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Tumor budding is an independent prognostic factor in stage III colon cancer patients: A *post-hoc* analysis of the IDEA-France phase III trial (PRODIGE-GERCOR)

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Running head: Tumor budding as a prognostic factor in stage III CC

Highlights

- Tumor budding (Bd) is an emerging prognostic biomarker in colon cancer (CC).
- Bd currently influences decision-making in patients with pT1 and stage II CC.
- To date, the prognostic impact of Bd in patients with stage III CC has been limited to small and retrospective cohorts.
- In this post-hoc analysis of IDEA-France, categories B2 and B3 are strongly associated with poor DFS and OS in stage III CC.
- Bd could provide additional and clinically relevant prognostic information beside risk groups and Immunoscore®.

ABSTRACT

Purpose: Histological characteristics at the invasive front may reflect tumor aggressiveness; specifically, tumor budding (Bd) is an emerging prognostic biomarker in colon cancer (CC). We explored further the significance of Bd for risk stratification by evaluating survival of stage III CC patients included in the IDEA-France phase III trial.

Patients and Methods: This *post-hoc* study was conducted on tissue slides from 1,048 stage III CC patients. Bd was scored by central review by the Bd criteria of the 2016 International Tumor Budding Consensus Conference (ITBCC 2016) and classified as Bd1 (0-4 buds/0.785 mm²), Bd2 (5-9 buds), and Bd3 (≥ 10 buds) categories. Disease-free survival (DFS) and overall survival (OS) were analyzed by log-rank test. Clinicopathologic features and Immunoscore® were correlated with Bd.

Results: Overall, Bd1, Bd2, and Bd3 were observed in 39%, 28%, and 33% of CC, respectively. Bd2 and Bd3 were associated with vascular ($P = .002$) and perineural invasions ($P = .0009$). The 3-year DFS and the 5-year OS rates for Bd (1 *versus* 2-3) was of 79.4% *versus* 67.2% ($P = .001$) and 89.2% *versus* 80.8% ($P = .001$), respectively. This was confirmed after adjustment for relevant clinicopathological features for DFS (HR, 1.41; 95% CI, 1.12 to 1.77; $P = .003$) and OS (HR, 1.65; 95% CI 1.22 to 2.22; $P = .001$). When combined with pTN stage and Immunoscore® subgroups, Bd significantly improved disease prognostication.

Conclusion: Bd demonstrated its independent prognostic value for DFS and OS. Given these findings, Bd per the ITBCC 2016 should be mandatory in every pathology report in stage III CC patients. Bd and Immunoscore® could play a complementary role in personalized healthcare in this setting.

Key words: tumor budding, colon cancer, stage III colon cancer, prognostic

INTRODUCTION

Colon cancer (CC) is the third most commonly diagnosed malignancy worldwide [1]. In patients with stage III CC, oxaliplatin-based adjuvant chemotherapy (FOLFOX or CAPOX) is the current standard of care [2]. However, only 30% of patients at the most will actually benefit from adjuvant treatment, leaving 70% to unnecessary hazards and toxicity from multi-agent chemotherapy, especially oxaliplatin-related cumulative sensory neuropathy. [3,4] Recently, the International Duration Evaluation of Adjuvant Chemotherapy (IDEA) study analyzed data from six randomized phase III studies demonstrating the non-inferiority of shortened adjuvant therapy duration in the low-risk group (T1-3 and N1) associated with broadly better tolerance of treatment and quality of life.[5] However, even in this good prognostic group, approximately 20% of patients will experience disease recurrence.[1] Therefore, customizing a specific algorithm to establish intensity and duration of adjuvant treatment for more precise risk stratification is necessary to reduce proportions of patients exposed at potential toxicities of chemotherapy and, conversely, not to undertreat patients at high-risk of recurrence.[6, 7]

The tumor, nodes, metastasis (TNM) staging system that was published simultaneously by the Union for International Cancer Control (UICC) and by the American joint Committee on Cancer (AJCC), remains the gold standard for the classification of malignant tumors. Although clinically useful, it represents yet incomplete data set for patient-specific prognostic prediction. Therefore, further classification beyond pT and pN staging and other pathological features influencing stage III CC outcomes, remains of interest to refine risk classification and to guide adjuvant treatment. [3,8, 9]

Tumor budding (Bd), defined as a single cancer cell or up to four cancer cells at the tumor invasive margin, has emerged as a promising independent prognostic biomarker in colorectal cancer (CRC). Biologically, Bd could reflect an aggressive growth pattern.[10] Namely, it plays a key role in the tumor microenvironment as surrogate of morphological expression of epithelial-mesenchymal transition (EMT) representing the dynamic process of cancer infiltration.[11] In 2016, the International Tumour Budding Consensus Conference (ITBCC) recommended standardized Bd assessment and its reporting by pathologists in CRC.[10] Consequently, Bd was adopted as an additional tumor-related prognostic factor for this disease.[12] The prognostic role of Bd has been widely investigated and currently influences decision-making in patients with pT1 and stage II CC.[13–15] In particular, in those with pT1 CC, intermediate-high grade Bd (Bd2-3) is associated with node involvement and it is used to decide whether or not to perform complementary radical surgery following endoscopic resection. In stage II CC, high grade Bd (Bd3) represent a poor prognostic factor that should be considered for guidance of adjuvant treatment decision.[16] However, to date, the prognostic impact of Bd in patients with stage III CC has been limited to small and retrospective cohorts.[17]

The present *post-hoc* analysis of IDEA-France phase III trial aimed to assess the prognostic role of Bd applying the ITBCC 2016 criteria in stage III CC patients. The predictive value of Bd for survival benefit according to duration of adjuvant treatment has been further investigated. Amongst the future perspectives for the management of stage III CC is the use of Immunoscore®, whose prognostic value (corresponding to total [CD3+] and cytotoxic [CD8+] infiltrating T-cell counts in the tumor and its invasive margin) for DFS was confirmed in the IDEA-France trial.[18] The interactions between tumor buds and the immune system unleashed against them has been

referred to as an attacker-defender model [19]: tumor buds reflect an aggressive disease phenotype while CD8+ T-cells mediate the anti-cancer response. Hence, in this study we also explored the association between Bd and Immunoscore® based on the idea that the combination of Bd and immune infiltrate could better refine the prognosis of patients with CC.

