

# Immunoscore in mismatch repair-proficient and -deficient colon cancer

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## Abstract

The aim of this study was to investigate immune response and its prognostic significance in colon carcinomas using the previously described Immunoscore (IS). A population-based series of 779 colorectal cancers, operated on between 2000 and 2010, were classified according to tumour, node, metastasis (TNM) status, mismatch repair (MMR), and *BRAF* mutation status. Rectal cancer cases ( $n = 203$ ) were excluded as a high proportion of these patients received preoperative neoadjuvant chemoradiotherapy. Tissue microarray (TMA) samples collected from the tumour centre and invasive front were immunostained for CD3 and CD8. Lymphocytes were then digitally calculated to categorize IS from grade 0 to 4. Samples adequate for IS were available from 510 tumours. IS was significantly associated with AJCC/UICC stage, T stage, lymph node and distant metastases, perineural and lymphovascular invasion, MMR status, and *BRAF* mutation status. For IS0, IS1, IS2, IS3 and IS4, respectively, the 5-year disease-free survival (DFS) rates were 59, 68, 78, 83 and 94% ( $p < 0.001$ ); 5-year disease-specific survival (DSS) rates were 47, 55, 75, 80, and 89% ( $p < 0.001$ ); and 5-year overall survival (OS) rates were 40, 44, 66, 61, and 76% ( $p < 0.001$ ). IS was also prognostic for DFS, DSS, and OS within subsets of microsatellite-stable (MSS) and microsatellite-unstable (MSI) disease. Multivariable analysis showed that IS, AJCC/UICC stage, lymphovascular invasion, and lymph node ratio in AJCC/UICC stage III disease were independent prognostic factors for DFS, DSS, and OS. Age was an independent prognostic factor for DSS and OS. Gender and *BRAF* mutation were independent prognostic factors for OS. In conclusion, IS differentiated patients with poor versus improved prognosis in MSS and MSI disease and across AJCC/UICC stages. IS, AJCC/UICC stage, lymphovascular invasion, and lymph node ratio in AJCC/UICC stage III disease were independent prognostic factors for DFS, DSS, and OS.

**Keywords:** immunoscore; mismatch repair status; BRAF; AJCC/UICC stage; colon cancer

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No conflicts of interest were declared.

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## Introduction

Colorectal cancer (CRC) is the fourth most common malignancy worldwide, occurring with an estimated annual incidence of approximately 1.3 million cases

and causing over 600 000 deaths per year [1]. The American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) has published the most reliable histopathological method for CRC staging, which shows great prognostic

significance even though prognosis can vary substantially even among patients within the same stage [2]. It is estimated that 30 to 50% of colon cancers recur during follow-up despite optimal primary treatment [3]. Biological differences reportedly influence tumour behaviour and prognosis [4].

Recent findings indicate that CD3<sup>+</sup>, CD8<sup>+</sup>, and CD45RO<sup>+</sup> lymphocytes play important roles in tumour growth suppression, both in the tumour centre and at the invasive front of primary and metastatic CRCs [5–8]. Therefore, investigations of immune responses may provide more detailed information regarding CRC prognosis.

The Immunoscore (IS) has been developed to describe immune responses in CRC. The IS classification is based on CD3<sup>+</sup> and CD8<sup>+</sup> lymphocyte cell counts in representative areas of the tumour centre and invasive front. The Immunoscore is reportedly a significant prognostic factor for colon and rectal cancer [7–9]. It has been suggested that use of the IS in combination with AJCC/UICC staging could lead to better determination of tumour prognosis [10].

CRCs can also be classified according to their mismatch repair (MMR) capacity into MMR-deficient and MMR-proficient tumours, also termed microsatellite-unstable (MSI) or microsatellite-stable (MSS) cancers. The MMR deficiency pathway induces hypermutated MSI cancers that differ from MSS cancers in their mutation profile and biological behaviour [11]. MSI colorectal cancers show improved prognosis compared to MSS cancers [12–14]. Due to their abundant mutations, MSI tumours exhibit aberrant neoantigens that can induce immune responses against cancer cells [15]. *BRAF* mutation plays a specific role in several tumour types and has been associated with worse prognosis in MSS colon carcinoma [16–18]. Other factors also reportedly have prognostic significance in CRC, such as perineural and lymphovascular invasion [19,20], as well as lymph node ratio (LNR) in stage III disease [21].

The aim of our study was to define the prognostic significance of IS in a large population-based cohort of colon cancer patients classified into four AJCC/UICC stages using current histopathological criteria. We particularly wanted to assess the association of IS with other known prognostic factors, such as MMR/*BRAF* mutation status, perineural or lymphovascular invasion, and lymph node ratio.

## Material and methods

### Patients

This study was performed at the Central Hospital of Central Finland, which serves a defined catchment area

of about 274 000 people. Tissue microarray (TMA) data were available for representative tumour samples from 799 patients who underwent operations at our hospital from 2000 to 2010. For 779 of these cases, good quality immunohistochemical (IHC) staining of a representative tumour sample for MSI was also available. Patients with rectal cancer ( $n = 203$ ) were excluded as a high proportion of these patients underwent preoperative neoadjuvant chemoradiotherapy, which may influence immune response. Tissue cores adequate for IS determination were available from 510 colon cancers.

