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Human leukocyte antigen-G in hepatocellular carcinoma driven by chronic viral hepatitis or steatotic liver disease

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Hepatocellular carcinoma (HCC) is the sixth most common cancer globally and the third leading cause of cancer-related mortality, primarily driven by viral infections (HCV, HBV) and steatotic liver diseases (SLD). Despite advances in treatment, early detection and accurate prognosis remain challenging. The Human leukocyte antigen G (HLA-G) molecule is dysregulated in various conditions, including cancers and viral infections. This study aimed to investigate HLA-G's role in viral-related and SLD-driven HCC. We analyzed a cohort of 116 HCC patients and 140 healthy controls to assess HLA-G genetic variants and soluble levels. Results showed significantly higher levels of soluble HLA-G in HCC patients compared to controls (Pc = 0.003). Moreover, overall survival (OS) was significantly lower in patients with the extended *HLA-G*01:01:01/UTR-1* haplotype (Log-rank test, p = 0.002), a trend consistent in both HCV and/or HBV-related HCC (p = 0.025) and SLD-related HCC (p = 0.018). Elevated sHLA-G levels were associated with shorter OS across both subgroups (p = 0.034 (HBV/HCV) and p = 0.010 (SLD), respectively). The findings suggest that elevated levels of soluble HLA-G and specific genetic variants are associated with poor prognosis in HCC patients, highlighting the potential of HLA-G as a prognostic biomarker in both viral-related and steatotic liver disease-related HCC.

Keywords HLA-G, CC, SLD, sHLA-G, HBV, HCV

Liver cancer ranks as the 6th most common cancer globally and the second leading cause of cancer-related deaths, with approximately 866,136 new cases and 758,725 deaths reported in 2022, accounting for 7.8% of all cancer cases[1]. Hepatocellular carcinoma (HCC) accounts for about 90% of primary liver cancers and represents a significant public health concern worldwide[2]. HCC exhibits higher prevalence in males, with a male-to-female ratio estimated at 2–3:1[2]. Incidence generally rises with age across populations, peaking around 70[3]. However, the average age varies among ethnic groups, with Chinese and black Africans exhibiting lower averages compared to Japanese populations[4,5]. Geographic disparities in incidence are notable, with East Asia and sub-Saharan Africa reporting the highest rates, while Europe generally shows lower incidence except in southern regions where it accounts for approximately 9.7% of cases[6]. Approximately 90% of hepatocellular carcinoma (HCC) cases have identifiable underlying causes, predominantly linked to chronic viral hepatitis. In Italy, around 35% of them are attributed to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections[7].

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The second most frequent other possible risk factor for HCC is represented by steatotic liver diseases (SLD)[8], which include alcohol consumption (ALD), metabolic disorders (MASLD), and other less frequent diseases such as hemochromatosis, Wilson's disease, and alpha-1-antitrypsin deficiency, or exposure to aflatoxins[9,10]. All these elements may eventually lead to hepatic cirrhosis[11]. Indeed, this condition can represent the intermediate stage and the primary background of HCC development[12]. Cirrhosis is globally the 9thleading cause of death in Europe and, overall, one-third of cirrhotic patients will develop HCC in their lifetime[13]. Long-term follow-up studies have found that approximately 1–8% of cirrhosis patients develop HCC per year (e.g. 2% in cirrhotic patients with HBV infection and 3–8% in cirrhotic patients with HCV infection)[14]. Although therapeutic advancements have been made, researchers are exploring new prognostic markers for early disease detection. Recent genome-wide association studies (GWAS) reported that genetic polymorphisms in the *HLA* region may contribute significantly to the development of HCC[15]. Several candidate genes, such as *HLA-A* and *HLA-G*, have been investigated to evaluate their roles in susceptibility to HCC. However, due to insufficient evidence and lack of independent validation, these findings are inconclusive[15]. Other studies have shown that different molecular levels of soluble HLA-G (sHLA-G) may be implicated in multiple liver diseases[16].

The HLA-G is encoded by Human leukocyte antigen-G, a non-classical HLA class I gene located on chromosome 6p21.3. This tolerogenic protein plays a key role in fetal-maternal tolerance. Unlike other *HLA* genes, *HLA-G* has low levels of polymorphisms in the coding region. Currently, 110 alleles, 36 proteins, and 6 non-coding regions have been identified (https://www.ebi.ac.uk/ipd/imgt/hla/about/statistics/, January 2023). Seven different HLA-G isoforms can be produced through alternative splicing: four membrane isoforms (G1-G4) and three soluble isoforms (G5-G7)[17]. In the past, the most widely studied polymorphism is the 14-base pair deletion-insertion (*14-bp INS/DEL*) (*rs66554220*) located in the 3′-untranslated region (3′UTR)[18]. This polymorphism can influence mRNA stability and protein expression and has been associated with several pathological conditions[18].

Moreover, variation in the secretion levels of soluble HLA-G appears to be linked to different *HLA-G 3'UTR* haplotypes[19]. Indeed, from the literature, it is known that two UTRs (*UTR-5* and *UTR-7*) are correlated with low sHLA-G secretion, whereas *UTR-1* is associated with high sHLA-G secretion. The other UTRs are associated with intermediate sHLA-G expression[20,21].

Recent studies based on *HLA-G* whole gene amplification reveal a linkage disequilibrium between the *HLA-G* 3' UTR and allelic coding sequence. These extended haplotypes (*HLA-G*alleles linked with 3' UTR haplotypes) influence the expression and function of the protein, and therefore their analysis is useful for studies on the role of HLA-G in transplantation, pregnancy, and cancer[22].

HLA-G molecules exert their tolerogenic activity through interaction with the immunoglobulin (Ig)-like transcript 2 (ILT2) and ILT4 inhibitory receptors expressed on natural killer (NK) cells, T and B lymphocytes, dendritic cells, and neutrophils. Therefore, the HLA-G/ILT interaction is considered a promising immune checkpoint[23].

The immunosuppressive role of HLA-G molecules has been investigated in numerous inflammatory and autoimmune diseases[24,25], in infectious conditions[16,26–34], and also in organ transplantations[35–40].

Moreover,HLA-G plays an active role in liver homeostasis and immune response to liver injury and cancer[41,42]. The presence of HLA-G can promote immunosuppression through receptor binding, impaired chemotaxis, and trogocytosis[33], and has been shown to exacerbate liver fibrosis by producing a shift in the immune response towards a TH2 cytokine profile[16,29].

Recent studies have reported elevated levels of sHLA-G in HCC patients[43]. This tolerogenic molecule may play a role in shielding cancer cells from immune system activators, such as CD8-positive T cells, NK cells, B cells, and dendritic cells (DCs)[44]. The heightened expression of HLA-G has been linked to tumor evasion from immune surveillance and, consequently, poor prognosis in HCC patients[43,45]. Previous studies have investigated the association between HCC and specific *HLA-G* polymorphisms and/or HLA-G expression[43,46], although the findings have occasionally been inconsistent[47]. To date, no studies have specifically examined the impact of *HLA-G* extended haplotypes on HCC.

In this study, our objective is to elucidate the relationship between different *HLA-G* haplotypes and sHLA-G levels with the risk of developing HCC. Additionally, patients with HCC were divided into two subgroups based on the two most frequent risk factors[7,8]: viral infection (HBV and/or HCV) or SLD (ALD or MASLD/MASH), to evaluate whether HLA-G could influence the outcome of HCC differently in the two patient subgroups. Our study seeks to enhance our understanding of hepatocarcinoma progression and could pave the way for more effective preventive strategies for patients. This will be achieved by comparing patients with a control population from the same geographical region (South Sardinia, Italy).

