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Impact of clonal hematopoiesis of indeterminate potential on hepatocellular carcinoma in individuals with steatotic liver disease

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Abstract

Background and Aims: Metabolic dysfunction–associated steatotic liver disease (MASLD) is a global epidemic and is the most rapidly rising cause of HCC. Clonal hematopoiesis of indeterminate potential (CHIP) contributes to neoplastic and cardiometabolic disorders and is considered a harbinger of

Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; MASLD, metabolic dysfunction–associated steatotic liver disease; PRS-5, Polygenic Risk Score 5; SLD, steatotic liver disease; T2D, type 2 diabetes; VAF, variant allele frequency.

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tissue inflammation. CHIP was recently associated with increased risk of liver disease. The aim of this study was to examine whether CHIP is associated with HCC development in patients with SLD.

Approach and Results: We considered individuals with MASLD-HCC (n = 208) and controls with (n = 414) and without (n = 259) advanced fibrosis who underwent whole exome sequencing. CHIP was diagnosed when ≥ 2 variant callers identified a known myeloid mutation with variant allele frequency ≥ 2%. CHIP was observed in 116 participants (13.1%), most frequently in *DNMT3A, TET2, TP53*, and *ASXL1*, and correlated with age ($p < 0.0001$) and advanced liver fibrosis (p = 0.001). Higher aspartate aminotransferase levels predicted non-*DNMT3A*-CHIP, in particular with variant allele frequency ≥ 10% (OR: 1.14, 1.03 −1.28 and OR: 1.30, 1.12 −1.49, respectively, $p < 0.05$). After adjustment for sex, diabetes, and a polygenic risk, a score of inherited MASLD predisposition CHIP was associated with cirrhosis (2.00, 1.30 −3.15, $p = 0.02$), and with HCC even after further adjustment for cirrhosis (OR: 1.81, 1.11 −2.00, 1.30 −3.15, $p = 0.002$). Despite the strong collinearity among aging and development of CHIP and HCC, non-*DNTM3A*-CHIP, and *TET2* lesions remained associated with HCC after full correction for clinical/genetics covariates and age (OR: 2.45, 1.35 −4.53; OR: 4.8, 1.60 -17.0, $p = 0.02$).

Conclusions: We observed an independent association between CHIP, particularly related to non-*DNTM3A* and *TET2* genetic lesions and MASLD-HCC.

INTRODUCTION

Steatotic liver disease (SLD), most often associated with insulin resistance (nonalcoholic or now redefined metabolic dysfunction-associated steatotic liver disease, NAFLD/MASLD), is now the leading cause of liver disease worldwide.[1] MASLD encompasses a range of disease severity, from simple steatosis to steatohepatitis, which can lead to liver fibrosis and HCC.[2] Advanced fibrosis is the main predisposing condition for the development of HCC and decompensated cirrhosis, which are the main liver-related events.[3] HCC is the fifth most common cancer and third most common cause of cancer-related death globally. While progress has been made in treating viral hepatitis, the increasing prevalence of obesity and diabetes is driving a surge in HCC incidence, with MASLD being the most rapidly increasing cause of HCC, particularly in women.[4,5] Insulin resistance and type 2 diabetes are major factors in the development of advanced fibrosis and HCC.[6] MASLD is a highly heritable condition, with common and rare genetic risk variants predisposing hepatocellular fat accumulation and lipotoxicity increasing the risk of steatohepatitis, fibrogenesis, and HCC,[7,8] acting in synergy with insulin resistance.[8,9] Inflammation triggered by lipotoxicity, as well as altered intestinal flora, and gut permeability, play a key role in MASLD progression.[10,11] The development of acquired genetic mutations, selected by a lipotoxic environment, can also contribute to the evolution of hepatic parenchymal damage and multistage carcinogenesis.[12]

Clonal hematopoiesis of indeterminate potential (CHIP) is defined as the presence of somatic mutations in hematopoietic stem cells with a variant allele frequency (VAF) ≥ 2% located in genes affected in hematologic malignancies. CHIP prevalence increases with age and is associated with an increased risk of hematologic cancers, cardiovascular, and other aging-related disorders.[14] Recently, CHIP has also been linked to increased susceptibility to SLD and severe liver disease.[13] The association of CHIP with liver inflammation and fibrosis was independent of steatosis but related to acquired mutations in some CHIP-related genes (especially *TET2*) in myeloid cells homing to the liver.[13] However, it is not yet known whether CHIP can facilitate the progression of liver disease to HCC independently of liver fibrosis.

The aim of this study was therefore to examine whether CHIP is associated with HCC independently of cirrhosis and other major clinical determinants in a

cross-sectional case-control multicenter cohort of patients with MASLD and controls.

METHODS

Study cohorts

The EPIDEMIC-NAFLD (now MASLD) ("Exome sequencing for the identification of genetic mutations promoting HCC development in NAFLD") is a cross-sectional multicenter case-control study cohort aimed at the identification of genetic variants predisposing to the development of HCC in individuals with MASLD. It enrolled European patients with MASLD-HCC (n = 208) and 2 groups of controls: patients with MASLD and advanced fibrosis without HCC (n = 414), individuals with MASLD without advanced fibrosis (n = 107) as well as locally and ethnically matched healthy individuals (n = 152). The diagnosis of NAFLD was based on the demonstration of fatty liver by imaging at the time of study inclusion or a previous positive clinical history in patients with advanced disease,[15] daily alcohol intake <30/20 g/d in males/females, and absence of concurrent liver diseases and other hepatotoxic factors (including chronic viral or autoimmune hepatitis; genetic liver diseases, including hereditary hemochromatosis, Wilson disease, alpha-1-antitrypsin deficiency, and use of steatogenic/hepatotoxic drugs). All patients fulfilled the metabolic criteria for MASLD. Advanced liver fibrosis and HCC were diagnosed according to the European Association for the Study of the Liver (EASL) criteria.[16,17] Age, sex, presence of type 2 diabetes (T2D), advanced liver fibrosis, AST, and ALT levels at the time of study enrollment were available in all patients. In addition, the main common germline risk variants for HCC in MASLD and a polygenic risk score (PRS) summarizing their effect were available for all.[18] Part of this cohort has been described contributing to the identification of new genetic risk loci for HCC.[8,19]

