Karger OPEN ACCESS

Pathobiology

Pathobiology , DOI: 10.1159/000539564
Received: November 20, 2023
Accepted: May 25, 2024
Published online: June 11, 2024

ATF4 as a Prognostic Marker and Modulator of Glutamine Metabolism in Estrogen Receptor Positive Breast Cancer

Patel R, Alfarsi LH, El-Ansari R, Masisi BK, Erkan B, Fakroun A, Ellis IO, Rakha EA, Green AR

ISSN: 1015-2008 (Print), eISSN: 1423-0291 (Online)
https://www.karger.com/PAT
Pathobiology

Disclaimer:
Accepted, unedited article not yet assigned to an issue. The statements, opinions and data contained in this publication are solely those of the individual authors and contributors and not of the publisher and the editor(s). The publisher and the editor(s) disclaim responsibility for any injury to persons or property resulting from any ideas, methods, instructions or products referred to the content.

Copyright:
This article is licensed under the Creative Commons Attribution 4.0 International License (CC BY) (http://www.karger.com/Services/OpenAccessLicense). Usage, derivative works and distribution are permitted provided that proper credit is given to the author and the original publisher.

© 2024 The Author(s). Published by S. Karger AG, Basel

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

ATF4 as a Prognostic Marker and Modulator of Glutamine Metabolism in Estrogen Receptor Positive Breast Cancer

Roshni Patel[1], Lutfi H. Alfarsi[1], Rokaya El-Ansari[1], Brendah K. Masisi[1], Busra Erkan[1], Ali Fakroun[1], Ian O. Ellis[1,2], Emad A. Rakha[1,2] and Andrew R. Green[1*]

[1] Nottingham Breast Cancer Research Centre, Academic Unit for Translational Medical Sciences, School of Medicine, University of Nottingham Biodiscovery Institute, University Park, Nottingham NG7 2RD.

[2] Cellular Pathology, Nottingham University Hospitals NHS Trust, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB.

***Correspondence:**
Dr. Andrew R. Green (ORCID ID 0000-0002-0488-5913). Nottingham Breast Cancer Research Centre, Academic Unit for Translational Medical Sciences, School of Medicine, University of Nottingham Biodiscovery Institute, University Park, Nottingham NG7 2RD
Tel: +44 115 8231407
Email: andrew.green@nottingham.ac.uk

Running head: Activating Transcription Factor 4 in breast cancer

Keywords: activating-transcription factor 4, breast cancer, glutamine metabolism, amino-acid transporters

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

ABSTRACT

Introduction: ATF4, a stress-responsive transcription factor that upregulates adaptive genes, is a potential prognostic marker and modulator of glutamine metabolism in breast cancer. However, its exact role remains to be elucidated.

Methods: ATF4 expression was evaluated at genomic and transcriptomic levels using METABRIC (n=1980), GeneMiner (n=4712) and KM-Plotter datasets. Proteomic expression was assessed via immunohistochemistry (n=2225) in the Nottingham Primary Breast Cancer Series. ATF4 genomic copy number (CN) variation and mRNA/protein in association with clinicopathological parameters, amino acid transporters (AATs), and patient outcome was investigated.

Results:
Genomic, transcriptomic, and proteomic overexpression of ATF4 was associated with more aggressive ER-negative tumours. ATF4 mRNA and protein expression were significantly associated with increased expression of glutamine related AATs including SLC1A5 ($p<0.01$) and SLC7A11 ($p<0.02$). High ATF4 and SLC1A5 protein expression was significantly associated with shorter breast cancer-specific survival ($p<0.01$), especially in ER+ tumours ($p<0.01$), while high ATF4 and SLC7A11 protein expression was associated with shorter survival ($p<0.01$).

Conclusion: These findings suggest a complex interplay between ATF4 and AATs in breast cancer biology and underscore the potential role for ATF4 as a prognostic marker in ER+ breast cancer, offering a unique opportunity for risk stratification and personalised treatment strategies.

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

INTRODUCTION

Metabolic reprogramming is a well-documented 'cancer hallmark' that allows cancer cells to produce energy, proliferate rapidly, metastasise, and survive in harsh tumour microenvironments [1].

Glutamine is the second primary metabolite, after glucose, to support cancer cell proliferation [2]. The importance of glutamine is highlighted throughout its numerous functions, namely; facilitating macromolecule synthesis of nucleotides, lipids and proteins [3] and supporting redox balance [4]. Furthermore, the metabolism of glutamine via glutaminolysis aids the replenishment of intermediates within the tricarboxylic acid (TCA) cycle [5].

Numerous studies support the role of amino acid transporters (AATs) in breast cancer (BC). Solute Carriers (SLC): SLC1A5, SLC3A2, and SLC7A5, which have high affintiy to glutamine, are associated with the aggressive nature of ER-positive breast cancer [6-9]. However, the regulation of AAT expression associated with glutamine transport within BC has yet to be explored. In this respect, there is a need to further explore the specific mechanisms behind transporter expression and consequent effects in BC. Activating Transcription Factor 4 (ATF4/CREB-2) has previously been implicated in the control of AAT within autophagy deficit tumour cells [10].

ATF4 is a stress responsive gene, belonging to the ATF/cyclic adenosine monophosphate response element binding protein (ATP/CREB) family [11]. *ATF4* gene, also known as Cyclic AMP-Responsive Element-Binding Protein 2 (CREB-2), is located on *22q13.1*, a region frequently associated with loss of heterozygosity in BC [12]. ATF4 protein is a DNA binding transcription factor and also involved in protein-protein intereractions. It consists of a DNA binding, dimersation and a c-terminal regulatory domains. ATF4 forms part of the Integrated Stress Response (ISR) pathway, underlying the downregulation of protein synthesis during cellular stress and amino acid starvation [13]. Previous studies by Ye *et al*. revealed upregulation of the ATF4 ISR pathway within tumours; implicating ISR and potential ATF4 necessity in cancer cell adaptation to tumour microenvironment [14].

There is increasing evidence that ATF4 upregulation is vital to long-term cell survival through promoting the expression of adaptive genes. ATF4 regulates the expression of genes associated with metabolism, oxidative stress, protein synthesis and amino acid transport [15]. Furthermore, ATF4 is linked to angiogenesis [16] and metastasis [17, 18]. In this respect, ATF4 expression is shown to be advantageous to cancer cells, enabling cell proliferation and preservation despite cellular stresses caused by this heightened activity.

