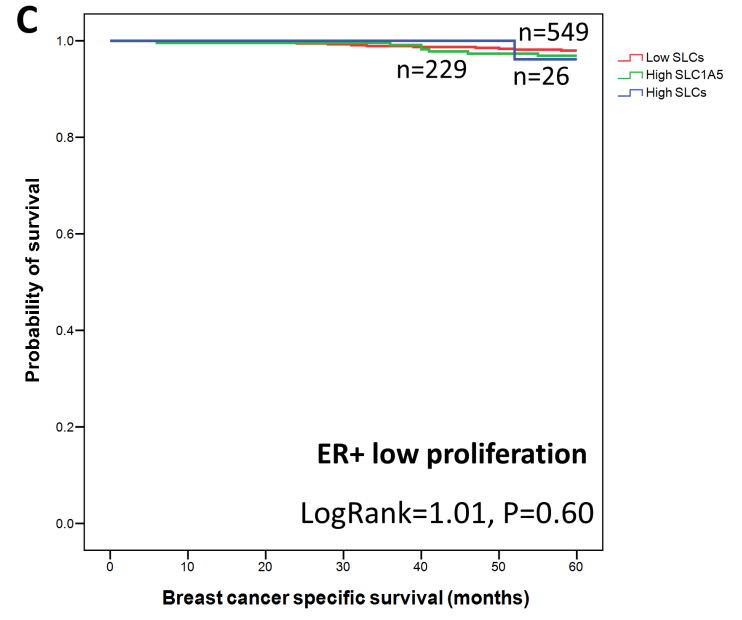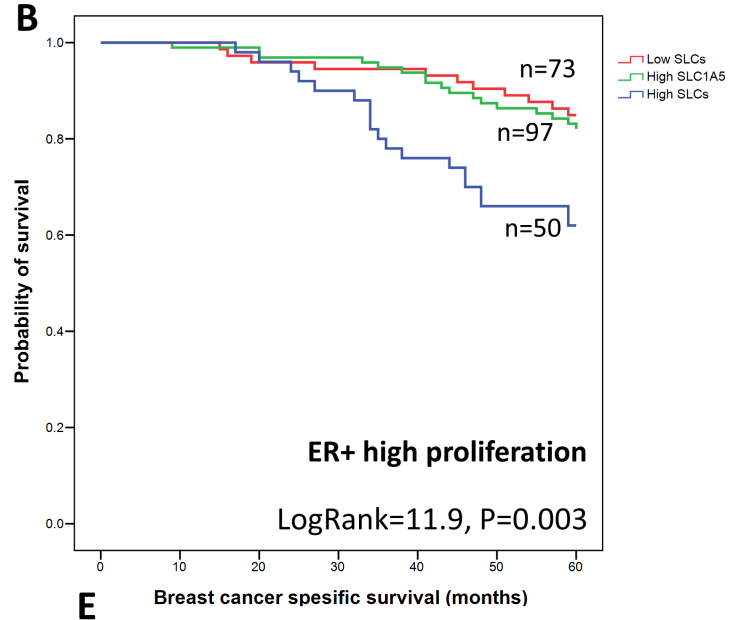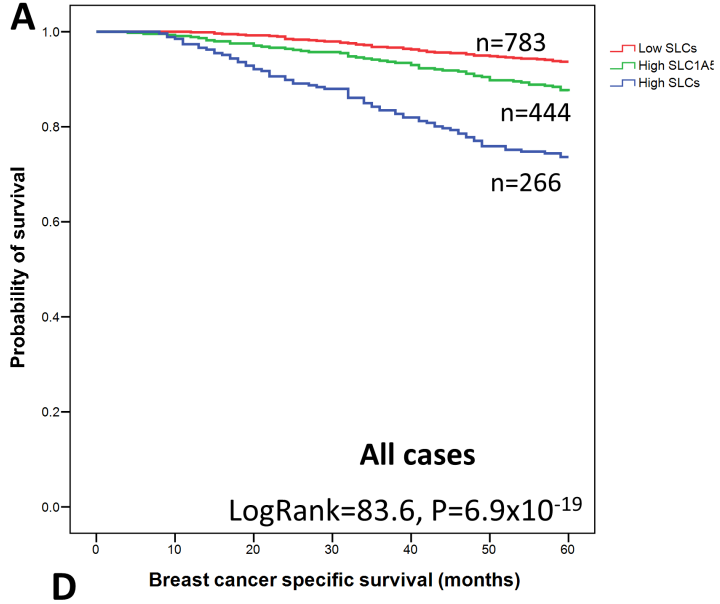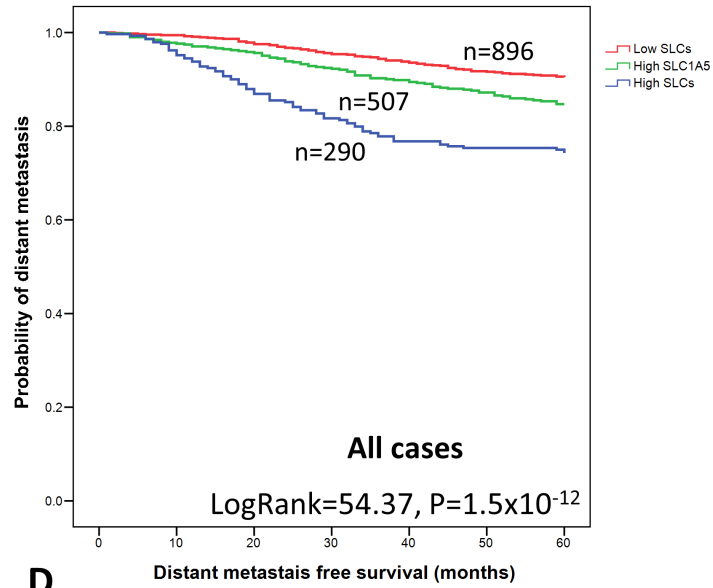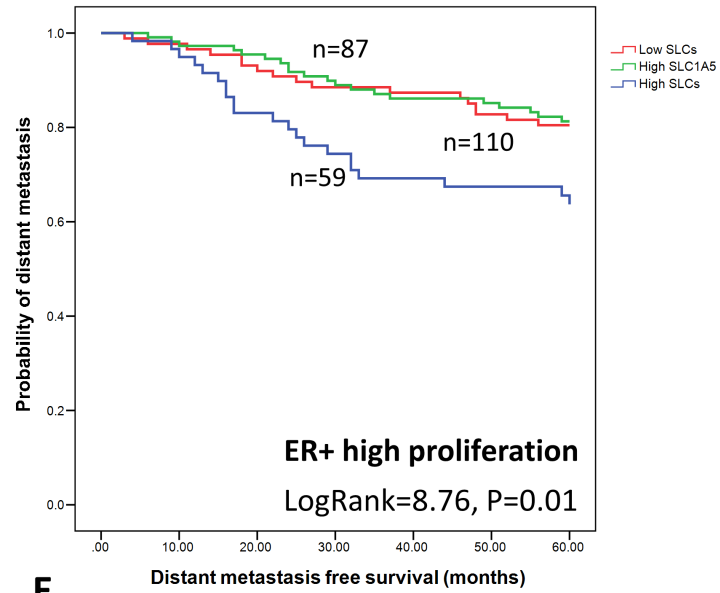