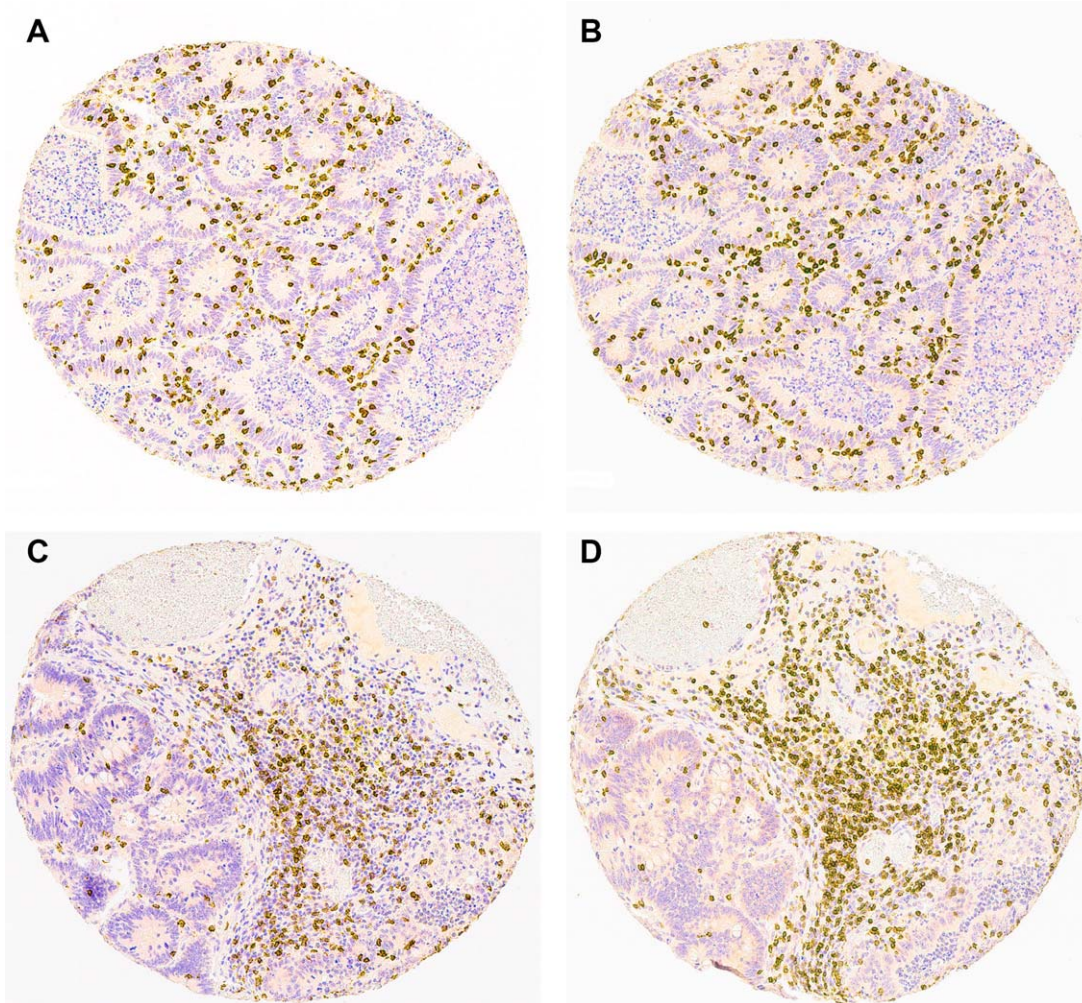
We reviewed hospital records to extract information regarding treatment and tumour recurrence. Causes of death were reviewed and updated in December 2015 from the Finnish Cause of Death Registry. Macroscopic and histological examination of tumour tissue was performed by an experienced histopathologist following AJCC guidelines (5th and 6th edition). The lymph node ratio (LNR) was calculated as the proportion of metastatic lymph nodes from the total number of lymph nodes examined. AJCC/UICC stage III patients were divided into three similar-sized groups based on LNR: <0.12, 0.12 to 0.30, and >0.30. A 6-month course of adjuvant postoperative chemotherapy with either 5-fluorouracil (FU) and oral folinic acid, oral capecitabine with or without oxaliplatin, or the FOLFOX regimen was prescribed to medically fit patients having stage III tumours or high-risk stage II disease (T4 or T3 with perineural or lymphovascular invasion).

### Tumour sampling

TMA blocks were prepared from formalin-fixed paraffin-embedded tissue (FFPE) samples. Tissue cores (diameter, 0.6 mm) were punched out and were set into a recipient paraffin block using the Manual Tissue Microarrayer MTA-1 (Beecher Instruments, Inc.). We obtained one core from normal tissue, two cores from the tumour centre, and two cores from the tumour invasive front. The TMA blocks were cut into 2- $\mu$ m-thick sections.

### Immunohistochemistry

For all included tumours, we performed a universal IHC screening for loss of MMR protein expression and *BRAF* mutation, as previously described [12]. For 21 cases, the *BRAF* staining was unsuccessful or left indifferent. The following monoclonal antibodies for were used immunohistochemical staining: MLH1 (Novocastrol NCL-L-MLH1, clone ES05), MSH2 (Oncogene Research Products NA27, clone FE11), MSH6 (Cell Marque 287M-16, clone 44), PMS2 (BD Pharmingen 556415, clone A16-4), *BRAF* V600E monoclonal antibody (Spring Bioscience E19292, clone VE1).



**Figure 1.** Examples of (A) CD8 staining from tumour centre, (B) CD3 staining from tumour centre, (C) CD8 staining from tumour invasive front, and (D) CD3 staining from tumour invasive front.

TMA block sections were immunohistochemically stained using CD3 (Novocastra, NCL-L-CD3, clone PS1) and CD8 (Thermo Scientific, RM-9116, clone SP16) antibodies, both diluted 1:100, following standard procedures using the BOND-III staining instrument (Leica Biosystems) and Bond Polymer Refine Detection Kit (Leica Biosystems). Samples were incubated with antibody dilutions for 30 min at room temperature. CD3 and CD8 antigen retrieval was performed for 20 min using Bond Epitope Retrieval Solution 2 (Leica Biosystems). Example of CD8 and CD3 staining are shown in Figure 1.