Whilst ATF4 is associated with a poor patient survival in triple negative BC [19], the importance of ATF4 as a prognostic biomarker as well as its specific role on AATs in ER+ BC remains undetermined.

Therefore, it is hypothesised that high ATF4 expression regulates glutamine-associated AATs in aggressive breast cancer subtypes. This study aims to assess ATF4 expression and its prognostic value within BC, in association with gluatmine-assocated AAT expression.

MATERIAL AND METHODS

ATF4 genomic and transcriptomic analysis

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset was used to generate data on genomic and transcriptomic profiling in n=1,980 breast cancer cases using the Affymetrix SNP 6.0 and Illumina HT-12 v3 platforms. The association between *ATF4* gene copy

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

number variations (CNV) and mRNA expression were investigated. *ATF4* mRNA expression was dichotomised into a high and low expression group using median cut-off log2 intensity value of 7.53, subsequent associations between expression groups and various clinicopathological parameters, molecular BC subtypes, and patient outcome were subsequently evaluated. The online breast cancer molecular datasets, BC Gene Expression Miner v4.4 (n=4712) (http://bcgenex.centregauducheau.fr) [20] and the KM plotter (http://kmplot.com) [21] were used as an external validation datasets. *ATF4* gene expression was correlated with gene expression of AAT showing high affinity for glutamine: *SLC1A5*, *SLC3A2*, *SLC3A2*, *SLC6A14*, *SLC6A15*, *SLC6A19*, *SLC7A5*, *SLC7A6*, *SLC7A8*, *SLC7A8*, *SLC7A9*, *SLC7A11*, *SLC28A1, SLC28A2, SLC28A3, SLC28A5, SLC28A7, SLC28A8*.

ATF4 proteomic analysis

Patient cohort

A cohort of n=1341 patients younger than 70 years, with early-stage operable BC were enrolled into the Nottingham Primary Breast Carcinoma Series and presented to Nottingham City Hospital, UK between 1986 and 2006. Patients were managed in accordance with a uniform protocol. Survival data was maintained on a prospective basis which included breast cancer specific survival (BCSS) defined as the time in months from primary surgery to the time of BC related death. Full patient characteristics of the cohort are summarised in Supplementary Table 1. Protein expression for ER, PR, HER2, and amino acid transporters (AAT) with high affinity for glutamine: SLC1A5, SLC3A2, SLC7A5, SLC7A8, SLC7A11, SLC28A2 were previously determined [9, 22, 23]. BC molecular subtypes were defined, based on tumour IHC profile and the Elston–Ellis mitotic score as ER+/HER2− low proliferation (mitotic score 1), ER+/HER2− high proliferation (mitotic scores 2 and 3), HER2-positive class: HER2+ regardless of ER status, Triple-Negative (TN): ER-, PR-, and HER2-.

Western blot

Western Blot was used prior to immunohistochemistry to ensure specificity of anti-ATF4 antibody (EPR18111, Ab184909, Abcam, UK) in ZR-75-1 (BC) and HeLa cell lysates (cervical adenocarcinoma) (American Type Culture Collection; Rockville, MD, USA). The primary antibody was diluted to 1:500, whilst the secondary antibody (IRDye 800CW Donkey Anti-Rabbit, LI-COR Biosciences, UK) was diluted to 1:15,000. Blocking solution, to prevent non-specific staining, was 5% milk (Marvel Original Dried Skimmed Milk, Premier Food Group Ltd., UK) in PBS-Tween 20 (0.1%). Mouse monoclonal anti-beta-actin antibody (Sigma-Aldrich, A5441) was used as a loading control. The A-Fluorescence detection method, using wavelengths at 800nm using Odyssey Fc imaging studio 4.0 (LI-COR Biosciences, UK) was used to detect ATF4. A single specific band at 50kDa was visualised at the correct predicted size for ATF4 (Supplementary Figure 1).

Immunohistochemistry

Immunohistochemistry was performed on 4 µm full-face invasive breast cancer tissue sections (n=21), to determine pattern of tissue staining, and tissue microarray (TMA) sections incorporating 0.6mm cores of invasive BC tissue (n=2225) as previously described [24]. The Novolink Max Polymer Detection System (RE7280-K, Leica Biosystems, UK) was used to stain protein expression according to manufacturer instructions. Heat-induced epitope retrieval was performed using citrate buffer (pH 6.0) for 20 minutes in a microwave (Whirlpool JT359 Jet Chef 1000 W). Tissues were incubated with ATF4 primary antibody, diluted at 1:300 overnight at 4°C. High resolution digital images of TMA slides were scanned using a NanoZoomer (Hamamatsu Photonics, Welwyn Garden City, UK) and visualised using Xplore (Philips, UK) at a magnification of 20x.

Scoring of ATF4 protein expression

Only TMA cores containing more than 15% invasive tumour tissue were assessed. To evaluate the extent of ATF4 expression, the semi-quantitative modified H-score was used, assessing both the intensity and percentage of nuclear staining. The intensity of staining was measured on a scale of 0-

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Accepted Manuscript

3, 0 indicating no staining, 1 indicating weak staining, 2 indicating moderate staining and 3 indicating strong staining. The percentage of nuclear staining within the cores was then subjectively evaluated. The final H-score was calculated by multiplying the percentage of positive cells (0–100) by the intensity (0–3), producing a total range of 0–300.

An independent scorer (RE), blind to clinical data and scores, scored 10% of cases. An inter-scorer correlation was calculated using Pearson's 2-tailed correlation coefficient producing a coefficient of $r=0.8$ with $p=3.6 \times 10^{-39}$, thus suggesting high inter-scorer reliability. ATF4 protein expression was dichotomised into high and low expression groups using the median H-score value of 130.

