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# Comparison of the prognostic value of measures of the tumor inflammatory cell infiltrate and tumor-associated stroma in patients with primary operable colorectal cancer

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**Running head:** Examining the tumor microenvironment in colorectal cancer

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**Keywords:** colorectal cancer, tumor stroma, tumor inflammatory cell infiltrate, tumor microenvironment

**Abbreviations:** KM - Klintrup-Mäkinen, TSP – tumor stroma percentage, H&E – haematoxylin & eosin

## Abstract

The aim of the present study was to compare the clinical utility of two measures of the inflammatory cell infiltrate - a H&E-based assessment of the generalised inflammatory cell infiltrate (the Klintrup-Mäkinen (KM) grade), and an immunohistochemistry-based assessment of combined CD3<sup>+</sup> and CD8<sup>+</sup> T-cell density (the “Immunoscore”), in conjunction with assessment of the tumor stroma percentage (TSP) in patients undergoing resection of stage I-III colorectal cancer (CRC). 246 patients were identified from a prospectively maintained database of CRC resections in a single surgical unit. Assessment of KM grade and TSP was performed using full H&E sections. CD3<sup>+</sup> and CD8<sup>+</sup> T-cell density was assessed on full sections and the Immunoscore calculated. KM grade and Immunoscore were strongly associated ( $P<0.001$ ). KM grade stratified cancer-specific survival (CSS) from 88% to 66% ( $P=0.002$ ) and Immunoscore from 93% to 61% ( $P<0.001$ ). Immunoscore further stratified survival of patients independent of KM grade from 94% (high KM, Im4) to 60% (low KM, Im0/1). Furthermore, TSP stratified survival of patients with a weak inflammatory cell infiltrate (low KM: from 75% to 47%; Im0/1: from 71% to 38%, both  $P<0.001$ ) but not those with a strong inflammatory infiltrate. On multivariate analysis, only Immunoscore (HR 0.44,  $P<0.001$ ) and TSP (HR 2.04,  $P<0.001$ ) were independently associated with CSS. These results suggest that the prognostic value of an immunohistochemistry-based assessment of the inflammatory cell infiltrate is superior to H&E-based assessment in patients undergoing resection of stage I-III CRC. Furthermore, assessment of the tumor-associated stroma, using TSP, further improves prediction of outcome.

## Introduction

Colorectal cancer is the second most common cause of cancer death in the United Kingdom and Europe.<sup>1</sup> Following potentially curative surgery, prognosis and the need for adjuvant therapy is primarily based on pathological assessment of the depth of invasion of the primary tumor and the presence of lymphatic and distant organ metastases.<sup>2</sup> However, this TNM-based staging may fail to accurately stage all patients, and in particular those with earlier stage, lymph node negative disease, where other tumor characteristics may identify patients at increased risk and who may benefit from adjuvant treatment.

In addition to such tumor-based characteristics, assessment of the tumor microenvironment may similarly inform prognosis. For example, it is now accepted that the host local inflammatory response is an important determinant of disease progression and oncological outcome in patients with colorectal cancer, with the presence of a conspicuous inflammatory cell infiltrate identified as an independent predictor of improved survival.<sup>3,4</sup> Although semi-quantitative, histopathological assessment of the density of the generalised inflammatory cell infiltrate using routine pathological specimens has been validated as a stage-independent prognostic characteristic,<sup>5,6</sup> the prognostic value of immunohistochemistry-based assessment of immune cell type and location within the tumor microenvironment, such as the Immunoscore, has been of interest.<sup>7,8</sup> Although initially describing the density of cytotoxic (CD8<sup>+</sup>) and memory (CD45R0<sup>+</sup>) T-lymphocytes within the tumor microenvironment, the Immunoscore has recently been refined to reflect a cumulative score based on the density of the overall mature CD3<sup>+</sup> T-lymphocyte population in addition to the CD8<sup>+</sup> T-lymphocyte population within the tumor invasive margin and tumor core, and has been validated as a prognostic marker with superior prognostic ability compared to TNM staging in colorectal cancer.<sup>8-10</sup> However, whether the Immunoscore is

superior to the Klintrup-Mäkinen (KM) assessment of the generalised inflammatory cell infiltrate remains to be determined.

Together with the inflammatory cell infiltrate, the tumor-associated stroma has also been characterised as an important determinant of disease progression in colorectal cancer.<sup>11</sup> In particular, assessment of the proportion of stroma, or tumor stroma percentage (TSP), within the tumor microenvironment has been shown to predict survival independent of TNM stage for patients undergoing potentially curative resection of colorectal cancer as well as other cancers.<sup>12-16</sup> Furthermore, in patients with colorectal cancer, TSP has been shown to hold prognostic value independent of the inflammatory cell infiltrate as assessed using the KM grade.<sup>13</sup> Indeed, combined assessment of KM grade and TSP, termed the Glasgow Microenvironment Score (GMS), stratified survival greater than either measure alone, and in particular further stratified survival of those patients with a weak local inflammatory cell infiltrate.<sup>17</sup> However, whether inclusion of a potentially more detailed measure of the inflammatory cell infiltrate, such as the Immunoscore, may alter the prognostic value of the tumor-associated stroma is not clear. Therefore, the present study of patients undergoing potentially curative resection of stage I-III colorectal cancer has two aims: to compare the prognostic value of assessment of the inflammatory cell infiltrate using the KM grade and the Immunoscore, and to examine the clinical utility of combined assessment of the inflammatory cell infiltrate and TSP.

## Patients and Methods

### Patients and clinicopathological characteristics

Patients were identified from a prospectively collected database of all elective and emergency colorectal cancer resections performed in a single surgical unit in Glasgow Royal Infirmary since 1997. For the purposes of the present study, patients who had undergone primary resection between 1997 and 2008 for stage I-III colorectal cancer were included. Patients who had undergone localised or endoscopic resection, surgery with palliative intent, neoadjuvant chemoradiotherapy or who had died within 30 days of surgery were excluded. Only patients with rectal cancer who, after discussion at local colorectal multidisciplinary meetings comprised of colorectal surgeons, pathologists, radiologists and oncologists, were deemed to not require neo-adjuvant therapy prior to curative surgery were included. Approval was obtained from the West of Scotland Ethics Committee.

Pathological staging was performed according to the TNM 5<sup>th</sup> edition.<sup>2</sup> Venous invasion was identified using elastica staining as has been routine in our department since 2003; patients who had undergone resection prior to 2003 underwent retrospective reporting of venous invasion with elastica staining. Patients were routinely discussed at local colorectal cancer multidisciplinary meetings following resection; patients with stage III disease or high-risk stage II disease were considered for adjuvant chemotherapy when not precluded by advanced age, significant co-morbidity, or patient preference. Use of anti-angiogenic and anti-EGFR inhibitors was not routine during the study period.

The systemic inflammatory response, a stage-independent predictor of survival in patients with colorectal cancer, was reported using the modified Glasgow Prognostic Score (mGPS) as previously described.<sup>18</sup> Briefly, patients with serum C-reactive protein (CRP)  $\leq$  10mg/L were given a score of 0, patients with CRP > 10mg/L and serum albumin  $\geq$  35g/L a

score of 1, and patients with CRP > 10mg/L and albumin < 35g/L a score of 2. Serum CRP and albumin were recorded within 30 days prior to surgery for patients undergoing elective resection and on admission for patients undergoing emergency resection.

