



Immune Profiling of Combined Hepatocellular-Cholangiocarcinoma Reveals Distinct Subtypes and Activation of Gene Signatures Predictive of Response to Immunotherapy

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ABSTRACT

Purpose: Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare malignancy associated with an overall poor prognosis. We aimed to investigate the immune profile of cHCC-CCA and determine its impact on disease outcome.

Experimental Design: We performed a multicenter study of 96 patients with cHCC-CCA. Gene expression profile was analyzed using nCounter PanCancer IO 360 Panel. Densities of main immune cells subsets were quantified from digital slides of IHC stainings. Genetic alterations were investigated using targeted next-generation sequencing.

Results: Two main immune subtypes of cHCC-CCA were identified by clustering analysis: an “immune-high” (IH) subtype (57% of the cases) and an “immune-low” (IL) subtype (43% of the cases). Tumors classified as IH showed overexpression of genes related to immune cells recruitment, adaptive and innate immunity, antigen

presentation, cytotoxicity, immune suppression, and inflammation ($P < 0.0001$). IH cHCC-CCAs also displayed activation of gene signatures recently shown to be associated with response to immunotherapy in patients with HCC. Quantification of immunostainings confirmed that IH tumors were also characterized by higher densities of immune cells. Immune subtypes were not associated with any genetic alterations. Finally, multivariate analysis showed that the IH subtype was an independent predictor of improved overall survival.

Conclusions: We have identified a subgroup of cHCC-CCA that displays features of an ongoing intratumor immune response, along with an activation of gene signatures predictive of response to immunotherapy in HCC. This tumor subclass is associated with an improved clinical outcome. These findings suggest that a subset of patients with cHCC-CCA may benefit from immunomodulating therapeutic approaches.

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Translational Relevance

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare primary liver cancer associated with an overall poor prognosis. We identify two main immune subtypes of cHCC-CCA (immune-high and immune-low). Tumors classified as immune-high show overexpression of genes related to adaptive and innate immunity, antigen presentation, cytotoxicity, immune suppression, and inflammation. They are also associated with an improved overall survival and display activation of gene signatures that were recently shown to be associated with response to anti-PD1 blocking antibodies in patients with HCC. Our results may have important implications for the design of future clinical trials using immunomodulating agents and/or for the stratification of patients with this highly aggressive malignancy.

Introduction

Primary liver cancer (PLC) is the third cause of cancer-related deaths worldwide and consists in a heterogeneous group of malignant tumors with different clinical, histologic, and molecular features (1, 2). Among these neoplasms, hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (CCA), respectively, demonstrate hepatocytic and biliary differentiation and thus represent the two ends of the spectrum of PLCs.

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare subtype (incidence ranging from 1.0% to 4.7% of all PLCs) that shows histopathologic features of both HCC and CCA (3–6). Its clinical outcome is considered to be poorer than that of HCC and equal to or worse than that of CCA (5, 7–10). The biological mechanisms underlying this particular phenotype remain largely unknown, and they may indeed result from the malignant transformation of a progenitor cell or the dedifferentiation of a HCC or an CCA (11–13). Therapeutic strategies for patients with cHCC-CCA are limited. The mainstay of treatment is surgical resection with most series reporting a 3-year survival rate of 25%–50% (3, 6, 9, 14). No systemic treatment has so far demonstrated any significant antitumor activity for patients with recurrent and/or advanced disease.

Immunotherapy, hailed as a major breakthrough for the treatment of numerous solid or hematologic malignancies, is now providing new approaches for the treatment of PLCs. Indeed, compared with sorafenib, the combination of a monoclonal anti-programmed death ligand 1 (PDL1) antibody (atezolizumab) and an anti-angiogenic agent (bevacizumab) was recently shown to improve both overall and progression-free survival for patients with advanced HCC (15). Interestingly, Sangro and colleagues investigated tumor samples from patients with HCC treated with the anti-programmed death 1 (PD1) antibody nivolumab and showed a positive correlation between particular expression profiles and treatment response (16). Indeed, four immune/inflammatory gene signatures (Inflammatory, Gajewski 13-Gene Inflammatory, 6-Gene Interferon Gamma, and T-cell Exhaustion) were associated with both objective response rate and overall survival (16). These signatures noticeably include immune checkpoint inhibitors (*CD274*, *LAG3*), cytolytic effectors (*GZMA*, *PRF1*), and molecules involved in the recruitment of immune cells subsets (*CXCL9*, *IFNG*, *CXCL10*) and antigen processing/presentation (*HLA-DMA*, *HLA-DMB*). Altogether with numerous other studies in other human malignancies, these results strongly suggest that only certain subsets of “immune-hot” tumors show enhanced sensitivity to

immunotherapy (17). In this line, a significant degree of intertumor heterogeneity regarding HCC microenvironment has been reported, with approximately 25% of HCCs belonging to a particular subclass characterized by a massive infiltration by immune cells (18, 19).

There is unfortunately very limited data on the immunity of cHCC-CCA (20, 21). In particular, the existence of an immune-high (IH) subtype similar to that reported in HCC remains to be determined. The impact of immunity on cHCC-CCA outcome is also unknown.

We therefore aimed in the current study to investigate the immune characteristics of a large series of cHCC-CCA samples by means of gene expression profiling, gene sequencing, and digital quantitative IHC. This approach has allowed us to identify an IH subtype of cHCC-CCA characterized by an increased infiltration and activation of immune cells, an enhanced antigen presentation and cytolytic activity, and an improved clinical outcome. We also show an enrichment of gene signatures related to response to immunotherapy.

Materials and Methods

Patients and samples

We retrospectively included cHCC-CCA samples from patients who underwent surgical resection or liver transplantation in five University Hospitals (Henri Mondor, Créteil, France; Beaujon, Clichy, France; Pitié-Salpêtrière, Paris, France; Reims University Hospital, Reims, France; National Cancer Hospital, Hanoi, Vietnam; **Table 1**). Inclusion criteria were as follows: (i) available baseline data and histologic material, (ii) unequivocal histologic features of cHCC-CCA, and (iii) lack of preoperative antitumor treatment.

