The relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer

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Background: Tumour budding has previously been reported to predict survival in several solid organ tumours, including breast; however, whether this is independent of other aspects of the tumour microenvironment is unknown. In the present study, the relationship between tumour budding, the tumour microenvironment and survival was examined in patients with invasive ductal breast cancer.

Methods: Patients presenting between 1995 and 1998 were studied ($n = 474$). Using routine pathological sections, tumour budding was measured at the invasive margin and its association with clinicopathological characteristics and cancer-specific survival (CSS) was examined.

Results: Tumour budding was associated with several adverse pathological characteristics, including lymph node involvement, lymph vessel invasion (LVI), increased tumour stroma percentage (TSP) and weaker local inflammatory infiltrative. Tumour budding was associated with reduced CSS (hazard ratio (HR) 2.08, 95% confidence interval (CI) 1.14–3.09, $P = 0.004$), independent of nodal status, molecular subtypes, tumour necrosis, CD8 +, CD138 +, LVI, blood vessel invasion and TSP. Further, tumour budding was independently associated with reduced CSS in node-negative patients (HR 2.63, 95% CI 1.16–5.92, $P = 0.020$) and those who have low TSP (HR 1.98, 95% CI 1.09–3.57, $P = 0.024$) and high-grade local inflammatory infiltrative (HR 2.27, 95% CI 1.35–5.36, $P = 0.014$).

Conclusions: Tumour budding was a significant predictor of survival in patients with invasive ductal breast cancer, independent of adverse pathological characteristics and components of tumour microenvironment. The present study further confirms the clinical utility of both tumour and host-based factors of tumour microenvironment.

In the United Kingdom, > 49 000 women are diagnosed with breast cancer in 2011 and approximately 80% survive at least 5 years (Cancerstats, 2014). Breast cancer is a heterogeneous disease and a number of different molecular subtypes have emerged; however, standard histopathological characteristics remain the most useful prognostic factors. It is clear for such heterogeneous disease that the need to effectively stratify patients according to likely outcome remains important. This should be done against a comprehensive characterisation of the tumour and its microenvironment. For example, there is now increased recognition of the importance of the tumour microenvironment, including tumour necrosis, host local inflammatory responses and tumour stroma, in cancer progression and survival (Richards et al, 2011; Mohammed et al, 2012a; Freeman et al, 2013).

Recently, the tumour budding, which refers to detachment of single or cluster of up to five cancer cells scattered in stroma at the invasive front of tumour (Ueno et al, 2002; Prall et al, 2005; Lugli et al, 2009), has been proposed as an important determinant of

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progression and survival in a number of solid cancers (Hase *et al*, 1993; Ueno *et al*, 2002; Prall *et al*, 2005; Choi *et al*, 2007; Koike *et al*, 2008; Masugi *et al*, 2010; Koyuncuoglu *et al*, 2012; Taira *et al*, 2012). In particular, tumour budding is thought to be an early step in cancer metastasis as budded cells have the characteristics of epithelial–mesenchymal transition (EMT) (Masugi *et al*, 2010; Zlobec and Lugli, 2010; Koyuncuoglu *et al*, 2012; Lugli *et al*, 2012; Taira *et al*, 2012; Liang *et al*, 2013; Dawson and Lugli, 2015), which is a crucial step during carcinoma progression and metastasis (Kalluri and Weinberg, 2009).

In breast cancer, there is still limited information about the role of tumour budding in breast cancer (Liang *et al*, 2013; Salhia *et al*, 2015). Liang *et al* (2013) has reported the significance of budding in small breast cancer cohort ($n = 160$) with limited follow-up and only reported budding effect on overall survival but did not report on cancer-specific survival (CSS) as an end point. The second report examined the association of tumour budding and clinicopathological characteristics. However, survival analysis was not reported.

Moreover, it is not clear whether the effect of an increased tumour budding on survival is independent of host inflammatory response and other components of the tumour microenvironment. Therefore, the present study aims to examine the relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer.

MATERIALS AND METHODS

Patients presenting with invasive ductal breast cancer at Glasgow Royal Infirmary, Western Infirmary and Stobhill Hospital, at West of Scotland, between 1995 and 1998 and had formalin-fixed paraffin-embedded blocks of the primary tumour available for evaluation were studied ($n = 474$). The study was approved by the Research Ethics Committee of the West Glasgow University Hospitals NHS Trust (REC reference is 07/s0704/61).

Clinicopathological data that included age, tumour size, tumour grade, lymph node status, type of surgery and use of adjuvant treatment (chemotherapy, hormonal therapy and/or radiotherapy) were retrieved from the routine reports. Tumour grade was assigned according to Nottingham Grading System. ER and PR status were assessed on tissue microarrays (TMAs) using immunohistochemestry (IHC) with Dako (Glostrup, Denmark) ER antibody and Leica (Wetzlar, Germany) PR antibody and scored according to the American Society of Clinical Oncology and College of American Pathologists guidelines with a cutoff value of 1% positive tumour nuclei (Hammond *et al*, 2010). Her2 status were assessed visually using TMAs as previously described, that is, a score $3 +$ is regarded as positive; $2 +$ is regarded as equivocal, leading to referral for Her-2 FISH; and 0 and $1 +$ are regarded as negative (Mohammed *et al*, 2012b).

