



Unveiling the prognostic role of blood inflammatory indexes in a retrospective cohort of patients undergoing liver resection for intrahepatic cholangiocarcinoma

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Background: Systemic inflammation is relevant in intrahepatic cholangiocarcinoma (iCCA), but controversial results exist on the prognostic role of inflammatory indexes and their correlation with tumor microenvironment. The authors aimed to explore the biological and prognostic values of these indexes.

Materials and methods: A retrospective cohort study involving iCCA patients who underwent hepatic resection between 2010 and 2021 was conducted. The neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), and clinic-pathological factors were recorded. Immune-cell subpopulations, isolated from surgical specimens, were analyzed by flow cytometry. NLR and LMR cut-offs were calculated by X-Tile software. Linear regression, Kaplan–Meier, and Cox regression analyses were conducted.

Results: A total of 101 iCCA patients were considered. $NLR \geq 3.83$ and $LMR < 2.28$ correlated with worse survival. Patients were divided into groups: 67 (66.3%) in the low-risk and 34 (33.7%) in the high-risk (having at least one worse prognostic ratio). The 5-year overall survival was 49.8 and 18.9% for low- and high-risk groups, respectively ($P = 0.003$). An elevated CA19-9 in the high-risk group gives 2.148 HR (95% CI: 1.060–4.349) of mortality and 2.182 HR (95% CI: 1.206–3.948) of disease recurrence. Flow cytometry analysis of 20 surgical specimens highlighted that NLR was associated with tumor-derived NLR ($P = 0.026$) and LMR with tumor-infiltrating lymphocytes ($P = 0.002$). In a subset of five high-risk vs five low-risk patients, T-cell evaluation showed a higher prevalence of CD4+ compared to CD8+ cells in the high-risk group (78.5 vs. 21.5%, $P < 0.0001$). Conversely, low-risk patients demonstrated a noteworthy infiltration of CD8+ cells compared to the high-risk group (21.5 vs. 48.7%, $P = 0.037$).

Conclusions: The combination of blood inflammatory indexes determined two survival-risk profiles. The correlation between the blood scores and the iCCA microenvironment suggests a link between immune-cell infiltration and the risk group. These findings open the possibility of patient stratification with the chance to identify subgroups suitable for dedicated follow-up and targeted immuno-chemotherapy protocols.

Keywords: biliary tract cancer, intrahepatic cholangiocarcinoma, lymphocyte-to-monocyte ratio, neutrophil-to-lymphocyte ratio, tumor-infiltrating lymphocyte

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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International Journal of Surgery (2024) 110:7088–7096

Received 15 April 2024; Accepted 25 June 2024

Supplemental Digital Content is available for this article. Direct URL citations are provided in the HTML and PDF versions of this article on the journal's website, www.com/international-journal-of-surgery.

Published online 5 July 2024

<http://dx.doi.org/10.1097/JS9.0000000000001924>

Introduction

Intrahepatic cholangiocarcinoma (iCCA) is a rare tumor representing the second most common primary liver cancer, with an increasing incidence and mortality^[1]. While surgical resection is the mainstay of curative treatment, the challenges of late diagnosis and high rates of recurrence, despite advancements in systemic treatment protocols, make survivals dismal^[2,3].

Over the past decades, numerous tumoral prognostic factors have been identified, with most of them relying on histopathological characteristics (e.g. vascular invasion, tumor margin, and nodal status), which are only available after surgical resection^[4,5]. Consequently, significant efforts have been dedicated to exploring and validating preoperative criteria to stratify tumor aggressiveness and guide the decision process. In this context, several scores based on preoperative immune and nutritional laboratory values have been proposed^[6–8], yet their precise role in characterizing tumor behavior remains controversial^[9]. Numerous reports on iCCA prognostic scores have been derived from studies concerning other solid tumors, such as lung^[10], breast^[11], colon-rectal^[12], and hepatocellular carcinoma^[13]. Despite exhibiting some fluctuating association with survival data, no comprehensive explanation has been provided regarding how these systemic scores may be influenced by the tumoral mass itself and whether any association exists. While inflammation is closely linked to tumor onset and progression^[14], no conclusive evidence has demonstrated links between the inflammatory score and cancer-associated leukocyte populations.

This study aimed to investigate the role of systemic inflammatory indexes in iCCA and explore how tumor biology may lead to systemic leukocyte alterations, thereby affecting patient recurrence and prognosis. To achieve this, a single-center iCCA patient cohort was analyzed, focusing on the possible cause-effect relationship between the inflammation identified locally in the tumor and systemically, as reflected by the inflammatory indexes.

Methods

Study design and patients' selection

Patients undergoing liver resection for iCCA in a tertiary referral center between February 2010 and February 2021 were retrospectively identified from a prospectively maintained database. iCCA was defined following the Japanese classification of biliary tract cancer^[15] and staged in conformity with the American Joint Committee against Cancer (AJCC) staging system, 8th edition^[5]. Demographic, clinical, and laboratory data of the patients were analyzed as common baseline characteristics.

