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ORIGINAL ARTICLE

Local Inflammatory Response Can Predict Clinical Outcome in Patients with Curatively Resected Stage-IIB Colon Cancer: An Advanced Methodological Study

Mehmet Zengin[1]

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Abstract

Purpose Although local inflammatory response (LIR) is a reliable survival marker in colon cancers (CCs), there is no consensus on its use in daily practice. We investigated the prognostic value of LIR in a highly homogeneous population with a well-designed methodology.

Methods Eighty stage-IIB CC patients operated between 2002 and 2012 were included in the study. Standardization was investigated for extra-biopsy evaluation methods (magnification, staining, and counting). Model A was used for intra-biopsy evaluation methods (block, section, and focus). So, this study makes important contributions to the standardization of pathological evaluations.

Results In method 1, the following analyzes showed more successful results for LIR: relationship with prognostic factors [tumour deposits (p=0.017), Crohn's-like reaction (p=0.019), advanced grade, (p=0.012), positive surgical margin (p=0.019), perineural invasion (p=0.025), mismatch repair proteins-proficiency (p=0.031)], reproducibility of the study (Kappa=0.49–0.73, Intra-class correlation=0.442–0.724), and correlation of estimates (r=0.704). The cut-off value was also quite useful (area of under ROC=0.820 [0.694-0.920]). In univariate analysis, low LIR was related to poor overall survival (OS; p<0.001) and poor relapse-free survival (RFS, p=0.001) . Multivariate analysis confirmed that low LIR is an independent poor survival marker for OS (Hazard Ratio [HR]=1.32 [1.08-1.61, p=0.005) and RFS (HR=1.50 [1.22-1.85], p<0.001).

Conclusions Our results showed that low LIR had an independent prognostic significance in stage -IIB CCs. We also recommend using model A and method 1 for successful results and standardization.

Keywords Local inflammatory response · tumour biomarkers · colon cancers · stage -IIB

Introduction

Colon cancers (CCs) are the second most common cancer in the Western world and accounts for 10% to 15% of all cancers [1]. The early-stage disease accounts for approximately 20-30% of all CC patients and overall survival is generally good in this patient population. Estimation of prognosis for CCs is currently performed using the TNM staging system, which combines histopathological and clinical findings [1, 2]. Even this system, it is difficult to predict the clinical course individually. This is especially true for patients with stage II CCs because this patient population is not homogeneous in prognosis, has a poor postoperative survival of approximately 20% to 30%, and adjuvant treatment is generally not recommended [3]. Although current National Comprehensive Cancer Network guidelines describe some risk factors such as lymphatic/vascular invasion, localized perforation, poorly differentiated histology, close or positive margins, and perineural invasion [4], these parameters are inadequate in selecting the ideal patients for adjuvant therapy and new prognostic parameters are needed for better clinical management of stage II CC patients.

Since the processes in cancer development are quite complex, it is not enough to explain the results observed in cancers only with the characteristics of cancer. In other words, the interaction between host and tumour plays an important role in the progression and metastasis of cancers [5]. Local inflammatory response (LIR), recently described as the seventh hallmark of cancer, plays a key role in host resistance [5]. Also, the presence of LIR around and inside the tumour has significant effects on the survival of primary and metastatic CC [6].

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✉ Mehmet Zengin
mz1379@hotmail.com

[1] Kırıkkale University, Department of Pathology, Kırıkkale, Turkey

Recently, the distribution and subtypes of inflammatory cells observed in CCs have received great attention in the literature and promising findings have been reported as a good clinical outcome in a dominant inflammatory response [6–11]. As a result, LIR can provide important prognostic information for CCs, but the standardization of pathological evaluation methods in published studies for LIR is quite low.

In this study, a reliable prognostic marker (LIR) was investigated methodologically in a very homogeneous patient population (stage-IIB CC). Also, the prognostic role of many inflammatory cells subtypes was investigated. So, this study is quite suitable for standardization and provides a broad perspective on the survival effect of the inflammatory response.

Materials and Methods

In the literature, publications on LIR show significant differences in the terms of population and methodology [5–11]. This study was based on model A [12], which is a successful method for extra-biopsy evaluation methods. Also, a reliable intra-biopsy evaluation method was investigated. Thus, an advanced level was reached for standardization of pathological evaluation methods.

This study was reported according to REMARK [13] and was summarized in Supplementary Fig. S1.

Patient Selection and Study Design

This study was approved by the Kırıkkale University Health Research Ethics Committee (2019/11) and was carried out in accordance with the 1964 Helsinki declaration and the ethical standards of the national/institutional research committee. All volunteer patients were informed about the content of the study and informed consent was obtained individually.

This research was performed in a single tertiary care university hospital in Kırıkkale, Turkey. Five hundred twenty-five patients who were operated for CRC between 2002 - 2012 were included in this study. Clinical and pathological information of patients was obtained from the archives of Kırıkkale University. In our population, there were no patients with known distant metastasis, double tumours, and death or recurrence within 1 month. A summary of the exclusion criteria is as follows: missing tumour block in archive (n=4), died and relapsed within 1 month (n=5), diagnosed with another cancer before the primary CC (n=2), rectal cancers (n=144), insufficient tissue in the blocks (n=2), diagnosed with different stages of disease (n=280), stage IIB disease not identified in the new sections (n=4), and treated with adjuvant therapy (n=4). Finally, we had a population of 80 patients.

Data Collection

Clinical, pathological and survival information was obtained from the archive records of Kırıkkale University. CCs were categorized according to the following criteria: age (<75 and ≥75), size (<5 cm and ≥5 cm), Crohn's-like reaction (yes and no), localization (right and left), perineural invasion (yes and no), lymphatic invasion (yes and no), surgical margin (yes and no), invasive pattern (yes and no), tumour deposits (yes and no), microsatellite instability (yes and no), and grade (low/moderate grade and high grade).

