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ORIGINAL ARTICLE

Validation of the prognostic value of CD3 and CD8 cell densities analogous to the Immunoscore® by stage and location of colorectal cancer: an independent patient cohort study

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Abstract

In addition to the traditional staging system in colorectal cancer (CRC), the Immunoscore® has been proposed to characterize the level of immune infiltration in tumor tissue and as a potential prognostic marker. The aim of this study was to examine and validate associations of an immune cell score analogous to the Immunoscore® with established molecular tumor markers and with CRC patient survival in a routine setting. Patients from a population-based cohort study with available CRC tumor tissue blocks were included in this analysis. CD3+ and CD8+ tumor infiltrating lymphocytes in the tumor center and invasive margin were determined in stained tumor tissue slides. Based on the T-cell density in each region, an immune cell score closely analogous to the concept of the Immunoscore® was calculated and tumors categorized into IS-low, IS-intermediate, or IS-high. Logistic regression models were used to assess associations between clinicopathological characteristics with the immune cell score, and Cox proportional hazards models to analyze associations with cancer-specific, relapse-free, and overall survival. From 1,535 patients with CRC, 411 (27%) had IS-high tumors. Microsatellite instability (MSI-high) was strongly associated with higher immune cell score levels ($p < 0.001$). Stage I–III patients with IS-high had better CRC-specific and relapse-free survival compared to patients with IS-low (hazard ratio [HR] = 0.42 [0.27–0.66] and HR = 0.45 [0.31–0.67], respectively). Patients with microsatellite stable (MSS) tumors and IS-high had better survival (HR_{CSS} = 0.60 [0.42–0.88]) compared to MSS/IS-low patients. In this population-based cohort of CRC patients, the immune cell score was significantly associated with better patient survival. It was a similarly strong prognostic marker in patients with MSI-high tumors and in the larger group of patients with MSS tumors. Additionally, this study showed that it is possible to implement an analogous immune cell score approach and validate the Immunoscore® using open source software in an academic setting. Thus, the Immunoscore® could be useful to improve the traditional staging system in colon and rectal cancer used in clinical practice.

Keywords: colorectal cancer; immune infiltration; microsatellite instability; survival

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No conflicts of interest were declared.

Introduction

Colorectal cancer (CRC) is a heterogeneous disease that can present with varying expression of molecular biomarkers, tumor size, and lymph node invasion, which serve as prognostic and predictive factors. Microsatellite instability (MSI) is a known marker for better survival and lower stage of disease [1]. The frameshift mutations produced during the development of MSI-high tumors elicit a local immune response, making these cancers highly immunogenic [2], and providing a plausible mechanism to explain the better clinical course that these patients present.

In recent years, research on the role of the immune infiltration of the tumor has shown promising results regarding survival of CRC patients [3]. There is strong evidence that patients with a higher number of tumor infiltrating lymphocytes tend to have better survival outcomes than those who do not present a strong tumor immune response, independently of the MSI status and TNM stage [4]. The Immunoscore® methodology, which was introduced in 2006 and validated by an international consortium, provides a scoring system that characterizes tumor immune infiltration by calculating the density of CD3+ and CD8+ T-lymphocytes in the tumor center and its invasive margin [3–6].

Current evidence on the evaluation of the Immunoscore® is largely based on selected clinical populations with limited follow-up time [7], and further research is needed on the prognostic value of the Immunoscore® when investigated in combination with other clinical and molecular features. Additionally, the application of the method is dependent on commercially available software, which might prevent universal use and independent evaluation in routine clinical settings. Thus, this study aimed to describe the association of clinical and molecular characteristics with an immune cell score closely analogous to the Immunoscore® and to investigate and validate its association with patient survival in a large independent population-based cohort of CRC patients.