The following clinical, biological characteristics were systematically recorded: age, gender, etiologies of liver disease [hepatitis B (HBV) or hepatitis C (HCV) infection, alcohol intake, and metabolic syndrome], follow-up (overall survival), alpha-fetoprotein serum levels, tumor size, multinodularity, satellite nodules, vascular invasion (macrovascular/microvascular), and surgical margins. Fibrosis in the non-tumor

Table 1. Baseline clinical, biological, and pathologic features of the patients and tumors.

Variables	Available data	n (%)
Gender (male)	96	70 (72.9)
Age at surgery [median (min-max)]	96	59 (22–84)
Underlying etiologies		
Alcohol	82	37 (45.1)
HCV	93	22 (23.6)
HBV	90	20 (22.2)
NASH	80	11 (13.7)
AFP level $\geq$ 20 mg	54	27 (50)
Pathologic features		
Largest nodule diameter [median (min-max)]	96	45 (10–250)
Metastasis at time of surgery	96	8 (8.3)
Satellite nodules	96	28 (29.1)
Multinodular tumor	96	23 (23.9)
Positive surgical margin	96	13 (13.5)
Macrovascular	96	13 (13.5)
Microvascular	96	50 (52.1)
Non-tumor liver fibrosis		
F0–F1	96	16 (16.7)
F2–F3	96	26 (27.0)
F4	96	54 (56.3)

liver was assessed using the METAVIR scale (F0: no fibrosis, F1: portal fibrosis without septa, F2: portal fibrosis with few septa, F3: portal fibrosis with many septa, F4: cirrhosis).

To confirm the diagnosis of cHCC-CCA, histologic slides stained by hematoxylin and eosin were reviewed by three expert liver pathologists. Reviewing was performed according to the most recent consensus recommendations (12). The percentage of each morphologic component was assessed: HCC, CCA, equivocal (histologic appearance without clear-cut hepatocytic or biliary differentiation, or closely intermingled hepatocytic and biliary contingents) and cholangiolocellular (1). Cases with a cholangiolocellular morphologic appearance throughout the whole tumor were not included in the study. Indeed, the cholangiolocellular pattern consists in tumor cells arranged in anastomosing cords reminiscent of cholangioles or canals of Hering and it has been clearly demonstrated that they are a distinct biliary-derived entity. They are therefore now classified as a cholangiocarcinoma subtype (22). Cases with intermediate features throughout the whole tumor (intermediate cell tumors) were also excluded as they are now classified as a distinct entity. The presence of so-called “stem cell features” (small cells with hyperchromatic nuclei and a high nuclear-cytoplasmic ratio) was assessed using hematoxylin eosin-stained slides (1). A representative paraffin-embedded tissue block was further selected for each case. The study was performed according to the declaration of Helsinki and the local legal requirements of each center. It was approved by an Institutional Review Board. All necessary written and signed informed consents were obtained from patients.

A total of 96 patients matched our inclusion criteria. They were predominantly male (73%) and had a median age at surgery of 59 years. Main underlying etiologies were alcohol intake ($n = 37$, 45%), HCV infection ($n = 22$, 24%), HBV infection ($n = 20$, 22%), and non-alcoholic steatohepatitis ($n = 11$, 14%). High preoperative α -fetoprotein (AFP) levels (≥ 20 ng/mL) were detected in 50% (27/54) of patients. A subset of patients had multinodular disease ($n = 23$, 24%; **Table 1**). The median size of the tumors was 45 mm. Satellite nodules, macrovascular invasion, microvascular invasion were observed in 29% (28/96), 13% (13/96), and 52% (50/96) of the cases, respectively. The vast majority of the tumors occurred in a background of significant fibrosis (METAVIR stage F2-F3 or F4, $n = 80$, 83%; **Table 1**). The predominant histologic pattern of the lesions was hepatocellular, biliary, and equivocal in 43%, 25%, and 27% of the cases, respectively.

Gene expression profiling

For each case, 5- μ m-thick sections were cut from selected formalin-fixed paraffin-embedded (FFPE) tissue blocks. Tumor and non-tumor tissue were then macrodissected. Total RNA was further isolated using the Recover All Total Nucleic Acid Isolation Kit (Invitrogen, Thermo Fisher Scientific) according to manufacturer's instructions. All total RNAs were monitored to ensure their quality, purity, and integrity by performing electrophoresis on an Agilent 2100 Bioanalyzer device with the Pico assay (Agilent).

Gene expression profile was analyzed using the nCounter PanCancer IO 360 Panel (NanoString Technologies) that includes more than 700 genes involved in the main biological pathways of human immunity. Experiments were performed at the Genomics platform of Institut Curie (Paris, France). For each sample, 500 ng of total RNA extracted from the FFPE tumor blocks was used as a template. A human Universal Reference RNA including a no-template control (water) and 10 cell lines was also hybridized in parallel with the tumor samples of interest. NanoString positive and negative controls were also added to samples as spikes in controls. Probes and mRNAs were hybridized overnight at 65°C, and samples were further processed on the Nano-

String nCounter preparation station (NanoString Technologies) to immobilize biotinylated hybrids on a cartridge coated with streptavidin and remove probes in excess. The nCounter Digital Analyzer (NanoString Technologies) was used to scan the cartridges at maximum resolution (555 fields of view), count the individual fluorescent barcodes and quantify the RNA molecules. The Nanostring nSolver 4.0 software (NanoString Technologies) and “NanoStringNorm” package in R were used to control and normalize raw data. In particular, data were normalized against the geometric mean of 20 housekeeping genes in combination with a positive control normalization, which uses the geometric mean of six synthetic positive targets, to control technical sources of variability. The obtained gene expression values were then $\log_2$ transformed. Gene sets to investigate gene expression of different immune signatures and immune cell subsets were established on the basis of gene annotations of nCounter PanCancer IO 360 Panel (NanoString Technologies) and the web-based application Gorilla (<http://cbl-gorilla.cs.technion.ac.il>; ref. 23).

Gene sequencing

DNA and RNA extractions were performed from FFPE tissue sections of cHCC-CCA blocks using the Maxwell RSC Plus DNA FFPE Kit IVD and the Maxwell RSC RNA FFPE Kit IVD (Promega) according to the manufacturer's instructions. They were further quantified using a Qubit fluorimeter in combination with the Qubit dsDNA HS Assay Kit and Qubit RNA HS Assay Kit (Thermo Fisher Scientific).