The study was approved by the local Independent Ethics Committee at the enrolling center (registration number 1341/14). Written informed consent was obtained from the patients. Data collection and analysis were conducted following the ethical guidelines of the Declaration of Helsinki^[16]. The results are reported following the principles of Strengthening the Reporting of cohort, cross-sectional, and case-control studies in surgery (STROCSS, Supplemental Digital Content 1, <http://links.lww.com/JS9/D47>)^[17].

HIGHLIGHTS

- Elevated neutrophil-to-lymphocyte ratio and decreased lymphocyte-to-monocyte ratio identify high-risk patients with worse prognosis.
- Tumor microenvironment analysis revealed correlations between inflammatory indexes and tumor-infiltrating leukocytes.
- Blood inflammatory indexes may serve as prognostic markers for survival-risk stratification in intrahepatic cholangiocarcinoma patients.
- The link with immune cell infiltration opens possibilities for targeted immuno-chemotherapy protocols and tailored follow-up strategies for specific risk groups.

Study endpoint

The primary endpoint of the study was defining the role of inflammatory indexes in stratifying patients in terms of overall survival (OS) and disease-free survival (DFS). The secondary endpoint was deciphering the associations between immune cell infiltration in both tumoral and peritumoral liver tissue with the corresponding blood indexes.

Study eligibility criteria

The study included patients undergoing resection for iCCA. The inclusion criteria were: 1) confirmed histological diagnosis of iCCA, 2) age \geq 18 years, and 3) a minimum follow-up of 12 months, unless an earlier death event occurred. The exclusion criteria encompassed: 1) any systemic inflammatory condition before the surgical resection, and 2) incomplete laboratory data within 1 month before surgery. Patient surveillance was closed at the end of June 2022.

Surgical technique and follow-up protocol

All patients were deemed resectable at diagnosis. Treatment decisions were made in a multidisciplinary setting. Each patient's case was thoroughly evaluated, considering their underlying condition, oncologic and medical history, and local protocols. In our center, liver surgery follows a strict parenchyma-sparing surgical policy^[18].

After resection, patients were monitored with regular measurements of serum tumor markers (CA19-9 and CEA), abdominal ultrasound, and computed tomography scan or MRI, according to local outpatient visit protocols.

Inflammatory blood indexes

The inflammatory blood indexes analyzed in this study were the Neutrophil-to-Lymphocyte Ratio (NLR), Lymphocyte-to-Monocyte Ratio (LMR), and Platelet-to-Lymphocyte ratio (PLR), as previously reported in the literature^[9].

Sample collection, flow cytometry, and immunohistochemistry

The iCCA surgical samples ($n=20$) were prospectively collected between 2018 and 2020. Patients were included in the study based on the availability of sufficient material from surgical specimens of both healthy and tumoral tissues. This small cohort corresponded

to 20% of the whole patients' cohort and was thought to be representative at least for a preliminary study. The samples were digested as previously described^[19,20].

After the complete digestion of the healthy and tumor specimens, the immunophenotyping of circulating intrahepatic immune cells, intrahepatic immune cells, and tumor-infiltrating immune cells was performed using the 7-Color Immunophenotyping kit (Miltenyi Biotec) according to manufacturer protocol.

Flow cytometry data were analyzed using BD FACSCanto II (BD Biosciences), and the results were analyzed using FlowJo software (v10.8.2).

The schematic representation of sample digestion and the gating strategy used to analyze the immune cells are provided in SDC Fig. S1 (Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>).

Immunohistochemistry (IHC) was performed on paraffin-embedded iCCA tissue from samples obtained from the Pathology Department, as already described^[19,20]. The tissue sections were incubated with the following primary antibody: antihuman CD4 (1:300, clone UMAB64; Origene) and antihuman CD8 (1:500, clone C8/144B; Dako).

Statistical analysis

Normal distribution was assessed using the Kolmogorov–Smirnov test. Continuous variables were reported as median and interquartile range (IQR), while categorical variables were presented as frequency and percentage. The cut-off values for NLR, LMR, and PLR were determined using the bioinformatic tool X-Tile Software (Copyright: Yale University, Version 3.6.1). The choice of this software was based on its proven validity through the examination of several other established prognostic markers^[21]. Baseline characteristics between the groups were compared using Mann–Whitney and χ^2 or Fisher tests, as appropriate. The OS was measured as the difference in months between the date of surgery and the date of the death or last follow-up available. The DFS was measured as the difference in months between the date of treatment and the date of the disease recurrence. In case of no recurrence, or death, data were censored at the date of the last available follow-up visit. The analyses were made by the Kaplan–Meier method and comparisons among the two groups were assessed using the log-rank test. The adjusted Cox proportional hazard model was utilized to identify independent prognostic factors for survival, and the results were reported as hazard ratio (HR) with the corresponding 95% CI. Univariate and multivariate linear regression analyses were conducted to examine the association between systemic score, tumoral histopathological data, and leukocyte infiltrating cells. All the variables resulting in association with the dependent variable with a *P*-value <0.10 at the univariate analysis were inserted in the multivariate models. All statistical tests were two-tailed and a 5% significance level was adopted. All the analyses were computed by using the GraphPad Prism software (v. 9.5.1) and IBM-SPSS statistics software (v. 24.0).