Materials and methods

Study design and population

The darmkrebs: chancen der verhuetung durch screening (DACHS) study is a population-based case–control study conducted in more than 20 clinics in southwestern Germany in which cases with a confirmed diagnosis of CRC are followed-up as a cohort after diagnosis [8]. Sociodemographic information and medical and family history were collected at baseline by trained interviewers using a standardized questionnaire, and clinicopathological characteristics were obtained from medical records and pathology reports after surgical resection of the tumor. Long-term follow-up was performed at 3, 5, and 10 years after diagnosis including information on recurrence of disease. Vital status, and date and cause of death were determined from population registries and death certificates issued by the health authorities. Patients included in this analysis were recruited between 2003 and 2010, had available tumor tissue blocks and molecular tumor marker characterization. Among these, patients who received neoadjuvant treatment were excluded from the analysis, due to treatment-related changes in the local tumor area. The study was approved by the ethics committees of the University of Heidelberg and the state medical boards of Baden-Wuerttemberg and Rhineland-Palatinate. Written informed consent was obtained from each participant at inclusion.

Tumor marker characterization

Tumor tissue analyses were performed on formalin-fixed, paraffin-embedded (FFPE) blocks. Details of tumor marker characterization have been previously reported [9–11]. In brief, MSI status was determined using a mononucleotide panel (BAT25, BAT26, and CAT25). CpG island methylator phenotype (CIMP) status was determined using a five-marker methylation panel (*MLH1*, *MINT1*, *MINT2*, *MINT31*, and *MGMT*) and classified into negative (no hypermethylated loci), low (1–2 loci) or high (3 or more loci). BRAF V600E mutations were determined using immunohistochemical (IHC) analyses in tissue microarray (TMA) blocks or by mutational analysis using Sanger sequencing (exon 15). *KRAS* mutations were determined by single stranded conformational polymorphism technique on DNA samples or by Sanger sequencing (exon 2).

Determination of the immune cell score

The determination of the immune cell score was derived and adapted from previous publications of the international Immunoscore® consortium [3–6,12,13]. Two consecutive sections of tumor FFPE blocks were stained using IHC detection of CD3+ and CD8+ with rabbit monoclonal antibodies (2GV6 and SP57, respectively – Roche Deutschland Holding, Mannheim, Germany). Staining was performed using the automated immunostainer Ventana BenchMark Ultra with the corresponding visualization system OptiView

DAB Kit (Roche Deutschland Holding GmbH, Mannheim, Germany) according to the manufacturer's protocol. Subsequently, the CD3+ and CD8+ stained slides were digitalized using the Aperio AT2 slide scanner (Leica Biosystems, Nussloch, Germany) with the manufacturer's acquisition software suite using a ×20/40 objective.

On each scanned slide, the tumor invasive margin (IM) and the tumor center (TU) were manually annotated by a trained medical doctor (EA) using open source digital pathology software (QuPath v1.0.2) [14]. A subset of the annotations was discussed and validated in collaboration with an expert in CRC immune pathology (MK). The IM was manually delineated as a 1-mm region centered at the edge of where invasive tumor glands infiltrated healthy tissue. The tumor center was defined as the tumor mass consisting of adjacent CRC glands; in cases where the FFPE blocks had been previously sampled for TMAs, the punch areas were excluded from the annotations (supplementary material, Figure S1A,B). The number of lymphocytes per square millimeter was determined using fixed parameters with the software's automated cell detection function (supplementary material, Table S1 and Figure S2). Finally, the immune cell score was determined by converting the CD3+ and CD8+ cell densities into percentiles and calculating the mean of the four values (CD3+/IM, CD3+/TU, CD8+/IM, and CD8+/TU), as previously described [3,7]. Scores with mean percentiles of 0–25%, >25–70%, and >70% were categorized into low (IS-low), intermediate (IS-int), or high immune cell score (IS-high), respectively [7]. Additionally, a two-category score was defined as low when mean percentiles were less than 25% and high otherwise.

To study the association between the immune cell score in combination with the MSI status of the tumor and patient survival, four subgroups were defined based on the possible combinations (i.e. MSS/IS-low, MSS/IS-high, MSI/IS-low, and MSI/IS-high).