Statistical analysis

To determine associations between ATF4 expression and various ATTs, statistical analysis was performed using the SPSS v26.0 Statistical Software (IBM SPSS Statistics, SPSS Inc., Chicago, IL, USA). *ATF4* CNV/mRNA and ATF4 protein correlation within continuous variables was calculated using Pearson's correlation coefficient. Meanwhile, categorical data was evaluated through use of Pearson's chi-squared analysis. Wherein multiple statistical analyses were run, p-values were adjusted using the Bonferroni-Holm correction to account for multiple testing. Significant differences within multiple continuous variables were evaluated using one-way analysis of variance (ANOVA). Survival curves were evaluated using Kaplan Meier and log-rank testing, in relation to BCSS. Furthermore, Cox regression was used in multivariate analyses to identify independent prognostic factors. Within all analyses, p values <0.05 were considered statistically significant.

A summary of datasets/expertimental cohort together with analyses are shown in Figure 1.

RESULTS

ATF4 in breast cancer

A total of 2.7% (54/1980) BC showed gain of *ATF4* gene CN, whilst 11.8% (234/1980) showed *ATF4* CN loss. *ATF4* gene copy number gain and mRNA expression were strongly correlated in BC, with higher mRNA levels associated with gene CN gain (p<0.001, Figure 2).

ATF4 immunoreactivity in full-face breast tissue sections showed nuclear, homogenous staining of invasive BC cells, indicating that TMA cores are representative for the whole tumour. Within TMA cores, there were variable levels of staining intensities in invasive BC cells, ranging from absent to strong (Figure 3). Due to different cohorts, it was not possible to correlate ATF4 protein expression with either *ATF4* CNV or mRNA.

ATF4 and association with breast cancer clinicopathological parameters

ATF4 CN gain was significantly associated with high grade tumours, while *ATF4* CN loss was linked to low grade tumours (p<0.001; Table 1). High *ATF4* mRNA expression was associated with younger age at diagnosis, high tumour grade, and the poor NPI prognostic group (all p<0.001; Table 1). These associations were confirmed using Breast Cancer Gene-Expression Miner (p<0.05; Supplementary Figure 2). ATF4 protein expression however was not significantly associated with any of the clinicopathological parameters (Table 1).

ATF4 and biological breast cancer subtypes

At the genomic level, findings revealed a striking link between *ATF4* gene alterations and BC molecular subtypes. *ATF4* CN gain was predominant in ER-, PR-, and triple-negative tumours while *ATF4* CN loss was prevalent in ER+ and PR+ BC (all p<0.001, Table 2). These findings were further corroborated by significantly higher *ATF4* mRNA expression in ER- and PR- BC in the METABRIC (both p<0.001, Table 2) and Breast Cancer Gene Expression Miner (p<0.05; Supplementary Figure 2)

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Accepted Manuscript

datasets. High *ATF4* mRNA expression was observed in the basal-like immunosuppressed (BLIS) compared with basal-like immune-activated (BLIA), luminal androgen receptor (LAR), and mesenchymal (MES) subtypes (p<0.001; Supplemetnary Figure 2H).

High ATF4 protein expression was significantly more prevalent in Triple Negative BC, while low ATF4 protein expression was characteristic of ER+ and PR+ tumours (p=0.0002; Table 2).

Within PAM50 subtypes, *ATF4* CN gain was predominantly observed in basal tumours, while *ATF4* CN loss was prevalent in luminal tumours (p<0.001; Table 2). This pattern was mirrored by high *ATF4* mRNA expression in basal tumours and low expression in luminal tumours (p<0.001; Table 2). These findings were further validated using Breast Cancer Gene Expression Miner (p<0.05; Supplementary Figure 2). *ATF4* CN gain and high mRNA was associated with Cluster 10 (Triple Negative) whereas *ATF4* CN loss and low mRNA expression were associated with METABRIC Integrative Cluster 8 (Luminal A) (p<0.001; Table 2).

ATF4 expression and AATs

A remarkably strong correlation was observed between *ATF4* and *SLC3A2* mRNA in the METABRIC dataset (r=0.81, p=0.0003; Table 3), while moderate positive correlations were found between *ATF4* and *SLC1A5, SLC3A2, SLC6A15, SLC7A5, SLC7A7, SLC7A11, SLC38A2,* and *SLC38A8* in both METABRIC and Breast Cancer Gene Expression Miner datasets (all p≤0.012; Table 3). Intriguingly, weak negative correlations were observed between *ATF4* and *SLC7A8* mRNA in both datasets (r>-0.23, p<0.0001; Table 3). *ATF4* and *SLC1A5* and *SLC7A11* mRNA were correlated similarly in all TNBC subtypes (p<0.0001, Supplementary Figure 2I).

ATF4 protein expression exhibited a positive relationship with SLC1A5 and SLC7A11 protein (p=0.04, p=9.8×10^{-11} respectively; Table 4), while no significant association was observed with SLC3A2, SLC7A5, SLC7A8 or SLC38A2.

ATF4 and patient outcome

Contrary to expectations, neither *ATF4* CNV nor *ATF4* mRNA expression were associated with patient overall survival in the entire cohort (Figure 4) or within specific biological subtypes (data not shown). This lack of association is further corroborated by independent analyses using Breast Cancer Gene Expression Miner and Kaplan Meier Plotter datasets (Supplementary Figure 3). In addition, there was not significant difference in those tumours showing *ATF4* CN loss and low *ATF4* mRNA (p=0.465). Similarly, ATF4 protein expression showed no correlation with BCSS in the overall cohort (Figure 4) or in biological subtypes (data not shown).

The interplay between ATF4 and glutamine metabolism-related AATs were further investigated. There was no association between *ATF4* CNV or *ATF4* mRNA expression and any of the AATs with patient outcome (data not shown). However, ATF4 protein together with SLC1A5 or SLC7A11, revealed a differential impact on patient outcome. Co-expression of ATF4 and SLC1A5 protein was significantly associated with poor BCSS in ER+ tumours only (p<0.001, Figure 5A-C). Conversely, high ATF4 and high SLC7A11 expression conferred a better survival in the whole cohort (p=0.003; Figure 5D) irrespective of ER status (data not shown).

Multivariate analysis revealed that ATF4 alone or in combination with SLC1A5 was not an independent prognostic factor for breast cancer, beyond tumour grade, size, and nodal stage. (data not shown).

