

# Detecting tumor-associated peptides from the plasma-derived soluble immunopeptidome in human hepatocellular carcinoma patients: a pilot study

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## Short Report

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# Abstract

**Background and aims.** Hepatocellular carcinoma (HCC) shows limited response to checkpoint inhibitor therapy, leaving opportunities for antigen-based immunotherapy and improved disease monitoring. This study evaluates the plasma-derived soluble HLA-I (sHLA-I) peptidome as a minimally invasive source of tumor-associated antigens (TAAs) for personalized treatment and diagnostics in HCC. **Methods.** Plasma-derived sHLA-I peptides and membrane HLA-I peptides from paired tumor and adjacent liver tissues of HCC and non-HCC control patients were isolated and analyzed by LC-MS/MS. Identified peptides from the different HLA sources were subjected to patient-specific HLA-binding prediction and interrogated for the presence of TAA-derived peptides. **Results.** The plasma-derived sHLA-I peptidomes contained 1,553 to 9,421 unique peptides identified per sample after contaminant filtering. Peptides were enriched for canonical 8–11-mer lengths and mostly predicted to bind patient-specific HLA-I alleles. The HCC sHLA-I peptidome was derived from 3,907 source proteins and peptides from 815 of these were uniquely carried by HCC patient sHLA-I. Protein interaction analysis revealed significantly increased network connectivity of HCC unique source proteins centered around tumor-associated proteins. Comparison with mHLA-I peptidomes from tumor and adjacent tissues yielded 20 peptides from 17 TAAs that were shared between sHLA-I and mHLA-I. Nine of these were only identified from HCC plasma and not from non-HCC controls, five overlapped with tumor mHLA-I. **Conclusions.** This pilot study demonstrates that the plasma-derived sHLA-I immunopeptidome in HCC patients contains peptides derived from TAAs, partially also presented on tumor tissue. These findings support the potential of sHLA-I profiling as a minimally invasive strategy for identifying actionable targets and tumor-specific signatures in HCC. Future improvements in analytical sensitivity and throughput, and validation in larger cohorts are now needed to establish its clinical utility.

## Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death [1]. In advanced-stage disease, first-line treatment consists of an immune checkpoint inhibitor (ICI)-containing regimen [2]. Yet, durable responses remain limited. Considering that most HCC patients exhibit a non-inflamed tumor microenvironment, the introduction of novel tumor antigen-specific T cells by antigen-specific immunotherapy might be needed [3]. Multiple T cell epitopes derived from viral, tumor-associated, and tumor-specific antigens have been assessed as targets for such therapies in HCC with promising results [4–7].

Identification of targetable T cell epitopes relies on liquid chromatography–mass spectrometry (LC–MS/MS) analysis of human leukocyte antigen (HLA)-presented peptides (or *immunopeptidomics*) [8]. Current approaches focus on tumor-derived membranous HLA (mHLA), but tissue scarcity and *inter/intra*-patient heterogeneity limit comprehensive profiling [9–11]. Plasma-contained soluble HLA class I (sHLA-I) may overcome these constraints, as its peptide cargo can present tumor-associated antigens across various tumors [12–16]. This “liquid biopsy” enables non-invasive detection of

therapeutic epitopes and tumor monitoring. So far, interrogation of the sHLA-I cargo has not been performed for HCC.

We hypothesize that in HCC, sHLA-I and mHLA-I immunopeptidomes overlap, allowing sHLA-I to reflect tumor presence and antigen presentation. We compare HCC sHLA-I immunopeptidomes with (1) paired mHLA-I immunopeptidomes from tumor and adjacent non-tumor hepatocytes, and (2) sHLA-I and hepatic mHLA-I immunopeptidomes from non-HCC patients. This study suggests sHLA-I immunopeptidome analysis in HCC may enable detection of tumor-presented T cell epitopes, potentially aiding diagnosis and personalized immunotherapy strategies.

## Patients and Methods

### Patient Cohort and Sample Processing

Patients (age > 18 years) undergoing surgical resection or liver transplantation between 2017 and 2023 for HCC (HCC patient cohort) or end-stage liver disease (Control patient cohort) were included from the Erasmus MC University Medical Center (EMC)(**Tables S1-S2**). In accordance with the pathologist, tumor and paired adjacent liver tissues ( $\geq 2$ cm distant of the tumor) were obtained and freshly frozen and processed as described in De Beijer et al. [17]. Clinical data were retrieved from medical records. Ethical approval with informed consent were obtained (MEC2014-060, MEC2009-012).

### Plasma collection and processing

Patient blood was collected in heparin tubes and stored no longer than 24h at room temperature (RT) before processing. Plasma was separated from whole blood by centrifugation at 120xG for 15min at RT, and was sequentially depleted from platelets by centrifugation at 3220xG for 15 min at 4°C. Plasma was stored at -80°C until analysis.