Results

Clinical characteristics of HCC patients

HCC patients included in the study were enrolled according to the criteria outlined in the workflow diagram (Fig. 1). A total of 132 HCC patients were recruited between 2017 and 2022 and were followed at the Liver Unit of the University Hospital and the Division of Gastroenterology of ARNAS G. Brotzu (Cagliari, Italy). HCC was diagnosed according to the American Association for the Study of Liver Diseases (AASLD) guidelines[48]. The study aimed to assess the influence of extended *HLA-G* haplotypes and their associated sHLA-G levels on the onset and progression of HCC, and to examine potential differences between two patient subgroups: i) HCC driven by viral hepatitis (HBV and/or HCV) and ii) HCC associated with SLD (ALD or MASLD/MASH). For this reason, 6 patients were excluded from the initial 132 as their HCC developed due to autoimmune hepatitis type 1 (n = 2), hemochromatosis (n = 2), Wilson's disease (n = 1), or cryptogenic SLD (n = 1). An additional 10 patients were excluded due to the absence of plasma samples at diagnosis or incomplete clinical and follow-up data. The clinical and demographic characteristics of the remaining 116 HCC patients are shown in Table 1.

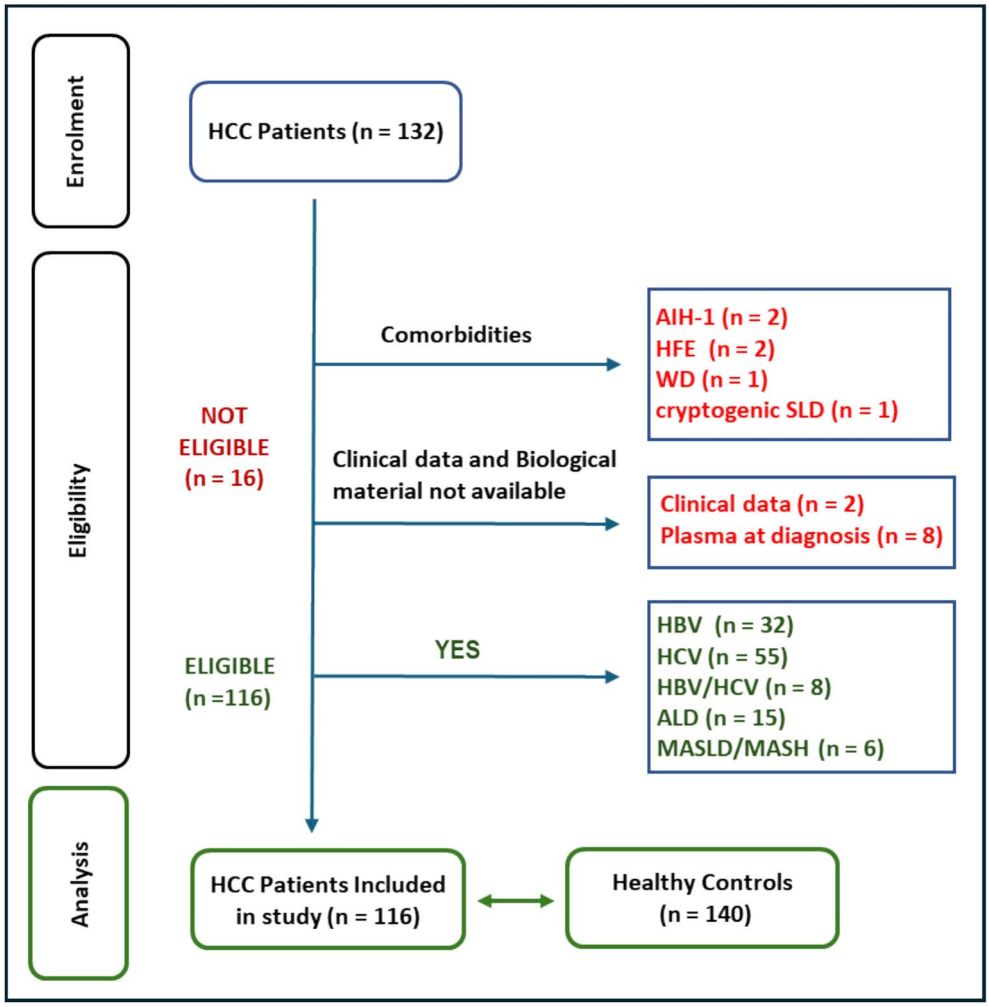


Fig. 1. HCC Patients' enrollment. A total of 132 HCC patients were recruited between 2017 and 2022 and were followed at the Liver Unit of the University Hospital and the Division of Gastroenterology at ARNAS G. Brotzu (Cagliari, Italy). HCC was diagnosed according to the American Association for the Study of Liver Diseases (AASLD) guidelines[49]. The aim of the study was to evaluate the influence of extended *HLA-G* haplotypes and their associated sHLA-G levels on the onset and progression of HCC, and to investigate potential differences between two subgroups of patients: i) HCC driven by viral hepatitis (HBV and/or HCV) and ii) HCC associated with SLD (ALD or MASLD/MASH). For this reason, 6 patients were excluded from the initial cohort of 132 as their HCC had developed due to autoimmune hepatitis type 1 (n = 2), hemochromatosis (n = 2), Wilson's disease (n = 1), or cryptogenic SLD (n = 1). An additional 10 patients were excluded due to the absence of plasma samples at diagnosis or incomplete clinical and follow-up data. AIH-1: Autoimmune Hepatitis type 1; HFE: Hereditary Hemochromatosis; WD: Wilson's Disease; SLD: Steatotic Liver Disease; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; ALD: Alcoholic Liver Disease; MASLD: Metabolic Dysfunction Associated Steatotic Liver Disease; MASH: Metabolic Dysfunction Associated Steatohepatitis. *Note*: New fatty liver disease nomenclature as defined in a multisociety Delphi consensus statement[8].

The mean age at diagnosis was 65.2 years (mean ± SD: 65.2 ± 9.25). Results indicated that most of the patients were male (83.62%, n = 97), followed by females (16.38%, n = 19). In our cohort, the majority of HCC cases (81.90%, 95 patients) arose from viral hepatitis. Specifically, within this subgroup, 55 patients (47.41%) had HCV infection, 32 patients (27.59%) had HBV infection, and 8 patients (6.90%) had HBV-HCV co-infection.

The second subgroup consisted of 21 patients (18.10%) in whom HCC was associated with SLD. Of these, 15 patients (71.4%) had SLD of alcoholic origin (ALD), and 6 patients (28.6%) had SLD secondary to metabolic dysfunction (MASLD), with or without steatohepatitis (MASH).

In most patients, HCC occurred in the context of liver cirrhosis (109 patients, 93.97%). Conversely, 7 cases (6.03%) had a complete response to treatment. At the time of diagnosis, the stage of liver disease was assessed according to the Child–Pugh score, with 97 patients (83.62%) in class A, 15 patients (12.93%) in class B, and 4 patients (3.45%) in class C.

According to the BCLC staging system at diagnosis, 15 patients (12.93%) had very early-stage HCC (BCLC 0), 68 patients (58.62%) had early-stage HCC (BCLC A), 26 patients (22.41%) were in intermediate-stage HCC

Clinical and demographic parameters		
Age at diagnosis (yrs): mean ± σ	65.21 ±9.25	
	n	**%**
Male	97	83.62
Female	19	16.38
Etiology		
Viral infections	95	81.91
HBV	32	27.59
HBV + HCV	8	6.90
HCV	55	47.41
SLD (ALD or MASLD/MASH)	21	18.10
Cirrhosis at diagnosis	109	93.97
Child–Pugh Score		
A	97	83.62
B	15	12.93
C	4	3.45
BCLC staging system		
0	15	12.93
A	68	58.62
B	26	22.41
C	4	3.45
D	3	2.59
Therapy		
RFA	42	36.21
TACE	35	30.16
Surgical resection	13	11.21
Sorafenib	13	11.21
Other treatment	13	11.21
Radiological response to therapy at 1 year		
CR	78	67.24
PR	7	6.03
PD	24	20.68
SD	7	6.03
Overall survival		
1 year	93	80.17
3 years	54	46.55
5 years	30	25.86

Table 1. Comparisons of baseline clinical parameters of the 116 HCC patients. HBV: Hepatitis B virus, HBV + HDV: co-infection Hepatitis B virus-Hepatitis D virus, HCV: Hepatitis C virus, SLD :Steatotic liver disease, ALD: Alcoholic liver disease, MASLD:Metabolic dysfunction associated steatotic liver disease, MASH: Metabolic dysfunction associated steatohepatitis, BCLC Barcelona clinic liver cancer, RFA: Radiofrequency ablation, TACE: transarterial chemoembolization, CR: complete response, PR: partial response, PD: progression disease, SD: stable disease. P values were calculated for comparisons between HCC patients and healthy controls. Abbreviations: σ = standard deviation;.

