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Hepatic CD4 T Cells Predict Hepatocellular Carcinoma Risk on Metabolic Dysfunction-Associated Steatohepatitis Patients

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ABSTRACT

Background & Aims: Metabolic dysfunction-associated steatohepatitis (MASH) increasingly drives hepatocellular carcinoma (HCC) development. We characterized inflammatory infiltrates in liver biopsies from MASH patients who developed HCC versus controls to identify predictive immune signatures.

Method: Formalin-fixed paraffin-embedded (FFPE) liver biopsies from MASH patients were categorized as pre-HCC MASH ($n = 10$) or control MASH ($n = 13$) by the ESCALON consortium. Standardized histological analysis and multiplexed immunohistochemistry were performed targeting CD4, CD8, PD1, PDL1, FoxP3, CXCR6, CD3, CD68, and CD20 using a PhenoImager Fusion scanner. Single-cell RNA-seq datasets characterized hepatic CD4 T cell heterogeneity. Clinical parameters measured included ALT, AST, GGT, alkaline phosphatase, platelets, and INR.

Results: Pre-HCC MASH showed inflammation extending from portal to periportal areas versus portal-only distribution in controls. Analysis of 291,908 cells revealed significantly higher CD4+ density ($p = 0.0243$) and CD4+PD1+ cells ($p = 0.017$) in pre-HCC patients, while CD8+ and regulatory T cell densities remained unchanged. Single-cell RNA-seq identified potential phenotypic shifts from Th1 cytotoxicity toward tissue-repair and Th17 CD4+ T cells in MASH livers. Combined immunological and clinical variables (sex, age, CD4+ T cell numbers, ALT, alkaline phosphatase and platelets) achieved excellent predictive performance (ROC-AUC = 0.944) for HCC development.

Conclusions: Increase in liver CD4+ T cell infiltration characterizes MASH-to-HCC progression. These immune signatures combined with clinical parameters demonstrate remarkable predictive value for identifying high-risk MASH patients.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; EASL, European Association for the Study of the Liver; HCC, Hepatocellular carcinoma; MASH, Metabolic dysfunction-associated steatohepatitis.

The last two authors share senior authorship.

Key Summary

- Summarise the established knowledge on this subject
  - Metabolic dysfunction-associated steatohepatitis (MASH) represents a growing etiology of hepatocellular carcinoma (HCC).
  - Animal models suggest that CD8+ T cells drive hepatic inflammation in MASH, while CD4+ T cells may both promote inflammation and serve tumor suppression functions through heterogeneous subpopulations.
  - Current blood-based biomarkers demonstrate insufficient predictive capabilities for early-stage HCC detection, particularly in resource-limited regions where late detection restricts treatment options.
  - Liver inflammation is the primary driver of HCC development, yet immunological mechanisms underlying HCC progression in MASH patients remain poorly defined.
- What are the significant and/or new findings of this study?
  - CD4+ T cell infiltration, particularly PD1+ expressing cells, significantly characterizes MASH patients who subsequently develop HCC, with two-fold higher density compared to controls.
  - Single-cell analysis suggested potential phenotypic shifts from cytotoxic Th1 toward tissue-repairing and Th17-resident CD4+ T cell subpopulations in MASH livers.
  - Combined immunological signatures with clinical parameters (CD4+ cells, ALT, alkaline phosphatase and platelets) achieved exceptional predictive performance (ROC-AUC = 0.944) for HCC development.
  - International normalized ratio (INR) emerged as a novel standalone biomarker with remarkable discriminatory power, particularly in non-cirrhotic, non-diabetic MASH patients.

1 | Introduction

Hepatocellular carcinoma (HCC) represents the third leading cause of cancer-related deaths worldwide [1, 2]. Patients with HCC experience notably high mortality rates, particularly in low-income regions, with only an 18% 5-year survival rate due to late-stage detection [3–5]. While current blood-based protein biomarkers provide valuable diagnostic insights, they nonetheless demonstrate insufficient predictive capabilities, especially for early-stage HCC [6–8]. Modern HCC treatment modalities have significantly improved patient outcomes through surgical and interventional approaches [3]. However, systemic therapies for advanced-stage disease continue to demonstrate limited efficacy, presenting a significant clinical challenge. Furthermore, recent clinical trials investigating checkpoint inhibitors in HCC patients have shown unresponsiveness or potentially negative outcomes [9]. Consequently, understanding the pathophysiological mechanisms underlying liver cancer development is critical for developing better early detection strategies and targeted therapeutic interventions to enhance patient survival.

Liver inflammation emerges as the primary driver of HCC development [10, 11]. The etiology of inflammatory processes leading to HCC has undergone substantial evolution, transitioning from traditionally prevalent infectious or toxic causes—such as hepatitis B virus (HBV) and hepatitis C virus (HCV), chronic alcohol consumption, and dietary toxin exposure—to an increasing prevalence of non-infectious, non-toxic etiologies. Notably, Metabolic dysfunction-associated steatotic liver disease (MASLD) progressing to Metabolic dysfunction-associated steatohepatitis (MASH) now represents a critical precursor to HCC, particularly in South American populations [5, 12]. Animal model studies have suggested multiple inflammatory mechanisms underlying HCC pathogenesis. Experimental models induced by a high-fat diet and choline-deficient conditions or fah-deficient suggest a pivotal role for autoreactive CD8+ T cells in driving hepatic inflammation [13, 14], observations subsequently corroborated in human HCC patients [15]. Conversely, genetic models with intrinsic hepatocyte alterations for HCC development indicate that CD4+ T cells can promote inflammation [16] but may simultaneously serve tumor suppression functions and revealed heterogenous CD4 T cell subpopulations characterized by single-cell RNAseq techniques involved in the progression of MASH into HCC [9, 17]. Moreover, the spatial distribution of inflammatory cells within liver architecture and the potential influence of microbiota composition may differentially impact HCC development contingent upon the underlying inflammatory etiology [18–20]. Understanding the complex pathogenic mechanisms of HCC development therefore necessitates a comprehensive, multifaceted evaluation of inflammatory changes associated with disease progression.

We performed the first precise quantification of cellular inflammatory components in liver biopsies from MASH patients who subsequently developed HCC. We hypothesized that inflammatory infiltrate, particularly CD4 T cells, in liver biopsy from MASH patients predicts HCC development. Here, we show that CD4 T cell numbers integrated with routine clinical measurements—including liver enzyme levels, platelet counts, and coagulation capacity—could predict future HCC occurrence in MASH patients.