### Enrichment of HLA-I-peptide complexes

Pan-HLA-I affinity immunopurification beads were generated as previously described<sup>2</sup>. In short, the pan-HLA-I antibody (clone: W6/32; Biolegend) was conjugated and crosslinked to nProtein A Sepharose Fast Flow beads (Cytiva) at a concentration of 2.5mg/mL packed beads. Pan-HLA-I affinity beads were incubated with 'blocking buffer' (1% bovine serum albumin (BSA), 1% fish skin gelatin in phosphate buffered saline (PBS)) for minimally 15 minutes before usage. A starting volume of 5mL platelet-depleted plasma was used for all HLA-I immunoprecipitations. Plasma was prepared for incubation with pan-HLA-I affinity beads as follows: dilution with 5mL RT PBS, centrifugation at 3220xG for 10 min at 4°C, and addition of a 1:1 volume of '2x detergent buffer' (100mM Tris-HCl pH8, 300mM NaCl, 10mM EDTA, 1% N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent, Merck), 2mM PMSF (Sigma), and one tablet cOmplete™ Mini EDTA-free 6 Protease Inhibitor Cocktail (Roche) / 25mL).

Plasma samples were incubated with 2x detergent buffer for 1h on ice and vortexed every 15 minutes before final centrifugation at 3220xG for 10 min at 4°C. sHLA-I complexes were immunoprecipitated with the mini-column set-up as previously described [18]. Levels of sHLA-I in patient samples prior and after immunoprecipitation were determined by sandwich ELISA. In short, plates were coated twice overnight with 25µg/mL anti-pan-HLA-I monoclonal antibody (clone: W6/32) and blocked prior to sample incubation for 1h at 37°C with detergent-treated plasma that was sampled prior and after sHLA-I immunoprecipitation. Plates were washed and subsequently incubated with 0.3µg/mL HRP-conjugated anti-human  $\beta$ 2-microglobulin antibody for 1h at 37°C. After incubation, plates were again washed and incubated with substrate solution (0.01M citric acid monohydrate / tri-sodium citrate dihydrate pH 4.0 + 0.006% H<sub>2</sub>O<sub>2</sub> + 0.8 mM ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) for 8 min at room temperature in the dark prior to addition of stop solution (2% (w/v) oxalic acid dihydrate). Absorbance was measured at 414nm. All samples were analyzed at the same time.

## Sample preparation for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

Samples were prepared and measured as described previously [18]. In short, sHLA-I peptides were eluted with 0.15% TFA, separated from larger fragments by 10kD molecular weight cut-off filters (Microcon-10, MRCPRT010, Millipore) and desalted on 1cc Sep-Pak columns with 10mg C18 and 10mg HLB resin. Elution after desalting was performed with 28% acetonitrile containing 0.1% TFA prior to drying of the peptides in a vacuum centrifuge.

Immunopeptidomics data was acquired in data dependent acquisition (DDA) mode on an Orbitrap Eclipse Tribrid mass spectrometer (Thermo) operating in positive mode coupled to an EASY-nLC 1200. Peptides were trapped on a 2 cm × 100 µm Pepmap C18 column (Thermo Fisher) before separation on a packed 50 cm × 75 µm column containing 2.4µm Reprosil-Pur C18 beads (Dr. Maisch). A flow rate of 275nL/min and a gradient of 0–32% acetonitrile in 0.1% formic acid was used. MS1 spectra were recorded in the Orbitrap at 120K resolution in a m/z window between 375-1,200. Fragmentation was performed by HCD at NCE30% and MS2 spectra were recorded in the Orbitrap at 30K resolution.

## Data analysis

Raw MS data were analyzed using PEAKS studio (v11.0, Bioinformatics Solutions Inc.) and searched against a concatenated protein sequence database of the human reference proteome (UniProt, UP000005640) and 4 hepatitis B reference proteomes (UP000008591, UP000121470, UP000007930, UP000172538) with a false discovery rate (FDR) threshold of 5% [19]. HLA binding prediction was performed with NetMHCpan 4.1 [20] and peptides with a binding rank to patient-specific HLA types (**Table S3**)  $\leq 2\%$  were annotated as binders. A list of source proteins likely to be contaminants was compiled using the decision tree depicted in **Fig. S1** and was used to clean up and enhance the quality of the sHLA-I and mHLA-I data sets. In short, peptides were annotated if derived from contaminant source

proteins. Both contaminant and non-contaminant peptide data sets were subjected to HLA-I binding prediction as described above. Analyses as shown in this manuscript were performed with all pooled predicted binders. Previously published datasets were used to enlist TAAs [21–27], TEIPPs [28], and neoAgs [29]. Experimentally recorded spectra were extracted from the acquired mass spectrometry data. For peptides of interest, predicted spectra were generated by PEAKS and mirrored to the recorded spectra that were visualized by PEAKS for spectral comparison. For each peptide identified in either the sHLA or the tumor ligandome, the recorded spectrum and the corresponding mirrored PEAKS-predicted spectrum are provided.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted using the DAVID Bioinformatics Resources, with the *Homo sapiens* gene set used as background [29, 30]. Protein IDs were converted into gene names using UniProt [31]. Identification of protein-protein association networks was carried out using STRING, with a minimal confidence level of 0.7 [32].

## Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 9.0 (GraphPad, La Jolla, the U.S.A.). Statistically significant differences between two groups were calculated with the Mann-Whitney U test for independent samples or the Wilcoxon signed-rank test for paired samples. Correlation analysis was performed according to Spearman. P-values lower than 0.05 were considered statistically significant (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ). For GO and KEGG pathway analysis, p-values were adjusted following a Bonferroni test to correct for multiple testing.

## Results

### Generation of a high-quality sHLA-I-bound immunopeptidome.

First, we analyzed the sHLA-I and mHLA-I peptidomes of HCC patients and end-stage liver disease patients without a tumor (non-HCC patients; **Fig. 1A**). For the sHLA-I analysis, we used plasma from these patients (**Table S1**,  $n = 5$  each). For three out of five HCC patients and two out of five non-HCC patients, the tumor and tumor-free liver mHLA-I immunopeptidomes had been obtained previously (**Fig. 1B**) [17].

Soluble HLA-I levels in HCC patients were slightly, but not significantly, lower compared to non-HCC patients (**Fig. 1C**). HLA-I immunoprecipitation (IP) of 5 mL plasma was highly efficient (93–98%; data not shown). Each sample yielded several thousands of peptides but the peptide length distribution was not dominated by 8-11-mer peptides typical for HLA-I peptides. We suspected contamination with proteolytic fragments of highly abundant plasma proteins. To clean up our data, peptides derived from four categories of likely contaminating proteins (**Fig. S1**) were removed (38% and 34% of unique peptides from the HCC and non-HCC cohorts, respectively; (**Fig. S2A**).

Excluded peptides did not display the typical length for HLA-peptides confirming that these unlikely derived from HLA-I (**Fig. S2B**). Importantly, percentages of 8-11-mers and predicted HLA-binders increased vastly after data clean-up (**Fig. S2C-D**). The cleaned dataset contained between 1,553 and 9,421 unique sequences for each sample (**Fig. 1D**). One sample from a non-HCC patient (cHBV/HDV co-infected) yielded a remarkably high number of unique peptides. Overall, there was no statistically significant difference in peptide numbers between cohorts. Length distributions showcased a strong enrichment for 8-11-mers (**Fig. 1E-F**) and most of these were predicted to bind source patient DNA-encoded HLA-I alleles (**Table S3**) by NetMHCpan4.1[20] ( $\leq 2\%$  binding rank). In contrast, peptides with the same length and amino acid composition but scrambled in sequence, were not (**Fig. 1G**). To prevent negligence of potential therapeutic targets, removed contaminant peptides that were predicted to bind patient expressed HLA types were retrieved (**Fig. S2E**; 7.4% (32)-26.7% (161) per sample (**Fig. S2F**)).

Correlation as determined with a Spearman correlation analysis between sHLA-I levels and the number of predicted binders ( $r$  value = 0.6848,  $p$ -value = 0.0347).

Plasma sHLA-I levels correlated with the number of predicted HLA-binding peptides (**Fig. 1H**) but neither significantly correlated with liver damage (ALT; **Fig. S3**) or tumor burden (not shown).

### **Tumor antigens identified from plasma-derived sHLA-I peptidomes of HCC patients resemble the hepatocyte-derived mHLA-I peptidomes.**

Next, we zoomed in on the source proteins of plasma sHLA-I cargo. The HCC patient-derived sHLA-I peptidome consisted of 6,415 peptides from a total of 3,907 source proteins (**Fig. 2A, Sup. Data 1**) of which 3,092 were shared with non-HCC patients. HCC patient-specific source proteins were significantly enriched for 135 GO terms and 22 pathways (**Fig. S4A, Fig. S5A**). Unique to HCC-patient sHLA-I were 815 source proteins (**Fig. 2A, Sup. Data 1**). Annotated to GO terms such as cytoplasm and nucleus (**Fig. S4B**). No pathways were overrepresented (**Fig. S5B**), but the top three covered included metabolism and oncogenesis. Lastly, the HCC-restricted sHLA-I peptidome was significantly higher connected than expected ( $p = 0.001$ ) and centered around nine proteins with  $\geq 10$  interactions (**Fig. S6**). Of note, a few HBV-derived peptides were identified in the mHLA-I immunopeptidome of infected individuals, but not in their sHLA-I immunopeptidome (**Table S4**).

Subsequently, we exploited a dataset of 1,678 known TAAs to identify TAAs in our dataset. First, we narrowed down the number of mHLA eluted TAA (peptides) to those unique to HCC patient tissues, resulting in a total of 397 unique peptides from 207 TAAs (**Fig. 2B, Sup. Data 1**; i.e., 75 + 79 + 53). Next, we directly compared tissue-derived mHLA-I and plasma-derived sHLA-I of 3 sets of matched samples in a paired fashion (**Fig. 2C, Sup. Data 1**). Across all individuals, 20 peptides from 17 different TAAs could be detected in both the mHLA-I and sHLA-I cargo, but not all TAAs were identified based on the same peptide (**Table S5**). For one patient (HCC2), peptides of three tumor-specific mHLA TAAs were found back on sHLA-I (i.e., DCD2, ESM1, and MFGE8).