Results

Patients

Between February 2010 and February 2021, a total of 101 patients met the selection criteria and were enrolled in the study.

The median age at the time of surgery was 70.6 years (IQR 62.9–75.3). Of these patients, 49 (48.5%) were male. Fifty-three (52.5%) patients were affected by T2–T4 iCCA and 29 (28.7%) cases were N0. Notably, a total of 42 (41.6%) patients underwent complete lymph node dissection. A negative resection margin (R0) was achieved in 66 (65.3%) patients. The median tumor size was 5.7 cm (IQR 3.9–8.7), and 28 (27.7%) cases showed multinodular disease. Additionally, 42 (41.6%) patients exhibited an elevated level of CA19-9. The baseline clinical and pathological characteristics of the population are summarized in SDC Table S1 (Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). After a median follow-up time of 44.6 (95% CI: 25.9–63.2) months the cohort had a median OS of 52.6 (95% CI: 31.5–73.7) months with a 1-year, 3-year, and 5-year OS rate of 82.2, 52.4, and 41.7%, respectively (SDC Fig. S2A, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). The median DFS was 12.6 (95% CI: 10.0–15.2) with a 1-year and 3-year DFS rate of 54.6 and 21.9% (SDC Fig. S2B, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>).

Inflammatory score assessment and prognostic significance

According to the preoperative laboratory exam conducted within a month before the surgical procedure, the population was classified based on the aforementioned blood inflammatory scores, namely: NLR, LMR, and PLR. The optimal cut-off values were determined using the graphical method of X-Tile Software, depending on OS, as shown in SDC Figure S3 (Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). The resulting

Table 1

Tumor aggressiveness characteristics and population's score.

Histopathological data and tumor markers	Low-risk score n 67 (% ^a)	High-risk score n 34 (% ^a)	<i>P</i>
T stage			0.011
T1a-b	37 (55.2)	9 (26.5)	
T2-4	30 (44.8)	23 (67.5)	
G stage			0.001
G1-2	54 (80.6)	16 (47.1)	
G3	13 (19.4)	17 (50)	
N stage ^b			0.481
N0	21 (50.0)	8 (19.1)	
N1	8 (19.1)	5 (11.9)	
Microvascular invasion			0.448
No	45 (67.2)	19 (55.9)	
Yes	22 (32.8)	13 (38.2)	
Tumor nodule > 1			0.687
No	49 (73.1)	24 (70.6)	
Yes	18 (26.9)	10 (29.4)	
Tumor size > 5 cm			0.001
No	39 (58.2)	8 (23.5)	
Yes	28 (41.8)	26 (76.5)	
Increased CA19-9			1
No	36 (53.7)	18 (52.9)	
Yes	28 (41.8)	14 (41.2)	
Increased CEA			0.344
No	54 (80.6)	29 (85.3)	
Yes	8 (11.9)	2 (5.9)	

^aPercentages refer to the total amount of patients considering missing values.

^bN stage is based on 42 patients undergone to lymphadenectomy.

CA 19.9, carbohydrate antigen 19.9; CEA, carcinoembryonic antigen; G, grade; N, lymph nodal; n, number; T, tumor.

