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Review Article

Prognostic role of tumour-infiltrating T lymphocytes in stage IIA (T3N0) colon cancer: A broad methodological study in a fairly homogeneous population

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ABSTRACT

Aim: Tumour-infiltrating T lymphocytes (TIL) are considered to be a reliable prognostic marker in CC, but the use in daily practice is unclear. We investigated the survival effect of TIL methodologically in a highly homogeneous population.

Methods: Seventy-two stage IIA (T3N0) CC patients who underwent surgical resection from 2000 to 2014 were included. CD3 and CD8 were separately scored for different blocks, areas and foci. To the best of our knowledge, this study has the most comprehensive methodology in the literature.

Results: Foremost, we searched for the optimal evaluation method. We found better results with Model A (deepest invasive block&hot spot area&invasive margin focus), e.g. for CD3, the relationship with prognostic factors [Crohn's-like reaction ($p = 0.015$), positive surgical margin ($p = 0.019$), Mismatch repair proteins deficiency ($p = 0.003$), advanced grade ($p = 0.015$)], the correlation of distinct estimates ($r = 0.708$), the reproducibility of research (Kappa = 0.60–0.71), and the usefulness of cut-off value (area of under ROC = 0.800 [0.683–0.917]) were best. Then, survival analysis was performed with two better methods including Model A. In univariate analysis, low TIL with Model A was associated with worse OS (CD3, $p < 0.001$; CD8, $p = 0.023$) and RFS (CD3, $p < 0.001$; CD8, $p = 0.005$). Multivariate analyses confirmed low TIL with same method as an independent worse prognostic marker for OS (CD3, Hazard ratio [HR] = 1.42 [1.10–1.85], $p = 0.005$) and RFS (CD3, HR = 1.46 [1.17–1.83], $p = 0.001$; CD8, HR = 1.32 [1.05–1.64], $p = 0.032$).

Conclusions: Our results confirm that low TIL is an independent worse prognostic marker in stage IIA (T3N0) CC and that the use of CD3 with Model A can contribute to improving the prognostication of early CCs.

1. Introduction

Colon cancer (CC) is one of the most common cancers in the Western world. It is the third most frequent cancer entity in men and the second most common in woman [1]. Recognition of CCs in early stage increased with the development of imaging modalities and approximately one-third of the patients are diagnosed with stage II disease. This patient subgroup has a good prognosis with a 5-year overall survival of approximately 70–80% after surgery alone [1] and only a minority of cases can be attributed to adjuvant chemotherapy [2]. Previous studies have shown that chemotherapy could improve survival in these patients, but absolute improvement in survival was < 5% [3]. Furthermore, given that adjuvant chemotherapy adversely affects the quality of patients' life [4], and that risk factors are inadequate for ideal patient selection for adjuvant therapy [5]. Therefore, it is clear that additional new prognostic markers for these patients are needed for better clinical management.

Cancer formation is a complex process in which the immune response plays an important role [6]. Tumour-infiltrating inflammatory cells are increasingly recognized as responsible for the production of inflammatory mediators that induce angiogenesis, tumour growth, invasion and metastasis [6]. T lymphocytes isolated directly from the tumour environment are called tumour-infiltrating T lymphocyte (TIL) [12]. In the tumour microenvironment, pre-existing T lymphocyte cells can attack cancer cells by recognizing abnormally expressed neoantigens, and play an important role in tumour regression [7]. In many studies in the literature, the prognostic value of TIL has been confirmed for many tumours [8-12] and it has been documented that high TIL intensity is associated with a better prognosis in CC [13-19]. However, the standardization of published studies is quite low and the evaluation methods are highly variable.

The aim of this study was to investigate the survival effect of TIL in

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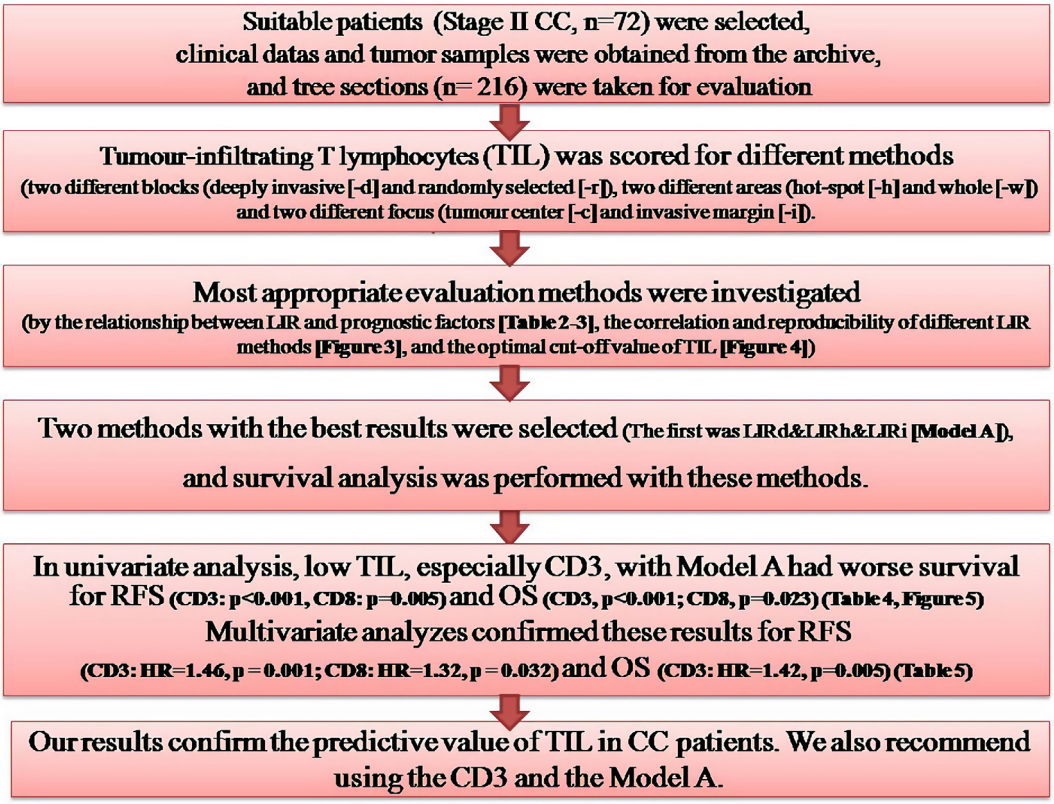

Fig. 1. Flowchart of the study.
Abbreviations: CC: colon cancer, H&E: Hematoxylin and eosin, CD3: Cluster of differentiation 3, CD8: Cluster of differentiation 8, IHC: Immunohistochemistry HR: Hazard ratio, OS: Overall survival, RFS: Relapse-free survival.

stage IIA (T3N0) CC by focusing on CD3 and CD8 T-cells. A distinctive feature of this study is that it represents a very homogeneous group of patients and tries to provide standardization to the methods of evaluation by investigating many different techniques.