Full-section haematoxylin and eosin (H&E) slides for the 474 patients were used to score local inflammatory infiltrate according to Klintrup criteria. Klintrup scoring of slides was carried out as previously described. Briefly, tumours were scored on four-point scores based on appearances at the tumour invasive margin. A score of 0 signified that there were no inflammatory cells at tumour's invasive margin; score 1 indicated a mild and patchy inflammatory cells; score 2 denoted a prominent band-like inflammatory reaction at the invasive margin; and score 3 revealed a florid cup-like inflammatory infiltrate at the invasive edge (Klintrup *et al*, 2005; Mohammed *et al*, 2012a). Individual immune cell types were assessed using IHC staining on TMA sections for macrophages, helper and cytotoxic T-lymphocytes and plasma cells using CD68, CD4, CD8 and CD138 antibodies, respectively (Mohammed *et al*, 2013).

Full-section H&E slides were also used to score the tumour stroma percentage (TSP) as previously reported (Gujam *et al*, 2014b). Briefly, at $\times 5$ magnification, an area representative of the tumour invasive margin was selected, and then a single field of $\times 10$ magnification was examined, ensuring that tumour cells were present at all four sides of the image and the area of stroma was calculated as a percentage.

Lymph and blood vessel invasion (LVI and BVI, respectively) were assessed, on 2.5-μm thick sections, using IHC staining with the lymphatic endothelial marker D2-40 (Covance, Monoclonal Antibody, SIG-3730, Princeton, NJ, USA) diluted $1:100$ and vascular endothelial marker Factor VIII (Mouse Monoclonal Antibody, NCL-L-Vwf, Leica, Newcastle, UK) diluted $1:100$ as previously described (Gujam *et al*, 2014a).

The molecular subtypes were defined as follows: Luminal A: oestrogen (ER) and/or progesterone receptor (PR) positive, Her-2 negative, low proliferative index ($\leqslant 15\%$); Luminal B: hormone receptor positive, Her-2 positive, high proliferative index ($>15\%$); Her-2 subtype: Her-2 positive and hormone receptor negative, any proliferative index; and triple negative: Her-2 negative, hormone receptor negative, any proliferative index.

Assessment of tumour budding. Full H&E-stained sections were used to assess tumour budding at the deepest tumour invasion margin as previously described (Ueno *et al*, 2002). Tumour sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at $\times 20$ magnification, and visualisation was carried out using the Slidepath Digital Image Hub, version 4.0.1 (Slidepath, Leica Biosystems, Milton Keynes, UK). At $\times 5$ magnification, an area representative of the tumour invasive margin was selected. A grid of 0.385 mm^2 size at the five highest budding areas was drawn. Using a $\times 20$ magnification, a tumour budding was counted. A bud was identified as an isolated single cancer cell or a group of up to five cancer cells (Ueno *et al*, 2002; Prall *et al*, 2005; Lugli *et al*, 2009; Figure 1). The highest bud count per field was used as the number of buds. Areas of necrosis or mucin were excluded from the field. To ensure reliability, co-scoring of 60 randomly selected cases was carried out by FJG and the consultant pathologist JJG. The interobserver intraclass
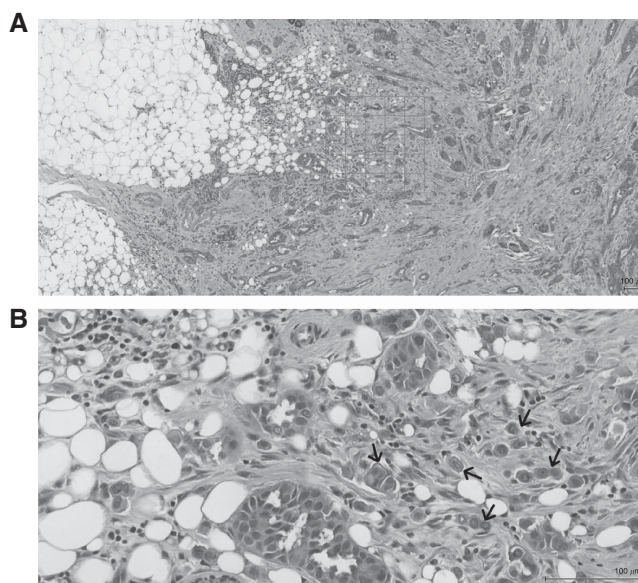

Figure 1. Haematoxylin and eosin stained section of invasive ductal breast cancer. (**A**) Shows a grid of high tumour budding area at the invasive margin, (**B**) shows single and clusters of tumour budding (arrows). Original magnification $\times 20$, scale 100 μm.

correlation coefficient (ICCC) for the raw continuous scores was 0.813 ($P < 0.001$). All the slides were then scored by FJG.

Patients were routinely followed up following surgery. Date and cause of death was cross-checked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31 May 2013 and that served as the censor date.

CSS was measured from the date of primary surgery until the date of death from breast cancer.

Statistical analysis. To identify the cutoff value of tumour budding for survival analysis, the highest budding count per five fields were split into tertiles, and survival analysis between each group using Kaplan–Meier log-rank test was performed (Figure 2A) (Choi *et al*, 2007; Sy *et al*, 2010). Subsequently, the first and second tertiles (highest tumour budding count $\leqslant 20$) were considered as the low budding group and the third tertile (highest tumour budding > 20) was considered as the high budding group. To simplify all further analysis, patients were subsequently grouped into low tumour budding ($\leqslant 20$) and high tumour budding (> 20).

When ROC analysis was carried out with CSS as an end point, the optimal number of tumour buds was between 15 (sensitivity = 0.55, specificity = 0.70) and 20 buds (sensitivity = 0.63, specificity = 0.60) per 5 fields. Therefore, the threshold was set at 20 buds. At this threshold, the AUC was 0.625 ($P < 0.001$). This was consistent with the threshold derived from the plot of the tertiles (see Figure 2A).