Blood samples to evaluate the presence of CHIP were collected at the time of study inclusion.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and Istanbul. The EPIDEMIC and SERENA study were approved by the Ethical Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milan and participating centers (EPIDEMIC-TERT study Ethical approval n. 1882_2013; Perspective-SERENA multicenter Study approval n. 485_2017, Fondazione IRCCS Ca' Granda Ethical Committee). Informed consent was obtained from each participant. The fatty liver inhibition of progression study was approved by the Newcastle Hepatopancreatobiliary Research Tissue Bank (REC reference: 10/H0906/41), with patient samples and data shared from the Newcastle Academic Health Partners Bioresource.

The clinical features of the study cohorts are presented in Table 1.

All research was conducted in conducted in accordance with both the Declarations of Helsinki and Istanbul. Written informed consent was obtained from study participants.

Next-generation sequencing and CHIP analysis

DNA sequencing was performed as described.[8] Briefly, DNA libraries were enriched for exome sequencing by the SureSelect Human All Exon v5/7 kits (Agilent, Cernusco sul Naviglio, Milan, Italy). Sequencing was subsequently performed on the HiSeq 4000/NextSeq2000 platforms (Illumina). Raw reads quality control was performed using FastQC software (Brabaham bioinformatics, Cambridge, UK). Reads mapping on the human GRCh37 genome was performed using the MEM algorithm of Burrows Wheeler Aligner version 0.7.10.[20] Reads with low-quality alignments and duplicate reads were filtered out using Samtools to generate high-quality bam files.[21] Mapping quality control was performed using Picard-tools (http://broadinstitute.github.io/picard) and Bedtools.[22] Sequencing mean depth was ×73, and no samples exhibit a mean depth lower than ×50.

Somatic mutations were identified, accepting a minimum variant coverage of 20, a minimum alternative allele count of 3, and a VAF between 0.02 and 0.46 in concordance with established variant filtering algorithms; the only exception was the *JAK2* V617F mutation, for which no VAF limitation was set.[23,24] Variants with a population mean allelic frequency higher than 1 in 1000 were treated as polymorphisms. Variants were then curated to include only variants known to be somatic and associated with either malignancy or CHIP with the use of a semi-automatic pipeline.[24,25,26] Genes evaluated for CHIP attribution included: *NADK*, *GNB1*, *CBL*, *KMT2D*, *RHEBL1*, *PPM1D*, *JMJD6*, *MFSD11*, *DNMT3A*, *SF3B1*, *ASXL1*, *NOL4L*, *GNAS*, *RUNX1*, *AF015262.1*, *RPL34P3*, *U2AF1*, *TET2*, *CUX1*, *SH2B2*, *BCOR*, *BCORL1*, *MCAM*, *TP53*, *METTL23*, *SRSF2*, *NPM1P46*, *LINC01426*, *EZH2P1*, *ELF4*, *ATP1B2*, *ZNF316*, *ANAPC1*, *JAK2*. Three variant callers were employed for variant identification: Mutect, Vardict, and Freebayes.[27–29] Only variants recognized by at least 2 variant callers were then considered for further analysis.

Study design

The principal study's aim was to assess the impact of CHIP and CHIP not driven by *DNMT3A* variants on the main outcome, that is, the risk of developing HCC in the cross-sectional EPIDEMIC cohort. The choice of selecting non-DNMT3A is based on recent evidence

TABLE 1 Clinical features of the 881 individuals included in the NAFLD-EPIDEMIC (MASLD) cross-sectional study cohort

		No HCC		
	HCC	Advanced fibrosis	No advanced fibrosis	
n	208	414	259	p^a
Age, y	70.0 (64.5, 76.0)	63.0 (56.0, 69.0)	51.0 (41.7, 60.2)	< 0.001
Sex, male	166 (79.8)	226 (54.6)	164 (63.3)	< 0.001
T2D, yes	111 (53.4)	150 (36.4)	4 (1.5)	< 0.001
BMI, kg/m^2	29.1 (26.1, 33.0)	29.8 (26.9, 34.0)	23.9 (22.0, 25.61)	< 0.001
AST, IU/L	44.50 (33.0, 58.3)	37.0 (27.0, 49.0)	22.0 (19.0, 29.0)	< 0.001
ALT, IU/L	37.0 (27.0, 52.0)	38.0 (28.0, 55.0)	19.0 (15.0, 27.0)	< 0.001
Cirrhosis, yes	148 (82.7)	176 (66.9)	0	< 0.001
PNPLA3, p.148M/M	58 (28.0)	135 (32.7)	13 (12.0)	< 0.001
PRS-5, score	0.44 (0.22-0.66)	0.40 (0.25, 0.60)	0.33 (0.13-0.49)	0.007
CHIP, yes	51 (24.5)	54 (13.0)	11 (4.2)	< 0.001
CHIP w VAF ≥ 10%	14 (6.7)	20 (4.8)	2 (0.8)	ns
DNMT3A, yes	16 (7.7)	32 (7.7)	3 (1.2)	0.1
TET2, yes	14 (6.7)	4 (1.0)	2 (0.8)	0.001
TP53, yes	8 (3.8)	5 (1.2)	2 (0.8)	0.06
ASXL1, yes	6 (2.9)	4 (1.0)	0	ns
JAK2, *yes*	1 (0.5)	0	2 (0.7)	ns

Note: Data are shown as N (%), or median (IQR), when appropriate.
[a]*p*-values were calculated among pairs through Kruskal-Wallis test for continuous variables (non-normality assumed) and Fisher test for categorical variables and corrected for multiple testing through false discovery rate. ns: not statistically significant ($p > 0.05$).
Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; LSM, liver stiffness measurement by Fibroscan (available in 28% of cases); MASLD, metabolic dysfunction–associated steatotic liver disease; PRS-5, Polygenic Risk Score 5; T2D, type 2 diabetes.