RNA was further reverse transcribed to cDNA using SuperScript IV VILO Master Mix (Thermo Fisher Scientific). Fifty nanograms of DNA and RNA (as measured by fluorimetry) were amplified using the Oncomine Comprehensive Library Assay v3C with IonChef (Thermo Fisher Scientific), a multiplex PCR-based library-preparation method. Amplicons were then digested, barcoded, and amplified by using Ion Ampliseq Library Kit Plus (ion ampliseq kit for chef DL8, Thermo Fisher Scientific) and Ion Xpress barcode adapter kit (Thermo Fisher Scientific). After DNA and RNA quantification, 50 pmol/L of each library was multiplexed and clonally amplified on ion-sphere particles (ISP) by emulsion PCR performed on Ion Chef (Thermo Fisher Scientific), according to the manufacturer's instructions. The ISP templates were loaded onto an Ion-540 chip and sequenced on an Ion S5 sequencer with the Ion 540 Kit-Chef, according to the manufacturer's instructions. Run performance was assessed and data analyzed with the Ion Reporter™ Software (Thermo Fisher Scientific) using specific stringent filters (allele frequency between 5% and 90%; only exonic location, read depth greater than 300 $\times$).

IHC and *in situ* quantification of immune cells

The FFPE tissue blocks were cut into 4- μ m-thick slides, and all immunochemical stainings were further performed on an automated immunostainer (Leica Bond-Max; Leica Biosystems), according to the manufacturer's instructions. Blocking endogenous peroxidases was performed using Peroxide Block (Bond Polymer Refine Detection Kit, Leica Biosystems). Antigen retrieval was performed with E1 (ph6) (CD20, CD68, MPO) and E2 (ph9) (CD3, CD8) reagents. The concentrations and sources of the antibodies used in this study were as follows: anti-CD3 (Dako, Clone F7.2.38, dilution 1/50, ABCAAB17143-500), anti-CD8 (Dako, Clone C8/1448, Dilution 1/200, M7103), anti-CD20 (Dako, Clone L26, Dilution 1/500, M0755), anti-MPO (Dako, Polyclonal rabbit, Dilution 1/2,000, A0398), anti-CD68 (Dako, Clone KP1, Dilution 1/5,000, GA609), and PDL1 (Cell Signaling Technology, rabbit monoclonal E1L3N, dilution 1/100, 13684S). Immunodetection was performed

using Polymer and 3,3'-diaminobenzidine (Bond Polymer Refine Detection Kit, Leica Biosystems). PDL1 expression was evaluated on tumor cells as well as immune cells, as described previously (24). We also assessed the densities of tertiary lymphoid structures (TLS) by reviewing standard hematoxylin eosin-stained slides (25).

Digital pathology and quantification of main immune cell subsets

Slides were scanned in brightfield mode using the NanoZoomer HT scanning device (Hamamatsu Photonics) at a 20× magnification and a 0.45 μm/pixel resolution. Whole-slide images were further analyzed using the Qupath digital software 0.1.2 version (<https://qupath.github.io/>; ref. 26). Tumoral (CT) and non-tumoral (NT) liver areas were manually annotated by a pathologist (C.T. Nguyen). Optical density sum and Cytoplasm DAB OD mean were applied as parameters to detect positive cells in both CT and NT. Cell densities of immune cells were expressed as the mean number of positive cells per mm².

Statistical analysis

Data visualization and statistical analysis were performed using R software version 4.0.2 (R Foundation for Statistical Computing, <https://www.R-project.org/>) and Bioconductor version 3.4. Unsupervised hierarchical clustering was done using Cosine distance and Ward's linkage method. Principal component analysis (PCA) using the main two components was also performed in a series of matched tumor/NT sample pairs. Heatmaps were generated using the R packages "ComplexHeatmap" (27). Bioconductor limma package was used to test for differential gene expression between two groups (28). Differentially expressed genes were defined by a *q*-value threshold of ≤0.05 (according to the FDR method from Benjamini-Hochberg) and a log fold-change >|1|.

Data were compared using Wilcoxon rank test or Fisher exact test according to the type of variable. The comparison of multiple dependent variables in two groups was performed using a multivariate ANOVA (MANOVA). To test the differences regarding the activated immune pathways and the specific gene signatures, we extracted gene lists from the nCounter PanCancer IO 360 Panel - Annotations and Sangro and colleagues (16), respectively. For each gene list, we then defined a score as the average gene expression. Scores in the two groups were compared using the Wilcoxon rank test.

To compare the gene expression profile of cHCC-CCA and HCC and CCA, we also included in an integrated analysis 355 HCC samples from TCGA-LIHC and 36 CCA samples from TCGA-CHOL (29). RNA-sequencing data from The Cancer Genome Atlas (TCGA) were obtained using the "TCGAbiolinks" package in R (30). Then, we standardized gene expression separately to have mean 0 and SD 1 per gene in each dataset. Only genes in common between the three datasets were considered for the analysis. Unsupervised hierarchical clustering of the integrated data was performed as described above.

Survival analysis was performed in patients treated by R0 liver resection with available survival data (*n* = 73). Overall survival was defined by the interval between surgery (resection or liver transplantation) and death. Survival curves were represented using the Kaplan-Meier method compared with log-rank statistics. Univariate analysis was performed using the Cox proportional-hazards model and variables with a *P* value < 0.05 were selected for multivariate analysis. All tests were two tailed and *P* values < 0.05 were considered significant.