Consistency between the observers was analysed using the ICCC value. The relationships between variables were assessed using contingency table analysis with the χ^2 test for linear trend. Kaplan–Meier analysis was used to examine the effect of tumour budding on CSS. Univariate survival analysis was performed using Cox proportional hazards regression. Variables with *P*-value of < 0.1 were entered into a multivariable model using a backwards conditional method for all patients, node-negative patients and those who have low TSP and high K–M score. All statistical analyses were two-sided and significance defined as *P*-value < 0.05. All statistical analysis was performed using the SPSS software version 22 (IBM SPSS, Chicago, IL, USA).

RESULTS

Table 1 summarises clinicopathological characteristics of patients ($n = 474$). The majority of patients (70%) were > 50 years, had small tumour size $\leqslant 20$ mm (60%), had grade II and III tumours (80%) and negative lymph node (54%). The majority had ER-positive (69%) tumours, PR-positive tumours (61%) and Her-2-negative tumours (80%). In all, 182 (38%) had lumpectomy and radiotherapy, and 292 (62%) had mastectomy and radiotherapy. Also, 243 (51%) patients received endocrine therapy only, 101 (21%) patients received adjuvant chemotherapy only and 95 (20%) had both.

A high tumour budding was identified in 167 (35%) patients. From the distribution chart of the frequency of the buds/5 fields per patient, the number of patients around the cutoff 18, 19, 20, 21 and 22 buds was 15, 14, 17, 42 and 36 patients, respectively.

The relationship between tumour budding, clinicopathological characteristics, local host inflammatory response and TSP is presented in Table 1. Tumour budding was not significantly
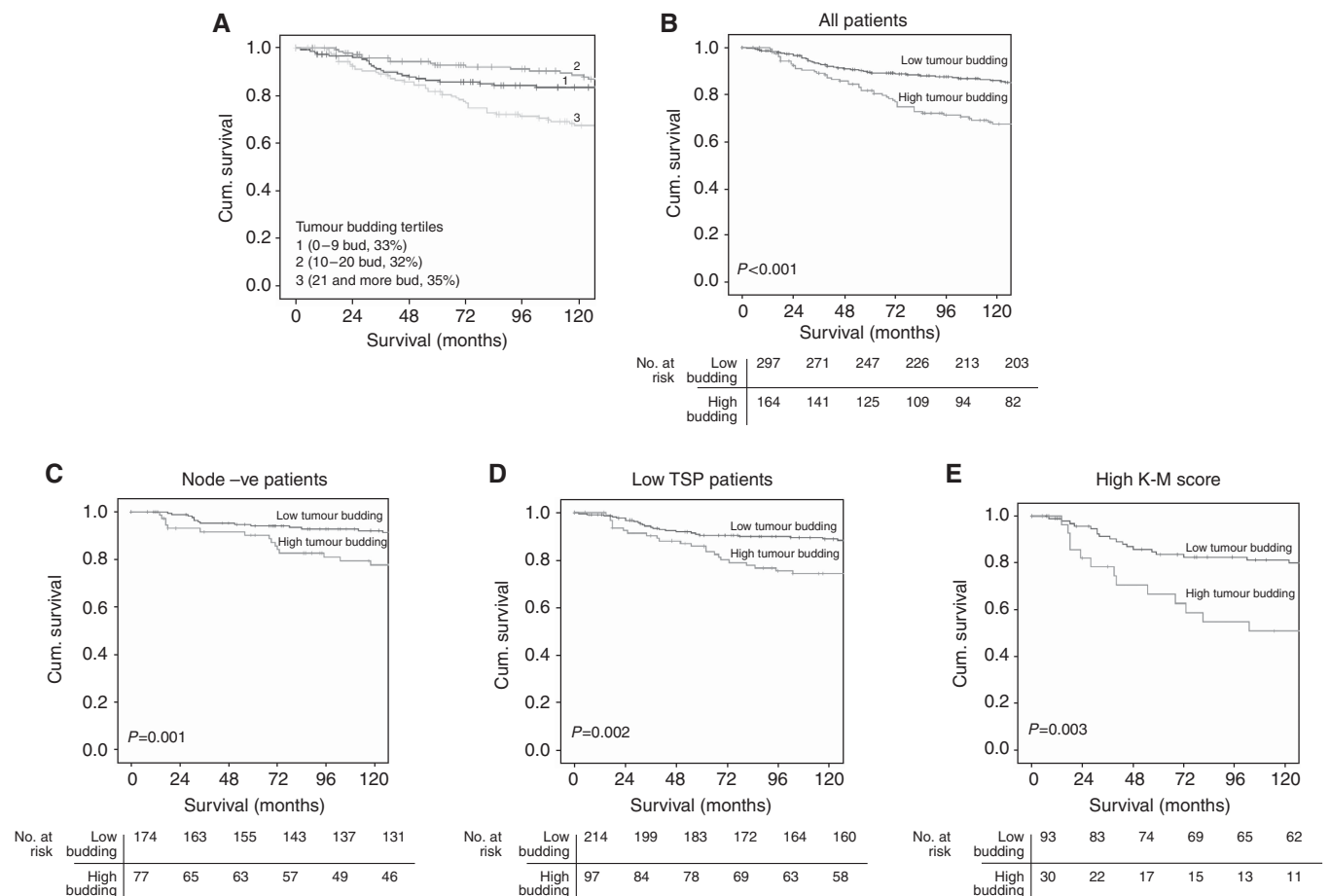

Figure 2. Kaplan-Meier survival curves (Log rank) of cancer specific survival (**A**) according to the tumour budding tertiles, (**B**) in all patients, (**C**) in patients with node negative tumours, (**D**) in patients with low TSP and (**E**) in patients with high K–M score.

