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Immunotherapy-induced cholestasis in cancer: insights from the two real-world pharmacovigilance databases of FAERS and VigiBase

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Background: The US Food and Drug Administration (FDA) recently issued a safety alert regarding cholestasis as a potential adverse reaction to immune checkpoint inhibitor (ICI) therapy. However, the underlying mechanisms of ICI-induced cholestasis remain poorly elucidated.

Methods: This study analyzed adverse event reports of cancer patients treated with ICIs, extracted from the FAERS (2013–2023) and VigiBase (1968–2023) databases. The reporting odds ratio (ROR) and information component (IC) methods were employed to evaluate the association between cholestasis and ICIs therapy, while time-to-onset (TTO) analysis was conducted. Clinical data from hospitals and results from mouse experiments were integrated to validate the analysis findings.

Results: Both ROR and IC analyses demonstrated a statistically significant elevation in cholestasis risk among ICI-treated patients compared to those receiving conventional chemotherapy. A heightened risk was observed in the 0–65 age cohort, with no significant gender-specific disparities noted. The TTO analysis revealed a delayed onset of cholestasis in both ICI-treated patients and female subjects compared to their respective counterparts. Gene expression profiling elucidated multiple cholestasis-associated signaling pathways, encompassing biliary inflammation, bile acid metabolic disorders, and impairment of hepatocellular drug metabolism.

Conclusion: ICI-treated patients exhibit a higher cholestasis risk compared to conventional chemotherapy. Long-term liver function monitoring is crucial for patient safety. ICI-related cholestasis may result from immune-mediated bile duct injury or metabolic disorders, potentially influenced by baseline liver function. This comprehensive article provides crucial evidence for the risk assessment and management of ICI-related cholestasis, thereby contributing to safe medication use and enhanced patient care in clinical practice.

Keywords: cholestasis, data mining, FAERS and VigiBase, ICI, mechanism

Introduction

Immune checkpoint inhibitors (ICIs) have revolutionized cancer immunotherapy by demonstrating remarkable efficacy in treating various solid tumors through the enhancement of anti-tumor immune responses[1-4]. ICIs have been extensively employed in the treatment of melanoma, non-small cell lung cancer, renal cell carcinoma, and urothelial carcinoma[5-12], resulting in significant improvements in patient outcomes. The expanding clinical applications of ICIs, however, have led to an increase in reported

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immune-related adverse events (irAEs) affecting multiple organ systems[13-16]. Among these irAEs, hepatotoxicity emerges as a common and potentially severe complication that warrants special attention[17]. ICI-induced hepatic adverse reactions typically manifest as immune-mediated hepatitis, characterized by elevated serum transaminase and/or bilirubin levels. Although the majority of cases are manageable, a subset of patients may develop severe hepatitis, which can potentially progress to fatal liver failure[18,19]. Consequently, rigorous monitoring and prompt intervention strategies are imperative in clinical applications.

Cholestasis, a condition characterized by the abnormal accumulation of bile, is a rare but potentially severe irAE associated with the use of ICIs[20-22]. Despite its rarity, cholestasis can lead to treatment interruption, necessitate the administration of immunosuppressive therapy, and potentially impact overall treatment outcomes. Furthermore, it may serve as a harbinger of more severe liver injuries[23], potentially compromising patients' long-term prognosis. Despite the reported cases of ICIs-induced cholestasis, systematic studies investigating its precise mechanisms, predictive factors, and long-term prognosis remain scarce. Existing research is primarily limited to small-scale retrospective analyses, with a notable absence of large-scale prospective studies necessary for accurately assessing incidence and identifying potential risk factors. These research limitations pose significant challenges for clinicians in the diagnosis and management of ICIs-induced cholestasis.

Given the potential severity of ICIs-related cholestatic adverse reactions, a thorough understanding of their clinical manifestations, underlying mechanisms, and the development of effective management strategies is crucial for minimizing patient risk and optimizing treatment outcomes. By leveraging real-world data from the FDA Adverse Event Reporting System (FAERS)[24] and the World Health Organization Collaborating Center for International Drug Monitoring Individual Safety Reporting database (VigiBase)[25], researchers can comprehensively explore the characteristics of cholestasis occurrence in ICIs therapy, including incidence rates, patient clinical features, and potential risk factors. Further analysis utilizing The Cancer Genome Atlas (TCGA) database can elucidate potential molecular mechanisms and identify key molecular pathways and genes associated with ICIs-induced cholestasis. The findings from these bioinformatics analyses need to be validated in animal models by observing the process of cholestasis development and changes in molecular marker expression, thus enabling the refinement of hypotheses based on TCGA data. These research findings will assist clinicians in more accurately identifying and managing ICIs-related cholestasis, while providing a foundation for developing precise prevention and treatment strategies, ultimately enhancing the safety and efficacy of ICI therapy.

HIGHLIGHTS

- A significantly higher risk of cholestatic adverse events in ICI-treated patients compared to those receiving conventional chemotherapy, based on the analysis of FAERS and VigiBase databases.
- Identification of age-related risk factors and gender differences in the onset of ICI-induced cholestasis.
- A comprehensive approach, combining pharmacovigilance data analysis, clinical validation, and experimental studies, provides novel insights into the characteristics and mechanisms of ICI-induced cholestasis.
- Elucidation of potential biological mechanisms underlying ICIs-induced cholestasis through pan-cancer transcriptomic analysis and experimental validation in mouse models.
- Proposal of clinical strategies for early detection and management of ICI-related cholestasis.

Methods

Data sources and processing

This study systematically examined the characteristics of ICIs-related cholestasis utilizing the FAERS and VigiBase databases. The data screening and processing procedures were conducted as follows:

Adverse event reports were extracted from the FAERS database (2013Q1–2023Q4) and the VigiBase database (1968Q1–2023Q4), thereby establishing a large-scale retrospective study. For both the FAERS and VigiBase databases, ICIs were used as drug keywords, encompassing anti-Programmed-Death-1 (anti-PD-1) drugs (nivolumab, pembrolizumab, and cemiplimab), anti-Programmed-Death-Ligand 1 (anti-PD-L1) drugs (atezolizumab, avelumab, and durvalumab), and anti-Cytotoxic-T-Lymphocyte-Associated-Protein-4 (anti-CTLA-4) drugs (ipilimumab and tremelimumab). Simultaneously, "Cholestasis," "Cholestatic liver injury," and "Hepatitis cholestatic" were designated as keywords for cholestasis-related adverse reactions (Supplementary Method: http://links.lww.com/JS9/E288).

Given the potential for duplicate reports in the FAERS and VigiBase databases, this study implemented a rigorous deduplication process. This process utilized a combination of patient identification number, report date, drug name, and adverse reaction description[26] to mitigate the potential impact of duplicate reports on the study outcomes. Following a stringent screening process, a total of 488 cases from the FAERS database and 146 cases from the VigiBase database were ultimately incorporated into the analysis. The comprehensive data processing workflow is delineated in Figure 1.

Time-to-onset analysis

In the FAERS database, the date of adverse event (AE) occurrence is defined as EVENT_DT, whereas the start date of drug administration is defined as START_DT. The time interval between these two dates is designated as the time to onset. For records with incomplete date information, when only the year and month were available, we imputed the date as the first day of the corresponding month. Utilizing the FAERS data, we employed cumulative distribution curves to elucidate the time-to-onset characteristics of cholestasis adverse reactions in gender and age subgroups following immunosuppressant therapy, concurrently comparing the differences in adverse event occurrence times between ICIs and chemotherapy. Statistical analysis was conducted using the Mann–Whitney *U* test for intergroup comparisons.

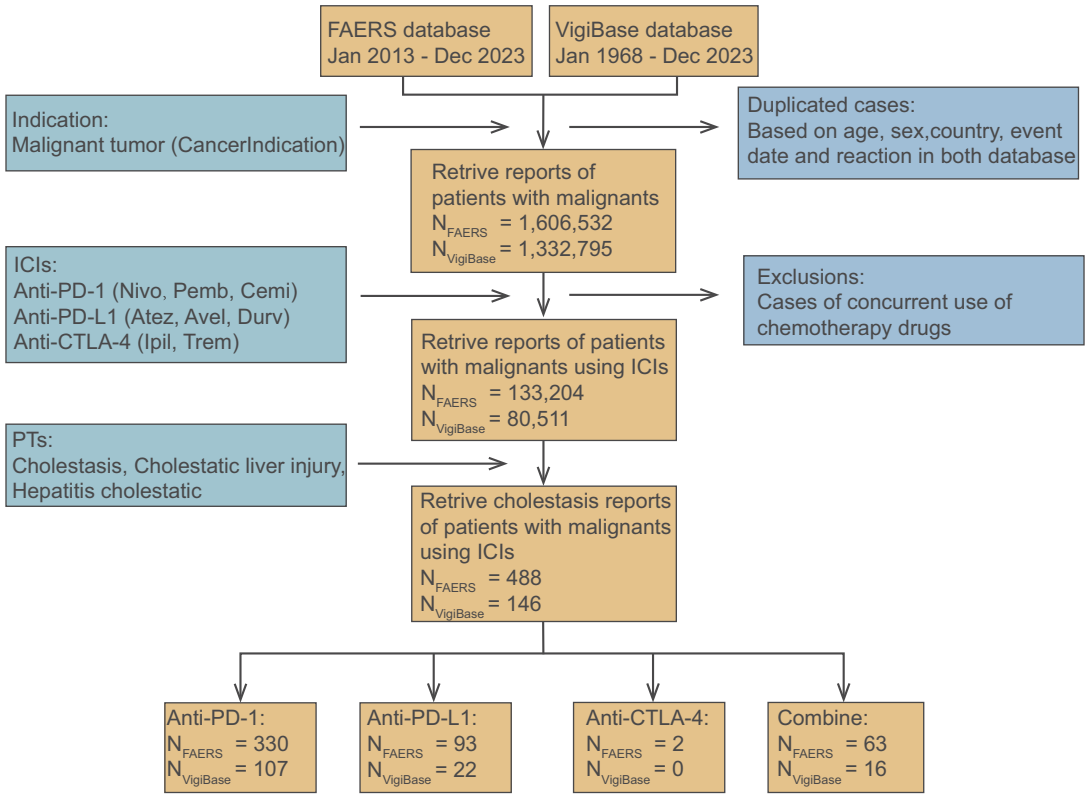

Figure 1. Data processing flowchart delineating the selection process of CirAEs post-ICIs therapy. The flowchart provides a detailed description of the selection process for ICIs-related cholestatic adverse events identified in the US Food and Drug Administration Adverse Event Reporting System (FAERS) and VigiBase databases. Atez: atezolizumab; Avel: avelumab; Cemi: cemiplimab; CirAEs: cholestatic immune-related adverse reactions; Durv: durvalumab; ICIs: immune checkpoint inhibitors; Ipil: ipilimumab; Nivo: nivolumab; Pemb: pembrolizumab; Trem: tremelimumab.