Statistical analyses

Clinicopathological and tumor characteristics were described by category of the immune cell score. Multinomial logistic regression was used to evaluate the association between molecular tumor markers and the immune cell score. The associations of the immune cell score with patient survival were explored with Kaplan–Meier plots and quantified by Cox proportional hazard models to calculate CRC-specific survival (CSS), relapse-free survival (RFS), and overall survival (OS). Multivariable models were adjusted for potential confounders including age, T-stage, N-stage, and adjuvant treatment, and additionally stratified by stage (UICC TNM classification system 6th edition for diagnoses from 2003 to 2009, 7th edition for diagnoses in 2010) and tumor location. All analyses were performed in R v4.0.2.

Results

Patient and tumor characteristics

Among 1535 patients, 21% (n = 326), 52% (n = 798), and 27% (n = 411) had IS-low, IS-int, or IS-high, respectively. Several tumor characteristics were associated with IS-high, including lower cancer stage and proximal location, but not age or sex (supplementary material, Table S2).

Molecular tumor markers and immune cell score

MSI-high tumors were present in 13% of patients, of which 7%, 42%, and 51% were IS-low, IS-int, or IS-high, respectively ($p < 0.001$) (supplementary material, Table S2). MSI-high tumors had significantly higher immune infiltration compared to MSS tumors (supplementary material, Figure S1C–F). After adjusting for stage, location, and the other markers (*BRAF* mutation, *KRAS* mutation, and CIMP-status), only MSI-high was significantly associated with IS-int and IS-high compared to MSS (OR_{int} = 2.29 [1.0–5.2] and OR_{high} = 4.81 [2.1–11], respectively) (Table 1).

Immune cell score and patient survival

Out of 1535 included patients, who were followed-up over a median of 9.6 years, 779 (51%) died, of whom 379 (49%) died from CRC. Among 1311 stage I–III patients, there were 585 (47%) deaths from any cause, 203 (35%) of which from CRC, and 267 (20%) relapse events.

Kaplan–Meier curves showed better CSS with higher immune cell score categories (supplementary material, Figure S3). The combination of MSI/IS status showed significant differences in CSS survival, with very similar CSS for the MSS/IS-high and MSI/IS-high subgroups (Figure 1).

In adjusted analyses, stage I–III patients with IS-int and IS-high had better CSS compared to patients with IS-low (hazard ratio [HR] = 0.68 [0.49–0.94] and HR = 0.42 [0.27–0.66], respectively). In analyses combining MSI status with the binary immune cell score, patients with MSS/IS-high and MSI/IS-high tumors had better CSS (HR = 0.60 [0.42–0.88] and

Table 1. Association of established molecular tumor markers with the immune cell score in stage I–IV colorectal cancer patients

		Model 1*		Model 2†	
Association with …	Immune cell score	OR (95% CI)	*p*	OR (95% CI)	*p*
MSI-high	Low	1 (Ref)		1 (Ref)	
	Intermediate	2.10 (1.0–4.3)	**0.044**	2.29 (1.0–5.2)	**0.045**
	High	4.87 (2.3–10)	**<0.001**	4.81 (2.1–11)	**<0.001**
CIMP-high	Low	1 (Ref)		1 (Ref)	
	Intermediate	1.41 (0.9–2.2)	0.130	1.27 (0.8–2.1)	0.400
	High	2.51 (1.6–4.1)	**<0.001**	1.46 (0.8–2.6)	0.200
KRAS mutation	Low	1 (Ref)		1 (Ref)	
	Intermediate	0.91 (0.7–1.2)	0.500	0.78 (0.6–1.1)	0.200
	High	0.74 (0.5–1.1)	0.110	0.79 (0.5–1.2)	0.300
BRAF mutation	Low	1 (Ref)		1 (Ref)	
	Intermediate	1.02 (0.5–1.9)	0.900	0.55 (0.3–1.2)	0.140
	High	2.33 (1.2–4.6)	**0.015**	0.69 (0.3–1.6)	0.400

Multinomial logistic regression comparing immune cell score high and intermediate with immune cell score low. *p*-values <0.05 in bold.
*Model 1: Adjusted for tumor stage and location.
†Model 2: Adjusted for tumor stage, location and remaining molecular markers.