Results

Identification of two main subtypes of cHCC-CCA using gene expression profiling

We investigated the gene expression profile of all 96 cHCC-CCA samples by unsupervised hierarchical clustering analysis and identified two distinct immune subtypes of cHCC-CCA that we designated immune-low (IL) and IH (Fig. 1A). Tumors classified as IH accounted for 57% of the cases (*n* = 55). Significantly upregulated genes in the IH cHCC-CCA subclass included cytokines/chemokines and their receptors (e.g., *IFNG*, *CCL19*, *CXCL9*, *IL1B*, *IL2*, *IL33*, *IL4*, *IL10*, *IFNA1*, *CXCR3*), markers of various subsets of immune cells (e.g., *CD8A*, *CD79A*, *CD19*, *KIR2DL3*, *CD209*, *FOXP3*, *KLRK1*, *KLRD1*), immune modulators (e.g., *PDL1*, *IDO1*, *LAG3*, *TIGIT*, *CTLA4*), cytolytic effectors (e.g., *PRF1*, *GZMB*, *GZMM*), components of the HLA system (e.g., *HLA-A*, *HLA-DRB1*, *HLA-RB5*) and cancer testis-related antigens (e.g., *MAGEA1*, *MAGEA4*; Fig. 1B; Supplementary Table S1). We further analyzed the association of cHCC-CCA immune subtypes and clinical, biological, and pathologic features, and observed very few correlations. The IH subclass was noticeably associated with the type of surgery (transplantation, *P* = 0.05), the existence of cirrhosis in the adjacent liver (*P* = 0.01), a lower frequency of microvascular invasion (*P* = 0.01), and the predominance of a hepatocellular contingent (*P* = 0.005; Supplementary Table S2). No other statistical differences were identified (in particular no association with stem cell features; Fig. 1A; Supplementary Table S2; Supplementary Fig. S1). Although unsupervised hierarchical clustering revealed two main cHCC-CCA subclasses we observed a certain degree of intracluster heterogeneity, with two subgroups being identified in the IH tumors: subcluster IH1 (left side of the main IH cluster) and subcluster IH2 (right side of the main cluster; Fig. 1A). IH1 cases accounted for approximately half of all IH cHCC-CCA (*n* 26/55, 47%) and, compared to sub-cluster IH2, showed overexpression of genes related to innate immunity and natural killer (NK) cells (*IFNA1*, *KIR3DL2*, *KIR2DL3*, *KIR3DL1*; Supplementary Table S3). Comparison between the subclusters IL1 (left side of the main IL cluster) and subcluster IL2 (right side of the main cluster) showed overexpression of cancer-testis antigens (*MAGEA3/A6*, *MAGEA4*, *MAGEC1*, *MAGEC2*; Supplementary Table S4). Further understanding of this heterogeneity will however require the study of additional cases.

To compare the immune profile of cHCC-CCA with that of conventional HCC and CCA, we further performed an integrative transcriptomic analysis with two independent datasets from TCGA (TCGA-LIHC *n* = 355 and TCGA-CHOL *n* = 36) and showed that cHCC-CCA retained their immune class and clustered close to HCC and CCA with similar transcriptomic features (Supplementary Fig. S2; refs. 31, 32).

We then aimed to validate our findings on a public dataset of 20 cases of cHCC-CCA (33). As observed in our series, unsupervised hierarchical clustering also showed two main immune clusters (high and low), with two subclusters of each main class (Supplementary Fig. S3). We observed in the IH subset upregulation of genes involved in various immune processes such as adaptive immunity or antigen processing (Supplementary Table S5), due to the low number of cases of this dataset, differences remained significant in only nine genes after adjustment for multiple tests). Compared with IH2, the IH1 cluster interestingly showed trends toward upregulation of genes related to NK cells, as identified in our series (Supplementary Table S6). We also observed trends toward dysregulation of cancer-testis antigen genes between IL1 and IL2 (Supplementary Table S7), due to the very low number of cases no difference remained significant after correction for

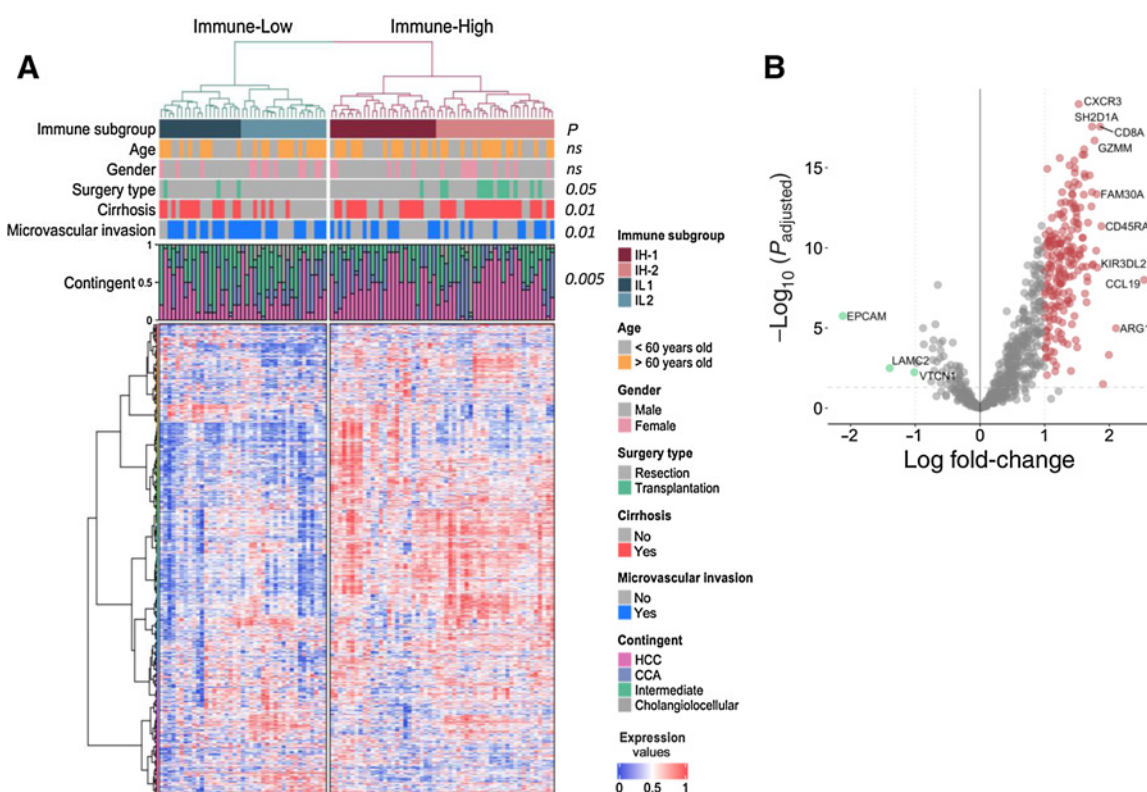


Figure 1.