### **Assessment of the tumor microenvironment**

The generalised inflammatory cell infiltrate was assessed using the KM score as previously described.<sup>6</sup> Briefly, haematoxylin and eosin-stained (H&E) section of the deepest point of tumor invasion were retrieved and the generalised inflammatory cell infiltrate at the invasive margin assessed in a semi-quantitative fashion using a four-point scale (0- no increase, 1- mild or patchy increase in inflammatory cells, 2- prominent inflammatory reaction forming a band at the invasive margin, and 3- florid cup-like infiltrate at the invasive edge with destruction of cancer cell islands). For the purposes of statistical analysis, patients were subsequently classified as low grade (0/1) or high grade (2/3) response (Figure 1a-b).

Full sections of the invasive margin were stained using antibodies for CD3<sup>+</sup>, a mature T-lymphocyte marker, and CD8<sup>+</sup>, a marker of cytotoxic T-lymphocytes, as previously described.<sup>19</sup> The Immunoscore was then calculated as previously described.<sup>8,19</sup> Briefly, the density of CD3<sup>+</sup> and CD8<sup>+</sup> T-cells within the invasive margin and tumor centre were separately semi-quantitatively graded as high or low; the Immunoscore was calculated from the number of regions with a high CD3<sup>+</sup> and CD8<sup>+</sup> cell density, giving five potential groups (Im0, Im1, Im2, Im3, Im4), ranging from all regions low density (Im0) to all regions high density (Im4).

Tumor stroma percentage was examined as previously described.<sup>13</sup> Briefly, using H&E sections of the invasive margin, TSP was calculated as the percentage of the visible

field comprised of stroma, excluding areas of necrosis and mucin deposition. Patients were graded as either low ( $\leq 50\%$ ) or high TSP ( $> 50\%$ ) (Figure 1c-d).

All assessments of the tumor microenvironment were performed by a single investigator blinded to clinical outcomes with co-scoring of at least 10% to ensure consistency of scoring. As previously reported, the intra-class correlation coefficient was greater than 0.8 for all assessments, indicating excellent agreement (KM grade – 0.81; TSP – 0.81; CD3<sup>+</sup> margin – 0.83; CD3<sup>+</sup> centre – 0.87; CD8<sup>+</sup> margin – 0.83; CD8<sup>+</sup> centre – 0.87)<sup>6, 13, 19</sup>

## **Survival**

Following resection patients were routinely followed-up for five years according to local treatment and surveillance guidelines. Date and cause of death were crosschecked with the cancer registration system and the Registrar General (Scotland). Cancer-specific survival was measured in months as the date from surgery until date of death from recurrent or metastatic colorectal cancer. Death records were complete until March 31, 2014, which acted as the censor date.

## **Statistical analysis**

The relationship between components of the tumor microenvironment and cancer-specific survival was examined using Kaplan-Meier log-rank analysis, with five-year survival presented as percentage surviving (standard error). The relationship between components of the tumor microenvironment, clinicopathological characteristic and survival was examined using multivariate Cox proportional hazards regression to calculate hazard ratios (HR) and 95% confidence intervals (CI). Variables with a *P*-value  $\leq 0.05$  on univariate regression analysis were entered into a multivariate model using a backward conditional approach. All



statistical analyses were performed using SPSS version 22.0 (IBM SPSS). A *P*-value  $\leq 0.05$  was considered statistically significant.

## Results

The study population was comprised of 246 patients undergoing potentially curative resection of stage I-III colorectal cancer. Clinicopathological characteristics are displayed in Table 1. Approximately two thirds of patients were 65 years of age or older at time of surgery and 52% were male. Fifteen patients (6%) underwent emergency resection, and just over two thirds of patients underwent resection of colon cancer. Histopathological reporting confirmed stage I, stage II and stage III disease in 7%, 52% and 41% of patients respectively. Mismatch repair status has previously been reported in a subset of this cohort and was available for 205 patients;<sup>17</sup> 30 patients (15%) had mismatch repair deficient colorectal cancer.

The mean follow-up of survivors was 150 months (range 87-206 months) with 76 colorectal cancer-related deaths and 76 non-cancer deaths. Mean and five-year survival was 40 months and 74% for cancer-specific survival, and 79 months and 63% for overall survival. In total, 71 patients (29%) received adjuvant chemotherapy; one patient with stage I (6%) disease, 19 patients with stage II disease (15%), and 51 patients with stage III disease (51%) received adjuvant chemotherapy. Compared to patients who did not receive adjuvant therapy, patients with node negative (stage I/II) disease who received adjuvant chemotherapy were younger ( $P=0.004$ ), more likely to have venous invasion ( $P=0.009$ ), margin involvement ( $P=0.045$ ) or peritoneal involvement ( $P=0.046$ ) and showed a trend towards emergency presentation ( $P=0.078$ ) and poor tumor differentiation ( $P=0.062$ ). Patients receiving adjuvant chemotherapy showed a non-significant trend towards poorer survival ( $P=0.158$ ); patients with node negative (stage I/II) disease receiving adjuvant therapy showed a trend towards poorer five-year survival (69% vs. 85%;  $P=0.196$ ), whereas patients with node positive (stage III) disease receiving adjuvant therapy showed a trend towards increased five-year survival (69% vs. 51%;  $P=0.153$ ).

## Relationship between local inflammatory cell infiltrate and cancer-specific survival

The relationship between the inflammatory cell infiltrate and cancer-specific survival is displayed in Figure 2. The KM score was associated with cancer-specific survival ( $P=0.015$ ; Figure 1a). When classified as low grade or high grade, a low KM grade was associated with poorer five-year survival (66% vs. 88%;  $P=0.002$ ; Figure 1b). When stratified by tumor site (Supplementary Table 1, Supplementary Figure 1), low KM grade was associated with poorer survival in patients with colon cancer ( $P<0.05$ ) and showed a trend towards poorer survival in patients with rectal cancer ( $P=0.068$ ). When stratified by TNM stage, low KM grade showed a trend towards poorer survival in patients with node negative (TNM I/II) disease ( $P=0.053$ ) and node positive (TNM III) disease ( $P=0.057$ ). Finally, low KM grade was associated with poorer survival in both patients who did not receive and who received adjuvant chemotherapy (both  $P<0.05$ ).

A decrease in Immunoscore was associated with a decrease in cancer-specific survival (Figure 1c): five-year cancer-specific survival ranged from 93% for patients with an Immunoscore=4 to 61% for patients with an Immunoscore=0 ( $P<0.001$ ). The survival of patients with Im0 and Im1, or Im2 and Im3 did not differ significantly ( $P=0.788$  and  $P=0.599$ , respectively). As such, for further statistical analysis the Immunoscore was refined to stratify patients into three prognostic groups (Figure 1d): Im4, with five-year survival of 93%; Im2/3, with five-year survival of 84%; and Im0/1, five-year survival of 61% ( $P<0.001$ ). When stratified by tumor site or TNM stage (Supplementary Table 1, Supplementary Figure 2), the Immunoscore was associated with survival of patients with both colon and rectal cancer (both  $P<0.01$ ) and of patients with both node negative ( $P<0.01$ ) and node positive ( $P<0.05$ ) disease. Finally, low Immunoscore was associated with poorer survival in patients

who did not receive adjuvant chemotherapy ( $P<0.001$ ) and showed a trend towards poorer survival in patients who did receive adjuvant therapy ( $P=0.059$ ).

