

PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes

H. R. Ali^{1,2*}, S.-E. Glont¹, F. M. Blows³, E. Provenzano^{4,5}, S.-J. Dawson^{1,3,5,†}, B. Liu¹, L. Hiller⁶, J. Dunn⁶, C. J. Poole⁶, S. Bowden⁷, H. M. Earl^{3,5}, P. D. P. Pharoah^{3,8} & C. Caldas^{1,3,5}

¹Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Cambridge; ²Department of Pathology, University of Cambridge, Cambridge;

³Department of Oncology, University of Cambridge, Addenbrooke's Hospital, Cambridge; ⁴Department of Histopathology, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge; ⁵Cambridge Experimental Cancer Medicine Centre and NIHR Cambridge Biomedical Research Centre, Cambridge;

⁶Warwick Clinical Trials Unit, University of Warwick, Coventry; ⁷Cancer Research UK Clinical Trials Unit, Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham; ⁸Strangeways Research Laboratory, University of Cambridge, Cambridge, UK

Received 23 March 2015; revised 8 April 2015; accepted 13 April 2015

Background: Expression of programmed death ligand 1 (PD-L1) in solid tumours has been shown to predict whether patients are likely to respond to anti-PD-L1 therapies. To estimate the therapeutic potential of PD-L1 inhibition in breast cancer, we evaluated the prevalence and significance of PD-L1 protein expression in a large collection of breast tumours.

Patients and methods: Correlations between *CD274* (PD-L1) copy number, transcript and protein levels were evaluated in tumours from 418 patients recruited to the METABRIC genomic study. Immunohistochemistry was used to detect PD-L1 protein in breast tumours in tissue microarrays from 5763 patients recruited to the SEARCH population-based study ($N = 4079$) and the NEAT randomised, controlled trial ($N = 1684$).

Results: PD-L1 protein data was available for 3916 of the possible 5763 tumours from the SEARCH and NEAT studies. PD-L1 expression by immune cells was observed in 6% (235/3916) of tumours and expression by tumour cells was observed in just 1.7% (66/3916). PD-L1 was most frequently expressed in basal-like tumours. This was observed both where tumours were subtyped by combined copy number and expression profiling [39% (17/44) of IntClust 10 i.e. basal-like tumours were PD-L1 immune cell positive; $P < 0.001$] and where a surrogate IHC-based classifier was used [19% (56/302) of basal-like tumours were PD-L1 immune cell positive; $P < 0.001$]. Moreover, *CD274* (PD-L1) amplification was observed in five tumours of which four were IntClust 10. Expression of PD-L1 by either tumour cells or infiltrating immune cells was positively correlated with infiltration by both cytotoxic and regulatory T cells ($P < 0.001$). There was a nominally significant association between PD-L1 and improved disease-specific survival (hazard ratio 0.53, 95% confidence interval 0.26–1.07; $P = 0.08$) in ER-negative disease.

Conclusions: Expression of PD-L1 is rare in breast cancer, markedly enriched in basal-like tumours and is correlated with infiltrating lymphocytes. PD-L1 inhibition may benefit the 19% of patients with basal-like tumours in which the protein is expressed.

NEAT ClinicalTrials.gov: NCT00003577.

Key words: breast cancer, PD-L1, *CD274*, immune checkpoint, lymphocytes, basal-like

introduction

The potential of enlisting the immune system as an endogenous tumouricidal therapy has been realised in recent clinical trials of

inhibitors of immune checkpoint proteins such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4) [1], programmed cell death 1 (PD-1) [2] and programmed death ligand 1 (PD-L1) [3, 4]. PD-L1 binds PD-1 [5, 6] and CD80 [7] to reduce the cellular immune response by inducing T-cell tolerance which, among other roles, helps prevent autoimmunity in certain organs and is thought to act as a mechanism of immune evasion by tumours [8]. Therefore, inhibition of PD-L1 will, in a sense, 'release the brakes' on the immune response enabling an unfettered T-cell-mediated attack. In solid tumours, PD-L1 has been found

*Correspondence to: Dr Hamid R. Ali, CRUK Cambridge Institute, University of Cambridge, Robinson Way, Cambridge CB2 0RE, UK. Tel: +44-1223-769650; E-mail: raza.ali@cruk.cam.ac.uk

†Present address: Division of Cancer Medicine, Division of Research, Peter MacCallum Cancer Centre, Melbourne, Australia.

to be expressed by both tumour cells and infiltrating immune cells [4, 9], and its inhibition has been found to result in an enduring clinical response in a variety of solid tumours including melanoma [1], renal cell carcinoma [3], lung carcinoma [3] and, most recently, bladder carcinoma [4]. Breast cancer has not generally been thought of as an immunogenic malignancy and, unlike bladder cancer, no therapy designed to enhance the anti-tumour immune response is currently used. However, breast cancer is an exceptionally heterogeneous disease and it has become apparent that a subset of tumours is subject to an effective immune response. In a large-scale analysis of four studies, we found that the presence of infiltrating cytotoxic T cells was associated with improved outcome but that this effect was limited to ER-negative and HER2-positive disease [10].

Since clinical trials have found that expression of PD-L1 on both tumour and immune cells predicts whether a patient is likely to respond to its inhibition [9], we used large clinical studies to investigate the prevalence of PD-L1 expression in breast cancer in order to estimate the proportion of the population that may benefit from these therapies.

methods

patient population

Primary tumour samples from three clinical studies were used for this analysis: the SEARCH observational study [11], the NEAT randomised, controlled trial [12] and the METABRIC genomic study [13, 14]. Patient characteristics are summarised in supplementary Table S1, available at *Annals of Oncology* online. Details of clinical studies are provided in supplementary Methods, available at *Annals of Oncology* online.

molecular subtyping

Tumour molecular subtype was assigned using both combined copy number and expression profiling for tumours from the METABRIC study [13, 15] and a surrogate immunohistochemical classifier for the SEARCH and NEAT studies [16] which is summarised in supplementary Table S2, available at *Annals of Oncology* online. Genomic instability and *TP53* status were determined as previously described [13, 17, 18]. Further details are provided in the supplementary Methods, available at *Annals of Oncology* online.

PD-L1 assay validation and scoring

A rabbit monoclonal antibody raised against residues near the carboxy terminus of PD-L1 (Cell Signalling Technology, catalogue #13684) was investigated for use in immunohistochemistry (IHC). Two breast cancer cell lines (MDA-MB-231 and MCF7) were used to assess the specificity of the antibody by western blot analysis. To modulate PD-L1 expression, cell lines were treated with interferon- γ and knockdown experiments were conducted using siRNA directed against human *CD274* (PD-L1). Specificity of the immunohistochemical assay was tested using formalin-fixed paraffin-embedded (FFPE) pellets of the cell lines with concurrent quantitative western blot analysis of fresh cell lysates for comparison. Detailed experimental procedures are described in supplementary Methods, available at *Annals of Oncology* online.

Tumour samples were represented by a single 0.6-mm core in tissue microarrays. Stained slides were scanned using an Aperio Scanscope AT2 digital slide scanner. PD-L1 was scored as reported in recent trials [9], where tumour and immune cells were attributed separate scores on a four-point scale as follows: 0, $\leq 1\%$ positive cells; 1, 1%–5% positive cells; 2, 5%–10% positive cells and 3, $\geq 10\%$ positive cells.

statistical analysis

Correlations between continuous and ordinal variables were assessed using Spearman's rank correlation coefficient. Associations between categorical variables were assessed using Pearson's χ^2 test. The Kruskal–Wallis rank test was used to investigate the relationship between *CD274* (PD-L1) copy number status and gene expression. The relationship between expression of PD-L1 and disease-specific survival was investigated using a Cox proportional hazards regression model stratified by study. Since expression of ER violates the proportional hazards assumption [16], analyses were conducted separately by ER status. To investigate whether expression of PD-L1 modified the association between infiltrating lymphocytes and outcome previously reported, an interaction term was included in survival models. Similarly, whether expression of PD-L1 could predict differential benefit from the addition of epirubicin to CMF was assessed using an interaction term in survival models. Estimates of absolute five and ten year survival were calculated using the Kaplan–Meier method. All analyses were conducted in Intercooled Stata version 11.2 (Stata Corp., College Station, TX). Further details are provided in supplementary Methods, available at *Annals of Oncology* online.

results

PD-L1 assay validation

Western blot analysis using a rabbit monoclonal antibody detected a protein of 50 kDa, approximately the size of glycosylated PD-L1 [19], in lysates of the MDA-MB-231 breast cancer cell line while no protein was detected in lysates of MCF7 (supplementary Figure S1, available at *Annals of Oncology* online). Treatment with interferon- γ increased the amount of protein detected in lysates of MDA-MB-231 and a band of similar atomic mass became detectable in lysates of MCF7 (supplementary Figure S1, available at *Annals of Oncology* online). IHC was applied to FFPE cell pellets of MDA-MB-231 cells revealing strong circumferential membranous staining. Antibody specificity was confirmed by knockdown using siRNA against PD-L1, resulting in a more than 70% reduction in the amount of protein detected by quantitative western blot analysis and a proportionate reduction in the signal observed by IHC (supplementary Figure S2A–D, available at *Annals of Oncology* online). IHC of human tonsil revealed weak membranous staining of macrophages and staining of human placenta showed strong expression by syncytiotrophoblasts (supplementary Figure S2E, available at *Annals of Oncology* online) consistent with previous observations [19]. Collectively these analyses confirmed the specificity of the immunohistochemical assay for PD-L1.