Table 1. Relationship between clinicopathological characteristics and tumour budding in patients with invasive ductal breast cancer (474)				
	Patients, n (%)	Tumour budding ⩽20 n = 307 (65%)	Tumour budding >20 n = 167 (35%)	P-value
Age (⩽50/>50 years)	140 (30%)/334 (70%)	99/208	41/126	0.080
Size (⩽20/21–50/>50 mm)	283 (60%)/178 (38%)/13 (3%)	186/114/7	97/64/6	0.469
Grade (I/II/III)	94 (20%)/190 (40%)/190 (40%)	62/108/137	32/82/53	0.099
Involved lymph node (negative/positive)[a]	257 (54%)/212 (45%)	180/124	77/88	0.009
ER status (no/yes)[a]	141 (31%)/330 (69%)	105/199	36/131	0.003
PR status (no/yes)[a]	180 (38%)/289 (61%)	126/176	54/113	0.054
Her-2 status (no/ yes)[a]	381 (80%)/74 (16%)	245/44	136/30	0.429
Molecular subtypes (Luminal A/Luminal B/Her-2 +ve/triple negative)[a]	197 (42%)/89 (19%)/46 (10%)/109 (23%)	118/52/28/82	79/37/18/27	0.102
Tumour necrosis (low/high)	226 (48%)/248 (52%)	138/169	88/79	0.107
Ki67 (low/high)[a]	345 (73%)/106 (22%)	225/64	120/42	0.364
Lymph vessel invasion (no/yes)	327 (69%)/147 (31%)	232/75	94/73	<0.001
Blood vessel invasion (no/yes)	419 (88%)/55 (12%)	271/36	150/17	0.610
Tumour inflammatory infiltrate				
Klintrup–Mäkinen grade (weak/strong)	345 (73%)/129 (27%)	209/98	136/31	0.002
CD68 + (low/moderate/high)[a]	145 (31%)/153 (32%)/153 (32%)	99/88/101	46/65/52	0.708
CD4 + (low/moderate/high)[a]	207 (44%)/90 (19%)/157 (33%)	132/53/104	75/37/53	0.675
CD8 + (low/moderate/high)[a]	151 (32%)/145 (31%)/158 (33%)	97/79/113	54/66/45	0.173
CD138 + (low/moderate/high)[a]	254 (54%)/55 (12%)/143 (30%)	164/27/97	90/28/46	0.687
Tumour-stroma percentage (low/high)	320 (68%)/154 (32%)	224/83	98/69	0.001
Locoregional treatment (lumpectomy + radiotherapy/ mastectomy + radiotherapy)	182 (38%)/292 (62%)	118/189	64/103	0.891
Systemic treatment (hormonal/hormonal + chemotherapy/ chemotherapy/none)	243 (51%)/95 (20%)/101 (21%)/27 (6%)	148/92/72/18	95/33/29/9	0.096
Alive/cancer death/non-cancer death[a]	275 (58%)/96 (20%)/90 (19%)	199/43/55	76/53/35	0.002
Cancer-specific survival (months)[b]		159 (153–164)	136 (127–145)	<0.001

Abbreviations: ER = oestrogen receptor; PR = progesterone receptor.
[a]Number of patients when incomplete data are available.
[b]Mean (95% confidence interval).

associated with age, size, grade, necrosis, Ki67 and BVI. A high tumour budding was associated with ER-positive status ($P = 0.003$), lymph node-positive tumours ($P = 0.009$), presence of LVI ($P < 0.001$) and high TSP ($P = 0.001$). A high tumour budding was inversely associated with local inflammatory response as measured by the K–M score ($P = 0.002$) but not by macrophage, plasma cells and T-cell lymphocyte subtypes.

The relationship between clinicopathological characteristics and tumour budding in node-negative patients is presented in Table 2. A high tumour budding was associated with presence of LVI ($P < 0.001$) and inversely associated with local inflammatory response as measured by the K–M score ($P = 0.038$). A high tumour budding showed a trend towards an association with TSP ($P = 0.080$).

The median survival of survivors was 164 months, with 96 deaths from breast cancer and 90 non-cancer deaths. In all, 13 (3%) patients do not have survival data and were excluded from all survival analysis. Mean CSS was shorter in patients with high tumour budding compared with those with low tumour budding (136 vs 159 months, $P < 0.001$; Figure 2B).

The relationship between tumour budding, clinicopathological characteristics and CSS is presented in Table 3. On univariate analysis, a high tumour budding was associated with shorter CSS ($P < 0.001$). On multivariate analysis, a high tumour budding was associated with reduced CSS (HR 1.96, 95% CI 1.14–3.09, $P = 0.004$), independent of nodal status, molecular subtypes, tumour necrosis, CD8 +, CD138 +, LVI, BVI and TSP.

In node-negative patients, a high tumour budding was associated with shorter mean CSS compared with a low tumour budding (150 vs 167 months, $P = 0.001$; Figure 2C). On multivariate survival analysis (Table 3), a high tumour budding was associated with reduced CSS

(HR 2.63, 95% CI 1.16–5.92, $P = 0.020$), independent of PR status, tumour necrosis, LVI, BVI and TSP.

In order to account for the high TSP and low cellular inflammatory infiltrate effects, sub-group survival analyses were performed based on low TSP and high K-M grade (Table 4). In stroma-low patients, a high tumour budding was associated with shorter mean CSS compared with a low tumour budding (144 vs 161 months, $P = 0.002$; Figure 2D). On multivariate survival analysis (Table 4), a high tumour budding was associated with reduced CSS (HR 1.98, 95% CI 1.09–3.57, $P = 0.024$), independent of molecular subtypes, tumour necrosis, LVI, BVI and CD68 +.