PATIENTS AND METHODS

Study Design and Patients

IDEA-France (ClinicalTrials.gov identifier: NCT00958737) is a multicenter, two-arm, open-label, randomized phase III trial that enrolled 2,022 patients with stage III CC or cancer of the upper rectum who were candidates to receive adjuvant oxaliplatin-based chemotherapy. The primary outcomes of the IDEA-France trial have been published before.[20] All patients provided written informed consent. The protocol was approved by all relevant institutional ethical committees or review bodies, and the study was conducted in accordance with the Declaration of Helsinki and good clinical practice.

This *post-hoc* analysis assessed Bd in subgroup of patients from the IDEA-France trial who had available pathological samples and who signed the study translational informed consent (n = 1,048). The Immunoscore® test was previously performed on IDEA-France trial and reported in a prior publication [18].

The association between Bd categories, survival, clinicopathologic features including vascular and perineural invasion, VELIPI (Vascular Invasion, lymphatic invasion, perineural invasion), and tumor deposits, has been tested. Patients were staged according to the 7th edition of the AJCC/TNM classification.

Tumor budding assessment

Bd was assessed on scanned (Pannoramic Desk Scan-3DHitech) hematoxylin and eosin (H&E) stained tissue samples slides (Ambroise Paré Hospital, Boulogne, France) and scored at a medium power (x10-x20 magnification) in a single hotspot field area normalized to 0.785 mm² at the invasive front according to ITBCC 2016. Bd was defined as a single tumor cell or tumor cluster of up to 4 cells at the invasive margin. Tumor buds were counted independently by two pathologists (D.B. and C.B.) Any discrepancies were resolved by a third expert gastro-intestinal pathologist (M.S.). Categories for Bd scoring were: Bd1 (0-4 buds: low), Bd2 (5-9 buds: intermediate), and Bd3 (≥ 10 buds: high).

Objectives

The primary objective of the study was to assess the association between Bd and disease-free survival (DFS), defined as the time from randomization to relapse or death, whichever occurred first. Secondary objective was the relationship between Bd and overall survival (OS), defined as the time between randomization and death from any cause.

Statistical analysis

Descriptive analysis was done for patients' clinicopathological features (Table 1). Continuous and categorical variables were summarized as median values with interquartile ranges (IQR) and frequencies with percentages, respectively. Medians and proportions were compared using the Wilcoxon test and the chi-squared test (or the Fisher's exact test, if appropriate), respectively. Disease-free survival and OS

were estimated using Kaplan-Meier method, described using median or rate at specific time points along with 95% CI, and compared with the log-rank test.

The Cox proportional hazards regression models were used to estimate hazard ratios (HRs) for factors associated with DFS and OS. The association of baseline parameters with DFS and OS was first examined using a univariate Cox analysis and then variables with a P value $< .1$ were entered into a Cox regression multivariate model (parameters with more than 10% of missing data were not included). The potential differential effect of Bd on DFS and OS between the subgroups was illustrated using a forest plot. A statistical interaction was considered significant if $P < .1$. All analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC) and R software version 4.0.3 (R Development Core Team, Vienna, Austria; <http://www.r-project.org>) with two-sided statistical testing.

RESULTS

Patient characteristics

Of 2,010 CC patients treated in the IDEA-France study, 1,048 were included in the current analysis (Fig. 1). Comparison between population with and without Bd data available reported a lower proportion of patients with low-risk of recurrence (pT1-3, N1), and higher proportion of lympho-vascular invasion, low-grade and perforated tumors in the population with Bd data (Supplementary Table 1).

Clinicopathologic characteristics are listed in Table 1. The frequency of Bd1, Bd2, and Bd3 were 39%, 28%, and 33%, respectively. Patients with high-risk tumors (T4 and/or N2) accounted for 38% and 43% of the Bd1 and Bd2-3 subpopulations, respectively ($P = .109$). Moreover, the lymph node ratio (LNR) has been evaluated. No significant difference was reported about LNR in the two groups ($P = .621$; Table

1). Bd1 and Bd2-3 were observed at rates of 53% and 49%, respectively, in the 3-month arm ($P = .176$; Table 1). Patients with low Immunoscore® accounted for 45% of Bd1 category and for 45% of Bd2-3 category ($P = .937$). Bd2-3 was associated with significantly reduced probability of perforation ($P = .025$), but higher probability of perineural ($P = 0.0009$), and vascular invasion ($P = .002$), and VELIPI ($P = 0.0005$, Table 1).

Tumor budding and survival

Regarding survival, Bd2 and Bd3 patients had a considerably worse 3-year DFS compared to Bd1 patients (69.3% [95% CI 63.6 to 74.3] and 65.4% [95% CI 60.1 to 70.1] versus 79.4% [95% CI 75.1 to 83.1]; $P = .002$; Supplementary Figure 1). When grouping Bd2-3 patients, 3-year DFS rate was 79.4% (95% CI 75.1 to 83.1) for Bd1 patients and 67.2% (95% CI 63.3 to 70.7) for Bd2-3 patients ($P = .0008$; Fig. 2A).