Discussion

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

ATF4 has been identified in BC subtypes, but its relevance as a prognostic marker is not well understood. Amino acids are essential for cell survival, especially in tumour cells, which have high proliferation rates and increased amino acid demand [25]. AATs are therefore vital for nutrient supply to cancer cells. Additionally, glutamine metabolism is associated with cancer cell metabolic reprogramming and is closely linked to AATs [4]. In this study, we aimed to assess ATF4 expression, its prognostic value, and potential association with glutamine-associated AATs in large cohorts of BC patients.
Overall, we confirm that *ATF4* CN gain, high *ATF4* mRNA and ATF4 protein are associated with aggressive BC. This is consistent with previous literature where Fan *et al.* showed high ATF4 expression in metastatic BC, whilst González-González *et al*. associated ATF4 with increased aggressiveness in Triple Negative BC [26, 19]. In some cases, where there is no CNV, increased ATF4 expression in cancer cells could be to due to activation of the ISR, which is necessary for cancer cell survival and proliferation, especially in aggressive tumours [15, 27]. ATF4 has also been shown to play a protective role in maintaining normal, healthy cell development. For example, ATF4 is a critical regulator of osteoblast differentiation and plasma cell viability [28, 29]. ATF4 overexpression also decreases proliferation and accelerates mammary gland involution in transgenic mice during pregnancy and lactation [30]. These studies demonstrate that ATF4 is essential for the differentiation and survival of rapidly proliferating cells, as well as for normal breast development. ATF4 expression is associated with several AATs at the transcriptomic level, but only SLC1A5 and SLC7A11 at the protein level in this cohort. This suggests that the relationship between ATF4 and AATs is complex and dependent on the transporter in question. The influence of ATF4 on AATs may reflect its dual role in promoting both survival and apoptosis [31]. For example, High expression of SLC1A5, SLC3A2, and SLC7A5 is associated with poor prognosis in highly proliferative ER+ BC [9]. This is likely due to their role in regulating intracellular amino acid concentrations. *ATF4* mRNA expression is positively correlated with these transporters, reflecting its pro-survival role. However, ATF4 is also strongly associated with SLC7A11, which exchanges intracellular glutamate for extracellular cystine [32]. This suggests that ATF4 may also promote cancer cell sensitivity to glucose starvation by decreasing intracellular glutamate. While this has not yet been documented in BC, it suggests that ATF4 may have pro-apoptotic effects in this disease.

Moreover, high ATF4 with low expression of SLC1A5 conferred longer survival rates within in ER+ BC. Whilst it could be suggested that the low expression of SLC1A5 is responsible for this result alone, both this study as well as numerous others have implicated elevated ATF4 expression with that of elevated SLC1A5 and consequent cancer cell survival [33]. Perhaps one explanation for this may be through post-translational modifications. Phosphorylation at various threonine residues have been shown by Bagheri-Yarmand *et al.* to reduce ATF4 activity at the promotors of pro-apoptotic targets NOXA and PUMA [34]. Whilst it is unclear if this interaction is specific to pro-apoptotic targets, it demonstrates the complex mechanisms by which ATF4 activity is modulated.

Conclusion: These findings suggest a complex interplay between ATF4 and AATs in breast cancer biology and underscore the potential role for ATF4 as a prognostic marker in ER+ breast cancer, offering a unique opportunity for risk stratification and personalised treatment strategies. Future investigations are needed to confirm this.

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Accepted Manuscript

Compliance with Ethical Standards

Conflict of interests: The authors declare no conflict of interests.

Ethical approval: This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer' and the North West – Greater Manchester Central Research Ethics Committee under the title 'Nottingham Health Science Biobank (NHSB)' reference number 15/NW/0685. This study was performed according to the REMARK guidelines for tumour prognostic studies [35].
Written informed consent was obtained from all individuals and all samples were anonymized.

Data availability: The data that support the findings of this study are not publicly available due to ethical reasons but are available from the corresponding author (ARG) upon reasonable request.

Funding: No funding was received for this study.

Acknowledgements: We thank the Nottingham Health Science Biobank and Breast Cancer Now Tissue Bank for the provision of tissue samples.

Author Contributions: R.P., L.H.A., R.E.: writing, review, editing, methodology, formal analysis and interpretation. B.K.M., B.E., A.F.: methodology and writing - original draft, reviewing and editing. I.O.E., E.A.R. supervision, writing - review and editing A.G: conceptualisation, formal analysis and interpretation, supervision, writing - review and editing.

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.
2. Eagle H. Nutrition needs of mammalian cells in tissue culture. Science. 1955 Sep 16;122(3168):501-14.
3. DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene. 2010 Jan 21;29(3):313-24.
4. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, et al. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A. 2007 Dec 4;104(49):19345-50.
5. Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature. 2011 Nov 20;481(7381):380-4.
6. Kim S, Kim DH, Jung WH, Koo JS. Expression of glutamine metabolism-related proteins according to molecular subtype of breast cancer. Endocr Relat Cancer. 2013 Jun;20(3):339-48.
7. El Ansari R, Craze ML, Diez-Rodriguez M, Nolan CC, Ellis IO, Rakha EA, et al. The multifunctional solute carrier 3A2 (SLC3A2) confers a poor prognosis in the highly proliferative breast cancer subtypes. Br J Cancer. 2018 Apr;118(8):1115-22.
8. El Ansari R, Craze ML, Miligy I, Diez-Rodriguez M, Nolan CC, Ellis IO, et al. The amino acid transporter SLC7A5 confers a poor prognosis in the highly proliferative breast cancer subtypes and is a key therapeutic target in luminal B tumours. Breast Cancer Res. 2018 Mar 22;20(1):21.
9. El-Ansari R, Craze ML, Alfarsi L, Soria D, Diez-Rodriguez M, Nolan CC, et al. The combined expression of solute carriers is associated with a poor prognosis in highly proliferative ER+ breast cancer. Breast Cancer Res Treat. 2019 May;175(1):27-38.
10. Zhang N, Yang X, Yuan F, Zhang L, Wang Y, Wang L, et al. Increased Amino Acid Uptake Supports Autophagy-Deficient Cell Survival upon Glutamine Deprivation. Cell Rep. 2018 Jun 5;23(10):3006-20.
11. Ameri K, Harris AL. Activating transcription factor 4. Int J Biochem Cell Biol. 2008;40(1):14-21.
12. Castells A, Gusella JF, Ramesh V, Rustgi AK. A region of deletion on chromosome 22q13 is common to human breast and colorectal cancers. Cancer Res. 2000 Jun 1;60(11):2836-9.
13. Hinnebusch AG. The eIF-2 alpha kinases: regulators of protein synthesis in starvation and stress. Semin Cell Biol. 1994 Dec;5(6):417-26.
14. Ye J, Kumanova M, Hart LS, Sloane K, Zhang H, De Panis DN, et al. The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. EMBO J. 2010 Jun 16;29(12):2082-96.
15. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell. 2003 Mar;11(3):619-33.
16. Wang Y, Alam GN, Ning Y, Visioli F, Dong Z, Nor JE, et al. The unfolded protein response induces the angiogenic switch in human tumor cells through the PERK/ATF4 pathway. Cancer Res. 2012 Oct 15;72(20):5396-406.
17. Nagelkerke A, Bussink J, Mujcic H, Wouters BG, Lehmann S, Sweep FC, et al. Hypoxia stimulates migration of breast cancer cells via the PERK/ATF4/LAMP3-arm of the unfolded protein response. Breast Cancer Res. 2013 Jan 7;15(1):R2.
18. Zeng P, Sun S, Li R, Xiao ZX, Chen H. HER2 Upregulates ATF4 to Promote Cell Migration via Activation of ZEB1 and Downregulation of E-Cadherin. Int J Mol Sci. 2019 May 6;20(9).
19. Gonzalez-Gonzalez A, Munoz-Muela E, Marchal JA, Cara FE, Molina MP, Cruz-Lozano M, et al. Activating Transcription Factor 4 Modulates TGFbeta-Induced Aggressiveness in Triple-Negative Breast Cancer via SMAD2/3/4 and mTORC2 Signaling. Clin Cancer Res. 2018 Nov 15;24(22):5697-709.