Specimens

Paraffin-embedded and formalin-fixed tumour specimens were collected from the archives of the Department of Pathology. The number of tumour blocks ranged from 2 to 15 per patients. Two tumour blocks were selected from each cases using an x10 lens (4.9 mm^2), one showing the deepest invasive area and one randomly selected. For immunohistochemistry (IHC), tumour blocks that could have enough tumour tissue and adjacent normal colonic tissue were included in the study. For each block, seven sections of 4-micron thick (n=560) were prepared by experienced technicians. Four of the slides were stained with IHC as leukocyte common antigen (LCA, for lymphocyte), cluster of differentiation 38 (CD38, for plasma cells) and CD15 (for granulocytes), and one was stained with hematoxylin and eosin (H&E). MSI status was determined by IHC as MLH1 and PMS2 and was classified into two groups as Mismatch Repair Proteins-proficiency (MMR-P) and MMR-deficiency (MMR-D). Scoring was blindly performed by three experienced and independent pathologists (M.Z, G.Ö, and S.A.) and the final score was given according to the average of the observers. The guidelines of the American Joint Committee on Cancer Classification (7th) were used for tumour evaluation [14].

Optimal Evaluation Method

Choosing the optimal assessment method is one of the most important challenges in diagnostic tests. In this study, extra-biopsy evaluation methods (magnification, staining, and counting) were evaluated according to the results of the following analyzes: relationship with prognostic parameters, usefulness of cut-off value, correlation of estimates, and reproducibility of the study. Then, two methods that gave the best results were selected and the study was continued with these methods. For intra-biopsy evaluation methods (block, section, and focus), model A was used as a standard method [12]. Model A means using the deepest invasive block, hotspot area, and invasive margin for pathological evaluation. In addition, optimal cut-off values of LIRs were evaluated by ROC analysis. The best cut-off value is the value with the lowest

false-negative ratio and the highest true-positive ratio. Since the usefulness of a test is usually measured by the area under a ROC curve (AUC), a larger area (AUC → 1) indicates that the benefit of the test is better [15].

Analysis of LIR

The evaluation was performed according to the recommendations of the International Tumour-Infiltrating Lymphocytes Working Group, 2014 [11]. LIR was visually predicted by conventional microscopy (Nikon Eclipse E600, Nikon AG Instruments, Switzerland) and was scored per 5 enhancement per magnification, e.g. 5, 10, 15. The inflammatory cells evaluated for LIR were lymphocytes, neutrophils, eosinophils, and plasma cells. For extra-biopsy evaluation methods, LIR was scored separately for different magnification (x20 and x40), different stainings (H&E and IHC) and different counting methods (qualitative and quantitative).

Firstly, all sections were scanned using an x10 objective to examine differences in the distribution of inflammatory cells within the tumour. An area containing predominantly inflammatory cells within the field of view was selected. Inflammatory cells should be present at all image borders in this selected area. Then, inflammatory cells were noted using an x 20 objective in 10 high-power fields (HPF) according to the methods described above. Cases with less than 10 HPFs, all available HPFs were counted and the mean value was given according to these areas. Finally, all patients were divided into two groups as high-density and low-density according to survival-related cut-off values.

To avoid counting of IHC stained brown cytoplasmic artefacts, LIR number was not counted unless a clearly defined blue hematoxylin stained nucleus was present. Other prognostic parameters associated with tumour morphology (invasive pattern and tumour deposits) were also evaluated according to the Model A.

Reproducibility of LIR

For reproducibility of study, agreement of observers and heterogeneity of tumours were considered. Three independent pathologists scored this parameter blindly from clinical and pathological information. Intra-Class Correlation (ICC) was used to investigate the inter- and intra-tumour heterogeneity [16]. ICC was considered as a ratio of variances indicating the distinction between examined tumour. If intra-tumoural heterogeneity causes the majority of the variation, e.g. heterogeneity, ICC will be low (ICC → 0). If inter-tumoural heterogeneity is responsible for the majority of the variation, e.g. biological variation, ICC will be high (ICC → 1). Kappa (κ) analysis was used to evaluate the inter-observer agreement. The κ value is a ratio of variances for the agreement of observers and was identified according to Landis et al. [17] as perfect, moderate and substantial for values of 0.81–1, 0.41–0.60 and 0.61–0.80, respectively.

Surveillance

In this study, survival and recurrence ratio were evaluated for outcome measures. Time-to-event endpoints were calculated from the day of primary surgery. The follow-up period was determined as a wide range (ten years, range: 12.5 to 128.5 months) to make a more reliable decision about the clinical outcome. All events after sixty months of follow-up and the last contact date more than sixty months after primary surgery were censored at sixty months. The time from the first surgery date to the date of death for any reason or the last follow-up date was determined as overall survival (OS). The time from the first surgery date to the date of death for any reason or to the distant/local-regional recurrence date was defined as relapse-free survival (RFS). Patients who were diagnosed with a secondary malignancy during follow-up were censored from the RFS at the time of diagnosis of this new cancer.

Immunohistochemistry

For immunohistochemistry, six sections of 4 μm (n=480) were cut from every two blocks and plated on to platinum-coated slides (Dako, K8020, Denmark). To obtain the target epitope, tissue slides were placed in Targeting solution (Dako), were incubated by a pressure cooker in a microwave at pH 9 and 97°C for 20 min, and were cooled for 40 min. To block the endogenous peroxidase activity, tissue slides were incubated in 3% Hydrogen peroxide for 20 min, Avidin Block (Dako) for 15 min and Biotin Block (Dako) for 15 min, respectively. Mouse monoclonal LCA (1: 100, Dako, clone 2B11 + PD7/26), mouse monoclonal CD38 (1: 100, Dako, clone AT13/5), mouse monoclonal CD15 (1: 100, Dako, clone Carb-3) were the primary antibody. Mouse monoclonal MLH1 (Dako, clone ES05, 1: 100) and PMS2 (Dako, clone EP51, 1: 500) antibodies were used for MMR (Since there was no family history of Lynch syndrome, MSH6 and MSH2 were not performed). These antibodies were diluted with antibody diluent (Dako) and were incubated overnight at 25°C. To detect bound antibody, secondary anti-mouse antibody (Dako) and Avidin-Biotin conjugate complex (Dako) was applied respectively. Then, sections were visualized with diaminobenzidine reaction (Dako) for 5 min and were stained with Mayer's hematoxylin for counterstain (Merck, Germany, Darmstadt). Finally, sections were coated with Pertex (Histolab, Sweden, Gothenburg). For each run test, tissues had positive and negative internal control.