CD274 (PD-L1) amplification occurs in a subset of almost exclusively basal-like tumours

We used the METABRIC dataset comprising 1980 tumours to investigate the relationship between *CD274* (PD-L1) copy number status, gene and protein expression and molecular subtype (Figure 1). *CD274* (PD-L1) gene expression was found to be positively associated with copy number status ($P = 0.0001$). The distribution of the ten integrative clusters of breast cancer (IntClust) [13, 14] also significantly differed according to *CD274* (PD-L1) copy number status ($P < 0.001$). Of the 10 clusters, IntClust 10, which approximately equates to basal-like tumours, was significantly enriched among tumours

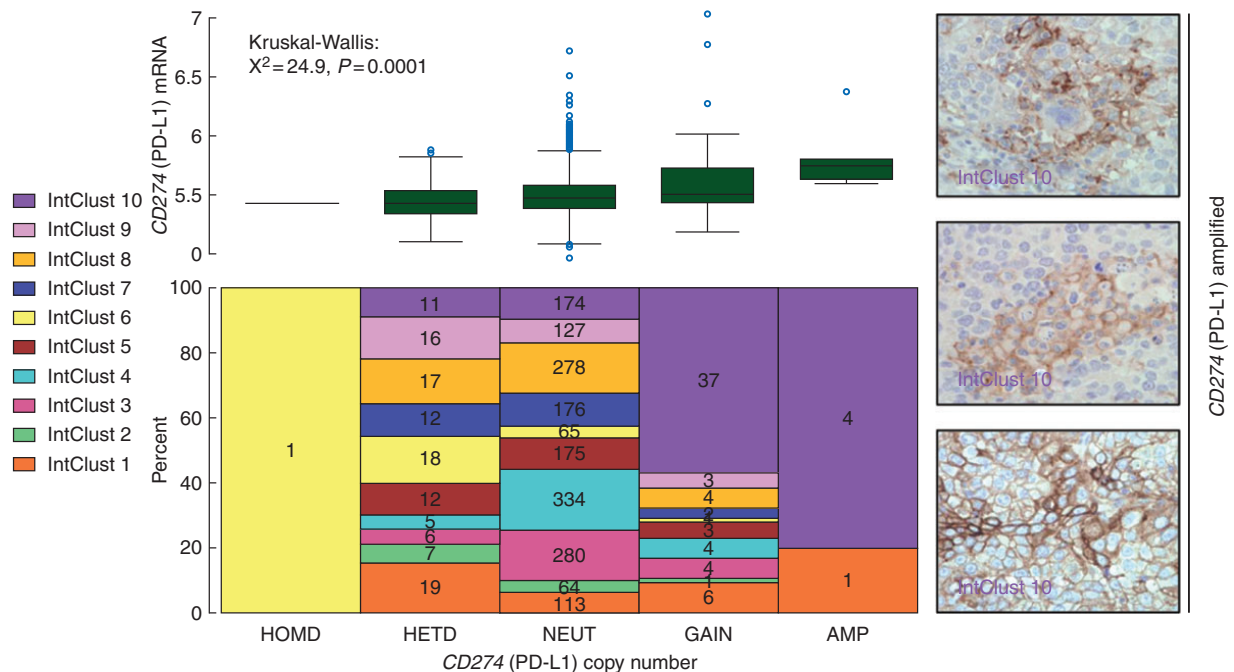


Figure 1. Distribution of PD-L1 (*CD274*) gene expression and molecular subtypes of breast cancer by PD-L1 (*CD274*) copy number status based on aCGH in the METABRIC study. Values within coloured boxes are the absolute number of cases. Right panel depicts immunohistochemistry applied to full-face sections from three IntClust 10 tumours shown to harbour PD-L1 amplification by aCGH.

with either a copy number gain or amplification of *CD274* (PD-L1). Only five tumours showed amplification and, of these, four belonged to IntClust 10 while of 65 tumours with copy number gain of *CD274* (PD-L1), 37 (57%) were IntClust 10. IHC for PD-L1 protein was conducted on full-face sections of three IntClust 10 tumours with *CD274* (PD-L1) amplification, and strong expression was observed in tumour cells. However, the distribution of expression differed between these cases with only one showing diffuse expression in a large proportion of the tumour, whereas the others showed focal PD-L1 expression largely limited to the tumour–stroma interface (Figure 1). IHC for PD-L1 was also conducted on a subset of 418 cases represented in TMAs from the METABRIC study. PD-L1 protein expression was modestly correlated with *CD274* (PD-L1) mRNA expression. The Spearman's rank correlation between tumour PD-L1 expression and gene expression was 0.17 ($P = 0.0005$) and 0.15 ($P = 0.002$) between PD-L1 immune cell expression and gene expression in bulk tumour samples (supplementary Figure S3, available at *Annals of Oncology* online). Supplementary Figure S4, available at *Annals of Oncology* online, depicts box-plots illustrating that both tumour and immune cell PD-L1 expression was significantly associated with a greater number of genomic breakpoints (PD-L1⁺ immune cells $P = 0.003$; PD-L1⁺ tumour cells $P = 0.004$) but not with a genome instability index. Supplementary Figure S5, available at *Annals of Oncology* online, depicts cross-tabulations of PD-L1 expression versus *TP53* mutation status, separately for ER-positive and ER-negative disease. PD-L1 expression by either tumour or immune cells was not significantly associated with mutation of *TP53*.

PD-L1 protein is rarely expressed in breast cancer

Data on expression of PD-L1 was successfully generated in 3916 tumours from the SEARCH ($N = 2453$) and NEAT ($N = 1463$) studies (supplementary Figure S6, available at *Annals of Oncology* online); data were not generated on the remaining 1847 owing to technical reasons such as core drop out or lack of adequate representation of tumour cells. Expression in >1% of immune cells was observed in 235 (6%) tumours while just 66 (1.7%) tumours expressed PD-L1 in >1% of tumour cells (supplementary Figure S6, available at *Annals of Oncology* online). Expression of PD-L1 in tumour and/or immune cells was observed in 245 tumours.

PD-L1 expression is associated with infiltrating lymphocytes and basal-like tumours

Expression of PD-L1 showed a significant positive correlation with levels of infiltrating intra-tumoral CD8⁺ and FOXP3⁺ lymphocytes (Spearman's rank correlation = 0.3; $P < 0.0001$ for both comparisons). Supplementary Figure S7, available at *Annals of Oncology* online, depicts the distribution of infiltrating intra-tumoral or stromal lymphocytes by level of PD-L1 expression. Of the 418 cases from the METABRIC study for which both PD-L1 protein data and genomic subtyping data were available, 50 showed expression in >1% of infiltrating immune cells and 17 of these belonged to IntClust 10, while only 15 showed expression in >1% of tumour cells and 9 of these belonged to IntClust 10. Thirty-nine percent (17/44) of IntClust 10 cases showed PD-L1 expression in >1% of immune cells (supplementary Figure S8, available at *Annals of Oncology* online). The

distribution of IHC-defined molecular subtypes also differed significantly according to the level of PD-L1 expression ($P < 0.00001$; Figure 2). The proportion of tumours which belonged to the basal-like (CBP, i.e. triple-negative tumours with expression of CK56 and/or EGFR) subtype steadily increased with the proportion of PD-L1 positive immune cells where the proportion of basal-like tumours at each level of $<1\%$, $1\%–5\%$, $5\%–10\%$ and $>10\%$ PD-L1-positive immune cells was 8% (246/2899), 20% (9/44), 33% (19/58) and 47% (28/59); collectively amounting to 19% (56/302) of basal-like tumours with PD-L1 protein expression in $>1\%$ of immune cells. In addition when limited to basal-like tumours, expression of PD-L1 was not significantly associated with grade, the number of positive lymph nodes, tumour size or patient age.

association with disease-specific survival

Expression of PD-L1, by either tumour cells or immune cells, was not significantly associated with outcome in either ER-positive or ER-negative breast cancer (supplementary Tables S3 and S4, available at *Annals of Oncology* online) irrespective of whether the variable was modelled as categorical, continuous or dichotomous. However, in ER-negative disease, a subgroup in which immune infiltration has previously been found to be associated with outcome [10], PD-L1 expression in $>10\%$ of immune cells was associated with reduced disease-specific mortality although this was nominally statistically significant

(hazard ratio 0.53, 95% confidence interval 0.26–1.07; $P = 0.08$). There was no significant interaction between the prognostic effect of cytotoxic or regulatory T cells and PD-L1 expression nor was any significant interaction between randomisation arm in the NEAT trial and PD-L1 (data not shown). Supplementary Table S5, available at *Annals of Oncology* online, details estimates of absolute survival at 5 and 10 years for patient subgroups defined by PD-L1 expression, the presence of intra-tumoral $CD8^+$ (iT-CD8) lymphocytes and ER status. These estimates require cautious interpretation owing to the rarity of PD-L1 expression.