In patients with high K-M score, a high tumour budding was associated with shorter mean CSS compared with a low tumour budding (110 vs 151 months, $P = 0.003$; Figure 2D). On multivariate survival analysis (Table 4), a high tumour budding was associated with reduced CSS (HR 2.27, 95% CI 1.35–5.36, $P = 0.014$), independent of molecular subtypes, LVI, BVI, CD68 +, CD8 + and TSP.

When survival analysis for tumour budding was performed across the different molecular subtypes, a high tumour budding was associated with shorter mean CSS compared with a low tumour budding in Luminal B patients (121 vs 152 months, $P = 0.008$), Her-2 patients (89 vs 142 months, $P = 0.019$) and triple-negative patients (110 vs 144 months, $P = 0.020$) (Figure 3).

DISCUSSION

In the present study, high tumour budding was associated with more tumour stroma and a weaker inflammatory cell infiltrate and was independently associated with reduced CSS. These results

Table 2. Relationship between clinicopathological characteristics and tumour budding in patients with node-negative invasive ductal breast cancer (n = 257)

	Tumour budding ⩽ 20 n = 180 (70%)	Tumour budding > 20 n = 77 (30%)	P-value
Age (⩽ 50/ > 50 years)	49/131	23/54	0.666
Size (⩽ 20/21–50/ > 50 mm)	125/54/1	56/20/1	0.696
Grade (I/II/III)	45/62/73	18/41/18	0.137
ER status (no/yes)	57/122	18/59	0.173
PR status (no/yes)	74/103	23/54	0.072
Her-2 status (no/yes)	151/21	66/10	0.835
Molecular subtypes (Luminal A/Luminal B/Her-2 + ve/triple negative)	77/27/13/50	43/13/6/11	0.065
Tumour necrosis (low/high)	93/87	49/28	0.078
Ki67 (low/high)	136/34	62/12	0.488
Lymph vessel invasion (no/yes)	153/27	50/27	< 0.001
Blood vessel invasion (no/yes)	162/18	69/8	0.925
Tumour inflammatory infiltrate			
Klintrup–Mäkinen grade (weak/strong)	133/47	66/11	0.038
CD68 + (low/moderate/high)	65/52/52	26/26/23	0.747
CD4 + (low/moderate/high)	80/31/59	38/14/24	0.623
CD8 + (low/moderate/high)	60/46/64	26/31/19	0.313
CD138 + (low/moderate/high)	102/15/52	45/15/15	0.393
Tumour-stroma percentage (low/high)	224/83	98/69	0.080
Locoregional treatment (lumpectomy + radiotherapy/mastectomy + radiotherapy)	84/96	37/40	0.832
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	105/20/36/17	54/6/8/8	0.142
Alive/cancer death/non-cancer death	126/15/33	45/17/15	0.184
Cancer-specific survival (months)[a]	167 (162–173)	150 (138–168)	0.001

Abbreviations: ER = oestrogen receptor; PR = progesterone receptor.
[a]Mean (95% confidence interval).

suggest a complex relationship between tumour budding and the tumour microenvironment and disease progression in patients with invasive ductal breast cancer.

Few studies have examined the prognostic value of tumour budding in breast cancer (Liang *et al*, 2013; Salhia *et al*, 2015). However, the prognostic value and method of assessment of tumour budding in colorectal cancer has recently been reviewed by van Wyk *et al* (2015). They concluded that IHC did not improve the detection rate or the prognostic value of tumour budding over that of H&E (van Wyk *et al*, 2015).Therefore, in the present study, the H&E approach was used.

In the present study, examination of tumour budding was by semiquantitative method and was reproducible (ICCC = 0.813). Patients in the present study were divided into three budding groups based on tertiles. The cutoff considered the best discriminator of CSS (Choi *et al*, 2007; Sy *et al*, 2010) was between groups 2 and 3 and yielded a cutoff consistent with previous reports (16–25 buds) (Prall *et al*, 2005; Wang *et al*, 2009). Furthermore, in the present study tumour budding was found in 35% of patients and is consistent with previous report in patients with breast cancer (Liang *et al*, 2013).

The results of the present study showed that high grade budding was significantly associated with ER-positive tumours. These results are consistent with the recent observations of Salhia *et al* (2015) using a pan-cytokeratin stain to assess tumour budding. The basis of these observations is not clear. However, it was recently reported that oestrogen is involved in EMT in breast cancer cell lines with stem cell properties (Sun *et al*, 2014) and that oestrogen is involved in disruption of tight junction and increased cell motility (Sanchez *et al*, 2010; Jiménez-Salazar *et al*, 2014). Therefore, this may suggest that ER-positive tumours with high tumour budding may be undergoing a higher degree of EMT and as a result more metastatic potential. If this were to be the case, then it might be expected that anti-oestrogen treatment would reduce the degree of budding in those patients.

Despite being associated with lymph node metastasis and lymph vessel invasion, tumour budding was not associated with blood vessel invasion. The basis of this observation was not clear; however, tumour buds may find their way of metastasis through invasion into lymph vessels than blood vessels, as it is the major route of metastasis in breast cancer (Mohammed *et al*, 2009; Gujam *et al*, 2014b). In the present study, there was a lack of any perceived association between tumour budding and tumour size, grade, necrosis or Ki67 in all cohort and in sub-group analysis. Previous breast and colorectal cancers studies reported that budded cells displayed lower proliferation activity rather than high proliferative activity (Palmqvist *et al*, 2000; Liang *et al*, 2013; Dawson and Lugli, 2015). This may suggest that detachment and dissociation of tumour cells are not influenced by increased tumour size, its differentiation or proliferation activities.