2 | Methods

This retrospective study analyzed MASH patients from three international centers (Argentina, Colombia, Netherlands) stratified into pre-HCC MASH (n = 10) and control MASH (n = 13) groups based on subsequent HCC development. Formalin-fixed paraffin-embedded liver biopsies underwent standardized histological analysis and multiplex immunohistochemistry using panels targeting CD3, CD68, CD20, CD4, CD8, PD1, PDL1, FoxP3 and CXCR6. Images were acquired using a PhenoImager Fusion scanner with automated cell segmentation and phenotyping via InForm software. Clinical parameters (ALT, AST, GGT, alkaline phosphatase, platelets, INR) were measured by routine diagnostics. Single-cell RNA-seq datasets (GSE159977) were reanalyzed using Seurat v4.0.5 to characterize hepatic CD4 T cell heterogeneity. Statistical analysis included Mann–Whitney U tests, ROC curves, machine learning approaches and logistic regression with $p < 0.05$ considered significant. Analysis and graphs were performed in R studio

(version 2024.04.02) and GraphPad Prism (version 9.4.1). The complete methods section can be found in the Supporting Information S1.

3 | Results

3.1 | Demographic and Histological Data of Patient Cohort

This study examined 23 individuals with MASH: 10 pre-HCC MASH patients and 13 control MASH patients. Demographic characteristics and histological findings from non-tumoral liver biopsies are detailed in Table 1. Median ages were 57 and 51 years, respectively, with male predominance in both groups (pre-HCC: 70%, control: 62%). Histological analysis of liver biopsies revealed varying degrees of steatosis across both groups. Notably, 70% (n = 7) of MASH pre-HCC patients exhibited fibrosis stage > F2 compared to 38.4% (n = 5) of controls. Cirrhosis presence was significantly higher in pre-HCC MASH (40% vs. 0%, $p = 0.024$). The average interval between biopsy and HCC diagnosis was 1.8 years for the pre-HCC MASH group, while the average follow-up time for the control MASH group was 4.28 years.

TABLE 1 | Demographic and histological features of the patient cohort.

	MASH control (n = 13)	MASH pre-HCC (n = 10)	p-value
Age (years)			
Median	51	57	0.391
Range	38–67	42–81	
25th percentile	44.50	46.75	
75th percentile	63.50	64.50	
Sex, n (%)			
Male	8 (62%)	7 (70%)	0.999
Female	5 (38%)	3 (30%)	
Country, n (%)			
Argentina	4 (30.8%)	5 (50%)	0.436
Colombia	4 (30.8%)	1 (10%)	
The Netherlands	5 (38.4%)	4 (40%)	
Steatosis, n (%)			
Minimal	0 (0%)	1 (10%)	0.139
Mild	3 (23.1%)	6 (60%)	
Moderate	6 (46.1%)	2 (20%)	
Severe	4 (30.8%)	1 (10%)	
Fibrosis > F2, n (%)			
Yes	5 (38.4%)	7 (70%)	0.213
No	8 (61.6%)	3 (30%)	
Cirrhosis, n (%)			
Yes	0 (0%)	4 (40%)	*0.024
No	13 (100%)	6 (60%)	
Diabetes, n (%)			
Yes	6 (46%)	5 (50%)	0.8584
No	7 (54%)	5 (50%)	
Treatment, n (%)			
Statins	7 (53.8%)	3 (30%)	0.999
Metformins	5 (38.5%)	3 (30%)	
Time from biopsy, mean ± SD (years)			
	4.28 ± 1.87 (FU)	1.81 ± 2.28 (HCC Dx)	*0.0169

Abbreviations: Dx: diagnosis, FU: Follow-up time, SD: standard deviation.
*$p < 0.05$.

3.2 | Pre-HCC MASH Patients Exhibited Higher Immune Cell Infiltration in the Liver

Control MASH patients presented low-to-moderate infiltration in portal areas (shown in Figure 1A), while pre-HCC MASH patients showed moderate-to-high infiltration extending from portal to periportal areas (shown in Figure 1B). Other histological differences included the presence of necrosis and multiple regenerative nodules in pre-HCC MASH patients in contrast to control MASH patients (shown in Figure 1A and B). To comprehensively characterize the infiltrating immune cell populations, we conducted multiplex immunostaining (shown in Figure 1C and D, Supporting Information S1: Figure 2).

Spatial distribution analysis demonstrated predominant immune cell accumulation in portal areas in both groups, with varying degrees of infiltrate diffusion into the periportal and lobular areas (shown in Figure 1E). No infiltrates were observed in central areas of pre-HCC MASH patients. Assessment of total CD3+ T cells (shown in Figure 1F), CD68+ macrophages (shown in Figure 1G) and CD20+ B cells (shown in Figure 1H) showed no significant differences between MASH control and MASH pre-HCC groups.

3.3 | CD4+ T Cells Are Elevated in the Immune Cell Infiltrate of Pre-HCC MASH

Multiplex immunofluorescence demonstrated the predominant presence of CD4 and CD8 T cells showing different intensities of PD1 and CXCR6 staining in the immune infiltrates of liver biopsies from pre-HCC and control MASH (shown in Figure 2A and B). While CD8+ T cell analysis showed no significant differences in total CD8+ cells, CXCR6+CD8+ cells, or CD8+PD1+ cells between groups (shown in Figure 2C–E), CD4+ cell analysis revealed significantly higher mean density in pre-HCC MASH compared to control MASH samples (p = 0.0243; shown in Figure 2F), representing a two-fold enrichment. The majority of CD4+ cells were confirmed as T lymphocytes rather than CD4+ macrophages (shown in Supporting Information S1: Figure 3). No significant differences between the MASH groups were observed in the presence of CD4+ cells expressing the CXCR6 marker (CD4+ CXCR6+)