TCGA database analysis

For the molecular mechanism study, we performed an analysis of cholestasis-related molecular mechanisms utilizing the TCGA database. Data acquisition and processing were conducted as follows: FPKM-formatted transcriptome data for 26 cancer types were obtained from the TCGA project through the UCSC Xena database[27]. Subsequently, the FPKM expression matrix was converted to a Transcripts Per Million (TPM) matrix[28]. Single-sample Gene Set Enrichment Analysis (ssGSEA) was applied to calculate pathway scores for each tumor sample[29]. The median was utilized as the representative value for pathway scores of each cancer type. A pan-cancer level analysis was conducted to investigate the correlation between the Reporting Odds Ratio (ROR) of ICIs-related cholestatic adverse reactions and the activation levels of biological pathways[30,31]. This analysis aimed to elucidate potential pathogenic mechanisms underlying ICIs-related cholestatic adverse reactions.

Local data analysis

To further validate the analytical results from the FAERS and VigiBase databases, this study conducted a retrospective analysis of clinical examination data from patients receiving ICI treatment at our hospital. The inclusion criteria for the study were as follows: (1) patients with a histologically confirmed cancer diagnosis; (2) administration of at least one ICI treatment; (3) availability of comprehensive clinical information records. The clinical data from the local database were utilized primarily to corroborate the findings derived from the FAERS and VigiBase analyses. This study was conducted in accordance with ethical standards and was approved by the Institutional Review Board. Furthermore, written informed consent was obtained from all patients participating in this study.

Establishment of an ICI treatment mouse model

This work has been reported in line with the ARRIVE criteria[32]. All animal experiments in this study were conducted in strict accordance with the protocol approved by the Institutional Animal Care and Use Committee. The 24 mice (male C57BL/6 mice, aged 5–6 weeks) were randomly assigned to six experimental groups, each consisting of four mice receiving four intraperitoneal injections administered at three-day intervals. The treatment regimens for each group were as follows: (1) anti-PD-1 antibody group: 200 μg of anti-PD-1 antibody (clone RMP1-14, BioXCell, West Lebanon, NH, USA); (2) Anti-PD-L1 antibody group: 200 μg of anti-PD-L1 antibody (clone 10 F.9, BioXCell, West Lebanon, NH, USA); (3) Anti-CTLA-4 antibody group: 200 μg of anti-CTLA-4 antibody (clone 9D9, InVivoGen, San Diego, CA, USA); (4) Anti-CTLA-4/PD-1 combination group: 100 μg of anti-CTLA-4 antibody (clone 9D9, InVivoGen, San Diego, CA, USA) combined with 100 μg of anti-PD-1 antibody (clone RMP1-14, BioXCell, West Lebanon, NH, USA); (5) Anti-CTLA-4/PD-L1 combination

group: 100 μg of anti-CTLA-4 antibody (clone 9D9, InVivoGen, San Diego, CA, USA) combined with 100 μg of anti-PD-L1 antibody (clone 10 F.9, BioXCell, West Lebanon, NH, USA); (6) Control group: an equal volume of phosphate-buffered saline (PBS). Seventy-two hours after the final administration, mice were euthanized humanely, peripheral blood samples were collected, and serum biochemical indicators, including ALT, AST, direct bilirubin (DBIL), and total bilirubin (TBIL), were measured using standard laboratory techniques. Additionally, mouse liver tissue samples were collected and processed for high-throughput sequencing analysis. Sequencing data were quantified based on the mouse reference genome (mm39) to obtain raw counts using standard bioinformatics pipelines. Subsequently, ssGSEA was employed to process the transcriptome data and calculate pathway activity scores for the mice, as previously described[33]. Simultaneously, we harvested liver tissues, which were first washed with sterile PBS solution and subsequently fixed in 4% paraformaldehyde solution for 24 hours, followed by standard tissue embedding and hematoxylin-eosin (H&E) staining.

Statistical analysis

This study utilized two established pharmacovigilance statistical methods, the ROR and Information Component (IC)[34-36], to assess the association between ICIs and cholestasis-related adverse events. A statistically significant signal was defined as meeting all of the following criteria: the number of reported cholestasis-related adverse events (a) was ≥ 3, the lower bound of the 95% confidence interval for ROR (ROR025) exceeded 1, and the lower bound of the 95% confidence interval for IC (IC025) was greater than 0[35,37]. These criteria suggest that the use of ICIs may significantly elevate the risk of cholestasis-related adverse events compared to conventional chemotherapy agents. All statistical analyses were conducted using R software (Version 4.3.3). Data visualization, including box plots, bar charts, and cumulative curves, was generated using the ggplot2 package in R. Statistical significance was defined as a two-sided P value <0.05 throughout this study.

Results

Characterization of cholestasis adverse reactions following ICIs treatment: an analysis of FAERS and VigiBase databases

This study systematically analyzed the FAERS and VigiBase databases, identifying 488 cases (FAERS) and 146 cases (VigiBase) of cancer patients who developed cholestasis following treatment with ICIs in the respective databases (Fig. 1). Statistical analysis using the ROR and IC methods demonstrated that, in both FAERS and VigiBase databases, the risk of cholestatic adverse reactions associated with ICIs use was significantly higher than that of chemotherapeutic agents (ROR_{FAERS} = 1.16, $ROR_{VigiBase}$ = 1.78; $IC025_{FAERS}$ = 0.003, $IC025_{VigiBase}$ = 0.33; Figs. 2A and B).

To further elucidate the impact of different ICI types on the risk of cholestatic adverse reactions, we conducted a drug subgroup analysis. The results indicated that, in both databases, the highest risk of cholestasis was associated with the use of anti-PD-1 drugs, either as monotherapy or in combination regimens. Additionally, monotherapy with anti-PD-L1 drugs also demonstrated a significant increase in cholestasis risk in the VigiBase database. Patients were stratified into two age groups (0–65 years and >65 years), and subgroup analyses were conducted based on age and gender. Results revealed that, in both databases, the 0–65 age group exhibited a higher risk of cholestasis compared to the >65 age group, while gender did not demonstrate a consistent impact (Figs. 2A and B). We also examined the differences in the occurrence of three predefined cholestasis-related adverse reactions among different ICI types (Fig. 2C).

Time-to-onset (TTO) analysis of cholestasis-related adverse events following ICIs treatment

TTO analysis of cholestasis-related adverse events in the FAERS database revealed that the median time for ICIs to induce adverse events was significantly longer than that of chemotherapy drugs (ICIs vs. Chemotherapy median [Q1–Q3]: 1.60 [0.67–4.07] vs. 0.70 [0.23–2.07] months; $P < 0.05$; Figure 2D). To further investigate potential factors influencing TTO in ICI-treated populations, we conducted additional analyses based on FAERS data, considering demographic factors such as patient sex and age. Results indicated that sex was a significant factor influencing the TTO of cholestasis-related adverse events induced by ICIs (Fig. 2E). Our analysis revealed that, compared to females, males experienced a significantly longer median time to adverse event onset (female vs. male median [Q1–Q3]: 1.17 [0.50–3.17] vs. 1.90 [0.70–5.12] months; $P < 0.05$; Fig. 2E). We stratified patients into two age groups (0–65 years and over 65 years), and the analysis revealed no significant difference in TTO for ICIs-related adverse events between these groups (Fig. 2F). The overall cohort exhibited peak incidence within the initial treatment month, whereas gender-stratified analysis demonstrated the highest frequency in the second week in both genders (Figs. 3A–C).

