

Morphometric Analysis of Mast Cells in Tumor Predicts Recurrence of Hepatocellular Carcinoma After Liver Transplantation

Nataliya Rohr-Udilova ¹,¹ Kaoru Tsuchiya,^{1,2} Gerald Timelthaler,³ Martina Salzmann,⁴ Tobias Meischl ¹,¹ Katharina Wöran,⁵ Judith Stift,⁵ Merima Herac,⁵ Rolf Schulte-Hermann,³ Markus Peck-Radosavljevic,⁶ Wolfgang Sieghart,⁷ Robert Eferl,³ Erika Jensen-Jarolim,^{4,8} Michael Trauner,¹ and Matthias Pinter ¹

Tumor-infiltrating immune cells are relevant prognostic and immunotherapeutic targets in hepatocellular carcinoma (HCC). Mast cells play a key role in allergic response but may also be involved in anticancer immunity. Digital morphometric analysis of patient tissue sections has become increasingly available for clinical routine and provides unbiased quantitative data. Here, we apply morphometric analysis of mast cells to retrospectively evaluate their relevance for HCC recurrence in patients after orthotopic liver transplantation (OLT). A total of 173 patients underwent OLT for HCC at the Medical University of Vienna (21 women, 152 men; 55.2 ± 7.9 years; 74 beyond Milan criteria, 49 beyond up-to-7 criteria for liver transplantation). Tissue arrays from tumors and corresponding surrounding tissues were immunohistochemically stained for mast cell tryptase. Mast cells were quantified by digital tissue morphometric analysis and correlated with HCC recurrence. Mast cells were detected in 93% of HCC tumors and in all available surrounding liver tissues. Tumor tissues revealed lower mast cell density than corresponding surrounding tissues ($P < 0.0001$). Patients lacking intratumoral mast cells (iMCs) displayed larger tumors and higher tumor recurrence rates both in the whole cohort (hazard ratio [HR], 2.74; 95% confidence interval [CI], 1.09–6.93; $P = 0.029$) and in patients beyond transplant criteria (Milan HR, 2.81; 95% CI, 1.04–7.62; $P = 0.01$; up-to-7 HR, 3.58; 95% CI, 1.17–10.92; $P = 0.02$). Notably, high iMC identified additional patients at low risk classified outside the Milan and up-to-7 criteria, whereas low iMC identified additional patients at high risk classified within the alpha-fetoprotein French and Metroticket criteria. iMCs independently predicted tumor recurrence in a multivariate Cox regression analysis (Milan HR, 2.38; 95% CI, 1.16–4.91; $P = 0.019$; up-to-7 HR, 2.21; 95% CI, 1.05–4.62; $P = 0.035$). **Conclusion:** Hepatic mast cells might be implicated in antitumor immunity in HCC. Morphometric analysis of iMCs refines prognosis of HCC recurrence after liver transplantation. (*Hepatology Communications* 2021;5:1939–1952).

Hepatocellular carcinoma (HCC) is one of the leading causes of death in the world, and its incidence is growing globally.^(1,2) Infection by hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, or fatty liver are important drivers of HCC development.^(3,4) Persistent liver inflammation is a common feature of the main HCC risk factors and is also significant for tumor development and progression.^(5,6)

Abbreviations: AFP, alpha-fetoprotein; AIC, Akaike information criteria; ALD, alcoholic liver disease; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; IgE, immunoglobulin E; IL, interleukin; iMC, intratumoral mast cell; NASH, nonalcoholic steatohepatitis; OLT, orthotopic liver transplantation; PEI, percutaneous ethanol injection; Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile; sMC, mast cell in surrounding tissue; TACE, transarterial chemoembolization.

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Therapeutic options for HCC include tumor resection or ablation, transarterial chemoembolization (TACE), treatment with tyrosine kinase inhibitors, immuno-oncologic agents, and liver transplantation.^(7,8) Liver transplantation is the only curative option for early HCC and requires mostly lifelong immunosuppressive medication.⁽⁹⁾ To select patients eligible for liver transplantation, current guidelines recommend applying Milan criteria or their extended variants,⁽¹⁰⁻¹⁶⁾ but no universally accepted consensus exists in this regard. For this reason, additional biomarkers for further refinement of transplant criteria are urgently needed, particularly in light of the worldwide dramatically increasing number of patients at risk for HCC in combination with the limited availability of transplant livers.⁽¹⁷⁾

The immune phenotype is a relevant prognostic factor in patients with cancer.^(18,19) We and others emphasized the differences in immune cell composition between tumor adjacent tissue and tumor tissue in HCC.^(20,21) Based on mathematical deconvolution of global gene expression data by the Cell-Type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) method,⁽²²⁾ we assessed the immune cell landscape of healthy human livers, HCC tumors, and HCC-adjacent tissues. By this approach, we found that patients with HCC lack or have decreased immunoglobulin E (IgE)-activated mast cells in their tumors.

An immunohistochemical mast cell staining in a small pilot cohort of 10 patients supported this finding.⁽²⁰⁾ However, additional comprehensive studies on the role of mast cells in HCC are still lacking.

Mast cells constitute less than 1% of all immune cells in humans and represent one of the most evolutionary conserved immune cell types.⁽²³⁾ Although mast cells are known as central players in allergic reactions,⁽²⁴⁾ they are also important for tissue homeostasis.^(25,26) IgE and microbial peptides as well as venoms can activate mast cells and cause degranulation with instant release of mediators, such as histamine, chymase, and tryptase.