#### Immunoscore determination

CD3<sup>+</sup> and CD8<sup>+</sup> cells were assessed by digital image analysis. Stained TMA block sections from

representative areas of the tumour centre and invasive front were scanned using an Aperio digital slide scanner (Leica Biosystems), followed by analysis using an ImageJ-based program to count the numbers of CD3<sup>+</sup> and CD8<sup>+</sup> cells. Cells were identified based on their size, shape, and staining intensity as described earlier by Väyrynen *et al* [22]. In order to minimize selection bias as well as the number of discarded patients from the study because of unrepresentative or inadequate samples (tissue cores), we took two cores from the tumour centre and two cores from the invasive front. From each core, we had one section cut for both lymphocyte stains. If the sample taken from the original tumour tissue block did not represent tissue area that was targeted (eg, core taken from invasion front) it was considered unrepresentative. Inadequate means that majority of the tissue

core was for example torn off from the TMA section. Each tissue core was 600 µm in diameter and from each patient we had a minimum of four representative core sections (one core section from tumour centre and one from invasive front for both stains). Eighty-nine and eighty-eight tumour centre core sections were abandoned for CD8 and CD3, and 38 and 42 invasive front core sections were abandoned for CD8 and CD3, respectively, as they were either unrepresentative or inadequate. The average area analyzed from each patient was 0.52 mm<sup>2</sup> for CD8 and CD3 at the tumour centre and 0.54 mm<sup>2</sup> for CD8 and CD3 at the invasive front, giving a total analyzed area of 2.12 mm<sup>2</sup> for each patient. All of the automatically analyzed sections were individually reviewed and values were corrected manually in cases with inaccurate results. Of the four acquired section pairs (two core sections from the tumour centre and two from the invasive front for both stains), we selected the sections with the highest lymphocyte count in order to minimize the number of false negative results. IHC stains were always assessed without knowledge of the clinical data.

The immunoscore was generated from four samples: one core section from the tumour centre and one core section from the invasive front for both lymphocyte stains (CD3 and CD8). The sections were determined to have either high or low lymphocyte count (number of cells/mm<sup>2</sup>). The cut-off values were obtained from receiver operating characteristic (ROC)-curves drawn for each group in relation to disease-specific 3-year mortality. ROC-curves give a likelihood ratio for disease-specific mortality for each lymphocyte count, so we can select the value with optimal sensitivity and specificity to separate patients

into those who are more likely to have the end point and those who would not. Table 1 shows the cut-off values with likelihood ratios and area under the curve (AUC) for the CD8 and CD3 tumour centre and CD8 and CD3 invasive front ROC-curves. One Immunoscore point is given for each section with a high lymphocyte count so that in IS 4 all the sections had a high lymphocyte count and in IS 0 none of the sections had a high count.

### Statistical analyses

Categorical data were compared using Pearson's chi-square or Mantel-Haenszel tests. The Kaplan-Meier method was used to calculate disease-free survival (DFS), disease-specific survival (DSS), and overall survival (OS), and the log-rank test was used to compare differences. Survival times for DSS and OS were calculated from the date of surgery to the time of death or the end of follow-up. For DFS, the endpoint was the date of cancer recurrence after radical surgery or the end of follow-up. Death within 30 days following surgery was considered postoperative, and metastasis within 6 months after surgery was considered synchronous. Univariable and multivariable Cox proportional hazards regression models were used to analyze prognostic factors for DFS, DSS, and OS. Only variables with a *P* value of <0.20 in univariable analysis were included in the multivariable analysis with age and gender. Among AJCC/UICC stage I patients there were no cancer deaths; thus, these patients were excluded from multivariable analysis for DSS. *P* values of <0.05 were considered significant. Statistical analyses were conducted using SPSS Statistics for Windows (release 23.0.0.0; SPSS, Inc.).

**Table 1.** Immunoscore cut-off values

	Cut-off value (cells/mm <sup>2</sup> )	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Likelihood Ratio
<b>CD8 Tumour centre:</b>					
3-year DSS	226	0.64 (0.57 to 0.70)	0.74 (0.64 to 0.82)	0.53 (0.48 to 0.58)	1.57 (1.34 to 1.83)
5-year DSS	226	0.64 (0.58 to 0.69)	0.72 (0.64 to 0.80)	0.54 (0.49 to 0.59)	1.57 (1.34 to 1.83)
<b>CD3 Tumour centre</b>					
3-year DSS	339	0.64 (0.59 to 0.70)	0.50 (0.40 to 0.60)	0.74 (0.69 to 0.78)	1.93 (1.50 to 2.48)
5-year DSS	339	0.64 (0.58 to 0.70)	0.50 (0.41 to 0.59)	0.75 (0.70 to 0.79)	1.98 (1.54 to 2.54)
<b>CD8 Tumour invasive margin:</b>					
3-year DSS	286	0.65 (0.59 to 0.71)	0.59 (0.49 to 0.69)	0.68 (0.63 to 0.73)	1.85 (1.49 to 2.29)
5-year DSS	290	0.65 (0.58 to 0.70)	0.58 (0.49 to 0.67)	0.68 (0.63 to 0.73)	1.83 (1.48 to 2.26)
<b>CD3 Tumour invasive margin:</b>					
3-year DSS	618	0.65 (0.59 to 0.70)	0.65 (0.55 to 0.74)	0.63 (0.58 to 0.67)	1.74 (1.44 to 2.10)
5-year DSS	618	0.65 (0.59 to 0.71)	0.63 (0.54 to 0.72)	0.63 (0.58 to 0.68)	1.73 (1.43 to 2.09)

Cut-off values for the tumour centre and invasive margin were selected from 3-year disease-specific survival (DSS) ROC curves to identify the most aggressive tumours. Only a few cancer deaths occurred between 3 and 5 years, and the cut-off values from the 5-year ROC curve did not significantly differ from the 3-year cut-off values. AUC, area under the curve; CI, confidence interval.