2. Materials and methods

This research was designed according to REMARK [20] and was summarized in Fig. 1.

2.1. Patient population

This study retrospectively reviewed the records of four hundred twenty-five stage I-IV CC patients who underwent radical surgery from 2000 to 2014 at a single tertiary university-based hospital in Kırıkkale, Turkey. Retrospective clinical information of patients was obtained from the Kırıkkale university archive. This database contains the following parameters: Age, size, gender, perineural invasion, lymphatic invasion, pathologic tumour stage (PT), invasive pattern, surgical margin, microsatellite instability (MSI), and grade. Patients were excluded from the study with no tumour blocks in the archive (n = 24), rectal cancer (n = 125), another cancer diagnosed prior to primary CC (n = 16), insufficient tissue detected for examination (n = 12), stage II not recognized in cut of new sections (n = 136), died or recurred within 1 month (n = 15), adjuvant therapy treated (n = 13), and loco-advanced disease diagnosed (n = 12). Finally, the study population comprised of seventy-two patients.

2.2. Samples

Archives, formal-fixed and paraffin-embedded tumour blocks were collected from the Pathology department archives in Kırıkkale University. The number of blocks ranged from 3 to 14 per patient (mean = 5.2). Two tissue blocks were selected from each patient using archived sections, one of which showed the deepest invasive region and the other was randomly selected. Tree sections of 4 microns thick (n = 216) were cut from each block, one section was stained with hematoxylin and eosin (H&E) and two sections were stained with immunohistochemistry (IHC) as Cluster of differentiation 3 (CD3) and Cluster of differentiation 8 (CD8). Tumour blocks containing adjacent normal colonic tissue and adequate tumour tissue were selected for IHC. For MSI status, we detected Mismatch Repair Proteins (MMR) proteins by IHC and classified as MMR deficiency (MMR-D) or MMR proficiency (MMR-P). Pathological evaluation of the primary tumour was performed according to the American Joint Committee on Cancer Classification, 8th [21]. All slides were separately and semi-quantitatively evaluated by three experienced pathologists (M.Z, M.E. and M.A.) and the final score was noted according to the mean number of these observers.

2.3. Histopathologic assessment

TIL was identified according to recommendations of the International TIL Working Group, 2014 [12]. Many different methods have been used in the literature for TIL evaluation [13,14]. In this research, TIL was noted separately for two different blocks (deepest invasive block [-d] and randomly selected block [-r]), two different areas (hot-spot area [-h] and whole area [-w]) and two different focus (tumour center [-c] and invasive margin [-i]).

The number of TIL was visually predicted semi-quantitatively on IHC stained slides by conventional microscopy. Firstly, we scanned all sections to select the highest and lowest density areas of TIL using the ×10 lens. Then, an area containing predominantly T-lymphocytes within the field of view is selected using an ×20 objective. T-

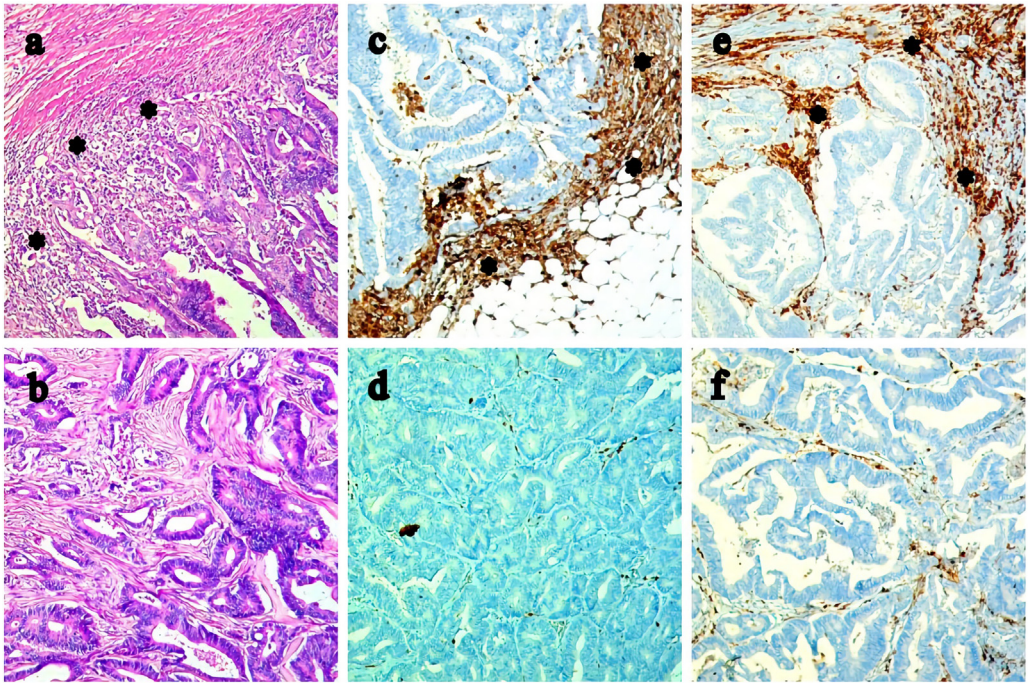

Fig. 2. Representative examples of TIL counting.
We scanned all the slides to determine the highest and the lowest areas of tumour-infiltrating T lymphocytes (TIL) using a ×10 objective. An area containing predominantly T lymphocytes within the field of view is selected using a ×20 lens. Then, TIL (asterisks) was scored separately in 10 high-power fields at ×20 magnification. Finally, cases were divided into two groups as low (< 50 lymphocytes) TIL (a–c–e) and high (≥50 lymphocytes) TIL (b–d–f).

Table 1
Descriptive statistics of immunohistochemical variables (n = 72).