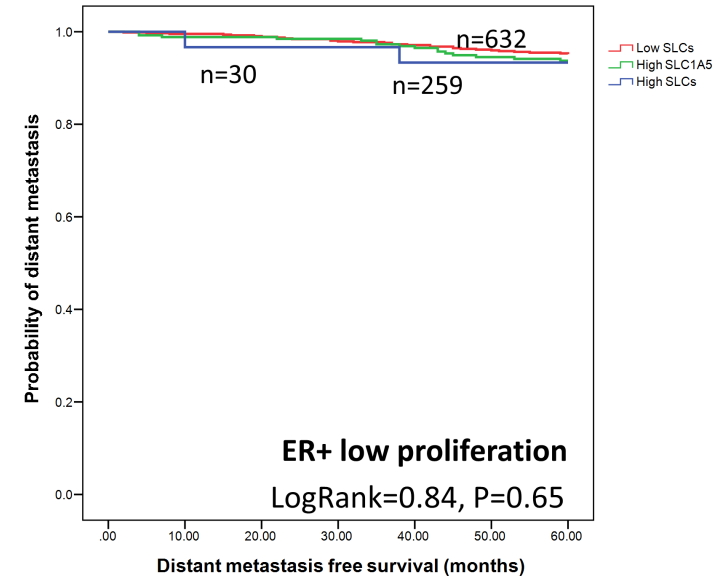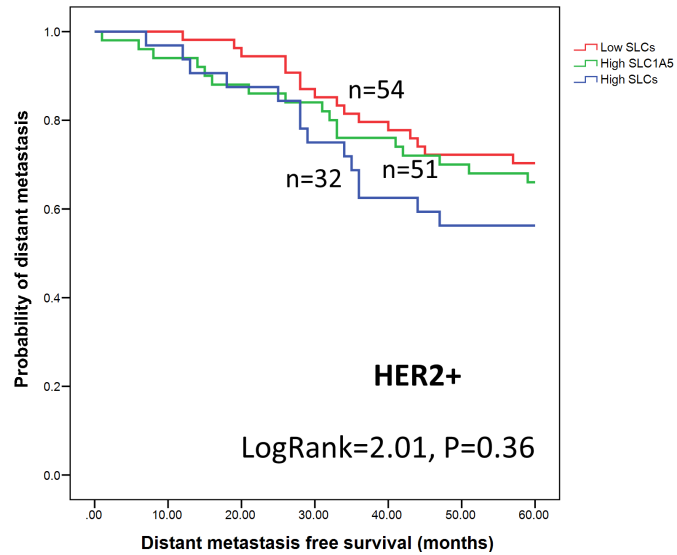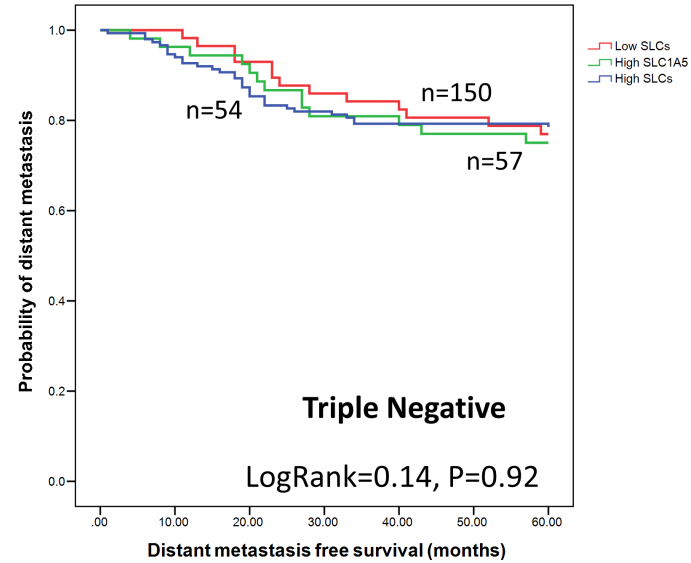
Supplementary Table 1. Clinicopathological parameters of the METABRIC and Nottingham breast cancer series.