discussion

We pooled large clinical studies to investigate the prevalence and significance of PD-L1 protein expression in breast cancer using a validated assay. Expression of PD-L1 by immune cells was observed in 6% of tumours, while expression by tumour cells occurred in just 1.7%. These frequencies significantly differed by tumour subtype. Basal-like tumours (CBP, i.e. triple-negative tumours with expression of CK56 and/or EGFR) were characteristically enriched in PD-L1 expression with 19% containing PD-L1-positive immune cells. Although expression of PD-L1 was significantly correlated with infiltrating lymphocytes, a large proportion of tumours which contained lymphocytic infiltration did not contain PD-L1-positive cells. There was,

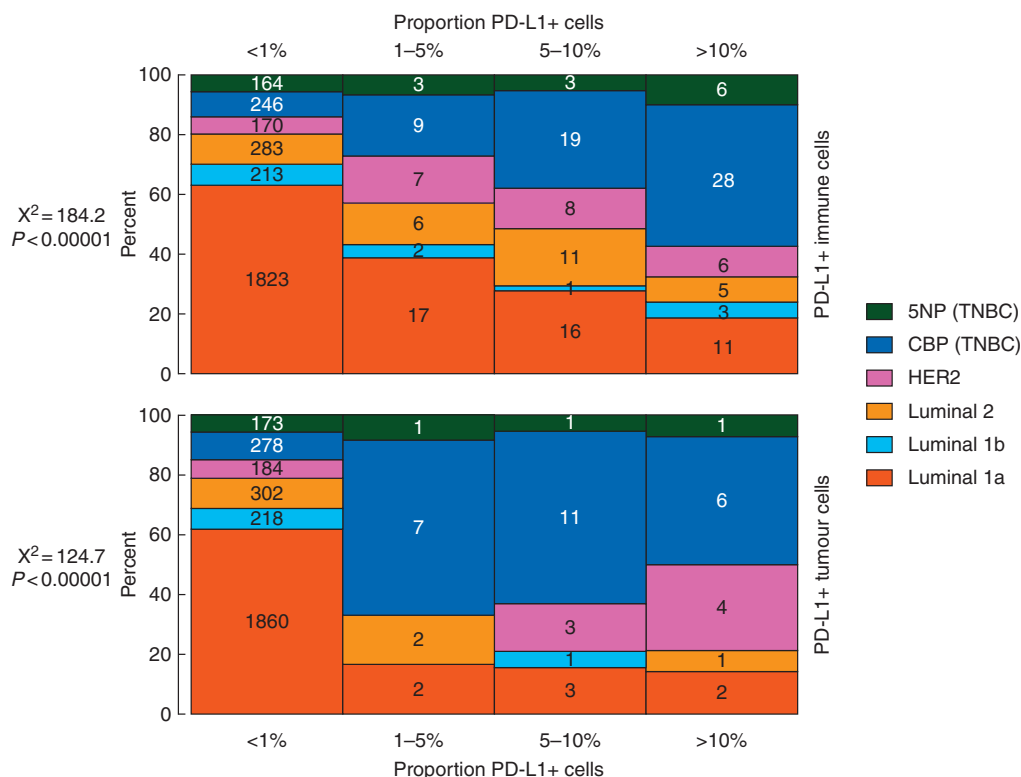


Figure 2. Distribution of molecular subtypes of breast cancer by PD-L1 protein expression level in pooled cases from the SEARCH and NEAT studies. Values within coloured boxes are the absolute number of cases. HER2, human epidermal growth factor receptor 2; CBP, core-basal phenotype; 5NP, five marker negative phenotype; TNBC, triple-negative breast cancer.

however, no interaction between the prognostic effect of infiltrating lymphocytes and expression of PD-L1. There was a nominal association between PD-L1 expression and longer disease-specific survival in ER-negative disease.

This study is the first large-scale analysis of PD-L1 protein expression in breast cancer. Previous studies have included many fewer patients [20] and their analytic validity has been questioned [21, 22]. We used a validated assay and two large clinical studies including a population-based cohort in order to derive reliable estimates of the probable prevalence of PD-L1 expression in the breast cancer population. We report lower frequencies of PD-L1 expression than previous studies. Two prior studies evaluated PD-L1 at the level of gene expression using RNA fluorescence *in situ* (FISH) in one [23], and pooled microarray data in the other [24]. Based on RNA FISH, Schalper et al. report that up to 60% of breast tumours show *CD274* (PD-L1) expression [23] whereas Sabatier et al. report that *CD274* (PD-L1) is up-regulated in 20% of tumours based on microarray data [24]. These estimates themselves differ enormously and both are far greater than the frequencies observed here. This disparity may be due to the poor correlation between PD-L1 protein and RNA, as well as differences between study populations.

We find that PD-L1 expression is rare overall and expressed in around one-fifth of basal-like tumours, however clinical benefit from inhibition of PD-L1 may extend beyond this population. Recent studies have shown, based both in mouse models [25] and in human tumour samples [26, 27], that mutational patterns can influence the efficacy of immune checkpoint inhibitors. Most notable among these characteristics are mutations which result in genetic alterations predicted to be immunogenic by encoding neo-antigens. Identifying the best predictor of immune checkpoint inhibition whether it's genomic, tissue based or a combination, will necessitate a head-to-head comparison in representative clinical cohorts of sufficient size.

We did not find a convincing association between PD-L1 expression by immune cells and clinical outcome. There was, however, a nominally significant association between high levels (>10%) of immune cell PD-L1 expression and improved survival ($P=0.08$). Given that both Schalper et al. and Sabatier et al. report a significant association between high *CD274* (PD-L1) gene expression and improved survival [23, 24], it is plausible that we did not robustly detect this effect owing, for example, to a different threshold for 'high' PD-L1 expression or due to insufficient power. This difference may be addressed by pooling data from additional studies and using a quantitative system for scoring PD-L1 expression. However, our findings are supportive of the association between improved survival and PD-L1 expression previously reported.

The main limitation of this study was the use of TMAs for representation of tumours. In some tumours, immune infiltration may be heterogeneous and this heterogeneity will not be captured by TMAs. However, the reduced power associated with this sampling error was here attenuated by a large sample size. Indeed, TMAs enable the conduct of large-scale pathology studies and in this way ultimately lead to more reliable conclusions. A second limitation is that we have not assessed PD-L1 protein expression in the metastatic setting in which trials of PD-L1 inhibitors have so far been conducted.

We find that PD-L1 protein expression is rare in primary breast cancer overall but that around one-fifth of basal-like tumours contain infiltrating immune cells that express it. Basal-like breast cancer is again implicated as subject to the effects of the PD-L1 axis by the observation that amplification of *CD274* (PD-L1), though rare, occurs almost exclusively in basal-like tumours. This implies that within the microenvironment of these tumours expression of PD-L1 confers a survival advantage. Moreover, amplification of *CD274* (PD-L1) has been observed in the setting of EBV-positive gastric cancer [28] and in samples of Hodgkin's lymphoma from patients who have had a clinical response to PD-1 inhibition [29]. Taken together, these observations have important implications for the potential of inhibitors of PD-L1 in breast cancer, particularly for the design and analysis of clinical trials. Our observations suggest that PD-L1 inhibitors may benefit a small subset of women with breast cancer with tumours that express PD-L1, most of which will prove to be basal-like. Previous observations, both in cell lines and in tumour samples are concordant with this conclusion [30].

conclusions

To our knowledge, we have conducted the first large-scale analysis of PD-L1 protein expression in breast cancer. We find that PD-L1 expression, both by immune and tumour cells is rare, associated with infiltrating lymphocytes and significantly enriched in basal-like tumours. Our findings together with those previously published [23, 24] suggest that high PD-L1 expression is associated with improved survival. These findings imply that clinical trials of PD-L1 inhibitors in breast cancer ought to be enriched for patients with basal-like breast cancer and, given the rarity of expression, may need to recruit across a large number of centres.

acknowledgements

We thank the participants of the SEARCH, NEAT and METABRIC studies who have permitted use of their tissue for research and the many individuals who have made this work possible. We thank Oscar M. Rueda for advice regarding bioinformatic analyses.

funding

This work was supported by Cancer Research UK (C490/A10119 and C490/A10124), the Cambridge Experimental Cancer Medicine Centre and the NIHR Cambridge Biomedical Research Centre. HRA is an NIHR Academic Clinical Lecturer and supported by a Career Development Fellowship from the Pathological Society of Great Britain and Northern Ireland and a Starter Grant for Clinical Lecturers from the Academy of Medical Sciences, UK.

disclosure

The authors have declared no conflicts of interest.