Statistical Evaluation

For statistical evaluation, frequency and percentage were used for categorical variables, standard deviation (SD), ranges and averages were used for continuous variables. The relationships between LIR and prognostic parameters were analyzed

by Chi-Square test. Spearman correlation analysis was used for correlations and Wilcoxon Signed-Rank test was used for differences. As mentioned above, the optimal cut-off value was evaluated by the ROC analysis, tumour heterogeneity was evaluated by the ICC analysis, and inter-observer agreement was evaluated by the κ test. Log-rank test was used to compare univariable survival groups, and Kaplan-Meier method was used to present survival curves. Cox regression analysis with a 95% confidence interval (CI) and a hazard ratio (HR) of 1.0 as a reference was used to define independent prognostic factors. P values less than 0.05 were considered statistically significant. All analyses were two-tailed and were performed by SPSS 21.0 (IBM Institute, Armonk, NY, USA).

Results

Patient Characteristics

In total, eighty patients were included in the study. Thirty-two (40.0%) cases were females, and 48 (60.0%) cases were males. The median age and size were 75.28±9.48 (range, 35 to 88 years) and 5.27 ± 1.56 (range, 2 cm to 9 cm), respectively. 35 patients (43.7%) had a low/moderately differentiated tumour, and 45 patients (52.3%) had a poorly differentiated tumour; 57 (64.7%) of the tumours were in the left colon, 23 (35.3%) were in the right colon.

Evaluation of LIR

When slides scanned at low-power magnification (x10 objective), it was seen that LIR was heterogeneously distributed within tumours. Inflammatory cells were often detected in the stroma, either in a diffuse manner or in lymphoid aggregates, and were often reduced at invasive front and deeply invasive areas. Two independent blocks with the best inflammatory cell homogeneity were selected for each case and three gastrointestinal pathologists evaluated LIR for the extra-biopsy evaluation methods described [12]. Representative examples of statistics and images for LIR are shown in Supplementary Table S1 and Fig. 1.

Relationship Between LIR and Prognostic Factors

As mentioned above, different magnification, staining and counting methods for LIR were investigated. According to the results of the analysis below, the two most suitable methods were decided and survival analysis was performed with these methods. The first was "x20 objective & IHC & quantitative [method 1]" and the second was "x40 objective & IHC & quantitative [method 2]. Also, lymphocytes reached the best results among LIR subtypes. In method 1, the relationship with the prognostic parameters for lymphocytes was as follows: tumour deposits (p=0.017), positive surgical margin (p=0.019), MMR-P (p=0.031), Crohn's-like reaction (p=0.019), advanced grade (p=0.012), and perineural invasion

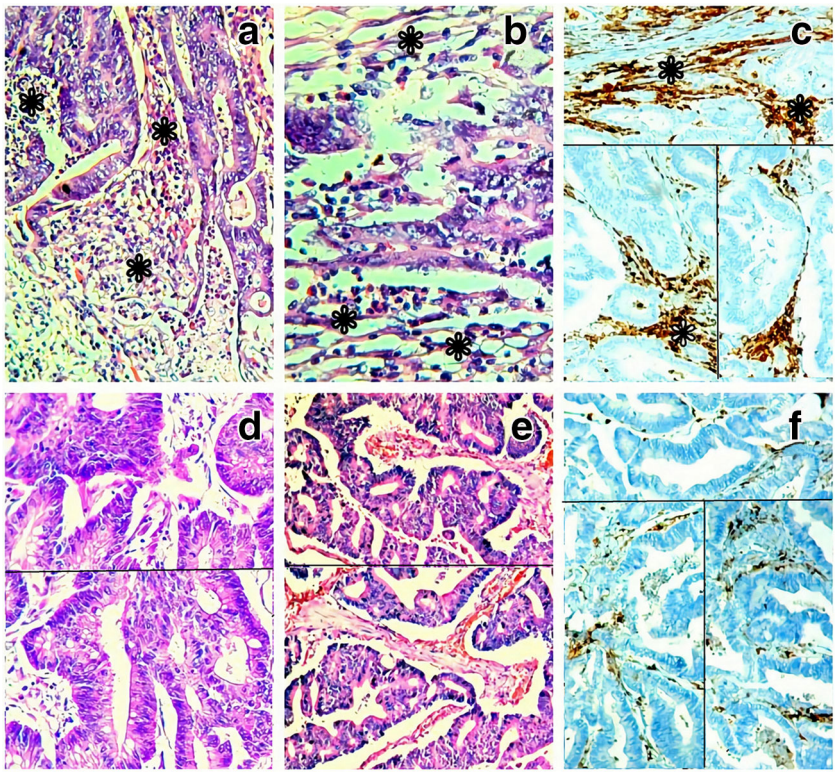

Fig. 1 We scanned all slides to determine the highest and lowest areas for Local inflammatory response (LIR). Then, we selected an area containing predominantly inflammatory cells within the field of view. LIR (asterisks) were scored using an x 20 objective in 10 HPFs according to the above-mentioned methods, and all cases were divided into two groups as high-density (a, b, c) and low-density (d, e, f) according to the survival-related cut-off values. Figures a (x10), b (x20), d (x20), and e (x10) are H&E stained sections, figures c (x10, LCA) and f (x10, CD38) are IHC stained sections. *H&E* Hematoxylin and eosin; *IHC* Immunohistochemistry; *LCA* leukocyte common antigen; *CD38* Cluster of differentiation 38; *HPF* High-power field

(p=0.025) (Table 1). Also, the results of correlation and difference were good (r=0.704, p=0.331) (Table 2). In addition, the cut-off value was more useful (ROC: 50.87; AUC: 0.820 [95% CI, 0.694 to 0.920]) (Fig. 2) (This value was considered to be 50 for ease to use). On the other hand, plasma cells had better results among inflammatory cells other than lymphocytes (Supplementary Table S2).