The KM grade was strongly associated with the Immunoscore ( $P<0.001$ ; Figure 3), however neither KM grade nor Immunoscore were associated with mismatch repair status ( $P=0.661$  and  $P=0.284$  respectively) or the systemic inflammatory response as measured by mGPS ( $P=0.999$  and  $P=0.214$  respectively). Comparison between the prognostic value of the KM grade and Immunoscore was subsequently performed (Table 2). The KM grade stratified five-year cancer-specific survival from 88% (high grade) to 66% and the Immunoscore stratified survival from 93% to 61%. The Immunoscore was able to further stratify the survival of patients with both a low and high KM grade; the survival of patients with a low grade KM ranged from 90% (Im4) to 60% (Im0/1) ( $P=0.015$ ), whereas the survival of patients with a high grade KM ranged from 94% (Im4) to 71% (Im0/1) ( $P=0.010$ ). In contrast, the KM grade did not further significantly stratify the Immunoscore.

### **Relationship between tumor stroma percentage, the tumor inflammatory cell infiltrate and cancer-specific survival**

The prognostic value of combined assessment of the inflammatory cell infiltrate and TSP was subsequently examined (Table 3). The TSP significantly stratified the survival of patients from 80% (low TSP) to 57% (high TSP) ( $P=0.001$ ). In combination with the inflammatory cell infiltrate, TSP significantly stratified survival of those with a weak infiltrate but not those with a strong infiltrate. In particular, TSP significantly stratified survival of patients with a low KM grade from 75% to 47% ( $P<0.001$ ), whereas in patients with a high KM grade, survival of patients with a low TSP was comparable to that of patients with a high TSP ( $P=0.485$ ). In combination with the Immunoscore, the effect of TSP on survival decreased as the Immunoscore increased; TSP stratified the survival of patients with

Im0/1 from 71% to 38% ( $P<0.001$ ) and patients with Im2/3 from 87% to 77% ( $P=0.069$ ), but not patients with Im4 ( $P=0.545$ ) (Figure 4). Conversely, assessment of the inflammatory cell infiltrate was able to stratify survival of patients with both a high and low TSP; KM grade stratified patients with a low TSP from 88% to 75% ( $P=0.081$ ) and patients with a high TSP from 87% to 47% ( $P=0.034$ ), whereas Immunoscore stratified survival of patients with a low TSP from 92% to 71% ( $P=0.002$ ) and patients with a high TSP from 100% to 38% ( $P=0.004$ ).

### **Relationship between the tumor microenvironment, clinicopathological characteristics and cancer-specific survival**

On univariate survival analysis (Table 4), emergency presentation, T stage, mGPS (both  $P<0.05$ ), N stage, venous invasion, margin involvement and peritoneal involvement (all  $P\leq 0.001$ ) were associated with cancer-specific survival. The KM grade ( $P=0.003$ ), Immunoscore and TSP were all associated with survival (both  $P<0.001$ ).

On multivariate analysis, after controlling for age, sex, tumor site and adjuvant therapy and considering all variables significant on univariate analysis, the Immunoscore and TSP (both  $P<0.01$ ), but not the KM grade, were associated with survival independent of venous invasion ( $P=0.001$ ) and mGPS ( $P<0.05$ ). When the Immunoscore was removed from the multivariable model, KM grade ( $P<0.05$ ) and TSP ( $P<0.01$ ) remained associated with survival independent of venous invasion ( $P=0.001$ ) and mGPS ( $P<0.01$ ).

## Discussion

In the present study, an immunohistochemistry-based assessment of the inflammatory cell infiltrate was superior to that of H&E-based assessment in predicting outcome of patients undergoing potentially curative resection of stage I-III colorectal cancer. Furthermore, the combination of assessment of the inflammatory cell infiltrate, using either KM grade or Immunoscore, and assessment of the tumor-associated stroma, using TSP, provided additional prognostic information.

The present study compared the prognostic utility of two validated measures of the tumor inflammatory cell infiltrate – the KM grade and the Immunoscore.<sup>5, 8</sup> Although both were associated with cancer-specific survival, the Immunoscore, an immunohistochemistry-based assessment of CD3<sup>+</sup> and CD8<sup>+</sup> T-lymphocyte density, was able to better stratify survival of patients than the KM grade, an H&E-based assessment of the generalised inflammatory cell infiltrate. In particular, the Immunoscore was able to stratify survival of patients with both a low and high KM grade; indeed, survival of patients with a low KM grade but high Immunoscore was comparable to that of patients with a high KM grade.

The relative difference in the prognostic value of both measures of the inflammatory cell infiltrate may be explained by the components of the immune response that each measures. Whereas the KM grade provides a measure of the overall, generalised inflammatory cell infiltrate, the Immunoscore measures the host adaptive T-lymphocytic response to cancer. Indeed, although an increase in KM grade is associated with an increase in the density of tumor-infiltrating T-lymphocytes,<sup>19-21</sup> it is also associated with an increase in the density of the innate immune infiltrate, and in particular neutrophils and macrophages.<sup>20</sup>

<sup>21</sup> For example, in the present study, within the group of patients with a high KM grade, the number of patients with a low (Im0/1, *n*=19) or high (Im4, *n*=21) Immunoscore was similar,

whereas of those patients with a low KM grade, a small number had a high Immunoscore. However, although the importance of host adaptive anti-tumor immune responses is recognised, it is now appreciated that myeloid-derived cells, such as neutrophils and macrophages, play an important functional role in promoting tumor progression.<sup>22</sup> Indeed, it remains to be determined whether immunohistochemical assessment of the innate immune infiltrate may increase the clinical and prognostic utility of measuring the inflammatory cell infiltrate in patients with colorectal cancer.

It was of interest in the present cohort that TSP, an assessment of the tumor-associated stroma, was associated with survival independent of either measure of the inflammatory cell infiltrate, and that combined assessment provided greater prognostic value. For example, it was possible to stratify five-year survival from 92% (Im4, low TSP) to 38% (Im0/1, high TSP). Furthermore, although the relationship between TSP and survival was strongest in patients with a poor inflammatory cell infiltrate, both the number of patients with a high TSP, and its prognostic value, decreased as the density of the inflammatory infiltrate increased. Although it has previously been suggested that the presence of a tumor-associated stroma precludes effective infiltration of the tumor microenvironment by an anti-tumor immune response,<sup>23</sup> the present results are consistent with our previous findings,<sup>17</sup> and may favour the alternative hypothesis that loss of the adaptive immune infiltrate predisposes to the development of a pro-tumor stromal compartment, potentially mediated by the residual innate immune infiltrate.<sup>21, 24, 25</sup>

We have previously proposed a novel prognostic score based on assessment of the KM grade and TSP, termed the Glasgow Microenvironment Score,<sup>17</sup> however the present results suggest that a similar scheme may be applied to the combination of the Immunoscore and TSP and may have even greater clinical utility. Indeed, such a combination may optimise

risk prediction in patients undergoing colorectal cancer resection by identifying both those with an excellent prognosis (Im4; five-year cancer-specific survival of 93%), and those with an extremely poor prognosis who may benefit from adjuvant therapy (Im0/1, high TSP; five-year cancer-specific survival of 38%).

In the present study, it was of interest that both the systemic inflammatory response as measured by mGPS, and the local inflammatory cell infiltrate as measured by KM grade or Immunoscore had independent prognostic value in the multivariate analysis. It is likely that these measures reflect the same underlying process and therefore it would be of interest to compare the local and systemic inflammatory responses and how they may be combined to form a prognostic score. Indeed, Turner and colleagues have recently combined measures of the local and systemic inflammatory response to give better risk stratification in patients with node negative colorectal cancer<sup>26</sup>. However, the rationale of their approach that combined the neutrophil: lymphocyte ratio (NLR) and assessment of the chronic inflammatory cell density, was not clear since different cell types were assessed locally and systemically. Indeed, only approximately 20% of patients had an elevated NLR and a low chronic inflammatory cell density, and therefore this score does not capture the same entity. Similarly, combinations of other systemic and local inflammatory measures, such as the mGPS and KM grade or Immunoscore, will have such limitations. Moreover, the numbers of patients included in the present study ( $n=246$ ) limits the value of such analysis and therefore was not formally examined.