Mast cells originate from hematopoietic precursors and become resident in organs where they get final differentiation.⁽²⁷⁾ The liver also contains hepatic mast cells, which are located close to hepatic arteries, veins, and bile ducts in the portal tracts.⁽²⁸⁻³⁰⁾

Data are insufficient with respect to the role of mast cells in HCC. Some authors have addressed correlations between mast cells and prognosis of patients with HCC but obtained controversial results.⁽³¹⁻³⁴⁾ Tu and colleagues⁽³¹⁾ described an inverse association of increased mast cell number with survival in a small cohort of 57 patients with HCC. In contrast, a comprehensive investigation in a larger cohort of 245 patients found a positive association between higher mast cell number and longer overall and disease-free survival after tumor resection.⁽³³⁾ All these studies

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ARTICLE INFORMATION:

From the ¹Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria; ²Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan; ³Institute of Cancer Research, Internal Medicine I, Medical University of Vienna and Comprehensive Cancer Center, Vienna, Austria; ⁴Institute of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology, and Immunology, Medical University of Vienna, Vienna, Austria; ⁵Clinical Institute of Pathology, Medical University of Vienna, Vienna, Austria; ⁶Internal Medicine and Gastroenterology, Central Admission, and First Aid, Public Hospital Klagenfurt am Woerthersee, Klagenfurt, Austria; ⁷IMed19, Vienna, Austria; ⁸Comparative Medicine, Interuniversity Messerli Research Institute of the University of Veterinary Medicine Vienna, Medical University of Vienna and University of Vienna, Vienna, Austria.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Nataliya Rohr-Udilova, Ph.D.
Division of Gastroenterology and Hepatology
Department of Internal Medicine III
Medical University of Vienna

Wachringuer Guertel 18-20
A-1090 Vienna, Austria
E-mail: nataliya.rohr-udilova@meduniwien.ac.at
Tel.: +43 1 40400 47440

focused mainly on patients infected with HBV after tumor resection, whereas other HCC etiologies were underrepresented or absent.⁽³¹⁻³⁴⁾

To the best of our knowledge, this is the first report on quantitative analysis of hepatic mast cells and their impact on HCC recurrence (and therefore prognosis) in a cohort of 173 patients after orthotopic liver transplantation (OLT).

Patients and Methods

The cohort comprised 173 patients with histologically confirmed HCC who underwent OLT between 1994 and 2014 at the Medical University of Vienna, Austria. The mean (\pm SEM) follow-up duration was 4.78 ± 0.39 years (95% confidence interval [CI], 3.81-5.33 years). Pretransplant alpha-fetoprotein (AFP) values were available for 103 patients and allowed calculations of HCC risk using AFP French⁽¹³⁾ and Metroticket 2.0⁽¹⁴⁾ scores. To identify patients at high risk, the cutoffs for AFP French score >2 and Metroticket score $>70\%$ were applied. The study protocol was approved by the local ethics committee and conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

TISSUE MICROARRAYS

Tissue arrays from HCC tumor tissues and corresponding surrounding tissues were constructed and included two cores per tumor tissue and one core per corresponding surrounding tissue for each patient. We calculated the mean mast cell density from the two tumor cores for each patient and used this value for further analysis. Tissue array core diameter was ~ 2 mm, and the mean core area was 4.1 ± 0.7 mm². One slide contained 48 tissue cores. Tissue arrays were stained immunohistochemically for the mast cell marker tryptase, as described.⁽²⁰⁾

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Mast cells were evaluated immunohistochemically by tryptase staining. After deparaffinization, we performed heat-induced epitope retrieval. The slides were cooled down, washed twice with phosphate-buffered saline (PBS), and permeabilized by 0.2% Tween in PBS. Unspecific background was blocked by 5% fetal bovine serum (FBS) in PBS for 30 minutes at room temperature.

Antibody mouse anti-human mast cell tryptase (clone AA1; BioRad) was diluted 1:10,000 in 5% FBS and incubated overnight. After the washing step, Dako polymer (horseradish peroxidase [HRP] Mouse Envision Kit; Dako, Agilent) was applied for 30 minutes at room temperature. 3,3'-Diaminobenzidine (Dako, Agilent) chromogen substrate was applied for 30 seconds, and the slides were washed with Aqua Dest. Counterstaining was performed by hematoxylin, and tryptase-positive cells were evaluated by tissue morphometric analysis of digitized slides, using Tissue Studio software (Definien, Munich, Germany). Slides were digitized using a Panoramic Midi Slide Scanner (3Dhistech, Budapest, Hungary) with 40 \times optical magnification. HCC tumor tissues from 173 patients were evaluated. Corresponding tumor adjacent tissues were available for 146 patients.

STATISTICS

Baseline characteristics were summarized using descriptive statistics. The chi-squared test was used to compare nominal data. A *t*-test or Wilcoxon test was used to compare metric data. Overall survival was defined as time from liver transplantation until date of death or last follow-up. Time to recurrence was defined as the time from liver transplantation until tumor recurrence; patients without recurrence were censored at the date of death or last follow-up.

The log-rank Mantel-Cox test was applied to compare Kaplan-Meier survival curves. Multivariate analyses were performed using Cox regression and presented with Akaike information criterion (AIC), which evaluates how the parameters (i.e., mast cell density, vascular invasion, tumor size, tumor number) affected the dependent variables as time to recurrence and patient survival. The lower the AIC, the more explanatory and informative the model is.⁽³⁵⁾ Statistical analyses were performed using SPSS 25.0 and GraphPad Prism 8 software (GraphPad Software, LLC).

Results

PATIENT CHARACTERISTICS

Our patient cohort included 152 men and 21 women. Of these, 74 patients were beyond Milan criteria and 49 patients were beyond up-to-7 extended criteria for liver transplantation. Sixty-six patients received locoregional therapies before liver transplantation, TACE

being the most frequent one. Clinical data and detailed patient characteristics are given in Table 1.

ASSOCIATION OF MAST CELL DENSITY WITH UNDERLYING ETIOLOGY

We detected mast cells in 93% of HCC tumor tissues (160 out of 173) and in all available corresponding tumor surrounding liver tissues ($n = 149$). Representative images of mast cell staining in tumor tissue and in corresponding surrounding tissue in patients with different etiologies (HCV, alcoholic liver disease [ALD], HBV, and nonalcoholic steatohepatitis [NASH]) are shown in Fig. 1. We applied digital tissue morphometric analysis in order to quantify mast cell density as the number of cells per mm^2 in each tissue core. Mast cell density within the tumor was lower than the corresponding surrounding tissue (9.1 ± 1.0 cells/ mm^2 in tumor vs. 20.3 ± 1.7 cells/ mm^2 in surrounding tissue, $P < 0.001$) (Fig. 2A).

Because the underlying etiology may affect immune cell distribution and composition, we further assessed mast cell density in patients with respect to underlying disease. Patients with the following etiologies revealed lower mast cell density in tumor than in surrounding tissue: ALD (10.1 ± 2.0 vs. 28.7 ± 3.5 cells/ mm^2 , $P < 0.01$), HCV (8.6 ± 1.3 vs. 19.5 ± 2.8 cells/ mm^2 , $P < 0.01$), and other minor etiologies (8.3 ± 3.4 vs. 21.2 ± 6.8 cells/ mm^2 , $P < 0.05$) (Fig. 2B-F). Patients with hepatitis B showed a similar trend (6.6 ± 1.5 vs. 13.7 ± 3.7 cells/ mm^2 , $P = 0.07$). In contrast, patients with NASH displayed no difference between mast cell density in tumor and in surrounding tissue (10.7 ± 3.5 vs. 10.3 ± 2.1 cells/ mm^2 , not significant).