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

20. Jezequel P, Frenel JS, Campion L, Guerin-Charbonnel C, Gouraud W, Ricolleau G, et al. bc-GenExMiner 3.0: new mining module computes breast cancer gene expression correlation analyses. Database (Oxford). 2013;2013:bas060.
21. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat. 2010 Oct;123(3):725-31.
22. El Ansari R, Alfarsi L, Craze ML, Masisi BK, Ellis IO, Rakha EA, et al. The solute carrier SLC7A8 is a marker of favourable prognosis in ER-positive low proliferative invasive breast cancer. Breast Cancer Res Treat. 2020 May;181(1):1-12.
23. Morotti M, Zois CE, El-Ansari R, Craze ML, Rakha EA, Fan SJ, et al. Increased expression of glutamine transporter SNAT2/SLC38A2 promotes glutamine dependence and oxidative stress resistance, and is associated with worse prognosis in triple-negative breast cancer. Br J Cancer. 2021 Jan;124(2):494-505.
24. Abd El-Rehim DM, Ball G, Pinder SE, Rakha E, Paish C, Robertson JF, et al. High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. Int J Cancer. 2005 Sep 1;116(3):340-50.
25. Bhutia YD, Babu E, Ramachandran S, Ganapathy V. Amino Acid transporters in cancer and their relevance to "glutamine addiction": novel targets for the design of a new class of anticancer drugs. Cancer Res. 2015 May 1;75(9):1782-8.
26. Fan CF, Mao XY, Wang EH. Elevated p-CREB-2 (ser 245) expression is potentially associated with carcinogenesis and development of breast carcinoma. Mol Med Rep. 2012 Feb;5(2):357-62.
27. Bi M, Naczki C, Koritzinsky M, Fels D, Blais J, Hu N, et al. ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. EMBO J. 2005 Oct 5;24(19):3470-81.
28. Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, et al. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. Cell. 2004 Apr 30;117(3):387-98.
29. Goldfinger M, Shmuel M, Benhamron S, Tirosh B. Protein synthesis in plasma cells is regulated by crosstalk between endoplasmic reticulum stress and mTOR signaling. Eur J Immunol. 2011 Feb;41(2):491-502.
30. Bagheri-Yarmand R, Vadlamudi RK, Kumar R. Activating transcription factor 4 overexpression inhibits proliferation and differentiation of mammary epithelium resulting in impaired lactation and accelerated involution. J Biol Chem. 2003 May 9;278(19):17421-9.
31. Wortel IMN, van der Meer LT, Kilberg MS, van Leeuwen FN. Surviving Stress: Modulation of ATF4-Mediated Stress Responses in Normal and Malignant Cells. Trends Endocrinol Metab. 2017 Nov;28(11):794-806.
32. Lim JC, Donaldson PJ. Focus on molecules: the cystine/glutamate exchanger (System x(c)(-)). Exp Eye Res. 2011 Mar;92(3):162-3.
33. van Geldermalsen M, Wang Q, Nagarajah R, Marshall AD, Thoeng A, Gao D, et al. ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer. Oncogene. 2016 Jun 16;35(24):3201-8.
34. Bagheri-Yarmand R, Sinha KM, Gururaj AE, Ahmed Z, Rizvi YQ, Huang SC, et al. A novel dual kinase function of the RET proto-oncogene negatively regulates activating transcription factor 4-mediated apoptosis. J Biol Chem. 2015 May 1;290(18):11749-61.
35. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast cancer research and treatment. 2006;100(2):229-35.
36. Galea MH, Blamey RW, Elston CE, Ellis IO. The Nottingham Prognostic Index in primary breast cancer. Breast Cancer Res Treat. 1992;22(3):20

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Figure legends

Fig 1 Summary of datesets/expertimental cohort and analyses used

Fig 2 *ATF4* gene copy number variation and relationship with *ATF4* mRNA expression in invasive breast cancer using the METABRIC dataset. Data represented with median ± standard deviation using one-way analysis of variance with the post-hoc Tukey test