		CD3		CD8	
		Mean ± SD	Median (range)	Mean ± SD	Median (range)
TIL	Deepest invasive block	61.3 ± 7.47	60.0 (35–75)	60.2 ± 7.18	60.0 (30–70)
	Randomly selected block	46.4 ± 7.43	45.0 (25–70)	49.8 ± 7.27	48.0 (25–65)
	Hot-spot area	60.0 ± 7.45	60.0 (30–75)	58.8 ± 7.05	58.0 (30–75)
	Whole area	45.7 ± 7.24	45.0 (25–65)	47.5 ± 7.12	47.0 (25–65)
	Tumour centre	54.5 ± 7.37	50.0 (30–70)	53.6 ± 6.94	53.0 (30–75)
	Invasive margin	49.8 ± 7.39	48.0 (25–75)	50.9 ± 7.13	50.0 (30–70)

Abbreviations: TIL: Tumour-infiltrating T lymphocytes, CD3: Cluster of differentiation 3, CD8: Cluster of differentiation 8, SD: Standard deviation.

lymphocytes should be present at most of the borders in the selected image area. Finally, TIL was scored in 10 high-power fields (HPF) at ×20 magnification (Nikon Eclipse E600, 0.785 mm^2, Nikon AG Instruments, Switzerland) (Fig. 2). The number of lymphocytes was noted per 10 enhancement per magnification, e.g. 10, 20, 30. In addition, all patients were divided into two groups as high-density and low-density according to the optimal survival-related cut-off value.

In this study, all CD3 and CD8 lymphocytes are evaluated, i.e. other mononuclear cells including plasma cells, granulocytes and polymorphonuclear leukocytes are excluded. To avoid the number of IHC stained brown artefacts and cytoplasmic fragments, TIL number were not be counted unless a clearly defined blue hematoxylin stained nucleus was present. In sections with < 10 HPFs available, TILs were scored in as many adjacent HPFs as possible and the mean number of lymphocytes was noted according to this number of areas.

2.4. Investigation of optimal assessment techniques

One of the difficulties of achieving successful results in diagnostic tests is the selection of the optimal evaluation method. In this study, this difficulty was exceeded with the following methods: the relationship with prognostic factors, the correlation between different TIL estimates, the reproducibility of the study, and the optimal cut-off value. Then, two techniques with the best results were chosen and survival analysis was performed with these methods. Receiver operating characteristic (ROC) analysis was used to determine the most appropriate cut-off value that affects the sensitivity and specificity of the test. The value with the lowest false positive rate together with the highest true positive rate is the best cut-off value. In addition, as the area under a ROC curve (AUC) is usually a measure of the usefulness of a test, a larger area (AUC → 1) means a more useful test [22].

2.5. Evaluation of reproducibility

The reproducibility of the study was assessed by the heterogeneity of tumours and the agreement of observers. To calculate these parameters, three experienced pathologists (MZ, ME, and MA) observed the score of the TIL, independently and without knowing the clinical and pathological information. The intra- and inter-tumoral heterogeneity were determined by Intra-Class Correlation (ICC) [23]. ICC was accepted as the ratio of total variance calculated by the distinction between examined tumours. If the majority of the variant of the estimator is attributable to inter-tumoral variation, e.g. biological variation, it will be high (ICC → 1) and the majority of the variation is caused by intra-tumoral variation, e.g. heterogeneity, the ICC will be low (ICC → 0). The inter-observer agreement was investigated by calculating weighted and simple Kappa test (K). K value is a ratio of variances and was described according to Landis et al. [24] as substantial, moderate, and perfect for K values of 0.41–0.60, 0.61–0.80, and 0.81–1, respectively.

Table 2
Association between CD3 T-lymphocytes and prognostic factors (n = 72).

	Randomly selected block						Deepest invasive block					
CD3	Whole area			Hot-spot area			Whole area			Hot-spot area		
	Tumour c centre	Invasive margin	P value	Tumour centre	Invasive a margin	P value	Tumour centre	Invasive margin	P value	Tumour centre	Invasive margin	P value
Age			0.413			0.190			0.299			0.136
< 75	20 (66)	10 (34)		21 (70)	9 (30)		12 (40)	18 (60)		16 (53)	14 (47)	
≥75	24 (57)	18 (43)		16 (38)	26 (62)		22 (52)	20 (48)		15 (35)	27 (65)	
Size			0.429			0.335			0.2590.1530.153			0.133
< 5 cm	9 (52)	8 (48)		7 (41)	10 (59)		6 (35)	11 (65)		10 (58)	7 (42)	
≥5 cm	35 (63)	20 (37)		30 (54)	25 (46)		28 (51)	27 (49)		21 (38)	34 (62)	
Localization			0.295			0.247			0.281			0.150
Right	29 (65)	15 (35)		25 (56)	19 (44)		23 (52)	21 (48)		16 (36)	28 (64)	
Left	15 (53)	13 (47)		12 (42)	16 (58)		11 (39)	17 (61)		15 (53)	13 (47)	
Lymphatic invasion			0.117			0.195			0.180			0.113
No	25 (54)	21 (46)		21 (45)	25 (55)		19 (41)	27 (59)		23 (50)	23 (50)	
Yes	19 (73)	7 (27)		16 (61)	10 (39)		15 (57)	11 (43)		8 (30)	18 (70)	
Perineural invasion			0.624			0.357			0.564			0.575
No	29 (59)	20 (40)		27 (55)	22 (45)		22 (44)	27 (56)		20 (40)	29 (60)	
Yes	15 (65)	8 (35)		10 (43)	13 (57)		12 (52)	11 (48)		11 (47)	12 (53)	
Crohn's-like reaction			0.342			0.191			***0.031****			***0.015****
No	37 (66)	21 (34)		32 (55)	26 (45)		31 (53)	27 (47)		29 (50)	29 (50)	
Yes	7 (50)	7 (50)		5 (35)	9 (65)		3 (21)	11 (79)		2 (14)	12 (86)	
Invasive pattern			0.282			0.242			0.331			0.208
No	21 (55)	17 (45)		22 (57)	16 (43)		20 (52)	18 (48)		19 (50)	19 (50)	
Yes	23 (67)	11 (33)		15 (44)	19 (56)		14 (41)	20 (59)		12 (35)	22 (65)	
Surgical margin			0.248			0.158			***0.009****			***0.019****
Negative	25 (67)	12 (33)		22 (59)	15 (41)		23 (62)	14 (38)		23 (62)	14 (38)	
Positive	19 (54)	16 (46)		15 (42)	20 (58)		11 (31)	24 (69)		8 (32)	17 (68)	
MSI status			0.767			0.348			0.232			***0.003****
MMR-P	22 (59)	15 (41)		21 (56)	16 (44)		20 (54)	17 (46)		22 (59)	15 (41)	
MMR-D	22 (62)	13 (38)		16 (45)	19 (55)		14 (40)	21 (60)		9 (25)	26 (75)	
Grade			0.295			0.501			0.118			***0.015****
Low grade	15 (53)	13 (47)		13 (46)	15 (54)		10 (35)	18 (65)		17 (60)	11 (30)	
Moderate/high grade	29 (65)	15 (35)		24 (54)	20 (46)		24 (54)	20 (46)		14 (31)	30 (69)	