In univariable analysis, Bd2-3 was associated with shorter DFS (HR, 1.46; 95% CI 1.2 to 1.8; $P = .0009$; Table 2). Moreover, male gender (HR 1.24; 95% CI 0.99 to 1.53; $P = .056$), age over 70 years (HR 1.33; 95% CI 1.07 to 1.66; $P = .011$), high-risk tumors (T4 and/or N2 vs T1-2-3 and N1, HR 1.97; 95% CI 1.60 to 2.43, $P < 0.0001$), shorter treatment duration (HR 1.24; 95% CI 1.003 to 1.52; $P = .046$), Immunoscore® low (HR, 1.53; 95% CI 1.21 to 1.93; $P = .0004$), vascular invasion (HR, 1.52; 95% CI 1.20 to 1.92; $P = .0005$), perineural invasion (HR, 1.60; 95% CI 1.25 to 2.04; $P = .0002$), VELIPI (HR, 1.67; 95% CI 1.31 to 2.13, $P < 0.0001$) and high grade tumors (HR 1.51; 95% CI 1.01 to 2.25, $P = 0.045$) were associated with a poor prognosis in univariate model (Table 2). The prognostic role of Bd2-3 for DFS (HR, 1.41; 95% CI 1.12 to 1.77; $P = .003$, Table 2) was confirmed in multivariable analysis adjusted for age, gender, risk group, histological grade, and treatment duration (3 versus 6 months).

Further multivariate models including T stage and lymph node ratio (LNR; instead of TN stage; Supplementary Table 2A), exact histological grading (subdivided into G1 to G4; Table S3B), Immunoscore (IS; Supplementary Table 2B), and VELIPI (Supplementary Table 2C) were conducted. These multivariate analyses were exploratory since the inclusion of such data in the main model was questionable due to the high rate of missing data (>10%) and the statistically significant correlation between Bd with vascular, perineural invasion, and VELIPI.

Patients with Bd2 and Bd3 had poorer OS; the 5-year OS was 81.6% (95% CI 76.5 to 85.7) in Bd2 group and 80.2% (95% CI 75.5 to 84.0) in Bd3 group versus 89.1% (95% CI 85.6 to 91.9) in Bd1 group ($P = .0063$; Supplementary Figure 2A). When grouping Bd2-3 the 5-year OS rate was 89.1% (95% CI 85.61 to 91.87) for Bd1 patients and 80.8% (95% CI 77.5 to 83.7) for Bd2-3 patients ($P = .001$; Supplementary Figure 2B)

In univariable analysis for OS, Bd2-3 was associated with worse survival outcome (HR 1.59, 95% CI 1.19 to 2.12; $P = .002$; Supplementary Table 3). This association was also confirmed by multivariable analysis (HR, 1.65; 95% CI 1.22 to 2.22; $P = .001$) conducted with potential confounders (Supplementary Table 4A and 4B). Other significant variables were age (>70 years), male gender, sidedness (right colon), high-risk tumors (Supplementary Table 4A), as well as T stage and LNR (Supplementary Table 4B).

Subgroup analysis

Forest plots summarizing HR of DFS and OS according to Bd1 and Bd2-3 groups are provided in Supplementary Figure S3 and S4. Immunoscore® ($P = .006$) and vascular invasion ($P = .026$) were significant variables for DFS. Interestingly,

subgroups with high-risk tumor and high grade Bd (Bd2-3) and low Immunoscore® showed shorter DFS (Fig. 2B and C). The 3-year DFS was 56.6% (95% CI 50.5 to 62.3) for T4-N2/Bd2-3 ($P < 0.0001$; Fig. 2B) and 57.7% (95% CI 50.9 to 62.3) for Immunoscore® low/Bd2-3 ($P < 0.0001$; Fig. 2 C and D). Bd category was not associated with DFS in patients with Immunoscore® intermediate or high. A significant interaction was observed between OS and pTN stage ($P = .050$) and vascular invasion ($P = .007$). There was also a correlation between the Bd category between pTN stage and Immunoscore® subgroups in terms of OS. The OS was poor for high-risk tumors T4-N2/Bd2-3 and Immunoscore® low/Bd2-3 with 5-years OS of 70.8% (95% CI 64.9 to 76.0) and 74.0% (95% CI 67.5 to 79.4), respectively (Supplementary Figure S2C and S2D).

Predictive value of tumor budding for the duration of FOLFOX-based adjuvant treatment

Of 1,048 patients, 518 and 530 received 6 months and 3 months of mFOLFOX6-based chemotherapy, respectively.

Clinicopathological characteristics and Immunoscore according to the duration of chemotherapy are shown in Supplementary Table S5. No significant interaction in terms of DFS was observed for predictive value of Bd category for adjuvant chemotherapy duration (3 months versus 6 months; Supplementary Figure S5). A numerical increasing trend for better DFS with 6 months of the mFOLFOX6 regimen was observed in the Bd2-3 subgroup (HR 0.78; 95% CI 0.60 to 1.02; $P = .073$; the 3-year DFS: 65.9% versus 61.8%; Supplementary Figure S6). Moreover, the predictive role of Bd was also tested in the low and high-risk groups of patients. No significant differences were observed between the different groups stratified by pTN stage and

Bd according to duration of treatment received. Interestingly, the Kaplan-Meier curves showed no benefit among patients with low-risk group and Bd1 receiving 6 months of therapy ($P = .11$; Supplementary Figure S7A). While a numerical beneficial effect could be detected in patients with low-risk tumors and Bd 2-3 receiving FOLFOX for 6 months compared to 3 months (Supplementary Figure S7B), as well as in the high-risk group, regardless of the Bd (Supplementary Figure S7C and S7D).