suggesting that only selected DNMT3A mutations confer an inflammatory phenotype.[27] We considered unadjusted analyses, adjusted for main confounders available in the cohort (sex, presence of T2D and advanced fibrosis, genetic predisposition due to carriage of common germline mutations as summarized by PRS-5 [Polygenic Risk Score 5]) excluding age (in the hypothesis that CHIP may be involved in mediating the effect of age on HCC risk), and with further adjustment by age. As secondary aims to investigate the possible mechanism underlying the epidemiological association, we tested the impact of CHIP on liver damage: AST and ALT, correlating with inflammation and fibrosis and liver fat, respectively, and the presence of advanced fibrosis, and whether CHIP defined by VAF ≥ 10% was more robustly associated with the study outcomes.

Finally, to explore whether genetic lesions in the most frequently mutated genes may specifically be associated with HCC, we also tested the impact of mutations at specific genes defining CHIP on the main outcome.

Statistical analysis

For descriptive statistics, categorical variables are shown as number and proportion, while continuous variables are shown as mean and SD or median and IQR, as appropriate. The association of CHIP with AST and ALT levels was assessed by multivariable generalized linear models, whereas the association with cirrhosis and HCC through multivariable logistic regression models. As one of the main aims of the study was to examine the impact of CHIP on HCC, we included as covariates in multivariable models, sex, presence of T2D, and advanced fibrosis and PRS-5. Analyses were then further adjusted for age.

Statistical analysis was carried out using the JMP 16.0 Pro Statistical Analysis Software (SAS Institute, Cary, NC), and R statistical analysis software version 4.1 (http://www.R-project.org). *p*-values <0.05 (two tailed) were considered significant. *p*-values were corrected with a false discovery rate approach, where appropriate Supplemental Materials, http://links.lww.com/HEP/I480.

RESULTS

Study cohort

The cross-sectional NAFLD-EPIDEMIC study cohort comprised 881 individuals: 208 patients had MASLD-HCC, 414 MASLD and advanced liver fibrosis without HCC, and 259 controls without advanced fibrosis (Table 1).

There was a progressive increase in age ($p < 0.001$), AST levels ($p < 0.001$), and prevalence of cirrhosis ($p < 0.001$) from nonadvanced fibrosis to advanced

fibrosis and HCC. Patients with advanced fibrosis and HCC had higher body mass index ($p < 0.001$), prevalence of T2D ($p < 0.001$), ALT levels ($p < 0.001$), and prevalence of *PNPLA3* p.I148M variant homozygosity ($p < 0.001$), and higher PRS-5 score ($p = 0.007$) than those without advanced fibrosis. Males were overrepresented in all 3 cohort subgroups (63.3%, 54.6%, and 79.8%, respectively).

Prevalence of CHIP

CHIP-defining genetic lesions were identified in 116 out of 881 participants (13.1%). CHIP was found in 51 (24.5%) of patients with HCC, and among those without HCC in 54 (13.0%) of those with advanced fibrosis, and 11 (4.2%) of those without advanced fibrosis (Table 1; $p < 0.001$). As expected, the prevalence of CHIP was age-dependent (OR 2.02 per year, 95% CI 1.05–3.87; $p < 0.0001$) and increased consistently across diagnosis groups, with the spike in CHIP incidence being observed after the age of 60 (Figure 1).

The oncoplot showing the frequency of specific CHIP-defining genetic lesions is shown in Figure 2. Across the cohort, 116 (13.1%) patients showed at least one CHIP-defining mutation. Forty-three (37%) carried at least an additional CHIP lesion. The most frequently mutated gene was *DNMT3A*, followed by *TET2*, *TP53*, and *ASXL1* (Table 1). The spectrum of mutations was as expected, with 7.8% (4/51) of *DNMT3A* mutations occurring at codon R882, 40% (8/20) of *TET2* mutations being truncating, and 90% (9/10) of *ASXL1* mutations were in exon 13, whereas JAK2 lesions included the V617F lesion only. Overall, the median VAF was 5.6% (range min-max 2%–86%), with 31% (36/116) of patients showing at least 1 variant with VAF $\geq 10\%$. CHIP with an allele burden $\geq 10\%$ was observed predominantly in the advanced fibrosis (20/36) and in the HCC groups (14/36). *TET2* and *TP53* variants were enriched in patients with HCC ($p < 0.001$, 0.022, respectively), while prevalence of *DNMT3A* was not significantly different between the advanced fibrosis and the HCC group. The *JAK2* V617F mutation appeared in 3 patients, one with HCC carrying a lesion with a high