Lastly, to eliminate peptides from source proteins also presented on sHLA-I in individuals without HCC, the HCC-restricted sHLA-I TAA cargo and mHLA-I cargo of the pooled paired samples (n = 3) were compared (Fig. 2D, **Sup. Data 1**). In total, peptides derived from nine TAAs were uniquely identified in HCC samples. Of these, peptides from five TAAs were also detected in tissue mHLA-I (Fig. 2D, **Fig. S7**). Notably, DCDC2- and ESM1-derived peptides were identified only in tumor and not adjacent tissue.

## Discussion

Here, we present the first characterization of the plasma-derived soluble immunopeptidome of HCC patients. This sHLA-I immunopeptidome partially overlapped with the mHLA-I immunopeptidome and hence may carry valuable clues about HCC presence and could serve a minimally invasive ‘liquid biopsy’.

sHLA-I levels and peptide yield were comparable to those reported in other cancers [14–16]. Unexpectedly, no significant difference in sHLA-I levels was observed between HCC patients and non-HCC controls. This contrasts the elevated sHLA-I levels reported in other cancers [15]. Possibly this can be explained by the notion that controls were transplant recipients with fulminant liver disease while HCC patients were treated with resection and had relatively low disease burden. While we did not find liver damage (ALT) to be significantly associated with sHLA levels, our small dataset cannot exclude HLA is shed from dying cells. Another plausible sHLA source could be liver resident or infiltrated HLA<sup>high</sup> myeloid cells. Consequently, sHLA-I levels may reflect hepatocellular disease rather than tumor-specific biology, limiting use as a quantitative biomarker. The composition of the sHLA-I immunopeptidome, however, may still reveal valuable tumor-associated signals.

To demonstrate the utility of sHLA immunopeptidomics, it is essential to characterize HCC patient-restricted antigen presentation. We uncovered 815 proteins uniquely presented on HCC patient sHLA highly connected by nine proteins of which five HCC carcinogenesis-associated. Given the small heterogeneous cohort, however, we likely missed a significant proportion of sHLA-I peptides that could be presented and any possible effects of HLA genotype. Therefore, our study should be considered hypothesis-generating and requires larger follow-up studies to comprehensively grasp the HCC-specific sHLA immunopeptidome.

We analyzed the immunopeptidome for TAA-related peptides. Comparison of sHLA-I and mHLA-I cargo revealed 17 shared TAAs that were only presented on HCC patient but not control mHLA-I. The (per patient) overlap between the sHLA-I and mHLA TAA in this study matches that reported in the other studies, typically ranging 40–65% [12, 14, 16]. Nine TAAs were not identified in eluates from plasma sHLA-I obtained from non-HCC patients, of which five overlapped with HCC mHLA-I including ESM1, MAGED4, and H3F3A. ESM1 is a proteoglycan upregulated in various cancers, including HCC, and associated with tumor progression [33, 34]. MAGED4, a cancer germline antigen, was related to HCC and explored as a vaccine target [35]. Histone variant H3F3A, has been implicated in the progression of lung cancer [36]. These are potential therapeutic targets and their identification supports the utility of sHLA-I profiling in identifying clinically actionable antigens. While TAAs DCDC2 and ESM1 were restricted to

tumor mHLA-I, DDX25, H3F3A and MAGED4 were also detected in tumor adjacent (but not non-HCC patient) tissue. Whether this reflects presentation in non- or pre-malignant tissue or micro-metastatic disease requires further investigation [24]. No TEIPP or neoantigens derived from genetic alterations could be detected among sHLA peptides (not shown); the latter could not be assessed due to the lack of tumor genomic data. For this purpose, the recently proposed biomarker candidate, circulating tumor DNA (ctDNA) [37], reflecting the genomic status of the tumor, might be useful. On its own it is of limited value for antigen-based immunotherapy design because it does not prove protein presence nor inform on T cell epitopes. The latter can be derived from sHLA. Combining ctDNA with sHLA immunopeptidomics might allow for more sensitive and accurate prognostic and diagnostic screening, as well as for neoantigen-based therapeutic target identification.

Plasma sHLA-I immunopeptidome preparations are characterized by high levels of proteolytic peptides derived from abundant plasma proteins. We therefore introduced an additional step to remove such peptides using a structured, transparent decision tree retaining bona fide, potentially tumor-related, HLA-peptides. The proportional increase and consistent high level of both 8-11-mers and predicted binders after contaminant removal, underscores the quality of our workflow. In contrast, fluctuating proportions of 8-11-mers have been reported using various other approaches for data analysis [13, 14].

A key limitation of our study is the sensitivity with which sHLA-I cargo can be analyzed, partially explaining the low overlap between tumor and sHLA TAA cargo. Besides increasing the size of the plasma sample, analytical depth may be improved by a recently introduced semi-automated protocol for mid-throughput sHLA-I immunopeptidomics [38] or targeted mass spectrometry strategies for HCC-associated antigens [17, 24, 39, 40]. Furthermore, validation studies in larger and more diverse patient cohorts, including those currently amenable for immunotherapeutic approaches, will be critical to now assess generalizability and clinical applicability.