(BCLC B), 4 patients (3.45%) were in advanced stage (BCLC C), and 3 patients (2.59%) were in terminal stage (BCLC D).

Treatment modalities included radiofrequency thermal ablation (RFA) for 42 patients (36.21%), transarterial chemoembolization (TACE) for 35 patients (30.17%), liver resection surgery for 13 patients (11.21%), and systemic therapy with Sorafenib (Nexavar®) for 13 patients (11.21%). The remaining 13 patients (11.21%) received other treatments: 4 patients (3.45%) with percutaneous ethanol injection (PEI), 1 patient (0.86%) with microwave ablation (MWA), and 8 patients (6.90%) were referred to palliative care.

Radiological response to therapy was evaluated at one year: 78 patients (67.24%) had a complete response (CR), 7 patients (6.03%) had a partial response (PR), and 24 patients (18.97%) had a poor response or disease progression (PD). In 7 patients (6.03%), the disease remained stable (SD).

Additionally, patients' overall survival at 12, 36, 60 months was 80.17% (93/116), 46.55% (54/116) and 25.86% (30/116) respectively.

Analysis of the HLA-G locus

The comparison of all extended haplotypes (*HLA-G* alleles linked with 3' UTR haplotypes) is presented in Table 2. No significant differences in the frequencies of extended haplotypes were observed between patients and healthy controls (P > 0.05). The *HLA-G*01:01:01:01/UTR-1* was the most prevalent extended haplotype in both patients and controls [21.1% (49/232) vs. 27.5% (77/280), respectively]. Considering only the coding portion of the *HLA-G*01:01:01* (3-digit) alleles (*G*01:01:01:01, G*01:01:01:08 and G*01:01:01:09*), the frequency of *HLA-G*01:01:01/UTR*-1 was 32.8% (76/232) in patients vs. 34.3% (96/280) in controls (P = 0.778).

Extended haplotypes with frequencies above 10% were also observed for *HLA-G*01:03:01:02/UTR-5* [12.5% (29/232) vs. 15.4% (43/280)] and *HLA-G*01:01:02:01/UTR-2* [15.5% (36/232) vs. 11.8% (33/280)]. No statistically significant differences were observed when comparing the two subgroups of HCC patients (Table 3). Notably, the extended haplotype *HLA-G*01:01:01:08/UTR-1* was more frequent in the subgroup of patients with HCC driven by SLD compared to the subgroup of patients with HCC related to HBV and/or HCV [19.0% (8/42) and 9.5% (18/190), *P* = 0.101].

HLA-G 3'UTR haplotype frequencies were compared between 116 HCC patients and 140 healthy controls (Table S1). None of these *HLA-G UTR* haplotypes showed significantly different in frequencies between patients and control individuals (P > 0.05).

In both groups (patients vs controls) the most prevalent haplotype was *UTR-1* [34.48% (80/232) and 34.30% (96/280), respectively] followed by the *UTR-2* [27.16% (63/232) and 26.1% (73/280)] and *UTR-5* [15.7% (44/232) vs 12.50% (29)]. The remaining UTR haplotype groups were marginally represented in both control and patients' groups.

Even within the two subgroups of HCC patients, the *HLA-G 3'UTR* haplotype frequencies did not show significant or relevant differences (Table S2). The largest difference was observed for the *UTR-1* haplotype,

Extended haplotypes		140 Healthy controls				116HCC patients		Patients vs Controls
Alleles	3'UTR haplotype	2 N = 280	*f*	2 N = 232	*f*	*P* value	OR	95% CI
G*01:01:01:01	UTR-1	77	0.275	49	0.211	0.100	0.706	0.468–1.064
G*01:03:01:02	UTR-5	43	0.154	29	0.125	0.374	0.787	0.474–1.307
G*01:01:02:01	UTR-2	33	0.118	36	0.155	0.243	1.375	0.827–2.285
G*01:01:03:03	UTR-7	19	0.068	22	0.095	0.327	1.439	0.759–2.730
G*01:01:01:08	UTR-1	18	0.064	26	0.112	0.059	1.837	0.980–3.443
G*01:01:22:01	UTR-2	18	0.064	10	0.043	0.333	0.656	0.297–1.450
G*01:01:01:05	UTR-4	14	0.05	13	0.056	0.843	1.128	0.519–2.450
G*01:04:01:01	UTR-3	15	0.05	21	0.086	0.111	1.792	0.885–3.494
G*01:06:01:01	UTR-2	10	0.028	2	0.009	0.121	0.296	0.051–1.082
G*01:05 N	UTR-2	5	0.018	7	0.03	0.393	1.711	0.536–5.464
G*01:04:04	UTR-3	5	0.018	1	0.0043	0.228	0.238	0.028–2.053
G*01:01:01:04	UTR-18	5	0.018	0	0	0.067	0.000	-
G*01:04:01:01	UTR-10	3	0.011	0	0	0.256	0.000	-
G*01:06:01:02	UTR-2	2	0.0071	3	0.0129	0.663	1.821	0.302–10.991
G*01:01:01:06	UTR-4	2	0.0071	0	0	0.503	0.000	-
G*01:04:01:02	UTR-3	2	0.0071	1	0.0043	1.000	0.602	0.054–6.678
G*01:01:01:01	UTR-4	1	0.0036	0	0	1.000	0.000	-
G*01:01:01:04	UTR-6	1	0.0036	0	0	1.000	0.000	-
G*01:01:01:06	UTR-2	1	0.0036	0	0	1.000	0.000	-
G*01:06:02:02	UTR-2	1	0.0036	0	0	1.000	0.000	-
G*01:01:01:01	UTR-3	1	0.0036	0	0	1.000	0.000	-
G*01:03:01:01	UTR-5	1	0.0036	0	0	1.000	0.000	-
G*01:02:02	UTR-2	1	0.0036	0	0	1.000	0.000	-
G*01:03:01:01	UTR-2	1	0.0036	0	0	1.000	0.000	-
G*01:01:01:09	UTR-1	1	0.0036	1	0.0043	1.000	1.208	0.075–19.416
G*01:01:01:13	UTR-6	0	0	2	0.0086	0.205	-	-
G*01:01:01:01	UTR-6	0	0	1	0.0043	0.453	-	-
G*01:01:17	UTR-2	0	0	1	0.0043	0.453	-	-
G*01:04:01:01	UTR-2	0	0	2	0.0086	0.205	-	-
G*01:03:01:02	UTR-13	0	0	2	0.0086	0.206	-	-
G*01:01:22:01	UTR-10	0	0	1	0.0043	0.453	-	-

Table 2. Extended haplotypes (*HLA-G* alleles and 3'UTR haplotypes) frequencies in healthy controls and HCC patients. P values were calculated for comparisons between HCC patients and the healthy control group. Abbreviations: OR, odds ratio; CI,confidence interval; *f* = allele frequencies expressed as decimals.