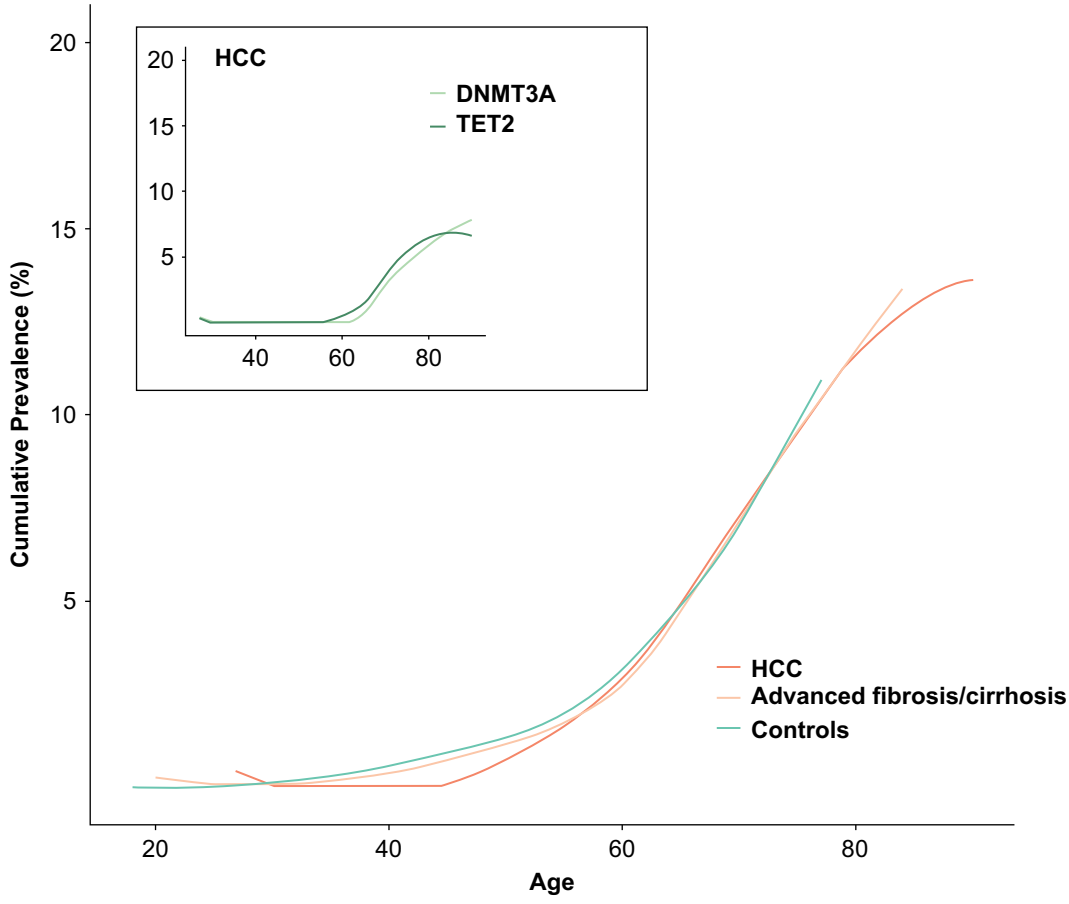

FIGURE 1 Clonal hematopoiesis of indeterminate potential (CHIP) prevalence as a function of age ($p < 0.0001$). A steep expected increase is seen after 60 years of age and appears to be consistent across patient groups. The cumulative prevalence of clonal hematopoiesis of indeterminate potential in the overall study cohort settled around 13.1%. Cumulative prevalence of DNMT3A and TET2 mutations showing divergent age dependency in the HCC cohort with TET2 plateauing after 75 years of age.

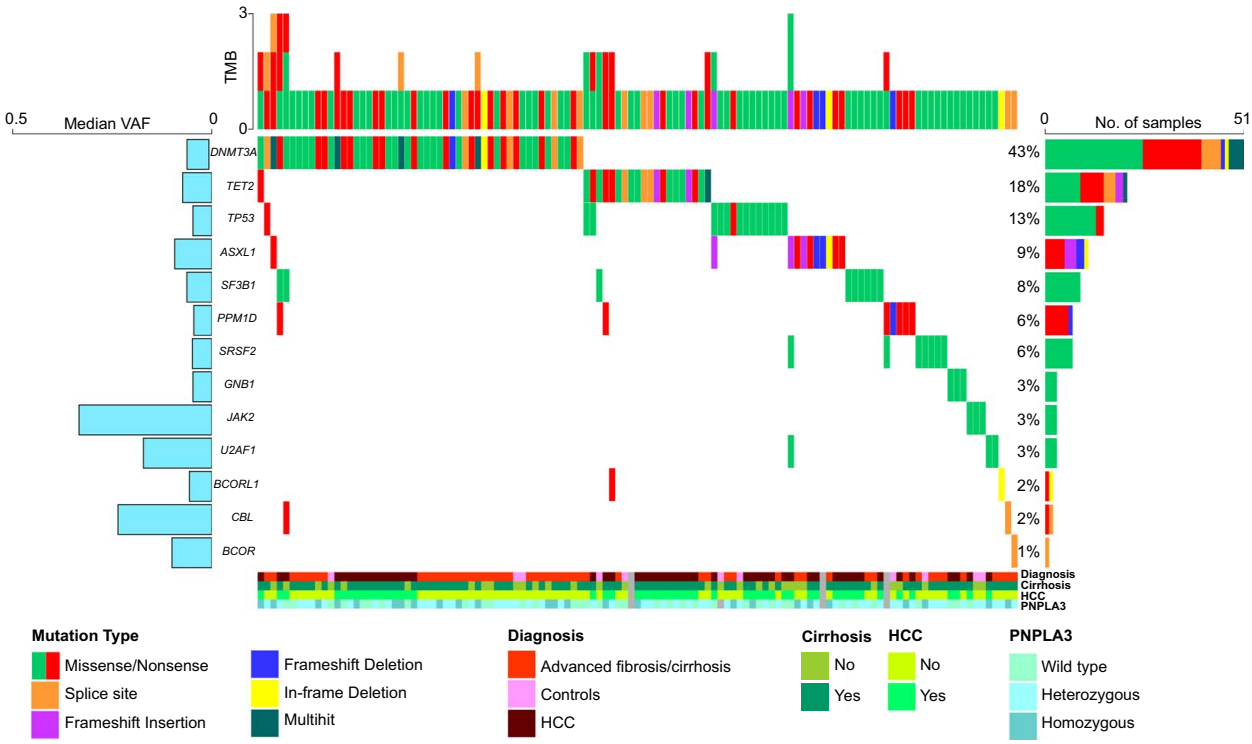

FIGURE 2 Oncoplot showing clonal hematopoiesis of indeterminate potential (CHIP) defining lesions across patient cohorts. Genes are ordered according to cohort frequency. Variants are color coded according to legend. Median lesion variant allele frequency is shown in the left barplot. Bottom ribbons identify disease groups, presence or absence of cirrhosis, and HCC. Abbreviation: TMB, total mutation burden.