DISCUSSION

In patients with stage III CC, there is a need to refine the risk of recurrence, which may help to better personalize the duration of adjuvant chemotherapy in this setting. The IDEA international collaboration study has shown that in stage III CC patients adjuvant treatment duration could be adjusted according to T/N staging (low and high risk). Indeed, 3 months of CAPOX seemed as efficient and less toxic in T1-3/N1 patients [5,20]. The two IDEA *post-hoc* analyses, the IDEA-France and CALGB/SWOG 80702 studies, demonstrated that combining the number of tumor deposits and the number of lymph-node metastases improves the prognostication accuracy of TNM staging [21, 22] . Therefore, restaging of some tumors from low-risk pN1 to high-risk pN2 could potentially optimize the chemotherapy duration in some patients. The Immunoscore® assessment also represent a promising perspective for the management of stage III CC. The Immunoscor®e analysis in the IDEA-France trial confirmed its prognostic value in stage III CC and showed a beneficial effect of 6 months of FOLFOX/CAPOX in tumor patients with high or intermediate Immunoscore® [1]. In addition to these parameters, Bd could also be a pathological marker clinically useful to further refine risk classification and guide decision-making in stage III CC. Of note, Bd has been described as a robust and independent adverse

prognostic biomarker in CRC [23, 24], which has been emphasized by inclusion of this factor in the last edition of the TNM classification [25]. However, implementation of Bd into the TNM staging system relies on establishing a standardized, reproducible, and evidence-based scoring method. The ITBCC grading recommendations (ITBCC 2016) published in 2017 have led to the standardization of the Bd assessment and reporting by pathologists in the context of CRC [10]. In the ITBCC 2016, consensus for Bd assessment was reached for the following four criteria based on a review of the existing retrospective studies and meta-analyses: (i) it is defined as single tumor cells or cell clusters of up to four tumor cells; (ii) it is counted on hematoxylin and eosin; (iii) it is assessed in one hotspot at the invasive front (in a field area of 0.785 mm²); (iv) it is categorized into low (0–4 buds), intermediate (5–9 buds), and high (≥ 10 buds) levels, termed tumor Bd grades Bd1, Bd2, and Bd3, respectively [10].

Currently, Bd is used to determine the risk of lymph-node involvement in patients with early (pT1) CC (specifically Bd2 and Bd3) and decide subsequent surgery and to identify among stage II CC patients those at high-risk of recurrence and mortality to better guide adjuvant treatment decision [19]. However, its use in clinical practice is limited in stage III CC to tailor treatment decisions, notably due to the lack of relevant data in this setting [17]. Our study, which is the first to our knowledge to evaluate the prognostic impact of Bd in a large therapeutic trial according to the ITBCC 2016 criteria, validated Bd as an independent prognostic factor, improving the multivariate prognostic model both in terms of DFS (79.4% versus 67.2% for Bd1 and Bd2-3, respectively) and OS (89.1% versus 80.8%). These results are in line with those of a single-center retrospective study of 237 patients reported by Abakane et al. [17], which showed that Bd1 patients demonstrate significantly better DFS. The number of

Bd1 and Bd2-3 cases was quite similar to that in our study (42% and 58% versus 39% and 61%). In the Akabane et al. study, a two-tier system (low Bd; 0-4 buds, versus high Bd; >5 buds) was used as we did in the current work. Because risk stratification according to the ITBCC grading system strongly depends on a clinical scenario, a three-tier system (Bd1, Bd2, and Bd3) was favored over a two-tier system (low versus high) in the ITBCC 2016 recommendations. Our results showed that, in stage III CC, both Bd2 and Bd3 should be considered as risk factors for poor DFS and OS.

Noteworthy, our analyses showed a higher proportion of patients with low-risk stage III CC in the Bd1 group and high-risk stage III in the Bd2-3 group. However, as shown in Fig. 2B, combining both risk stage groups and Bd allows an accurate and non-overlapping prognostication, with a wide difference of the 3-year DFS across subgroups ranging from 54.4% for T4-N2/Bd2-3 to 82.1% for T1-3N1/Bd1.

Bd is a dynamic process, in which E-cadherin represent the key regulator. Moreover, it is often considered as a surrogate feature of EMT, characterized by cytoskeletal rearrangements, cell migration, and invasion with extracellular matrix degradation.[26–28] Data from immunohistochemistry and RNA sequencing conducted on CC, showed that Bd cells could degrade the extracellular matrix and invade and migrate through the surrounding stroma.[19] Notably, the interaction between tumor buds and immune cells plays a crucial role reflecting the attacker–defender model, with potential prognostic and therapeutic implications.[29] A study by Nearchou et al.[30], conducted on stage II CC revealed the absence of tumor infiltrating lymphocytes in tumors with high Bd grade. Epithelial-mesenchymal transition has already been described as an immune-escape mechanism, with tumor cells losing their major histocompatibility complex expression during this transition

making them invisible to effector immune cells.[31] Interestingly, several reports have also shown that T-lymphocytes at the tumor front close to Bd were associated with better outcomes as high lymphocyte to the Bd ratio.[28],[32] In our analyses, a significant interaction was reported between Bd and Immunoscore® ($P = .005$), showing a lower proportion of patients with the 3-year DFS and the 5-year OS for Immunoscore® low/Bd2-3, which is in accordance with the previously mentioned findings. However, when Immunoscore® was high, the prognostic value of Bd was no longer observed.