* P value is significant at the 0,05 level. Significant results in italics. Abbreviations: CD3: Cluster of differentiation 3, CD8: Cluster of differentiation 8, PT: Pathologic tumour stage, MMR-D: Mismatch repair proteins deficiency, MMR-P: Mismatch repair proteins proficiency.

2.6. Follow-up

In this research, outcome measures were determined using relapse and survival rates. The follow-up period was chosen as a wide range of fourteen years in order to make a more reliable decision about the recurrence of the patients. The time between the primary surgery date and the death date from any cause or the last follow-up date was defined as overall survival (OS). The time from the primary surgery date until the death date of any cause or the first loco-regional or distant recurrence date was defined as relapse-free survival (RFS). All patients with the last date contact > 60 months after diagnosis and events after 60 months of follow-up were censored at 60 months. Patients diagnosed with other malignancies after diagnosis of primary disease were also identified and subsequently censored from the RFS analysis on the date of their new cancer diagnosis.

2.7. Immunohistochemical study

Two sections of 4 μm (n = 288) were cut and placed on a Dako's platinum coated slide (K8020, Denmark, Glostrup). We performed the pretreatment methods using Dako's PT link. We obtained the retrieval epitope which was induced by heat with Dako's targeting solution (EnVision Flex) at Ph 9, 97 °C for 20 min. We performed the staining using Dako's Autostainer link 48. We blocked the endogenous peroxidase activity by Dako's peroxidase blocking reagent (EnVision Flex). The primary antibodies were mouse monoclonal CD3 (1: 50, Dako, clone F7.2.38) and CD8 (1: 100, Dako, clone C8/144B) diluted with Dako's antibody diluent (EnVision Flex). IHC staining of mismatch repair proteins was performed using monoclonal mouse MLH1 (1: 100, Dako, clone ES05) and PMS2 (1: 500, Dako, clone EP51) antibodies. Since there were no cases with Lynch syndrome in our cases, MSH2 and MSH6 were not performed. We incubated these antibodies at room temperature for 30 min, and Dako's mouse linker (EnVision Flex) was used for amplification at 20 min. We detected the bound antibody by Dako's HRP reaction (EnVision Flex) and visualized by Dako's DAB reaction (EnVision Flex) with diluted chromogen in Dako's substrate buffer (EnVision Flex). We used the hematoxylin of Meyer's (Merck, Germany, Darmstadt) for counterstain and finally, we covered the slides with pertex.

2.8. Statistical evaluation

SPSS 21. 0 (IBM institute, North Castle, ABD) was used in the analysis. All tests were two-sided and P values < 0.05 were noted significantly. Descriptively variables were noted using ranges, average and standard deviation for continuous variables, and percentage and frequencies for the categorical variable. In categorical variables, the Chi-square test was used for the comparisons between TIL groups and clinicopathological features. In continuous variables, the Spearman correlation analysis was used for the correlation of TIL estimates and the Wilcoxon signed rank test was used for the differences. The optimal survival-related cut-off value was assessed by the ROC analyses.

Table 3
Association between CD8 T-lymphocytes and prognostic factors (n = 72).

	Randomly selected block						Deepest invasive block					
CD8	Whole area			Hot-spot area			Whole area			Hot-spot area		
	Tumour c centre	Invasive margin	P value	Tumour centre	Invasive a margin	P value	Tumour centre	Invasive margin	P value	Tumour centre	Invasiv margin	P value
Age			0.553			0.834			0.457			0.222
< 75	21 (44)	26 (56)		20 (42)	27 (58)		25 (53)	22 (47)		24 (51)	23 (49)	
≥75	13 (52)	12 (48)		10 (40)	15 (60)		11 (44)	14 (56)		9 (36)	16 (64)	
Size			0.194			0.155			0.471	0.1530.153		0.112
< 5 cm	23 (53)	20 (47)		15 (34)	28 (66)		20 (47)	23 (53)		23 (53)	20 (47)	
≥5 cm	11 (37)	18 (63)		15 (51)	14 (49)		16 (51)	13 (49)		10 (36)	19 (64)	
Localization			0.101			0.102			0.098			0.061
Right	20 (57)	15 (43)		18 (51)	17 (49)		21 (60)	14 (40)		20 (57)	15 (43)	
Left	14 (37)	23 (63)		12 (32)	25 (68)		15 (40)	22 (60)		13 (35)	24 (65)	
Lymphatic invasion			0.380			0.467			0.151			0.118
No	16 (53)	14 (47)		14 (46)	16 (54)		18 (60)	12 (40)		17 (56)	13 (44)	
Yes	18 (42)	24 (58)		16 (38)	26 (62)		18 (42)	24 (58)		16 (38)	26 (62)	
Perineural invasion			0.782			0.072			0.478			0.138
No	19 (48)	20 (52)		20 (51)	19 (49)		18 (46)	21 (54)		21 (53)	18 (47)	
Yes	15 (45)	18 (65)		10 (30)	23 (70)		18 (54)	15 (46)		12 (36)	21 (64)	
Crohn's-like reaction			0.178			0.102			0.413			0.124
No	16 (57)	12 (43)		15 (53)	13 (47)		10 (35)	18 (65)		16 (57)	12 (43)	
Yes	18 (40)	26 (60)		15 (35)	29 (65)		20 (45)	24 (55)		17 (38)	27 (62)	
Invasive pattern			0.863			0.658			0.079			0.070
No	15 (48)	16 (52)		12 (38)	19 (62)		11 (35)	20 (65)		18 (58)	13 (42)	
Yes	19 (46)	22 (54)		18 (43)	23 (57)		19 (46)	22 (54)		15 (36)	26 (64)	
Surgical margin			0.232			0.216			***0.007****			***0.004****
Negative	20 (54)	17 (47)		18 (48)	19 (52)		21 (56)	16 (44)		23 (62)	14 (38)	
Positive	14 (40)	21 (60)		12 (34)	23 (66)		9 (25)	26 (75)		10 (28)	25 (72)	
MSI status			0.299			0.467			0.089			***0.041****
MMR-P	12 (40)	18 (60)		14 (46)	16 (54)		16 (53)	14 (47)		18 (60)	12 (40)	
MMR-D	22 (52)	20 (48)		16 (38)	26 (62)		14 (33)	28 (67)		15 (35)	27 (65)	
Grade			0.979			0.174			0.345			0.105
Low grade	16 (47)	18 (53)		17 (50)	17 (50)		15 (44)	19 (56)		19 (55)	15 (45)	
Moderate/high grade	18 (47)	20 (53)		13 (34)	25 (66)		21 (55)	17 (45)		14 (36)	24 (64)	