The association between clinical factors and the occurrence of ICI-related cholestasis

Through screening of ICI reports in the FAERS and VigiBase databases, we identified 488 and 146 reports of cholestatic adverse reactions associated with ICI use, respectively. Subsequently, we conducted a comprehensive statistical analysis of the clinical characteristics of these reported cases. The median age of patients in the FAERS database was 67 years (interquartile range [IQR]: 17–88 years, n = 488). We stratified patients into three age groups using thresholds of 45 and 65 years. In both databases, patients over 65 years of age constituted the largest proportion (FAERS: n = 318, 65.2%; VigiBase: n = 68, 46.8%; Fig. 3D–E). Male patients were more frequently reported than females in both databases (FAERS: males n = 241, 49.4%, females n = 216, 44.3%; VigiBase: males n = 98, 67.1%, females n = 42, 28.8%; Figs. 3F and G). The peak reporting year in the FAERS database was 2023 (n = 148, 30.3%), whereas, in the VigiBase database, it was 2020 (n = 35, 24.0%) (Figs. 3D and F). With respect to reporting regions, France predominated in the FAERS database, while the European region comprised the vast majority in the VigiBase database (FAERS: n = 301, 61.7%; VigiBase: n = 130, 89.0%). Among ICI treatment strategies, anti-PD-1 monotherapy and ICI combination therapy were predominant (anti-PD-1: FAERS

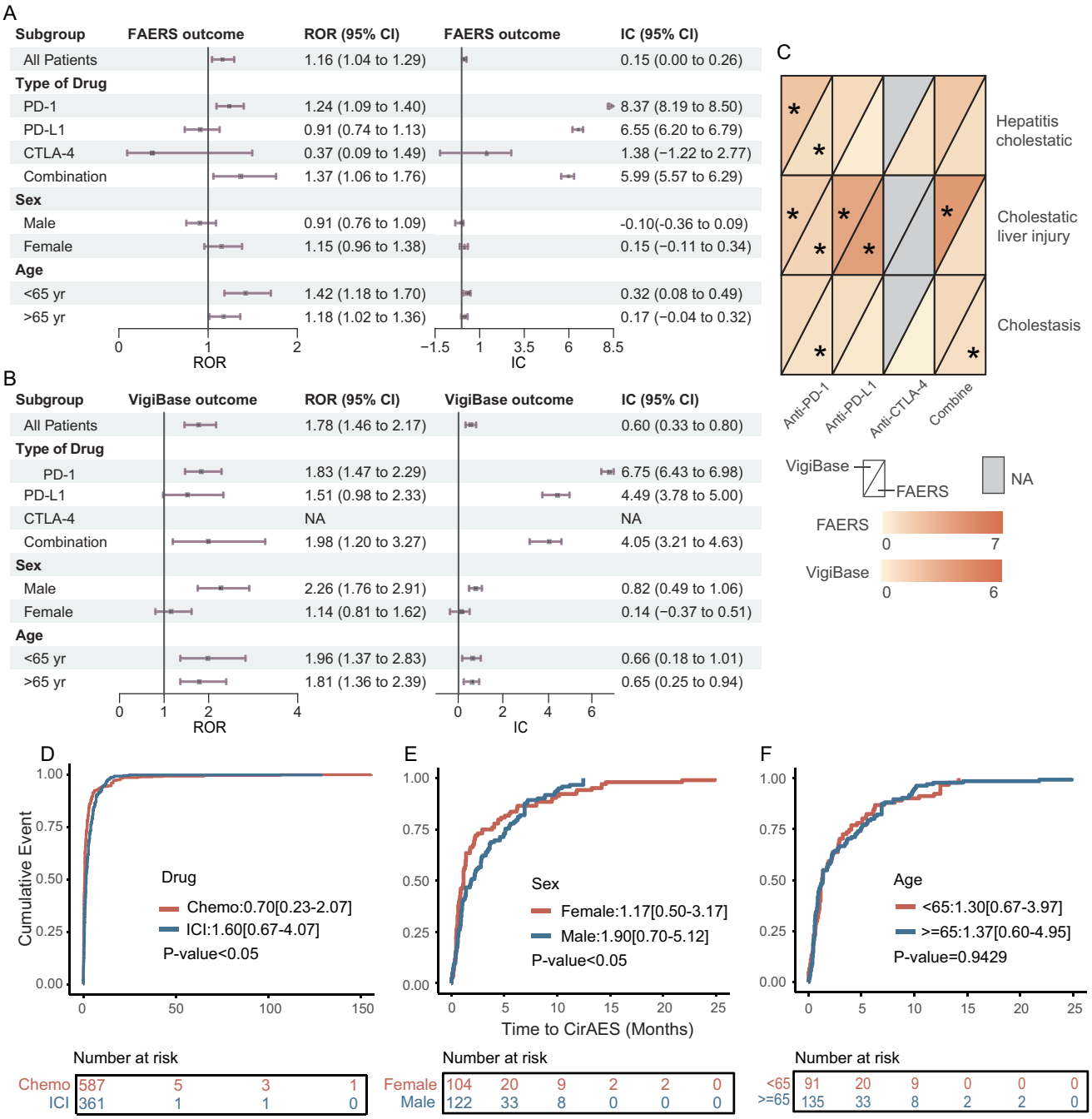

Figure 2. A comprehensive investigation of ICIs-related cholestatic adverse events based on data from the FAERS and VigiBase databases. (A and B) Forest plots depict the ROR and IC for cholestasis associated with ICIs in the FAERS (A) and VigiBase (B) databases, calculated using ROR and IC methodologies. The plots also incorporate results of subgroup analyses for ICI drug types, gender, and age groups, providing a comprehensive overview of the data. Error bars represent 95% confidence intervals (CI) for ROR and IC. (C) A heatmap visualizes ROR values for three Preferred Terms (PTs) ("Cholestasis," "Cholestatic liver injury," "Hepatitis cholestatic") across different treatment strategies (anti-PD-1, anti-PD-L1, anti-CTLA-4, and their combinations) in both the FAERS and VigiBase databases. The intensity of colors correlates with ROR values, with darker shades representing higher values. An asterisk (*) denotes an ROR025 > 0 for the corresponding PT in conjunction with the specified treatment strategy. (D) Cumulative distribution curves illustrate the time to onset of cholestatic adverse reactions following immunosuppressive and chemotherapy treatments, as reported in FAERS. (E and F) Cumulative distribution curves demonstrate the time to onset of cholestatic adverse reactions following immunosuppressive treatment, stratified by gender (E) and age group (F), based on data from FAERS. Age groups were dichotomized into "<65 years" and "≥65 years" cohorts. Statistical comparisons were performed using the Mann–Whitney *U* test to assess differences between groups. CirAEs: cholestatic immune-related adverse events; ICIs: immune checkpoint inhibitors; ROR: reporting odds ratio; IC: information component.

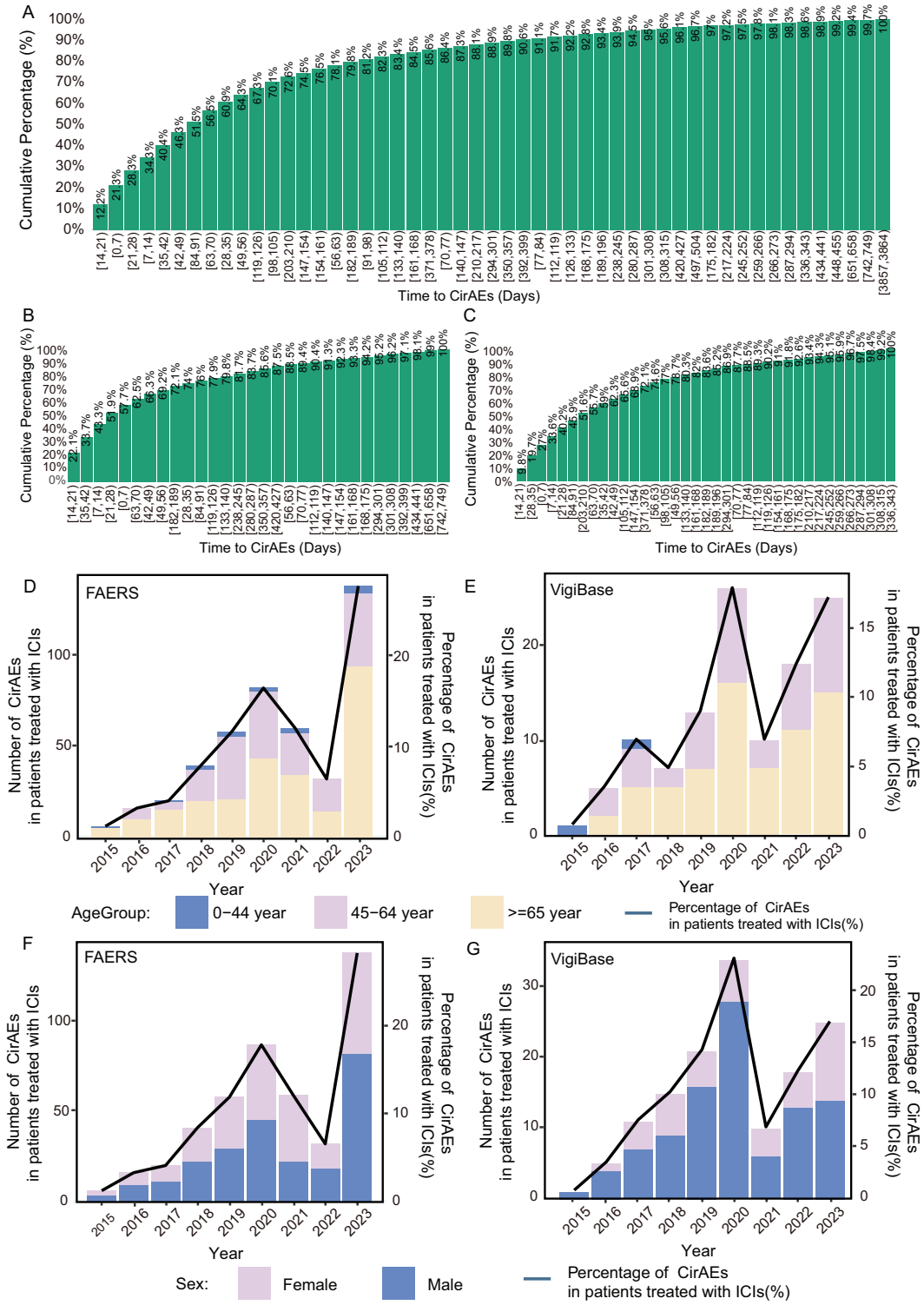

Figure 3. Cumulative Incidence of CirAEs onset and incidence and distribution of cholestatic adverse reactions associated with ICIs as reported in the FAERS (2013–2023) and VigiBase (1968–2023) databases. (A–C) Cumulative percentage charts depict the proportion of the TTO of cholestatic adverse reactions in patients with malignant neoplasms undergoing ICIs therapy, as reported in the ICI regimen (A), female subgroup (B), and male subgroup (C). (D and E) Stacked bar graphs depict the annual distribution of cholestatic adverse reactions in patients with malignant neoplasms undergoing ICIs therapy, as reported in FAERS (D) and VigiBase (E), with color-coding representing age stratification. (F and G) Stacked bar graphs present the annual distribution of cholestatic adverse reactions in patients with malignant neoplasms receiving ICIs therapy, as reported in FAERS (F) and VigiBase (G), with color-coding representing gender stratification. Overlaid line graphs demonstrate the annual incidence rates, computed as the proportion of ICIs-associated cholestatic events relative to the total cholestatic events reported in each database per year. CirAEs: cholestatic immune-related adverse events.

n = 330, 67.6%, VigiBase *n* = 107, 73.3%; anti-PD-L1: FAERS *n* = 93, 19.1%, VigiBase *n* = 22, 15.1%).