Some reports have suggested that tumors undergoing EMT might resist conventional chemotherapy.[33] Trinh et al.,[34] demonstrated that Bd is associated with a consensus molecular subtype 4, which represents EMT and cancer stem cell phenotype. In the study by Abakane et al. [17] Bd1 was associated with a favorable prognosis in patients with low-risk stage III CC, thus indicating that Bd could play a role in selecting patients who could benefit from 5-fluorouracil monotherapy in that setting. Our analysis shown no significant association between Bd and treatment duration, although a numerically better outcome in Bd2-3 patients when treated for 6 months was observed. Interestingly, further analysis conducted to evaluate the benefit of 3-month or 6-month arm showed no significant statistical differences among Bd and risk groups. Notably, no benefit was observed among patients with low-risk group and Bd1 receiving 6 months of therapy, whereas a numerical beneficial effect could be reported in patients with low-risk tumors and Bd2-3 receiving FOLFOX for 6 months compared to 3 months, as well as in the high-risk group, regardless of the Bd. With the limits of the *post-hoc* analysis and the lack of statistical significance probably due to the low statistical power about treatment duration, 3 months of treatment could be considered in the low-risk population with

Bd1, while the 6 months of treatment could be proposed for the remaining patient groups. Nevertheless, the present study represents a starting point for future research that could allow to identify patients among low-risk group who could still benefit from more prolonged treatment.

There are potential limitations to our study. First, patient number attrition with a study population not representative of the overall IDEA-France cohort could introduce some bias and we observed indeed significant difference between patients with and without available Bd data, particularly about risk stage ($P = .0084$) that could not guarantee fully representative groups of the study population. Although the findings should be interpreted with caution, this study has several strengths that include: i/ the large number of patients included in the final analysis; ii/ the great reliability of the Bd classification owing to the assessment performed by two independent pathologists with a third independent evaluation in case of contradictory results; iii/ the estimation, probably for the first time, of the prognostic impact of Bd in a large phase III trial according the ITBCC 2016 criteria. Finally, we believe that the *post-hoc* nature of our study leads to power impairment and therefore do not compromise the accuracy of our results.

In conclusion, our analysis revealed that both Bd2 and Bd3 categories are strongly associated with worse OS and DFS in stage III CC patients treated with oxaliplatin-based standard adjuvant chemotherapy in IDEA France. Bd seems to provide additional and clinically relevant prognostic information beside risk groups and Immunoscore®. Its association with the duration of treatment remains to be better defined by prospective studies stratified according to Bd1 and Bd2-3. Based on these results, Bd according to the ITBCC 2016 recommendations should be reported as a

microscopic biomarker in routine practice in every pathology report of patients resected for a stage III CC.