HR = 0.51 [0.26–0.97], respectively) compared to MSS/IS-low patients. In stage-specific analyses, the associations were stronger among stage III patients, whereas no statistically significant differences were observed among stage II patients, for whom the number of events was much lower (Figure 2).

Among stage I–III patients with proximal and distal colon cancer, IS-high was associated with better CSS (HR = 0.37 [0.16–0.69] and HR = 0.34 [0.11–1.03], respectively) (supplementary material, Figure S4). Analyses of the combination of MSI status and binary immune cell score showed better CSS for MSI/IS-high in the proximal colon only. For MSS/IS-high patients, however, the association with better CSS was found only in the rectum (HR = 0.47 [0.25–0.91]), and not in the proximal or distal colon (HR = 0.56 [0.30–1.07] and HR = 0.74 [0.38–1.45], respectively).

Similar results were observed for associations of the immune cell score with RFS. Among stage I–III patients, those with IS-int and IS-high had better RFS

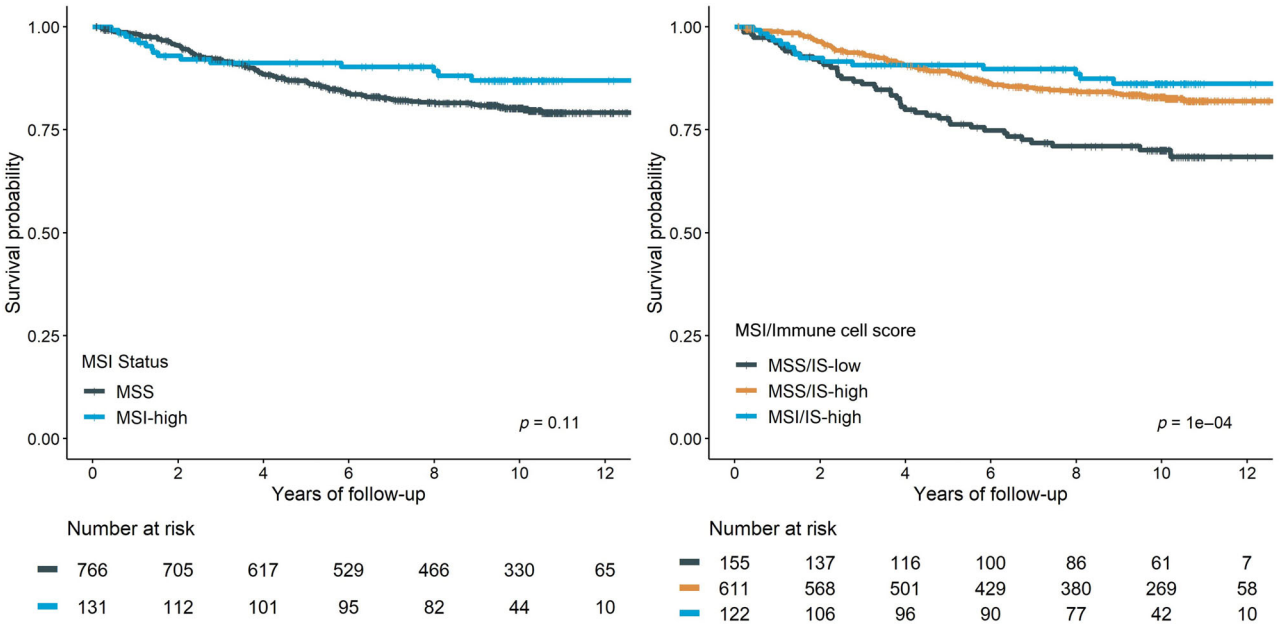

Figure 1. Kaplan–Meier plots for CSS among stage I–III patients by MSI status (left) and combinations of MSI/immune cell score status (right). The MSI/IS-low subgroup was too rare for an evaluation and is therefore not included ($n = 9$, no events).