Reproducibility of Study

For reproducibility, LIR was analyzed separately for different extra-biopsy evaluation methods described above. Although both continuous and categorical variables were evaluated, the results were similar, so only better results are shown here. The reproducibility of the study was investigated as follows.

Heterogeneity of Tumours

Biological differences between tumours constituted most of the variation. For example, an ICC count of 0.724 in Table 2 means that 27.6% of the total heterogeneity was associated with variation in a single tumour. Therefore, inter-tumoural variation is considerably higher than intra-tumoural variation. When the analysis was carefully examined, it was found that the magnitude of ICCs at x40 magnification was significantly higher. This means that the detail increases at high magnification and gives more heterogeneity to the field of view (Table 2).

Agreement of Observers

The inter-observer agreement was in a clinically useful and generally ranged from moderate to substantial. When the analysis was examined in detail, it was seen that κ values increased in IHC stained sections, and reached a perfect level considering the quantitative method. In other words, since the presence of the inflammatory response was more pronounced on IHC stained sections, the interobserver agreement was at a higher level as expected (Table 2).

Surveillance

Survival analyses were more successful for lymphocytes in method 1, similar to other analyzes. In the follow-up period of ten years, thirty-one patients relapsed (38.7%; n=10 in high LIR, and n=21 in low LIR) and twenty-four patients died (30.0%; n=8 in high LIR, and n=16 in low LIR). The 5-year RFS and OS rates were 76% and 78% in low LIR population versus 88% and 90% in high LIR population, respectively (Table 3).

Univariate Survival Analyses

In univariate analysis, low lymphocytes in method 1 were significantly associated with poor prognosis for both OS (p<0.001) and RFS (p=0.001). Other prognostic parameters associated with poor prognosis were Crohn's like reaction, tumour perforation, surgical margin, and MMR-P. (Table 3, Figs. 3 and 4).

Multivariate Survival Analyses

In multivariate analysis, low lymphocytes in method 1 were significantly associated with poor RFS (HR: 1.50; 95% CI: 1.22 to 1.85; p<0.001) and OS (HR: 1.32; 95% CI: 1.08 to 1.61; p=0.005), independent of other parameters. Other parameters associated with independent poor survival were surgical margin and MMR-P (Table 3).

Discussion

In this study, the subtypes of inflammatory cells were investigated methodologically in a highly homogeneous CC population. It was concluded that lymphocytes are very important in independent survival for stage-IIB CC patients. Also, model A and method 1 had more successful results for pathological evaluation methods.

Although some studies have not reported prognostic significance [18], many large retrospective clinical studies in the literature have shown that low LIR in CC is an independent prognostic marker as consistent with our study [10, 19–25]. On the other hand, we should know that patient populations were highly variable in these studies. For example, most involved different stages of the disease and some had patients with rectum tumours. However, it is not clear in the literature whether the survival value of LIR is different between rectal cancers and CCs. This also applies to diseases of different stages. We investigated a quite uniform population of patients resected only for stage-IIB CC. Moreover, patients with other known malignancies and treated with adjuvant chemotherapy were excluded from the study to avoid possible confusion. Therefore, unlike other studies, our patient population is quite homogeneous.

Neutrophils, eosinophils, and plasma cells are natural immune system cells involved in the non-specific early immune response. There are very few reports on the clinical significance of LIR subpopulations in patients with stage-II CC, especially other than lymphocytes. Although most studies have reported a significant relationship between these cells and survival, some studies have not found any relationship [26–28]. In our study, no significant relationship was found between these inflammatory cells and survival. These differences in results may reflect variations in the evaluation

Table 1 Relationship between lymphocytes and prognostic factors (n=80)

Lymphocytes		H&E						IHC					
		×40 objective			×20 objective			×40 objective			×20 objective		
		QL	QN	*P*-value	QL	QN	*P*-value	QL	QN	*P*-value	QL	QN	*P*-value
Age				0.921			0.858			0.453			0.151
	<75	13	18		12	19		14	17		18	13	
		(41)	(59)		(38)	(62)		(45)	(55)		(58)	(42)	
	≥75	20	29		18	31		18	31		15	34	
		(40)	(60)		(36)	(64)		(36)	(64)		(30)	(70)	
Size				0.624			0.674			0.254			0.624
	≥5 cm	20	31		20	31		18	33		20	31	
		(39)	(67)		(39)	(67)		(35)	(65)		(39)	(67)	
	<5 cm	13	16		10	19		14	15		13	16	
		(44)	(56)		(34)	(66)		(48)	(52)		(44)	(56)	
Localization				0.806			0.225			0.686			0.080
	Right	24	33		19	38		22	35		27	30	
		(42)	(58)		(33)	(67)		(38)	(62)		(47)	(53)	
	Left	9	14		11	12		10	13		6	17	
		(39)	(61)		(47)	(53)		(43)	(57)		(26)	(74)	
Lymphatic invasion				0.813			0.182			0.146			0.077
	No	23	31		14	32		15	31		15	31	
		(42)	(58)		(30)	(70)		(32)	(68)		(32)	(68)	
	Yes	10	16		12	14		13	13		14	12	
		(38)	(62)		(46)	(54)		(50)	(50)		(53)	(47)	
Crohn's-like reaction				0.947			0.951			0.562			0.019*
	No	22	31		20	33		20	33		17	36	
		(41)	(59)		(37)	(63)		(37)	(63)		(32)	(68)	
	Yes	11	16		10	17		12	15		16	11	
		(40)	(60)		(37)	(63)		(44)	(66)		(59)	(41)	
Tumour deposits				0.151			0.588			0.036*			0.017*
	Negative	18	33		18	33		16	35		16	35	
		(35)	(65)		(35)	(65)		(31)	(69)		(31)	(69)	
	Positive	15	14		12	17		16	13		17	12	
		(51)	(49)		(41)	(59)		(55)	(45)		(58)	(42)	
Invasive pattern				0.769			0.720			0.637			0.445
	No	20	30		18	32		19	31		19	31	
		(40)	(60)		(36)	(64)		(38)	(62)		(38)	(62)	
	Yes	13	17		12	18		13	17		14	16	
		(43)	(57)		(40)	(60)		(43)	(57)		(46)	(54)	
Surgical margin				0.736			0.755			*0.013**			*0.019**
	Negative	22	33		20	35		27	28		28	27	
		(40)	(60)		(36)	(64)		(49)	(51)		(51)	(49)	
	Positive	11	14		10	15		5	20		5	20	
		(44)	(56)		(40)	(60)		(20)	(80)		(20)	(80)	
MSI Status				0.571			0.109			*0.049**			*0.031**
	MMR-P	19	30		15	34		16	33		15	34	
		(38)	(62)		(30)	(60)		(32)	(68)		(30)	(60)	
	MMR-D	14	17		15	16		17	14		17	14	