Parameters	Nottingham TMA series n (%)
Age	
≥ 50 years	1784 (67.4)
< 50 years	864 (32.6)
Tumour size	
≥ 2cm	1176 (44.4)
< 2cm	1471 (55.6)
Grade	
1	421 (15.9)
2	988 (37.4)
3	1235 (46.7)
Tumour type	
Ductal (including mixed)	2257 (85.3)
Lobular	221 (8.4)
Medullary-like	39 (1.5)
Miscellaneous	16 (0.6)
Special type	113 (4.2)
Vascular Invasion	
Definite	833 (31.5)
Negative/Probable	1808 (68.5)
Lymph node stage	
1	674 (61.0)
2	341 (30.8)
3	91 (8.2)
Follow-up Status	
Alive	1679 (63.4)
Died from Breast Cancer	647 (24.4)

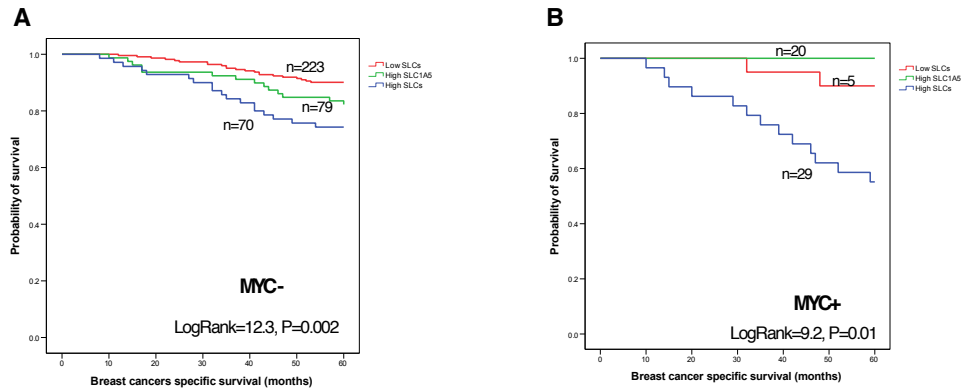|	
Died from other causes	321 (12.2)
ER	
Negative	570 (21.6)
Positive	2067 (78.4)
PgR	
Negative	1047 (41.4)
Positive	1483 (58.6)
HER2	
Negative	2297 (88.5)
Positive	298(11.5)

Supplementary table 2: multivariate analysis of prognostic variables and SLC clusters, in relation to BCSS, in different BC subtypes.