VAF (>80%) and in 2 cases of patients with nonfibrotic liver disease with a VAF of 33 and 7%, respectively.

Impact of CHIP on disease severity

We first sought to assess the impact of CHIP on liver disease severity by analyzing the association of mutations with biomarkers of liver damage. Although at univariate analysis in the overall cohort no significant association was found between AST or ALT levels and the presence of CHIP ($p > 0.05$), after correcting for the main clinical confounders, namely, age, sex, T2D, and body mass index, and CHIP cases with VAF ≥ 10% were associated with higher ALT (OR: 1.1, CI: 95% 1.002–1.2) and AST levels (OR: 1.1, CI: 95% 1.01–1.2) (Figure 3A). Regardless of allele frequency, CHIP, in the absence of *DNMT3A* mutations, was associated with higher AST levels (OR: 1.1, CI: 95% 1.02–1.2) but not with ALT, suggesting a sequenced relation with liver enzyme elevation.

Even higher levels of both AST and ALT were observed in patients with CHIP defined by mutations other than *DNMT3A* and VAF ≥ 10% when corrected for the aforementioned variables (OR: 1.20, CI: 95% 1.10–1.30 and OR: 1.21, CI: 95% 1.10–1.35, respectively, Figure 3).

The association of CHIP with the risk of cirrhosis is presented in Table 2. At univariate logistic regression analysis, CHIP was associated with cirrhosis (OR: 2.64, 95% CI: 1.73–4.13, $p < 0.001$). At multivariate analysis, the association of CHIP with cirrhosis remained independent of sex, T2D, and inherited genetic predisposition to SLD as captured by the PRS-5 (OR: 1.70, 95% CI: 1.10–2.73, $p = 0.02$). The impact of CHIP appeared more consistent in men (OR: 1.85, 95% CI: 1.06–3.37, $p = 0.035$) than in women (OR: 1.41, 95% CI: 0.66–3.27, $p = 0.38$). However, the association between CHIP and cirrhosis was attenuated and lost statistical significance after correction for age (OR: 1.26, 95% CI: 0.78–2.10, $p = 0.35$). Neither CHIP with VAF ≥ 10% nor specific genetic lesions was associated with cirrhosis independently of age (not shown).

Impact of CHIP on HCC risk

The impact of CHIP on the risk of HCC is presented in Table 3. Logistic regression highlighted a significant association between CHIP and HCC (OR: 3.04, 95% CI: 2.01–4.55, $p < 0.001$). Remarkably, the association remained significant after correction for sex, T2D, and PRS-5 (OR: 2.21, 95% CI: 1.42–3.41, $p < 0.001$), and even after further correction for the presence of cirrhosis (OR: 2.01, 95% CI: 1.30–3.15, $p = 0.002$), suggesting it