* P value is significant at the 0,05 level. Significant results in italics. Abbreviations: CD3: Cluster of differentiation 3, CD8: Cluster of differentiation 8, PT: Pathologic tumour stage, MMR-D: Mismatch repair proteins deficiency, MMR-P: Mismatch repair proteins proficiency.

Univariable survival groups were evaluated with the Log-rank test and survival curves were presented with the Kaplan-Meier method. Multivariable survival groups were evaluated with the Cox-regression model with a 95% confidence interval and a hazard ratio (HR) of 1.0 as a reference. The heterogeneity of tumours was examined by the ICC using the mixed-effects model and the observer agreement was evaluated the by K test.

3. Results

3.1. General features

The mean age and size were 75.37 ± 10.48 (SD) (range: 39–92) and 5.44 ± 1.92 (range: 2–10), respectively. 30 (41.7%) of the cases were female and 42 (58.3%) were male. 43 (59.7%) of the cases had a Crohn's-like reaction, 29 (40.3%) had not; 31 (43.0%) of the patients were low/moderately differentiated and 41 (57.0%) were poorly differentiated.

3.2. Estimates of TIL

CD3 and CD8 were separately scored within different slides, areas and foci on IHC stained sections, as described above. At low-power magnification, the distribution of lymphocytic cell infiltration was not relatively homogeneous within slides. Lymphocytes infiltrated CC tissue in a diffuse manner or in lymphoid aggregates, and heavy lymphocytic infiltrates were detected in stromal areas. Two independent blocks that were selected from each tumour had a good level of homogeneity for the density of the lymphocytic cell. Representative statistics and images on TIL variables are shown in Table 1 and Fig. 2.

3.3. Optimal assessment method

In this research, two models with the best results (The first was to use the 'deeply invasive blocks&hot-spot area&invasive margin [Model A]' as a method, and the second was to use the 'deeply invasive blocks& whole area&tumour centre [Model B]' as a method) were selected, and survival analysis was performed with these methods. In model A, there was a better relationship with poor prognostic parameters (Crohn's-like reaction [CD3: p = 0.015], positive surgical margin [CD3: p = 0.019; CD8: p = 0.004], MMR-D [CD3: p = 0.003; CD8: p = 0.041], advanced grade [CD3: p = 0.015]) (Tables 2–3). Also, with the same method, the correlation between different TIL estimates was the best (CD3: r = 0.708; CD8: r = 0.512) and no significant differences were found (CD3: p = 0.678; CD8: p = 0.433) (Fig. 3a–b). In addition, the most usefulness cut-off value was obtained with CD3 (ROC: 50.32; AUC = 0.800 [0.683–0.917]) (Fig. 4). This ROC value was considered as 50 to be easy to implement, and all specimens were classified into low-density and high-density groups by using this value.

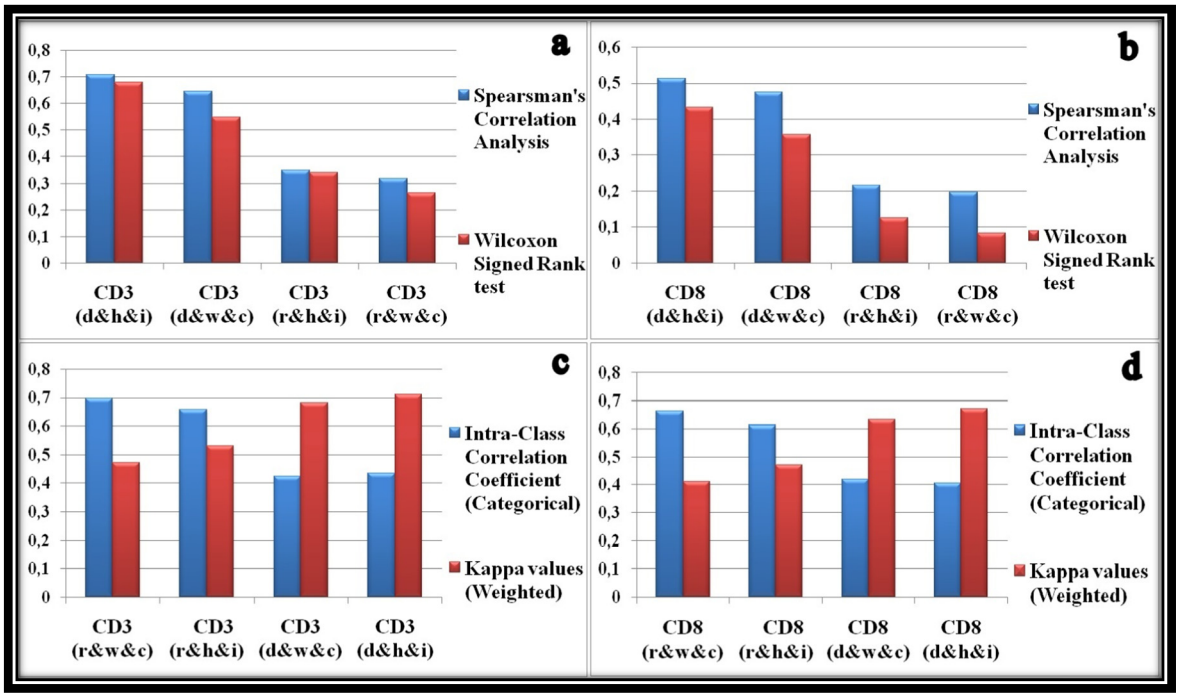

Fig. 3. Correlation and reproducibility of different TIL evaluation techniques.
Correlation (a–b) and reproducibility (c–d) for different TIL assessment methods are shown here. Since similar results are observed with categorical and continuous variables, only the best results are shown here. Abbreviations: CD3: Cluster of differentiation 3, CD8: Cluster of differentiation 8, -d: Deepest invasive block, -r: Randomly selected block, -h: Hot-spot area, -w: Whole area, -c: Tumour centre, -i: Invasive margin.