Table 1 (continued)

		H&E						IHC					
Lymphocytes		×40 objective			×20 objective			×40 objective			×20 objective		
		(45)	(65)	0.858	(48)	(52)	0.218	(54)	(46)	*0.026**	(54)	(46)	*0.012**
Grade	Low grade	19	28		15	32		14	33		14	33	
		(40)	(60)		(31)	(69)		(29)	(71)		(29)	(71)	
	Moderate/ High grade	14	19		15	18		18	15		19	14	
		(46)	(54)	0.646	(43)	(57)	0.109	(57)	(43)	0.112	(59)	(41)	*0.025**
Perineural invasion	No	22	32		17	37		19	35		17	37	
		(40)	(60)		(31)	(69)		(35)	(65)		(31)	(69)	
	Yes	12	14		13	13		14	12		15	11	
		(46)	(54)		(50)	(50)		(53)	(47)		(57)	(43)	

*P-value is significant at the 0.05 level. Significant results are in italics

H&E: Hematoxylin and eosin, *IHC*: Immunohistochemistry, *MMR-D*: Mismatch repair proteins deficiency, *MMR-P*: Mismatch repair proteins proficiency, *QN*: Quantitative, *QL*: Qualitative

methods as well as tumour heterogeneity. In this study, LIR was scored in 10 HPFs and this counting method may partially explain the different results. Also, to avoid false staining, only inflammatory cells with a clearly identifiable nucleus were counted and our LIR count may be different due to this counting rule. In addition, heterogeneity between different tumours was determined in this study and the use of different tumour sites may change the results obtained. As a result, differences may arise from the variability of methods and heterogeneity of tumours. Further comprehensive studies are needed to standardize these different methods.

MSI has received considerable attention in the literature due to its prognostic effects on CCs. Most studies have shown that CC patients with MMR-D have a better survival rate [19–29]. Also, MMR-D tumours exhibit specific features for host-related immune response, such as the Crohn's -like reaction. In addition, the presence of dense chronic inflammatory cells is another commonly reported feature [31]. In this study, a strong relationship was found between poor survival, low inflammatory cell density and MMR-R tumours, as reported in the literature. This information can considerably extend the spectrum of current prognostic parameters and can provide crucial survival information from resection specimens [24, 25]. For example, stage-IIB CC patients with low inflammatory cell infiltration may be considered a high-risk group and may benefit from adjuvant chemotherapy after curative surgery.

A disadvantage of LIR assessment is the lack of standardization and reproducibility. Sources of variability include the selection of the visualization (x20 magnification, x40 magnification), staining (IHC, H&E), and scoring (qualitative, quantitative) for extra-biopsy evaluation methods, and selection of the optimal block, section, and focus for intra-biopsy evaluation methods. There are over a hundred publications in the literature about LIR that differ in methodology. In some studies, inflammatory cells have been investigated in stromal and intraepithelial compartments [30]. Other studies have evaluated inflammatory cells in the centre and invasive front of tumours [31]. Also, some studies have evaluated inflammatory cells in the neoplastic epithelium, not in the tumour-associated stroma [18]. In this study, we used model A as a standard method for intra-biopsy evaluation methods [12]. Also, we found that method 1 gives reliable results for extra-biopsy evaluation methods. Therefore, unlike the aforementioned studies, significant improvements have been achieved in the standardization of LIR.

Although most of the previous studies routinely used H&E stained sections for the evaluation of inflammatory cells, the current consensus suggests the use of IHC stained tissue sections [18, 30, 31]. However, it is not clear whether the use of the IHC stained sections is advantageous over the H&E stained sections. In this research, both H&E and IHC stained sections were used to evaluate inflammatory cells. A disadvantage of H&E stained sections is that many other

Table 2 Correlation, difference and reproducibility of LIR (n=80)

	Spearman correlation analysis (n=80)	Wilcoxon signed-rank test (n=80)	ICC (95% CI) (n=80)	Kappa Values (n=80)
x20&QN(IHC)	0.704	0.331	0.698 (0.621-0.744)	0.73 (A&B), 0.70 (B&C), 0.71 (A&C)
x40&QN(IHC)	0.686	0.374	0.724 (0.653-0.845)	0.69 (A&B), 0.67 (B&C), 0.69 (A&C)
x20&QN(H&E)	0.652	0.425	0.637 (0.528-0.703)	0.67 (A&B), 0.66 (B&C), 0.65 (A&C)
x20&QL(IHC)	0.645	0.449	0.594 (0.488-0.683)	0.65 (A&B), 0.63 (B&C), 0.61 (A&C)

Only better results for LIR are given as examples.