In conclusion, this proof-of-concept study demonstrates the feasibility of sHLA-I immunopeptidome analysis in HCC and its potential as a minimally invasive source of clinically actionable tumor antigens. Ongoing improvements in sensitivity and standardization may soon enable widespread adoption of sHLA-I profiling in both research and clinical settings.

## Abbreviations

|     |                               |
|-----|-------------------------------|
| A   | Acute infection               |
| AFP | Alpha fetoprotein             |
| ALD | Alcohol-related liver disease |
| ALT | Alanine transaminases         |
| BSA | Bovine serum albumin          |

|          |                                         |
|----------|-----------------------------------------|
| C        | Chronic infection                       |
| CP       | Child Pugh                              |
| ctDNA    | Circulating tumor DNA                   |
| DDA      | Data dependent acquisition              |
| EMC      | Erasmus MC University Medical Center    |
| ENT      | Entecavir                               |
| GO       | Gene ontology                           |
| HBV      | Hepatitis B virus                       |
| HCC      | Hepatocellular carcinoma                |
| HCV      | Hepatitis C virus                       |
| HDV      | Hepatitis D virus                       |
| ICI      | Immune checkpoint inhibition            |
| IEDB     | Immune Epitope Database                 |
| IP       | Immunoprecipitation                     |
| KEGG     | Kyoto Encyclopedia of Genes and Genomes |
| LC-MS/MS | Mass spectrometry                       |
| mHLA     | Membranous human leukocyte antigen      |
| MVI      | Microvascular invasion                  |
| N(eg)    | Negative                                |
| ND       | Not determined                          |
| NR       | Not reported                            |
| ns       | Nonsignificant                          |
| P(os)    | Positive                                |
| PBS      | Phosphate buffered saline               |

|      |                                 |
|------|---------------------------------|
| PRM  | Parallel reaction monitoring    |
| PSC  | Primary sclerosing cholangitis  |
| PSM  | Peptide spectrum match          |
| R    | Resolved infection              |
| RT   | Room temperature                |
| sHLA | Soluble human leukocyte antigen |
| TAA  | Tumor-associated antigen        |
| TDF  | Tenofovir                       |
| TFL  | Tumor-free (adjacent) liver     |

## Declarations

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### Competing Interests

D.S. has received funding and/or collaborates with Pfizer, Merus, and ISA Therapeutics. S.I.B. has received funding and/or collaborates with Pfizer, Merus, NUMAB therapeutics, ISA Therapeutics, ISAbella Pharma, Imuno therapeutics and Vitroscan. All other authors declare to have no conflict of interest.

### Authors contributions:

Conceptualization: AK, JD, SB, YR; Data curation: AK, KB, LB, MB, RL, RP, SB, WD, YR; Formal analysis: AK, KB, LB, MB, RL, RP, WD, YR; Funding acquisition: AK, DS, JD, JIJ, SB, YR; Investigation: AK, KB, LB, MB, RL, WD, YR; Methodology: AK, SB, YR; Project administration: AK, YR; Validation: AK, KB, LB, MB, RL, WD, YR; Visualization: AK; YSR; Writing - original draft: AK; YR; Writing – review and editing: AK, YR, DS, JD, JIJ, KB, LB, MB, RL, SB, WD.

### Data availability

Raw mass spectrometry data have been deposited to the ProteomeXchange (PRIDE/EBI) database with identifier PXD066255.

## Ethics approval

All study procedures were approved by the local ethics committee (MEC2014-060; MEC2009-012).

## Consent to participate

Patients had given informed consent for tissue donation as well as usage of medical data.