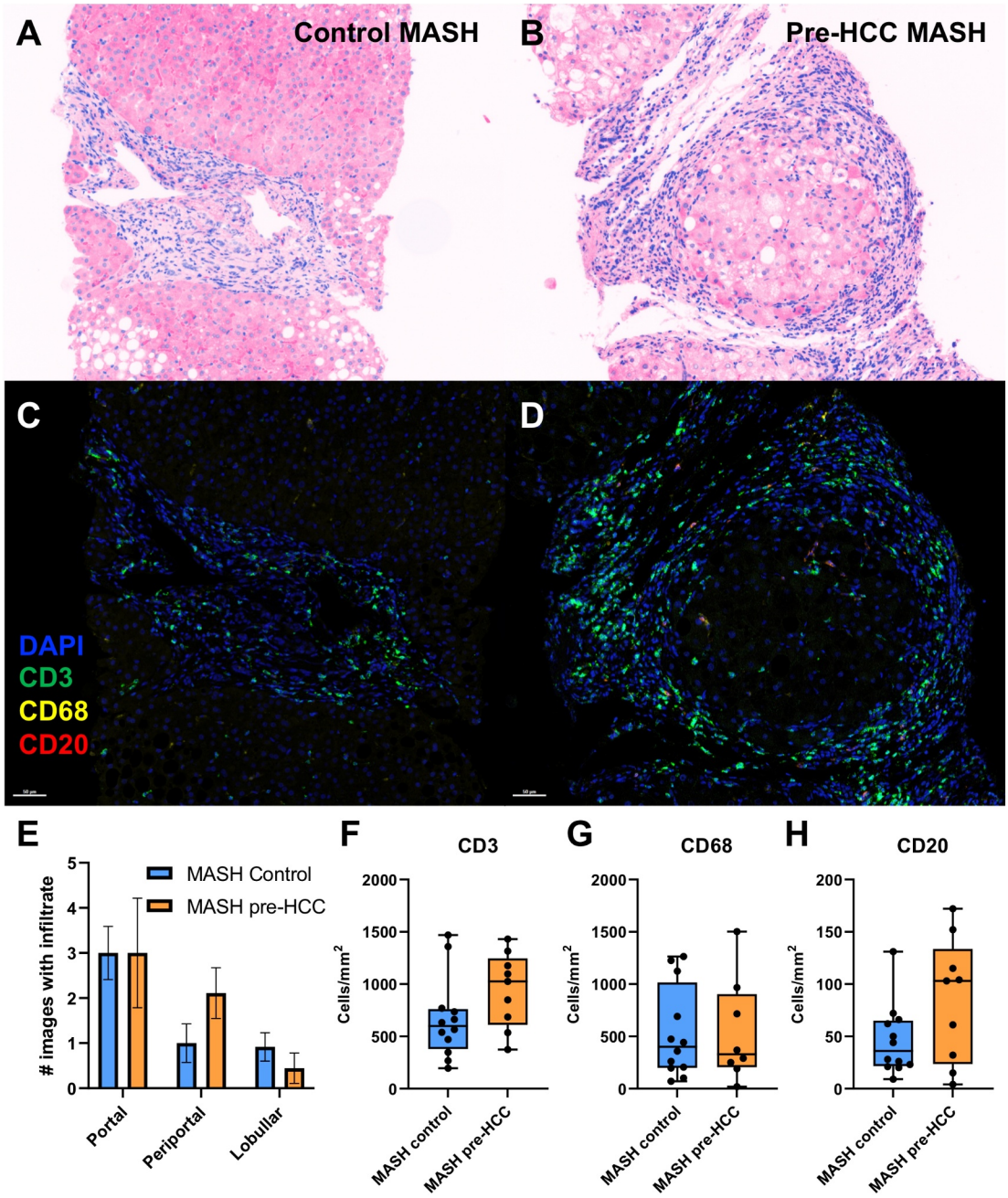


FIGURE 1 | MASH pre-HCC patients show increased liver infiltration. H&E staining of representative liver biopsies from control MASH patient (A) or pre-HCC MASH patient (B). CD3 (green), CD68 (yellow), CD20 (red), and DAPI staining on representative microphotographs of MASH control (C) and MASH pre-HCC liver biopsies (D). 200X magnification. Scale bar 50 μm. (E) Location of immune cell infiltrates within liver tissue. The number of images in which cells were found in each corresponding location for each patient is displayed. Cell density (cells/mm^2) of CD3+ cells (F), CD68+ macrophages (G) and CD20+ B cells (H).

(shown in Figure 2G) or in the regulatory T cell population (CD4+ Foxp3+ cells) (shown in Figure 2H). In fact, the majority of CD4 T cells detected by microscopy did not express CXCR6+ or FoxP3+ markers. CD4+ PD1+ cells showed a notable increase (p = 0.0169; shown in Figure 2I) and CD4+PD1+CXCR6+ (p = 0.0485, shown in Figure 2J) in the liver biopsies of MASH pre-HCC patients, with a 3-fold and an 8-fold increase, respectively. No significant differences were observed in CD4+CXCR6+ cells or regulatory CD4+Foxp3+ cells or CD4+PD1+Foxp3+ cells (shown in Figure 2G, H and K).

3.4 | CD4+ T Cell Heterogeneity in MASH Patients

To characterize the phenotype and functionality of the CD4 T cells present in the liver of MASH patients, re-analysis of publicly available single-cell RNAseq datasets was performed on CD4 T cells from liver biopsies of healthy individuals versus MASH patients (shown in Figure 3A–D). Differential gene expression analysis of CD4+ T cells from MASH patients versus healthy individuals revealed distinct transcriptional signatures characterized by upregulation of ribosomal proteins (*rps26*,