H&E Hematoxylin and eosin; *IHC* Immunohistochemistry; *QL* Qualitative; *QN* Quantitative; *ICC* Intra-Class Correlation; *CI* Confidence interval; *A* First observer; *B* Second observer; *C* Third observer

inflammatory cells may have a lymphocyte-like appearance such as polymorphonuclear leukocytes. Also, IHC stained sections may show broad reactivity in cell types other than inflammatory cells such as histiocytes and endothelial cells of vascular neoangiogenesis. On the other hand, the use of IHC-stained sections was found to be more related to survival.

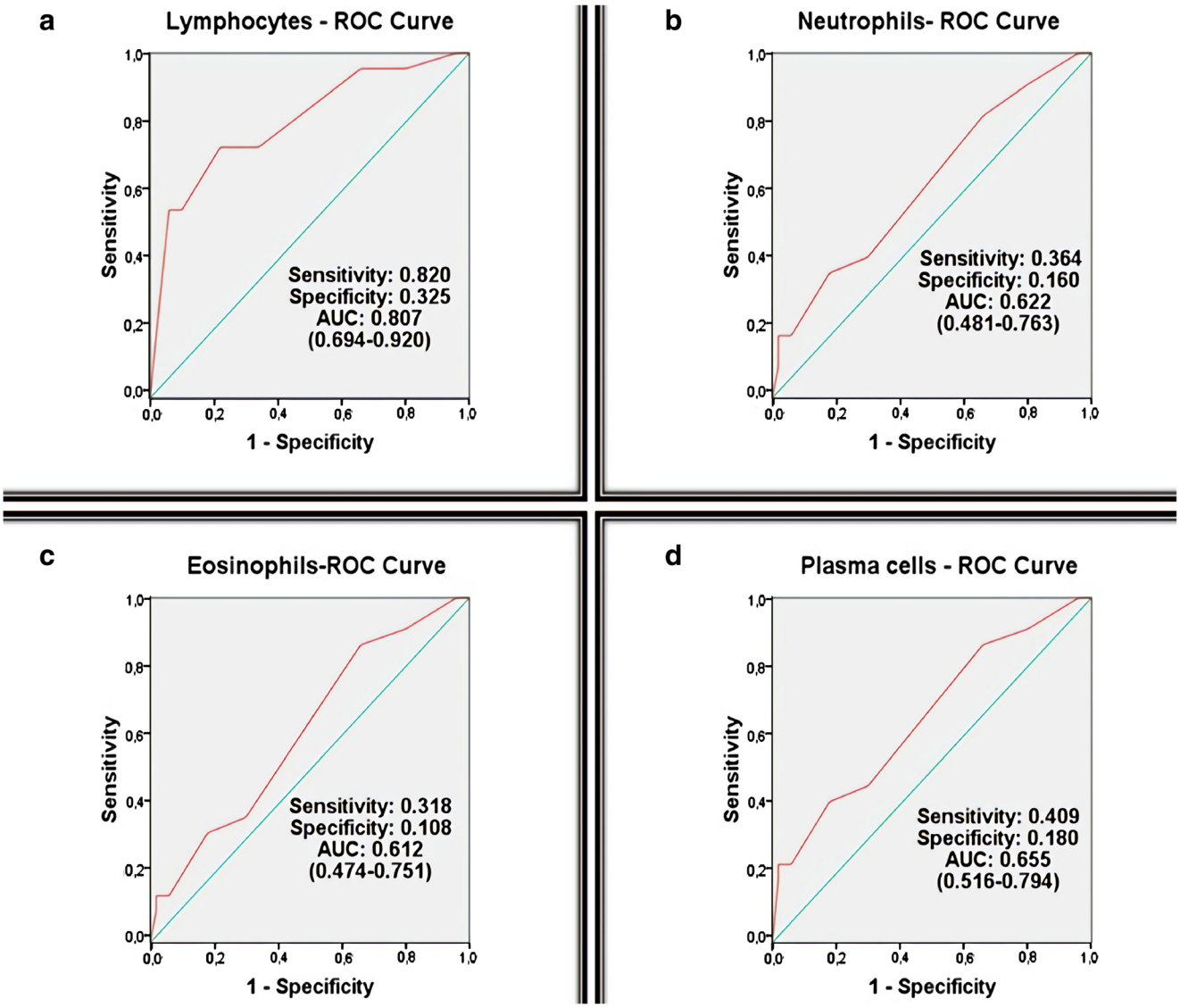

Fig. 2 ROC curves for lymphocytes (a), neutrophils (b), eosinophils (c), and plasma cells (d). AUC analyzed by manual methods. *AUC* Areas under the ROC curves; *ROC* Receiver Operating Characteristic

Table 3 Univariate and multivariate survival analysis of LIR (n=80)

		Univariate Analysis		Multivariate Analysis	
		OS	RFS	OS	RFS
		P-value (5-year survival)	P-value (5-year survival)	P-value (HR %95 CI)	P-value (HR %95 CI)
Age		0.935	0.465	-	-
	<75	%85	%84		
	≥75		%80		
Size		0.236	0.755	-	-
	<5	%90	%83		
	≥5	%83	%82		
Localization		0.823	0.565	-	-
	Right	% 86	%87		
	Left	% 85	%83		
Lymphatic invasion		0.624	0.641	-	-
	No	%87	%86		
	Yes	%84	%84		
Crohn's-like reaction		0.075	*0.038**	0.135	0.113
	No	%80	%77	1.89	2.06
	Yes	%89	%89	(0.82-4.35)	(0.84-5.07)
Tumour deposits		0.121	*0.038**	0.597	0.541
	Negative	%89	%77	1.31	1.32
	Positive	%81	%89	(0.48-3.57)	(0.55-3.17)
Invasive pattern		0.765	0.068	-	-
	No	%86	%88		
	Yes	%84	%79		
Surgical Margin		*0.028**	*0.001**	0.066	*0.043**
	No	%89	%87	2.54	2.67
	Yes	%79	%77	(1.00-6.40)	(1.14-6.27)
MSI		*0.001**	*0.001**	*0.042**	*0.046**
	MMR-D	%90	%87	2.68	2.99
	MMR-P	%78	%76	(1.01-6.91)	(1.12-7.25)
Grade		0.236	0.254	-	-
	Low grade	%86	%88		
	Moderate/ High grade	%84	%81		
Perineural invasion		0.121	0.201	-	-
	No	%89	%88		
	Yes	%81	%80		
Lymphocytes (Method 1)		*<0.001**	*0.001**	*0.005**	*<0.001**
	High	%90	%88	1.32	1.50
	Low	%78	%76	(1.08-1.61)	(1.22-1.85)
Lymphocytes (Method 2)		*0.028**	0.153	0.080	0.087
	High	%89	%88	3.66	3.65
	Low	%79	%78	(0.85-15.6)	(0.86-16.3)
Neutrophils (Method 1)		0.424	0.346	-	-
	High	%89	%88		
	Low	%84	%80		
Eosinophils (Method 1)		0.624	0.465	-	-
	High	%87	%88		