A retrospective analysis of the effects of ICI therapy on hepatic function and bilirubin metabolism

We conducted a retrospective study on patients undergoing ICIs therapy. We analyzed serum biomarkers associated with hepatic function (AST, ALT, and alkaline phosphatase [ALP]) and bilirubin metabolism (TBIL, DBIL, and indirect bilirubin [IBIL]). We stratified these biomarkers into pre- and post-treatment groups based on the timing of ICIs administration. We then calculated mean values for each patient during both periods and conducted statistical analyses using the paired Wilcoxon signed-rank test. Our findings revealed that patients' hepatic function markers and bilirubin levels were generally elevated following ICIs therapy compared to baseline measurements (AST: mean 43.40 vs. 57.90 U/L, $P < 0.05$; ALT: mean 36.74 vs. 39.64 U/L, $P > 0.05$; ALP: mean 115.89 vs. 131.32 U/L, $P < 0.05$; TBIL: mean 14.77 vs 19.07 μmol/L, $P < 0.05$; IBIL: mean 6.44 vs 7.44 μmol/L, $P < 0.05$; DBIL: mean 7.96 vs 11.48 μmol/L, $P > 0.05$; Fig. 4A-F).

ICIs-induced cholestasis significantly affected patients' clinical outcomes. Analysis of approximately 2200 cases of ICIs-induced cholestasis from our institutional database demonstrated that cholestasis was associated with significant hepatic dysfunction, characterized by marked elevations in AST, ALT, ALP, TBIL, IBIL, and DBIL levels (Fig. 4A-F). In these cases, ICI therapy is typically suspended, and hepatoprotective interventions are initiated. However, despite these management strategies, patients' clinical outcomes often remain suboptimal.

Mouse experiments for validation

In our experimental validation using an ICIs-treated mouse model (Fig. 5A), we systematically evaluated the effects of various ICIs, administered either as monotherapy or combination therapy, on murine hepatic function. The primary endpoints

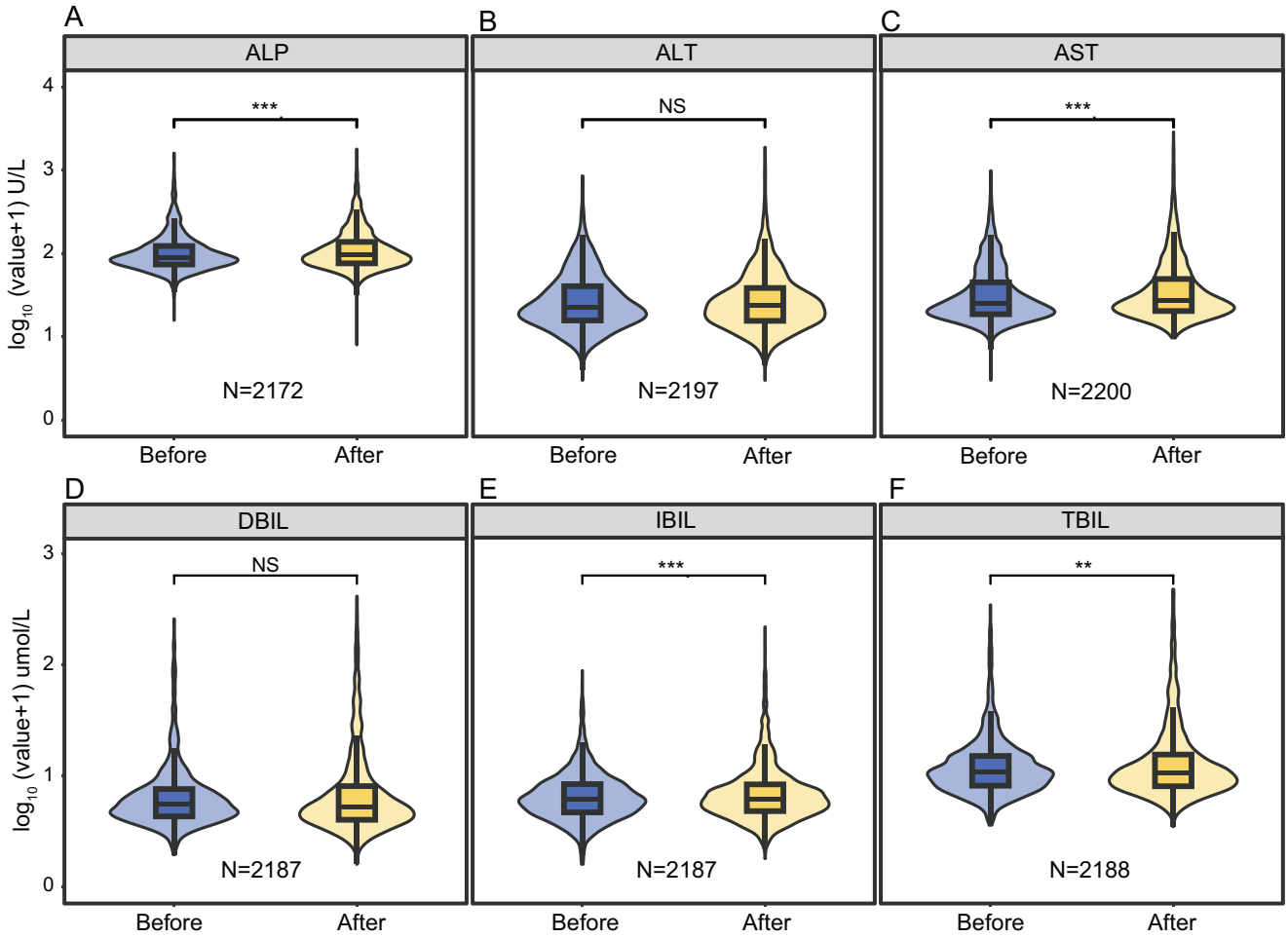

Figure 4. Alterations in hepatic function parameters and bilirubin fractions in patients with malignant neoplasms following ICIs therapy: analysis of the local database. (A–F) Box and violin plots are employed to visualize changes in hepatic function markers and bilirubin fractions in patients with malignant neoplasms following ICIs therapy. Figure A depicts ALP levels, Figure B shows ALT levels, and Figure C presents AST levels. Figure D presents DBIL levels, Figure E shows IBIL levels, and Figure F depicts TBIL levels. Statistical analysis was performed using paired Wilcoxon signed-rank tests. Significance levels: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBIL, direct bilirubin; IBIL, indirect bilirubin; TBIL, total bilirubin.