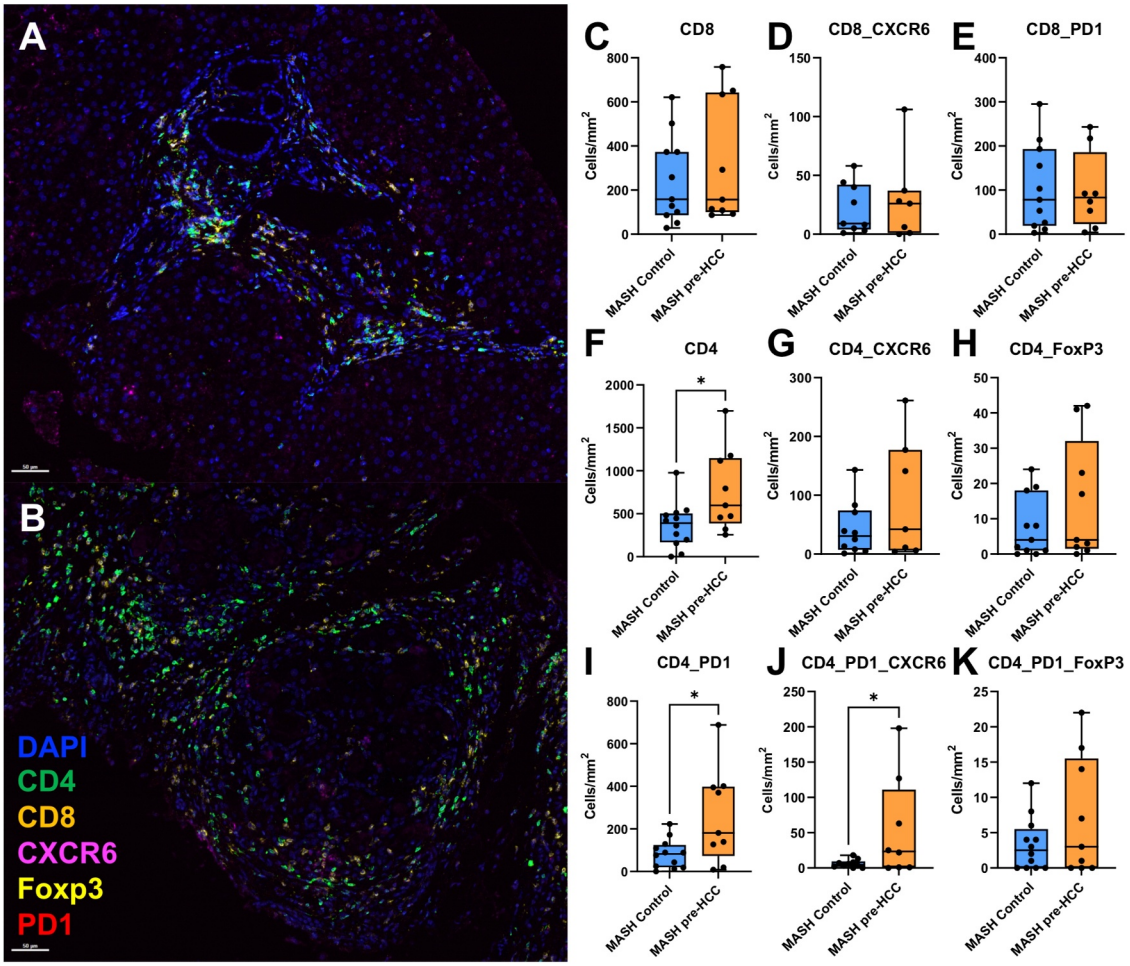


FIGURE 2 | Elevated CD4 T cell Infiltrate in liver biopsies from MASH pre-HCC patients. CD4 (green), CD8 (orange), CXCR6 (magenta), foxp3 (yellow), PDL1 (red) and DAPI on a representative liver biopsy from control MASH patient (A) or pre-HCC MASH patient (B). 200X magnification. Scale bar 50 μm. (C) CD8+ cells, (D) CD8+ CXCR6+ cells, (E) CD8+ PD1+ cells (F) CD4+ cells ($p = 0.0243$) (G) CD4+ CXCR6+ cells, (H) CD4+ foxP3+ cells. (I) CD4+PD1+ cells ($p = 0.0169$), (J) CD4+PD1+ CXCR6+ ($p = 0.0485$), (K) CD4+PD1+foxp3+ cell density values. Unpaired Student t test or nonparametric Mann–Whitney test was performed. Outliers were excluded. *$p < 0.05$. **$p < 0.01$.

rpl39, *rps21*) and mitochondrial respiratory chain components (*atpse*, *cox7c*, *atpsl, mt-nd3, cox6B1, uqcr11*) (shown in Figure 3A). In contrast, CD4 T cells from MASH patients showed a reduction in mitochondrial ribosomal RNA (*mtrnr1L12*), decreased expression of nuclear organization genes (*gltscr2, neat1, prrc2c*), downregulation of the non-classical MHC molecule hla-f and reduced expression of the heat shock protein gene *dnajb1* (shown in Figure 3A).

Single-cell RNA sequencing analysis using established definitions of CD4 T cell subpopulations [17] revealed distinct CD4 T cell subpopulations with altered proportions and specialized functions in MASH patients compared with healthy individuals (Figure 3B–D). Although all CD4 T cells expressed tissue residency markers (*Cd69*), interferon-stimulated genes (*mx1*, *ifi6*, *isg15*), inflammation (*ccl5*), and cytotoxicity (*nkg7*) markers, differential expression patterns distinguished naive/memory populations (*ccr7*, *lef1*, *cd62l* and *cd44*) from effector/regulatory populations (*cxcr6* and *il18r1*) (Figure 3D). The effector compartment revealed four distinct inflammatory subsets: cytotoxic Th1 cells expressing exhaustion markers (cluster 2 and 6), Th17 cells (cluster 5), an exhausted/dysfunctional population (cluster 3), and regulatory T cells (cluster 4) (shown in Figure 3B–D). Proportional changes showed that MASH patients had predominance of tissue-repairing naïve cells (cluster 0 and 1) and inflammatory Th17 effector cells (cluster 5), while showing a reduction in cytolytic effector (cluster 2 and 6), regulatory (cluster 4), and effector memory subpopulations (cluster 7 and 8) (Figure 3C). Gene expression profiles within individual subpopulations showed no significant changes between healthy and MASH patients (Figure 3D). These dynamic changes in immune cell populations suggest that the hepatic immune environment is reshaped during MASH progression, which may impact disease advancement toward HCC.

3.5 | PD-L1 Expression Was Predominantly Observed on CD3+ Cells Interacting With CD4+ Cells in MASH Liver Biopsies

While PD-L1+ cell density showed no significant difference between groups (shown in Figure 4A–D); T cells (CD3+ cells) were the primary identified contributor to PD-L1 expression in both MASH pre-HCC and control samples (shown in Figure 4E), most