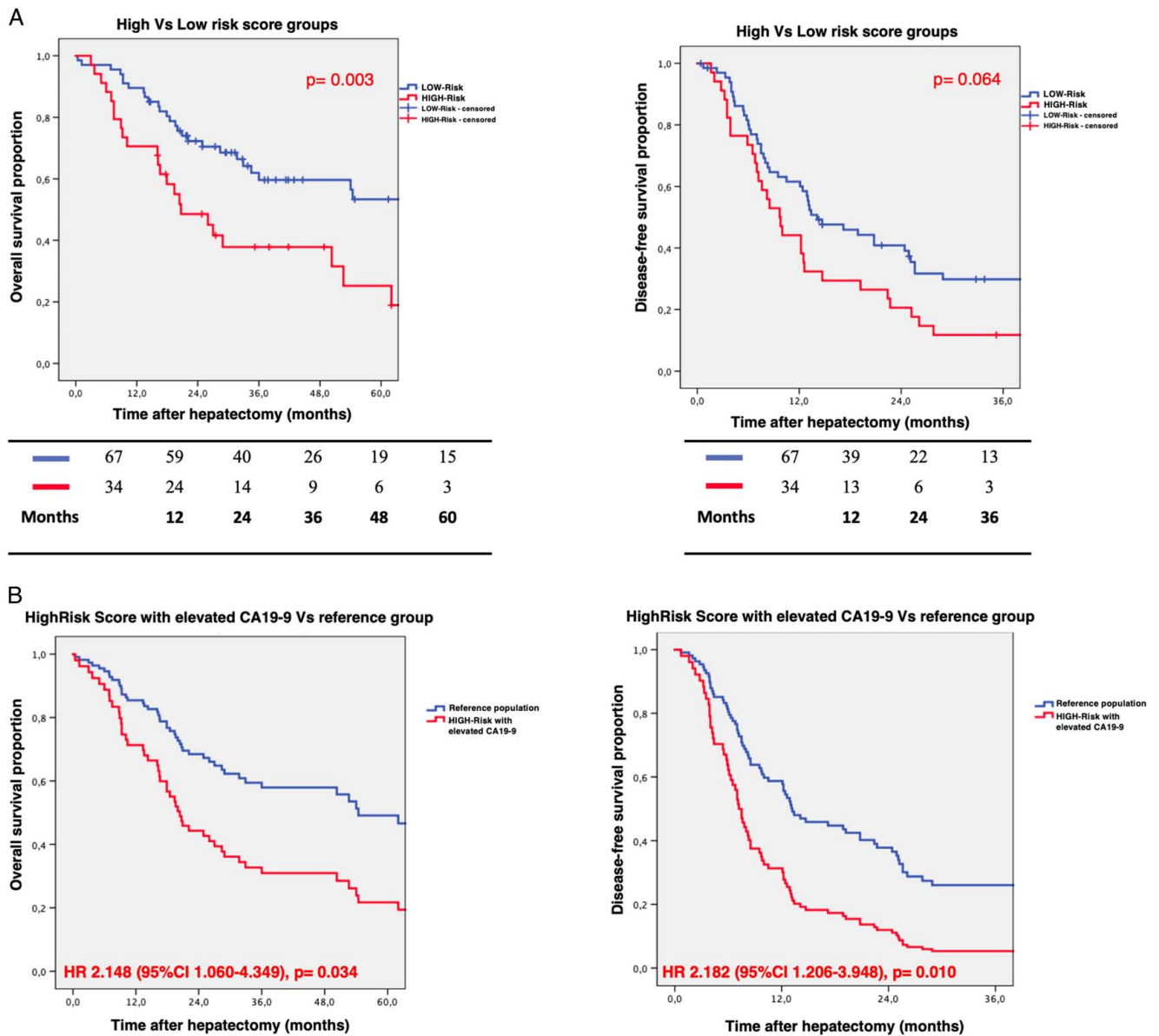


Figure 1. Survival of the analyzed cohort according to the risk score. A) Overall survival and disease-free survival comparisons between the high-risk and the low-risk score populations. B) Overall survival and disease-free survival comparisons between high-risk and CA19-9pos versus reference group.

cut-offs were as follows: 3.83 for NLR, 2.28 for LMR, and 285.00 for PLR. Details regarding the distribution of patients according to their respective scores are provided in SDC Table S2 (Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). Univariate comparisons between groups in terms of OS and DFS were run. The NLR was found to be significantly associated with OS: patients with a preoperative NLR score <3.83 had a favorable median OS of 54.4 (95% CI: 26.2–82.6) months compared to 26.0 (95% CI: 12.9–39.1) months for others ($P=0.036$) (SDC Fig. S4A, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). Similarly, an LMR score of ≥ 2.28 resulted in a better median OS of 54.4 (95% CI: 21.3–87.4) months compared to 20.7 (95% CI: 7.2–34.1) months for the other group ($P=0.035$) (SDC Fig. S4B, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). However, no significant association was found

between NLR and LMR scores with DFS (SDC Fig. S4C,D, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>), and there was no association observed by dividing the population based on the PLR score cut-off (data not shown). Subsequently, a comprehensive score considering both NLR and LMR cut-offs was calculated. The high-risk score (HRS) group was considered comprehensive of patients presenting with at least one among the prognostic unfavorable score values ($NLR \geq 3.83$ or $LMR < 2.28$) with 34 (33.7%) patients belonging to this group (SDC Table S2, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). The HRS and low-risk score (LRS) groups were then compared based on characteristics known to better represent tumor aggressiveness (Table 1). The HRS patients showed an increased number of tumor sizes > 5 cm ($P=0.001$), as well as higher tumor stages ($P=0.011$). Conversely, patients in the LRS group

presented with a higher proportion of well-differentiated (G stage) tumors ($P=0.001$).

Prognostic value of high-risk profile and survival analyses

The HRS population exhibited OS rates of 70.6, 37.8, and 18.9% at 1-year, 3-year, and 5-year, respectively, while the LRS group had higher OS rates of 88.1, 59.6, and 49.8% at the same intervals ($P=0.003$). In terms of recurrence, the 1-year and 3-year DFS were 38.2 and 11.8% for the HRS group and 60.0 and 27.7% for the LRS group, although the difference did not reach statistical significance ($P=0.064$). The survival curves are depicted in Figure 1A. Following the univariate analysis, several factors were identified as associated with OS, including T stage, tumor size, risk score (referring to HRS vs. LRS), and CA19-9. The multivariate analysis recognized an increased CA19-9 (HR: 1.969, 95% CI: 1.072–3.618, $P=0.029$) and the HRS (HR: 2.138, 95% CI: 1.153–3.967, $P=0.016$) as statistically significant factors associated with OS. The results of Cox univariate and multivariate regression analyses for OS are reported in Table 2. When the referred outcome was DFS, only multinodular disease was confirmed to be a relevant prognostic factor in terms of recurrence in the multivariate analysis (SDC Table S2, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). As depicted in Figure 1B, a combination of the HRS population with an abnormal CA19-9 level identified a specific subgroup at increased risk, not only for OS (HR: 2.148, 95% CI: 1.060–4.349, $P=0.034$) but also in terms of recurrence (HR: 2.182, 95% CI: 1.206–3.948, $P=0.010$).