Table 3 (continued)

		Univariate Analysis		Multivariate Analysis	
		OS	RFS	OS	RFS
		P-value (5-year survival)	P-value (5-year survival)	P-value (HR %95 CI)	P-value (HR %95 CI)
	Low	%84	%82		
Plasma cells (Method 1)		0.121	0.068	-	-
	High	%89	%88		
	Low	%81	%79		

Only better results for LIR are given as examples

*. P-value is significant at the 0.05 level. Significant results are in italics

RFS Relapse-free survival; *OS* Overall survival; *MMR-D* Mismatch repair proteins deficiency; *MMR-P* Mismatch repair proteins proficiency; *CI* Confidence interval; *HR* Hazard ratio; *Method 1* Using the " x20 objektive&IHC&quantitative"; *Method 2* Using the "x40 objektive&IHC&quantitative"

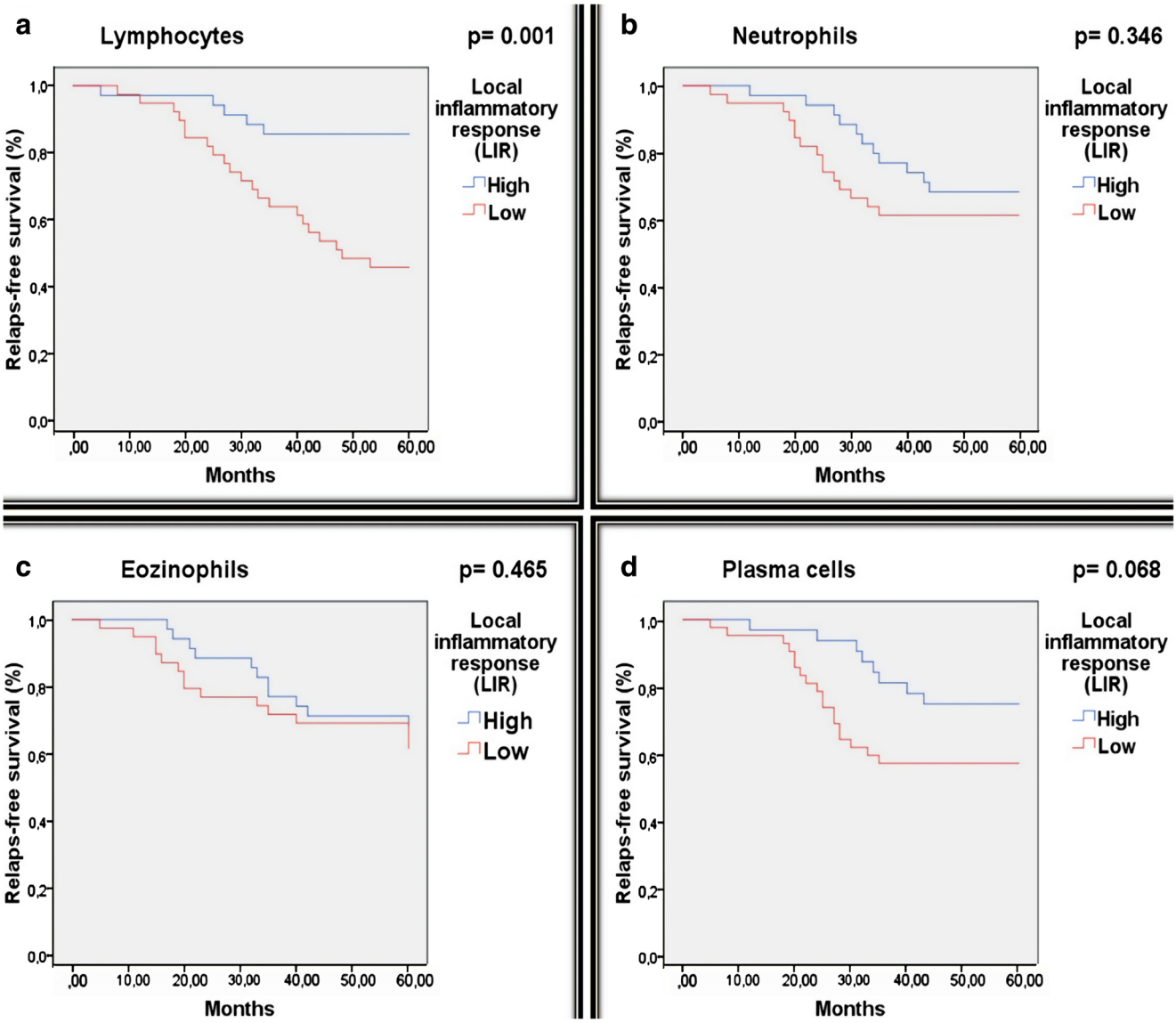

Fig. 3 Relapse-free survival curves of Kaplan-Meier for lymphocytes (a), neutrophils (b), eosinophils (c), and plasma cells (d). P-value is significant at the 0.05 level