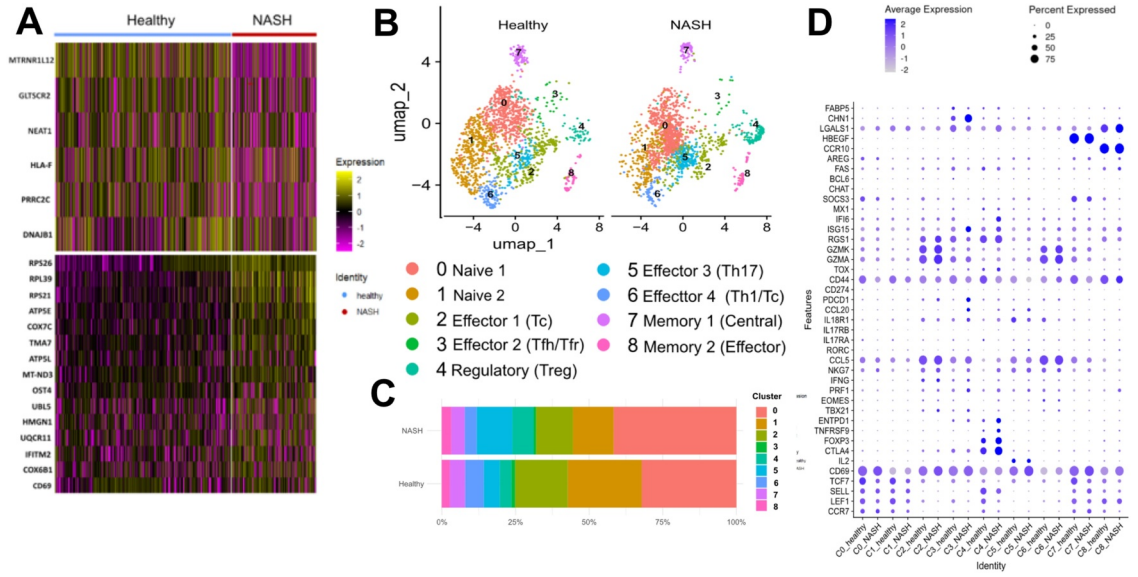


FIGURE 3 | High CD4+ T cell heterogeneity is found in MASH patients. (A) Heatmap constructed using genes that changed in the majority (> 80%) of single-cell RNAseq sequences from CD4+ T cells isolated from liver biopsies of healthy controls ($n = 4$) or MASH patients ($n = 3$), (B) U-map plot comparing subpopulations defined based on gene expression from healthy control versus MASH patients. (C) Proportion of subpopulations of CD4 T cells in healthy controls versus MASH patients. (D) Specific gene expression profile for each defined subpopulation of isolated CD4 T cells in healthy controls versus MASH patients.

predominantly CD4+ T cells (shown in Figure 4F). Spatial analysis revealed significant close proximity between CD3+PDL1+ and CD3+CD4+ cells in MASH pre-HCC liver biopsies (shown in Figure 4G), suggesting that T:T cell interactions might play a role in immune response suppression.

3.6 | Clinical Biomarkers Distinguished MASH Pre-HCC Patients

Pre-HCC patients showed significantly lower ALT levels, increased alkaline phosphatase, reduced platelet counts, and elevated INR levels compared with controls (Figure 5A–F), possibly indicating impaired liver synthetic function and coagulation deficiency. These changes were associated with higher cirrhosis and diabetes rates (Supporting Information S1: Figure 4A–D, F-I, K-N), though INR remained significantly elevated in non-cirrhotic, non-diabetic patients (Supporting Information S1: Figure 4E, J, O). Machine learning models identified key predictors: Lasso regression highlighted platelets and INR, while random forest analysis emphasized INR, platelets, ALT, and alkaline phosphatase (Supporting Information S1: Table 2). When controlling for confounders such as cirrhosis and diabetes (DM2) through individual logistic regression, only CD4+ T cell density and INR remained statistically significant predictors of HCC development (Supporting Information S1: Table 2), establishing these as independent early biomarkers of disease progression.

3.7 | Immune and Clinical Profile Define HCC Development in MASH Patients

Multivariate analysis including CD4+ cell numbers, ALT, alkaline phosphatase, platelet levels, and INR clearly discriminated MASH pre-HCC from controls (shown in Figure 6A). The separation between patients was mainly driven by the number of CD4 T cells aided by the levels of Alk Phos, platelets and INR in contrast to the ALT levels (shown in Figure 6B). ROC curves using CD4+ cell numbers alone showed an AUC of 0.768 (shown in Figure 6C), while the aggregate of all variables (Age, Sex, CD4 numbers, ALT, Alkaline Phosphatase, platelets) excluding INR showed an AUC of 0.944 (shown in Figure 6D). Comparing AUC values revealed a gradient of importance for discriminating MASH patients at risk of developing HCC (shown in Figure 6E), with the highest AUC achieved when all variables are included. Interestingly, INR had the highest AUC value as a single discrimination variable (shown in Figure 6E). Additionally, predicted probabilities of developing HCC differed significantly between the observed control and pre-HCC patients (shown in Figure 6F).

4 | Discussion

MASH represents a growing risk factor for HCC, yet the immunological mechanisms driving this progression have remained poorly defined. In this cross-sectional retrospective study spanning three countries, we identified profound alterations in the hepatic inflammatory landscape up to 2 years before clinical HCC detection. Our analysis revealed marked CD4+ T cell infiltration with over half expressing PD1, likely reflecting localized T cell exhaustion. These expanded CD4+ populations exhibited a distinct profile suggestive of a phenotypic shift. They were not characterized by inflammatory (CXCR6+) or regulatory (FOXP3+) markers. Instead, they were enriched for tissue-repair cells and actively proliferating Th17 effector cells, whereas cytotoxic Th1 subpopulations were depleted, as observed in MASH by publicly available single-cell RNA-seq studies. Spatial analysis demonstrated frequent