Of note in the present study, the use of adjuvant chemotherapy was not associated with a significant improvement in survival and, in patients with node negative disease, showed a non-significant trend towards poorer survival. However, given the effect size of adjuvant chemotherapy in patients with node negative disease is modest (3.6% absolute improvement in survival of patients with stage II disease at five years in the QUASAR trial



( $n=2983$ )),<sup>27</sup> the present study did not have sufficient statistical power to examine the impact of adjuvant chemotherapy on survival of patients with colorectal cancer.

The present study is perhaps limited by its use of manual, semi-quantitative assessment of the inflammatory cell infiltrate as opposed to automated assessment as has been recommended for routine assessment of the Immunoscore.<sup>8</sup> However, the manual techniques employed showed excellent inter-operator agreement<sup>6, 19</sup> and manual assessment of the inflammatory cell infiltrate has been shown to correlate strongly with automated assessment.<sup>28, 29</sup> Furthermore, manual assessment of immunohistochemical staining may allow for greater discrimination of non-specific, background staining and provide superior prognostic value compared to automated assessment.<sup>29</sup> Furthermore, meaningful statistical analysis was precluded by the small number of patients in particular subgroups, such as those with stage II disease and high-risk pathological characteristics, or patients with stage I disease. Finally, the results of the present study, and in particular the prognostic utility of combined assessment of the inflammatory cell infiltrate and tumor-associated stroma, remain to be validated in an independent patient cohort from an independent centre.

In conclusion, the present results suggest that the prognostic value of an immunohistochemistry-based assessment of the inflammatory cell infiltrate is superior to H&E-based assessment in patients undergoing potentially curative resection of stage I-III colorectal cancer. Furthermore, TSP improves the prediction of survival by either measure of the inflammatory cell infiltrate.

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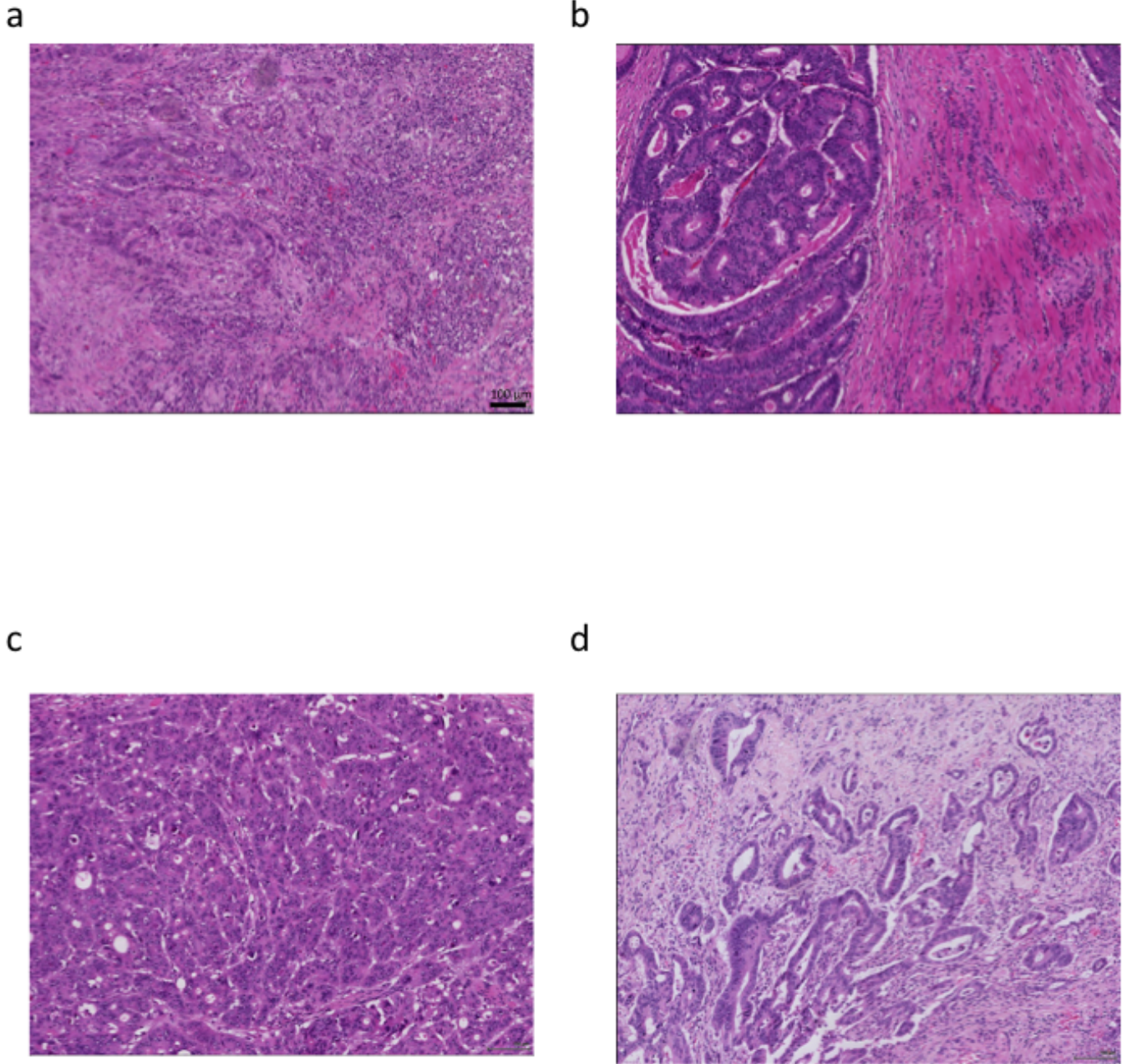
## Figure Legends

**Figure 1.** Haematoxylin and eosin-based assessments of the tumor microenvironment of patients undergoing potentially curative resection of stage I-III colorectal cancer (a) low Klintrup-Mäkinen grade, (b) high Klintrup-Mäkinen grade, (c) low tumor stroma percentage, and (d) high tumor stroma percentage (x80 magnification).