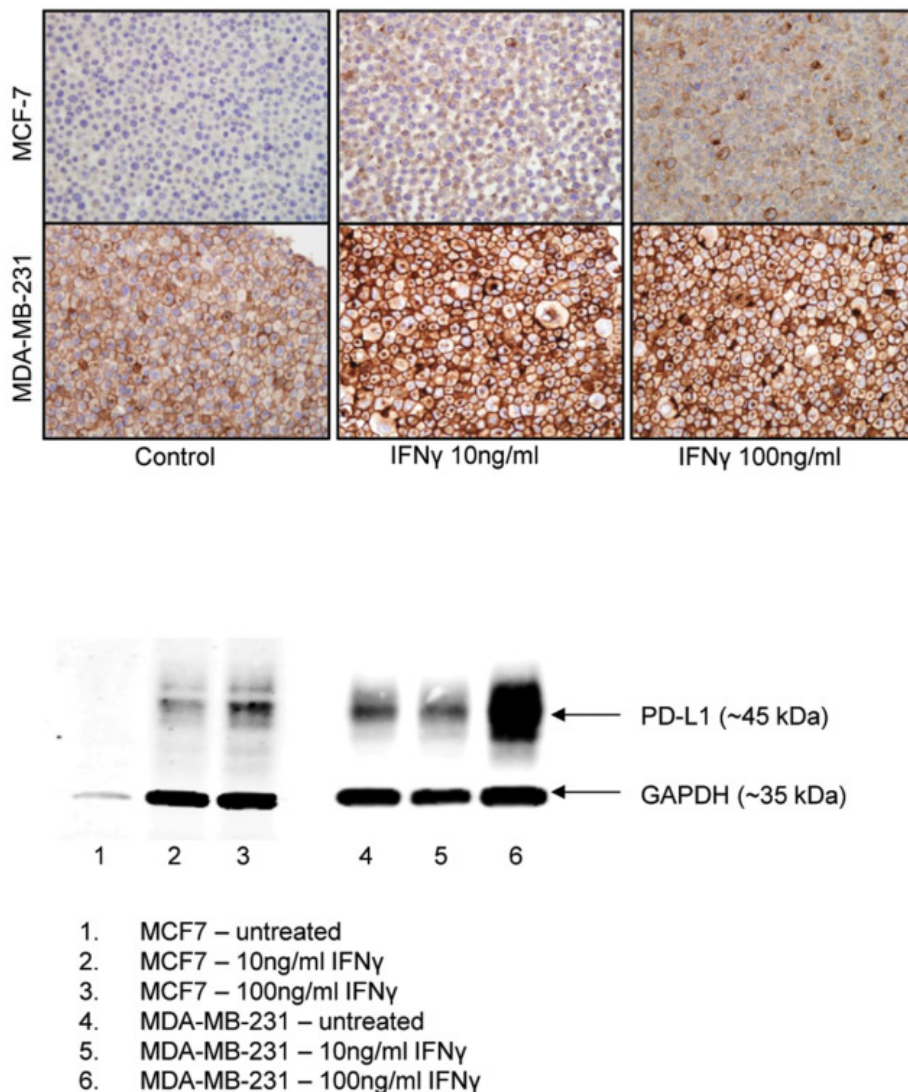
references

- Hodi FS, O'Day SJ, McDermott DF et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; 363: 711–723.
- Topalian SL, Hodi FS, Brahmer JR et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; 366: 2443–2454.
- Brahmer JR, Tykodi SS, Chow LQ et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; 366: 2455–2465.
- Powles T, Eder JP, Fine GD et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* 2014; 515: 558–562.
- Yang J, Riella LV, Chock S et al. The novel costimulatory programmed death ligand 1/B7.1 pathway is functional in inhibiting alloimmune responses in vivo. *J Immunol* 2011; 187: 1113–1119.
- Butte MJ, Keir ME, Phamduy TB et al. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 2007; 27: 111–122.
- Park JJ, Omiya R, Matsumura Y et al. B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance. *Blood* 2010; 116: 1291–1298.
- Dong H, Strome SE, Salomao DR et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002; 8: 793–800.
- Herbst RS, Soria JC, Kowanz M et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 2014; 515: 563–567.
- Ali HR, Provenzano E, Dawson SJ et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients. *Ann Oncol* 2014; 25: 1536–1543.
- Lesueur F, Pharoah P, Laing S et al. Allelic association of the human homologue of the mouse modifier *Ptprj* with breast cancer. *Hum Mol Genet* 2005; 14: 2349–2356.
- Poole CJ, Earl HM, Hiller L et al. Epirubicin and cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy for early breast cancer. *N Engl J Med* 2006; 355: 1851–1862.
- Curtis C, Shah SP, Chin SF et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012; 486: 346–352.
- Ali HR, Rueda OM, Chin SF et al. Genome-driven integrated classification of breast cancer validated in over 7,500 samples. *Genome Biol* 2014; 15: 431.
- Dvinge H, Git A, Graf S et al. The shaping and functional consequences of the microRNA landscape in breast cancer. *Nature* 2013; 497: 378–382.
- Blows F, Driver K, Schmidt M et al. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. *PLoS Med* 2010; 7: e1000279.
- Vollan HK, Rueda OM, Chin SF et al. A tumor DNA complex aberration index is an independent predictor of survival in breast and ovarian cancer. *Mol Oncol* 2015; 9: 115–127.
- Silwal-Pandit L, Vollan HK, Chin SF et al. TP53 mutation spectrum in breast cancer is subtype specific and has distinct prognostic relevance. *Clin Cancer Res* 2014; 20: 3569–3580.
- Chen BJ, Chapuy B, Ouyang J et al. PD-L1 expression is characteristic of a subset of aggressive B-cell lymphomas and virus-associated malignancies. *Clin Cancer Res* 2013; 19: 3462–3473.
- Muenst S, Schaefer AR, Gao F et al. Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treat* 2014; 146: 15–24.
- Rimm D, Schalper K, Pusztai L. Unvalidated antibodies and misleading results. *Breast Cancer Res Treat* 2014; 147: 457–458.
- Muenst S, Tzankov A, Gillanders WE, Soysal SD. Author's response to "Letter to the editor: unvalidated antibodies and misleading results". *Breast Cancer Res Treat* 2014; 147: 459–462.
- Schalper KA, Velcheti V, Carvajal D et al. In situ tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. *Clin Cancer Res* 2014; 20: 2773–2782.
- Sabatier R, Finetti P, Mamessier E et al. Prognostic and predictive value of PDL1 expression in breast cancer. *Oncotarget* 2015; 6: 5449–5464.
- Gubin MM, Zhang X, Schuster H et al. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. *Nature* 2014; 515: 577–581.
- Snyder A, Makarov V, Merghoub T et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 2014; 371: 2189–2199.
- Rizvi NA, Hellmann MD, Snyder A et al. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015; 348: 124–128.
- Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014; 513: 202–209.
- Ansell SM, Lesokhin AM, Borrello I et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med* 2015; 372: 311–319.
- Soliman H, Khalil F, Antonia S. PD-L1 expression is increased in a subset of basal type breast cancer cells. *PLoS One* 2014; 9: e88557.

Supplementary Figure 1

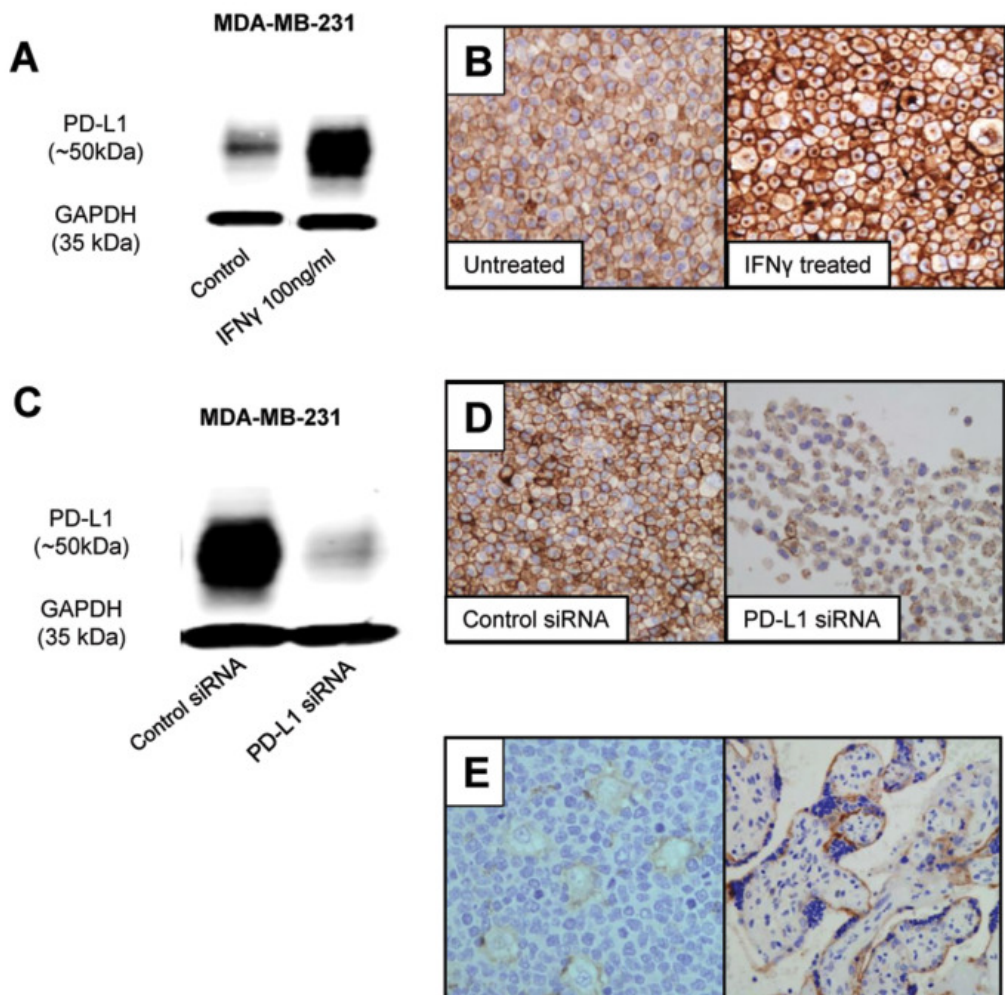
Top panel: Formalin-fixed paraffin-embedded cell pellets of breast cancer cell lines (MDA-MD-231 and MCF7) variably treated with interferon gamma as indicated with PD-L1 expression detected by immunohistochemistry.