Table 2. Clinicopathological variables and their associations with Immunoscore

	Immunoscore 0 & 1 (N = 142) N (% of row)	Immunoscore 2 & 3 (N = 208) N (% of row)	Immunoscore 4 (n = 160) N (% of row)	Total (n = 510) N (% of column)	p
Age:					
<65 years	44 (31)	55 (39)	43 (30)	142 (28)	0.913*
65 to 75 years	32 (22)	67 (46)	48 (33)	147 (29)	
>75 years	66 (30)	86 (40)	69 (31)	221 (43)	
Gender:					
Male	66 (29)	95 (42)	65 (29)	226 (44)	0.520
Female	76 (27)	113 (40)	95 (34)	284 (56)	
Tumour location:					
Right hemicolon	73 (25)	113 (39)	101 (35)	287 (56)	0.094
Left hemicolon	69 (31)	95 (43)	59 (27)	223 (44)	
T stage					
1	5 (21)	10 (42)	9 (38)	24 (5)	0.001*
2	15 (20)	32 (43)	28 (37)	75 (15)	
3	77 (24)	136 (43)	104 (33)	317 (62)	
4	45 (48)	30 (32)	19 (20)	94 (18)	
Lymph node metastasis:					
No	59 (20)	121 (41)	113 (39)	293 (58)	<0.001
Yes	83 (38)	87 (40)	47 (22)	217 (42)	
Distant Metastasis:					
No	112 (25)	184 (41)	150 (34)	446 (88)	<0.001
Yes	30 (47)	24 (38)	10 (16)	64 (12)	
AJCC/UICC Stage:					
I	16 (21)	32 (41)	30 (39)	78 (15)	<0.001*
II	40 (20)	83 (41)	79 (39)	202 (40)	
III	55 (34)	68 (42)	38 (24)	161 (32)	
IV	31 (45)	25 (36)	13 (19)	69 (14)	
Lymph node ratio:					
<0.12	11 (20)	28 (52)	15 (28)	54 (11)	0.127*
0.12 to 0.30	23 (42)	20 (36)	12 (22)	55 (11)	
>0.30	21 (40)	20 (39)	11 (21)	52 (10)	
Perineural invasion:					
No	116 (25)	193 (42)	151 (33)	460 (90)	<0.001
Yes	26 (52)	15 (30)	9 (18)	50 (10)	
Lymphovascular invasion:					
No	110 (26)	178 (41)	144 (33)	432 (85)	0.009
Yes	32 (41)	30 (38)	16 (21)	78 (15)	
MMR status:					
MSS	129 (31)	181 (43)	109 (26)	419 (82)	<0.001
MSI	13 (14)	27 (30)	51 (56)	91 (18)	
BRAF status:					
BRAF wild-type	122 (30)	180 (44)	108 (27)	410 (80)	<0.001
BRAF mutation	17 (22)	21 (27)	41 (53)	79 (15)	
MMR/BRAF combinations					
MSS/BRAFwt	118 (31)	168 (44)	96 (25)	382 (75)	<0.001
MSS/BRAFmut	9 (38)	8 (33)	7 (29)	24 (5)	
MSI/BRAFwt	4 (14)	12 (43)	12 (43)	28 (5)	
MSI/BRAFmut	8 (15)	13 (24)	34 (62)	55 (11)	

\*Mantel-Haenszel test used.

MMR, mismatch repair; MSS, micro-satellite stable; MSI, micro-satellite instable; AJCC, The American Joint Committee on Cancer; UICC, Union for International Cancer Control; BRAFwt, BRAF wild-type; BRAFmut, BRAF mutation.