The inflammatory score reflects the tumor microenvironment in iCCA patients

To evaluate the relationship between blood inflammatory indexes and tumor characteristics, an in-depth analysis was conducted on the immune infiltrate derived from circulating intrahepatic blood, intrahepatic, and intratumoral tissue, isolated from 20 patients' surgical specimens (SDC Fig. S1, Supplemental Digital Content 2, <http://links.lww.com/JS9/D48>). In this subpopulation, seven (35%) patients belong to the HRS group. Using the infiltrating lymphocytes, monocytes, and neutrophils from FACS analysis, we computed 'tissue immune-infiltrating' scores for NLR and LMR in the circulating, intrahepatic, and intratumoral samples. Univariate and multivariate linear regression analyses were employed to explore the potential association between the systemic inflammatory score and the infiltrating immune cell populations.

In multivariate analysis, aside from the tumor size > 5 cm, the infiltrating immune cells demonstrated a significant association with both the systemic NLR and LMR (Table 3a-b). Specifically, an increase in the systemic NLR was positively associated with the tumor NLR, with a beta coefficient of 0.424 ($B=0.568$ 95% CI: 0.075–1.061, $P=0.026$). Conversely, the increase in systemic LMR showed a positive correlation with tumor lymphocyte infiltration, with a beta coefficient of 1.118 ($B=0.090$, 95% CI: 0.039–0.141, $P=0.002$), other than tumor neutrophils with a beta coefficient of 0.872 ($B=0.077$, 95% CI: 0.017–0.138, $P=0.016$). In both cases, the presence of a tumor size > 5 cm was associated with the NLR ($P=0.015$) and the LMR ($P=0.001$), with a positive or negative correlation depending on the respective prognostic significance of the two ratios.

Table 2

Univariate and multivariate Cox-regression analysis for overall survival (OS).

Variable	Univariate (OS)		Multivariate (OS)	
	HR (95% CI)	P	HR (95% CI)	P
Age > 70 years	1.219 (0.717–2.073)	0.463		
Sex (male)	0.907 (0.536–1.534)	0.716		
G stage				
G1	Ref	0.916		
G2	1.053 (0.406–2.732)	0.143		
G3	2.104 (0.778–5.690)			
T stage				
T1a	Ref	0.178	Ref	0.970
T1b	1.794 (0.767–4.194)	0.011	1.022 (0.332–3.149)	0.332
T2	2.575 (1.244–5.329)	0.009	1.646 (0.602–4.505)	0.527
T3	3.019 (1.325–6.880)	0.031	1.451 (0.459–4.591)	0.446
T4	5.367		2.019	
	(1.165–24.720)		(0.332–12.293)	
N stage ^a				
N0	Ref	0.181		
N1	0.563 (0.242–1.307)			
MVI	1.403 (0.814–2.416)	0.223		
Surgical margins (R1)	1.243 (0.708–2.184)	0.449		
n nodule (> 1)	1.445 (0.814–2.565)	0.209		
Size (> 5 cm)	2.085 (1.204–3.610)	0.009	1.266 (0.576–2.782)	0.558
RiskScore (High)	2.055 (1.197–3.530)	0.009	2.138 (1.153–3.967)	0.016
Increased CEA	1.702 (0.755–3.837)	0.200		
Increased CA19-9	2.191 (1.279–3.753)	0.004	1.969 (1.072–3.618)	0.029
Liver substrate ^b	0.691 (0.407–1.172)	0.171		
Viral infection ^c (yes)	1.057 (0.546–2.048)	0.869		

^aN stage is based on patients undergone to lymphadenectomy.

^bLiver substrate is intended for patients having normal liver background versus those with hepatopathy (from steatosis to cirrhosis).

^cViral infections are considered both HBV/HCV.

CA 19.9, carbohydrate antigen 19.9; CEA, carcinoembryonic antigen; G, grade; HR, Hazard Ratio; MVI, microvascular invasion; N, lymph nodal; n, number; R1, microscopic residual tumor; T, tumor.

To gain deeper insights into the prognostic significance of the identified systemic scores concerning the immune cell composition within the tumor microenvironment (TME), we evaluated the percentages of CD4+ and CD8+ T cells in high-risk ($n=5$) and low-risk ($n=5$) groups, as determined by systemic scores. As shown in Figure 2, low-risk patients demonstrated a comparable percentage of CD4+ and CD8+ T cells within the tumor niche (51.6 vs. 48.7%, $P=0.998$). On the contrary, high-risk patients displayed a significantly higher prevalence of CD4+ T cells, compared to the CD8+ T cells (78.5 vs. 21.5%, $P<0.0001$). Moreover, low-risk patients exhibited a noteworthy infiltration of CD8+ cells in comparison to the high-risk group (21.5 vs. 48.7%, $P=0.037$), suggesting a potential association between the distinct infiltration patterns of CD4+ and CD8+ cells and the risk stratification observed in iCCA patients based on the identified inflammatory scores.