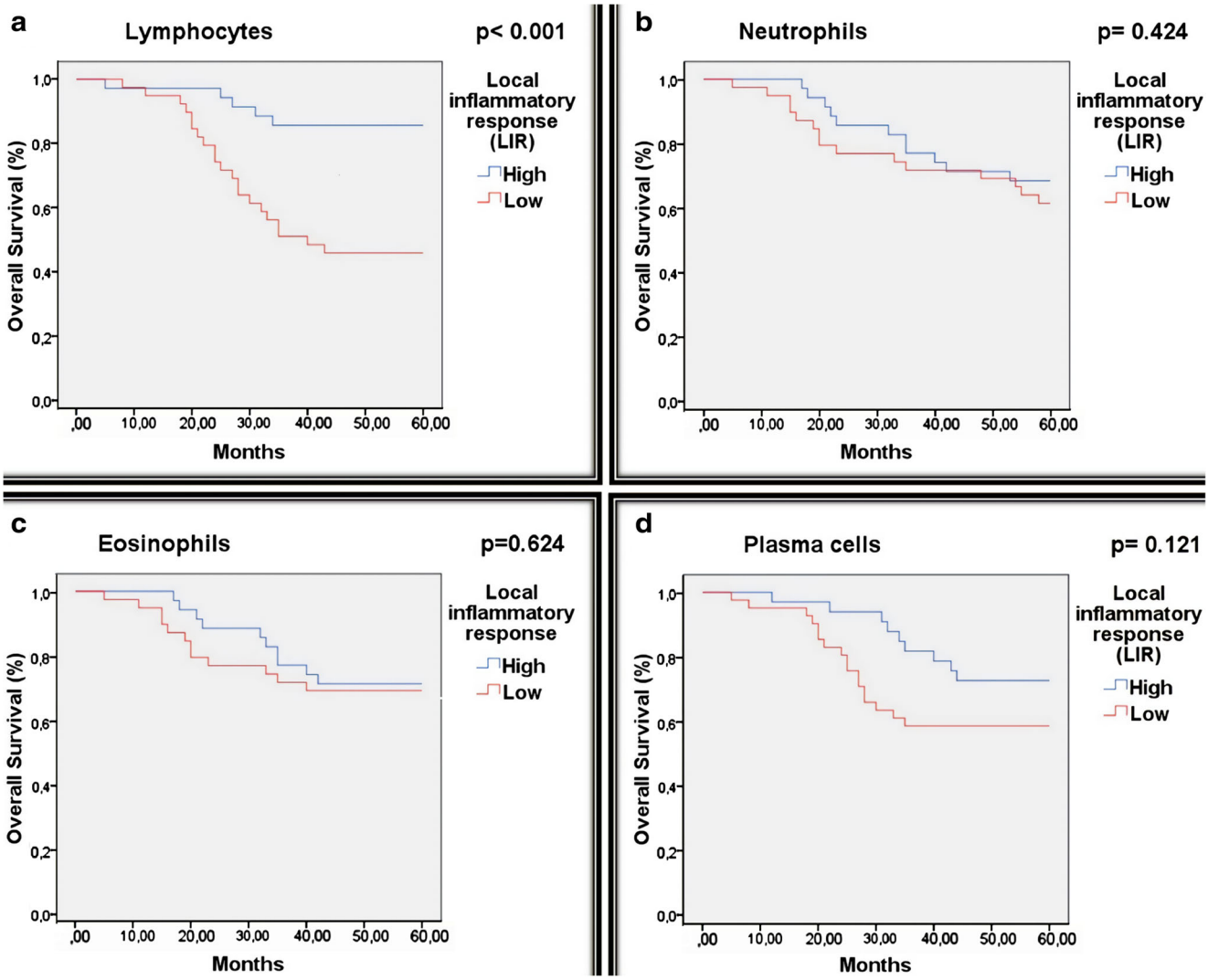

Fig. 4 Overall survival curves of Kaplan-Meier for lymphocytes (a), neutrophils (b), eosinophils (c), and plasma cells (d). P-value is significant at the 0.05 level

Therefore, we recommend the use of IHC stained sections for successful results in future studies.

There are many important features in this research. Firstly, we have studied a reliable parameter that gives promising findings in many studies in the literature but showed low standardization. Our study represents a highly homogeneous patient population, i.e. stage-IIB CC patients, without receiving adjuvant chemotherapy. We also standardized extra- and intra-biopsy evaluation methods with method 1 and model A. In other words, this study provided further improvements in the standardization of pathological assessment. Finally, this study was conducted in accordance with REMARK guidelines.

There were some limitations in our study. Firstly, there are internal limitations in the nature of the retrospective analysis. For example, it was impossible to overcome sampling differences because the existing tissue was previously sampled for diagnosis. Although we evaluated many different areas of each tumour, these samples represented only a small portion of a whole tumour. Also, since treatment protocols prior to 2012 are applied to our patients, so there may be differences in treatment compared to current protocols.

Conclusion

Our study confirms that low lymphocytes are an independent poor prognostic factor in patients with stage-IIB CC. In our opinion, the presence of lymphocytes should be routinely included in pathology reports of CC. This is particularly important when deciding adjuvant therapy in stage-IIB CC patients. To obtain more successful results, we recommend using model A and method 1 for pathological evaluations.

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Abbreviations *AJCC:* American Joint Cancer Committee*, LIR*: Local inflammatory response*, CC:* Colorectal cancer, *H&E:* Hematoxylin and eosin*, HPF:* High-power field*, SD*: Standard deviation, *IHC*: Immunohistochemistry*, CI*: Confidence interval, *SD*: Standard deviation, *ICC*: Intra-Class Correlation Coefficient, *HR*: Hazard ratio, *K*: Kappa, *OS*: Overall survival, *RFS*: Relapse-free survival, *MSI*: Microsatellite instability, *MMR*: Mismatch repair proteins, *Method 1*: Using the 'x20 objektive&IHC&quantitative', *Model A*: Using the 'deeply invasive blocks&hot-spot area&invasive margin'.

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Compliance with Ethical Standard

Conflicts of Interest The author does not report a conflict of interest.

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