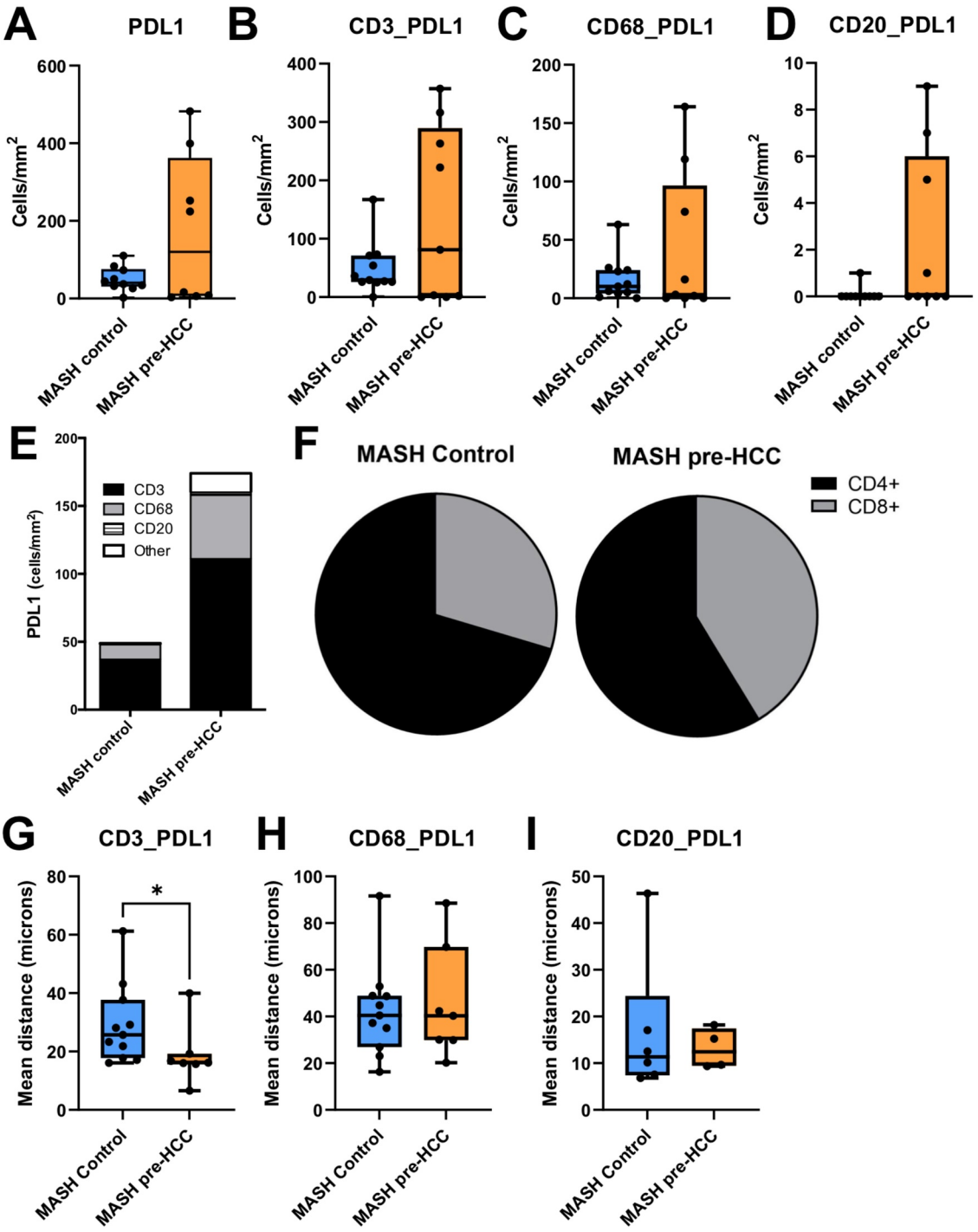


FIGURE 4 | PDL1 expression was mainly observed in T cells. Cell density (cells/mm^2) of PDL1+ (A), CD3+ (B), CD68+ (C), or CD20+ PDL1+ cells (D). Proportion of PDL1+ cells detected in relation to the average cell number per mm^2 per subpopulation (E). Proportion of CD4+ and CD8+ of total CD3+ cells expressing the PD-L1 marker (F). Mean distance of CD3+PDL1+ (G, $p = 0.0031$), Mean distance of CD68 + PDL1+ cells (H) and CD20 + PDL1+ cells (I) to CD3+CD4+ cells. Unpaired Student t test or nonparametric Mann–Whitney test. Outliers were excluded. *$p < 0.05$.

colocalization between CD4+ and PDL1-expressing T cells, suggesting T cell-to-T cell interactions as a possible pathogenic mechanism in the MASH progression to HCC. When integrated with clinical parameters, these immunological signatures yielded exceptional predictive performance (AUC = 0.944), establishing CD4+ T cell increase as both a possible pathophysiological driver and an early biomarker for MASH-associated HCC development.

The shifting etiology of HCC from viral to metabolic causes has fundamentally altered the immunological landscape preceding malignancy [5]. Historically, viral infections (HBV and HCV) dominated HCC pathogenesis, characterized by senescent PD1+CD8+ T cells that respond favorably to checkpoint blockade therapy [21]. However, widespread HBV vaccination and improved HCV treatments have dramatically reduced viral-associated HCC, with MASLD progressing to MASH now