Discussion

iCCA is an aggressive tumor associated with short-term and long-term survival even after surgical resection^[3,22]. Efforts have been made to stratify the iCCA prognosis by using biomarkers^[2,23]. Traditionally, CEA and CA 19-9 have been correlated with clinical outcomes with disparate results^[24]. Recently, attention

Table 3**Univariate and multivariate regression analyses for a) neutrophil-to-lymphocyte ratio (NLR) and b) lymphocyte-to-monocyte ratio (LMR) with healthy, peri-tumoral, and tumoral immune infiltrate**

Variable	Univariate				Multivariate			
	B	Beta	95% CI	P	B	Beta	95% CI	P
a. Univariate and multivariate regression analyses for neutrophil-to-lymphocyte ratio (NLR) with healthy, peri-tumoral, and tumoral immune infiltrate.								
Tumor lymphocytes	-0.034	-0.533	-0.060--0.007	0.016^a				
Tumor neutrophils	0.035	0.507	0.006-0.065	0.023^a				
Tumor monocytes	0.079	0.267	-0.065-0.221	0.255				
Healthy tissue lymphocytes	0.007	0.106	-0.024-0.037	0.657				
Healthy tissue neutrophils	0.003	0.052	-0.025-0.031	0.826				
Healthy tissue monocytes	-0.021	-0.065	-0.180-0.138	0.785				
Circulating lymphocytes	-0.004	-0.094	-0.028-0.020	0.711				
Circulating neutrophils	0.011	0.222	-0.015-0.038	0.376				
Circulating monocytes	-0.043	-0.186	-0.162-0.077	0.461				
G stage	0.428	0.233	-0.457-1.312	0.323				
T stage	0.217	0.176	-0.383-0.818	0.457				
N stage ^b	0.044	0.019	-2.023-2.111	0.961				
MVI	0.331	0.157	-0.703-1.364	0.510				
Tumor nodule > 1	-0.800	-0.349	-1.864-0.264	0.132				
Size > 5 cm	1.398	0.634	0.554-2.241	0.003	1.045	0.474	0.234-1.855	0.015
Liver substrate	-0.762	-0.355	-1.755-0.231	0.124				
Increased CEA	-0.763	-0.301	-2.147-0.620	0.275				
Increased CA19-9	-0.454	-0.209	-1.603-0.695	0.414				
NLRscore (tumor)	0.808	0.603	0.279-1.336	0.005	0.568	0.424	0.075-1.061	0.026
NLRscore (Healthy tissue)	-0.060	-0.131	-0.287-0.166	0.582				
NLRscore circulating	0.027	0.068	-0.183-0.237	0.790				
b. Univariate and Multivariate regression analyses for lymphocyte-to-monocyte ratio (LMR) with healthy, peri-tumoral, and tumoral immune infiltrate.								
Tumor lymphocytes	0.045	0.566	0.013-0.078	0.009	0.090	1.118	0.039-0.141	0.002
Tumor neutrophils	-0.039	-0.439	-0.078-0.001	0.053	0.077	0.872	0.017-0.138	0.016
Tumor monocytes	-0.022	-0.058	-0.208-0.165	0.808				
Healthy tissue lymphocytes	-0.004	-0.055	-0.043-0.034	0.817				
Healthy tissue neutrophils	0.001	0.012	-0.034-0.036	0.960				
Healthy tissue monocytes	0.046	0.113	-0.155-0.247	0.636				
Circulating lymphocytes	0.004	0.071	-0.025-0.033	0.780				
Circulating neutrophils	-0.008	-0.130	-0.041-0.025	0.608				
Circulating monocytes	-0.013	-0.045	-0.160-0.135	0.860				
G stage	-0.256	-0.110	-1.402-0.890	0.644				
T stage	-0.195	-0.125	-0.962-0.573	0.601				
N stage ^c	0.949	0.303	-1.719-3.617	0.428				
MVI	0.384	0.144	-0.928-1.697	0.546				
Tumor nodule > 1	0.604	0.208	-0.804-2.013	0.379				
Size > 5 cm	-1.750	-0.626	-2.828--0.671	0.003	-1.961	-0.702	-2.930--0.991	0.001
Liver substrate	-0.026	-0.010	-1.373-1.321	0.968				
Increased CEA	1.371	0.396	-0.454-3.195	0.129				
Increased CA19-9	0.028	0.010	-1.457-1.514	0.968				
LMRscore (tumor)	0.017	0.224	-0.020-0.054	0.342				
LMRscore (Healthy tissue)	-0.018	-0.162	-0.073-0.037	0.496				
LMRscore circulating	-0.006	-0.201	-0.023-0.010	0.424				

^aData not considered for multivariate linear regression analysis since multicollinearity with NLRscore (tumor).^bCalculated on patients undergone lymphadenectomy.^cCalculated on patients undergone lymphadenectomy.