Although the inter-relationships between the tumour budding, tumour microenvironment and gross pathological characteristics are likely complex, tumour budding remained independently associated with CSS in different patient sub-groups. In high-risk patients with node-negative disease, tumour budding was significantly associated with reduced CSS alongside with tumour necrosis, LVI and BVI. Indeed, the present results further confirm the importance of both tumour and host-based factors of the tumour microenvironment in determining cancer outcome.

Although there is now increased appreciation of the importance of the tumour budding in cancer progression and survival in several previous reports (Hase *et al*, 1993; Ueno *et al*, 2002; Prall *et al*, 2005; Koike *et al*, 2008; Masugi *et al*, 2010; Koyuncuoglu *et al*, 2012; Taira *et al*, 2012; Liang *et al*, 2013), its relationship with other components of the tumour microenvironment has yet to be fully characterised. It was of interest that the present study found an association between tumour budding and increased amount of TSP. Earlier reports in colorectal cancer have shown an association between tumour budding and the presence of an immature stroma and a high density of stromal myofibroblasts (Ueno *et al*, 2004).

Table 3. Relationship between clinicopathological characteristics and cancer-specific survival in patients with invasive ductal breast cancer

	Univariate analysis		Multivariate analysis	
All patients (_n_ = 461)	**Hazard ratio (95% CI)**	_P_-value	**Hazard ratio (95% CI)**	_P_-value
Age (⩽50/>50 years)	1.22 (0.77–1.91)	0.397		
Size (⩽20/21–50/>50 mm)	2.11 (1.49–2.97)	<0.001		0.717
Grade (I/II/III)	1.87 (1.38–2.53)	<0.001		0.491
Involved lymph node (no/yes)	2.76 (1.80–4.23)	<0.001	1.54 (0.95–2.45)	0.081
ER status (no/yes)	0.617 (0.41–0.93)	0.021		0.905
PR status (no/yes)	0.54 (0.36–0.81)	0.003		0.646
Her-2 status (no/yes)	2.02 (1.27–3.22)	0.003		0.216
Molecular subtypes (Luminal A/Luminal B/Her-2 +ve/triple negative)	1.61 (1.34–1.94)	<0.001	1.50 (1.22–1.84)	<0.001
Tumour necrosis (low/high)	1.97 (1.48–8.59)	0.005	2.53 (1.41–4.53)	0.002
Lymph vessel invasion (no/yes)	4.14 (2.75–6.29)	<0.001	2.09 (1.28–3.40)	0.003
Blood vessel invasion (no/yes)	3.39 (2.14–5.39)	<0.001	2.23 (1.35–3.69)	0.002
Klintrup–Mäkinen grade (weak/strong)	1.48 (0.96–2.26)	0.069		0.488
CD68 + (low/moderate/high)	0.86 (0.67–1.09)	0.222		
CD4 + (low/moderate/high)	1.00 (0.80–1.25)	0.983		
CD8 + (low/moderate/high)	0.69 (0.54–0.88)	0.004	0.54 (0.41–0.71)	<0.001
CD138 + (low/moderate/high)	1.38 (1.11–1.71)	0.003	1.01 (1.01–1.02)	0.002
Tumour stroma percentage (low/high)	2.19 (1.46–3.27)	<0.001	1.74 (1.14–2.66)	0.010
Tumour budding (low/high)	2.53 (1.69–3.78)	<0.001	1.96 (1.14–3.09)	0.004
Node-negative patients (_n_ = 251)				
Age (⩽50/>50 years)	1.22 (0.55–2.71)	0.632		
Size (⩽20/21–50/>50 mm)	2.49 (1.25–4.97)	0.010		0.323
Grade (I/II/III)	1.67 (1.03–2.72)	0.038		0.884
ER status (no/yes)	0.48 (0.24–0.97)	0.040		0.693
PR status (no/yes)	0.39 (0.19–0.81)	0.010	0.41 (0.19–0.87)	0.020
Her-2 status (no/yes)	1.75 (0.72–4.26)	0.221		
Molecular subtypes (Luminal A/Luminal B/Her-2 +ve/triple negative)	1.57 (1.12–2.18)	0.008		0.276
Tumour necrosis (low/high)	3.75 (1.73–8.11)	0.001	3.02 (1.31–6.92)	0.009
Lymph vessel invasion (no/yes)	4.67 (2.33–9.36)	<0.001	3.11 (1.39–6.97)	0.006
Blood vessel invasion (no/yes)	3.95 (1.77–8.80)	0.001	2.66 (1.09–6.45)	0.030
Klintrup–Mäkinen grade (weak/strong)	1.45 (0.67–3.13)	0.347		
CD68 + (low/moderate/high)	0.52 (0.39–1.35)	0.643		
CD4 + (low/moderate/high)	1.04 (0.63–1.21)	0.872		
CD8 + (low/moderate/high)	0.653 (0.324–1.15)	0.132		
CD138 + (low/moderate/high)	1.13 (0.48–1.63)	0.625		
Tumour stroma percentage (low/high)	1.46 (1.84–3.66)	0.014		0.087
Tumour budding (low/high)	2.83 (1.46–5.86)	0.003	2.63 (1.16–5.92)	0.020

Abbreviations: CI = confidence interval; ER = oestrogen receptor; PR = progesterone receptor.