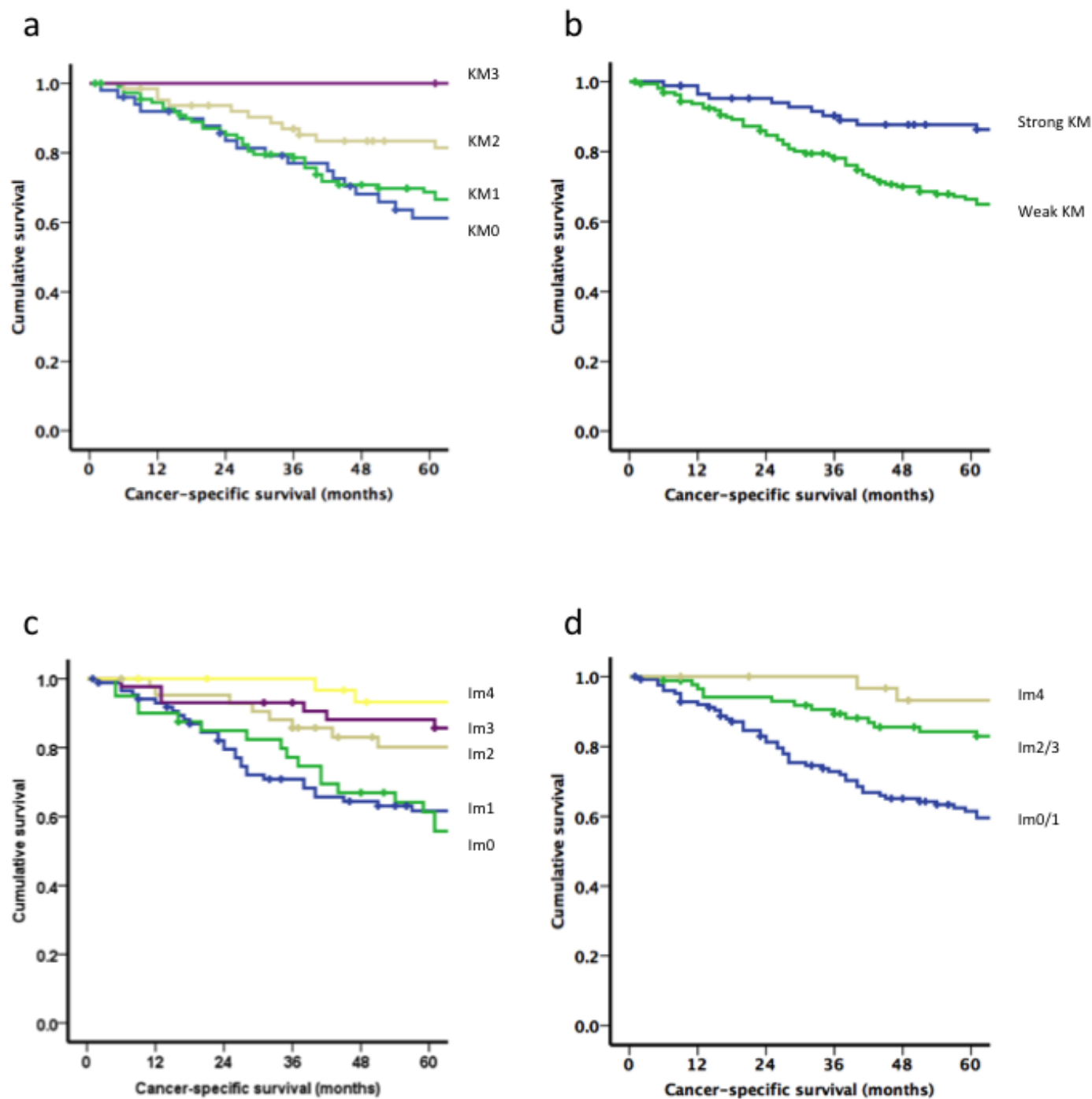
**Figure 2.** Relationship between tumor inflammatory cell infiltrate and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer (a) Klintrup-Mäkinen score ( $P=0.015$ ), (b) Klintrup-Mäkinen grade ( $P=0.002$ ), (c) Immunoscore ( $P<0.001$ ), and (d) Immunoscore stratified in to three groups ( $P<0.001$ ).

**Figure 3.** Relationship between Klintrup-Mäkinen grade and Immunoscore in patients undergoing potentially curative resection of stage I-III colorectal cancer ( $P<0.001$ ).

**Figure 4.** Relationship between Immunoscore, tumor stroma percentage and cancer-specific survival in patients undergoing potentially curative resection of stage I-III colorectal cancer (a) Im0/1 ( $P<0.001$ ), (b) Im2/3 ( $P=0.069$ ), (c) Im4 ( $P=0.545$ )



**Figure 1. Haematoxylin and eosin-based assessments of the tumor microenvironment in patients undergoing potentially curative resection of stage I-III colorectal cancer (a) Strong Klintrup-Mäkinen grade, (b) weak Klintrup-Mäkinen grade ( $P=0.002$ ), (c) low Tumor Stroma Percentage, and (d) high Tumor Stroma Percentage(x80 magnification)**



**Figure 2. Relationship between tumor inflammatory cell infiltrate and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer (a) Klintrup-Mäkinen score ( $P=0.015$ ), (b) Klintrup-Mäkinen grade ( $P=0.002$ ), (c) Immunoscore ( $P<0.001$ ), and (d) Immunoscore stratified in to three groups ( $P<0.001$ ).**