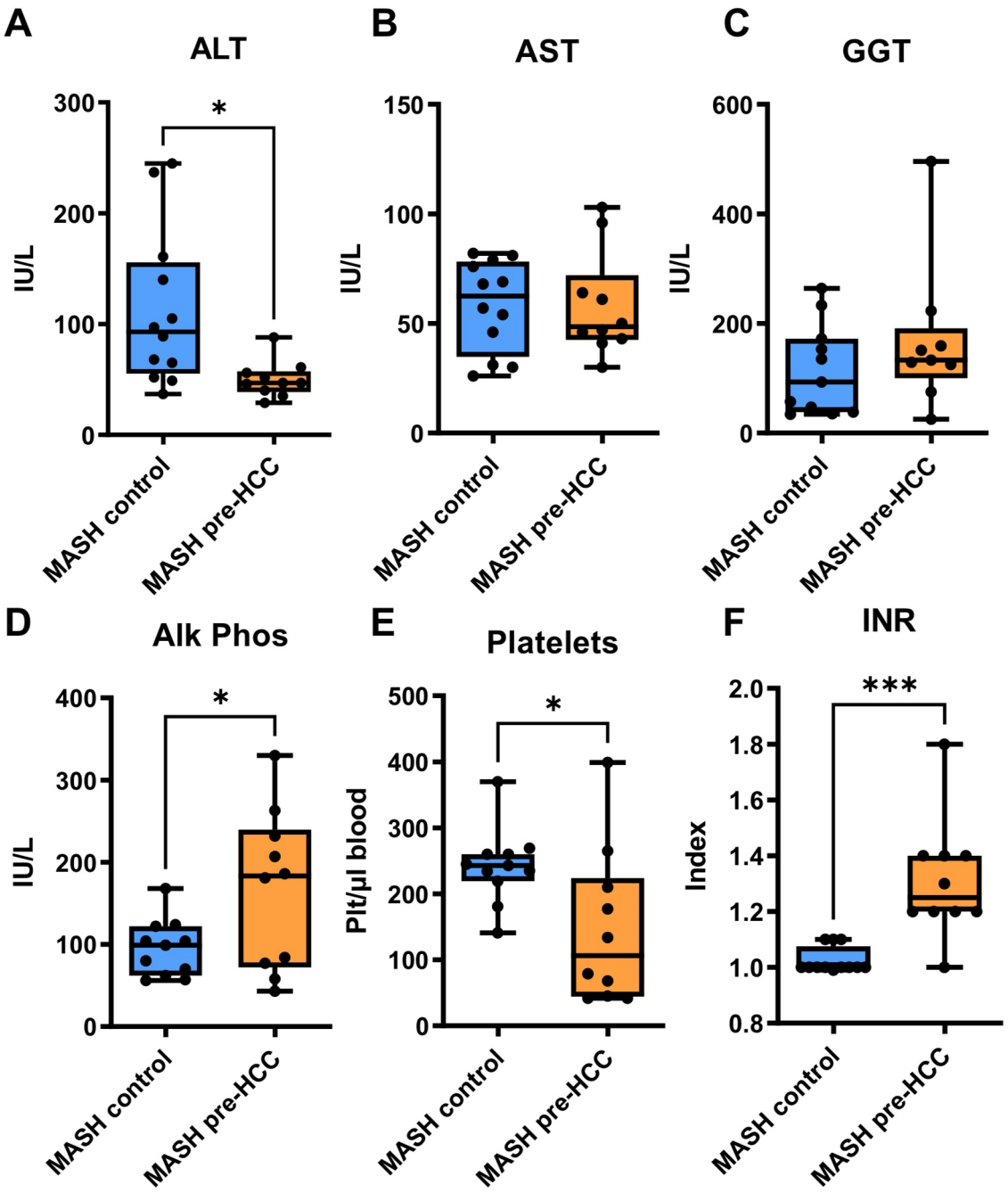
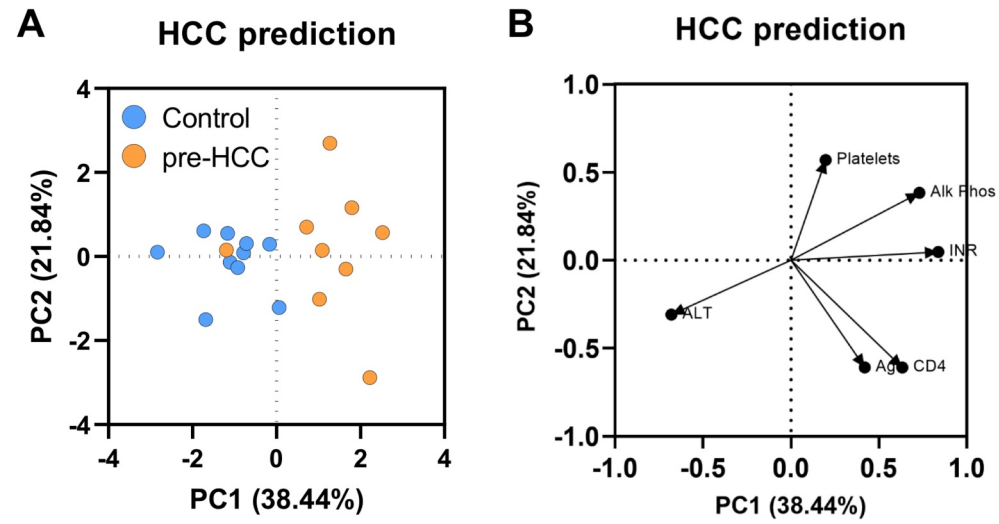
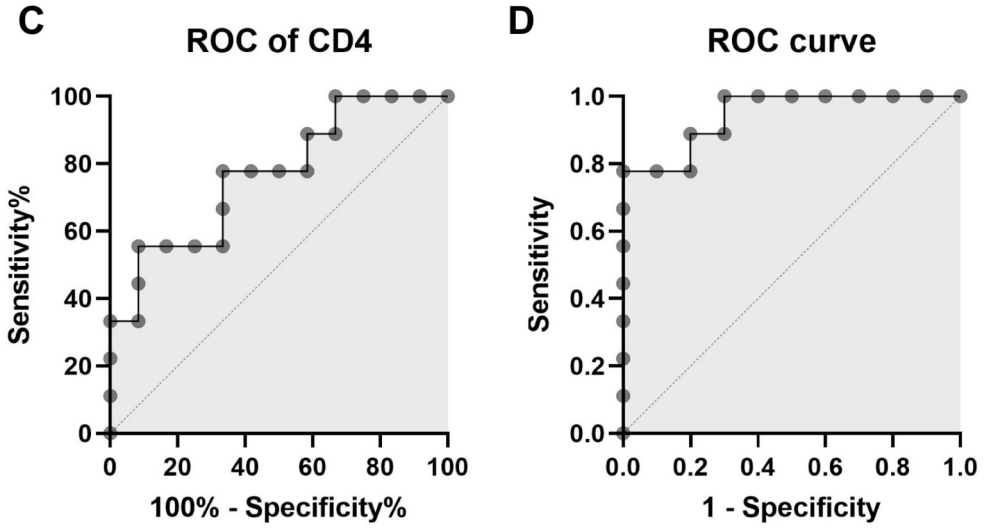


FIGURE 5 | Clinical biomarkers distinguished MASH Pre-HCC Patients. MASH pre-HCC patients showed distinctive liver enzymes levels in serum, platelet counts, and coagulation times compared to MASH control. (A) Alanine aminotransferase (ALT, p-value = 0.0135); (B) Aspartate aminotransferase (AST); (C) Gamma-glutamyl transferase (GGT) and (D) Alkaline Phosphatase (Alk Phos, p-value = 0.0342) levels determined in serum from MASH control and MASH pre-HCC patients. (E) Platelets count performed in peripheral blood from MASH control and MASH pre-HCC patients (p-value = 0.0271). (F) International normalized ratio (INR) index (p-value = 0.0002). Unpaired t test. *$p < 0.05$, ***$p < 0.001$.

representing the predominant etiological pathway. This etiological shift has revealed fundamentally different immunological mechanisms. While animal models initially implicated inflammatory CXCR6+ PD1+ CD8+ T cells in MASH-associated HCC development [9], clinical evidence suggests a more complex picture. CD8+ T cell depletion reduces HCC development, yet increased PD1+ CD8+ T cells following immunotherapy fail to improve survival in MASH-associated HCC patients [9], indicating an auto-reactive rather than protective role for CD8 T cells. CD4+ T cells may also contribute to HCC development by promoting inflammation in MASH [16, 22]. CD4+ T cell increases were detected alongside CD8+ T cell increases in MASH patients [9], potentially supporting immune surveillance or control of autoreactive T cells [23]. While both T cell subsets may interact during MASH pathogenesis, CD4+ T-cell depletion —particularly when combined with PD1+ cell depletion—significantly reduced HCC development [9, 24], suggesting CD4+ T cells as key drivers of malignant transformation in the MASH context. Importantly, our findings contrast previous animal studies that suggested CD8 T cells





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Biomarker	AUC	95% CI	P-value
Platelets	0.678	0.3883-0.9672	0.1914
Alk Phos	0.767	0.5234-1.000	0.005
CD4	0.768	0.5614-0.9756	0.0393
ALT	0.843	0.6681-1.000	0.0098
INR	0.934	0.8012-1.000	0.0011
Age + Sex+ CD4+ALT+AlkPh +Platelets	0.944	0.8478-1.000	0.0011

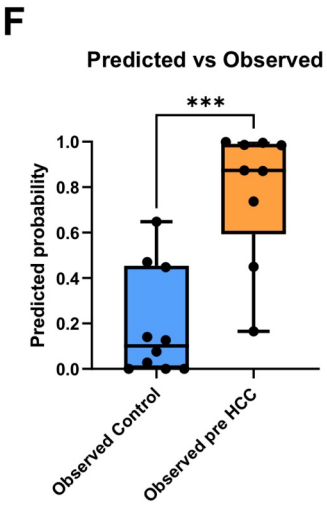

FIGURE 6 | Immune and clinical profiles define HCC development in MASH patients. (A) PCA plot showing the multivariate analysis of MASH control and MASH pre-HCC samples including clinical and histological variables. (B) Loading plot. Vectors indicate the direction and strength of each variable to the overall distribution. Area under the curve (AUC) for potential biomarkers for HCC development in MASH patients: (C) CD4 only or (D) Age, Sex, CD4, ALT, Alk Phos and Platelets. (E) ROC curve by multiple logistic regression analysis. (F) Predicted versus Observed probability of developing HCC (p-value = 0.0001). Unpaired Student t test was performed. ***$p < 0.001$.

were increased in MASH because we demonstrated that CD4+ T cells, not CD8+ T cells, represent the predominant expanding population during MASH-to-HCC progression.