Furthermore, tumour stroma have been implicated to facilitate EMT, one of the features of budded cells (Masugi _et al_, 2010; Zlobec and Lugli, 2010; Koyuncuoglu _et al_, 2012; Lugli _et al_, 2012; Taira _et al_, 2012; Liang _et al_, 2013), and metastasis of tumour cells into normal tissue (De Wever and Mareel, 2003; Hemmings 2013). Therefore, the present finding may support an important role of the tumour stroma in facilitating tumour cell de-differentiation and dissemination, perhaps providing suitable energy substrate and reducing the build-up of metabolic waste (Koukourakis _et al_, 2006).

Of interest, the present study has characterised the relationship between tumour budding and local host inflammatory infiltrate. There was a weaker peritumoural inflammatory infiltrate, as measured by K–M score but not by individual subtypes of innate or adaptive immune cells, in patients with high-grade tumour budding. This may suggest that tumour budding may promote the development of a pro-tumour rather than antitumour immune response. It is of interest that the prognostic value of the ratio of CD8 and budding has recently been reported in primary operable colorectal cancer and showed that a high tumour budding and a low CD8 + T-lymphocyte index was associated with tumour

progression and worse survival (Lugli _et al_, 2009), confirming the pro-tumour impact of the tumour budding. However, when we examined CD8/budding index in the present breast cancer cohort, the CD8/budding index did not show additional prognostic value to that of tumour budding alone. Therefore, further work is required to establish the prognostic value of the CD8/budding index in patients with cancer.

Given that tumour budding has independent prognostic value in patients with primary operable ductal breast cancer, it would be of interest to examine the prognostic value of intra-tumoural budding (ITB), as if this was the case then it may be applied to the initial diagnostic biopsy samples to better predict likely outcome and plan treatment prior to surgery. For example, if ITB was strongly associated with lymph node metastases, then it may be that the corresponding sentinel lymph nodes should be analysed carefully on frozen sections in preoperative biopsies. Indeed, Zlobec _et al_ (2014) reported that ITB in preoperative biopsies predicts the presence of lymph node and distant metastases in colorectal cancer patients. However, Salhia _et al_ (2015) reported that, in breast cancer, ITB in preoperative core biopsies was associated with blood

Table 4. Relationship between clinicopathological characteristics and cancer-specific survival in patients with low TSP and high K–M score

	Univariate analysis		Multivariate analysis	
Stroma-low patients (n = 311)	**Hazard ratio (95% CI)**	**P-value**	**Hazard ratio (95% CI)**	**P-value**
Age (⩽50/>50 years)	1.04 (0.59–1.86)	0.885		
Size (⩽20/21–50/>50 mm)	2.73 (1.69–4.40)	<0.001		0.224
Grade (I/II/III)	1.93 (1.27–2.94)	0.002		0.956
Involved lymph node (no/yes)	2.52 (1.45–4.39)	0.001		0.302
ER status (no/yes)	0.52 (0.30–0.92)	0.023		0.814
PR status (no/yes)	0.65 (0.32–1.06)	0.072		0.437
Her-2 status (no/yes)	1.96 (1.04–3.70)	0.036		0.960
Molecular subtypes (Luminal A/Luminal B/ Her-2 +ve/triple negative)	1.66 (1.28–2.16)	<0.001	1.58 (1.18–2.13)	0.002
Tumour necrosis (low/high)	4.59 (2.30–9.14)	<0.001	2.89 (1.35–6.27)	0.007
Lymph vessel invasion (no/yes)	4.59 (2.63–8.02)	<0.001	2.76 (1.49–5.12)	0.003
Blood vessel invasion (no/yes)	5.49 (3.13–9.64)	<0.001	4.43 (2.38–8.25)	<0.001
Klintrup–Mäkinen grade (weak/strong)	1.64 (0.93–2.08)	0.076		0.105
CD68 + (low/moderate/high)	0.99 (0.99–1.00)	0.028	0.95 (0.92–0.99)	0.003
CD4 + (low/moderate/high)	0.01 (0.09–1.01)	0.337		
CD8 + (low/moderate/high)	0.99 (0.99–1.02)	0.243		
CD138 + (low/moderate/high)	1.01 (0.99–1.01)	0.092		0.261
Tumour budding (low/high)	2.29 (1.32–3.95)	0.002	1.98 (1.09–3.57)	0.024
High Klintrup–Mäkinen grade patients (n = 123)				
Age (⩽50/>50 years)	0.73 (0.36–1.45)	0.365		
Size (⩽20/21–50/>50 mm)	1.86 (0.93–3.74)	0.081		0.735
Grade (I/II/III)	1.75 (0.71–4.32)	0.226		
Involved lymph node (no/yes)	2.35 (1.09–5.09)	0.030		0.836
ER status (no/yes)	0.60 (0.29–1.23)	0.165		
PR status (no/yes)	0.49 (0.22–1.10)	0.496		
Her-2 status (no/ yes)	1.22 (0.59–2.53)	0.598		
Molecular subtypes (Luminal A/Luminal B/Her-2 +ve/triple negative)	1.55 (1.05–2.30)	0.029	2.49 (1.50–4.12)	<0.001
Tumour necrosis (low/high)	23.78 (0.16–34.34)	0.213		
Lymph vessel invasion (no/yes)	6.25 (2.65–14.49)	<0.001	5.84 (2.39–14.25)	<0.001
Blood vessel invasion (no/yes)	3.94 (1.89–8.20)	<0.001	4.15 (1.76–9.74)	0.001
CD68 + (low/moderate/high)	0.95 (0.92–0.99)	0.004	0.95 (0.94–0.99)	0.027
CD4 + (low/moderate/high)	0.99 (0.99–1.01)	0.300		
CD8 + (low/moderate/high)	0.99 (0.98–0.99)	0.006	0.99 (0.98–1.00)	0.092
CD138 + (low/moderate/high)	1.01 (0.99–1.01)	0.164		
Tumour stroma percentage (low/high)	2.62 (1.26–5.45)	0.010	2.41 (1.09–5.30)	0.030
Tumour budding (low/high)	2.82 (1.39–5.72)	0.003	2.27 (1.35–5.36)	0.041