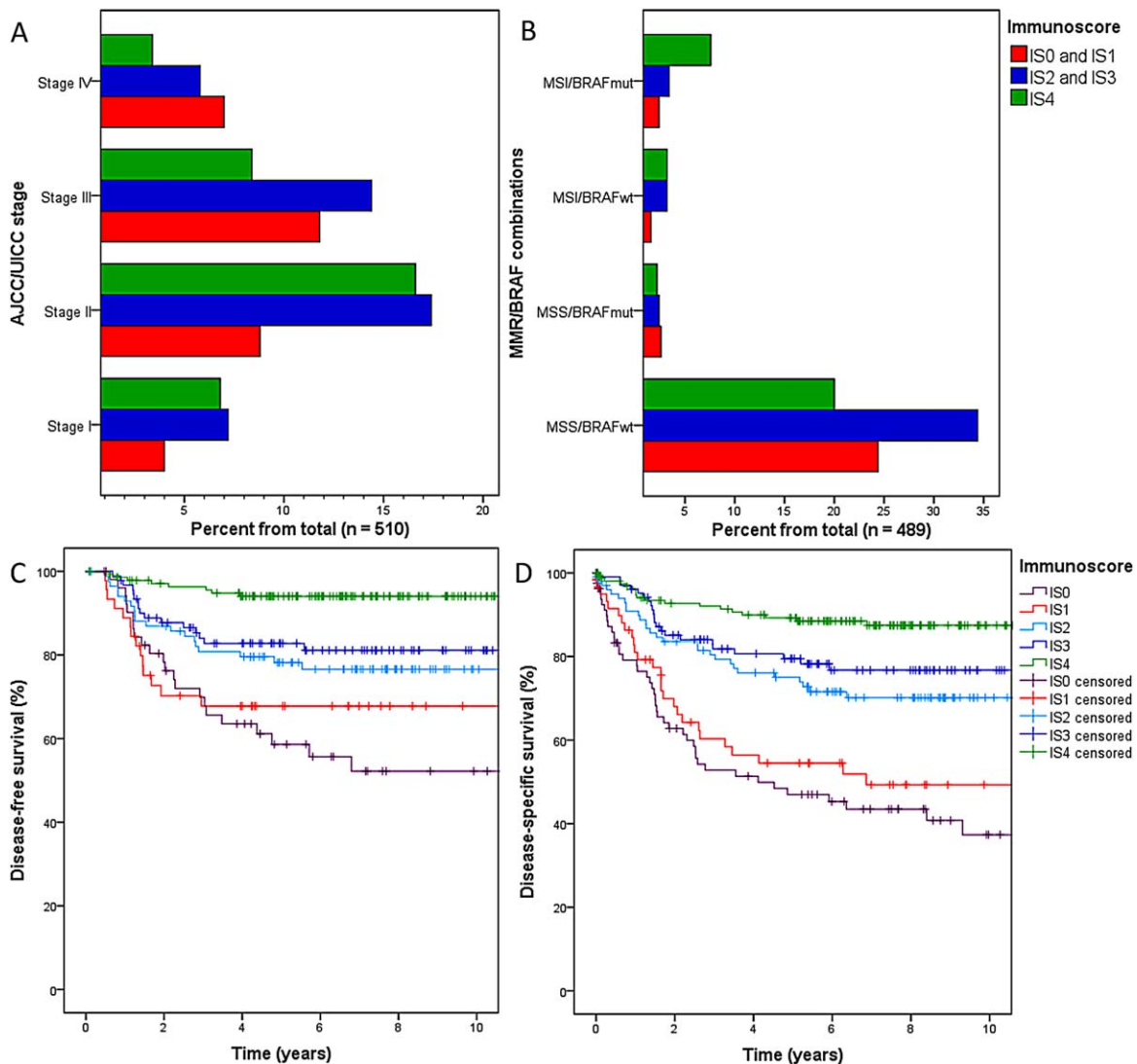
Ethical aspects

The study was approved by the ethical committee of the Central Finland Central Hospital. Authorization for use of the patient registry was obtained from the National Supervisory Authority for Welfare and Health (Valvira).

Results

Clinicopathological characteristics

Table 2 shows the patients' clinicopathological details including AJCC/UICC stage and MMR/BRAF mutation status distribution. Within the study



**Figure 2.** Distributions of (A) AJCC/UICC stage and (B) MMR/*BRAF* mutation status between different Immunoscoring (IS) classes; and (C) disease-free survival and (D) disease-specific survival for different IS classes.

population, the median follow-up time was 6.0 years (IQR, 1.7 to 9.0 years) and the median age at the time of surgery was 73 years (IQR, 64 to 79 years). R0 resection of the primary tumour was achieved in 435 of the 510 patients (85%). Non-radical operation was performed in 75 patients (15%), including 63 with stage IV disease with carcinosis or unresectable distant metastasis, nine with stage II or III disease showing local invasion to adjacent tissues, and one patient with abdominal wound recurrence. The median number of examined lymph nodes was 11 (IQR, 6 to 17). Postoperative death was recorded for 27 patients (5%). Of the 483 patients alive after surgery, 162 (34%) received adjuvant chemotherapy.

### Immunoscoring and association with other clinicopathological parameters

IS distribution in our series was as follows: IS0, 16%; IS1, 12%; IS2, 20%; IS3, 21%; and IS4, 31%. Lower IS was associated with increasing AJCC/UICC stage, as well as with increasing T stage, presence of lymph node and distant metastasis, and perineural or lymphovascular invasion (Table 2, Figure 2A). IS was not significantly associated with LNR, age, sex, or tumour location. IS distribution is shown in relation to MMR status and in relation to MMR/*BRAF* mutation sub-classification in Table 2 and Figure 2B, respectively. Most MSI tumours had high IS, with 56% belonging to the IS4 group. *BRAF* mutation occurred predominantly in MSI tumours with IS4 (Table 2).

Table 3. Survival according to clinicopathological variables and Immunoscore

	Disease-free survival (N = 417)			Disease-specific survival (N = 510)			Overall survival (N = 510)		
	Total N	5-year survival	p	Total N	5-year survival	p	Total N	5-year survival	p
Age:									
<65 years	117	77%	0.103	142	74%	0.102	142	71%	<0.001
65 to 75 years	125	87%		147	80%		147	71%	
>75 years	175	78%		221	68%		221	48%	
Gender:									
Male	183	82%	0.925	226	71%	0.415	226	56%	0.047
Female	234	80%		284	76%		284	66%	
AJCC/UICC stage:									
I	75	99%	<0.001	78	100%	<0.001	78	77%	<0.001
II	190	84%		202	89%		202	73%	
III	146	69%		161	71%		161	62%	
IV	6	33%		69	9%		69	7%	
Lymph node ratio:									
<0.12	54	83%	<0.001	54	89%	<0.001	54	80%	<0.001
0.12 to 0.30	49	75%		55	77%		55	69%	
>0.30	43	43%		52	45%		52	37%	
Perineural invasion:									
No	390	83%	<0.001	460	78%	<0.001	460	64%	<0.001
Yes	27	44%		50	39%		50	36%	
Lymphovascular invasion:									
No	374	84%	<0.001	432	81%	<0.001	432	68%	<0.001
Yes	43	52%		78	32%		78	24%	
Immunoscore:									
0	54	58%	<0.001	81	47%	<0.001	81	40%	<0.001
1	45	68%		61	55%		61	44%	
2	86	78%		102	75%		102	66%	
3	92	82%		106	80%		106	61%	
4	140	94%		160	89%		160	76%	
IS within adjuvant chemotherapy group:									
0 to 1	45	51%	0.003	47	55%	<0.001	47	55%	0.009
2 to 3	72	72%		73	82%		73	77%	
4	40	84%		42	85%		42	76%	
MMR status:									
MSS	337	78%	0.003	419	71%	0.003	419	60%	0.558
MSI	80	93%		91	86%		91	66%	
BRAF status:									
BRAF wild-type	339	78%	0.029	410	74%	0.941	410	62%	0.037
BRAF mutation	61	91%		79	72%		79	54%	
MMR/BRAF mutation status:									
MSS/BRAFwt	315	77%	0.043	382	73%	<0.001	382	62%	<0.001
MSS/BRAFmut	11	82%		24	42%		24	29%	
MSI/BRAFwt	24	90%		28	88%		28	68%	
MSI/BRAFmut	50	93%		55	85%		55	66%	