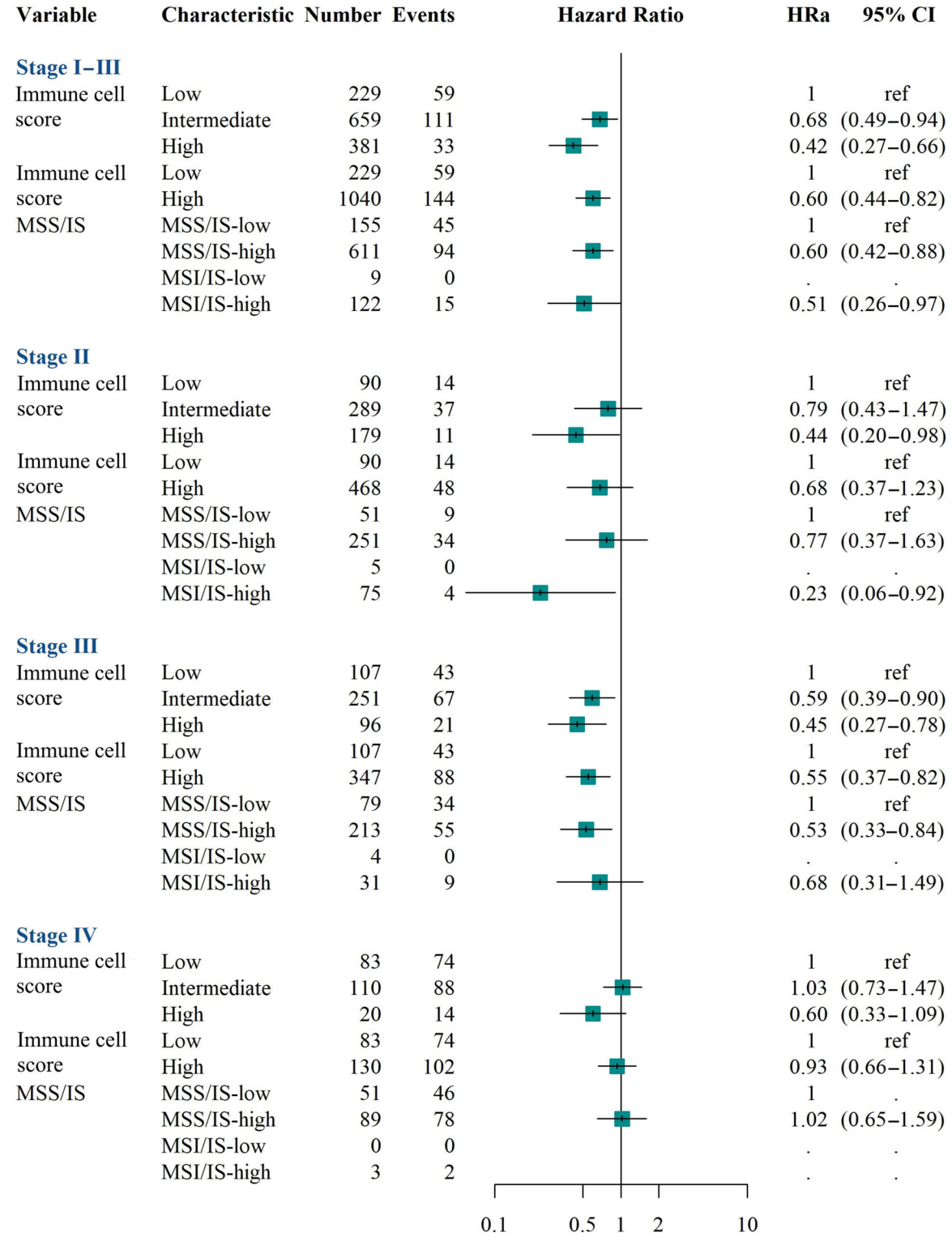

Figure 2. CSS for categories of immune cell score and combinations with MSI status stratified by stage at diagnosis. Models adjusted for age, location, T-stage, N-stage (except stage II), and adjuvant therapy. HRs were not calculated in subgroups with fewer than five patients or where no events occurred. HRa, adjusted hazard ratio.

than those with IS-low (HR = 0.71 [0.53–0.95] and HR = 0.45 [0.31–0.67], respectively) (supplementary material, Figure S5). Significantly better RFS was also observed for the MSS/IS-high group (HR = 0.60 [0.43–0.83]). No significant associations were observed for any of the immune cell score categories or combinations with MSI status and OS (supplementary material, Figure S6).

Discussion

In this population-based study, high tumor immune cell infiltration was strongly associated with MSI-high, early-stage, and proximal CRC. Among patients with stage I–III CRC, a higher immune cell score was prognostic for better CSS and RFS, independent of MSI status, tumor location, and to a certain extent of disease stage, since among stage II patients the lower number of events mostly hindered statistically significant results. These findings, based on a large patient cohort, confirm previously reported results [3,13] for the trade-marked Immunoscore® and highlight that the immune cell score is also a prognostic marker of disease-related CRC outcomes among patients with MSS tumors, and that its implementation could be feasible in an independent setting.

Similar to previous studies, the proportion of patients with an IS-high tumor was higher among MSI-high than among MSS patients, usually reported around 75% [7,15,16]. MSI-high tumors are known to be highly immunogenic [2], which is reflected by the correlation between MSI-high tumors and the response to immunotherapy treatments [17]. In analyses adjusted for stage and location, CIMP-high and *BRAF* mutated tumors were also associated with a higher immune cell score. However, these associations vanished after adjusting for the other tumor markers, in particular for MSI. This reflects the high complexity of the different molecular phenotypes of CRC, as it has been