Extended haplotypes		95 HCC correlate with HBV/HCV				21 HCC driven by SLD		SLD vs HBV/HCV
Alleles	3'UTR haplotype	2 N = 190	*f*	2 N = 42	*f*	*P* value	OR	95% CI
G*01:01:01:01	UTR-1	38	0.200	11	0.262	0.405	1.417	0.588–3.227
G*01:03:01:02	UTR-5	22	0.116	7	0.167	0.438	1.524	0.510–4.070
G*01:01:02:01	UTR-2	27	0.142	9	0.214	0.245	1.643	0.621–4.024
G*01:01:03:03	UTR-7	20	0.105	2	0.048	0.383	0.426	0.046–1.875
G*01:01:01:08	UTR-1	18	0.095	8	0.190	0.101	2.239	0.777–5.963
G*01:01:22:01	UTR-2	9	0.047	1	0.024	0.695	0.492	0.011–3.7240
G*01:01:01:05	UTR-4	13	0.068	0	0.000	0.133	0	0.000–1.453
G*01:04:01:01	UTR-3	19	0.100	2	0.048	0.383	0.451	0.049–1.997
G*01:06:01:01	UTR-2	2	0.011	0	0.000	1	0	0.000–24.259
G*01:05 N	UTR-2	6	0.032	1	0.024	1	0.749	0.016–6.434
G*01:04:04	UTR-3	0	0.000	1	0.024	0.181	-	> 0.116
G*01:06:01:02	UTR-2	3	0.016	0	0.0129	1	0	0.000–11.061
G*01:04:01:02	UTR-3	1	0.005	0	0.0043	1	0	0.000–175.946
G*01:01:01:09	UTR-1	2	0.011	0	0.0043	1	0	0.000–24.259
G*01:01:01:13	UTR-6	2	0.011	0	0.0086	1	0	0.000–24.259
G*01:01:01:01	UTR-6	1	0.005	0	0.0043	1	0	0.000–175.946
G*01:01:17	UTR-2	1	0.005	0	0.0043	1	0	0.000–175.946
G*01:04:01:01	UTR-2	2	0.011	0	0.0086	1	0	0.000–24.259
G*01:01:01:04	UTR-1	1	0.005	0	0.0043	1	0	0.000–175.946
G*01:03:01:02	UTR-13	2	0.011	0	0.0086	1	0	0.000–24.259
G*01:01:22:01	UTR-10	1	0.005	0	0.0043	1	0	0.000–175.946

Table 3. Extended haplotype frequencies (*HLA-G* alleles and 3′UTR haplotypes) in HCC patients, divided into two subgroups based on etiology: HCC associated with viral infection and HCC driven by steatotic liver disease (SLD), including Alcoholic Liver Disease (ALD) and Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD/MASH). Extended haplotypes (*HLA-G* alleles and 3'UTR haplotypes) frequencies in HCC patients divided into two subgroups based on etiology: HCC associated with viral infection and HCC driven by steatotic liver disease (SLD): including Alcoholic Liver Disease (ALD) and Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD/MASH). P values were calculated for comparisons between SLD-HCC and viral-HCC patients. OR, odds ratio; CI,confidence interval; *f* = allele frequencies expressed as decimals.

which was more represented in HCC driven by SLD compared to the subgroup of patients with HCC related to HBV and/or HCV [45.2% (19/42) and 32.1% (61/190), $P = 0.110$]. Furthermore, even when subdividing the patients with viral-related HCC, no significant differences were observed in either allele frequencies (Table S3) or sHLA-G levels (Figure S1). However, the limited number of patients in this subgroup may have affected the ability to detect subtle differences.

In addition, we examined the genotype and allele frequency distribution of the *HLA-G* 14 bp *Ins/Del* polymorphism in both controls and patients (Table S4). The *Del/Ins* polymorphism variants showed a distribution in Hardy–Weinberg equilibrium (HWE) both in patients and control. Indeed, the X^2_{HWE} p-values did not reach statistical significance in the two groups ($X^2_{HWE} = 0.271$, $P = 0.603$ and $X^2_{HWE} = 0.042$, $P = 0.837$, respectively).

Moreover, patients were divided into according to the etiology leading to HCC: I) viral hepatitis and II) SLD (ALD or MASLD/MASH) to examine the possible influence of the *Del/Ins* allele. However, also in this case no significant difference was observed ($X^2_{HWE} = 0.007$, $P = 0.932$ and $X^2_{HWE} = 0.069$, $P = 0.793$, respectively).

Soluble HLA-G dosage

Soluble HLA-G (sHLA-G) levels were measured in HCC patients at the time of diagnosis and in healthy controls at the time of enrollment. The results showed that sHLA-G levels were significantly higher in HCC patients compared to controls [Median (IQR): 31.70 (18.30) U/mL vs 14.50 (15.20) U/mL, respectively; $P = 1.9 \times 10^{-8}$, $Pc = 0.003$] (Fig. 2A).

Interestingly, HCC patients in both subgroups (HCV/HBV and SLD) had significantly higher sHLA-G levels than the control group. Specifically, the SLD subgroup showed [Median (IQR): 53.30 (41.00) U/mL vs. 14.50 (15.20) U/mL; SLD vs. Controls $P = 0.00043$, $Pc = 0.008$], and the HBV/HCV subgroup had [Median (IQR): 31.70 (18.00) U/mL vs. 14.50 (15.20) U/mL; HBV/HCV vs. Controls $P < 0.0001$, $Pc = 0.013$] (Fig. 2B). Although the sHLA-G levels of SLD patients were higher than those of HBV/HCV patients, this difference did not reach statistical significance [Median (IQR): 53.30 (41.00) U/mL vs. 31.70 (18.00) U/mL, respectively; $P > 0.05$].