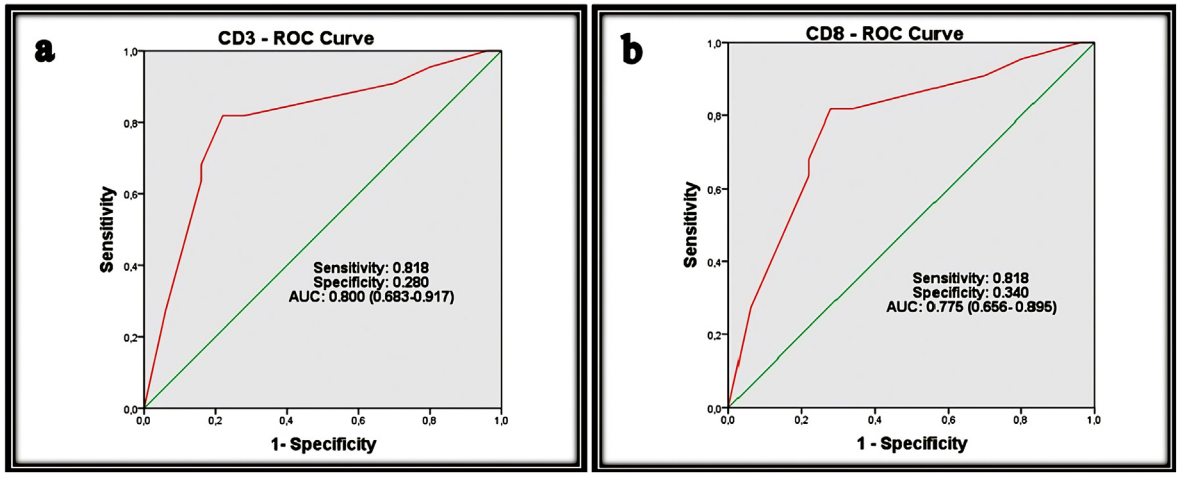

Fig. 4. Optimal cut-off value of TIL.
ROC curves were used to determine the most appropriate cut-off value for CD3 (a) and CD8 (b). AUC analyzed by manual methods. Abbreviations: TIL: Tumour-infiltrating T lymphocytes, ROC: Receiver Operating Characteristic, AUC: Areas under the ROC curves.

3.4. Reproducibility of TIL

TIL estimates were analyzed separately for different blocks, areas and focus described above. Both categorical and continuous variables were analyzed and similar results were found. Therefore, only the best results were given here as a sample The reproducibility of the study was evaluated as follows:

3.5. Agreement of observers

The inter-observer agreement was generally in a clinically useful range varying from moderate to substantial (CD3: K = 0.47–0.71; CD8: K = 0.41–0.67). Also, we found the inter-observer agreement for TILr was significantly lower than TILd. In addition, the K values increased when considering TILh and improved to almost perfect when choosing TILi (Fig. 3c–d).

3.6. Heterogeneity of tumours

ICC values of TILr were significantly higher than TILd for tumoral heterogeneity. So, the majority of the variation can be attributed to biological differences between tumours. For instance, an ICC count of 0.698 in Fig. 3c means that 30.2% of the total variance is due to the variation in a single tumour (measurement noise and variability). Therefore, intra-tumoral variation is considerably lower than inter-tumoral variation (Fig. 3c–d).

Table 4
Univariate survival analysis of TIL.

	OS		RFS	
	5-year survival (%)	P value	5-year survival (%)	*P* value
Age		0.359		0.153
< 75	84		84	
≥75	86		80	
Size		0.468		0.844
< 5 cm	83		76	
≥5 cm	87		88	
Localization		0.735		0.653
Right	80		78	
Left	90		86	
Lymphatic invasion		0.562		0.257
No	82		82	
Yes	88		82	
Perineural invasion		0.238		0.078
No	85		85	
Yes	85		79	
Crohn's-like reaction		0.645		0.854
No	81		76	
Yes	89		88	
Invasive pattern		0.836		0.574
No	79		79	
Yes	91		85	
Surgical margin		***0.023****		***0.001****
No	88		87	
Yes	82		77	
MSI status		0.174		***0.005****
MMR-P	86		86	
MMR-D	84		78	
Grade		0.238		0.475
Low grade	85		80	
Moderate/high grade	85		84	
CD3 (Model A)		***< 0.001****		***< 0.001****
Low	90		88	
High	80		76	
CD3 (Model B)		0.097		0.318
Low	87		81	
High	83		83	
CD8 (Model A)		**0.023***		***0.005****
Low	88		86	
High	82		78	
CD8 (Model B)		0.174		0.775
Low	86		77	
High	84		87	

* P value is significant at the 0,05 level. Significant results in italics. Abbreviations: CD3: Cluster of differentiation 3, CD8: Cluster of differentiation 8, PT: Pathologic tumour stage, MMR-D: Mismatch repair proteins deficiency, MMR-P: Mismatch repair proteins proficiency, OS: Overall survival, RFS: Relapse-free survival, Model A: Using the 'deeply invasive blocks&hot-spot area &invasive margin' as a method, Model B: Using the 'deeply invasive blocks&hot-spot area&tumour centre' as a method.

3.7. Follow-up

The results were better with Model A similar to other analyzes, so only the results of this method are given in survival analyses. Both CD3 and CD8 groups were examined, and similar results were found. Twenty-two patients (30.5%; n = 8 in high TIL, and n = 14 in low TIL) died in the follow-up period of fourteen years (range 2.7–96.7 months), four of which were in the first year (5.5%; n = 3 in low TIL, and n = 1 in high TIL), and twenty-six patients had relapsed (38.8%; n = 17 in low TIL, and n = 9 in high TIL). For CD3, the 5-year RFS rates were 88% in the high TIL population versus 76% in the low TIL and OS ratios were 90% in high TIL versus 80% in low TIL. For CD8, RFS and OS ratios were 86% and 87% in high groups, versus 78% and 83% in low groups, respectively (Table 4).