Intratumoral mast cell (iMC) density remained similar between etiologies; however, the density of mast cells in surrounding tissues (sMCs) varied (Fig. 2G). The highest sMC density was observed in patients with ALD followed by HCV and minor etiologies. Patients with NASH showed the lowest sMC density, without any difference to the tumor tissue (Fig. 2G).

ASSOCIATION OF MAST CELL DENSITY WITH TUMOR CHARACTERISTICS

We further explored whether the lower density of mast cells in tumor had any correlates with clinical patient characteristics and outcome. Because Milan

TABLE 1. PATIENT CHARACTERISTICS

Parameter	Value*
Males, n	152 (88%)
Females, n	21 (12%)
Mean age, years	55.2 ± 7.9
Mean tumor size, cm	3.84 ± 3.56
Mean number of tumors	2.44 ± 1.65
Tumor grading	
G1	28 (16.2%)
G2	121 (70%)
G3	23 (13.3%)
Underlying disease	
HCV	71 (41.0%)
ALD	49 (28.3%)
NASH	22 (12.7%)
HBV	16 (9.3%)
AIH	7 (4.1%)
HBV/HCV coinfectd	4 (2.3%)
PBC/PSC	4 (2.3%)
Microvascular invasion	15 (8.7%)
Beyond Milan criteria	74 (42.8%)
Beyond up-to-7 criteria	49 (28.3%)
Locoregional therapies (yes/no/n.a.):	66 (38.2%)/101 (58.4%)/6 (3.4%)
TACE	21 (12.1%)
Radiofrequency ablation	9 (5.2%)
PEI	16 (9.2%)
Chemotherapy	8 (4.6%)
Resection	10 (5.8%)
Others	2 (1.2%)

*Unless indicated differently, values show number (% of all patients, $n = 173$) or number \pm SD.

Abbreviations: AIH, autoimmune hepatitis; n.a., no information available; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

criteria or their extended variants (the up-to-7 criteria, AFP French model, and Metroticket 2.0) represent valid tools to evaluate the risk of HCC recurrence following liver transplantation, we compared mast cell density between tumor and surrounding tissue for patients within and beyond these transplant criteria. Irrespective of transplant criteria, density of mast cells in tumors was consistently lower than in surrounding tissues (Fig. 3A-D). While the application of the Milan criteria did not further change the net decrease found in iMC density (Fig. 3A), patients beyond up-to-7 criteria showed lower iMC density than patients within (Fig. 3B). Thus, the mast cell gradient between surrounding tissue and tumor tissue persists independently of meeting transplant criteria.

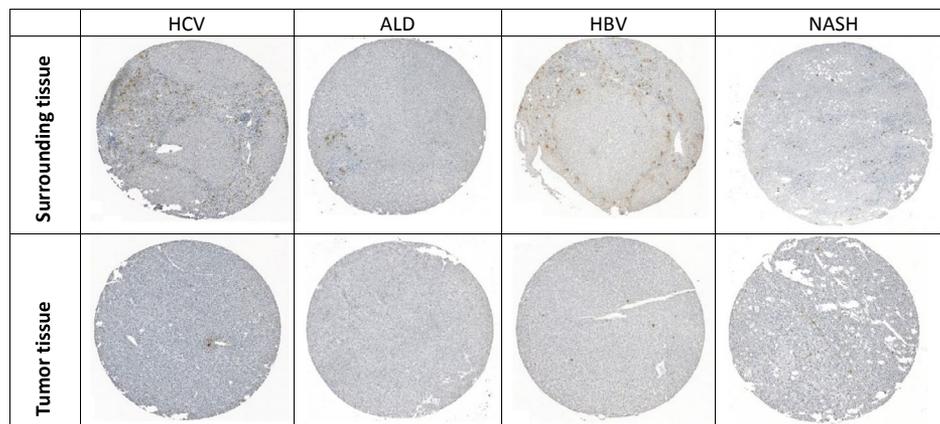


FIG. 1. Quantification of mast cells in HCC tumors and corresponding surrounding tissue in patients with different underlying diseases. Representative images of mast cell tryptase staining in patients with HCC with HCV, ALD, HBV, and NASH.

ASSOCIATION OF MAST CELL DENSITY WITH RECURRENCE

In our cohort, about 70% of all recurrences were registered during the first 3 years after transplantation (Supporting Fig. S2). Both sexes showed similar recurrence-free survival (data not shown). To further explore the clinical relevance of iMCs, we stratified patients according to the quartiles of iMC density (from Q₁, the lowest to Q₄, the highest) and analyzed recurrence rates. Patients within the lowest iMC quartile (iMC Q₁, low iMC) revealed the highest tumor recurrence of 46% within the first 3 years after liver transplantation (Fig. 3E). In contrast, patients from Q₂ to Q₄ iMC (high iMC) showed a 3-year recurrence rate between 9% and 17%. In comparison, 38.4% of patients beyond and 11.9% of patients within the Milan criteria developed tumor recurrence 3 years after transplantation. Tumor recurrence was significantly increased in patients with low iMC (Fig. 3F).

We reanalyzed the association between intratumoral mast cells and recurrence free survival for men and women separately. The results remained essentially the same in men (RFS 5,626 ± 346 days in patients with high iMC vs. 2,971 ± 553 days in patients with low iMC, $P = 0.0005$; log-rank Mantel-Cox test) but did not reach statistical significance in women (RFS 5,882 ± 1,125 days in patients with high iMC vs. 4,938 ± 653 days in patients with low iMC, $P = 0.534$; log-rank Mantel-Cox test). Response to TACE was not associated with altered mast cell density in tumor or in surrounding tissue (data not shown).

Furthermore, tumor size was larger in patients with the lowest Q₁ than in the highest Q₄ iMC quartile, whereas tumor number and vascular invasion remained similar (Supporting Fig. S3A). *In silico* analysis of proinflammatory and anti-inflammatory cytokines revealed that levels of interleukin (IL)-1b and IL-10 were higher in patients with low mast cells, whereas tumor necrosis factor α and IL-6 showed no differences between iMC quartiles (Supporting Fig. S4).