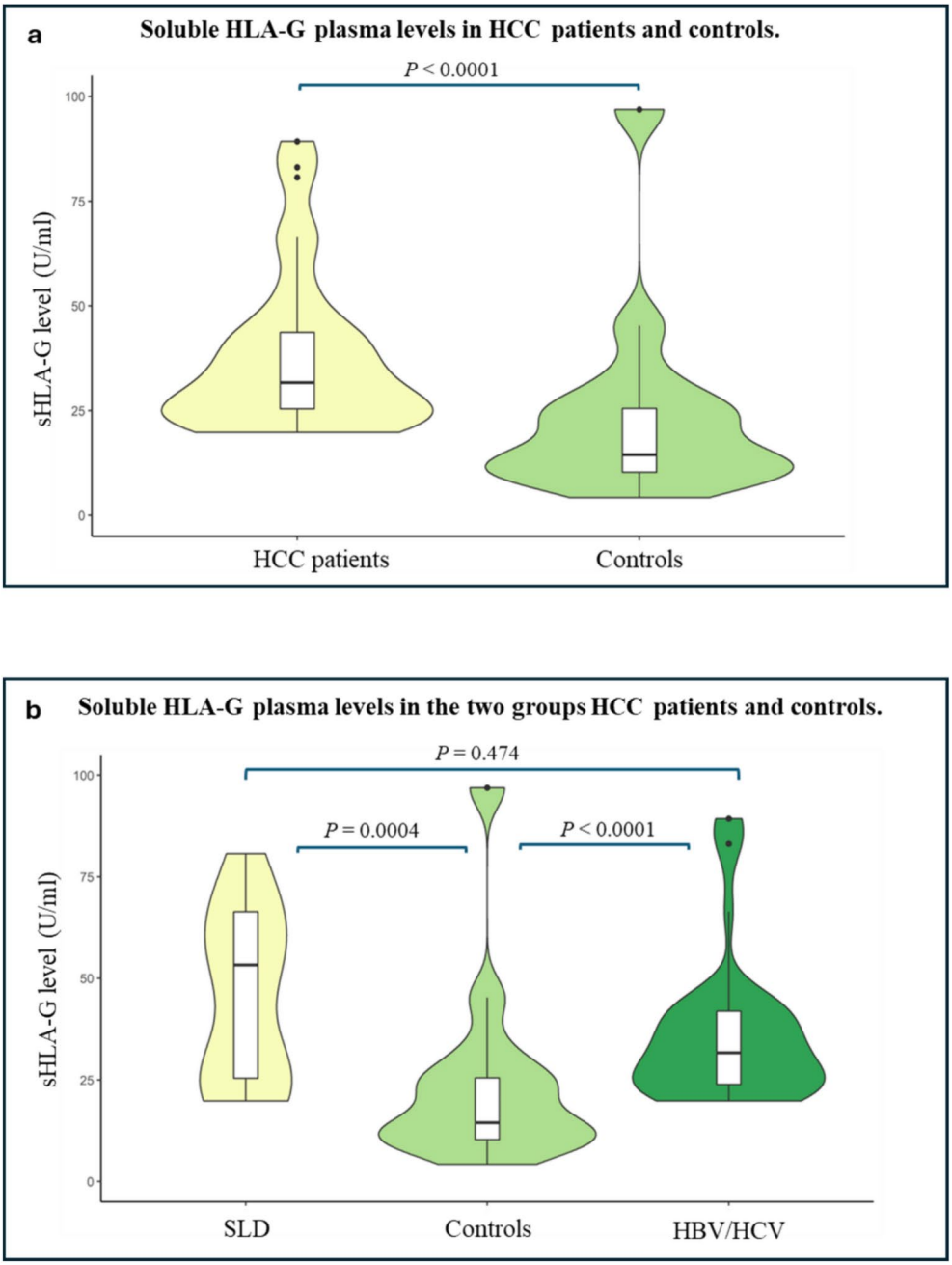


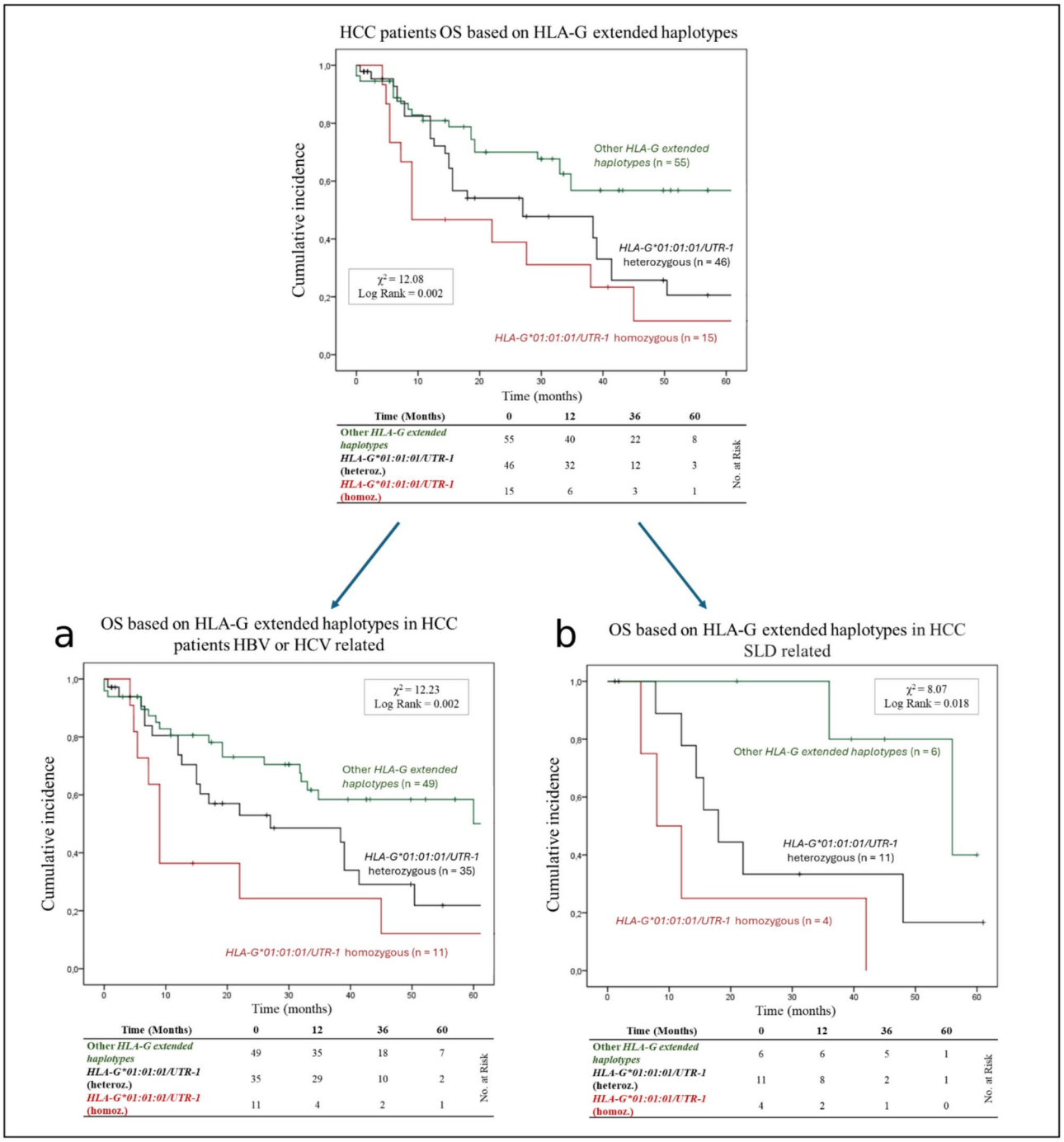
Fig. 2. Soluble HLA-G (sHLA-G) plasma levels (U/mL) were compared between HCC patients and the control group (**A**), and in the two groups of HCC patients (SLD vs HBV/HCV and vs Controls) (**B**). The boxplot is included in the violin to assess the median and interquartile range. The vertical bars represent the 95% confidence intervals. P values and mean differences, were obtained by the Student's t test. * Median and Pc value if the HLA-G levels greater than 100 U/ml are excluded.

HCC patients overall survival

Overall survival of HCC patients based on HLA-G genetic profile

We evaluated the overall survival (OS) of 116 HCC patients based on the extended *HLA-G* haplotype HLA-*G*01:01:01/UTR-1,* which is the most frequent in the studied population and has been associated by some authors with increased HLA-G expression. Results over a 60-month period are presented in Fig. 3.

Patients were categorized according to their *HLA-G* extended haplotypes into three groups: *HLA-G*01:01:01/UTR-1* homozygous, *HLA-G*01:01:01/UTR-1* heterozygous, and *HLA-G* other extended haplotypes. The survival curves reveal significant differences in OS among the different *HLA-G* extended haplotype groups. Specifically, patients with the *HLA-G*01:01:01/UTR-1* homozygous genotype exhibited a shorter overall survival compared to those with the *HLA-G*01:01:01/UTR-1* heterozygous and *HLA-G* other extended haplotypes (Log-rank test, p = 0.002) (Fig. 3). The median OS for the homozygous group was approximately 17.3 months, 22.1 months



for the heterozygous group, and 31.4 months for those with other haplotypes. Supporting these findings, we also observed that sHLA-G levels were significantly higher in the homozygous *HLA-G*01:01:01/UTR-1* patients compared to the other extended haplotypes, but not to the *HLA-G*01:01:01/UTR-1* heterozygote patients [94.1 ± 44.9 vs 65.74 ± 48.13; P-value = 0.043 and 94.1 ± 44.9 vs 72.32 ± 46.35; P-value = 0.486, respectively] (Figure S2).

After examining the entire cohort, we analyzed OS within two specific HCC subgroups: those with HCV and/or HBV-related HCC and those with SLD-driven HCC. Figure 3A illustrates the Kaplan–Meier survival curves for HCC-specific mortality in a cohort of 95 patients with HCC related to HCV and/or HBV infection, observed over 60 months. The survival curves reveal notable differences in OS among the *HLA-G* extended haplotype groups. Specifically, patients with the *HLA-G*01:01:01/UTR-1* homozygous genotype demonstrated worse overall survival compared to those with the *HLA-G*01:01:01/UTR-1* heterozygous genotype and *HLA-G* other extended haplotypes (Log-rank test, p = 0.002). The median OS for patients with the *HLA-G*01:01:01/UTR-1* homozygous genotype was approximately 17.5 months. In contrast, patients with the *HLA-G*01:01:01/UTR-1* heterozygous genotype had a median OS of 22.4 months, and the *HLA-G* other extended haplotypes group, with a median OS of 31.0 months.