CA 19.9, carbohydrate antigen 19.9; CEA, carcinoembryonic antigen; G, grade; MVI, microvascular invasion; N, lymph nodal; T, tumor.

has been concentrated on the role of inflammation, the immune system, and the TME in the development and progression of cancer^[25,26]. Indeed, inflammatory and immune biomarkers are emerging to predict the clinical outcome of cancer patients.

This study demonstrates the efficacy of a novel score in predicting the prognosis of patients undergoing resection for iCCA. It stratified the OS and, combined with CA19-9, identified patients at higher risk of recurrence. The prediction was based on

preoperative information, clinically available to every patient awaiting surgery for iCCA. This practicability represents an added value to this work. Besides, for the first time, the significance of this preoperative laboratory score was correlated with the immune contexture of the corresponding TME, as measured with FACS and IHC in the surgical specimens of the same patients.

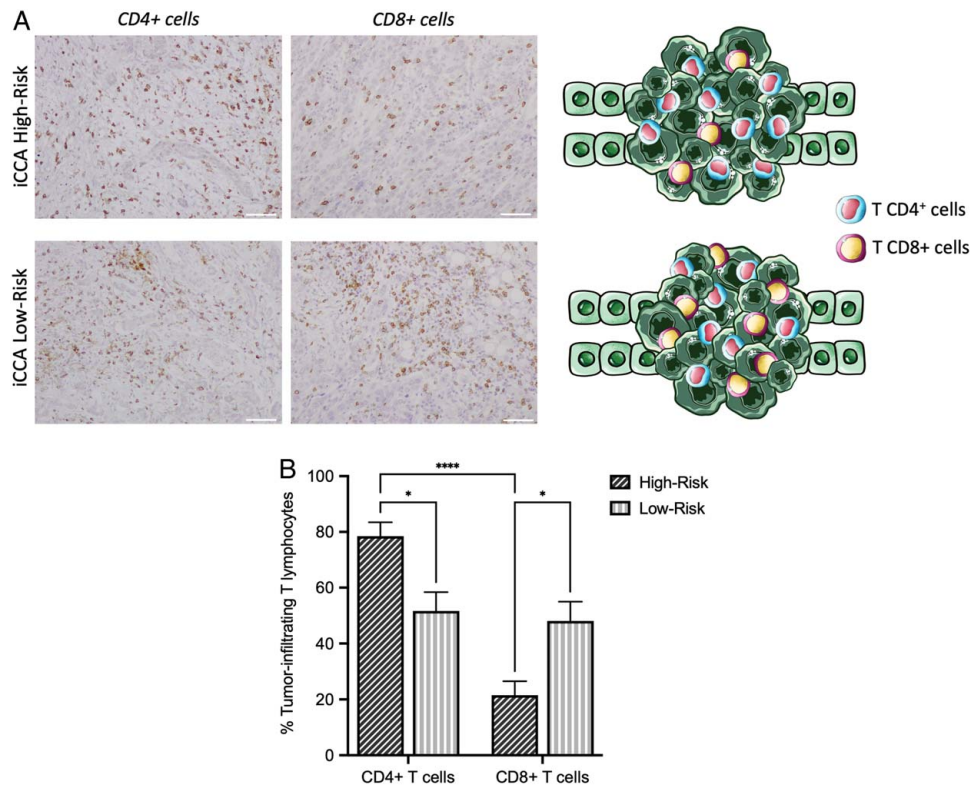


Figure 2. T cell subpopulation analysis of iCCA high-risk and low-risk patients. A) Immunohistochemistry (IHC) analysis and graphical representation of CD4 + and CD8 + cells in iCCA high-risk and low-risk patients. Scale bar 50 μ m. B) Percentage of infiltrating T cell subpopulations in iCCA high-risk ($n = 5$) and low-risk ($n = 5$) patients. Two-way ANOVA (mean \pm SEM). * $P < 0.05$; **** $P < 0.0001$.

Prior research has extensively emphasized the prognostic value of blood biomarkers in various types of cancer^[10,13], and also in iCCA^[9]. In particular, Cui *et al.*^[19] validated the role of pre-operative scores but also revealed a significant association between higher NLR and poorer overall and recurrence-free survival. Moreover, further corroborating our results, despite some heterogeneity among the reported studies, recent meta-analysis findings underscore that low LMR is associated with poor overall and recurrence-free survival^[27]. Over the years, other laboratory scores, such as the LabScore^[8], IFS^[7], and IS^[28], based on these simple ratios, have been reported as prognostic tools for iCCA, each exhibiting different performance characteristics.

Despite the recognition of the role of inflammation in iCCA prognosis, the biological explanation behind those scores has never been thoroughly investigated. The herein reported study represents the first attempt to comprehend the physio-pathological value of those prognostic scores highlighting that the blood inflammatory indexes closely reflect the iCCA TME, thus suggesting a rationale beyond their potential application for stratifying patients' prognoses. These results further suggest that, in iCCA, the blood peripheral circulating immune cells could be influenced by the TME, explaining the prognostic significance of laboratory-derived scores.