3.8. Univariable survival analyses

In univariate analysis, low TIL was significantly related to an adverse outcome for both RFS (CD3, $p < 0.001$; CD8, $p = 0.005$) and OS (CD3, $p < 0.001$; CD8, $p = 0.023$). In addition, other parameters that significantly related to worse survival were MSS and surgical margin (Table 4, Fig. 5).

3.9. Multivariable survival analyses

In multivariate analysis, low CD3 was significantly associated with both worse RFS (CD3, HR = 1.46 [1.17–1.83], p = 0.001; CD8, HR = 1.32 [1.05–1.64], p = 0.032) and OS (CD3, HR = 1.42 [1.10–1.85], p = 0.005), independent of other parameters. Surgical margin and MSS were the other parameters that independent significantly associated with worse survival (Table 5).

4. Discussion

In this study, we investigated the prognostic value of TIL methodologically in stage IIA (T3N0) CC patients treated with surgery only. We demonstrate that this parameter plays a significant role in the progression of CC and the evaluation of CD3 is more beneficial. We also evaluated the optimal assessment method for TIL and found that Model A was more successful.

Many large retrospective studies in the literature have shown that low T-cell infiltrates in CC are associated with shorter survival in accordance with our study [25-29], although few studies reported conflicting results as the densities of TILs were not found to be related to survival [30]. But, these studies are highly variable in terms of study populations, e.g. most of them include the different stage of diseases and rectal cancer patients. In addition, it is not clear in the literature whether the prognostic value of TIL in a different stage of diseases and rectal cancers is different from TIL in stage II and CCs. In this study, we investigated a patient population resected for only stage IIA CC, and to avoid possible confusion, we excluded patients treated with adjuvant chemotherapy and patients with other known malignancies. As a result, our patient group is highly homogeneous in contrast to other studies.

In the literature, there are several reported data on the clinical relevance of TIL subpopulations [13,25,30]. Recently, Turksma et al. [31] investigated TILs in stage II and III CC and demonstrated a survival value of both CD3 and CD8. In addition, Mei et al. [18], Galon et al. [32], and Deschoolmeester et al. [33] published data on multivariate analysis of a number of prognostic factors. And, these studies showed that CD3 and CD8 appeared to be the most informative markers. We showed that the intensity of these two markers, with CD3 being more prominent, is strongly associated with improved prognosis in accordance with these studies. This information significantly enlarges the spectrum of current prognostic indicators. In clinical practice, this may provide important prognostic information based on resection specimens in receiving chemoradiotherapy [27,28]. So, the CC patients with low immune infiltration may be considered as high-risk groups and may benefit from adjuvant therapy after surgical resection.

A disadvantage of TIL evaluation is the limited reproducibility and lack of standardization in different techniques. Sources of variability in the assessment of TIL include the optimal location for evaluation (invasive margin vs. tumour centre), staining (H&E staining vs. IHC), visualization (×20 objective vs. ×40 objective), and the method of scoring (quantitative vs. qualitative) [30-34]. In some studies, TILs were evaluated in the invasive front and the centre of the tumour [32,33]. Other studies were evaluated TILs in the neoplastic epithelial cells, not in the tumour-associated stroma [30]. Also, some of them evaluated TILs separately in the intraepithelial and stromal compartments [31,34]. In this research, we investigated TIL separately with many different methods to find the most appropriate evaluation technique. In Model A with the best results, low TIL was an independent