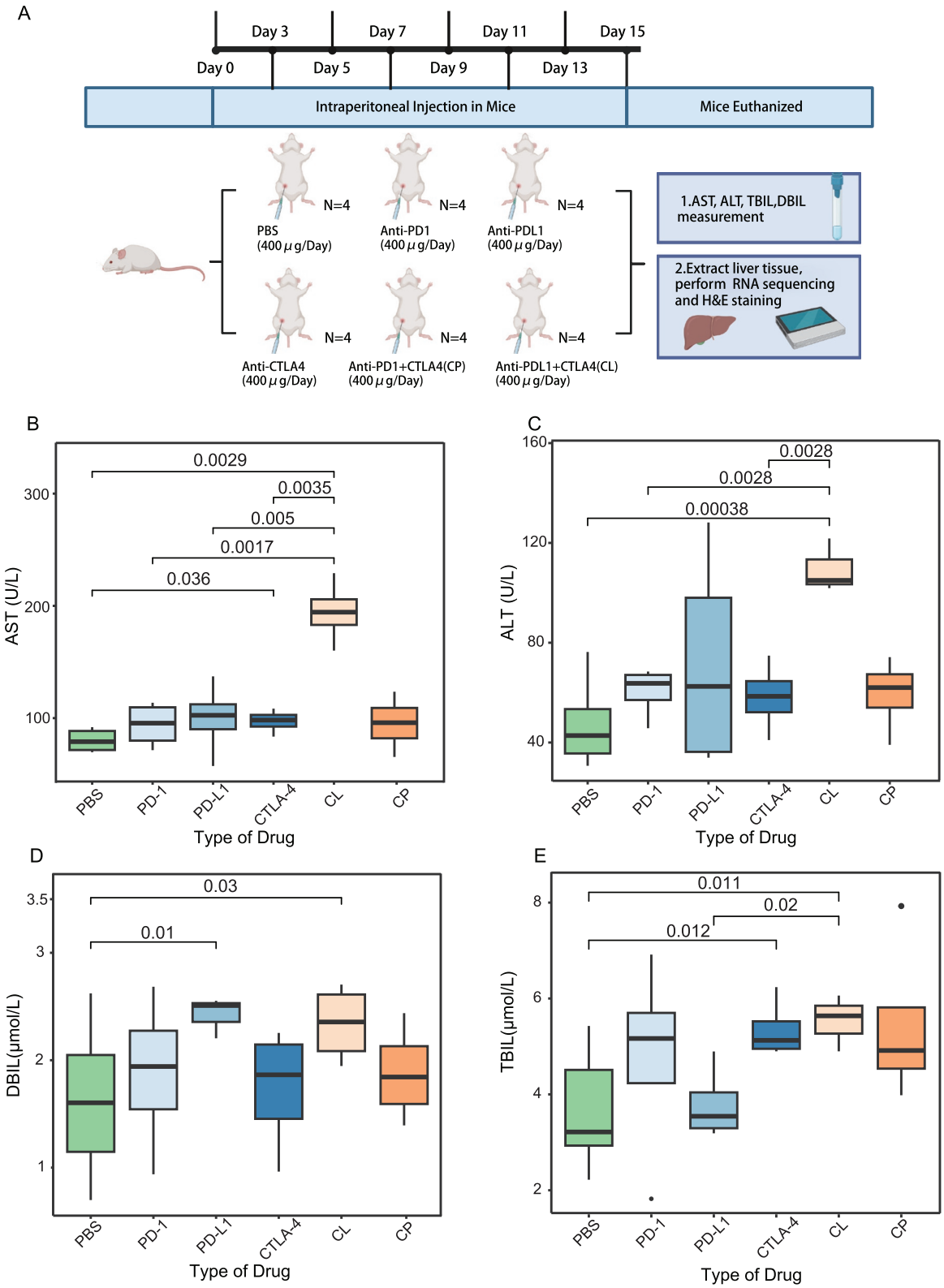

Figure 5. Alterations in hepatic function and bilirubin levels in mice following ICIs treatment: an experimental study. (A) This flow chart delineates the construction of the murine experimental model, followed by peripheral blood testing and RNA sequencing. (B–E) These box plots illustrate alterations in hepatic function and bilirubin levels in mice following treatment with various ICIs. The plots depict levels of (A) AST, (B) ALT, (C) DBIL, and (D) TBIL across treatment groups. Statistical analysis was conducted using the paired Wilcoxon signed-rank test, with corresponding p-values indicated in each plot. Treatment groups: anti-PD-1, anti-PD-L1, anti-CTLA-4, combination of anti-CTLA-4 and anti-PD-L1 (CL), and combination of anti-CTLA-4 and anti-PD-1 (CP). ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBIL, direct bilirubin; TBIL, total bilirubin.

monitored were AST, ALT, DBIL, and TBIL levels. Our findings revealed that, among all monitored parameters, the anti-CTLA-4 and anti-PD-L1 combination therapy group (CL group) demonstrated a higher propensity for elevated biomarkers compared to other treatment cohorts. This suggests that the concurrent administration of anti-CTLA-4 and anti-PD-L1 inhibitors may potentially induce more severe hepatocellular injury and cholestasis. Nevertheless, no statistically significant differences were observed in these biomarkers between the monotherapy and combination therapy groups (Fig. 5B–E).

Elucidation of putative biological mechanisms

Through the integration of bioinformatics analyses of TCGA data and differential pathway analyses of murine hepatic tissues, we have identified several signaling pathways significantly associated with cholestasis (Fig. 6). In our pan-cancer analysis, the ROR of ICIs-related cholestatic adverse events exhibited significant heterogeneity across diverse cancer types (Fig. 7A). Analysis of the FAERS revealed that the ROR for ICIs-related cholestatic adverse events was highest in patients with skin cutaneous melanoma (SKCM) (ROR = 3.96). In contrast, analysis of VigiBase demonstrated that this indicator peaked in patients with adrenocortical carcinoma (ACC) (ROR = 10.98) (Fig. 7A). Our analysis of TCGA data revealed that the gene expression profiles of patients with cholestasis exhibited significant correlations with pathways associated with bile acid metabolic dysregulation and impaired hepatocellular drug metabolism (Fig. 7B)[38,39].

Specifically, the primary pathways implicated in this process encompass the significant upregulation of both the monooxygenase activity (GO_MONOOXYGENASE_ACTIVITY) pathway[40] and the endogenous sterol (REACTOME_ENDOGENOUS_STEROLS) pathway, which are intricately involved in bile acid synthesis and metabolism (Fig. 7B-C)[41,42]. These pathways play pivotal roles in the pathophysiology of cholestasis, potentially disrupting normal bile excretion through their influence on drug metabolism. In our murine model treated with ICIs, we observed a significant enrichment of key pathways associated with

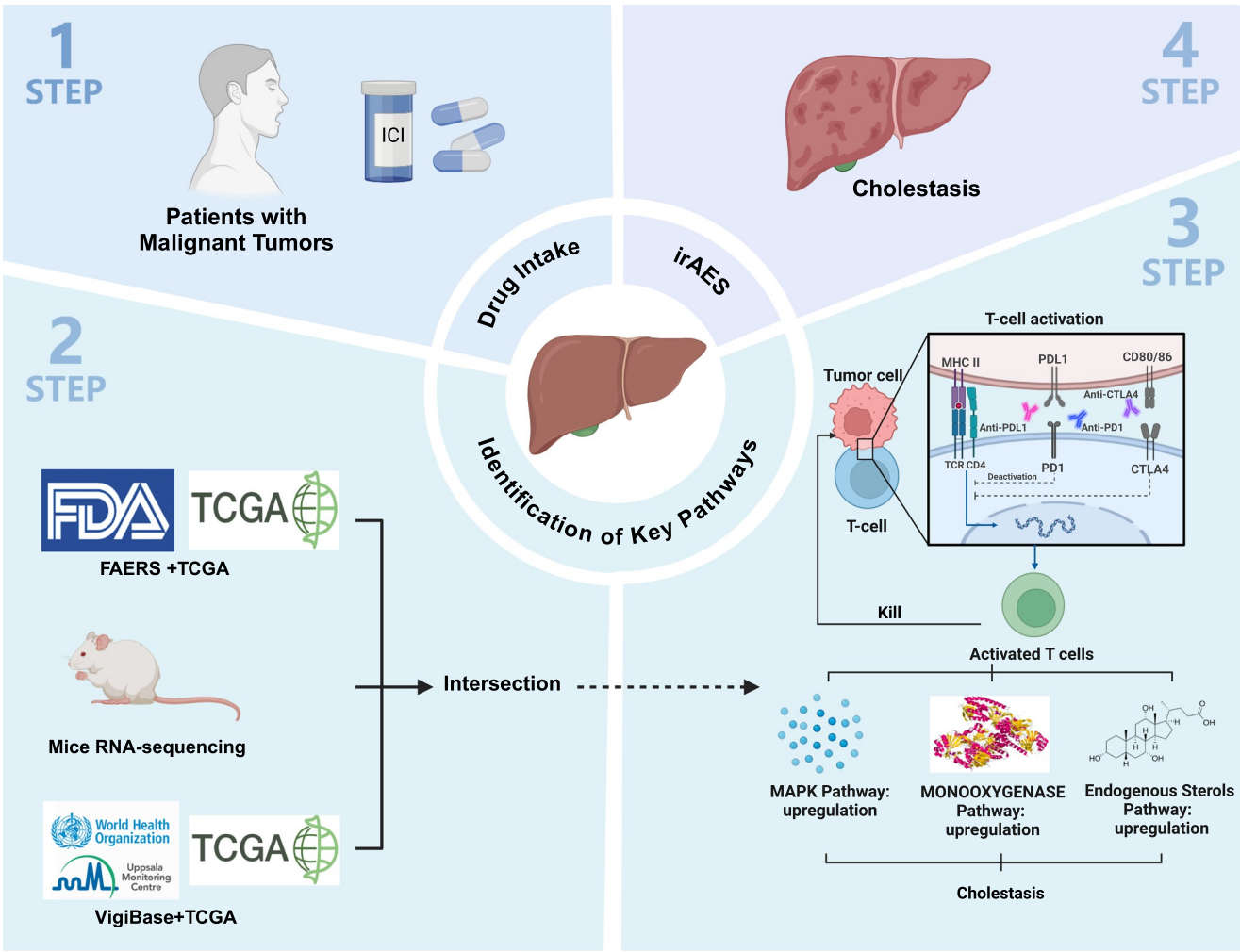

Figure 6. Elucidation of key signaling pathways associated with cholestasis. This schematic illustration delineates the systematic screening process for key signaling pathways implicated in ICI-induced cholestasis. Furthermore, it proposes putative mechanistic explanations for these cholestatic irAEs. irAEs, immune-related adverse events.