Importantly, clinicopathologic characteristics were similarly distributed between groups, except that women were overrepresented and patients within the AFP French score (≤ 2) were underrepresented in the low iMC group (Table 2).

To further explore the additional value of mast cells in HCC, we evaluated the impact of iMC density on tumor recurrence in patients within and beyond four common transplant criteria (Fig. 4A-D). Based on the percentage of patients meeting the criteria, rigor of transplant criteria for our cohort decreased in the following order: Milan > up-to-7 > AFP French > Metroticket, with Milan being the most rigorous (Fig. 4A-D). As expected, patients within the transplant criteria showed lower recurrence rates; however, significance was not reached for AFP French criteria.

We then stratified each group of patients either within or beyond the four transplant criteria according to low/high iMC (Fig. 4A-D). Notably, high iMC identified additional patients at low risk who were classified outside the rigorous criteria (Milan and up-to-7;

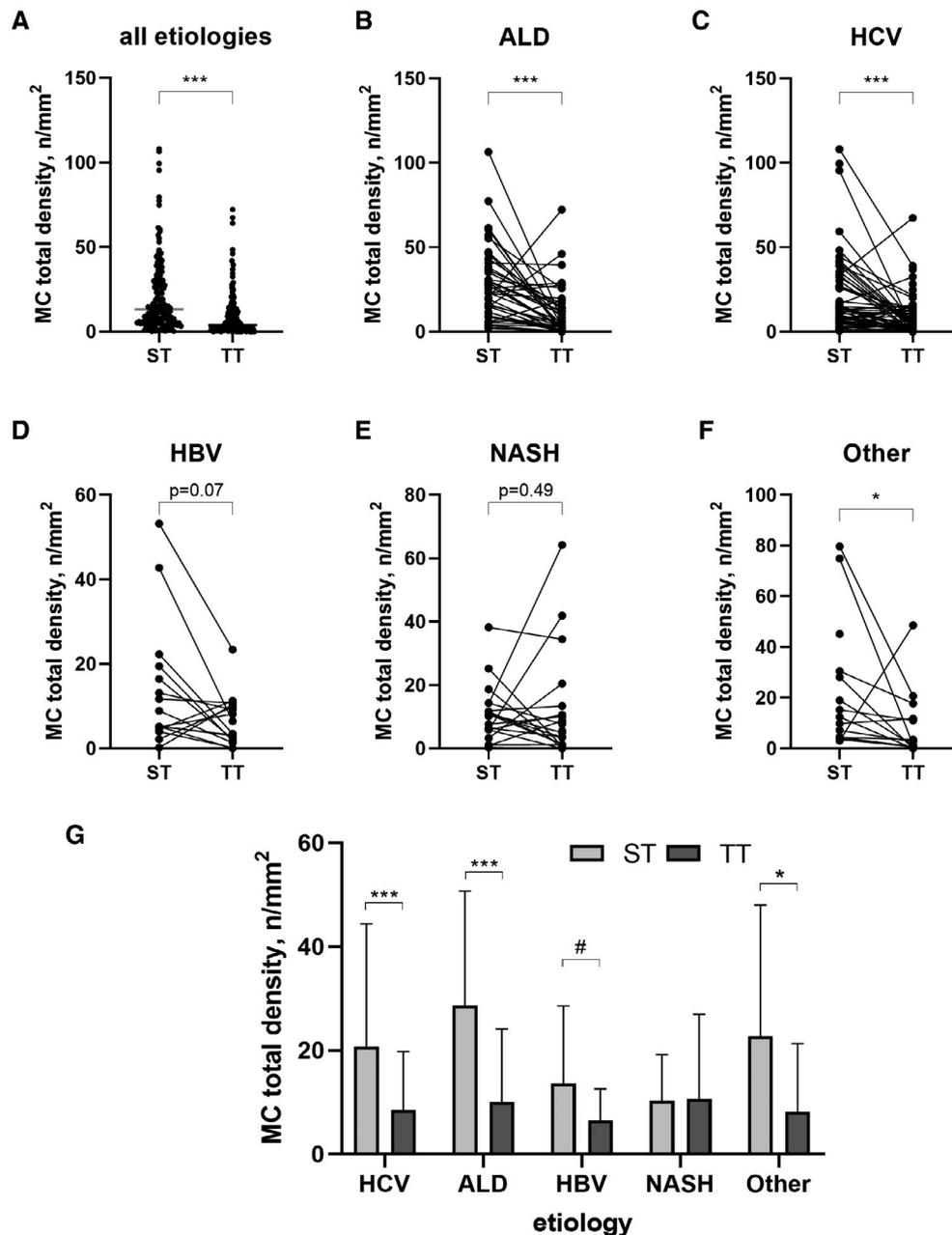


FIG. 2. Mast cell density in tumor tissue and in surrounding tissue of patients with HCC with different etiologies. (A) Whole patient cohort; (B-G) distinct etiologies (ALD, HCV, HBV, NASH, and minor etiologies). Mast cell density was quantified by tissue morphometric analysis in surrounding tissue and tumor tissue as number of cells per mm² tissue. Surrounding tissue and tumor tissue from the same patients are connected by a line. Wilcoxon matched-pairs signed-rank test was applied to compare mast cell density between ST and TT. *** $P < 0.0001$, * $P = 0.013$, # $P = 0.07$. Data show mean (box) and SD. Abbreviations: MC, mast cell; ST, surrounding tissue; TT, tumor tissue.

Fig. 4A,B), whereas low iMC identified additional patients at high risk who were classified within the less rigorous criteria (AFP French and Metroticket; Fig. 4C,D). Clinical patient characteristics, such as

age, sex, tumor size, tumor multiplicity, etiologies, tumor grading, and microvascular invasion, were similarly distributed between the high/low iMC groups (Supporting Tables S1 and S2).