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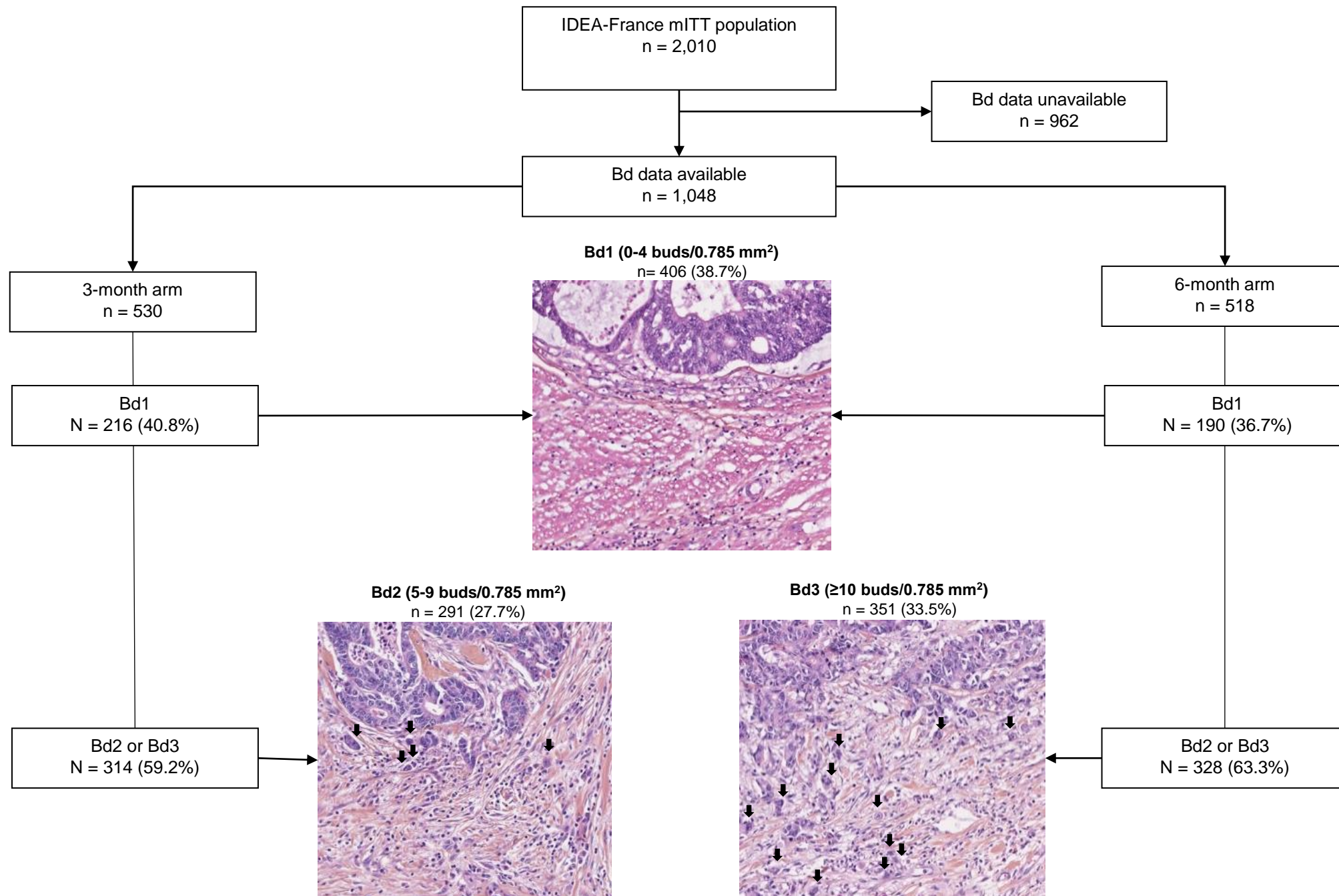
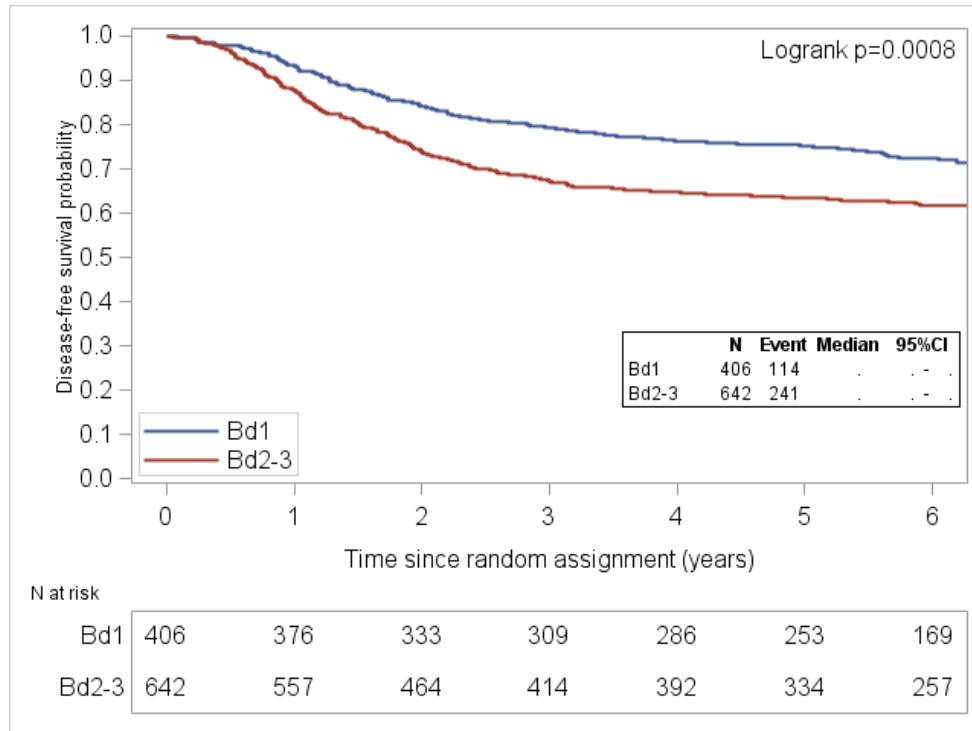
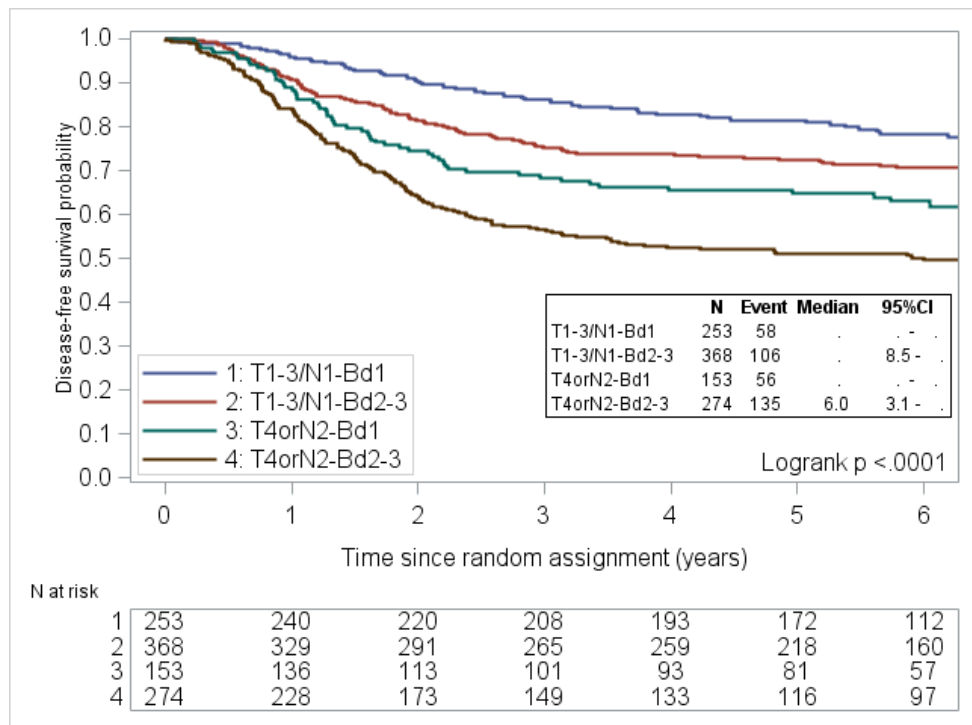


Figure 2. Kaplan-Meier estimates of disease-free survival by (A) budding group, (B) TN and tumor budding subgroups and by budding groups in (C) immunoscore low and (D) immunoscore intermediate or high groups.