described that CIMP status is correlated with the hypermethylation of the *MLH1* gene promoter, which in turn leads to MSI-high tumors, and that *BRAF* mutations are often prevalent in MSI tumors [16,18]. Other tumor characteristics, such as proximal location and lower T-stage of the tumor were associated with higher immune infiltration. As expected, a high immune cell score translated into a lower number of affected lymph nodes, since lower immune infiltration represents a higher chance for tumor cells to escape and therefore infiltrate the local lymphatic drainage [19].

Our results are consistent with an international validation study of the Immunoscore® in stage I–III colon cancer patients, where both IS-int and IS-high tumors showed better disease-free survival than IS-low tumors (HR = 0.62 [0.52–0.75] and HR = 0.51 [0.40–0.65]) [3]. Our results among stage I–III CRC patients are consistent with these findings, both for RFS (HR = 0.71 [0.53–0.95] and HR = 0.45 [0.31–0.67]) and CSS (HR = 0.68 [0.49–0.94] and HR = 0.42 [0.27–0.66]), with similar results among rectum cancer patients in stratified analyses.

A high immune cell score was also associated with better CSS and RFS among patients with the more frequently diagnosed MSS CRC, and the effect was comparable to that among MSI-high patients. Results from the present analyses are similar to those presented in the international validation study for stage I–III colon cancer patients [3], where MSS patients with a high Immunoscore® showed better disease-free survival (HR = 0.56 [0.46–0.68]); in our study, colon cancer patients with MSS/IS-high had a tendency toward better RFS, although not statistically significant (HR = 0.74 [0.49–1.13]). Tumor immune infiltration reflects the interaction between the tumor microenvironment and the host's native immune response and has increasingly gained importance in the era of personalized medicine, not only as a prognostic marker, but also as an emerging potential predictive marker. Particularly for early stage MSS colon cancer patients, the evaluation of the tumor immune microenvironment could be an important predictor for efficacy of treatment with immune checkpoint inhibitors [20].