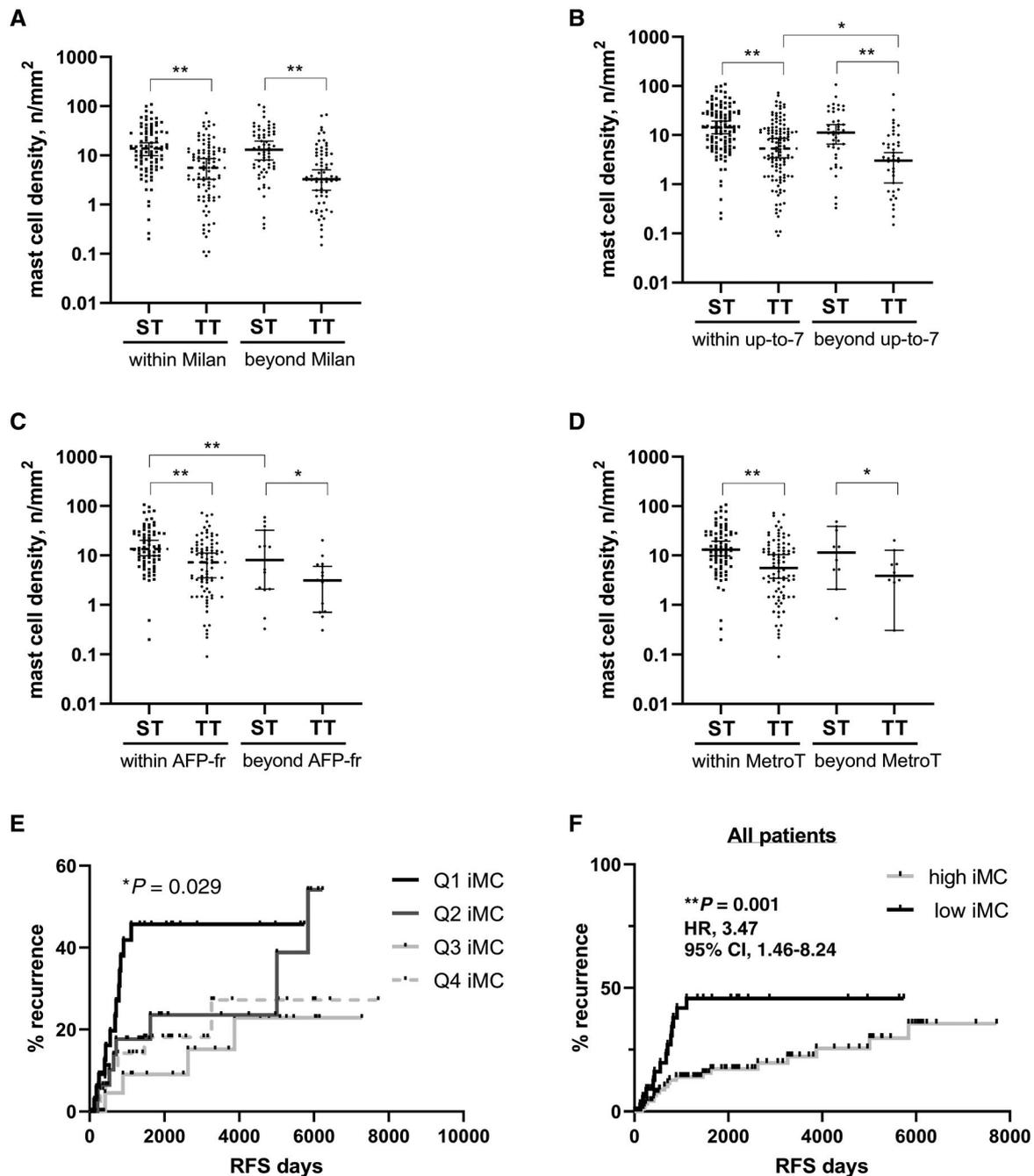


FIG. 3. Mast cell density in tumor tissue and surrounding tissue of patients with HCC in relation to the transplant criteria and clinical parameters. Mast cell density in tumor tissue and surrounding tissue in patients within and beyond (A) Milan criteria; (B) up-to-7 criteria; (C) AFP French criteria; (D) Metroticket criteria; $*P < 0.05$, $**P < 0.01$, analysis of variance Kruskal-Wallis test. Data show mean (horizontal line) \pm 95% CI. (E) RFS in patients stratified according to iMC density quartiles (Q₁, lowest to >Q₄, highest iMC; log-rank Mantel-Cox test. (F) RFS in all patients stratified according to low (Q₁)/high (Q₂-Q₄) iMC quartiles of mast cell density; log-rank Mantel-Cox test. Abbreviation: fr, French; RFS, recurrence-free survival; ST, surrounding tissue; TT, tumor tissue; TTR, time to response.

In addition to iMC density, we stratified patients according to mast cell density in surrounding tissues (sMC) and evaluated HCC recurrence. Both

recurrence-free survival and overall survival were similar between the sMC quartiles (Supporting Fig. S3B). Nevertheless, patients with low sMC showed a

TABLE 2. CORRELATIONS OF LOW AND HIGH iMCs WITH CLINICOPATHOLOGIC FEATURES IN THE WHOLE COHORT OF PATIENTS WITH HCC

Variable		iMC Low (Q1) n = 44	iMC High (Q2-Q4) n = 129	Chi-Quadrat Test P Value*
Sex	Female	10 (22.7%)	11 (8.5%)	0.029*
	Male	34 (77.3%)	118 (91.5%)	
Age, years	<55	16 (36.4%)	50 (38.8%)	0.813
	>55	28 (63.6%)	79 (61.2%)	
Milan criteria	Within	21 (47.7%)	78 (60.5%)	0.160
	Beyond	23 (52.3%)	51 (39.5%)	
Up-to-7 criteria	Within	26 (59.1%)	98 (76.0%)	0.051
	Beyond	18 (40.9%)	31 (24.0%)	
AFP French score*	Within	13 (65.0%)*	73 (88.0%)*	0.021*
	Beyond	7 (35.0%)*	10 (12.0%)*	
	n.a.	24	46	
Metroticket 2.0 score*	Within	18 (90%)*	75 (90.4%)*	0.999
	Beyond	2 (10%)*	8 (9.6%)*	
	n.a.	24	46	
Tumor size, cm	<5	33 (75.0%)	107 (82.9%)	0.270
	>5	11 (25.0%)	22 (17.1%)	
Tumor number	Single	16 (36.4%)	44 (34.1%)	0.855
	Multiple	28 (63.6%)	85 (65.9%)	
Etiology				0.346
HBV	No	42 (95.5%)	115 (89.1%)	
	Yes	2 (4.5%)	14 (10.9%)	
HCV	No	30 (68.2%)	72 (55.8%)	
	Yes	14 (31.8%)	57 (44.2%)	
ALD	No	29 (65.9%)	95 (73.6%)	
	Yes	15 (34.1%)	34 (26.4%)	
NASH	No	37 (84.1%)	114 (88.4%)	
	Yes	7 (15.9%)	15 (11.6%)	
Other etiologies	No	38 (86.4%)	120 (93.0%)	
	Yes	6 (13.6%)	9 (7.0%)	
T stage	1/2	19 (43.1%)	72 (55.8%)	0.278
	3/4	22 (50.0%)	53 (41.0%)	
	n.a.	3 (6.9%)	4 (3.2%)	
Tumor grading	1	11 (25.0%)	17 (13.2%)	0.095
	2/3	33 (75.0%)	112 (86.8%)	
Vascular invasion	No	40 (90.9%)	118 (91.5%)	0.999
	Yes	4 (9.1%)	11 (8.5%)	
Pretransplant locoregional therapy	No	26 (59.1%)	75 (58.1%)	0.857
	Yes	16 (36.4%)	50 (38.8%)	
	n.a.	2 (4.5%)	4 (3.1%)	
Art of locoregional therapy				0.053
TACE		2 (12.5%)*	19 (38%)*	
PEI		3 (18.8%)*	13 (26%)*	
Radiofrequency ablation		1 (6.3%)*	8 (16%)*	
Resection		5 (31.2%)*	5 (10%)*	
Chemotherapy		5 (31.2%)*	3 (6%)*	
Others		0 (0%)*	2 (4%)*	