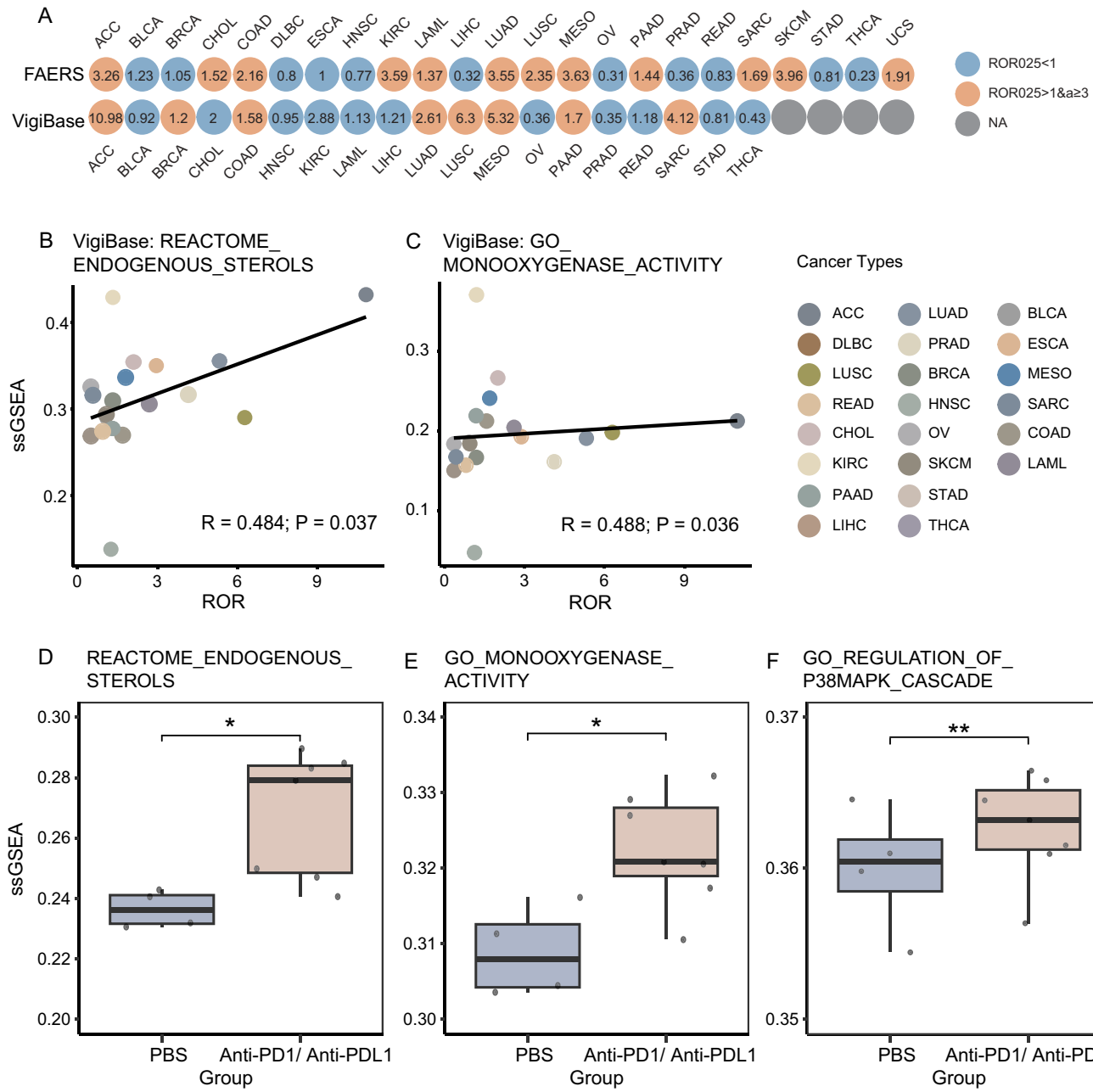

Figure 7. Exploration of the association between ICIs-induced cholestasis and relevant biological pathways. (A) Heatmap visualization of ROR values for ICIs-induced cholestasis across 23 distinct cancer types, derived from the FAERS and VigiBase databases. Cancer types with fewer than three reported cases were excluded from the analysis. Color coding: red denotes statistically significant results, blue indicates non-significant findings, and gray represents not available (NA) data. (B and C) Scatter plots illustrating the correlation between ROR values of ICIs-induced cholestasis and ssGSEA enrichment scores for the following pathways: (B) REACTOME_ENDOGENOUS_STEROLS, (C) GO_MONOOXYGENASE_ACTIVITY. Correlations were assessed using Spearman's rank correlation coefficient. (D–F) Box plots depicting differential ssGSEA enrichment scores between control and ICIs-treated mice for the following pathways: (D) REACTOME_ENDOGENOUS_STEROLS, (E) GO_MONOOXYGENASE_ACTIVITY, and (F) GO_REGULATION_OF_P38MAPK_CASCADE. Statistical comparisons were conducted using the paired Wilcoxon signed-rank test. *$P < 0.05$; **$P < 0.01$. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; HNSC, head and neck squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; LAML, acute myeloid leukemia; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma; UCS, uterine carcinosarcoma.

cholestasis in the hepatic tissues of ICIs-treated mice compared to the control group. Our principal findings encompass the upregulation of the inflammation-associated MAPK pathway and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway, which is implicated in cell apoptosis and autophagy[43,44]. Additionally, we observed significant upregulation of the

monooxygenase activity pathway, which is integral to drug metabolism[45], and the endogenous sterol pathway, which is closely linked to bile acid metabolism (Fig. 7D–F).

To assess the impact of ICIs on the integrity of liver and bile duct structures, we performed histopathological analyses on liver tissues from both experimental and control groups of mice. These histopathological findings were consistent with our transcriptomic data results: H&E staining revealed significant differences between the two groups. In control mice, liver sections exhibited mild structural disorganization, characterized by the irregular arrangement of hepatocytes and hepatic cords. Occasional focal portal inflammatory infiltrates were observed, with mild hepatocellular necrosis evident in localized areas (Fig. 8A and B). In contrast, ICI-treated mice exhibited exacerbated pathological features that demonstrated cholestatic liver injury. Beyond the changes observed in the control group, liver tissues from the experimental group also exhibited dense inflammatory cell infiltration in portal areas; marked bile duct dilatation accompanied by biliary epithelial cell proliferation; cholestasis; pronounced hepatocellular necrosis and intracellular lipid accumulation, with hepatic necrotic foci further observed in the anti-CTLA-4 and combination therapy groups (Fig. 8C-J).

Discussion

Despite the extensive research on ICI-related adverse events, comprehensive studies specifically addressing ICI-associated cholestasis remain limited. This study represents the first comprehensive pharmacovigilance investigation of ICI-associated cholestasis, integrating real-world data from the FAERS and VigiBase databases. Comparative analyses utilizing the ROR and IC methods identified three types of cholestasis events that were strongly associated with ICI therapy. Clinical characteristics were examined and validated through local hospital data and in vivo mouse experiments. Pan-cancer transcriptomics data analysis explored potential underlying biological mechanisms,

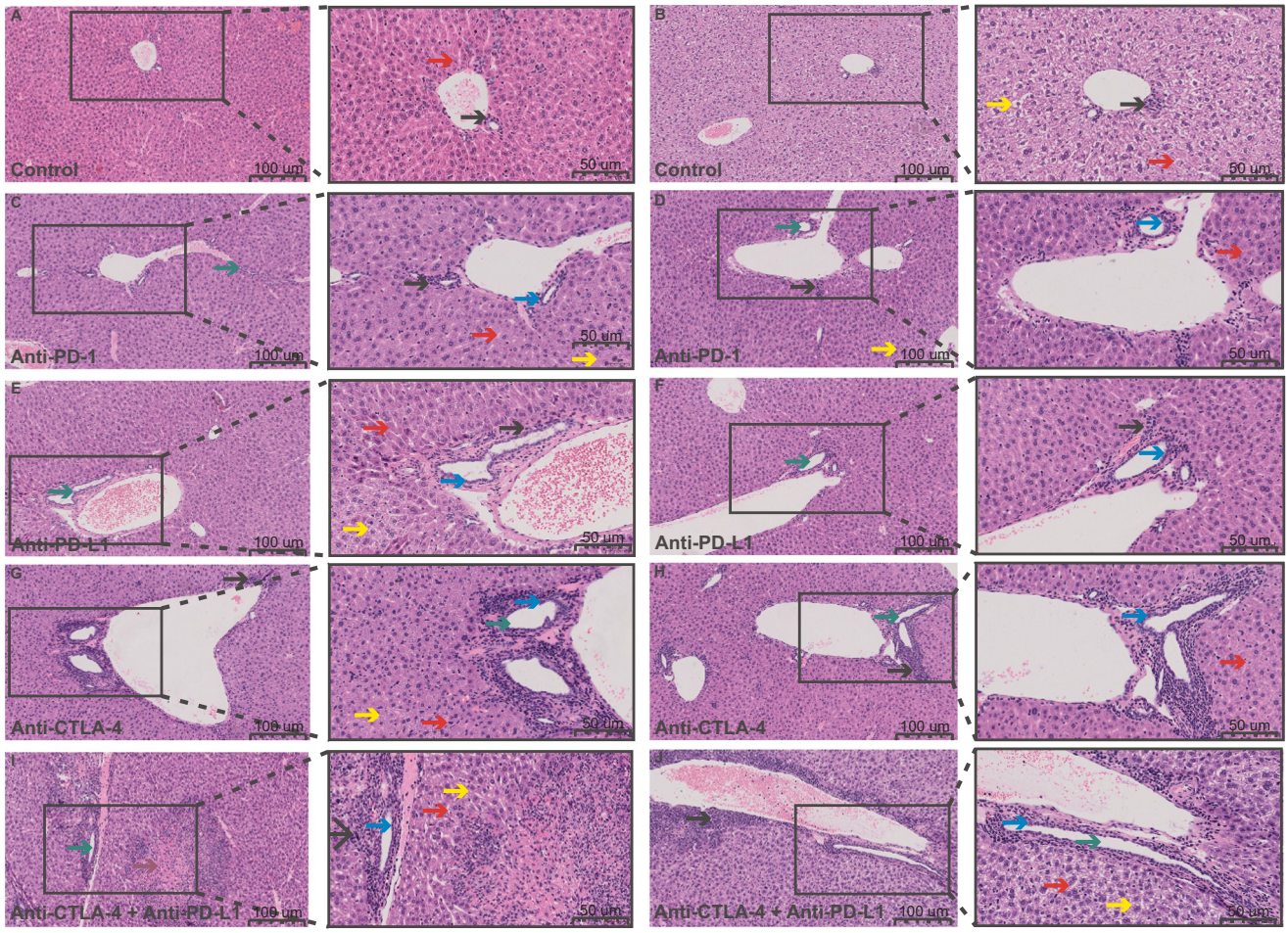

Figure 8. Histopathological evidence of immune checkpoint inhibitor-induced cholestasis in murine liver tissue. (A and B) Control group liver tissue exhibits mild architectural disorganization, including irregular arrangement of hepatocytes and hepatic cords, hepatocellular swelling, and focal hepatocyte necrosis (red arrows). Scattered inflammatory infiltrates are present in portal areas (black arrows). (C–J) Experimental group liver sections display, in addition to the pathological alterations observed in controls, portal inflammatory infiltration (black arrows), bile duct dilatation (green arrows) with epithelial hyperplasia (blue arrows), cholestasis, multifocal necrosis (purple arrows), and steatosis (yellow arrows). Liver sections from mice treated with anti-PD-1 (C and D), anti-PD-L1 (E and F), anti-CTLA-4 (G and H), and combined anti-CTLA-4 plus anti-PD-L1 therapy (I and J) demonstrated these pathological features. Scale bar: 100 μm (left row); 50 μm (right row). All sections were stained with hematoxylin and eosin.