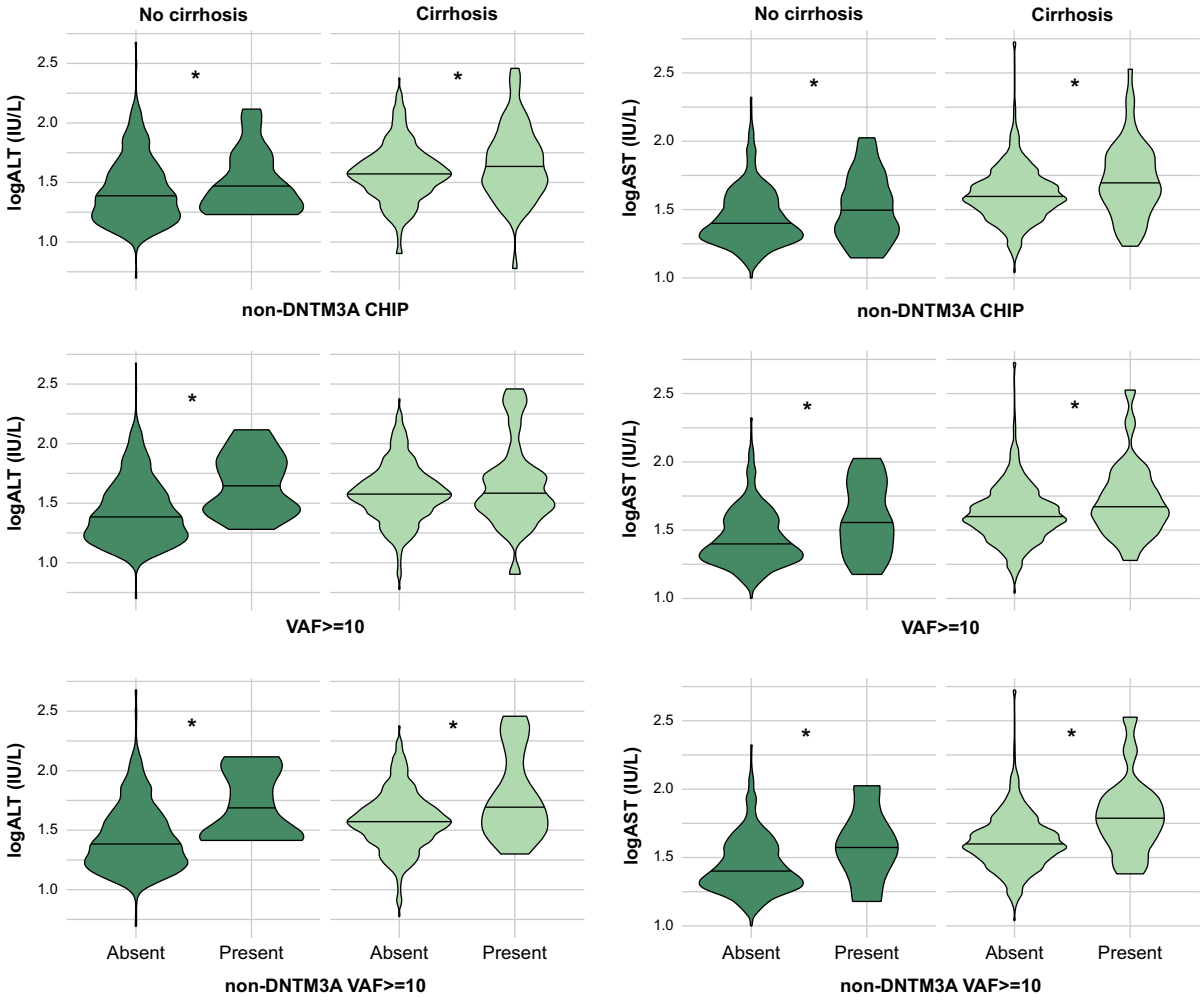

FIGURE 3 Violin plot displaying AST and ALT levels (log10 [IU/L]), as biomarkers of liver fat and AST of liver inflammation, respectively, between samples with detectable clonal hematopoiesis (CHIP; in the absence of *DNMT3A* mutations), CHIP with an allele fraction ≥10%, and nonmutated samples. Additional stratification according to cirrhotic status is shown. Statistically significant differences are marked by an asterisk ($p < 0.05$, at generalized linear models adjusted for type 2 diabetes, body mass index, age, and sex. Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; VAF, variant allele frequency.

is independent of the stage of liver disease. In this model, the association of CHIP with HCC was significant in men (OR 2.81, 95% CI: 1.65–4.83, $p < 0.001$), but not in women (OR: 0.71, 95% CI: 0.23–1.83, $p = 0.5$).

Despite a relevant proportion of patients with HCC were found to carry CHIP, the association of HCC and CHIP was not statistically significant when correcting for age ($p > 0.05$), due to the older median age in patients with HCC. However, CHIP associated with HCC even in

TABLE 2 Independent determinants of cirrhosis (n = 474) in 881 individuals in the NAFLD-EPIDEMIC (MASLD) study cohort

	Unadjusted OR, 95% CI	*p*	Model 1 OR, 95% CI	*p*	Model 2 OR, 95% CI	*p*
Age, y	1.10, 1.08–1.11	< 0.0001	—	—	1.06, 1.04–1.08	< 0.0001
Sex, M	1.03, 0.78–1.35	0.81	0.92, 0.66–1.28	0.63	1.00, 0.71–1.42	0.96
T2D, yes	3.71, 2.71–5.15	< 0.0001	1.98, 1.41 –2.80	< 0.0001	1.45, 1.01–2.10	< 0.041
PRS-5, unit	4.2, 2.25–8.00	< 0.001	4.27, 2.25–8.24	< 0.001	3.75, 1.90–7.54	0.0001
CHIP, yes	2.64, 1.72–4.14	< 0.001	1.70, 1.07–2.73	0.02	1.26, 0.77–2.10	0.35

Note: At logistic regression analysis adjusted for the covariates reported in the models. Unadjusted; model 1: adjusted for sex, T2D, polygenic risk score of fatty liver disease; model 2: further adjusted for age at enrollment.
Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; PRS-5, Polygenic Risk Score 5; T2D, type 2 diabetes.

TABLE 3 Independent determinants of HCC (n = 208) in 881 individuals in the NAFLD-EPIDEMIC (MASLD) study cohort

	Unadjusted OR, 95% CI	p	Model 1 OR, 95% CI	p	Model 2 OR, 95% CI	p	Model 3 OR, 95% CI	p	Model4 OR, 95% CI	p
Age, years	1.11, 1.10–1.13	< 0.0001	—	—	—	—	1.10, 1.07–1.12	< 0.0001	1.10, 1.07, 1.12	< 0.001
Cirrhosis, yes	6.35, 4.31–9.60	< 0.0001	—	—	3.50, 2.30–5.43	< 0.0001	3.04, 1.85–5.03	< 0.0003	2.32, 1.46, 3.73	< 0.001
Sex, M	2.87, 2.00–4.20	< 0.0001	2.90, 2.00–4.34	< 0.0001	3.10, 2.10–4.70	< 0.0001	3.80, 2.50–5.90	< 0.0001	3.82, 2.51–5.92	< 0.001
T2D, yes	3.84, 2.80–5.33	< 0.001	2.62, 1.86–3.70	< 0.0001	2.33, 1.64–3.33	< 0.0001	1.97, 1.35–2.88	0.0004	1.98, 1.36–2.89	< 0.001
PRS-5, unit	1.32, 0.70–2.50	0.38	1.44, 0.74–2.82	0.27	1.05, 0.52–2.12	0.88	0.90, 0.43–1.90	0.80	—	0.31
PRS-5, high risk (≥ 0.495)	0.76,0.55–1.06	0.10	—	—	—	—	—	—	1.22, 0.83–1.80	—
CHIP, yes	3.04, 2.02–4.56	< 0.0001	2.08, 1.26–3.26	0.0004	2.00, 1.30–3.15	0.002	1.41, 0.86–2.30	0.17	1.46, 0.89–2.40	0.13

Note: At logistic regression analysis adjusted for the covariates reported in the models. Unadjusted; model 1: adjusted for sex, T2D PRS-5; model 2: further adjusted for cirrhosis; model 3: further adjusted for age at enrollment; model 4 evaluated PRS-5 as a nominal variable, with high-risk PRS being ≥ 0.495. Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; PRS-5, Polygenic Risk Score 5; T2D, type 2 diabetes.

the context of age correction when *DNMT3A* lesions were excluded from the analysis (OR: 2.45, CI: 95% 1.34–4.53, $p = 0.02$).