* $P < 0.05$ is considered significant.

†103 patients were evaluated for Metroticket 2.0 and AFP French scores.

‡Percentages were calculated setting the number of patients with locoregional therapies as 100% (n = 16 for low iMC and n = 50 for high iMC).

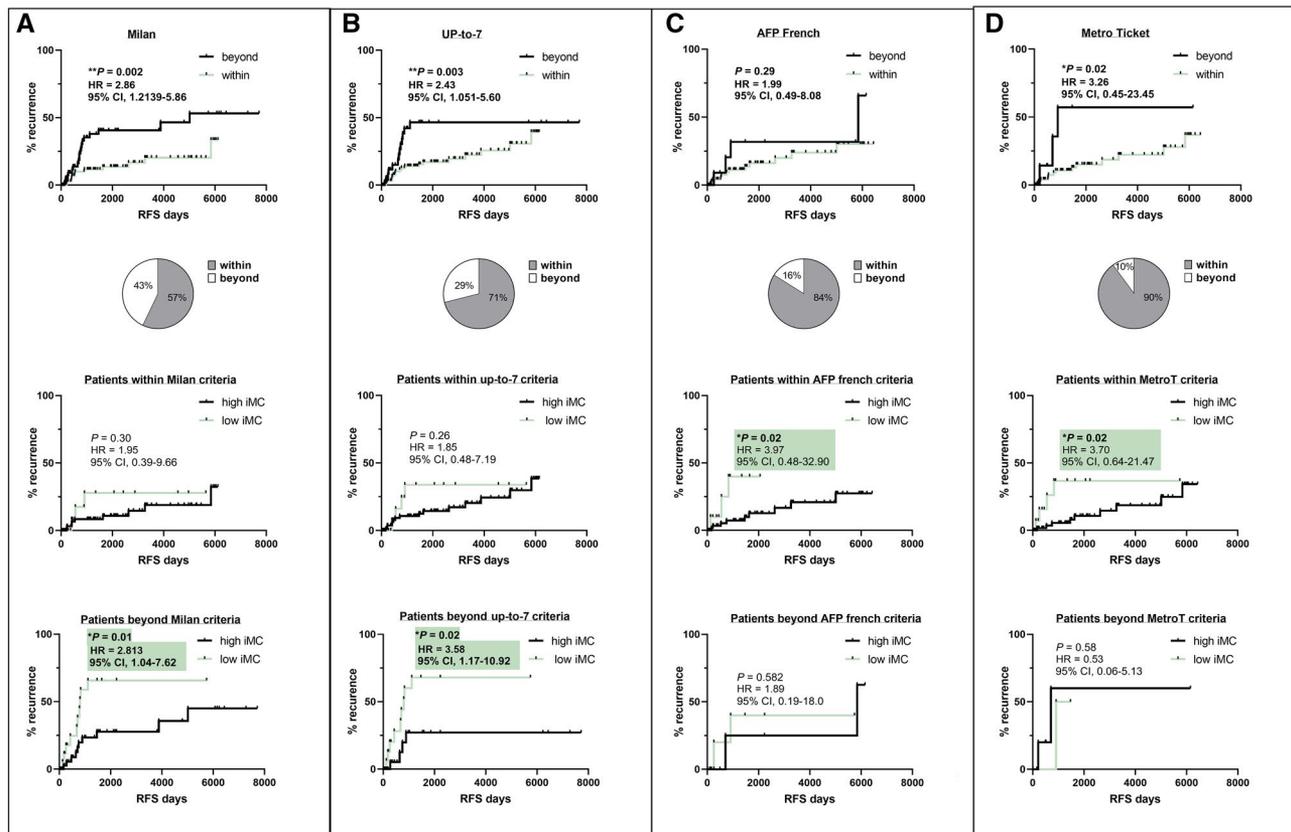


FIG. 4. iMC and recurrence-free survival of patients with HCC after OLT. (A) Milan criteria; (B) up-to-7 criteria; (C) AFP French criteria; (D) Metroticket criteria. Each panel shows RFS in the whole cohort of patients within and beyond the criteria, percentage of patients fitting the criteria, RFS of patients within criteria stratified into low and high iMC groups, and RFS of patients beyond criteria stratified into low and high iMC groups. The green shaded boxes mark significant recurrence differences ($P < 0.05$ Log-rank Mantel-Cox test) between patients with low and high iMC density. P value, HR, and 95% CI of ratio are shown to compare tumor recurrence among the groups. Abbreviation: RFS, recurrence-free survival.

trend to early tumor recurrence within 2 years after transplantation (median cut-off sMC, 13.0 cells/mm²; Supporting Fig. S3C). sMC density had a weak positive correlation with iMC density (Pearson $r^2 = 0.164$, $P = 0.046$, $n = 146$).

In order to evaluate whether mast cell density independently predicts tumor recurrence, we conducted univariate and multivariate Cox regression analysis. The performance of comparable models was quantified by means of AIC, with a lower AIC value indicating better performance.