Gene profiling of cHCC-CCA identifies two distinct immune subtypes. **A**, Hierarchical unsupervised clustering of 96 cHCC-CCA samples reveals two main subgroups characterized by different expression levels of immune-related genes (immune high-IH, immune low-IL). The IH subtype is associated with cirrhosis ($P = 0.01$), liver transplantation ($P = 0.05$), and lack of microvascular invasion ($P = 0.01$; Fisher exact test) as well as the amount of different histologic contingents ($P = 0.005$; MANOVA). We did not observe any association with other clinical, biological, and pathologic features. **B**, Volcano plot showing differentially expressed genes between IH and IL cHCC-CCA subtypes. Fold changes and P value of the full list of genes investigated are presented in Supplementary Table S1. CCA, cholangiocarcinoma; HCC, hepatocellular carcinoma; ns, no significance.

multiple tests). Altogether these observations strengthen the results obtained in our series of tumors.

Activation of immune pathways and increased infiltration by immune cells in IH cHCC-CCA

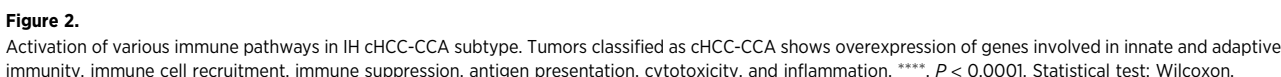
To have a better understanding of the biology of IH tumors, we selected and annotated a series of 161 genes related to major immune pathways (Fig. 2). We showed that gene signatures related to immune cells recruitment, adaptive and innate ($P < 0.0001$) immunity, antigen processing and presentation ($P < 0.0001$), cytotoxicity/killing of cancer cells ($P < 0.0001$), immune suppression and regulation ($P < 0.0001$), and inflammation ($P < 0.0001$) were upregulated in IH cHCC-CCA subtype (Fig. 2). However, there was no difference in the activation of these pathways between the two subclusters within each group. Nonetheless, these subclusters showed a different regulation of genes involved in cell proliferation (IL1 vs. IL2, $P = 0.003$; IH1 vs. IH2, $P = 0.004$; Supplementary Fig. S4).

We also aimed to confirm the increased immune infiltration in IH tumors by two distinct approaches: gene expression profiling and digital image analysis of IHC stainings. We first showed that gene sets related to T cells ($P < 0.0001$), CD8 T cells ($P < 0.0001$), B cells ($P < 0.0001$), exhausted CD8 T cells ($P < 0.0001$), macrophages ($P < 0.0001$), dendritic cells ($P < 0.0001$), neutrophils ($P < 0.0001$),

and NK cells ($P < 0.0001$) were upregulated in IH cHCC-CCA (Fig. 3A). Consistent with the intracluster heterogeneity described above, we observed that some IH cHCC-CCA show a lack of particular immune cell subsets, such as B cells, neutrophils, or NK cells (Fig. 3A).

We further performed IHC stainings of main immune cell subsets in 67 randomly selected cases and quantified the densities using Qupath software on the scanned digital slides. In line with the results obtained by gene expression profiling, we showed that intratumoral densities of CD3⁺ T cells ($P < 0.0001$), CD8⁺ T cells ($P = 0.0052$), CD20⁺ B cells ($P = 0.019$), and CD68⁺ macrophages ($P = 0.015$) were higher in IH tumors (Fig. 3B and C), correlations between mRNA expression levels and IHC are shown in Supplementary Fig. S5. A trend was observed for an increased density of MPO⁺ cells but it did not reach statistical significance (Fig. 3B). Given the associations between the IH subtype and the predominance of an HCC histologic contingent, we quantified, for 30 cases, the *in situ* immune infiltration within each contingent, but failed to detect any significant difference (Supplementary Fig. S6).

We finally quantified TLSs in intratumor tissues for all 96 cases. In line with our gene expression and IHC findings, we observed that the TLS densities were significantly higher in IH tumors ($P = 0.0039$; Supplementary Fig. S7).



We observed that IH tumors were characterized by a significant overexpression of all these gene signatures: 6-Gene Interferon Gamma signature ($P < 0.0001$), Gajewski 13-Genes Inflammatory signature ($P < 0.0001$), Sangro Inflammatory signature ($P < 0.0001$), and T-cell Exhaustion signature ($P < 0.0001$; **Fig. 4A**). They noticeably include various cytokines/chemokines (*CXCL10*, *CXCL9*, *CCL4*, *CCL2*), immune exhaustion/checkpoint inhibitors (*PDL1/CD274*, *LAG3*, *PDL2/PCDILG2*, *IDO1*) and components of the HLA system

We also investigated the relationship of our immune groups with the gene signature of the formerly reported immune class of HCC (18); among 110 genes of this signature, 33 genes are available in our panel (**Fig. 4A**). As expected, our IH group displayed a strong overexpression of these genes (**Fig. 4A**). We further aimed to determine whether our subgroups were related to active or exhausted subtypes. We compared the expression of key genes whose expression differs between these subclasses (*CD8A*, *CD3E*, *CD3G*, *IFNG*, *PRF1*, *CXCL9*, *CXCL10*, *TBX21*, *CXCL12*, *CCL2*; ref. 18). Apart from upregulation of *CXCL12* in IH2 compared with IH1 ($P < 0.001$), none of them was differentially expressed between our (i) IH1 and IH2 and (ii) IL1 and IL2 subgroups.