Differential gene expression analysis reveals that CD4+ T cells in MASH patients exist in a paradoxical state of hyperactivation coupled with functional exhaustion. These cells upregulate ribosomal proteins and mitochondrial respiratory chain components, indicating metabolic hyperactivity driven by increased energy demands for cytokine production and sustained inflammatory responses that characterize MASH pathogenesis. Simultaneously, downregulated cellular maintenance functions demonstrate metabolic exhaustion: reduced mitochondrial ribosomal RNA impairs protein synthesis capacity, disrupted nuclear organization genes compromise RNA processing and stress responses, downregulated non-classical MHC genes (*hla-f*) alter immune surveillance, and reduced heat shock protein (*dnjb1*) expression compromises cellular stress management. This gene profile defines CD4+ T cells that simultaneously drive inflammatory responses while losing essential maintenance functions, creating a dysfunctional state that promotes MASH pathogenesis that could potentially develop into HCC.

CD4+ T cell expansion creates a heterogeneous population with widespread PD1 expression, indicating localized exhaustion that compromises immune surveillance. This infiltrate includes tissue-repairing and cytolytic subpopulations alongside proliferating Th17 cells, consistent with evidence that IL-17A blockade ameliorates MASH-to-HCC progression [11]. Inflammatory CXCR6+ CD4+ T cells respond to microbiota changes, possibly increasing hepatic IL-17A secretion [22] and tissue damage that promotes MASH pathogenesis [25]. Increased IL-17-secreting CD4+ T cells that could impair immune surveillance have been shown to depend on increased IgA + B cells [26]. However, MASH patients that developed HCC had few B cells detected with no significant differences between the groups in our study. Single-cell RNA-seq analysis revealed depletion of protective cytotoxic Th1 subpopulations and absence of choline acetyltransferase (ChAT)-expressing CD4+ T cells [17], creating an immunological environment permissive for malignant transformation or activation of autoreactive T cells. The low abundance of regulatory FoxP3+ T cells and B cells indicates that classical immunosuppressive mechanisms do not drive progression toward HCC [27]. Instead, close spatial proximity between PDL1-expressing T cells and CD4+ T cells—predominantly T cell-to-T cell interactions rather than hepatocyte-T cell interactions [9]—could suggest an alternative pathogenic mechanism. This finding challenges current therapeutic approaches and establishes that strategies targeting T cell recruitment or T:T interactions may be more effective than traditional checkpoint inhibition in MASH-associated HCC.

Early HCC detection in MASH patients is critical yet challenging as patients remain asymptomatic until advanced stages [1, 28], particularly in non-cirrhotic cases inadequately addressed by current guidelines [29, 30]. While imaging modalities and blood-based biomarkers have evolved, existing biomarkers show MASH-specific limitations: AFP with ultrasound achieves high overall sensitivity (97%) but only moderate early-stage detection (63%), while GALAD score (AUC: 0.96) [31] and ASAP score (AUC: 0.80–0.86) [7] still require additional validation. Our integrated approach combining CD4+ T cell counts with clinical parameters (age, sex, ALT, alkaline phosphatase, platelets) achieved superior performance (AUC = 0.944) approximately 2 years before HCC detection. INR alone demonstrated exceptional discriminatory power (AUC = 0.934), particularly in non-cirrhotic, non-diabetic patients, representing a novel clinical association as INR has not previously been linked to liver cancer risk despite its role in MASLD-to-MASH progression [32]. This elevation likely reflects declining hepatic protein synthesis during early malignant transformation [33], offering a readily available, cost-effective biomarker. Multivariable regression controlling for relevant confounders (fibrosis and diabetes) confirmed only CD4+ T cell density and INR as statistically significant independent predictors of HCC. When cirrhosis was included as an additional confounder in the multivariable regression analysis instead of or in addition to fibrosis, only INR was statistically significant as a predictor of HCC development in MASH patients (shown in Sup. Table 2).

While the use of machine learning strategies such as LASSO regularization in the statistical analysis helps mitigate overfitting concerns, the small sample size of our exploratory study limits the statistical power of our conclusions; therefore, larger prospective cohorts are needed to validate our findings. This study establishes CD4+ T cell infiltration and INR elevation as critical pathophysiological mechanisms and powerful biomarkers for MASH-associated HCC development. Further single-cell RNA-seq studies of liver biopsies from MASH patients are needed to fully elucidate CD4+ T cell subpopulation functions and their mechanistic contributions to HCC progression. The combination of immune signatures with clinical parameters delivers exceptional predictive capability, enabling earlier intervention strategies that could significantly improve outcomes for high-risk MASH patients. These mechanistic insights into CD4+ T cell-mediated pathways could open new therapeutic avenues targeting the unique immunological features of MASH-associated hepatocarcinogenesis, positioning this biomarker panel as a potentially transformative tool for precision medicine in metabolic liver cancer prevention.

Author Contributions

Concept and design J.D.D., A.B., D.C.B. and P.A.R. Patient recruitment and sampling M.F., J.P., D.C.B. and A.B. Histological assessment P.C. and M.K. Experiments and multiplex microscopy procedures E.R. and P. S. Analysis of publicly available single-cell RNAseq data S.D. and N.N. Data analysis and statistics E.R., M.F. and P.A.R. Writing and revision of the article E.R., D.B., J.D., A.B. and P.A.R.

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Ethics Statement

The study was conducted in strict adherence to the principles of the Helsinki Declaration, was reviewed and approved by the local Ethics Committees: Protocol codes HP 4-315 (HPCMC, Argentina), R00781-19 (CEHD, Colombia) and MEC-2017-1140 (Erasmus, The Netherlands).

Consent

Since this was a retrospective study, written informed consent was not required and was approved by the local Ethics Committee.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Individual participant data that underlie the results reported in this article, after de-identification, are available from the corresponding authors upon reasonable request.

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Supporting Information

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