A



B



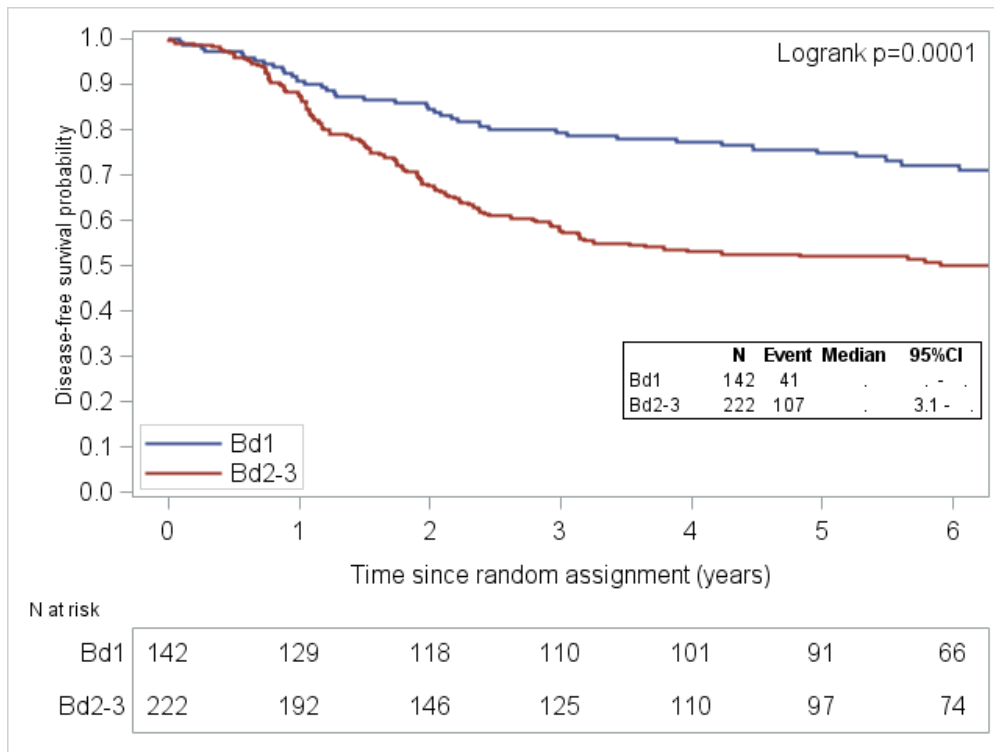
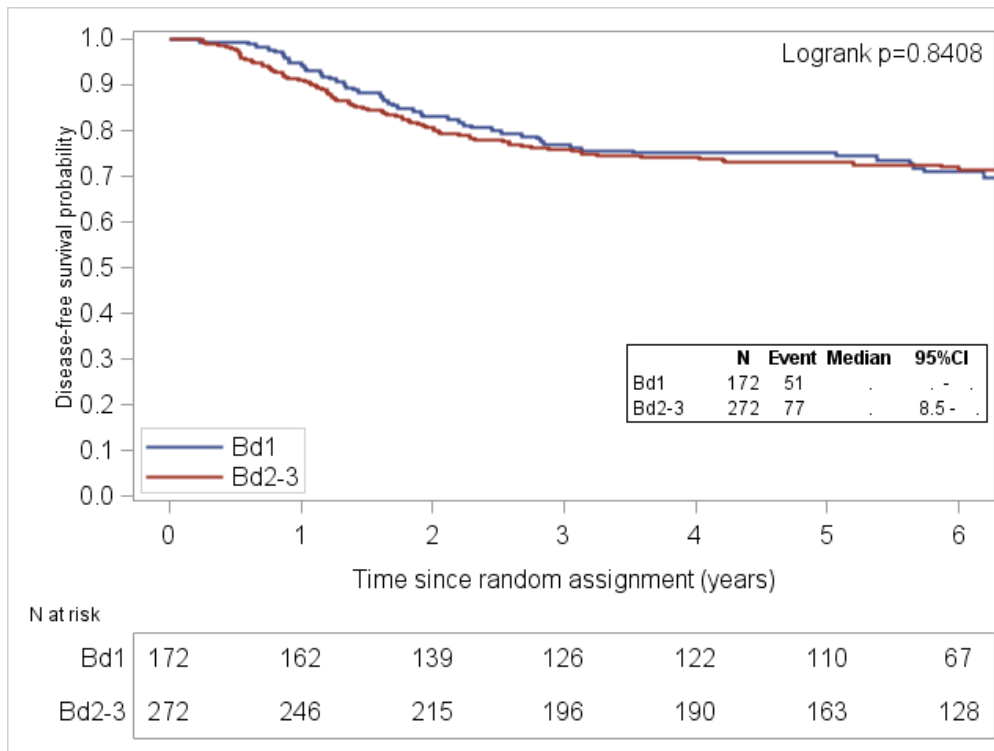
C**D**