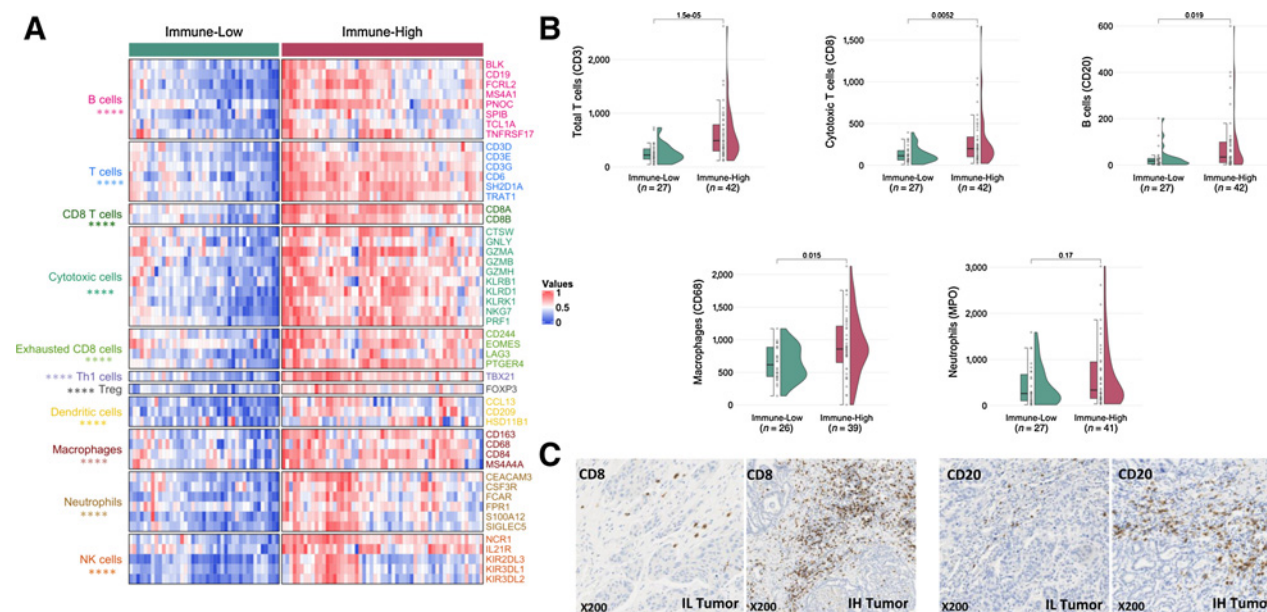


Figure 3. Combined hepatocellular-cholangiocarcinomas classified as IH display higher densities of intratumoral immune cells. **A**, Heatmaps showing overexpression of genes related to various immune cell subset in IH cHCC-CCA. B cells, T cells, CD8 T cells, cytotoxic T cells, exhausted T cells, Th1 cells, Tregs, dendritic cells, macrophages, neutrophils, NK cells ****, $P < 0.0001$, Statistical test: Wilcoxon. **B**, Quantification of immune cell densities by IHC and image analysis software shows higher infiltration by CD3 T cells ($P = 1.5 \times 10^{-5}$), CD8 T cells ($P = 0.0052$), B cells ($P = 0.019$), and macrophages ($P = 0.015$) in IH tumors. No difference was observed for neutrophils ($P = 0.17$). Densities are expressed as cell number/mm². Statistical test: Wilcoxon. **C**, Examples of anti-CD8 and anti-CD20 stainings in IH and IL cHCC-CCAs (X200, positive cells are displayed in brown).

suggesting that our subgroups do not align with these immune subsets of HCC (Supplementary Fig. S9).

Relationship of cHCC-CCA immune subtypes and underlying molecular alterations

Using targeted next-generation sequencing (Thermo Fisher Comprehensive Panel), we further screened 45 randomly selected samples for molecular alterations that are reported to recurrently occur in cHCC-CCA (20). Among the investigated samples, 29 belonged to the IH subclass and 16 to the IL subclass. The most frequent genetic defect was *TP53* mutations (58%) followed by *MYC* amplifications (18%), *TERT* promoter (16%), *BAP1* (9%) mutations, and *FGF19* amplifications (Supplementary Table S8). These findings are consistent with former studies that investigated the molecular profile of cHCC-CCA (20, 22). We did not identify any significant association between the immune subtype and the molecular alterations (Fig. 4B).

The intratumoral immune microenvironment of cHCC-CCA is not related to that of the adjacent NT liver

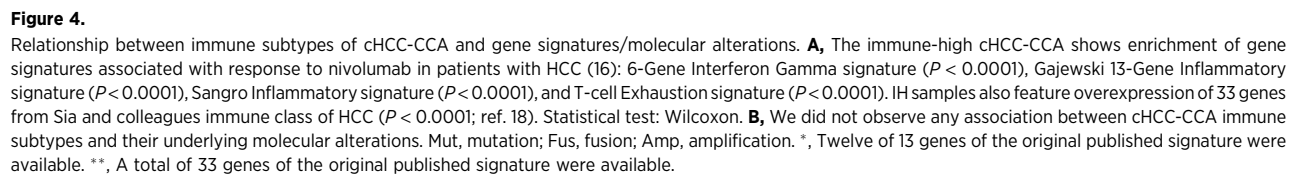
PLCs, including cHCC-CCA, most often develop in the context of chronic liver disease and inflammation that may impact the antitumor immunity (8, 34, 35). We therefore aimed to determine whether the immune subtype of the tumors was linked to the immune infiltration of the surrounding non-tumor parenchyma. We first extracted, for 19 randomly selected cases, mRNA from the adjacent liver and performed gene expression profiling using the same gene expression Panel. Using PCA, we investigated the relationships between the matched profiles. We observed that samples tended to cluster according to their tumor or non-tumor nature, and not according to the patient from whom they were obtained (Fig. 5A). The two-dimensional (2D) representation

using principal components 1 and 2 also showed that the tumor samples were most often located far from their non-tumor counterparts (Fig. 5A). We further quantified the immune cell densities in the 67 cases for which IHC was performed and compared their value according to the immune subtype of the adjacent cHCC-CCA (Fig. 5B). Consistent with the gene expression experiments, we failed to detect any significant difference between non-tumor immune cell densities according to the immune subtype of the tumor (Fig. 5B). Altogether, these results support the hypothesis that the subtype of cHCC-CCA is not related to the immune infiltration of the adjacent liver parenchyma.

Clinical impact of cHCC-CCA immune subtype

Finally, we assessed the prognostic impact of the cHCC-CCA immune subtype. Survival data were available for 73 patients, the median follow-up was 25 months (interquartile range, 12.2–48). Patients with non-curative resection were excluded (R1 or R2). Twenty-one deaths were recorded. The overall survival rates at 24 months, 60 months were 90%, 80% for patients with the IH subtype and 71%, 67% for patients with the IL subtype, respectively. Kaplan–Meier curves of survival times according to the immune subtype are represented in Fig. 6. Univariate analysis showed that IH subtype [HR = 0.37, 95% confidence interval (CI) = 0.14–0.96, $P = 0.04$], satellite nodules (HR = 2.96, 95% CI = 1.08–8.10, $P = 0.034$), and HBV infection (HR = 2.97, 95% CI = 1.06–8.34, $P = 0.039$) significantly influenced overall survival times of patients with cHCC-CCA (increased risk of death; Table 2). Multivariate analysis confirmed the IH was an independent prognostic factor (HR = 0.17, 95% CI = 0.05–0.53, $P = 0.002$).