Immunoscore (IS) includes all the patients from the study.

The IS within the adjuvant chemotherapy group includes patients who received adjuvant chemotherapy (medically fit patients having stage III tumours or high-risk stage II disease). Stage IV patients are excluded because they had chemotherapy that differs from standard adjuvant treatment.

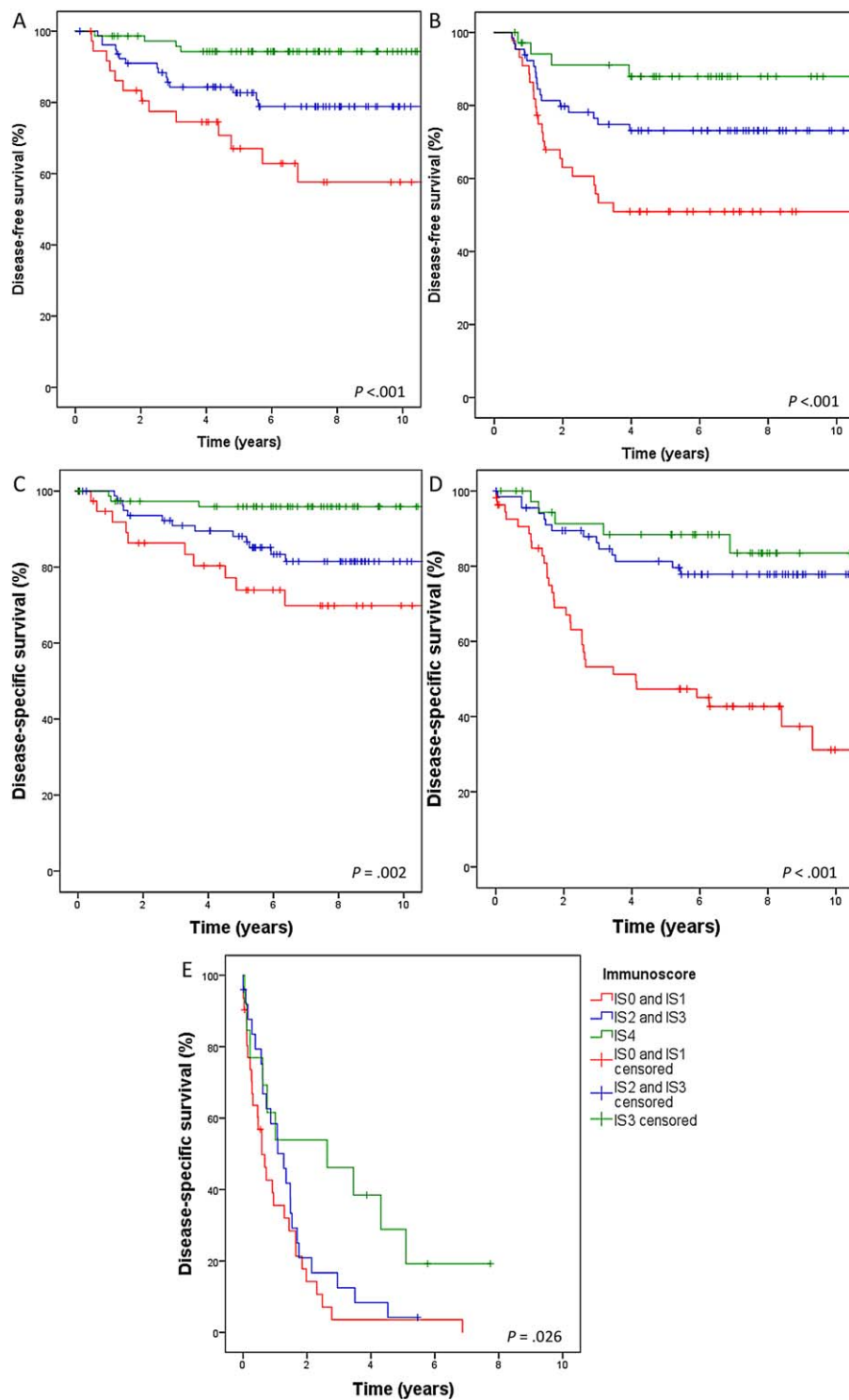
The Kaplan-Meier method was used to calculate disease-free survival (DFS), disease-specific survival (DSS), and overall survival (OS), and the log-rank test was used to compare differences.

CI, confidence interval; HR, hazard ratio; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; AJCC, The American Joint Committee on Cancer; UICC, Union for International Cancer Control; BRAFwt, BRAF wild-type; BRAFmut, BRAF mutation.