Finally, we examined the impact of specific CHIP genetic lesions on the risk of HCC. We highlighted an association between *TET2* and *TP53* mutations with HCC (Table 4). Strikingly, 70% of *TET2* mutations were observed in HCC cases. On the contrary, *DNMT3A*-driven CHIP, despite being the most represented event, was equally distributed with respect to HCC prevalence. The association between HCC and *TET2* remained statistically significant at multivariable logistic regression when correcting for covariates including age, sex, T2D, presence of cirrhosis, and PRS-5 score (OR: 4.8, 95% CI: 1.6–17.0, $p = 0.02$; Table 4 and Figure 4). Notably, 25% of *TET2* mutated HCC cases had VAF ≥ 10% with a trend for higher AST levels with respect to the low allelic burden counterpart (median AST 84 vs. 40 IU/l), reinforcing the concept that a higher fraction of CHIP circulating myeloid elements associates with a higher inflammatory burden.

DISCUSSION

Here, we report the prevalence of CHIP in a multicenter cross-sectional cohort of patients with severe MASLD and controls without advanced liver fibrosis and describe the association of CHIP with liver damage and HCC development, with the latter being the main study focus. Indeed, recent evidence suggests that the occurrence of somatic mutations in the liver and hematopoietic cells accompany and contribute to the progression of MASLD to steatohepatitis and fibrosis.[12,13] Importantly, clonal mutations defining CHIP, and particularly *TET2* mutations, have been shown to promote liver disease progression by inducing a proinflammatory phenotype in myeloid cells homing to the liver.[13] However, despite inflammation being involved in hepatic carcinogenesis, no data were yet available on the impact of CHIP on HCC onset.

First, we observed that 13% of the cohort population showed one or more genetic mutations defining CHIP, this being in line with the expected proportion of affected genes and types of mutations,[30] with a prevalence distribution sharply rising at the age of 60 across all study groups.

Second, patients who are CHIP-bearing, especially those with a higher clonal burden (≥ 10%), showed levels of AST higher than age and comorbidity-matched peers. The contribution of larger CHIP clones on liver inflammatory markers has been described in larger cohorts.[13] Whether this finding is the result of higher circulating CHIP clone fraction contributing more consistently to organ inflammation is not currently known. In the present cohort, elevated AST and less strict ALT levels correlated with CHIP, especially when *DNMT3A*-

TABLE 4 Impact of genetic lesions at specific genes defining CHIP on the risk of hepatocellular carcinoma (HCC, n = 179) in 530 individuals in the NAFLD-EPIDEMIC study cohort

	Model 1 OR, 95% CI	*p*	Model 2 OR, 95% CI	*p*
CHIP	3.04, 2.01–4.60	< 0.001	1.41, 0.86–2.31	0.24
CHIP without *DNMT3A*	4.47, 2.72–7.42	< 0.001	2.45, 1.35–4.53	0.02
DNM3TA	1.51, 0.80–2.76	0.18	0.60, 0.27–1.22	0.24
TET2	8.02, 3.17–22.90	0.002	4.8, 1.60–17.0	0.02
TP53	3.81, 1.35–11.00	0.045	1.93, 0.50–7.92	0.37
ASXL1	5.00, 1.41–19.60	0.015	2.00, 0.45–10.30	0.37

Note: At logistic regression analysis adjusted for the covariates reported in the models. Unadjusted; model 1: adjusted for CHIP-defining genetic lesions; model 2: further adjusted for age at enrollment, sex, T2D, cirrhosis and PRS-5 score. A false discovery rate approach was employed to account for multiple testing. Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; PRS-5, Polygenic Risk Score 5; T2D, type 2 diabetes.

driven cases were not considered. This observation is in line with evidence that *DNMT3A* mutations had a lower impact on the risk of progressive MASLD than those in *TET2* and *ASXL1*[13] and might subtend a different inflammatory potential of circulating CHIP clones according to their driving genetic lesion. In addition, the inflammatory potential of *DNMT3A* mutations seems to be lesion specific. In our case, only one patient carried the R882H variant, that has been associated with the higher inflammatory burden by recent studies.[31] Hence, we do not exclude a priori a role for *DNMT3A* in liver pathology, but if a role is present, it could be mutation specific. Evaluation of *DNMT3A* mutations in larger cohorts is required to define their role in liver disease. Individuals positive for CHIP were at almost 2-fold higher risk of being affected by cirrhosis independently of the main clinical covariates, such as sex and presence of T2D, and genetic predisposition toward progressive SLD. However, due to the strong link between CHIP and aging, the association was lost after correcting for age at enrollment. Therefore, we could not conclude whether CHIP may partially mediate the effect of aging on progressive liver disease or was an epiphenomenon.

The main study finding, however, was the evidence of an association between CHIP and the occurrence of HCC in the NAFLD-EPIDEMIC cohort. Importantly, as a whole, CHIP was associated with a 2-fold increase in the risk of HCC when corrected for other contributing factors, including the presence of cirrhosis. Notably, CHIP was able to discriminate between patients who developed HCC and those who did not better than the genetic predisposition intended as inheritance of common risk variants predisposing to severe SLD, which are associated with progression to cirrhosis. However, the association between CHIP and HCC was not independent of age when considering DNMT3A lesions, but it did so when DNMT3A lesions were excluded. This finding could be explained by the strong collinearity between development of CHIP and HCC with increasing age, but also by a mutation-specific effect, with most *DNMT3A* variants described in our series being neutral.