It has recently been shown that the immune infiltration in the NT liver may impact prognosis in HCC, and we also assessed whether that



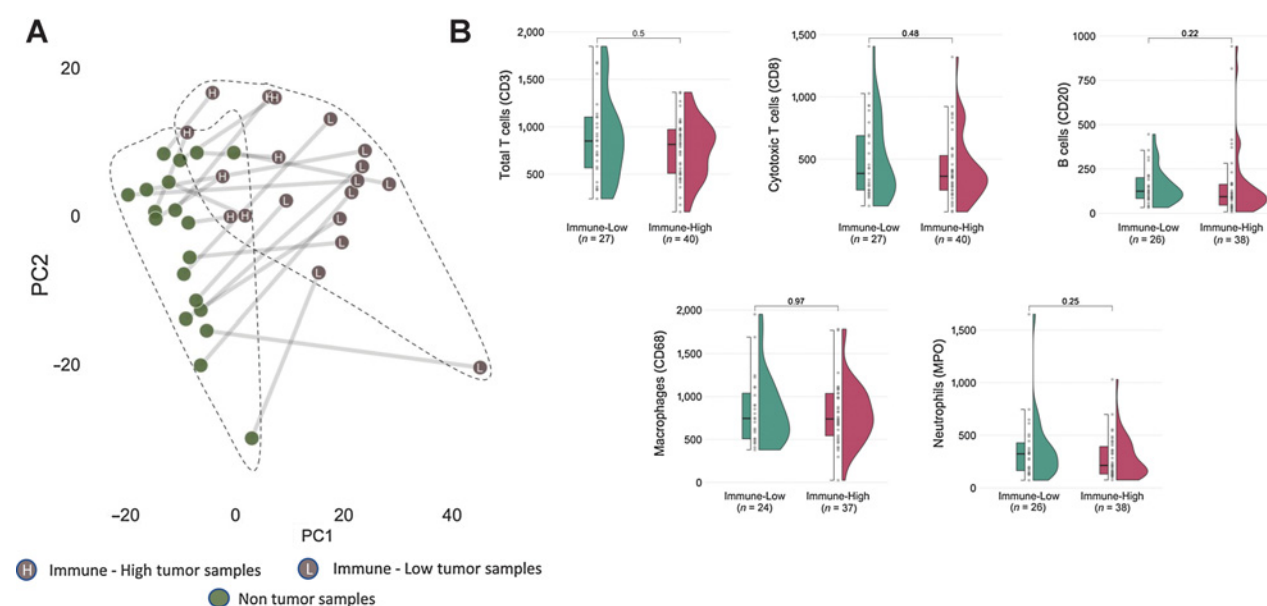


Figure 5. The immune subtype of cHCC-CCAs is not related to the immune infiltration of the surrounding NT liver. **A**, For 19 cases, we performed gene expression profiling on both the tumor and the adjacent liver parenchyma. PCA with 2D representation shows that the tumoral samples do not cluster next to their non-tumoral counterpart. Each pair of samples is connected by a gray line. **B**, There is no difference in immune cell densities in the NT liver according to the immune subtype of the tumor. Total T cells ($P = 0.5$), cytotoxic T cell ($P = 0.48$), B cells ($P = 0.22$), macrophages ($P = 0.97$), neutrophils ($P = 0.25$). Statistical test: Wilcoxon. H, immune-high; L, immune-low.

could also be the case for cHCC-CCA. We however did not observe any significant association between survival and $CD3^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells, $CD68^+$ macrophages, and MPO^+ cells densities (Supplementary Fig. S10).

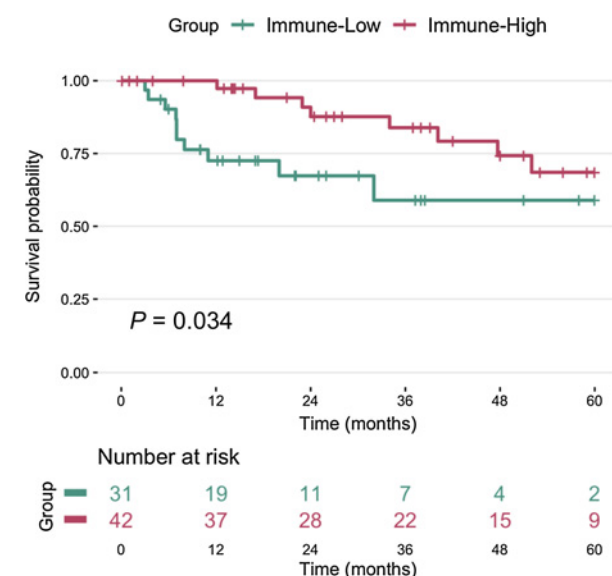


Figure 6. Prognostic impact of cHCC-CCA immune subtype. Kaplan-Meier survival curves according to the immune subtype of cHCC-CCA: patients with IH tumors display a significantly prolonged overall survival ($P = 0.034$; log-rank statistics).

Discussion

Accounting for approximately 1%–5% of PLCs, cHCC-CCA remains an underinvestigated tumor subtype and, given its composite and complex histologic features, cHCC-CCA is also probably underdiagnosed (1, 12). With its aggressive clinical behavior and the lack of guidelines for its management, cHCC-CCA poses greater challenges than HCC and iCCA for which promising therapeutic strategies are emerging (36, 37). The vast majority of cHCC-CCA lack so-called actionable genetic alterations, and other strategies should be considered (20, 38). Immunotherapy has shown impressive results in various solid tumors, and is now the standard of care (in combination with bevacizumab) for patients with advanced HCC (12).

We show in the current study that approximately 60% of cHCC-CCA belong to a particular subclass characterized by a significant immune infiltration, with activation of various pathways related to both innate and adaptive immunity. Although it will have to be investigated by clinical trials, we may hypothesize that patients with this subclass of cHCC-CCA may benefit from immunomodulating agents. Indeed, in other solid tumors, immune infiltration is usually associated with enhanced sensitivity to immunotherapy and we observed, in the IH class of cHCC-CCA, enrichment in several gene signatures associated with response to nivolumab in patients with advanced HCC (16, 17).