suggesting multiple pathways, including biliary inflammation, dysregulation of hepatocyte apoptosis and autophagy, and alterations in drug metabolism pathways[40,44,45].

Our research findings are largely consistent with previous studies, indicating an increased risk of hepatotoxicity with combination treatment regimens[46,47], although our cohort did not demonstrate the highest risk signal for anti-CTLA-4, which may reflect a limited sample size – a limitation we explicitly acknowledged in our discussion. Additionally, the higher incidence of cholestatic adverse events observed in younger patients (<65 years) aligns with reports of ICIs-related hepatotoxicity being more prevalent in the 56–63 age group as compared to elderly populations[48]. This age-related vulnerability may stem from reduced expression of costimulatory molecules required for T cell activation in elderly patients[48,49]. Regarding clinical management, current guidelines recommend liver function monitoring for grade 1 toxicity while continuing ICIs therapy, whereas corticosteroid administration and ICIs discontinuation are recommended for grades 2–4 toxicity, with liver biopsy serving as an adjunct diagnostic tool for atypical presentations[16]. These perspectives underscore the necessity of age-stratified and regimen-specific monitoring protocols for patient populations receiving ICIs therapy.

Substantial variations in cholestatic adverse event risks were identified across ICI treatment regimens. In our analysis, we observed that patients receiving PD-1 inhibitor monotherapy and combination therapy exhibited a significantly higher risk of cholestasis when compared to other treatment regimens. This phenomenon may be attributed to PD-1 inhibitors (such as pembrolizumab and nivolumab), which enhance T-cell activation, potentially leading to excessive immune responses against hepatic antigens and resulting in cholestatic liver injury[50]. Furthermore, studies indicate that patients receiving combination immunotherapy demonstrate a significantly increased risk of developing cholestasis. This elevated risk may be attributed to the synergistic effects of dual checkpoint blockade, which potentially leads to more pronounced activation of the immune system. Research suggests that the combination of nivolumab and ipilimumab can enhance T-cell responses, thereby potentially resulting in more severe hepatic inflammation and cholestatic injury[51,52]. Additionally, dual blockade may disrupt the delicate balance of immune tolerance in the liver, thereby exacerbating the risk of cholestasis[53,54]. However, given the limited sample size, these findings require further validation through large-scale prospective studies or randomized controlled trials.

The development of cholestasis is associated with multiple clinical factors, notably age and sex[55,56]. Upon further investigation of factors influencing ICIs-related cholestatic adverse events, we observed that female patients and individuals aged younger than 65 years exhibited higher incidences of adverse reactions. After reviewing previous research, we found that the impact of age on immune-related adverse reactions has remained controversial[57,58]. A comprehensive review discussed the complex relationship between age and immune-related adverse reactions, noting that toxicity profiles vary across different age thresholds[59]. These findings further support that younger patients have a significantly elevated risk of ICIs-related hepatotoxicity compared with elderly patients. This phenomenon may be attributed to the lower expression levels of costimulatory molecules required for T-cell activation in elderly patients[48,49]. Early identification and intervention should be emphasized to improve outcomes in high-risk populations.

The observed sex differences in the earlier onset of cholestatic adverse events may stem from two interconnected biological mechanisms. First, sex hormones (particularly estrogen) possess immunomodulatory effects that enhance immune activity[60]. Estrogen enhances T cell reactivity, thereby increasing female susceptibility to irAEs[61]. Notably, mouse studies indicate that estrogen upregulates PD-1 receptor expression on immune cells[62], thus potentially enhancing the targeting efficacy of ICIs while accelerating T cell-mediated hepatotoxicity. Second, immune-related genes encoded on the X chromosome frequently escape X inactivation[63], resulting in higher expression levels in female immune cells compared with males. X chromosome-associated immune genes, such as TLR7 and FOXP3, frequently escape X inactivation in females, leading to bi-allelic expression and higher baseline immune gene activity compared with males. These two mechanisms in combination may synergistically promote CD8 + T cell hyperactivation and biliary epithelial damage, resulting in earlier and more severe cholestatic complications in females.

To validate our database analysis results, we utilized local clinical data and mouse experimental models, selecting biochemical markers such as AST and ALT to evaluate the occurrence and severity of cholestasis in patients. AST and ALT, which serve as primary enzymatic markers for evaluating hepatocellular damage, are released into the bloodstream upon liver cell injury[64]. ALP is predominantly localized in bile duct epithelial cells, and its levels increase significantly in cases of biliary obstruction or impaired bile flow[65,66]. Cholestatic liver injury frequently presents with elevated levels of TBIL and DBIL, resulting from biliary obstruction hindering the normal excretion of bilirubin[67,68]. In the diagnosis of cholestasis, elevated IBIL levels may be indicative of bilirubin metabolism disorders [68,69] These markers provide essential evidence for elucidating pathological mechanisms and developing targeted diagnostic and therapeutic strategies.

Analysis of the TCGA database and murine RNA sequencing data revealed that the putative biological mechanisms underlying ICIs-induced cholestasis may encompass the effects of these agents on the biliary system, modulation of endogenous sterol metabolism, and alterations in drug metabolism-related enzymatic systems. ICIs may precipitate cholestasis by inducing immune-mediated injury to biliary epithelial cells and perturbing bile acid transport processes. The activation of mitogen-activated protein kinase (MAPK) signaling pathways plays a pivotal role in the biliary cell stress response and bile acid metabolism, potentially constituting one of the key mechanisms underlying cholestasis[70,71]. Activation of the MAPK pathway leads to the upregulation of proinflammatory cytokines and chemokines, which are critical mediators of hepatic inflammation[43,72]. This inflammatory cascade contributes to hepatocellular injury and impaired bile flow[73]. Moreover, the upregulation of monooxygenase pathways, particularly the activation of cytochrome P450 (CYP) family enzymes[45], may perturb bile acid metabolism or generate hepatotoxic metabolites, potentially compromising hepatocyte function and subsequently inducing cholestasis. Our identification of potential pathway involvement in ICIs-induced cholestasis suggests several potential interventions. Selective p38 MAPK inhibitors (e.g.,

ralimetinib) could target inflammatory signaling[51,74], while FXR agonists like obeticholic acid might counteract bile acid dysregulation[75,76]. Low-dose PI3K inhibitors could help maintain hepatocellular homeostasis without compromising antitumor effects[77,78]. These approaches could be tailored based on pre-treatment biomarkers of pathway activation, potentially offering personalized hepatoprotective strategies during immunotherapy. The histopathological changes observed in our mouse model, particularly bile duct dilatation, biliary epithelial proliferation, portal inflammation, and hepatocellular necrosis, provided crucial structural validation of the transcriptomic pathways involved in ICIs-induced cholestasis. The upregulation of bile acid metabolism-related pathways correlated with histological evidence of cholestasis and biliary proliferation. The histologically observed concurrent steatosis further supported the disruption of bile acid signaling, as impaired FXR activation is known to exacerbate lipid metabolic dysfunction[79]. Transcriptomic activation of monooxygenase pathways, particularly cytochrome P450 enzymes, correlated with both the extensive hepatocellular necrosis and oxidative damage observed in H&E-stained sections[80]. Histological evidence of biliary epithelial proliferation directly reflected the transcriptional upregulation of MAPK/PI3K signaling pathways[43]. In cholestatic models, PI3K/AKT activation promotes cholangiocyte proliferation, representing a maladaptive response to biliary obstruction[81], while MAPK signaling stimulates collagen deposition through hepatic stellate cell activation[82].

The discrepant ROR patterns between FAERS (highest in SKCM) and VigiBase (highest in ACC) likely reflect several underlying methodological and clinical factors. FAERS predominantly captures US data, where melanoma immunotherapy has experienced widespread adoption following early FDA approvals, potentially resulting in more comprehensive SKCM adverse event reporting[83]. Conversely, VigiBase's global scope encompasses diverse healthcare systems with heterogeneous reporting practices and standards[84]. These databases employ different reporting thresholds and case ascertainment methods, which may influence signal detection sensitivity. For rare cancers like ACC, the small denominator (total treated patients) can substantially amplify ROR signals even with relatively few reported cases. SKCM patients typically receive prolonged ICI treatment and combination therapy[85,86], potentially increasing cumulative drug exposure and subsequent hepatobiliary toxicity risk. ACC patients often present with altered steroid metabolism[87] and require concomitant medications affecting hepatobiliary function[88], possibly explaining their elevated cholestasis signal in VigiBase.