Bottom panel: Concurrent western blot analysis for PD-L1 protein.



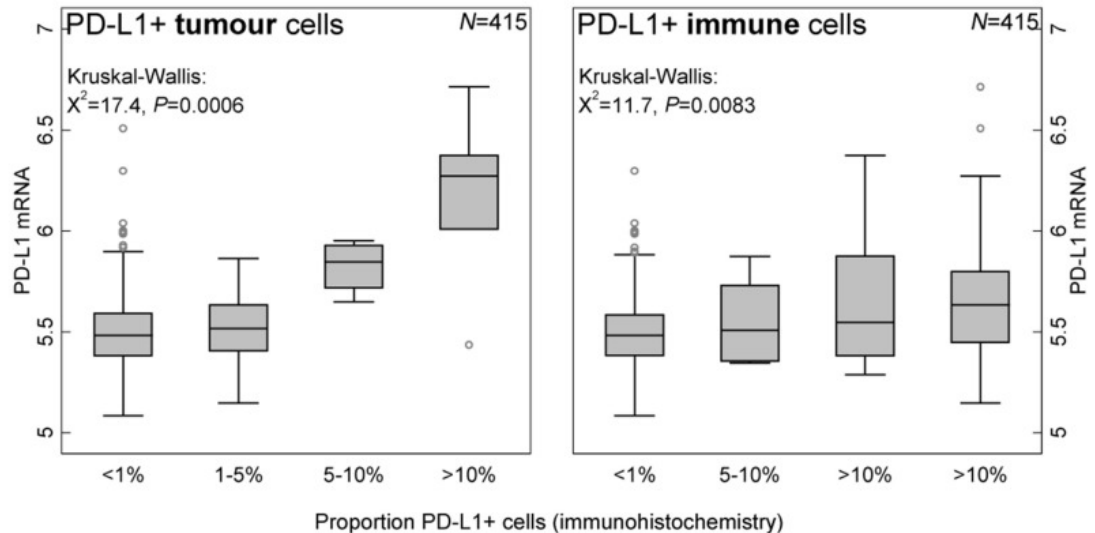
Supplementary Figure 2

- Western blot analysis for PD-L1 protein using lysates from MDA-MB-231 untreated and treated with interferon gamma.
- Immunohistochemistry for PD-L1 on formalin-fixed paraffin-embedded cell pellets of MDA-MB-231 untreated and treated with interferon gamma.
- Western blot analysis for PD-L1 protein using lysates from MDA-MB-231 following treatment with control (scramble) and PD-L1 targeting siRNA.
- Immunohistochemistry for PD-L1 on formalin-fixed paraffin-embedded cell pellets of MDA-MB-231 treated with control (scramble) and PD-L1 targeting siRNA.
- Immunohistochemistry for PD-L1 applied to human tonsil (left) revealing weak membranous expression by macrophages and on human placenta (right) revealing strong expression by syncytiotrophoblasts.



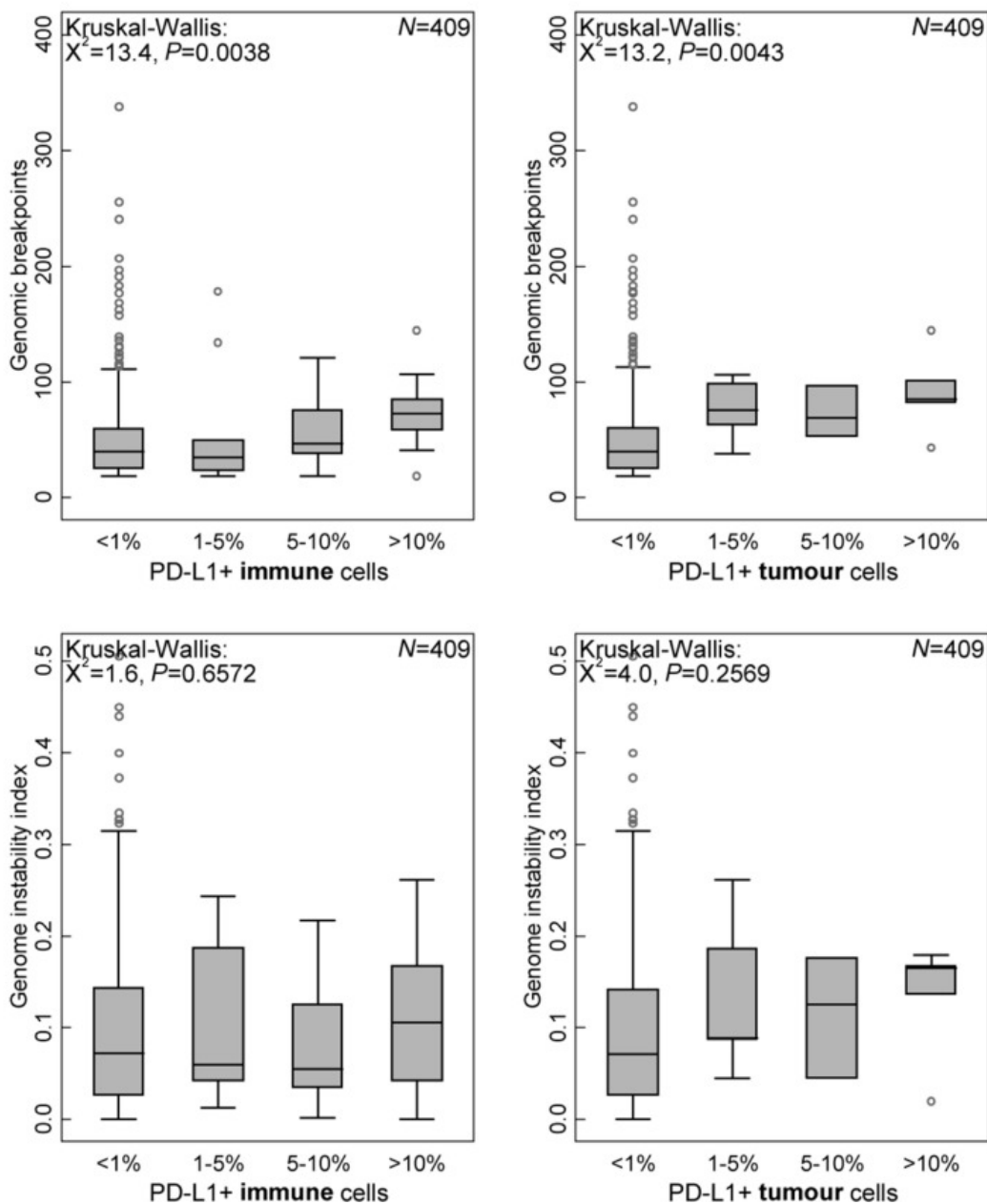
Supplementary Figure 3

Box-plots depicting the correlation between PD-L1 protein expression and PD-L1 mRNA expression in tumour cells and immune cells.

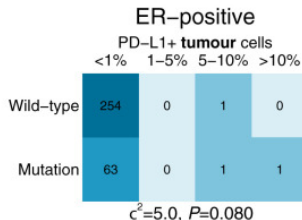
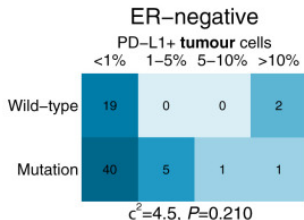
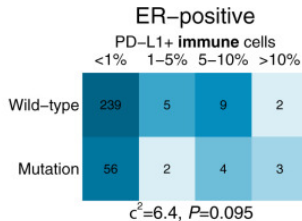
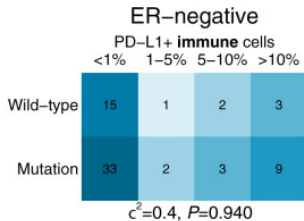


Supplementary Figure 4

Box-plots depicting the distribution of the number of genomic breakpoints (top) and of a genomic instability index (bottom) by the proportion of immune or tumour cells expressing PD-L1 protein by immunohistochemistry.