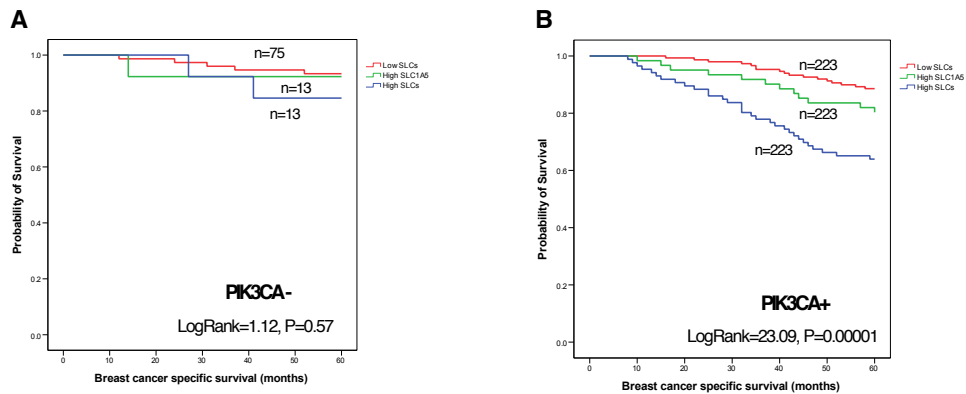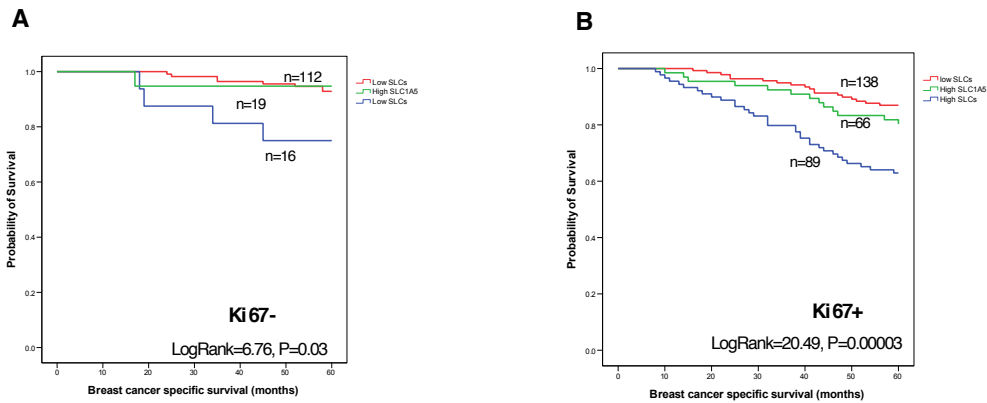| Variable	ER-low proliferation		ER-High proliferation		Triple Negative		HER2+	
	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
Clusters	1.11 (0.49-2.45)	0.80	1.38 (1.01-1.90)	**0.01**	0.97 (0.70-1.36)	0.89	1.40 (0.92-2.12)	0.11
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
Stage	3.82 (1.93-7.54)	**0.0001**	1.66 (1.14-2.41)	**0.007**	2.43 (1.72-3.44)	**5.2×10^{-7}**	1.88 (1.18-2.98)	**0.007**

Supplementary Figure 1. 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.
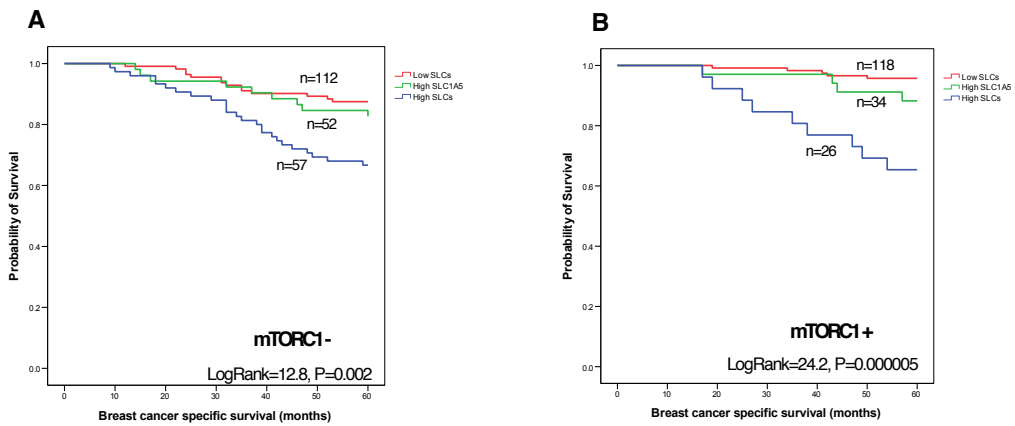
Supplementary Figure 2. SLC clusters and breast cancer patient outcome in A) MYC-tumours, B) MYC+ tumours.

Supplementary Figure 3. SLC clusters and breast cancer patient outcome in A) PIK3CA-tumours, B) PIK3CA+ tumours.

Supplementary Figure 4. SLC clusters and breast cancer patient outcome in A) Ki67-tumours, B) Ki67+ tumours.

Supplementary Figure 5. SLC clusters and breast cancer patient outcome in A) mTORC1-tumours, B) mTORC1+ tumours.