Our findings suggest that patients receiving ICIs therapy should undergo more rigorous hepatic function monitoring compared to standard chemotherapy protocols[16,89], particularly during the first 4 weeks of treatment when the majority of cholestatic events occur, as demonstrated in our time-to-onset analysis. This is especially important for patients in the 0–65 age cohort, who demonstrated a higher risk in our study. We recommend baseline liver function testing prior to ICIs initiation, followed by monitoring at 2-week intervals and monthly for the subsequent 6 months. Our study demonstrates a significantly shorter time to onset of ICIs-associated cholestasis in female patients compared to males (Female: 1.17 [0.50–3.17] vs. Male: 1.90 [0.70–5.12]), suggesting the need for sex-specific surveillance protocols with accelerated monitoring intervals in women. For high-risk populations – specifically patients receiving anti-PD-1 therapy and combination ICIs therapy (particularly anti-PD-L1 with anti-CTLA-4), those with pre-existing hepatobiliary disorders, and patients with melanoma or cholangiocarcinoma, where we observed higher reporting odds ratios – we recommend more intensive monitoring. Evidence-based clinical monitoring strategies are systematically outlined in Table 1. Our pathway analyses identifying involvement of bile acid metabolic dysregulation and impaired hepatocellular drug metabolism suggest that current guidelines should be updated to include specific biomarkers beyond standard liver enzymes. The monitoring of serum bile acids and drug metabolism capacity through phenotyping tests could provide early warning signs of developing cholestasis before clinical manifestations appear. This approach would represent a significant advancement over current guidelines, which primarily focus on transaminase monitoring. Prompt, comprehensive clinical evaluations and advanced imaging studies are strongly recommended when patients manifest clinical signs of cholestasis. Treatment strategies should be individualized based on the specific etiology and unique patient characteristics[67]. Furthermore, our findings on the molecular mechanisms suggest that early intervention strategies targeting the MAPK and PI3K/AKT pathways could potentially mitigate cholestatic injury. We recommend that current guidelines incorporate prophylactic hepatoprotective interventions for high-risk patients, which is not commonly practiced under existing protocols.

Our study has several notable limitations that merit careful consideration. First, spontaneous reporting systems such as FAERS and VigiBase harbor inherent limitations, including underreporting, misreporting, and regional reporting disparities. These issues may introduce significant bias into our findings, particularly when comparing cholestasis incidence across geographical regions. Secondly, these retrospective pharmacovigilance databases lack comprehensive clinical information, including baseline liver function data and concurrent medication use, which substantially limits our ability to distinguish immunotherapy-induced cholestasis from exacerbations of underlying conditions or potential drug interactions. Additionally, our study encountered sample size constraints in several key aspects. The clinical validation cohort, though substantial with over 2000 cases, represents a single-institution experience that may not adequately capture diverse patient populations and clinical practice patterns. Certain treatment subgroups, particularly anti-CTLA-4 monotherapy, had limited representation, potentially compromising the statistical robustness of subgroup analyses. Our murine model experiments included only four mice per treatment group, which may raise concerns regarding statistical power and reproducibility. Increasing the number of mice in future studies would enhance the statistical robustness of the experimental results. While our bioinformatic analyses identified several potential mechanistic pathways underlying ICIs-induced cholestasis, we were unable to functionally validate these findings through experimental approaches such as bile acid transport assays or protein-level validation of key pathway markers. Furthermore, the pharmacovigilance databases do not systematically record therapeutic interventions such as the use of corticosteroids, ursodeoxycholic acid, or other cholestasis-specific treatments. This data limitation prevents us from evaluating the effectiveness of various management approaches and providing evidence-based recommendations for clinical practice. Consequently, we are

Table 1

Evidence-based clinical monitoring recommendations for ICI-related cholestasis

Monitoring parameter	Frequency & threshold	Target population	Cost-benefit analysis
		Baseline assessment	
Liver function tests (LFTs)	ALT, AST, ALP, GGT, TBIL, DBIL, INR	**All patients** initiating ICIs therapy	Establish reference values for early detection
Serum bile acids (TBA)	Total bile acids (TBA): >10 μmol/L (female), >7 μmol/L (male)	**High-risk groups** (melanoma, cholangiocarcinoma, prior hepatobiliary disease, combined ICIs therapy)	Higher specificity for cholestasis than ALP alone[16,90]
		Early phase (0–4 weeks)	
LFTs + TBA	**Female: Weekly** **Male: Every 2 weeks** *Threshold*: ALP > 1.5 × ULN, ALT/ALP ≤ 2, or TBA > 20 μmol/L, or TBIL < 2 × ULN	**All patients** initiating ICI therapy, especially patients aged 0-65 years or undergoing anti-PD-1/PD-L1 ± anti-CTLA-4 therapy	Frequent monitoring in high-risk groups reduces hospitalization costs[61]
		Maintenance phase (1–6 months)	
LFTs	**Monthly** *Threshold*: ALP > 2 × ULN, ALT/ALP ≤ 2, or TBIL ≥ 2 × ULN, or INR ≥ 1.5	**All patients** sustaining ICIs therapy	Prevent progression to severe cholestasis.
Abdominal ultrasound	**Every 3 months** Evaluate bile duct dilation, gallbladder sludge	Patients with rising ALP/TBA or symptoms (pruritus, jaundice)	Avoid unnecessary ERCP/MRCP[16,91]
		Adjustments	
Accelerated monitoring	**Female: Double the frequency** in the first 8 weeks (e.g., weekly → biweekly after 4 weeks)	**Female patients** (Female: 1.17 [0.50–3.17] vs. Male: 1.90 [0.70–5.12], $P < 0.05$)	Reduce severe cholestasis risk
	Add **TBA + MRCP** at 2-week intervals during the induction phase	Combined ICIs therapy recipients, melanoma, cholangiocarcinoma	Early detection of severe cholestasis
	Hepatology consultation and FibroScan at baseline and three months	Pre-existing liver disease	

AASLD, American Association for the Study of Liver Diseases; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DILI, drug-induced liver injury; ERCP, endoscopic retrograde cholangiopancreatography; GGT, gamma-glutamyl transferase; ICIs, immune checkpoint inhibitors; INR, International normalized ratio; LFTs, liver function tests; MRCP, magnetic resonance cholangiopancreatography; NCCN, National Comprehensive Cancer Network; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TBA, total bile acids; TBIL, total bilirubin.

unable to assess whether early intervention with specific agents might mitigate the severity or duration of cholestasis, or whether certain management strategies are more effective for particular patient subgroups or ICIs classes. Future prospective studies specifically designed to capture detailed treatment data are necessary to fill this critical knowledge gap and optimize clinical management of ICIs-induced cholestasis. The pharmacovigilance databases also do not systematically record management strategies employed for patients who developed ICIs-induced cholestasis, thereby preventing us from evaluating the effectiveness of various treatment approaches or providing evidence-based recommendations for clinical practice. While our bioinformatic analyses identified several potential mechanistic pathways underlying ICIs-induced cholestasis, we acknowledge that we were unable to validate these findings using functional studies such as bile acid transport assays or protein-level validation of key pathway markers (e.g., CYP enzymes, MAPK, PI3K/AKT) via western blot or immunohistochemistry. These functional validations will be prioritized in our future research to establish causality and further characterize the precise molecular mechanisms involved[92,93]. Despite these limitations, we employed multiple analytical approaches and integrated data from diverse sources, including two independent pharmacovigilance databases, local clinical data, and experimental models (biochemical markers, transcriptomic data, and histological analysis), to strengthen the reliability of our findings. Future prospective, multi-center studies with standardized protocols for patient monitoring, comprehensive functional validation studies, and detailed treatment data collection are essential to address these limitations and further elucidate the risk factors, underlying mechanisms, and optimal management strategies for immunotherapy-induced cholestasis.

Conclusion

In summary, this study offers valuable insights into the risk and characteristics of ICIs-associated cholestasis through integrating the analysis of real-world pharmacovigilance data with clinical data, murine experiments, and structural pathology. These findings emphasize the importance of vigilant monitoring and early intervention for patients receiving ICIs therapy, especially those with identified risk factors. As the utilization of ICIs continues to broaden in cancer treatment, a more comprehensive understanding of their hepatobiliary effects is imperative for optimizing patient care and minimizing adverse outcomes.

Ethical approval

The animal experiment protocol of this study was in accordance with the Animal Care and Use Committee of the Second Military Medical University and the guiding principles of animal experimentation of the institute. This study strictly complied with relevant international and domestic ethical guidelines and legal regulations and was approved by the Ethics Committee of Zhujiang Hospital of Southern Medical University (ID: 2024-KY-129-01).

Consent

Written informed consent, approved by the institutional ethics committee, was obtained from each patient.

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Author contributions

All authors have read and agreed to the published version of the manuscript. Conceptualization: Q.C., J.Z., A.L., P.L. Investigation: Q.C., J.Z., A.L., P.L. Methodology: X.Y., A.L. Supervision: Q.C., J.Z., A.L., P.L. Formal analysis: X.Y., Z.L., A.J. Visualization: X. Y., Z.L., A.J., J.C., X.H., A.H., H.Z.H.W. Writing – original draft: X.Y., A.L., A.J., Z.L., J.C. Writing – review and editing: X.Y., Z.L., A.J., J.C., X.H., A.H., H.Z.H.W., Q.C., J.Z., A.L., P.L.

Conflicts of interest disclosure

The authors declare no conflicts of interest.

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Data availability statement

The datasets supporting the conclusions of this article are included within the article and its additional materials.

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