Notably, a strikingly clear signal has emerged, a specific enrichment of *TET2* and *TP53* lesions in HCC

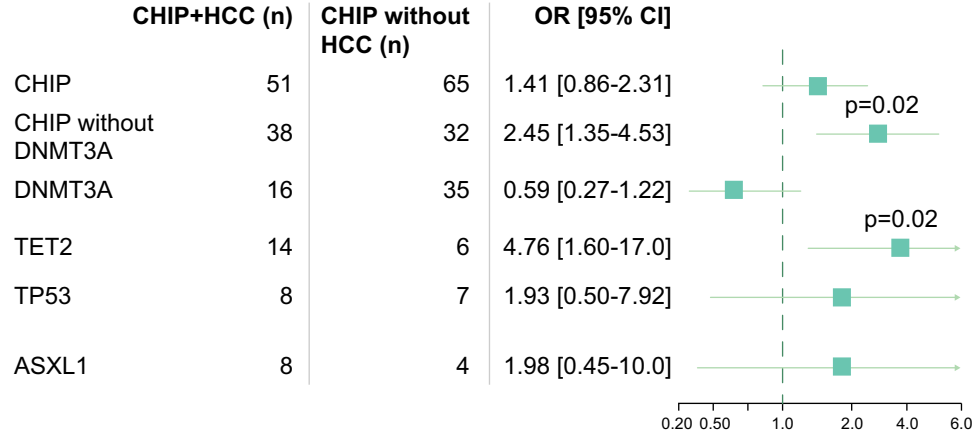

FIGURE 4 Forest plot displaying the association between HCC, CHIP, and CHIP subgroups multivariable at analysis, including type 2 diabetes, age at study enroll, cirrhosis, sex, and PRS-5 score as covariates. Absolute counts of patients with concomitant HCC and CHIP and HCC without CHIP are shown in the first 2 columns. OR and their 95% CI associating CHIP subtypes to HCC are shown in the third column. Rows represent different CHIP subtypes. Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; PRS-5, polygenic risk score of fatty liver disease.

samples. The association of *TET2* genetic lesions (and of non-*DNMT3A*-CHIP overall) with HCC remained significant even after adjusting for all covariates, including clinical and genetic factors as well as age at enrollment. The risk of developing HCC was found to be approximately 4.8 times higher in individuals with *TET2*-CHIP. This observation supports a diverse contribution to inflammation and organ damage from circulating myeloid cells bearing different CHIP lesions. Indeed, it has been suggested that different CHIP clones engage selected immune phenotypes, with *DNMT3A*-driven clonal hematopoiesis being associated with increased interferon activity and *TET2*-driven hematopoiesis with an NLRP3-dependent IL-1B production and elevated IL-6 and IL-8 levels. Supporting these findings, wild-type *TET2* gene seems required for suppression of IL-6 production.[31–33] While IL-6 activity has been reported to be higher also in *DNMT3A*-driven CHIP, this is the case for R882H mutations only, further corroborating our hypothesis.[31]

Overall, these data suggest that specific genetic lesions defining CHIP, and definitively those in *TET2*, may play a causal role in hepatic carcinogenesis by inducing liver inflammation and be at least partly involved in mediating the impact of aging on the risk of HCC development.

Current limitations include the lack of an independent validation cohort, inclusive of different ethnicities, with a prospective approach to assess the impact of CHIP incidence on HCC risk. The contribution of CHIP to HCC may be confounded by the strong age dependency of CHIP, with age being a determinant of HCC risk as well. In our study, non-DNMT3A-CHIP was associated with HCC even after adjusting for age in a multivariate model. However, only larger prospective cohort studies are likely to determine whether the impact of CHIP to HCC risk is substantial enough to become a clinically useful parameter. Not the least, there is a need for mechanistic demonstration of the impact of specific CHIP-defining genetic lesions on hepatic carcinogenesis.

In conclusion, in this cross-sectional multicenter case-control cohort of European patients with MASLD, we observed a high prevalence of CHIP, which was associated with more severe liver damage and a 2-fold higher risk of HCC. HCC association with CHIP was independent of clinical and genetic cofactors, and of age when considering non-DNMT3A lesions. In addition, when analyzing the specific drivers, a significant 4-fold impact of *TET2*-driven CHIP on HCC was observed, independent of age. However, given the relatively small size of our cohort and its retrospective nature, additional studies will be crucial in validating our findings. External validation with a prospective cohort is required to fully establish CHIP as a risk factor for liver disease progression and untangle the interaction between aging and clonal hematopoiesis. Furthermore, experimental and in vivo studies are needed to determine a pathogenic contribution of tumor infiltrating leukocytes in the inflammatory milieu of HCC.

AUTHOR CONTRIBUTIONS

Study design: Luca Valenti and Niccolò Bolli; funding and oversight: Luca Valenti, Niccolò Bolli, and Daniele Prati; writing of first manuscript draft: Alfredo Marchetti, Luca Valenti, Niccolò Bolli, and Serena Pelusi; coordination of clinical study: Serena Pelusi and Luca Valenti; clinical data collection: Serena Pelusi, Elisabetta Bugianesi, Luca Miele, Umberto Vespasiani-Gentilucci, Roberta D'Ambrosio, Alessandro Federico, Paola Dongiovanni, Giorgio Soardo, Misti V. McCain, Helen L. Reeves; data analysis: Alfredo Marchetti, Serena Pelusi, Alfredo Marchetti, Francesco Malvestiti, Antony Ricchiuti, Luisa Ronzoni, and Marta Lionetti. All authors read and approved the final manuscript version.

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CONFLICTS OF INTEREST

Roberta D'Ambrosio consults and is on the speakers' bureau for AbbVie and Gilead. Helen L. Reeves advises

Bayer, Boston Scientific, and Eisai. She received grants from AstraZeneca. Vincenzo La Mura consults for AlfaSigma, Biomarin, CSL Behring, and Pfizer. He is on the speakers' bureau for Gore. He received grants from Sanofi and Takeda. Daniele Prati consults, advises, is on the speaker's bureau, and received grants from Macropharma. He consults, is on the speaker's bureau, and received grants from Diamed, Diatech, Grifols, Immucor, Ortho Clinical Diagnostics, and Terumo. He is on the speakers' bureau for Diasorin. Niccolò Bolli consults and is on the speakers' bureau for Janssen. He consults for Pfizer. He is on the speakers' bureau for Amgen and Jazz. Luca Valenti consults is on the speakers' bureau, and received grants from Gilead. He consults for AstraZeneca, Boehringer Ingelheim, Diatech, Intercept, Ionis, Novo Nordisk, Pfizer, and Resalis. He is on the speakers' bureau for AbbVie, AlfaSigma, and MSD. The remaining authors have no conflicts to report.

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