As said, inflammation plays a crucial role in tumor growth, development, and cancer progression, with different cell populations exerting distinct roles in the systemic inflammatory response^[14,29]. In detail, neutrophils have been shown to

promote the survival and proliferation of cancer cells by releasing inflammatory mediators such as tumor necrosis factor, interleukin 1, interleukin 6, and vascular endothelial growth factor^[30,31]. Conversely, tumor-infiltrating lymphocytes (TILs), particularly T CD8+, have a recognized protective role, in inducing cancer cell apoptosis through the release of cytotoxic factors^[32,33]. As previously reported by our group and other studies, higher levels of CD8+ TILs have been associated with improved OS in iCCA patients^[34,35]. Our analysis revealed a significant increase in the abundance of CD8+ T cells within the TME of iCCA patients with low-risk profiles compared to those in the high-risk group. These findings suggest that the balance between CD4+ and CD8+ T cell infiltration in the TME plays a crucial role in determining survival. Altogether these findings reflect the presence of very complex interactions among the different immune cells in the TME that might be responsible for the wide array of clinical presentations and responsiveness to treatments. Understanding these interactions in general, and on an individual patient, is the way to investigate and apply immunotherapy as a therapeutic option for a larger number of iCCA patients^[36].

In terms of advantages, it is important to acknowledge that the proposed score holds strengths in clinical practice, generalizability, and personalized medicine. First, it is easily obtainable in clinical as it is cost-effective, derived from a standard blood examination that is affordable, accessible, and already performed on all patients at diagnosis or just before surgery. Second, it could significantly impact patient management by facilitating a truly

personalized approach. This would enable a more aggressive postoperative treatment and/or dedicated follow-up protocol, particularly for patients at a higher risk of recurrence. Third, unraveling the immune landscape within the TME presents tremendous translational opportunities. This includes gaining insights into the pathophysiological mechanisms influencing the controversial responsiveness to immunotherapy and uncovering novel treatment strategies^[37]. In this context, there is a significant advantage in having the surgical specimen available for translational research. Utilizing cutting-edge omics technologies, comprehensive translational research should be conducted to understand the relevant implications and identify potential predictive biomarkers that may help select which patients may truly benefit from immunotherapy.

Despite these encouraging future perspectives, our study has some limitations. This is a single-center, retrospective study with a relatively small sample size, which might have attenuated the association between prognostic factors and survival outcomes with potential biases from unmeasured confounders. The long-time frame of the study may also underestimate the impact of recent treatment advancements, such as the newest adjuvant protocols, and recent surgical implementations, as routine lymphadenectomy. Yet, the analyses of infiltrating immune cells were performed on a subpopulation of iCCA patients. Lastly, the study included all iCCAs without stratifying the disease based on the liver pathological substrate (e.g. metabolic and viral hepatitis) or pathological division of iCCA categories (e.g. large vs. small duct), which warrants further investigation focusing on specific pathological subtypes. Importantly, our findings require to be confirmed on an external larger patients' cohort. Nevertheless, the prognostic score developed in the context of this study allows for a prospective prognostic assignment of iCCA patients.

Conclusion

In conclusion, this study represents substantial progress in predicting iCCA prognosis by introducing a novel predictive risk score able to determine patients' sub-cohort with different clinical outcomes. The findings from this study offer a valuable understanding of how laboratory-derived scores are influenced by the TME, filling the gap between the clinical features and TME composition, and suggesting potential implications for enhancing personalized medicine approaches in managing iCCA patients.

Ethical approval

The study protocol was in accordance with the ethical guidelines established in the 1975 Declaration of Helsinki and compliant to the procedures of the local ethical committee of IRCCS Humanitas Research Hospital (Milan, Italy – registration number 1341/14).

Consent

Written informed consent was obtained from the patients.

Source of funding

This work was supported by 'PRIN: Progetti Di Ricerca Di Rilevante Interesse Nazionale (ID=2022SZT583). The funding

agency had no role in the design of the study or collection and analysis of data.

Author contribution

F.M., M.A.P., M.D., and G.T.: conceptualization; F.M., M.A.P., C.S., B.F., L.D.T., L.M.T., and A.L.: data curation; F.M., M.A.P., and S.F.: formal analysis; A.L., M.D., and G.T.: funding acquisition; F.M., M.A.P., C.S., B.F., and S.F.: investigation; F.M., M.A.P., M.D., and G.T.: methodology; M.D. and G.T.: project administration; M.A.P., C.S., B.F., and L.D.T.: resources; F.M., M.A.P., and S.F.: software; A.L., M.D., and G.T.: supervision; F.M. and M.A.P.: validation and visualization; F.M., M.A.P., and M.D.: writing – original draft; M.D. and G.T.: writing – review and editing. Then all authors read and approved the manuscript.

Conflicts of interest disclosure

The authors declare that they have no financial conflict of interest with regard to the content of this report.

Research registration unique identifying number (UIN)

Not applicable (retrospective observational study).

Guarantor

All the authors serve as guarantor.

Data availability statement

The data that support the findings of this study are available on request from the corresponding authors, [MD, GT]. The data are not publicly available.

Provenance and peer review

Not commissioned.

Acknowledgements

Assistance with the study: none.

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