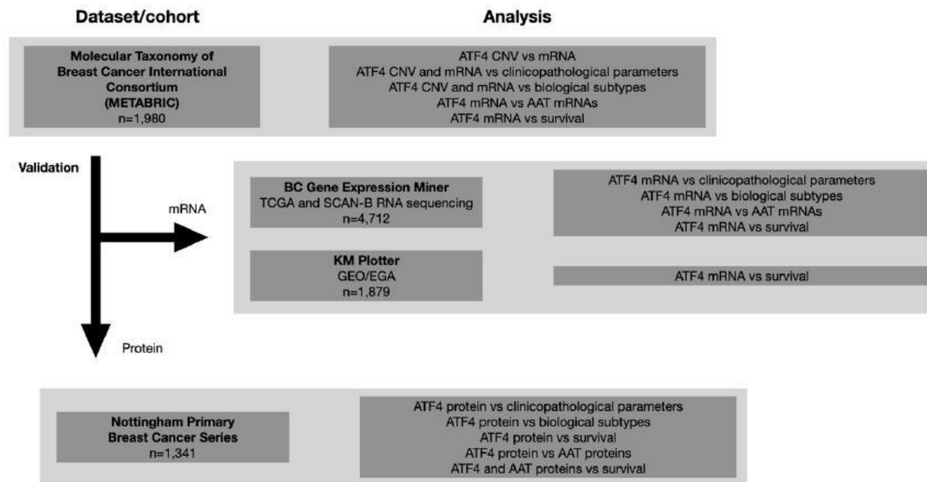
Fig 3 ATF4 protein expression in invasive breast cancer cells (Nottingham Primary Breast Cancer Series) using immunohistochemistry: A) TMA core showing negative staining, B) TMA core showing strong nuclear staining. x10 magnification.

Fig 4 ATF4 and survival in invasive breast cancer patients: A) *ATF4* gene copy number variation in the METABRIC dataset, B) *ATF4* mRNA expression in the METABRIC dataset, C) ATF4 protein expression in the Nottingham Primary Breast Cancer Series.

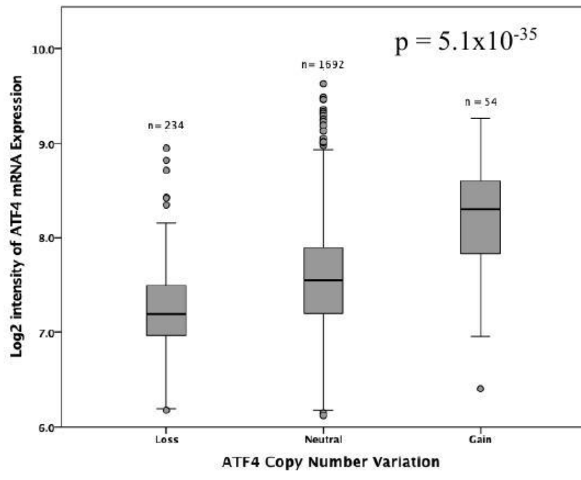
Fig 5 ATF4 and amino acid transporter protein co-expression in invasive breast cancer patient survival in the Nottingham Primary Breast Cancer Series: A) ATF4/SLC1A5, B) ATF4/SLC1A5 in ER+ tumours, C) ATF4/SLC1A5 in ER- tumours, D) ATF4/SLC1A11

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

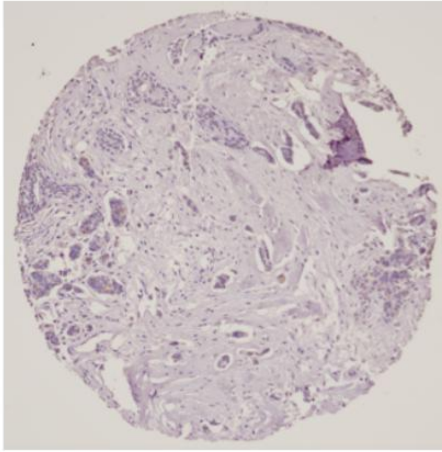


Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

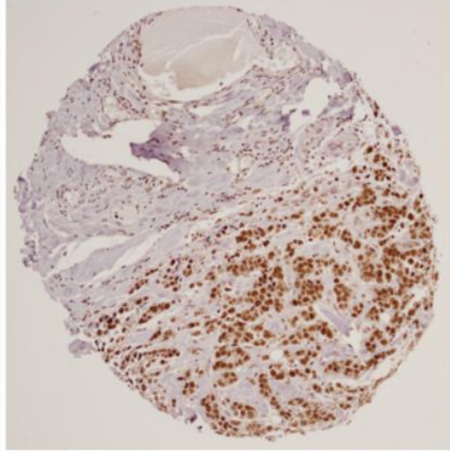


Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

A)

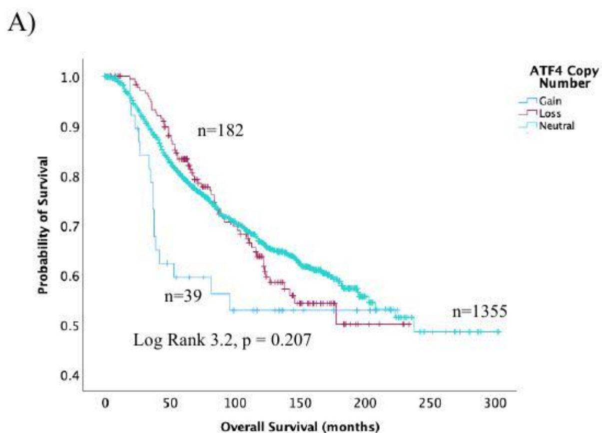


B)

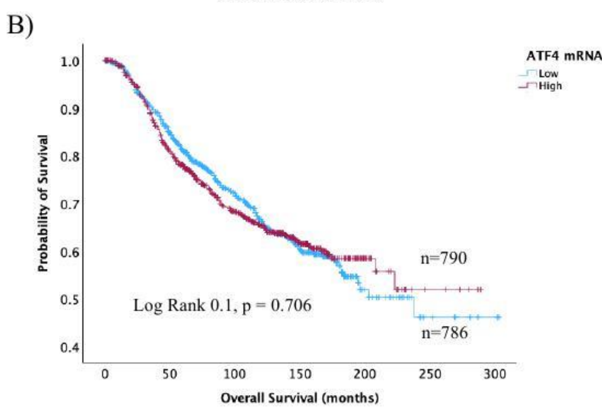


Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

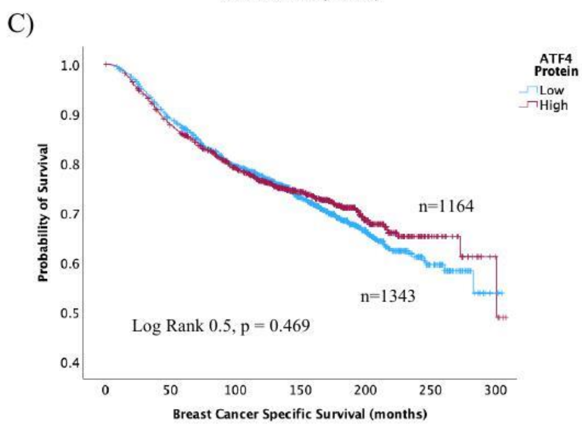
A)