One important perspective is to identify the main determinants of this antitumor immunity. Increased infiltration by immune cells has been shown to occur in the stroma of hypermutated tumors (1). It is however unlikely the case in cHCC-CCA as these tumors do not display this particular phenotype (20). Also, our molecular screening did not identify any relationship between cHCC-CCA immune subtypes and the underlying molecular alterations. However, apart from *TP53* mutations, the frequency of each event appears to be very low and it will be difficult, given the rarity of the disease, to reach sufficient

Table 2. Univariate and multivariate Cox regression analysis for overall survival.

Variable	Available data	Univariate analysis				Multivariate analysis			
		HR	95% CI (lower)	95% CI (upper)	P	HR	95% CI (lower)	95% CI (upper)	P
Age (> 60 years old)	73	0.44533	0.17507	1.13279	0.08947	—	—	—	—
Cirrhosis	73	1.42254	0.50311	4.02218	0.50630	—	—	—	—
Etiology (alcohol)	62	0.83412	0.31003	2.24415	0.71945	—	—	—	—
Etiology (hepatitis B)	68	2.97232	1.05907	8.34191	0.03855	5.1984	1.72374	15.6771	0.00343
Etiology (hepatitis C)	71	1.21478	0.39574	3.72889	0.73385	—	—	—	—
Etiology (NASH)	68	1.63295	0.46000	5.79673	0.44806	—	—	—	—
Gender (female)	73	0.37376	0.10798	1.29373	0.12031	—	—	—	—
Immune-high group	73	0.37040	0.14342	0.95658	0.04020	0.1655	0.05161	0.5306	0.00248
Largest nodule diameter	73	1.00338	0.99135	1.01556	0.58312	—	—	—	—
Macrovascular invasion	73	2.26810	0.65385	7.86762	0.19689	—	—	—	—
Microvascular invasion	73	1.95695	0.76283	5.02033	0.16249	—	—	—	—
Multinodular tumor	73	1.5870	0.6114	4.119	0.343	—	—	—	—
Preoperative AFP	43	0.99983	0.99934	1.00033	0.50762	—	—	—	—
Satellite nodules	73	2.96274	1.08364	8.10029	0.03430	6.7656	2.09553	21.8436	0.00139
Surgery type (transplantation)	73	0.62324	0.17924	2.16714	0.45711	—	—	—	—

numbers of altered cases for adequate statistical analyses. Of note is that both *CTNNB1*-mutated cases were classified as IH, whereas this alteration has been reported, in HCC, to suppress immune infiltration (2, 18, 39). Further studies will be needed to confirm that this is not the case for cHCC-CCA. Interestingly, cHCC-CCA are characterized by the expression of several progenitor/oncofetal genes that are usually only expressed during the embryonic or fetal development of the liver (11, 20, 40). The proteins are no longer produced once the immune system is fully developed and thus self-tolerance may not develop against these antigens (41). In this line, we observed that IH tumors display a higher expression of cancer testis-related antigens, which may contribute to induce the observed intratumor immune response. Finally, although we observed an association between the IH subtype and the predominance of an HCC contingent, we did not observe any difference in the *in situ* immune cell densities between each histologic contingent, suggesting that it does not have a direct impact on the intratumor immune infiltration.

It will also be important to have a better understanding of the potential intracluster heterogeneity. Indeed, although our analysis showed two main clusters, we also observed different “subclusters” in both IH and IL tumors. In particular, a subset of IH cHCC-CCA (IH1) with very high expression of markers related to NK cells and innate immunity was identified. NK cells have a well-documented antitumor effect and their presence within the intratumor stroma has been associated with an improved outcome in various human malignancies (42–44). They notably recognize the absence of self MHC I molecules as a way to discriminate normal from transformed cells. We did not observe any downregulation of HLA genes in this particular subcluster, and studies with enrichment in this particular subgroup are needed to determine the triggers of this response.

Consistent with the existence of an effective antitumor immunity, we also showed that the IH of cHCC-CCA was associated with prolonged survival, whereas common pathologic factors such as vascular invasion, tumor size, or satellite nodules did not show any impact on clinical outcome. Importantly, in contrast with former reports on smaller series, the extent of the biliary contingent was not identified as a prognostic factor (45, 46). The prognostic value of the IH subtype may thus have clinical implications for patients’ stratification and management. Indeed, due to its overall poor outcome, diagnosis of cHCC-CCA is currently a contraindication to liver transplantation.

Provided validation in additional cohorts, our results suggest that this therapeutic strategy may be considered for the subset of patients with an IH cHCC-CCA. In this line, some authors have reported long-term survival following liver transplantation for patients with cHCC-CCA (47, 48).

The major limitation of our work is the relatively low number of cases that were investigated, in particular in the validation set. The collection of a large number of cHCC-CCA samples is challenging due to the rarity of the disease, and our results therefore need to be further validated by the investigation of additional cases. The predictive value regarding treatment and prognosis will also need to be determined on biopsy samples in patients with more advanced disease.

In conclusion, we identified a subset of cHCC-CCA characterized by a significant immune infiltration, a more favorable clinical outcome and an enrichment in gene signatures shown to be predictive of response to nivolumab in patients with HCC. We believe that our findings may have important implications for the design of future clinical trials and the stratification of patients with this highly aggressive malignancy.

Authors’ Disclosures

J. Augustin reports personal fees from Servier outside the submitted work. V. Leroy reports personal fees and non-financial support from Abbvie, Intercept, and Gilead and personal fees from Mayoli outside the submitted work. J.C. Nault reports grants from IPSEN and BAYER during the conduct of the study. M. Allaire reports other support from Roche and Ipsen outside the submitted work. J. Zucman-Rossi reports grants from Ligue contre le Cancer and Inserm during the conduct of the study. J.-M. Pawlowsky reports personal fees from Gilead, Abbvie, Merck, Regulus, Memo Therapeutics, Assembly Biosciences, and Arbutus outside the submitted work. C. Tournigand reports grants from Bayer outside the submitted work. J. Calderaro reports grants from Fondation Bristol Myers Squibb pour la Recherche en Immunologie during the conduct of the study; personal fees from Ipsen outside the submitted work. No disclosures were reported by the other authors.

Authors’ Contributions

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writing—review and editing. **F. Lafdil:** Conceptualization, writing—review and editing. **V. Paradis:** Conceptualization, data curation, formal analysis, writing—review and editing. **J. Calderaro:** Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing.

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