TP53 mutation status

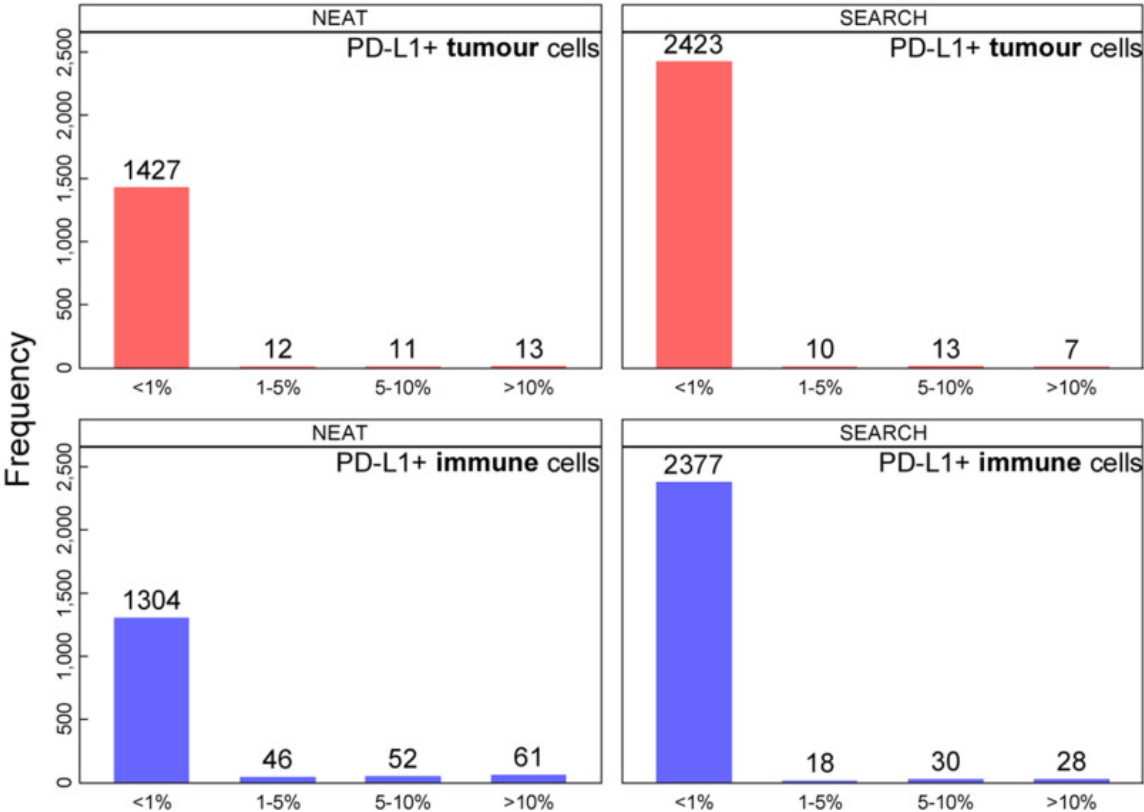


PETITIONER EXHIBIT 1022

Page 11 of 23

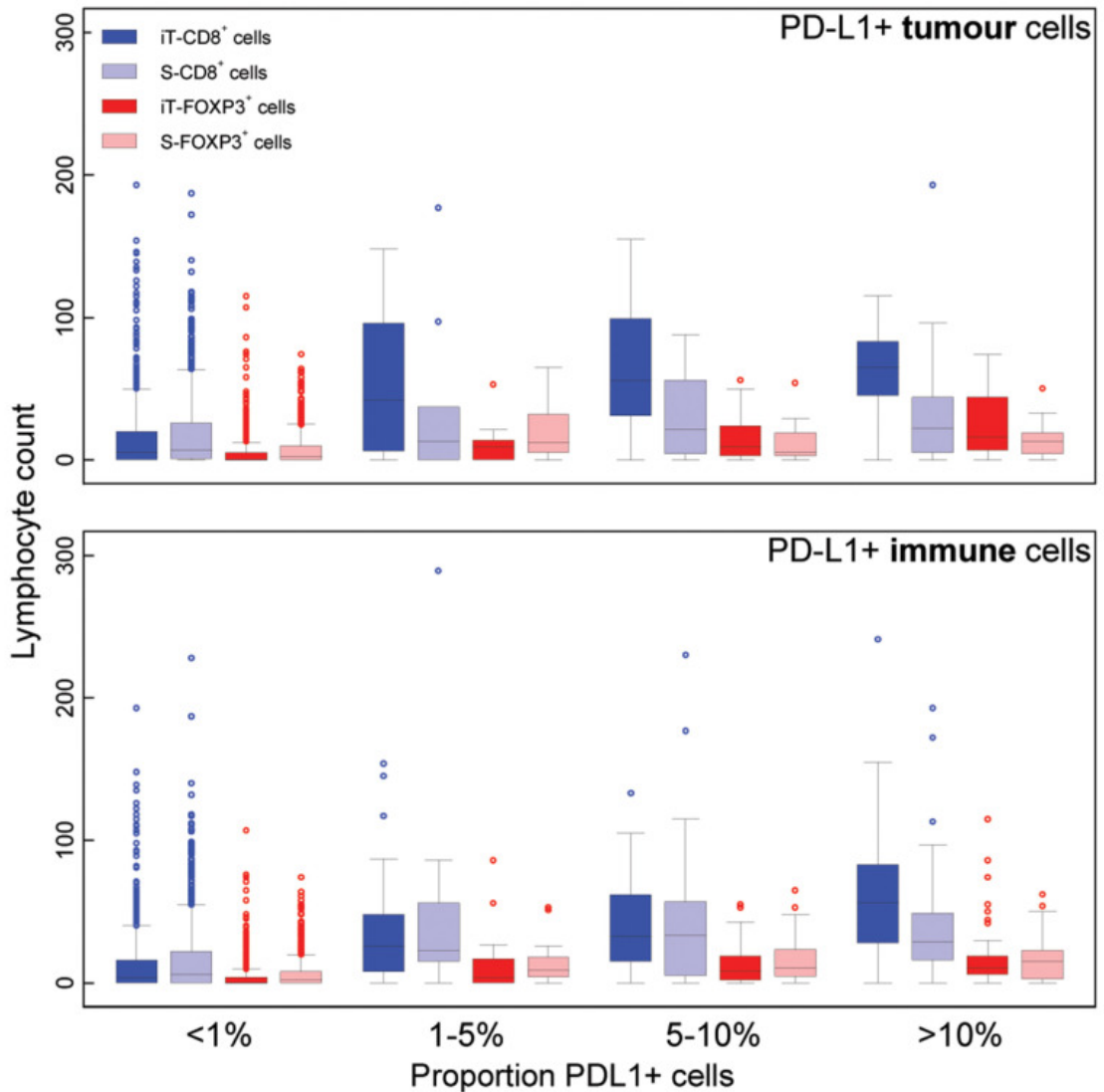
Supplementary Figure 6

Distribution of scores for PD-L1 protein expression by immunohistochemistry on immune and tumour cells in the SEARCH and NEAT studies.



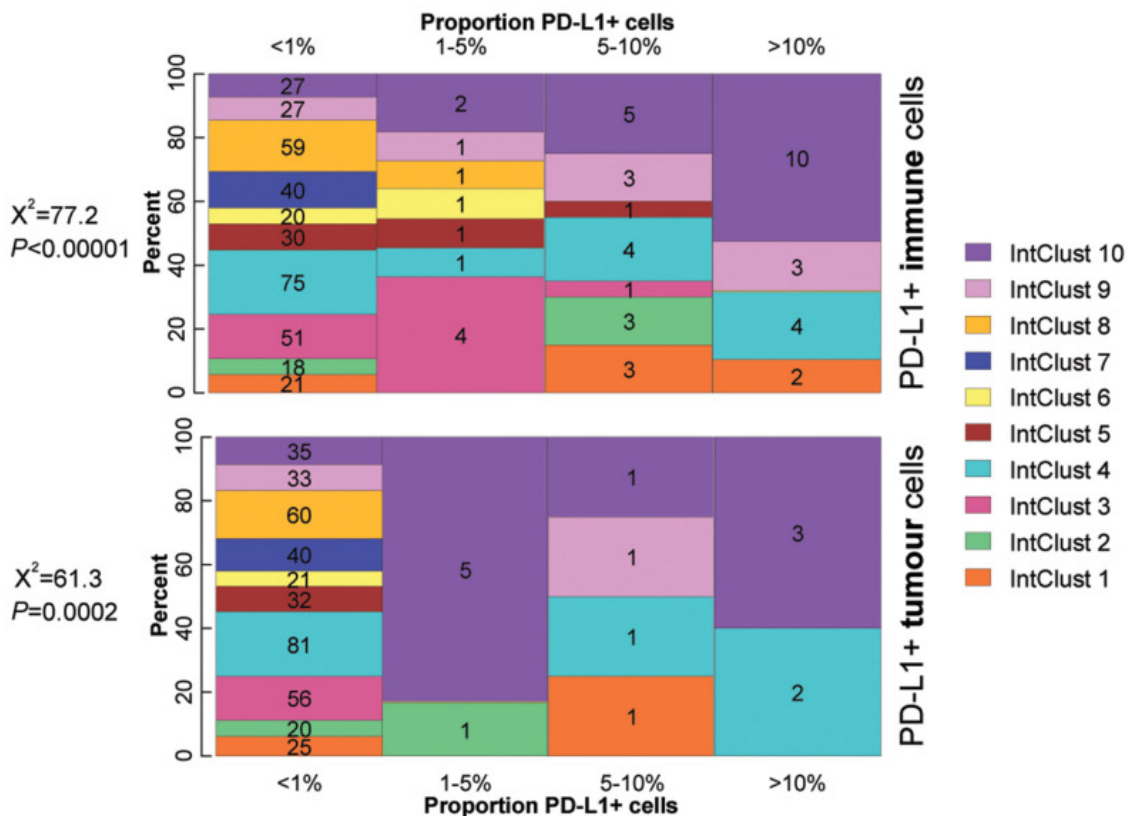
Supplementary Figure 7

Box-plots depicting the distribution of CD8⁺ and FOXP3⁺ lymphocytes by PD-L1 expression in tumour and immune cells in the cases pooled from the SEARCH and NEAT studies. Abbreviations: iT – intratumoural; S – stromal.