Table 1. Patient population characteristics according to tumor budding

Characteristics	Population with BD data		Bd1		Bd2-3		P
	n = 1,048		n = 406		n = 642		
	n	%	n	%	n	%	
Age (years)							0.990
Mean (SD)	64.0 (9.2)		64.0 (9.0)		64.0 (9.3)		
Median (Q1-Q3)	64.7 (58.1-70.9)		64.7 (58.0-70.5)		64.7 (58.2-71.2)		
Age (years)							0.849
≤70	758	72.33	295	72.66	463	72.12	
>70	290	27.67	111	27.34	179	27.88	
Gender							0.213
Male	623	59.45	251	61.82	372	57.94	
Female	425	40.55	155	38.18	270	42.06	
ECOG PS							0.351
0	773	73.76	293	72.17	480	74.77	
1-2	275	26.24	113	27.83	162	25.23	
Tumor and node stage							0.109
T1-3 and N1	621	59.26	253	62.32	368	57.32	
T4 and/or N2	427	40.74	153	37.68	274	42.68	
Tumor stage							0.178
T1-2-3	843	80.4	335	82.5	508	79.1	
T4	205	19.6	71	17.5	134	20.9	
Lymph Nodes Ratio							0.0962
Mean (sd)	0.17 (0.16)		0.16 (0.17)		0.17 (0.16)		
Median (Q1-Q3)	0.11 (0.06-0.21)		0.10 (0.06-0.20)		0.12 (0.06-0.23)		
Lymph Nodes Ratio							0.6210
≤0.3	899	85.8	351	86.5	548	85.4	
>0.3	149	14.2	55	13.5	94	14.6	
Chemotherapy duration (months)							0.176
3	530	50.57	216	53.20	314	48.91	
6	518	49.43	190	46.80	328	51.09	
Obstruction							0.079
Yes	160	15.30	52	12.84	108	16.85	
No	886	84.70	353	87.16	533	83.15	
Missing	2	-	1	-	1	-	
Perforation							0.025
Yes	59	5.63	31	7.64	28	4.36	
No	989	94.37	375	92.36	614	95.64	
Colon							0.246
Left	642	62.63	260	65.82	382	60.63	
Right	374	36.49	132	33.42	242	38.41	
Both	9	0.88	3	0.76	6	0.95	
Missing	23	-	11	-	12	-	
Histologic grade							0.981
Low	951	94.07	366	94.09	585	94.05	
High	60	5.93	23	5.91	37	5.95	
Missing	37	-	17	-	20	-	
Tumor deposits							0.196
Yes	96	9.31	31	7.83	65	10.24	
No	935	90.69	365	92.17	570	89.76	
Missing	17	-	10	-	7	-	
Immunoscore							0.937
Low	364	45.05	142	45.22	222	44.94	
Intermediate/High	444	54.95	172	54.78	272	55.06	
Missing	240	-	92	-	148	-	
Vascular invasion							0.003
Yes	542	59.04	185	52.86	357	62.85	
No	376	40.96	165	47.14	211	37.15	
Missing	130	-	56	-	74	-	
Perineural invasion							0.0009
Yes	251	31.53	75	24.59	176	35.85	
No	545	68.47	230	75.41	315	64.15	
Missing	252	-	101	-	151	-	
VELIPI							0.0005
Yes	592		202	56.11	390	67.36	

No	347	158	43.89	189	32.64
Missing	109	46	-	63	-

Table 2. Univariate and multivariate Cox hazards model analysis on DFS

	Univariate analysis n = 1,048					Multivariate analysis n = 1,011				
	N	events	HR	95% CI	P	N	events	HR	95%CI	P
Tumor budding						1011	335			
1	406	114	1					1		
2-3	642	241	1.46	1.17 to 1.82	0.0009			1.41	1.12 to 1.77	0.003
Age (years)										
≤70	758	239	1					1		
>70	290	116	1.33	1.07 to 1.66	0.011			1.33	1.06 to 1.67	0.014
Gender										
Female	425	127	1					1		
Male	623	228	1.24	0.99 to 1.53	0.056			1.31	1.05 to 1.64	0.019
ECOG PS										
0	773	254	1							
1-2	275	101	1.16	0.92 to 1.47	0.195					
Tumor and node stage										
T1-2-3 and N1	621	164	1					1		
T4 and/or N2	427	191	1.97	1.60 to 2.43	<0.0001			1.97	1.59 to 2.45	<0.0001
Chemotherapy duration (months)										
6	518	161	1					1		
3	530	194	1.24	1.00 to 1.52	0.046			1.39	1.12 to 1.73	0.003
Obstruction										
No	886	296	1							
Yes	160	57	1.10	0.83 to 1.47	0.492					
Perforation										
No	989	333	1							
Yes	59	22	1.19	0.77 to 1.83	0.437					
Colon										
Left	642	215	1							
Right	374	126	1.01	0.81 to 1.263	0.904					
Histological grade										
Low	951	309	1					1		
High	60	26	1.51	1.01 to 2.25	0.045			1.246	0.830 to 1.869	0.2884
Tumor deposits										
No	935	312	1							
Yes	96	37	1.20	0.86 to 1.69	0.287					
Immunoscore										
Intermediate/High	444	128	1							
Low	364	148	1.53	1.21 to 1.93	0.0004					
Vascular invasion										
No	376	104	1							
Yes	542	209	1.52	1.20 to 1.92	0.0005					
Perineural invasion										
No	545	161	1							
Yes	251	107	1.60	1.25 to 2.04	0.0002					
VELIPI										
No	347	90	1							
Yes	592	231	1.668	1.308-2.129	<0.0001					