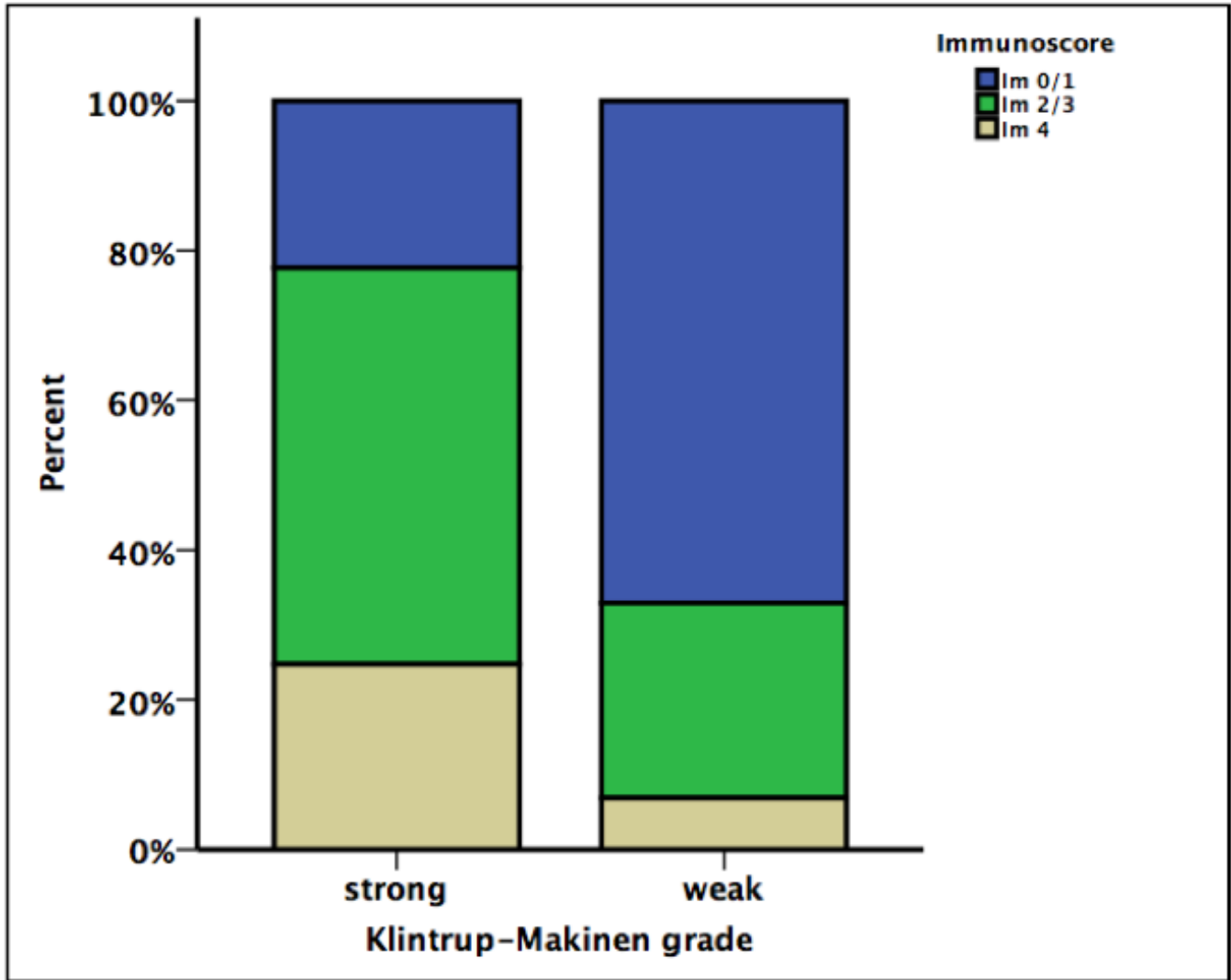
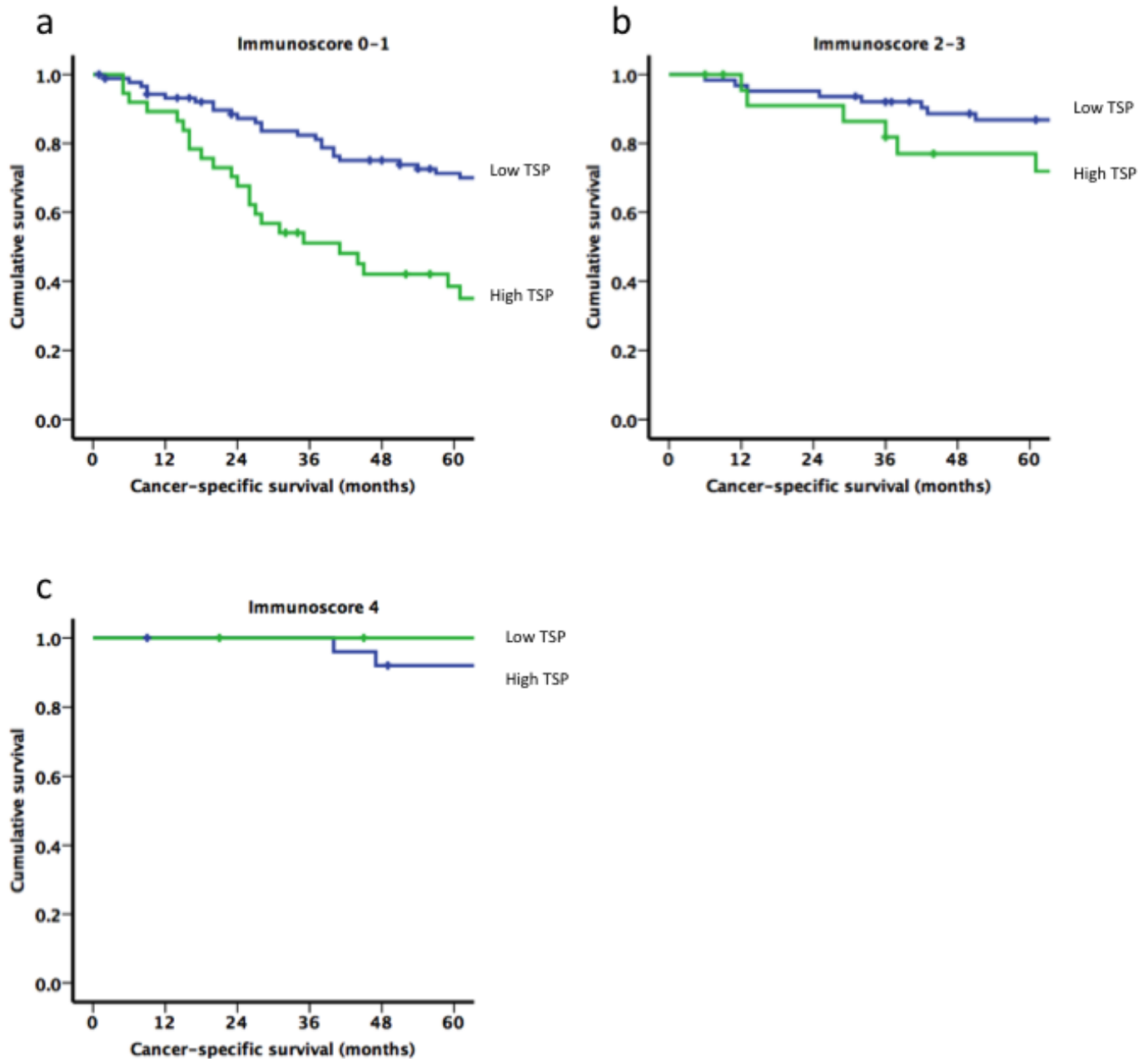
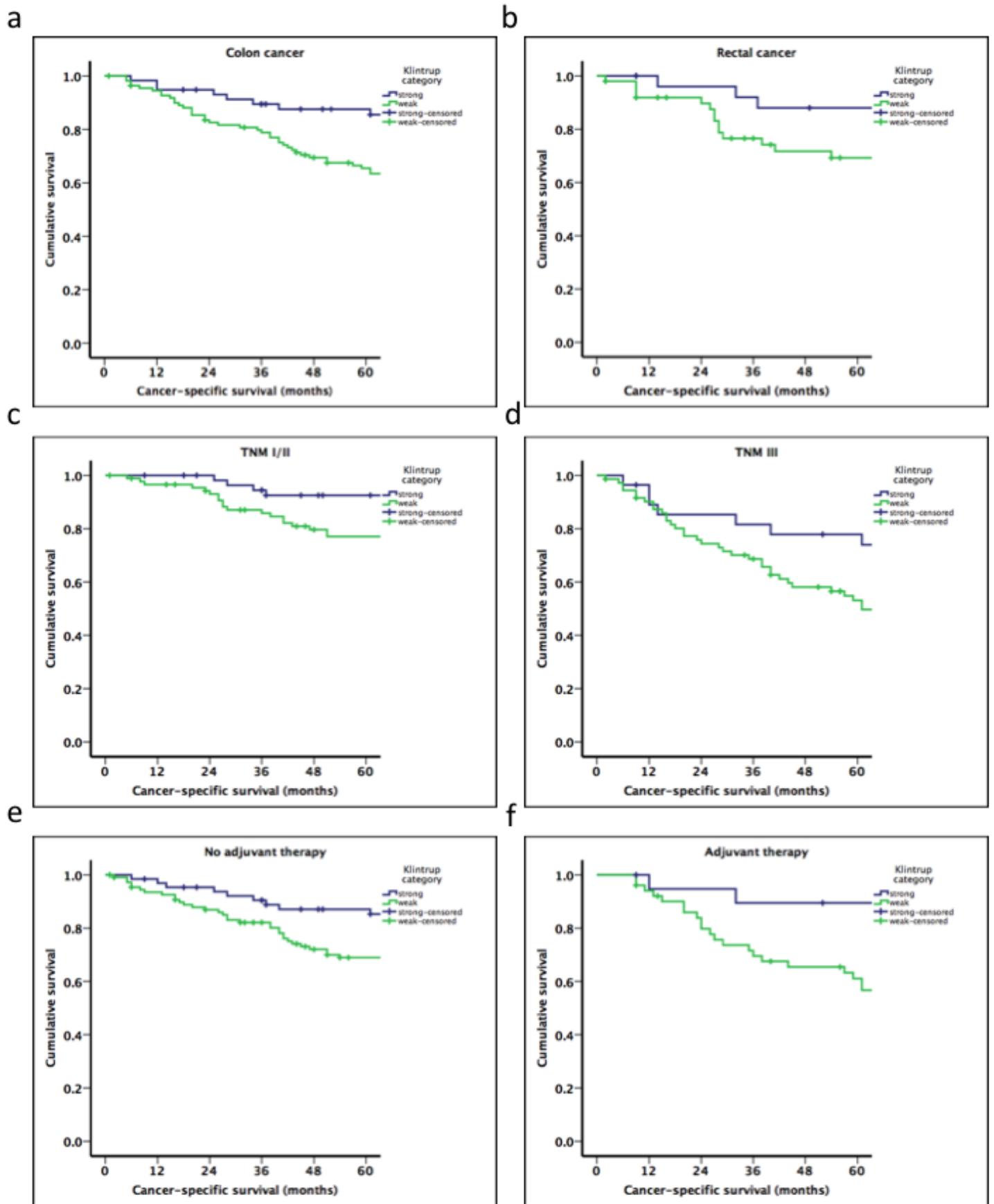