Supplementary Figure 8

Distribution of Integrative Clusters of breast cancer by PD-L1 protein expression in immune and tumour cells. Values within coloured boxes are the absolute number of cases.



Supplementary Methods

Patient population

Primary tumour samples from three clinical studies were used for this analysis: the SEARCH observational study[1], the NEAT randomised controlled trial[2] and the METABRIC genomic study[3, 4]. SEARCH (Studies of Epidemiology and Risk factors in Cancer Heredity) is an ongoing observational population-based study of women with primary breast cancer in East Anglia, England. Details of this study have been published previously[1]. It comprises prevalent and incident cases. Women already diagnosed with breast cancer (prevalent cases) at study commencement in 1996 were recruited providing they were under the age of 55 years and diagnosed between 1991 and 1996. After 1996 women newly diagnosed with breast cancer (incident cases) were also recruited to the study providing they were less than 70 years of age at diagnosis. NEAT (National Epirubicin Adjuvant Trial) was a phase III randomised controlled trial which compared the efficacy of cyclophosphamide methotrexate and fluorouracil (CMF) against Epirubicin in addition to CMF (E-CMF). Details and results of this trial have been published previously[2, 5]. METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) is a multi-centre observational genomics study of breast cancer. Gene expression and copy-number data were generated as previously described[3] using microarrays. All studies received ethics committee approval and patients provided written informed consent. Patient characteristics are summarised in Supplementary Table 1. Of the 418 cases included in analyses from the METABRIC study, 153 cases are also represented in the SEARCH study however no analyses pooling these studies together are presented.

Molecular subtyping and genomics

Details of the immunohistochemistry protocols and scoring methods for the five markers used for molecular subtyping have been published previously[6]. Two measures of genomic instability were used to investigate the relationship with PD-L1 expression: a genomic instability index based on the proportion of the genome affected by copy number alterations and the number of genomic breakpoints. Computation of both measures has been described previously[7]. In addition, *TP53* mutation status assayed as previously described[3] was also investigated as a correlate of PD-L1 expression.

Statistical Analyses

It has been shown that the inclusion of prevalent cases in survival analyses can bias estimates. To adjust for this bias we left truncated survival time for patients recruited to the SEARCH study, where patients were considered 'at risk' following their date of study entry rather than their date of diagnosis. It is important to note that date of diagnosis remained the commencement of survival time for these patients, however their inclusion in survival models was restricted (left truncated) to the

period during which they were under observation. This method has been shown to yield unbiased estimates providing the proportional-hazards assumption is not violated[8].

Cell culture and Manipulation of PD-L1 expression in cell lines

MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from American Type Culture Collection (ATCC) and cultured in DMEM media supplemented with 10% FBS and L-Glutamin.

For knock-down experiments, sub-confluent MDA-MB-231 cells were transfected with 25nM of either ON-TARGETplus Non-targeting siRNA (Control siRNA) (D-001810-01-20, Thermo Scientific Dharmacon) or the ON-TARGETplus Human CD274 (29126) siRNA - SMARTpool (L-015836-01, Dharmacon) using the DharmaFECT 4 Transfection Reagent (T-2004-02, Dharmacon). The transfections were conducted for 48 hours, in accordance with the protocol stated by Thermo Scientific Dharmacon.

For the induction of PD-L1 levels, sub-confluent MCF-7 and MDA-MB-231 cell lines were treated with Recombinant Human IFN-gamma (IFN γ) (285-IF-100, R&D systems), at a concentration of either 10ng/ml or 100 ng/ml. The treatment was maintained for 48 hours.

Cells were then harvested and protein lysates for Western blot as well as cell pellets for FFPE were collected. PD-L1 levels were assessed by Western blot analysis and Immunohistochemistry, respectively.

Western blot

Proteins were extracted using the Ripa buffer and quantified on the infrared (IR)-based biomolecular quantitation system Direct Detect® System (Millipore). They were then run a SDS-PAGE gel. Following electrophoresis, they were transferred onto a nitrocellulose membrane using the iBlot® 2 Dry Blotting System (Thermo Fisher Scientific). After blocking with Odyssey® Blocking Buffer (927-40000, Li-Cor), the membrane was probed with the PD-L1 (E1L3N®) XP® Rabbit mAb (#13684, Cell Signaling Technologies) diluted to 1:1,000 and the Mouse monoclonal GAPDH Antibody (#G8795, Sigma Aldrich) to 1:15,000.

Detection of the anti PD-L1 primary antibody was performed with the IRDye 800CW Goat (polyclonal) anti Rabbit (926-32211, Li-Cor Biosciences), 1:5,000, while the GAPDH was detected with the IRDye® 680LT Goat anti-Mouse (926-68020, Li-Cor), 1:15,000. All antibodies were diluted in Odyssey Buffer.

The proteins were visualised using the Odyssey CLx Infrared Technology (Li-Cor) and images were taken with the automated capture option from Image studio Version 4.0 software. The proteins were then quantified using the same software.

Immunohistochemistry

The 3 μ m paraffin sections were dewaxed in xylene and rehydrated through graded ethanol concentrations, on a Leica ST5020 system.

The PD-L1 (E1L3N®) XP® Rabbit mAb (#13684, Cell Signaling Technologies) was then validated for immunohistochemistry on the Leica Bond RX automated system (Leica Biosystems), using a BOND polymer refine detection kit (DS9800, Leica Microsystems). The bound primary antibody was detected using a polymer-conjugated secondary antibody that was then detected with the 3-3'-diaminobenzidine (DAB) chromogen.

Tissue pre-treatment conditions were optimised to heat induced antigen retrieval for 20 minutes in EDTA, pH 9 and the optimised dilution of the PD-L1 primary antibody was 1:100.

References

1. Lesueur F, Pharoah P, Laing S et al. Allelic association of the human homologue of the mouse modifier Ptp^{rrj} with breast cancer. *Hum Mol Genet* 2005; 14: 2349-2356.
2. Poole CJ, Earl HM, Hiller L et al. Epirubicin and cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy for early breast cancer. *N Engl J Med* 2006; 355: 1851-1862.
3. Curtis C, Shah SP, Chin SF et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012; 486: 346-352.
4. Ali HR, Rueda OM, Chin SF et al. Genome-driven integrated classification of breast cancer validated in over 7,500 samples. *Genome Biol* 2014; 15: 431.
5. Earl HM, Hiller L, Dunn JA et al. Adjuvant epirubicin followed by cyclophosphamide, methotrexate and fluorouracil (CMF) vs CMF in early breast cancer: results with over 7 years median follow-up from the randomised phase III NEAT/BR9601 trials. *Br J Cancer* 2012; 107: 1257-1267.
6. Ali HR, Provenzano E, Dawson SJ et al. Association between CD8⁺ T-cell infiltration and breast cancer survival in 12 439 patients. *Ann Oncol* 2014; 25: 1536-1543.
7. Vollan HK, Rueda OM, Chin SF et al. A tumor DNA complex aberration index is an independent predictor of survival in breast and ovarian cancer. *Molecular oncology* 2015; 9: 115-127.
8. Azzato E, Greenberg D, Shah M et al. Prevalent cases in observational studies of cancer survival: do they bias hazard ratio estimates? *Br J Cancer* 2009; 100: 1806-1811.

Supplementary Material

Supplementary Methods

Study populations, statistical analyses and experimental procedures for cell culture, *CD274* knockdown and immunohistochemistry.

Supplementary Tables

Supplementary Table 1

Summary of clinical characteristics of SEARCH and NEAT studies.

Supplementary Table 2

Scheme for molecular subtyping of tumours within the SEARCH and NEAT studies.

Supplementary Table 3

Univariate Cox-regression of PD-L1+ immune cells in ER-positive breast cancer for disease-specific survival, stratified by study.

Supplementary Table 4

Univariate Cox-regression of PD-L1+ immune cells in ER-negative breast cancer for disease-specific survival, stratified by study.

Supplementary Table 5

Survival estimates at five and ten years for subgroups defined by PD-L1 and iT-CD8 status, separately for ER-positive and ER-negative disease.

Supplementary Figures

Supplementary Figure 1

Top panel: Formalin-fixed paraffin-embedded cell pellets of breast cancer cell lines (MDA-MD-231 and MCF7) variably treated with interferon gamma as indicated with PD-L1 expression detected by immunohistochemistry.

Supplementary Figure 2

- A. Western blot analysis for PD-L1 protein using lysates from MDA-MB-231 untreated and treated with interferon gamma.
- B. Immunohistochemistry for PD-L1 on formalin-fixed paraffin-embedded cell pellets of MDA-MB-231 untreated and treated with interferon gamma.
- C. Western blot analysis for PD-L1 protein using lysates from MDA-MB-231 following treated with control (scramble) and PD-L1 targeting siRNA.