## References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I et al (2024) Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 74(3):229–263. 10.3322/caac.21834
2. Rimassa L, Finn RS, Sangro B (2023) Combination immunotherapy for hepatocellular carcinoma. *J Hepatol* 79(2):506–515. 10.1016/j.jhep.2023.03.003
3. Montironi C, Castet F, Haber PK, Pinyol R, Torres-Martin M, Torrens L et al (2023) Inflamed and non-inflamed classes of HCC: a revised immunogenomic classification. *Gut* 72(1):129–140. 10.1136/gutjnl-2021-325918
4. Yarchoan M, Gane EJ, Marron TU, Perales-Linares R, Yan J, Cooch N et al (2024) Personalized neoantigen vaccine and pembrolizumab in advanced hepatocellular carcinoma: a phase 1/2 trial. *Nat Med* 30(4):1044–1053. 10.1038/s41591-024-02894-y
5. Loffler MW, Gori S, Izzo F, Mayer-Mokler A, Ascierto PA, Konigsrainer A et al (2022) Phase I/II Multicenter Trial of a Novel Therapeutic Cancer Vaccine, HepaVac-101, for Hepatocellular Carcinoma. *Clin Cancer Res* 28(12):2555–2566. 10.1158/1078-0432.CCR-21-4424
6. Tan AT, Yang N, Lee Krishnamoorthy T, Oei V, Chua A, Zhao X et al (2019) Use of Expression Profiles of HBV-DNA Integrated Into Genomes of Hepatocellular Carcinoma Cells to Select T Cells for Immunotherapy. *Gastroenterology* 156(6):1862–76e9. 10.1053/j.gastro.2019.01.251
7. Meyer T, Finn RS, Borad M, Mahipal A, Edeline J, Houot R et al (2026) Phase I trial of ADP-A2AFP TCR T-cell therapy in patients with advanced hepatocellular or gastric hepatoid carcinoma. *J Hepatol* 84(1):113–121. 10.1016/j.jhep.2025.07.033
8. Shapiro IE, Bassani-Sternberg M (2023) The impact of immunopeptidomics: From basic research to clinical implementation. *Semin Immunol* 66:101727. 10.1016/j.smim.2023.101727
9. Jiang Y, Sun A, Zhao Y, Ying W, Sun H, Yang X et al (2019) Proteomics identifies new therapeutic targets of early-stage hepatocellular carcinoma. *Nature* 567(7747):257–261. 10.1038/s41586-019-0987-8
10. Zhang Q, Lou Y, Yang J, Wang J, Feng J, Zhao Y et al (2019) Integrated multiomic analysis reveals comprehensive tumour heterogeneity and novel immunophenotypic classification in hepatocellular carcinomas. *Gut* 68(11):2019–2031. 10.1136/gutjnl-2019-318912
11. Xing X, Hu E, Ouyang J, Zhong X, Wang F, Liu K et al (2023) Integrated omics landscape of hepatocellular carcinoma suggests proteomic subtypes for precision therapy. *Cell Rep Med*

- 4(12):101315. 10.1016/j.xcrm.2023.101315
12. Bassani-Sternberg M, Barnea E, Beer I, Avivi I, Katz T, Admon A (2010) Soluble plasma HLA peptidome as a potential source for cancer biomarkers. *Proc Natl Acad Sci U S A* 107(44):18769–18776. 10.1073/pnas.1008501107
  13. Ritz D, Gloger A, Weide B, Garbe C, Neri D, Fugmann T (2016) High-sensitivity HLA class I peptidome analysis enables a precise definition of peptide motifs and the identification of peptides from cell lines and patients' sera. *Proteomics* 16(10):1570–1580. 10.1002/pmic.201500445
  14. Shraibman B, Barnea E, Kadosh DM, Haimovich Y, Slobodin G, Rosner I et al (2019) Identification of Tumor Antigens Among the HLA Peptidomes of Glioblastoma Tumors and Plasma. *Mol Cell Proteom* 18(6):1255–1268. 10.1074/mcp.RA119.001524
  15. Kessler AL, Bruno MJ, Buschow SI (2020) The Potential of Soluble Human Leukocyte Antigen Molecules for Early Cancer Detection and Therapeutic Vaccine Design. *Vaccines (Basel)* 8(4). 10.3390/vaccines8040775
  16. Khazan-Kost S, Cafri G, Melamed Kadosh D, Mooshayef N, Chatterji S, Dominissini D et al (2022) Soluble HLA peptidome of pleural effusions is a valuable source for tumor antigens. *J Immunother Cancer* 10(5). 10.1136/jitc-2021-003733
  17. de Beijer MTA, Bezstarosti K, Luijten R, Doff WAS, Boor PPC, Pieterman RFA et al (2022) Immunopeptidome of hepatocytes isolated from patients with HBV infection and hepatocellular carcinoma. *JHEP Rep* 4(11):100576. 10.1016/j.jhepr.2022.100576
  18. Kessler AL, Pieterman RFA, Doff WAS, Bezstarosti K, Bouzid R, Klarenaar K et al (2025) HLA I immunopeptidome of synthetic long peptide pulsed human dendritic cells for therapeutic vaccine design. *NPJ Vaccines* 10(1):12. 10.1038/s41541-025-01069-1
  19. Bouzid R, de Beijer MTA, Luijten RJ, Bezstarosti K, Kessler AL, Bruno MJ et al (2021) Empirical Evaluation of the Use of Computational HLA Binding as an Early Filter to the Mass Spectrometry-Based Epitope Discovery Workflow. *Cancers (Basel)* 13(10). 10.3390/cancers13102307
  20. Reynisson B, Alvarez B, Paul S, Peters B, Nielsen M (2020) NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. *Nucleic Acids Res* 48(W1):W449–W54. 10.1093/nar/gkaa379
  21. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T et al (2009) CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. *Nucleic Acids Res* 37(Database issue):D816–D819. 10.1093/nar/gkn673
  22. Lu YC, Yao X, Crystal JS, Li YF, El-Gamil M, Gross C et al (2014) Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin Cancer Res* 20(13):3401–3410. 10.1158/1078-0432.CCR-14-0433
  23. Wang C, Gu Y, Zhang K, Xie K, Zhu M, Dai N et al (2016) Systematic identification of genes with a cancer-testis expression pattern in 19 cancer types. *Nat Commun* 7:10499. 10.1038/ncomms10499