### Survival

Higher IS was related to improved 5-year DFS, DSS, and OS (Table 3, Figure 2C,D) within the entire study population and in both the MSS and MSI subgroups. Among MSS cases (n = 419), 5-year DFS was 61%

for IS0 and IS1, 79% for IS2, and IS3 and 92% for IS4 (p < 0.001). For these respective IS groups, 5-year DSS was 49, 76, and 89% (p < 0.001), and 5-year OS was 42, 64, and 77% (p = 0.001). Among MSI cases (n = 91), 5-year DFS was 75% for IS0 and IS1, 90%



**Figure 3.** Disease-free survival according to Immunoscore (IS) in AJCC/UICC stage II (A) and stage III (B) disease. Disease-specific survival according to IS in AJCC/UICC stage II (C) and stage III (D) disease. (E) Disease-specific survival according to IS in AJCC/UICC stage IV disease.

for IS2 and IS3, and 98% for IS4 ( $p = 0.047$ ). For these respective IS groups, 5-year DSS was 71, 88, and 89% ( $p = 0.029$ ) and 5-year OS was 39, 63, and

75% ( $p = 0.008$ ). MMR status was a prognostic factor according to Kaplan-Meier survival analyses, with MSI patients showing better DFS and DSS (Table 3).



Table 4. Multivariable analysis with Cox proportional hazard model

	Univariable analysis	Disease-free survival (DFS) (n = 396)		Disease-specific survival (DSS) (n = 432)		Overall survival (OS) (n = 489)	
		HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
Age:							
<65 years	DFS: 0.109*	1	0.195*	1	0.023*	1	<0.001*
65 to 75 years	DSS: 0.105*	0.73 (0.39–1.39)		1.14 (0.70–1.87)		1.41 (0.96–2.06)	
>75 years	OS: <0.001*	1.27 (0.76–2.12)		1.72 (1.14–2.59)		3.14 (2.26–4.35)	
Gender:	DFS: 0.925						
Male	DSS: 0.416	0.93 (0.59–1.49)	0.769	0.98 (0.69–1.39)	0.891	1.29 (1.00–1.65)	0.049
Female	OS: 0.048	1		1		1	
AJCC/UICC stage:							
I	DFS: <0.001*	1	0.004*	Excluded	<0.001*	1	<0.001*
II	DSS: <0.001*	11.72 (1.60–86.08)		1		0.86 (0.58–1.29)	
III	OS: <0.001*	18.27 (2.48–134.37)		1.99 (1.22–3.25)		0.99 (0.65–1.53)	
IV		39.18 (4.18–367.28)		14.80 (8.88–24.67)		5.51 (3.45–8.80)	
Perineural invasion:							
No	DFS: <0.001	1	0.092	1	0.588	1	0.737
Yes	DSS: <0.001 OS: <0.001	1.74 (0.91–3.30)		1.12 (0.74–1.72)		1.07 (0.73–1.56)	
Lymphovascular invasion:							
No	DFS: <0.001	1	0.023	1	<0.001	1	<0.001
Yes	DSS: <0.001 OS: <0.001	1.90 (1.09–3.29)		2.72 (1.86–3.97)		2.30 (1.68–3.14)	
Immunoscore:							
0	DFS: <0.001*	5.68 (2.43–13.31)	0.001*	4.48 (2.49–8.05)	<0.001*	2.47 (1.66–3.67)	<0.001*
1	DSS: <0.001*	4.05 (1.64–9.97)		3.00 (1.58–5.70)		2.00 (1.29–3.08)	
2	OS: <0.001*	3.28 (1.41–7.64)		2.18 (1.19–4.02)		1.44 (0.98–2.12)	
3		2.51 (1.05–6.00)		1.44 (0.75–2.75)		1.41 (0.95–2.07)	
4		1		1		1	
MMR-status:	DFS: 0.005					Excluded	
MSS	DSS: 0.003	1	0.126	1	0.271		
MSI	OS: 0.558	0.42 (0.14–1.27)		0.70 (0.37–1.33)			
BRAF-status:	DFS: 0.036			Excluded			
BRAF wild-type	DSS: 0.941	1	0.760			1	0.031
BRAF mutation	OS: 0.038	0.84 (0.28–2.53)				1.46 (1.04–2.07)	
Tumour location:	DFS: 0.733			Excluded from all analyses			
Right hemicolon	DSS: 0.332						
Left hemicolon	OS: 0.345						

Univariate analyses were performed with the following reference categories: 65 years, male gender, Stage 1, no perineural invasion, no lymphovascular invasion, Immunoscore 4, MSS-status, BRAF wild type and tumour location in the left hemicolon. Only variables with a P value of <0.20 in univariable analysis were included in the multivariable analysis with age and gender. There were 396 cases available for analysis of DFS, 432 for DSS, and 489 for OS. Patients with pTNM stage I disease (n = 78) were discarded from DSS analysis because there was no disease-specific mortality. Ninety-three cases were excluded from DFS analysis due to either existing metastasis or non-radical surgery, 4 patients died before earliest event (recurrence) in a stratum, and an additional 17 cases had undetermined BRAF status. Due to missing data (BRAF status) 21 cases were excluded from OS analysis.

\*p for linearity.

CI, confidence interval; HR, hazard ratio; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; AJCC, The American Joint Committee on Cancer; UICC, Union for International Cancer Control.

Table 3 shows DFS, DSS, and OS according to AJCC/UICC stage. In AJCC/UICC stages II and III, higher IS was associated with improved DFS (Figure 3A,B) and DSS (Figure 3C,D). IS was also prognostic within the patient group with AJCC/UICC stage IIa disease (T3, N0; n = 171), with 5-year DFS rates of 73% for IS0 and IS1, 88% for IS2 and IS3, and 95%

for IS4 (p = 0.002), and respective 5-year DSS rates of 82, 94, and 97% (p = 0.018). Moreover, IS was prognostic for DFS, DSS, and OS among those patients with stage II or III disease who received adjuvant chemotherapy after surgery (n = 162). In AJCC/UICC stage IV disease (n = 69), higher IS was related to improved DSS (Figure 3E). The effect of IS by

AJCC/UICC stage on DFS and DSS was not investigated in stage I disease due to the low number of events (one recurrence, no cancer deaths). Increasing lymph node ratio was associated with lower DFS, DSS, and OS rates, but was not significantly associated with IS.