A recent analysis of stage III colon cancer patients treated with FOLFOX as part of a clinical trial [21] found significantly better disease-free survival among patients with intermediate to high Immunoscore® (HR = 0.59 [0.4–0.8]). Similarly, results from another randomized clinical trial among stage III colon cancer patients receiving 3 or 6 months of adjuvant oxaliplatin-based chemotherapy found better disease-free survival in patients with a high Immunoscore® (HR = 0.45 [0.3–0.8]) [7].

Evidence regarding the usefulness of the Immunoscore® among stage II CRC patients is currently lacking. In the present study, it was not possible to report additional stratified results for high- and low-risk subgroups due to the limited number of events among stage II patients.

Apart from expanding the information on prognosis of CRC, novel biomarkers need to have predictive value to aid in treatment decisions and to predict benefit from chemotherapy. Thus far, few studies including patients from clinical trials have generated evidence about the predictive capacity of the Immunoscore®, suggesting that patients with a high Immunoscore®

might benefit from adjuvant chemotherapy, while patients with a low Immunoscore®, who have no pre-existing strong immunity, might not find such a benefit [7,22]. These are highly interesting findings that need to be validated in larger, independent studies, before treatment guidelines are adapted accordingly. Thus far, neither the National Comprehensive Cancer Network (NCCN) guidelines, nor the European Society for Medical Oncology (ESMO) guidelines have considered the role of the Immunoscore® as certain enough to be definitely included as a predictive marker [23]. However, it is considered as an important emerging predictive factor among colon cancer patients, and the need for more detailed research on early stage patients to help identify the role of Immunoscore® as predictive of chemotherapy benefit is highlighted. Particularly for early stage MSS colon cancer patients, the evaluation of the tumor immune microenvironment could be an important predictor for efficacy of treatment with immune checkpoint inhibitors [20].

The large sample size of this well-characterized, unselected patient cohort with long term follow-up represents a major strength of this validation analysis, although case numbers were still limited in some of the subgroup analyses. Besides clinical and epidemiological information, detailed pathological information was available from histology reports and comprehensive tumor marker analyses. Although the software used to characterize the immune cell score was different from that used by the group that developed the Immunoscore®, the methodology was derived and adapted from published literature and the use of powerful open-source software yielded consistent results, thus demonstrating feasibility of its implementation and validation in an external academic setting.

In conclusion, in this independent population-based study, it was possible to confirm previously reported results [3,13] for the Immunoscore® as a strong prognostic factor of survival outcomes for CRC by using an adapted immune cell score approach.

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Author contributions statement

EA, MH and HB conceived and designed the study. EA, JNK, MK, AB, WR, KET, AE, ELA, JCC, HB and MH acquired data. EA and MH analyzed and interpreted data. All authors revised the manuscript for important content and approved the final version.

Data availability statement

Individual patient data and related tumor information underlying this article cannot be shared publicly due to data privacy protection laws. However, grouped data will be shared on reasonable request to the corresponding author.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Example illustrations of tumor slides and lymphocytic infiltration by MSI status

Figure S2. Example QuPath image analysis and CD3, CD8 cell quantification of a patient with a low number of tumor-infiltrating lymphocytes

Figure S3. Kaplan–Meier plot for cancer-specific survival among stage I–III patients by immune cell score category

Figure S4. CRC-specific survival for immune cell score categories and combinations with MSI status, stratified by tumor location among stage I–III patients

Figure S5. Relapse-free survival for immune cell score categories and combinations with MSI status, stratified by stage and location

Figure S6. Overall survival for immune cell score categories and combinations with MSI status, stratified by stage and location

Table S1. Annotation settings and settings for the cell counts in QuPath

Table S2. Patient and tumor characteristics by categories of immune cell score