◀**Fig. 3**. HCC Overall Survival based on *HLA-G* extended haplotypes. The overall survival (OS) of HCC-specific mortality is graphically presented for a cohort of 116 patients observed over a 60-months time frame. Patients were categorized based on their *HLA-G* extended haplotypes (*HLA-G* alleles with 3'UTR) in *HLA-G*01:01:01/UTR-1* homozygous, *HLA-G*01:01:01/UTR-1* heterozygous or *HLA-G* other extended haplotypes (*HLA-G* alleles with their UTR). P-values were calculated using the two-sided log-rank test. **A**) **Overall Survival based on *HLA-G* extended haplotypes in patients with HCC related to HCV and/or HBV infection.** The overall survival (OS) of HCC-specific mortality is graphically presented for a cohort of 95 patients observed over a 60-months time frame. Patients were categorized based on their *HLA-G* extended haplotypes (*HLA-G* alleles with 3'UTR) in *HLA-G*01:01:01/UTR-1* homozygous, *HLA-G*01:01:01/UTR-1* heterozygous or *HLA-G* other extended haplotypes (*HLA-G* alleles with their UTR). P-values were calculated using the two-sided log-rank test. **B**) **Overall Survival based on *HLA-G* extended haplotypes in patients with HCC SLD related.** The overall survival (OS) of HCC-specific mortality is graphically presented for a cohort of 21 patients observed over a 60-months time frame. Patients were categorized based on their *HLA-G* extended haplotypes (*HLA-G* alleles with 3'UTR) in *HLA-G*01:01:01/UTR-1* homozygous, *HLA-G*01:01:01/UTR-1* heterozygous or HLA-G other extended haplotypes (*HLA-G* alleles with their UTR). P-values were calculated using the two-sided log-rank test. This HCC patients subgroup included ALD and MASLD/MASH. Abbreviations: SLD = Steatotic liver disease; ALD = Alcoholic liver disease; MASLD = Metabolic dysfunction associated with steatotic liver disease; MASH = Metabolic dysfunction associated with steatohepatitis.

Similarly, in the remaining 21 patients with SLD-related HCC (Fig. 3B), a comparable trend was observed. Patients with the *HLA-G*01:01:01/UTR-1* heterozygous or homozygous genotype had significantly worse OS compared to those with other *HLA-G* extended haplotypes (Log-rank test, p = 0.018). The median OS for patients with the *HLA-G*01:01:01/UTR-1* homozygous genotype was approximately 16.8 months. In contrast, patients with the *HLA-G*01:01:01/UTR-1* heterozygous genotype had a median OS of 21.2 months, and the *HLA-G* other extended haplotypes group, with a median OS of 34.7 months.

Additionally, we analyzed the overall survival in the 116 patients, stratified by the *HLA-G* 14 bp *ins/del* polymorphism in the *HLA-G 3'UTR* region, which has been suggested to impact HCC outcomes in previous studies. Patients were grouped into three genotypes: homozygous insertion (*Ins/Ins*), heterozygous (*Ins/Del*), and homozygous deletion (*Del/Del*), (Figures S1). This analysis was also performed for two subgroups: 95 HCC patients with viral hepatitis (HCV and/or HBV) and 21 HCC patients with SLD (Figures S1-A, S1-B). However, no statistically significant differences in overall survival were found among the different genotypes in either subgroup (Log-rank test, P > 0.05).

Overall Survival of HCC Patients Based on sHLA-G Levels

The overall survival (OS) of HCC patients was assessed according to their sHLA-G levels over a 60-month period (Fig. 4). All 116 patients were categorized into three groups based on their soluble HLA-G (sHLA-G) levels: low (< 25 U/mL), medium (25–80 U/mL), and high (> 80 U/mL). The survival curves illustrate clear differences in OS among these groups. Notably, patients with high sHLA-G levels had significantly shorter OS compared to those with medium and low sHLA-G levels (Log-rank test, p = 0.024) (Fig. 4). The median OS for patients with sHLA-G low levels was approximately 26.4 months, whereas it was 25.3 months for those with medium levels, and 21.6 months for those with high levels.

In the subgroup of 95 patients with HCV and/or HBV-related HCC, a similar pattern was observed. Patients with medium and high sHLA-G levels had significantly reduced survival compared to those with low levels (Log-rank test, p = 0.034) (Fig. 4A). In this group, the median OS for patients with low sHLA-G levels was 25.6 months, while it was 24.4 months for medium levels, and 22.5 months for high levels.

For the remaining 21 patients with HCC driven by steatotic liver disease (SLD), the trend persisted (Fig. 4B). Patients with higher sHLA-G levels experienced lower survival, with a clear inverse relationship between sHLA-G levels and OS (Log-rank test, p = 0.010). The median OS for patients with low sHLA-G levels was 31.8 months, compared to 28.7 months for those with medium levels, and 15.4 months for those with high sHLA-G levels.

Discussion

One of the key findings from this study is the markedly elevated plasma levels of sHLA-G observed in HCC patients, which were on average 3–4 times higher than those in healthy controls. Interestingly, these elevated levels of sHLA-G were not limited to patients with HCC linked to viral hepatitis but were also present in cases where HCC was driven by ALD or MASLD/MASH.

Elevated sHLA-G levels in liver fibrosis and HCC have been previously documented in patients with HBV and HCV infections[29,43,50]. However, the functional significance of increased sHLA-G levels during HBV/HCV infection remains controversial. Bian et al. suggest that the upregulation of miR-152, triggered by HBV infection, could explain the increased expression of HLA-G in these cases[51]. This is the first study to document sHLA-G levels in patients with HCC related to SLD.

The exact mechanism responsible for the rise in sHLA-G levels in these patients remains unclear; however, it is plausible that this increase serves as a countermeasure against the chronic low-grade inflammation driven by the high levels of proinflammatory cytokines associated with excess adipose tissue[52]. Similarly, overexpression of soluble HLA-G is a common feature in many inflammatory conditions[53], such as systemic lupus erythematosus[54,55], systemic sclerosis[56], and antiphospholipid syndrome[57].

Additionally, factors such as Hypoxia-Inducible Factor 1 (HIF-1) and IFN-γ contribute to increased HLA-G expression through distinct mechanisms. HIF-1, a key transcriptional regulator activated under low oxygen