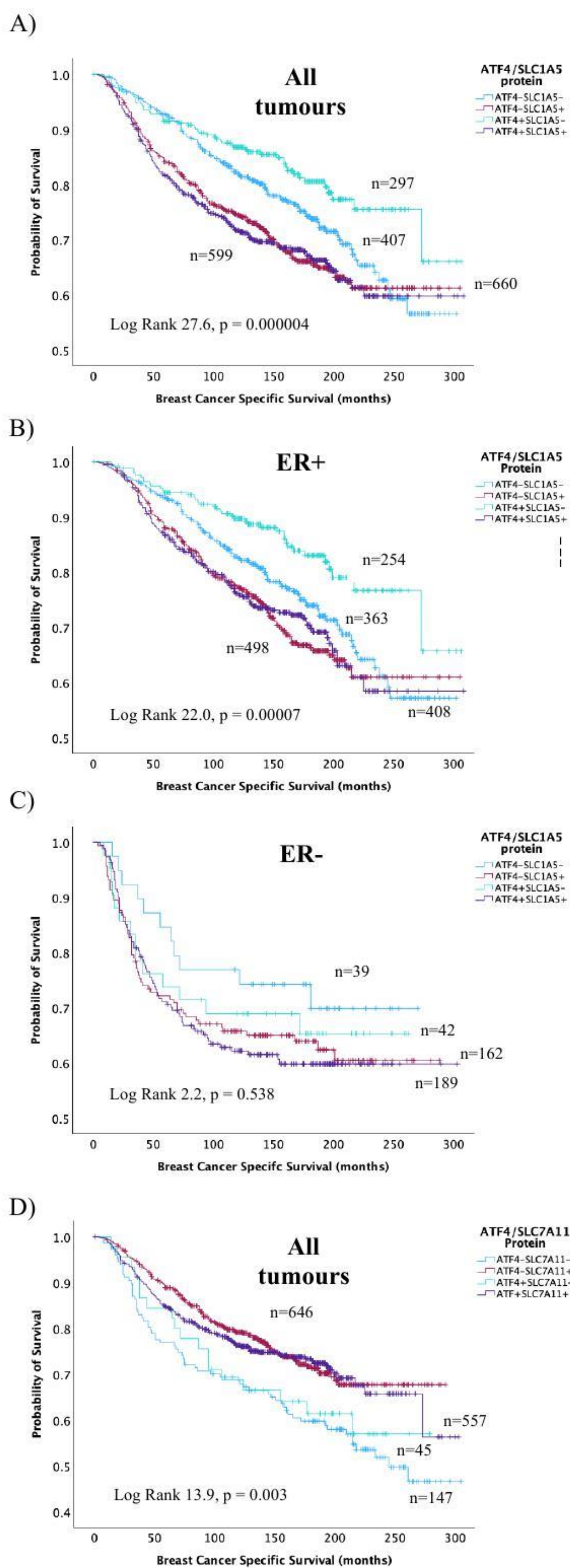
B)



C)



Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024



Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Table 1. *ATF4* gene copy number, mRNA (both METABRIC) and ATF4 protein (Nottingham Primary Breast Cancer Series) in relation to breast cancer clinicopathological parameters.

Parameter	Copy Number			Adjusted P-value	mRNA Expression		Adjusted P-value	Protein Expression		Adjusted P-value
	Gain n (%)	Loss n (%)	Neutral n (%)		Low n (%)	High n (%)		Low n (%)	High n (%)	
Size										
<2.0cm	17 (32)	81 (35)	524 (31)	1.13	328 (53)	291 (47)	0.79	817 (52)	746 (45)	0.66
≥2.0cm	36 (68)	151 (65)	1144 (69)		673 (51)	649 (49)		710 (55)	584 (45)	
Grade										
1	1 (2)	31 (14)	138 (9)	**$2.0x10^{-8}$**	103 (61)	65 (38)	**$1.0x10^{-6}$**	240 (54)	205 (46)	0.22
2	8 (16)	116 (52)	646 (40)		433 (57)	332 (43)		588 (56)	456 (44)	
3	42 (82)	78 (35)	832 (51)		425 (45)	522 (55)		699 (51)	666 (49)	
Lymph Node Stage										
1	31 (3)	121 (12)	883 (85)	0.91	541 (52)	491 (48)	0.52	927 (53)	839 (48)	0.56
2	15 (2)	72 (12)	535 (86)		312 (51)	302 (49)		446 (54)	374 (46)	
3	8 (3)	41 (13)	267 (85)		154 (49)	161 (51)		153 (57)	114 (43)	
NPI										
GPG	10 (2)	102 (15)	568 (84)	**0.012**	392 (58)	285 (42)	**0.0006**	481 (53)	419 (47)	0.81
MPG	36 (3)	112 (10)	953 (87)		530 (49)	562 (52)		777 (52)	706 (48)	
PPG	8 (4)	20 (10)	171 (86)		90 (45)	109 (55)		268 (57)	205 (43)	

Significant p-values highlighted in bold. NPI: Nottingham Prognostic Index, GPG: good prognostic group, MPG: moderate prognostic group, PGP: poor prognostic group. The NPI is derived from a combination tumour grade (1-3), lymph node stage (1-3) and tumour size (0.2 x size in cm). The final NPI scores are classified into 3 groups based on association with outcome as good moderate and poor prognostic groups [36].

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Table 2. *ATF4* gene copy number, mRNA (both METABRIC) and ATF4 protein (Nottingham Primary Breast Cancer Series) expression in breast cancer biological subtypes. Significant p-values highlighted in bold.