24. Noordam L, Ge Z, Ozturk H, Doukas M, Mancham S, Boor PPC et al (2021) Expression of Cancer Testis Antigens in Tumor-Adjacent Normal Liver Is Associated with Post-Resection Recurrence of Hepatocellular Carcinoma. *Cancers (Basel)* 13(10). 10.3390/cancers13102499
25. Zhang G, Chitkushev L, Olsen LR, Keskin DB, Brusica V (2021) TANTIGEN 2.0: a knowledge base of tumor T cell antigens and epitopes. *BMC Bioinformatics* 22(Suppl 8):40. 10.1186/s12859-021-03962-7
26. Yi X, Liao Y, Wen B, Li K, Dou Y, Savage SR et al (2021) caAtlas: An immunopeptidome atlas of human cancer. *iScience* 24(10):103107. 10.1016/j.isci.2021.103107
27. Carter JA, Matta B, Battaglia J, Somerville C, Harris BD, LaPan M et al (2023) Identification of pan-cancer/testis genes and validation of therapeutic targeting in triple-negative breast cancer: Lin28a-based and Siglece-based vaccination induces antitumor immunity and inhibits metastasis. *J Immunother Cancer* 11(12). 10.1136/jitc-2023-007935
28. Marijt KA, Blijleven L, Verdegaal EME, Kester MG, Kowalewski DJ, Rammensee HG et al (2018) Identification of non-mutated neoantigens presented by TAP-deficient tumors. *J Exp Med* 215(9):2325–2337. 10.1084/jem.20180577
29. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57. 10.1038/nprot.2008.211
30. Sherman BT, Hao M, Qiu J, Jiao X, Baseler MW, Lane HC et al (2022) DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Res* 50(W1):W216–W21. 10.1093/nar/gkac194
31. UniProt C (2025) UniProt: the Universal Protein Knowledgebase in 2025. *Nucleic Acids Res* 53(D1):D609–D17. 10.1093/nar/gkae1010
32. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R et al (2023) The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res* 51(D1):D638–D46. 10.1093/nar/gkac1000
33. Ziol M, Sutton A, Calderaro J, Barget N, Aout M, Leroy V et al (2013) ESM-1 expression in stromal cells is predictive of recurrence after radiofrequency ablation in early hepatocellular carcinoma. *J Hepatol* 59(6):1264–1270. 10.1016/j.jhep.2013.07.030
34. Calderaro J, Meunier L, Nguyen CT, Boubaya M, Caruso S, Luciani A et al (2019) ESM1 as a Marker of Macrotrabecular-Massive Hepatocellular Carcinoma. *Clin Cancer Res* 25(19):5859–5865. 10.1158/1078-0432.CCR-19-0859
35. Takami H, Kanda M, Oya H, Hibino S, Sugimoto H, Suenaga M et al (2013) Evaluation of MAGE-D4 expression in hepatocellular carcinoma in Japanese patients. *J Surg Oncol* 108(8):557–562. 10.1002/jso.23440
36. Park SM, Choi EY, Bae M, Kim S, Park JB, Yoo H et al (2016) Histone variant H3F3A promotes lung cancer cell migration through intronic regulation. *Nat Commun* 7:12914. 10.1038/ncomms12914
37. Campani C, Imbeaud S, Couchy G, Ziol M, Hirsch TZ, Rebouissou S et al (2024) Circulating tumour DNA in patients with hepatocellular carcinoma across tumour stages and treatments. *Gut*

73(11):1870–1882. 10.1136/gutjnl-2024-331956

38. Tanuwidjaya E, Lim Kam Sian TCC, Steele JR, Goncalves G, Woodhouse IB, Chang J et al (2025) SAPrlm 2.0: a semi-automated protocol for mid-throughput soluble HLA immunopeptidomics. *Front Immunol* 16:1546629. 10.3389/fimmu.2025.1546629
39. Loffler MW, Mohr C, Bichmann L, Freudenmann LK, Walzer M, Schroeder CM et al (2019) Multi-omics discovery of exome-derived neoantigens in hepatocellular carcinoma. *Genome Med* 11(1):28. 10.1186/s13073-019-0636-8
40. Dong LQ, Peng LH, Ma LJ, Liu DB, Zhang S, Luo SZ et al (2020) Heterogeneous immunogenomic features and distinct escape mechanisms in multifocal hepatocellular carcinoma. *J Hepatol* 72(5):896–908. 10.1016/j.jhep.2019.12.014