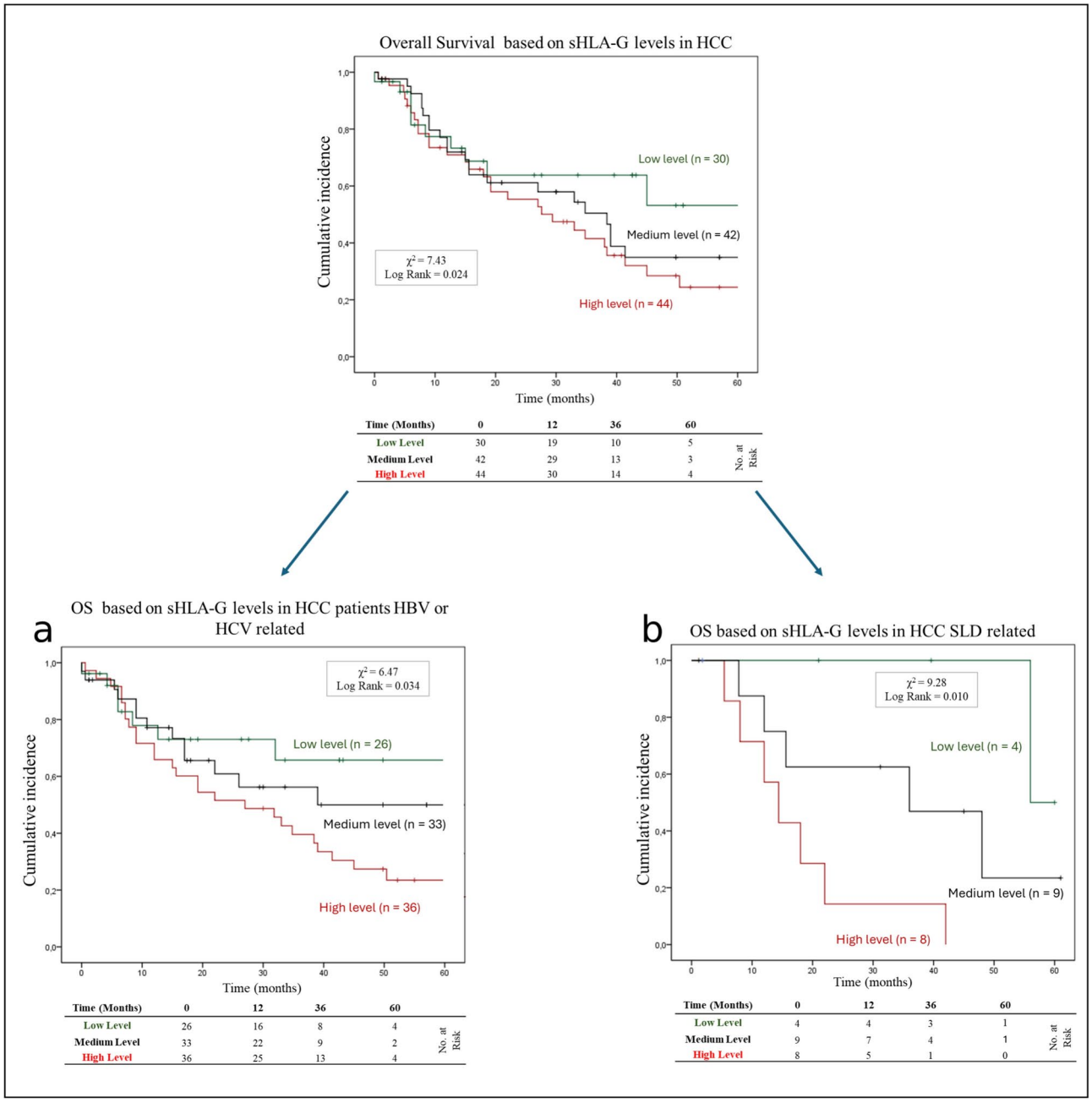


Fig. 4. HCC Overall Survival based on sHLA-G levels. The overall survival of HCC-specific mortality is graphically presented for a cohort of 116 patients observed over a 60-months time frame. Patients were categorized based on their soluble HLA-G (sHLA-G) in low (< 25 U/mL) medium (25–80 U/mL) or high (> 80 U/mL) levels. P-values were calculated using the two-sided log-rank test. **A**) **Overall Survival based on sHLA-G levels in patients with HCC related to HCV and/or HBV infection** The overall survival (OS) of HCC-specific mortality is graphically presented for a cohort of 95 patients observed over a 60-months time frame. Patients were categorized based on their soluble HLA-G (sHLA-G) in low (< 25 U/mL) medium (25–80 U/mL) or high (> 80 U/mL) levels. P-values were calculated using the two-sided log-rank test. **B**) **Overall Survival based on sHLA-G levels in patients with HCC SLD related** The overall survival (OS) of HCC-specific mortality is graphically presented for a cohort of 21 patients observed over a 60-months time frame. Patients were categorized based on their soluble HLA-G (sHLA-G) in low (< 25 U/mL) medium (25–80 U/mL) or high (> 80 U/mL) levels. P-values were calculated using the two-sided log-rank test. This HCC patients subgroup included ALD and MASLD/MASH. Abbreviations: SLD = Steatotic liver disease; ALD = Alcoholic liver 330 disease; MASLD = Metabolic dysfunction associated steatotic liver disease; MASH = Metabolic dysfunction associated steatohepatitis.

conditions, directly targets the *HLA-G* gene, leading to its upregulation[58]. Moreover, in vitro studies suggest that IFN-γ, secreted by infiltrating cytotoxic T cells, can enhance HLA-G expression in tumor cells[59]. Several studies have linked elevated sHLA-G levels in HCC patients to poor prognosis due to its role in promoting tumor progression[43,60]and strongly associated with advanced disease stages[61].

Consistent with these findings, we observed that higher sHLA-G levels significantly impacted overall survival (OS) in HCC patients, irrespective of the underlying cause, whether viral infection (HBV/HCV) or SLD. Specifically, approximately 35% of patients with low sHLA-G levels (< 25 U/mL) were alive at 60 months, compared to only 15% of those with medium-to-high levels. This supports the concept that HCC cells utilize HLA-G expression as a mechanism to evade immune surveillance, as observed in other malignancies[61,62]. HLA-G promotes tumor progression by expanding myeloid-derived suppressor cells and disrupting the balance between Th2 and Th1/Th17 responses[34,61].

Previous studies have demonstrated the utility of soluble HLA-G (sHLA-G) in both serum and saliva as a biomarker for various tumor types[63,64]. In our study, a clear correlation between sHLA-G levels and HCC progression was also observed, regardless of whether the disease was caused by viral or metabolic etiologies. However, the use of plasma sHLA-G measurement as an alternative biomarker for HCC appears to be of limited applicability in clinical practice, as its levels may be affected by various external factors such as viral infections, pharmacological treatments, or existing comorbidities[65]. Similar considerations apply to alpha-fetoprotein (AFP), the most widely used serum diagnostic biomarker for HCC; this biomarker has demonstrated limited sensitivity and specificity[66]. A considerable proportion of patients with advanced HCC do not exhibit AFP secretion, while individuals with chronic liver diseases, particularly cirrhosis, often present with persistently elevated AFP levels despite the absence of radiological evidence of HCC[67].

Additionally, novel biomarkers such as liquid biopsy are under development and show promise. However, their application in HCC remains challenging, particularly in the early disease phases, and still represents a field of ongoing investigation[68].

Despite the higher overall sHLA-G levels in HCC patients compared to controls, we observed considerable variability in sHLA-G expression, as highlighted by the violin plots (Figs. 3A and 3B). This variability is genetically determined and influenced by polymorphisms in both the coding and regulatory regions of *HLA-G*, including the 5' and 3' untranslated regions (3'UTR)[69]. For instance, the insertion/deletion polymorphism (*ins/del*) of 14 bp in the 3'UTR is known to regulate HLA-G expression, increasing its production[70].

Previous studies have suggested that protective polymorphisms, such as the 14 bp insertion homozygosity in the *HLA-G* 3'UTR, are associated with better outcomes, while increased HLA-G expression, linked to the 14 bp deletion polymorphism, correlates with reduced survival[46,71]. However, our study did not confirm this correlation. In our cohort, the *HLA-G del/del* genotype (14 bp of the 3'UTR) was not significantly associated with worse prognosis, as illustrated by the Kaplan–Meier survival curves (Figure S1). The log-rank test for overall survival did not show significant differences in either of the two groups (HBV/HCV and SLD-related HCC). However, patients with the *HLA-G ins/ins* genotype tended to have better survival outcomes. The small sample size and the unique genetic characteristics of the Sardinian population may explain the discrepancies with previous findings.

Consistent with our data, a recent study on 286 HCV-infected patients who developed fibrosis, cirrhosis, or HCC, and 129 healthy controls, did not find statistically significant differences when analyzing the *HLA-G 14 bp* polymorphism or the entire 3'UTR region[72].

A meta-analysis by Dhouioui et al., which included seven case–control studies on the *HLA-G 14-bp Ins/Del* polymorphism and 15 studies on soluble HLA-G (sHLA-G), also aligns with our findings[50]. Unlike the *HLA-G 14 bp Ins/Del* polymorphism, this study highlights how the extended *HLA-G*01:01:01/UTR-1* haplotype is a much more sensitive marker for predicting overall survival (OS) in HCC patients. Specifically, HLA-*G*01:01:01/UTR-1* homozygous patients have a significantly reduced life expectancy compared to patients with other extended HLA-G haplotypes (17.3 months vs. 31.4 months, Log-rank test, $p = 0.014$). Its prognostic value remains independent of the etiology of HCC. In fact, the significance of this marker is confirmed both in the group of 95 patients with HCC related to HCV and/or HBV infection and in the group of 21 patients with HCC driven by SLD.