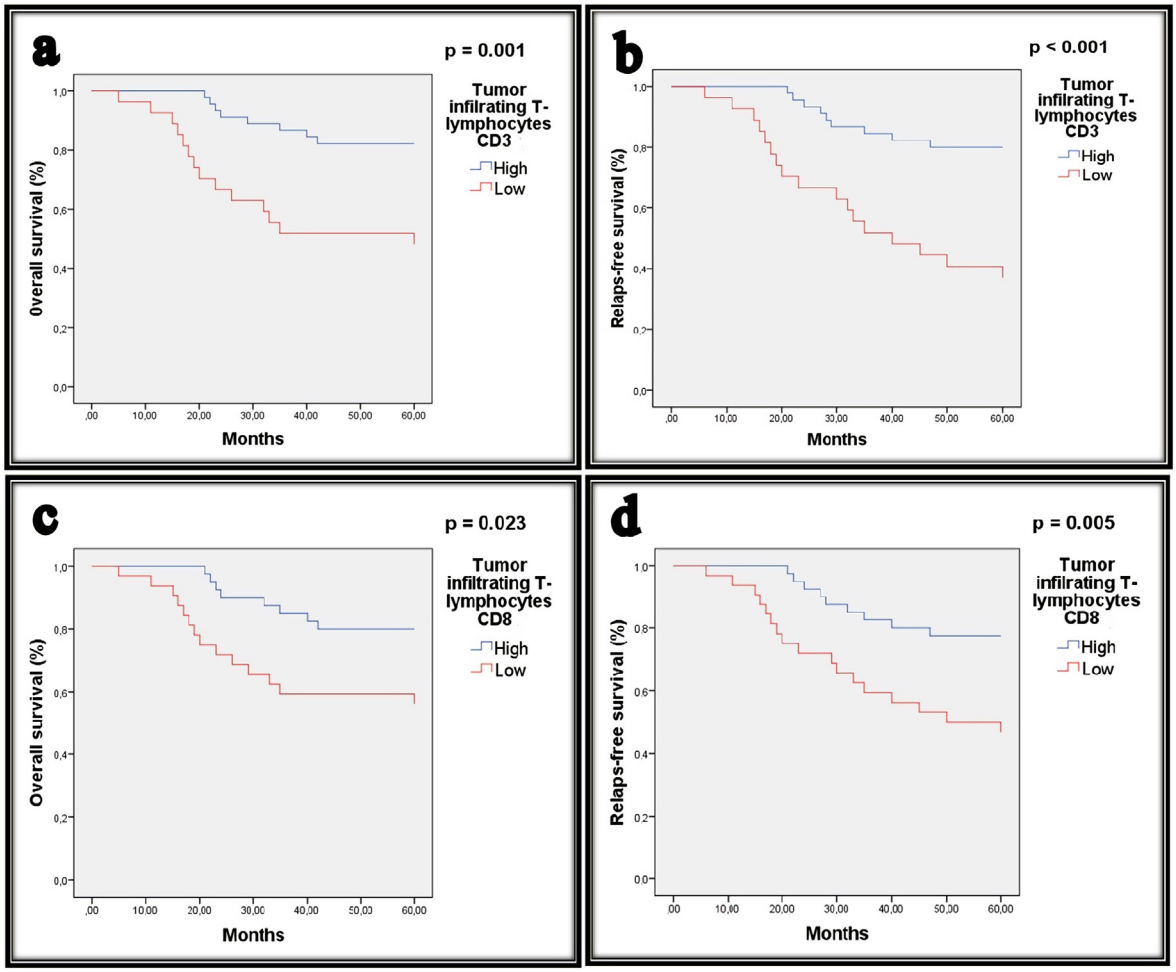

Fig. 5. Survival curves of TIL.
Kaplan-Meier survival curves were used for Overall survival (a) and Relapse-free survival (b). P value is significant at the 0,05 level.

Table 5
Multivariate survival analysis of five parameters.

	Overall survival (n = 72) (%)		Relaps-free survival (n = 72) (%)	
	HR (95% CI)	P value	HR (95% CI)	P value
Surgical margin	2.06 (0.84–5.07)	0.113	1.28 (1.03–1.60)	***0.013****
MMR-D	1.63 (0.80–3.83)	0.256	1.28 (1.03–1.59)	***0.035****
CD3 (Model A)	1.42 (1.10–1.85)	***0.005**** 1.46 (1.17–1.83)		***0.001****
CD3 (Model B)	2.13 (0.98–4.62)	0.254	2.54 (1.00–6.40)	0,686
CD8 (Model A)	1.45 (0.55–3.84)	0.150	1.32 (1.05–1.64)	***0.032****

* P value is significant at the 0,05 level. Significant results in italics. Abbreviations: CD3: Cluster of differentiation 3, CD8: Cluster of differentiation 8, MMR-D: Mismatch repair proteins deficiency, CI: Confidence interval, HR: Hazard ratio, OS: Overall survival, RFS: Relapse-free survival, Model A: Using the 'deeply invasive blocks&hot-spot area&invasive margin' as a method, Model B: Using the 'deeply invasive blocks&hot-spot area&tumour centre' as a method.

worse survival marker for both CD3 and CD8, and the reproducibility was well. Therefore, unlike these studies, many different methods were considered and the optimal one was chosen.

Although the assessment of TIL is based on routine H&E stained tissue sections in the majority of previous prognostic studies, the current consensus recommends the evaluation of TIL using IHC stained sections [30-34]. However, it is not clear whether TIL obtained by IHC stained sections has the same prognostic value as TIL obtained by H&E stained sections. In this study, we used mainly the IHC stained slides but also benefited from the H&E stained slides. We experienced a disadvantage of using IHC stained sections with staining in cell types other than lymphocytes, e.g. histiocytes and endothelial cells of vascular neoangiogenesis. We also experienced a drawback of the use of H&E stained slides with many other inflammatory cells can have a lymphocytes-like appearance, e.g. granulocytes and polymorphonuclear leukocytes. For the standardization of the techniques, we propose to investigate this subject with more comprehensive studies.

In this research, an increase in TIL was observed with the involvement of perineural and lymphatic invasion. This finding is consistent with Perez et al. [35], who showed that the number of TIL correlated statistically with the recognition of tumour-specific antigens and lymphocytic infiltration. However, most of the studies in the literature have shown that the presence of high TIL density in CC was associated with a lack of the characteristics of early metastatic processes, including lymphatic and perineural invasion [36,37]. These distinctions can be explained by differences in the evaluation methods as well as the

heterogeneity of tumours. We found the tumour heterogeneity in CC, and the use of different tumour sites may partly explain the disagreement in the obtained results. Also, we counted TILs in 10 HPFs, and this counting method may change the final average number. In addition, we counted TILs only when there is a clearly identifiable nucleus to avoid false staining, and our TIL score might be different due to this counting rule. So, future studies should standardize the counting method.

There are many important aspects of our research. We present a good parameter for the prognostication of CC patients, which has recently been discussed in a large number of large and statistically strong studies [13,25]. Our population is quite homogeneous because it is based on a well-characterized cohort of patients with stage IIA CC who are not receiving adjuvant treatment. In this study, many different methods have been tried and the most appropriate evaluation method has been found, i.e. this study has the most comprehensive methodology in the literature. And, this study was designed according to the REMARK guidelines for tumour marker prognosis studies, including the assessment of interobserver reproducibility [20].

Our study has some limitations. First of all, there are inherent restrictions in retrospective analysis. For example, it was impossible to overcome the sampling bias because the examined tissue was previously sampled for diagnosis. We have also evaluated many different areas of a tumour, but we are aware that this is only a small part of a whole tumour. In addition, since patients are treated according to guidelines before 2014, there may be differences in treatment compared to current stage IIA CC groups.

5. Conclusion

In this research, we demonstrate the prognostic effect of TIL in stage IIA (T3N0) CC patients. Our analyses show that the evaluation of TIL in resection specimens could provide additional good prognostic information to oncologists, gastroenterologists and surgeons. We also recommend the use of CD3 and Model A to achieve more successful results for future studies.

Abbreviations

CC	Colorectal cancer
AJCC	American Joint Cancer Committee
TIL	Tumour-infiltrating T lymphocytes
CD3	Cluster of differentiation 3
CD8	Cluster of differentiation 8
HPF	High power field
H&E	Hematoxylin and eosin
SD	Standard deviation
IHC	Immunohistochemistry
K	Kappa
ICC	Intra-Class Correlation Coefficient
PT	Pathologic tumour stage
MSI	Microsatellite instability
CI	Confidence interval
HR	Hazard ratio
OS	Overall survival
RFS	Relapse-free survival
Model A	Using ‘deeply invasive blocks&hot-spot area&invasive margin’ as a method

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Ethical standards

The study was carried out at Kırıkkale University and approved by Kırıkkale University Health Research Ethics Committee. All procedures performed in our study were consistent with the ethical standard of the national/institutional research committee and the 1964 Helsinki declaration and subsequent adjustment.

Declaration of Competing Interest

The author does not report a conflict of interest.

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