- D. Immunohistochemistry for PD-L1 on formalin-fixed paraffin-embedded cell pellets of MDA-MB-231 treated with control (scramble) and PD-L1 targeting siRNA.
- E. Immunohistochemistry for PD-L1 applied to human tonsil (left) revealing weak membranous expression by macrophages and on human placenta (right) revealing strong expression by syncytiotrophoblasts.

Bottom panel: Concurrent western blot analysis for PD-L1 protein.

Supplementary Figure 3

Box-plots depicting the correlation between PD-L1 protein expression and PD-L1 mRNA expression in tumour cells and immune cells.

Supplementary Figure 4

Box-plots depicting the distribution of the number of genomic breakpoints (top) and of a genomic instability index (bottom) by the proportion of immune or tumour cells expressing PD-L1 protein by immunohistochemistry.

Supplementary Figure 5

Cross-tabulations of *TP53* mutation status versus the proportion of immune or tumour cells expressing PD-L1, separately for ER-positive and ER-negative tumours. Boxes are shaded with an intensity in proportion to the depicted value.

Supplementary Figure 6

Distribution of scores for PD-L1 expression by immunohistochemistry on immune and tumour cells in the SEARCH and NEAT studies.

Supplementary Figure 7

Box-plots depicting the distribution of CD8⁺ and FOXP3⁺ lymphocytes by PD-L1 protein expression in tumour and immune cells in the cases pooled from the SEARCH and NEAT studies. Abbreviations: iT – intratumoural; S – stromal.

Supplementary Figure 8

Distribution of Integrative Clusters of breast cancer by PD-L1 protein expression in immune and tumour cells. Values within coloured boxes are the absolute number of cases.

Supplementary Tables

Supplementary Table 1

Summary of clinical characteristics of SEARCH and NEAT studies.

	NEAT		SEARCH		Total	
	No.	%	No.	%	No.	%
Deaths from breast cancer						
Censored	1295	77	3482	85	4777	83
Dead	389	23	597	15	986	17
Total	1684	100	4079	100	5763	100
Age (>55 years)						
<55	1291	77	2529	62	3820	66
≥55	393	23	1550	38	1943	34
Total	1684	100	4079	100	5763	100
No Positive Nodes						
0	531	32	2292	56	2823	49
1	341	20	569	14	910	16
2 to 4	514	31	508	12	1022	18
5 to 9	211	13	184	5	395	7
10+	80	5	120	3	200	3
Missing	7	0	406	10	413	7
Total	1684	100	4079	100	5763	100
Tumour size						
<10mm	76	5	444	11	520	9
10-19mm	478	28	1741	43	2219	39
20-29mm	576	34	1003	25	1579	27
30-49mm	383	23	497	12	880	15
50+mm	137	8	145	4	282	5
Missing	34	2	249	6	283	5
Total	1684	100	4079	100	5763	100
Grade						
1	97	6	799	20	896	16
2	569	34	1701	42	2270	39
3	999	59	1102	27	2101	36
Missing	19	1	477	12	496	9
Total	1684	100	4079	100	5763	100
Endocrine therapy						
No	0	0	837	21	837	15
Yes	0	0	3231	79	3231	56
Missing	1684	100	11	0	1695	29
Total	1684	100	4079	100	5763	100
Chemotherapy						
No	2	0	2472	61	2474	43
Yes	1682	100	1242	30	2924	51
Missing	0	0	365	9	365	6
Total	1684	100	4079	100	5763	100
ER status						
Negative	541	32	757	19	1298	23
Positive	819	49	2267	56	3086	54
Missing	324	19	1055	26	1379	24
Total	1684	100	4079	100	5763	100
HER2 status						
Negative	1272	76	2533	62	3805	66
Positive	327	19	345	8	672	12
Missing	85	5	1201	29	1286	22
Total	1684	100	4079	100	5763	100
PDL1+ immune cells						
<1%	1304	77	2377	58	3681	64
1-5%	46	3	18	0	64	1
5-10%	52	3	30	1	82	1
>10%	61	4	28	1	89	2
Missing	221	13	1626	40	1847	32
Total	1684	100	4079	100	5763	100
PDL1+ tumour cells						
<1%	1427	85	2423	59	3850	67
1-5%	12	1	10	0	22	0
5-10%	11	1	13	0	24	0
>10%	13	1	7	0	20	0
Missing	221	13	1626	40	1847	32
Total	1684	100	4079	100	5763	100

Supplementary Table 2

Scheme for molecular subtyping of tumours within the SEARCH and NEAT studies.

Molecular subtype	ER and/or PR	HER2	CK56 and/or EGFR
Luminal 1a	Positive	Negative	Negative
Luminal 1b	Positive	Negative	Positive
Luminal 2	Positive	Positive	NA
HER2	Negative	Positive	NA
CBP	Negative	Negative	Positive
5NP	Negative	Negative	Negative

Abbreviations: CBP – core basal phenotype, CK56 – cytokeratin 56, ER – estrogen receptor, EGFR – epidermal growth factor receptor, HER2 – human epidermal growth factor receptor 2, PR – progesterone receptor, 5NP – five marker negative phenotype.

Supplementary Table 3

Univariate Cox-regression of PD-L1+ immune cells in ER-positive breast cancer for disease-specific survival, stratified by study.

ER-positive						
Variable	Categories	Observations	Events	HR	95% CI	P-value
PDL1+ immune cells	<1%	2309	302		Referent	
PDL1+ immune cells	1-5%	25	3	0.6	0.19 - 1.87	0.4
PDL1+ immune cells	5-10%	22	5	1.55	0.64 - 3.76	0.3
PDL1+ immune cells	>10%	17	1	0.34	0.05 - 2.41	0.3
Total		2373	311			

Supplementary Table 4

Univariate Cox-regression of PD-L1+ immune cells in ER-negative breast cancer for disease-specific survival, stratified by study.

ER-negative						
Variable	Categories	Observations	Events	HR	95% CI	P-value
PDL1+ immune cells	<1%	869	194		Referent	
PDL1+ immune cells	1-5%	25	8	1.5	0.74 - 3.04	0.3
PDL1+ immune cells	5-10%	46	9	0.92	0.47 - 1.79	0.8
PDL1+ immune cells	>10%	60	8	0.53	0.26 - 1.07	0.08
Total		1000	219			

Supplementary Table 5

Survival estimates at five and ten years for subgroups defined by PD-L1 and iT-CD8 status, separately for ER-positive and ER-negative disease.

ER-NEGATIVE

PD-L1 immune cell status*	iT-CD8 status[†]	Time	Observations	Events	Survivor function	95% CI	
Negative	Negative	5	190	44	0.79	0.73	0.84
Negative	Positive	5	355	84	0.79	0.75	0.83
Positive	Negative	5	3	1	0.75	0.13	0.96
Positive	Positive	5	80	19	0.79	0.70	0.86
Negative	Negative	10	75	14	0.71	0.64	0.77
Negative	Positive	10	121	20	0.74	0.69	0.78
Positive	Negative	10	1	0	0.75	0.13	0.96
Positive	Positive	10	20	2	0.75	0.64	0.83

ER-POSITIVE

PD-L1 immune cell status*	iT-CD8 status[†]	Time	Observations	Events	Survivor function	95% CI	
Negative	Negative	5	781	62	0.92	0.90	0.94
Negative	Positive	5	753	90	0.89	0.86	0.91
Positive	Negative	5	6	1	0.86	0.33	0.98
Positive	Positive	5	39	2	0.95	0.81	0.99
Negative	Negative	10	330	39	0.86	0.83	0.88
Negative	Positive	10	277	63	0.79	0.76	0.82
Positive	Negative	10	3	1	0.73	0.29	0.93
Positive	Positive	10	7	3	0.82	0.59	0.92

ER-NEGATIVE

PD-L1 tumour cell status*	iT-CD8 status[†]	Time	Observations	Events	Survivor function	95% CI	
Negative	Negative	5	191	44	0.80	0.74	0.84
Negative	Positive	5	406	96	0.79	0.75	0.83
Positive	Negative	5	2	1	0.67	0.05	0.95
Positive	Positive	5	29	7	0.78	0.59	0.89
Negative	Negative	10	75	14	0.71	0.64	0.77
Negative	Positive	10	132	20	0.74	0.70	0.78
Positive	Negative	10	1	0	0.67	0.05	0.95
Positive	Positive	10	9	2	0.68	0.45	0.83

ER-POSITIVE

PD-L1 tumour cell status*	iT-CD8 status[†]	Time	Observations	Events	Survivor function	95% CI	
Negative	Negative	5	786	63	0.92	0.90	0.94
Negative	Positive	5	784	92	0.89	0.87	0.91
Positive	Negative	5	1	0	1.00		
Positive	Positive	5	6	0	1.00		
Negative	Negative	10	332	40	0.86	0.83	0.88
Negative	Positive	10	283	65	0.80	0.77	0.82
Positive	Negative	10	1	0	1.00		
Positive	Positive	10	1	1	0.67	0.05	0.95

*PD-L1 status: Negative $\leq 1\%$ cells, Positive $>1\%$ cells

[†]Intra-tumoural (iT)-CD8 status: Negative – no CD8-positive lymphocytes, Positive – any CD8-positive lymphocytes