This survival trend is further supported by the observation that patients carrying the extended *HLA-G*01:01:01/UTR-1* haplotype exhibit higher sHLA-G levels (Figure S2). It is important to note that several studies have shown that the 3'*UTR* region contains at least three polymorphic sites that influence HLA-G expression via different mechanisms[73,74]. In addition to the previously mentioned 14-bp polymorphism (*rs371194629*), there is an SNP at position +3142 (*rs1063320*)—with nucleotide +1 defined as the adenine of the first translated ATG—which may affect miRNA binding. Specifically, the presence of a guanine at this site increases the affinity for microRNAs such as *miR-148a-3p, miR-148b-3p,* and *miR-152, l*eading to enhanced mRNA degradation and reduced HLA-G production[75]. Moreover, the presence of an adenine at position +3187 (*rs9380142*) has been associated with decreased mRNA stability, likely due to its proximity to an AU-rich motif that promotes mRNA degradation[76]. The *UTR-1* region does not contain any of these polymorphisms and may therefore explain the increased sHLA-G levels observed. However, caution must be exercised due to the limited number of samples and the high variability in HLA-G expression, which is conditioned by possible external factors.

In conclusion, our study highlights the prognostic value of sHLA-G levels in HCC, showing significantly higher levels in patients compared to healthy controls, regardless of etiology. While elevated sHLA-G levels correlate with worse prognosis, this study is the first to document sHLA-G levels in SLD-related HCC, raising questions about inflammatory mechanisms. However, due to variability caused by infections and inflammation, sHLA-G appears less clinically viable than the *HLA-G*01:01:01/UTR-1* genetic marker, which is a more reliable predictor of overall survival in HCC patients, whether virus-related or SLD-driven.

Materials and methods

Patients and controls recruitment

We enrolled a cohort of 116 Sardinian outpatients with HCC whose onset was attributable to the two main risk factors: viral infection (HBV and/or HCV) or SLD (ALD or MASLD/MASH). Patients were recruited between 2017 and 2022 and were followed at the Liver Unit of the University Hospital and the Division of Gastroenterology of the ARNAS G. Brotzu (Cagliari, Italy). The diagnosis was performed according to the American Association for the Study of Liver Diseases (AASLD) guidelines[48]. In the setting of liver cirrhosis, international guidelines have set the non-invasive criteria for HCC diagnosis, represented by the detection of contrast hyperenhancement in the arterial phase (wash-in) and hyperenhancement in the portal or delayed phase (wash-out) with dynamic multi-detector computer tomography or magnetic resonance (MR) imaging. In some sporadic cases, liver biopsy was required. All cirrhotic patients were regularly and closely monitored every 6 months with ultrasound examination and serological marker testing (including AFP and markers of liver disease) to detect HCC. HCC patients were divided into two subgroups based on different etiopathogenesis: the first group comprised 95 viral-related HCC patients (HBV and/or HCV), and the second group consisted of 21 patients with HCC driven by steatotic liver disease (SLD). These 21 patients were reclassified according to the new fatty liver disease nomenclature[10]. The HLA-G allele and genotype frequencies and sHLA-G levels of the 116 HCC patients were compared with those of 140 unrelated healthy individuals recruited from the Sardinian Voluntary Bone Marrow Donor Registry. Both patients and controls were from South Sardinia, according to three-generation family trees.

Ethics statement

The study was performed following the Declaration of Helsinki and written informed consent was obtained from all participating patients and healthy subjects. The study protocol was approved by the responsible ethics committee (Ethics Committee of the Cagliari University Hospital; date of approval: 27/02/2017; protocol number PG/2017/3278. Records of written informed consent are kept on file and are included in the case history of each patient.

Genetic analysis

DNA was extracted from peripheral mononuclear blood according to standard procedures. (QIAGEN. QIAamp DNA mini blood mini handbook (2022) as described previously[77]. All 256 samples were analyzed for the *HLA-G* gene including the 3'UTR region by Next Generation Sequencing (NGS) technique following the method described by Nilsson et al.[49].

Primers were designed using Primer3web (version 4.1.0), based on *HLA-G* RefSeqGene version 191 NG_029039.1 (NCBI database), as previously described[49].

Long-range PCR was performed using GoTaq Long PCR Master Mix, 2X (Promega, Madison, Wisconsin), with the appropriate thermocycler program as specified by the manufacturer.

The long-range PCR products were purified using AMPure beads (Agilent Technologies, Santa Clara, California), and concentrations in the samples were evaluated with Qubit DNA High Sensitivity kit (ThermoFisher Scientific).

Libraries were prepared from 1 ng of PCR product using the Nextera XT DNA Library Preparation Kit. Sequencing was performed on Illumina MiSeq instrument using V3 flow-cells (600 cycles) paired-end. MiSeq Reporter v2.6 was used for FASTQ alignment and variant calling, and VariantStudio Software v3.0 (Illumina, Netherlands) was used for variant classification. Variants were validated individually and then entered into appropriate spreadsheets for statistical analysis. The 3'UTR haplotypes of *HLA-G* were determined using a method and nomenclature described elsewhere[78,79] using variations between + 2945 and + 3259 nucleotides.

The *HLA-G 14 bp Ins/Del* polymorphism (*rs66554220*) has been verified using PCR-SSP method as previously described[80].

Soluble HLA-G plasma quantification

Plasma samples were collected from 116 HCC patients and 180 controls during enrollment. The levels of sHLA-G were assessed using the sHLA-G ELISA assay kit (Exbio, Prague, Czech Republic) following the manufacturer's guidelines. The kit can detect both shedding HLA-G-1 and soluble HLA-G5 molecules. Upon separation, plasma samples were promptly frozen and stored at −80 °C until analysis. Prior to conducting the HLA-G assay, each sample (50 µl) was diluted 1:80 in a plasma-specific buffer. A six-point calibration curve was established using the human native HLA-G protein provided with the kit. The optical density at 450 nm was measured using a microplate reader after the reaction. The sensitivity limit of the assay was 0.6 U/ml. Duplicate assays were performed for all samples.

Statistical analysis

Mean and standard deviation (SD) were used to describe the clinical and biochemical parameters of HCC patients for continuous variables.

Continuous variables were compared between patients and controls using the Student's t test. We used Fisher's exact test for categorical data comparisons between patients and controls to calculate P values and odds ratios (ORs) with their 95% confidence intervals (CIs). Haldane correction method was used to avoid error if any of the cell values would cause a division by zero error[81].

Only P values lower than 0.05 were considered to be statistically significant. We computed X^2_{HWE} and P values to determine Hardy–Weinberg equilibrium (HWE) for the genotypes and alleles distribution of *HLA-G* 14 bp *Ins/Del* polymorphism. Deviation from HWE was performed using Haploview 4.0 software (Broad

Institute, Cambridge, MA, USA). All tests were two-sided and only values of $P < 0.05$ were considered statistically significant.

Kaplan–Meier curves were used to estimate Overall Survival (OS) over a 60-month time frame. HCC patients were censored at the date of diagnosis (clinical, radiological, histopathological and immunohistochemical), and at the last follow-up or death. Patients were stratified in three groups i) according to their HLA-G extended haplotypes (HLA-G alleles with specific UTR haplotypes): *HLA-G*01:01:01/UTR-1* homozygous, *HLA-G*01:01:01/UTR-1* heterozygous or other HLA-G extended haplotypes (other combinations of HLA-G alleles with UTR); ii) according to *HLA-G 3' UTR 14 bp Ins/Del* (*rs66554220*) genotypes: Ins/Ins and Ins/Del Del/Del. Additionally, the overall survival (OS) of patients was evaluated based on their plasma sHLA-G levels, categorized as low (< 25 U/mL), medium (25–80 U/mL), or high (> 80 U/mL) levels. The log-rank test was used for comparisons of the different gene profile combinations.

Statistical analysis was performed with the R programming language (R version 4.2.2) [R core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/].

Data availability

The datasets presented in this study can be found in online repositories: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1188533.

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Author contributions

R.L., S.M., A.P. and L.C. contributed significantly to the data analysis, interpretation and conceptualization. M.M. contributed significantly to statistical analysis. All authors actively participated in reviewing, revising, and approving the final version of the manuscript for submission.

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Declaration

Competing interests

The authors declare no competing interests.

Ethics approval

The study was conducted following the principles outlined in the Declaration of Helsinki, following the approval granted by the relevant local Ethics Committee (Ethics Committee of the G. Brotzu Hospital in Cagliari, Italy; approval date: January 23, 2014; protocol number NP/2014/456). Written informed consent was acquired from all patients and is securely stored in the appropriate Medical Record Office.

Additional information

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