In univariate analysis, lack of iMCs was associated with higher tumor recurrence rates (Table 3). Overall survival showed a similar trend that reached statistical significance only for absolute iMC values (Supporting

Table S3). Hazard ratios (HRs) for iMCs were comparable with that of transplantation criteria.

In multivariate analysis, inclusion of iMCs in addition to the established transplant criteria (Milan and up-to-7) always improved model precision, as shown by a drop in the corresponding AIC values (e.g., AIC, 273.371 for Milan criteria vs. AIC, 270.206 for a multivariate combination of Milan criteria with iMCs) (Table 3; Supporting Table S3). Performance of iMCs as a binary categorical variable, which indicates belonging to the Q1 low iMC group, was stronger than that of iMC absolute values. Belonging to the lowest iMC Q1 group was an independent predictor of tumor recurrence after liver transplantation as it significantly contributed to the multivariate Cox

TABLE 3. UNIVARIATE AND MULTIVARIATE ANALYSIS OF iMCs AS AN INDEPENDENT PREDICTOR OF RECURRENCE-FREE SURVIVAL IN PATIENTS WITH HCC AFTER LIVER TRANSPLANTATION

Variables	Exp(B) HR	95% CI for HR		PValue	Chi-Quadrat	df	PValue	AIC
		Exp(B)						
Univariate Analysis								
Milan	2.87	1.40-5.91		0.00*	8.69	1	0.00*	273.371
Up-to-7	2.45	1.21-4.99		0.01*	5.65	1	0.02*	276.416
AFP French	1.38	1.04-1.83		0.02*	5.32	1	0.04*	nd
Metroticket	0.97	0.94-0.99		0.04*	4.25	1	0.04*	nd
iMC absolute	0.98	0.95-1.02		0.28	1.41	1	0.24	280.653
iMC Q1	2.57	1.25-5.27		0.01*	6.10	1	0.01*	275.968
iMC quartiles				0.04*	9.01	3	0.03*	273.912
Q1 (low) vs. Q4 (high)	2.74	1.09-6.93		0.03*				
Q2 vs. Q4	1.36	0.49-3.76		0.55				
Q3 vs. Q4	0.68	0.20-2.31		0.53				
Multivariate Analysis								
Model 1: Milan	2.73	1.33-5.61		0.01*	15.21	2	0.00*	270.206
iMC Q1	2.38	1.16-4.91		0.02*				
Model 2: Up-to-7	2.10	1.01-4.34		0.04*	11.57	2	0.00*	274.235
iMC Q1	2.21	1.06-4.62		0.04*				
Model 3: AFP French	1.27	0.91-1.77		0.16	7.54	2	0.02*	nd
iMC Q1	2.23	0.65-7.69		0.20				
Model 4: Metroticket	0.97	0.944-1.01		0.23	7.31	2	0.03*	nd
iMC Q1	2.62	0.78-08.81		0.12				

*Significant at $P < 0.05$.

Abbreviations: df, degrees of freedom; Exp(B), exponentiation of the B coefficient; HR, hazard ratio; nd, not determined.

regression models for both established transplant criteria (Milan and up-to-7; Table 3). Multivariate Cox regression analysis, which combined iMCs with AFP French or Metroticket, also showed significant performance as whole models (Table 3). Thus, mast cell density provides complementary information relevant for HCC recurrence after liver transplantation.

Discussion

In this study, we describe for the first time a relevant association between iMCs and tumor recurrence in patients with HCC who underwent liver transplantation. To obtain quantitative results for mast cell density, we applied morphometric analysis of digitized microscopic images of immunohistochemically stained tissue microarray paraffin sections of liver tissue of patients with HCC. Previous studies on mast cells applied manual cell counting, which can be prone to subjective perception. In contrast, digital algorithm-based morphometric analysis allows determining cell numbers exactly and reproducibly.

Tumor microenvironment and specifically immune cells play a critical role in tumorigenesis.^(36,37) Moreover, immune cell infiltration and composition also stratify patients into responders and nonresponders to anticancer therapies.^(18,38-40) Our previous investigations suggested that the total extent of immune cell infiltration is altered in HCC and that relative abundance, specifically of mast cells, differs between surrounding tissue and tumor tissue.⁽²⁰⁾ In line with this, we show here that mast cell infiltration in tumors is lower than in surrounding tissues and may reflect general immunosuppression induced by the tumor, possibly paralleling progression. Ongoing mechanistic studies address the question of whether mast cells are the driver or bystander of antitumor immunity and can be targeted to overcome tumor-induced immunosuppression.

We also observed that HCC etiology may impact mast cell infiltration, as ALD, hepatitis C, and tendentially hepatitis B showed decreased mast cell infiltration into tumors while NASH did not. Although exploring potential reasons for these interesting

differences is beyond the scope of our study, these data suggest that HCC of NASH etiology may be particularly attractive for mast cell targeting.

Our results on the beneficial association between iMCs and tumor recurrence in HCC are in accordance with previous studies in other tumor types. In particular, patients with colon carcinoma and pulmonary adenocarcinoma with higher iMC numbers showed longer survival.⁽⁴¹⁻⁴³⁾ Similarly, enhanced levels of mast cells were also associated with better prognosis in breast cancer.⁽⁴⁴⁾ A comprehensive study using tissue arrays from patients with breast cancer revealed significantly prolonged disease-free survival in patients with at least one mast cell present per 0.6 mm² of tissue, a core surface of tumor sample on the array.⁽⁴⁵⁾ The analyzed core surface in our study was larger (4.1 ± 0.7 mm²), but the cut-off value reported by Dabiri et al.⁽⁴⁵⁾ is close to that absolute cutoff for the lowest iMC density Q1 quartile of 1.2 cells/mm² calculated in our study. The effect of mast cell density seems to be nonlinear dichotomous as the performance of the absolute iMC values is worse than that of categorical iMC. Our data indicate that reaching the threshold above 1.2 mast cells/mm² is crucial, while an increase of mast cell numbers above this threshold is not associated with further improvement in patient survival.

The beneficial role of mast cells in HCC after liver transplantation reported here does not necessarily contradict their protumorigenic role reported elsewhere.⁽²⁷⁾ Although mast cell-derived mediators can favor tumor cell proliferation, tissue remodeling, matrix degradation, and angiogenesis,⁽⁴⁶⁾ protumorigenic and antitumorigenic effects of mast cells in cancer may change depending on tumor site, mast cell differentiation, disease stage, and immune cell composition.