#### Prognostic factors for survival

Multivariable regression analyses revealed that IS, AJCC/UICC stage, and lymphovascular invasion were independent prognostic factors for DFS, DSS, and OS (Table 4). Additionally, age was independent prognostic factor for DSS and OS. Gender and *BRAF* mutation were independent prognostic factors for OS (Table 4).

In stage III disease, and with adjustment for age, gender, perineural, and lymphovascular invasion, IS, MMR status (in DFS and DSS), *BRAF* mutation status (in DFS and OS), we found that increasing LNR was an independent prognostic factor for worse DFS, DSS, and OS. With LNR < 0.12 used as the reference, the hazard ratio (HR) for DFS was 6.05 for LNR > 0.30 (95% CI, 2.52 to 14.49;  $p < 0.001$ ), the HR for DSS was 4.06 for LNR > 0.30 (95% CI, 1.82 to 9.09;  $p = 0.001$ ), and the HR for OS was 3.33 for LNR > 0.30 was (95% CI, 1.77 to 6.27;  $p < 0.001$ ).

## Discussion

Our present results reveal that Immunoscore is an independent prognostic factor for DFS, DSS, and OS regardless of tumour MMR status. This finding is in accordance with previous data [9]. The prognostic significance of IS for both DFS and DSS was evident in stages II and III. Importantly, among stage IIa patients who do not usually receive adjuvant chemotherapy, IS could identify patients with higher risk of recurrence and cancer-related death. Even among stage IV patients, higher IS was associated with improved DSS. We also found that high IS was significantly associated with a less invasive tumour phenotype (including reduced perineural and lymphovascular invasion rates) and with decreased risk of lymph node and distant metastases. This suggests that an improved cytotoxic immune response against tumour cells may also constrain metastatic progression. Among patients who received adjuvant chemotherapy, low IS corresponded with poor prognosis and high IS with good prognosis despite additional treatments.

There are two different aetiopathological pathways in CRC development: the MSI pathway that induces hypermutated cancers, and the chromosome instable pathway that generates MSS non-hypermuted cancers. In our study, IS was a prognostic factor for MSS colon cancer lacking the highly immunogenic response caused by

MSI. IS was also prognostic for better survival in MSI disease. These findings are similar to previously reported data [9]. Although CRCs with microsatellite instability generate more DNA mutations than stable CRCs [11], they have a better prognosis [11–14,23,24]. Our current results indicate that MSI induces enhanced cytotoxic immunogenic reactions. This improved immune response is likely explained by the high abnormal protein burden caused by hundreds of unpredictable mutations, and this strengthened host immune response may explain the survival benefits seen with MSI CRCs.

When categorizing IS, the selection of repeatable cut-off values is an important practical issue. In their preliminary study, Galon *et al* selected cut-off values with the smallest *P* value method, and found that the proportion of IS 0 cases was only 4% [7]. In our study, we determined cut-off values with optimal sensitivity and specificity based on ROC curves related to disease-specific 3-year mortality. This resulted in a more balanced distribution of patients among the five IS groups, and still demonstrated substantial predictive significance of IS. Several other factors may impact the distribution of IS categories and, therefore, cut-off values are case dependent to some extent. For example, the section thickness, representativeness of punch areas and immunohistochemical staining platforms may vary between different studies, potentially hindering the creation of universal standardized cut-off values.

To date, AJCC/UICC staging is the best CRC prognostic indicator. However, while it reflects tumour burden at the time of diagnosis, it does not provide information about the biological features of the cancer. In our study, IS was not superior to AJCC/UICC staging as an independent prognostic factor, but the lowest and highest IS groups could clearly differentiate the patients with the worst and best prognosis in each AJCC/UICC-stage. Thus, a combination of AJCC/UICC staging and IS might provide more exact prognostic information, allowing clinicians to better tailor treatment [10]. It has also been proposed that lymph node ratio may be an additional prognostic tool for stage III CRC patients [21]. Our results support the earlier findings on this subject; lymph node ratio was not associated with IS and could offer additional prognostic information.

As suggested in earlier studies, *BRAF* mutation is not associated with worse prognosis in MSI tumours [12,25,26], but is an independent risk factor for decreased survival in MSS disease [12,16–18]. In our study, most *BRAF* mutations (70%) were found in MSI tumours. High IS was associated with *BRAF* mutations in MSI tumours, but it is possible that immunogenicity develops due to the hypermutability of MSI tumours. It remains unclear whether *BRAF*

itself is immunogenic, since IS distribution was not influenced by *BRAF* mutation in MSS tumours.

In conclusion, our data demonstrate the use of IS in the classification of colon cancer prognosis across AJCC/UICC stages. Particularly among stage IIa colon cancer patients, IS may prove to be an important prognostic marker for identifying patients who are likely to benefit from adjuvant treatments. A strength of our study is that patients with colon cancers were classified and treated using a contemporary multidisciplinary approach. Moreover, the follow-up time was long and complete for each patient. Future randomized studies are needed to examine the combined use of AJCC/UICC stage and IS to select colon cancer patients for individualized adjuvant therapy.

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### Author contributions statement

EVW, TS, TK, IK, JPM, JB: conception and design; EVW, TS, MF, MA, JB: acquisition of data; EVW, JV, HK, TK, IK, JPM, JB: analysis and interpretation of data. All authors were involved in writing the paper and had final approval of the submitted version.

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