Figure 3. Relationship between Klintrup-Mäkinen grade and Immunoscore in patients undergoing potentially curative resection of stage I-III colorectal cancer ( $P < 0.001$ )

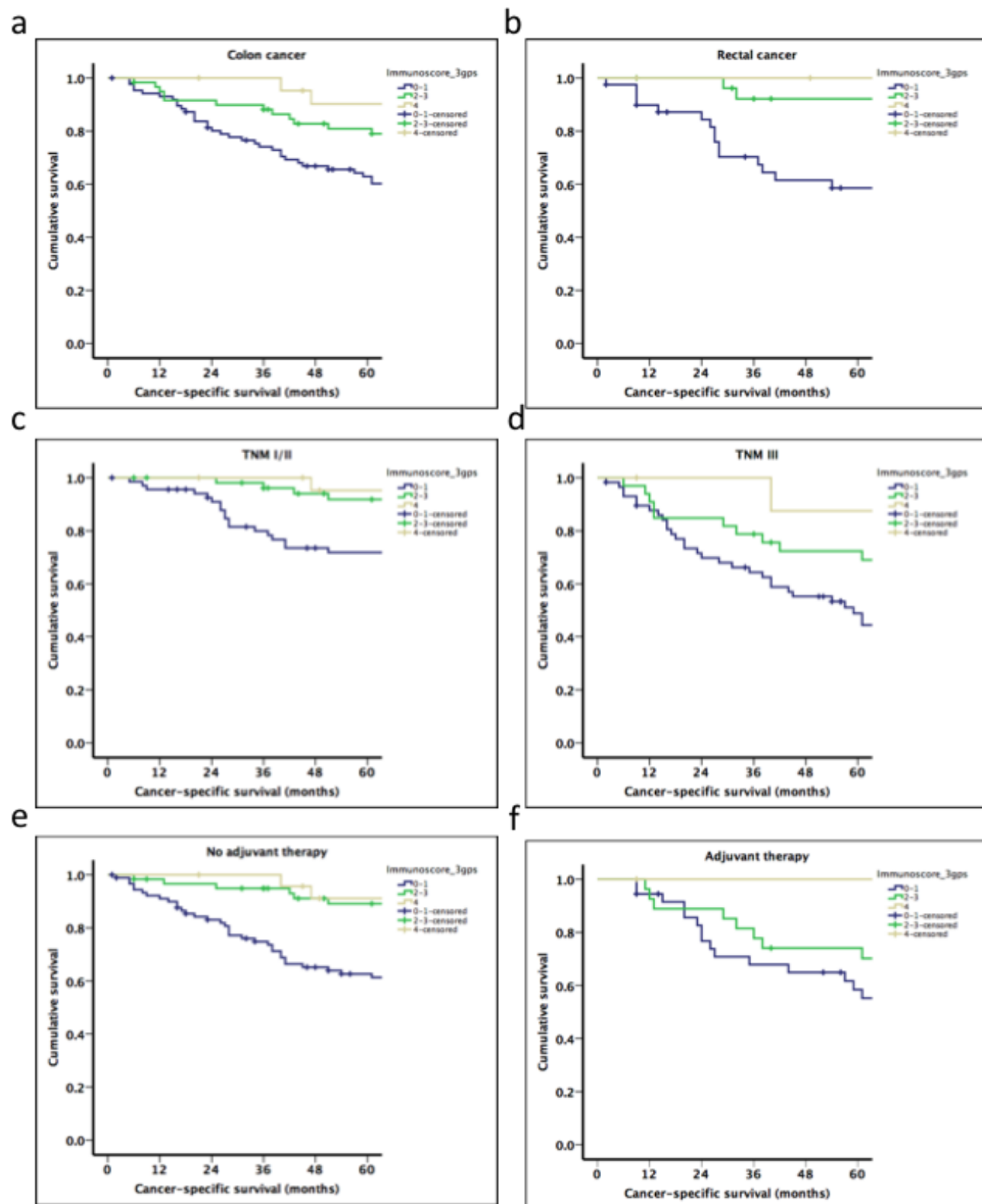




**Figure 4. Relationship between Immunoscore, tumor stroma percentage and cancer-specific survival in patients undergoing potentially curative resection of stage I-III colorectal cancer (a) Im0/1 ( $P<0.001$ ), (b) Im2/3 ( $P=0.069$ ), and (c) Im4 ( $P=0.545$ )**



**Supplementary Figure 1. Relationship between Klintrup-Mäkinen grade and cancer-specific survival of patients undergoing primary resection of colorectal cancer: (a) colon cancer ( $P=0.018$ ), rectal cancer ( $P=0.068$ ), (c) stage I/II disease ( $P=0.053$ ), (d) stage III disease ( $P=0.057$ ), (e) no adjuvant therapy ( $P=0.028$ ), and (f) adjuvant therapy ( $P=0.046$ ).**



**Supplementary Figure 1. Relationship between Immunosome and cancer-specific survival of patients undergoing primary resection of colorectal cancer: (a) colon cancer ( $P=0.003$ ), rectal cancer ( $P=0.001$ ), (c) stage I/II disease ( $P=0.002$ ), (d) stage III disease ( $P=0.011$ ), (e) no adjuvant therapy ( $P<0.001$ ), and (f) adjuvant therapy ( $P=0.059$ ).**

**Table 1. Clinicopathological characteristics of 246 patients undergoing potentially curative resection of stage I-III colorectal cancer**

	<i>N</i> (%)
<b>Age (&lt;65/ 65-74/ &gt;75)</b>	82 (33) / 84 (34) / 80 (33)
<b>Sex (female/ male)</b>	117 (48) / 129 (52)
<b>Presentation (elective/ emergency)</b>	231 (94) / 15 (6)
<b>Adjuvant therapy (no/ yes)</b>	175 (51) / 71 (29)
<b>Tumor site (colon/ rectum)</b>	169 (69) / 77 (31)
<b>TNM stage (I/ II/ III)</b>	18 (7) / 128 (52) / 100 (41)
<b>T stage (1-2/ 3/ 4)</b>	26 (11) / 152 (62) / 68 (28)
<b>N stage (0/ 1/ 2)</b>	146 (59) / 77 (31) / 23 (9)
<b>LN examined (&gt;12/ &lt;12)</b>	159 (65) / 87 (35)
<b>Differentiation (mod-well/ poor)</b>	216 (88) / 30 (12)
<b>Venous invasion (no/ yes)</b>	158 (64) / 88 (36)
<b>Margin involvement (no/ yes)</b>	230 (94) / 16 (7)
<b>Peritoneal involvement (no/ yes)</b>	178 (72) / 68 (28)
<b>Tumor perforation (no/ yes)</b>	238 (97) / 8 (3)
<b>Mismatch repair status (competent/ deficient) (205)*</b>	175 (85) / 30 (15)
<b>Tumor stroma percentage (low/ high)</b>	179 (73) / 67 (27)
<b>Klintrup-Mäkinen score (0/ 1/ 2/ 3)</b>	50 (20) / 111 (45) / 64 (26) / 21 (9)
<b>Klintrup-Mäkinen grade (low/ high)</b>	161 (65) / 75 (35)
<b>Immunoscore (0/ 1/ 2/ 3/ 4)</b>	87 (35) / 40 (16) / 44 (18) / 43 (18) / 32 (13)
<b>Alive/ cancer death/ non-cancer death</b>	94 (38) / 76 (31) / 76 (31)