Biology	*ATF4* Copy Number Variation				*ATF4* mRNA Expression			ATF4 Protein Expression		
	Gain n (%)	Loss n (%)	Neutral n (%)	Adjusted P-value	Low n (%)	High n (%)	Adjusted P-Value	Low n (%)	High n (%)	Adjusted P-Value
ER										
Negative	25 (5)	9 (2)	440 (93)	**2.3×10^{-15}**	176 (38)	295 (63)	**1.0×10^{-11}**	289 (47)	333 (53)	**0.0002**
Positive	29 (2)	225 (15)	1252 (83)		836 (56)	661 (44)		1234 (57)	991 (43)	
PR										
Negative	40 (4)	74 (8)	826 (88)	**5.7×10^{-9}**	428 (46)	507 (54)	**4.0×10^{-6}**	564 (49)	578 (51)	**0.0005**
Positive	14 (1)	160 (15.4)	866 (83)		584 (57)	449 (44)		931 (57)	718 (43)	
HER2										
Negative	41 (2)	222 (13)	1470 (85)	**0.0008**	920 (53)	803 (47)	**3.0×10^{-6}**	1308 (28)	1121 (46)	0.62
Positive	13 (5)	12 (5)	222 (90)		92 (38)	153 (62)		192 (53)	174 (48)	
Triple Negative										
No	38 (2)	227 (14)	1395 (84)	**5.0×10^{-9}**	893 (54)	757 (46)	**1.5×10^{-7}**	1310 (55)	1064 (45)	**0.0002**
Yes	16 (5)	7 (2)	297 (93)		119 (37)	199 (63)		199 (45)	246 (55)	
PAM50										
Luminal A	6 (1)	111 (15)	601 (84)	**2.5×10^{-15}**	435 (61)	283 (39)	**5.2×10^{-14}**			
Luminal B	17 (3)	90 (18)	381 (78)		223 (46)	265 (54)				
Basal	18 (5)	10 (3)	301 (91)		111 (34)	218 (66)		Not available		
HER2	10 (4)	11 (5)	219 (91)		110 (46)	130 (54)				
Normal-breast like	3 (2)	11 (6)	185 (93)		108 (54)	91 (46)				
METABRIC Integrative Clusters										
1	5 (4)	27 (19)	107 (77)	**1.3×10^{-20}**	59 (43)	77 (57)	**3.3×10^{-23}**			
2	2 (3)	20 (28)	50 (69)		44 (61)	28 (39)				
3	2 (1)	28 (10)	260 (90)		158 (55)	131 (45)				
4	5 (1)	14 (4)	324 (94)		178 (52)	163 (48)		Not available		
5	11 (6)	7 (4)	172 (91)		66 (35)	122 (65)				
6	4 (5)	15 (18)	66 (78)		46 (54)	39 (46)				
7	3 (2)	39 (21)	148 (77)		120 (63)	70 (37)				
8	4 (1)	61 (20)	234 (79)		211 (71)	85 (29)				
9	4 (3)	18 (12)	124 (85)		63 (43)	82 (57)				
10	14 (6)	5 (2)	207 (92)		67 (30)	159 (70)				
IHC subtypes										
ER low proliferation								805 (56)	640 (44)	**0.0005**
ER high proliferation								263 (57)	199 (43)	
HER2+								157 (53)	139 (47)	
TN								203 (45)	247 (55)	

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Table 3. Correlation of *ATF4* mRNA expression in relation to *AAT* genes in METABRIC and Breast Cancer Gene-Expression Miner datasets.

AAT	***ATF4 mRNA Expression*** **METABRIC r (p-value)**	**GeneMiner r (p-value)**
SLC1A5	0.23 (**$4.5x10^{-24}$**)	0.24 (<**0.0001**)
SLC3A2	0.81 (**0.0003**)	0.27 (<**0.0001**)
SLC6A14	Not available	0.20 (<**0.0001**)
SLC6A15	0.11 (**0.000003**)	0.19 (<**0.0001**)
SLC6A19	-0.001 (0.969)	0.06 (**0.0001**)
SLC7A5	0.16 (**$1.4x10^{-12}$**)	0.35 (<**0.0001**)
SLC7A6	-0.07 (**0.004**)	0.21 (<**0.0001**)
SLC7A7	0.10 (**0.00002**)	0.07 (<**0.0001**)
SLC7A8	-0.23 (**$3.6x10^{-24}$**)	-0.31 (<**0.0001**)
SLC7A9	-0.16 (**$9.9x10^{-13}$**)	0.13 (<**0.0001**)
SLC7A11	0.08 (**0.0003**)	0.28 (<**0.0001**)
SLC38A1	-0.29 (0.201)	-0.09 (<**0.0001**)
SLC38A2	0.46 (**$3.1x10^{-105}$**)	0.04 (**0.012**)
SLC38A3	0.02 (0.483)	0.10 (<**0.0001**)
SLC38A5	0.07 (**0.001**)	-0.05 (**0.0006**)
SLC38A7	-0.05 (**0.016**)	0.007 (<**0.0001**)
SLC38A8	0.10 (**0.00002**)	0.07 (<**0.0001**)

Significant p-values highlighted in bold.

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024

Table 4. ATF4 protein expression in relation to AAT protein expression (Nottingham Primary Breast Cancer Series).

AAT	ATF4 Protein Expression Low n (%)	ATF4 Protein Expression High n (%)	Adjusted P-Value
SLC1A5			
Low	471 (58)	340 (42)	**0.040**
High	750 (52)	685 (48)	
SLC3A2			
Low	801 (55)	653 (45)	1.06
High	290 (54)	252 (46)	
SLC7A5			
Low	1004 (57)	766 (43)	0.42
High	201 (53)	181 (47)	
SLC7A8			
Low	673 (58)	485 (42)	0.44
High	73 (51)	70 (49)	
SLC7A11			
Low	479 (66)	252 (35)	**9.8×10^{-11}**
High	418 (49)	440 (51)	
SLC38A2			
Low	743 (56)	575 (44)	0.98
High	72 (56)	56 (44)	

Significant p-values highlighted in bold.

Accepted Manuscript

Downloaded from http://karger.com/pat/article-pdf/doi/10.1159/000539564/4240686/000539564.pdf by guest on 25 June 2024