Abbreviations: CI = confidence interval; ER = oestrogen receptor; K–M = Kaplan–Meier; PR = progesterone receptor; TSP = tumour stroma percentage.

vessel invasion but not with lymphatic and nodal invasion. Nevertheless, prospective studies comparing the prognostic value of tumour budding in preoperative core biopsies and resection specimens would be of great interest.

Taken together, the present results suggest that tumour budding may promote disease progression through a direct effect on local and distant invasion into lymph nodes and lymphatic vessels. Indeed, budded cells have been shown to display epithelial–mesenchymal transition-like molecular phenotype in several cancers (Masugi *et al*, 2010; Zlobec and Lugli, 2010; Koyuncuoglu *et al*, 2012, Lugli *et al*, 2012; Taira *et al*, 2012; Liang *et al*, 2013), which is an early and critical step in cancer metastasis (Kalluri and Weinberg, 2009). Tumour with low inflammatory infiltrate may become more aggressive and allow tumour cells to detach and invade locally and systematically. Indeed, results from the present study and from our previous work (Gujam *et al*, 2014a) also suggest that both tumour budding and tumour stroma support pro-tumour rather than antitumour immune response.

The results of the present study suggest that tumour budding should be incorporated into routine clinical practice. However, in order for that to occur it has to be shown to be a reliable measure.

Although several studies have confirmed the prognostic value of tumour budding, several different methods have been described (Hase *et al*, 1993; Ueno *et al*, 2002; Prall *et al*, 2005; Wang *et al*, 2009). Therefore, there is a need for a standardised method to assess tumour budding in patients with cancer. In particular, if the standardised assessment of the tumour budding can reliably be performed in routine pathological sections and can offer useful prognostic information for clinicians, this would form the platform for the integration of tumour budding into existing staging systems.

With reference to patients with breast cancer, to date, tumour budding has been rarely examined, and therefore, the results of the present study need to be externally validated. Furthermore, whether tumour budding could be used as an additional morphological feature to stratify ER-positive into a high- and low-risk category has also to be validated.

CONCLUSION

In summary, the present study provides comprehensive assessment of the associations between tumour budding and the tumour microenvironment and, in a mature cohort of patients with long-
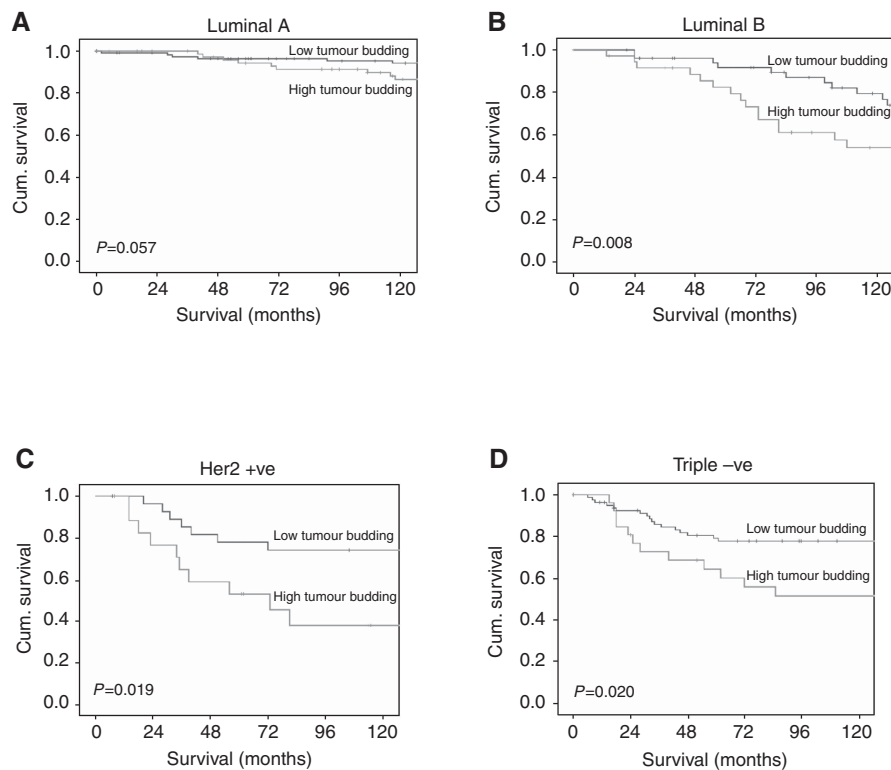
Figure 3. Kaplan-Meier survival curves (Log rank) of cancer specific survival in different molecular subtypes (**A**) patients with Luminal A, (**B**) patients with Luminal B, (**C**) patients with Her-2 positive tumours and (**D**) patients with Triple negative tumours.

term follow-up, further confirms the prognostic relevance of assessment of the tumour microenvironment in patients with invasive ductal breast cancer. Assessment of the tumour budding utilising routine pathological slides is relatively simple and may be readily incorporated into routine clinical pathology reporting to improve risk stratification, in particular for patients with node-negative breast cancer.

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