\*data not available for 41 patients



**Table 2 Relationship between Immunoscore, Klintrup-Mäkinen grade and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer.**

Immunoscore	Low grade KM		High grade KM		All KM	
	<i>N</i>	5-yr CSS	<i>N</i>	5-yr CSS	<i>N</i>	5-yr CSS
<b>Im0/1</b>	108	60% (5)	19	71% (11)	127	61% (4)
<b>Im2/3</b>	42	77% (7)	45	91% (4)	87	84% (4)
<b>Im4</b>	11	90% (9)	21	94% (5)	32	93% (5)
<b>All (Im0-4)</b>	161	66% (4) <sup>a</sup>	85	88% (4) <sup>a</sup>	246	74% (3)

Survival displayed as five-year cancer-specific survival (standard error)

<sup>a</sup> Log-rank survival analysis  $P < 0.05$

KM – Klintrup-Mäkinen, CSS – cancer-specific survival

**Table 3 Relationship between measures of the local inflammatory cell infiltrate, tumor stroma percentage and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer.**

	KM						Immunoscore							
	Low grade		High grade		All KM		Im0/1		Im2/3		Im4		All Immunoscore	
	N	5-yr CSS	N	5-yr CSS	N	5-yr CSS	N	5-yr CSS	N	5-yr CSS	N	5-yr CSS	N	5-yr CSS
<b>High</b>	50	47% (7)	17	87% (9)	67	57% (6) <sup>a</sup>	37	38% (8)	24	77% (9)	6	100% (0)	67	57% (6) <sup>b</sup>
<b>Low</b>	111	75% (4)	68	88% (4)	179	80% (3)	90	71% (5)	63	87% (4)	26	92% (5)	179	80% (3) <sup>b</sup>
<b>All</b>	161	66% (4) <sup>c</sup>	85	88% (4)	246	74% (3)	127	61% (4) <sup>c</sup>	87	84% (4)	32	93% (5)	246	74% (3)

Survival displayed as five-year cancer-specific survival (standard error)

<sup>a</sup>  $P < 0.05$ , <sup>b</sup>  $P < 0.01$ , <sup>c</sup>  $P < 0.001$ . KM- Klintrup-Mäkinen, TSP- tumor stroma percentage, CSS- cancer-specific survival

**Table 4 Relationship between the tumor microenvironment, clinicopathological characteristics and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer**

	Cancer-specific survival					
	Univariate HR (95% CI)	<i>P</i>	Multivariate HR (95% CI) (Model 1)	<i>P</i>	Multivariate HR (95% CI) (Model 2)	<i>P</i>
<b>Age (&lt;65/ 65-74/ &gt;75)</b>	1.18 (0.90-1.57)	0.237	-	0.444	-	0.091
<b>Sex (female/ male)</b>	0.93 (0.59-1.46)	0.762	-	0.065	-	0.308
<b>Presentation (elective/ emergency)</b>	2.22 (1.06-4.62)	0.034	-	0.724	-	0.369
<b>Adjuvant therapy (no/ yes)</b>	1.40 (0.88-2.24)	0.160	-	0.988	-	0.505
<b>mGPS (0/ 1/ 2)</b>	1.50 (1.10-2.05)	0.010	1.52 (1.09-2.11)	0.013	1.61 (1.16-2.24)	0.005
<b>Tumor site (colon/ rectum)</b>	0.82 (0.49-1.36)	0.433	-	0.479	-	0.316
<b>T stage (1-2/ 3/ 4)</b>	1.49 (1.07-2.07)	0.017	-	0.704	-	0.981
<b>N stage (0/ 1/ 2)</b>	1.78 (1.32-2.41)	<0.001	-	0.148	-	0.066
<b>Lymph nodes examined (&gt;12/ &lt;12)</b>	1.38 (0.87-2.17)	0.171	-	-	-	-
<b>Differentiation (mod-well/ poor)</b>	1.40 (0.72-2.72)	0.322	-	-	-	-
<b>Venous invasion (no/ yes)</b>	2.95 (1.87-4.66)	<0.001	2.20 (1.37-3.54)	0.001	2.35 (1.45-3.80)	0.001
<b>Margin involvement (no/ yes)</b>	3.15 (1.56-6.33)	0.001	-	0.067	-	0.096
<b>Peritoneal involvement (no/ yes)</b>	2.19 (1.38-3.46)	0.001	-	0.225	-	0.125
<b>Tumor perforation (no/ yes)</b>	2.52 (0.92-6.93)	0.072	-	-	-	0.060
<b>MMR status (competent/ deficient)</b>	0.42 (0.17-1.05)	0.064	-	-	-	-
<b>TSP (low/ high)</b>	2.46 (1.56-3.89)	<0.001	2.36 (1.44-3.84)	0.001	2.05 (1.28-3.30)	0.003
<b>KM grade (weak/ strong)</b>	0.44 (0.25-0.76)	0.003	-	0.469	0.50 (0.29-0.87)	0.015
<b>Immunoscore (Im0-1/ Im2-3/ Im4)</b>	0.66 (0.56-0.80)	<0.001	0.43 (0.28-0.66)	<0.001		

mGPS – modified Glasgow Prognostic Score, MMR- mismatch repair status, TSP – tumor stroma percentage, KM – Klintrup-Mäkinen



**Supplementary table 1. Relationship between components of the tumor microenvironment, tumor site, TNM stage and adjuvant chemotherapy use and cancer-specific survival of patients undergoing potentially curative resection of colorectal cancer**

		Tumor site				TNM stage				Adjuvant therapy			
		Colon		Rectum		TNM I/II		TNM III		No adjuvant therapy		Adjuvant therapy	
		5yr CSS	<i>P</i>	5yr CSS	<i>P</i>	5yr CSS	<i>P</i>	5yr CSS	<i>P</i>	5yr CSS	<i>P</i>	5yr CSS	<i>P</i>
<b>KM grade</b>	<b>Low</b>	65% (5)	0.018	69% (7)	0.068	77% (5)	0.053	53% (6)	0.057	69% (5)	0.028	61% (7)	0.046
	<b>High</b>	88% (4)		88% (6)		93% (4)		78% (8)		87% (4)		89% (7)	
<b>Immunoscore</b>	<b>Im 0/1</b>	63% (5)	0.003	58% (8)	0.001	72% (6)	0.002	49% (7)	0.011	62% (5)	<0.001	59% (8)	0.059
	<b>Im 2/3</b>	81% (5)		92% (5)		92% (4)		72% (8)		89% (4)		74% (8)	
	<b>Im 4</b>	90% (7)		100% (0)		95% (5)		88% (12)		91% (6)		100% (0)	
<b>TSP</b>	<b>High</b>	51% (8)	<0.001	67% (10)	0.102	73% (9)	0.073	45% (8)	0.007	62% (8)	0.005	52% (9)	0.016
	<b>Low</b>	80% (4)		80% (6)		85% (3)		70% (6)		79% (4)		82% (6)	

Survival displayed as five-year cancer-specific survival/% (standard error)

KM- Klintrop-Mäkinen, TSP- tumor stroma percentage, CSS- cancer-specific survival