The presence of iMCs in HCC was protective in our cohort, but an excess of mast cells can also be protumorigenic. In particular, introduction of mast cells increased hepatic vascular endothelial growth factor (VEGF)-A and VEGF-C formation in a transforming growth factor β 1-dependent manner,^(47,48) which can favor angiogenesis, epithelial-mesenchymal transition, and tumor growth. Because cholangiocarcinomas contain about 5 times more mast cells compared to HCC,⁽⁴⁹⁾ protumorigenic effects can prevail in cholangiocarcinoma.

Mast cells might have a particular role in our cohort of liver transplant recipients who receive

immunosuppression in order to prevent or to treat graft rejections.⁽¹⁰⁾ T and B lymphocyte proliferation in transplant recipients is pharmacologically impaired, whereas slow proliferating mast cells might become even more relevant for immune surveillance under such conditions.⁽⁵⁰⁾ Mast cells can act as effector cells, interact with other immune cell types, and be directly cytotoxic to tumor cells, thus leading to an antitumor immune response.⁽⁴⁶⁾

Our results raise the question of whether targeted manipulation of hepatic mast cells could improve current therapeutic options for patients with HCC. Some recent reports provide data in favor of this concept. One case report describes complete regression of metastatic HCC in a patient who experienced radiocontrast-induced anaphylactic shock.⁽⁵¹⁾ The authors hypothesized that activation of mast cells due to anaphylaxis might have stimulated activation of natural killer (NK) cells in this patient and subsequent tumor regression.⁽⁵¹⁾ Indeed, mast cells can induce selective chemotaxis of NK cells,⁽⁵²⁾ which are otherwise dysfunctional in HCC.⁽⁵³⁾ In addition, direct cytotoxicity of mast cells has been reported⁽⁵⁴⁾ and might have also contributed to tumor regression in this case.

Another indication for the potential involvement of mast cells in the antitumor response might come from studies on the tyrosine kinase inhibitor sorafenib. Sorafenib is an approved drug for HCC treatment and may cause side effects, like itch, mucositis, and rash.⁽⁵⁵⁾ More pronounced dermatologic side effects indicate better tumor response to sorafenib.^(56,57) In addition to its direct multikinase-inhibiting antitumorigenic effects, sorafenib is also known to induce degranulation of primary dermal mast cells.⁽⁵⁸⁾ It is attractive to speculate that hepatic mast cells also degranulate following sorafenib treatment and help to eliminate tumor cells by direct cytotoxic mechanisms. In our cohort, sorafenib did not influence the data as most of the patients from our cohort had received a transplant before 2009 and did not receive sorafenib medication. We also did not observe any significant correlations between pretransplant locoregional therapies and iMCs, although TACE and percutaneous ethanol injection (PEI) in radio frequency showed a trend toward enrichment in the high iMC group (Table 2).

We could not draw any statistically sound conclusions concerning mast cells and survival in women

because of the small number of female patients in our study. This is a limitation of our study. However, the sex distribution of our patients reflects the real HCC epidemiology. To address mast cells specifically in female patients with HCC after liver transplantation, multicentric efforts would be necessary.

Epidemiological associations between IgE and lower cancer incidence may provide another supportive argument in favor of an antitumorigenic role of mast cells. Mast cells express IgE receptor, and IgE stimulates mast cell degranulation in the allergic response. At the same time, IgE seems to protect against cancer.⁽⁵⁹⁻⁶¹⁾ IgE-induced mast cell degranulation might be one of the mechanisms behind the cancer protective function of IgE. Therapeutic strategies to recruit mast cells into tumors may improve recurrence-free survival of patients with HCC after liver transplantation.

The following three aspects define the strength of the current study: 1) unbiased digital morphometric mast cell quantification; 2) tumor tissue and corresponding surrounding tissue of the same patients in tissue array format; and 3) availability of clinical data with long term follow-up in a comprehensive cohort of 173 liver transplant recipients with HCC. Additional studies in independent cohorts of liver transplant recipients with HCC would provide further proof of our findings.

As to the practicability of iMC analysis presented here, at least a liver biopsy is a prerequisite. Here, we analyzed $4.1 \pm 0.7 \text{ mm}^2$ of tissue area per sample to quantify iMCs as it was the mean core area on the tissue array. In comparison, a liver biopsy with a 20-mm length and ~1-2-mm width would provide about 20-40 mm^2 tissue area, which is 4 times to 10 times more than what we used here and would be sufficient. Thus, although we conducted our study in the explanted livers, liver biopsy can also be suitable for iMC quantification.

When comparing the strictness of the iMC approach with other transplant criteria in our cohort, we place iMCs in the middle as follows: Milan > up-to-7 > iMCs > AFP French > Metroticket (compare with Fig. 4). Indeed, when criteria are rigorous, - as Milan and up-to-7, - some patients with low recurrence risk may be misclassified as being beyond the criteria; in this case, iMCs may help select some additional patients at low risk, HRs being 2.8 and 3.6, respectively (Fig. 4A,B). However, in the case when

criteria are less rigorous and allow inclusion of more patients, - as AFP French and Metroticket, - some patients with high recurrence risk may be misclassified as fitting into the criteria; in this case iMCs may help to select additional patients with a high risk of recurrence, HRs being 3.97 and 3.70, respectively (Fig. 4C,D).

As the data on neutrophil-to-lymphocyte ratio and AFP response to locoregional therapies were not complete in our cohort, we could not directly compare the power of iMCs with more recently developed liver transplant scores, such as Model of Recurrence After Liver Transplantation (MoRAL) and New York/California (NYCA).^(15,16,62) However, both, MoRAL and NYCA scores use AFP values, similar to AFP French and Metroticket criteria. As iMCs additionally refined patients within the two AFP-dependent scores (AFP French and Metroticket; Fig. 4C,D), we suggest that iMCs could bring additional value in liver transplantation as an AFP-independent prognostic marker. Further validation studies are required.

The iMC approach requires tumor material for immunohistochemical staining; it is invasive and cannot replace the well-established noninvasive transplant criteria. Rather, iMC quantification may help to refine the patients questionably classified beyond the Milan or up-to-7 criteria but within the AFP French or Metroticket criteria.

In summary, our current study confirms iMCs as positive prognostic markers in HCC treated by liver transplantation and provides the basis for further interventional studies on hepatic mast cells as a new potential therapeutic target in HCC.

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Autors names in bold designate shared (co-first) authorship.

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