## Figures

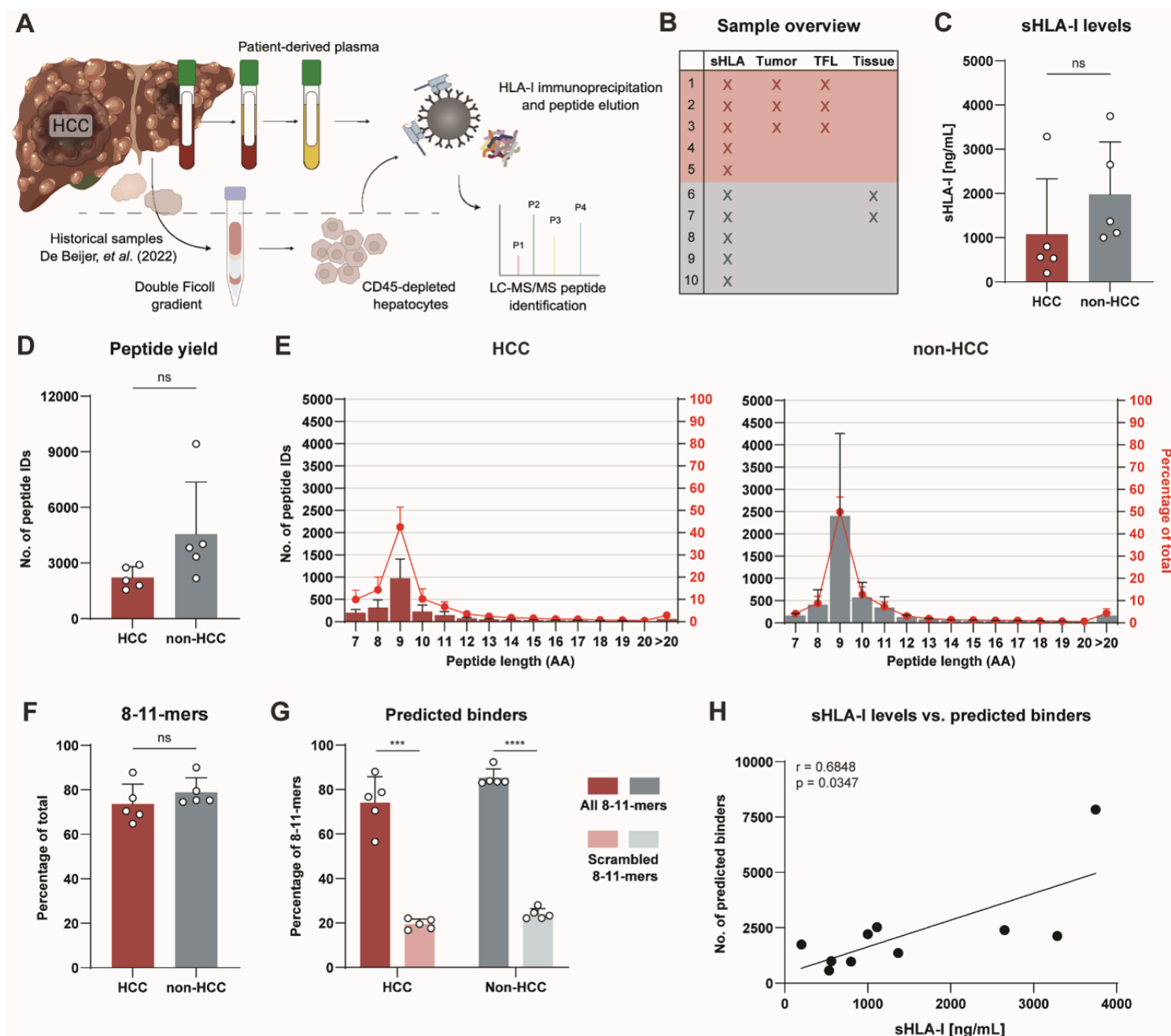


**Figure 1**

**Peptides eluted from plasma sHLA-I can be used as a liquid biopsy.** (A) Platelet-deprived plasma samples from HCC and non-HCC patients were subjected to HLA-I immunopurification followed by LC-MS/MS analysis. (B) Overview of paired historical tissue sample immunopeptidomes (tumor/TFL) previously obtained by De Beijer, *et al.*<sup>17</sup> of three HCC (red) and two non-HCC (blue) patients in the study cohort. From additionally two HCC and three non-HCC patients only plasma samples were analyzed. (C) The levels of sHLA-I in plasma (ng/mL) as read-out by HLA-I ELISA analysis. (D) The total number of unique peptide identifications per sHLA-I sample. (E) Length distribution analysis of unique peptides identified in HCC (red) and non-HCC (blue) sHLA-I immunopeptidomes. The right y-axis shows the relative proportion (%) of each n-mer to the total immunopeptidome. (F) The percentage of 8-11-mers in each sHLA dataset (HCC, red; non-HCC; blue). (C,D,F) Unpaired t-test, ns = non-significant. (G) The

percentage of predicted binders to patient-expressed HLA-types (**Table S3**; NetMHCpan 4.1, <2% binding rank) within the pool of 8-11-mers per sHLA sample (darker bars), or within a pool of sample-matched, scrambled 8-11-mers as a negative control (lighter bars). Paired t-test, \*\*\* = p-value<0.005, \*\*\*\* = p-value<0.001. **(H)** Correlation as determined with a Spearman correlation analysis between sHLA-I levels and the number of predicted binders (r value = 0.6848, p-value=0.0347).

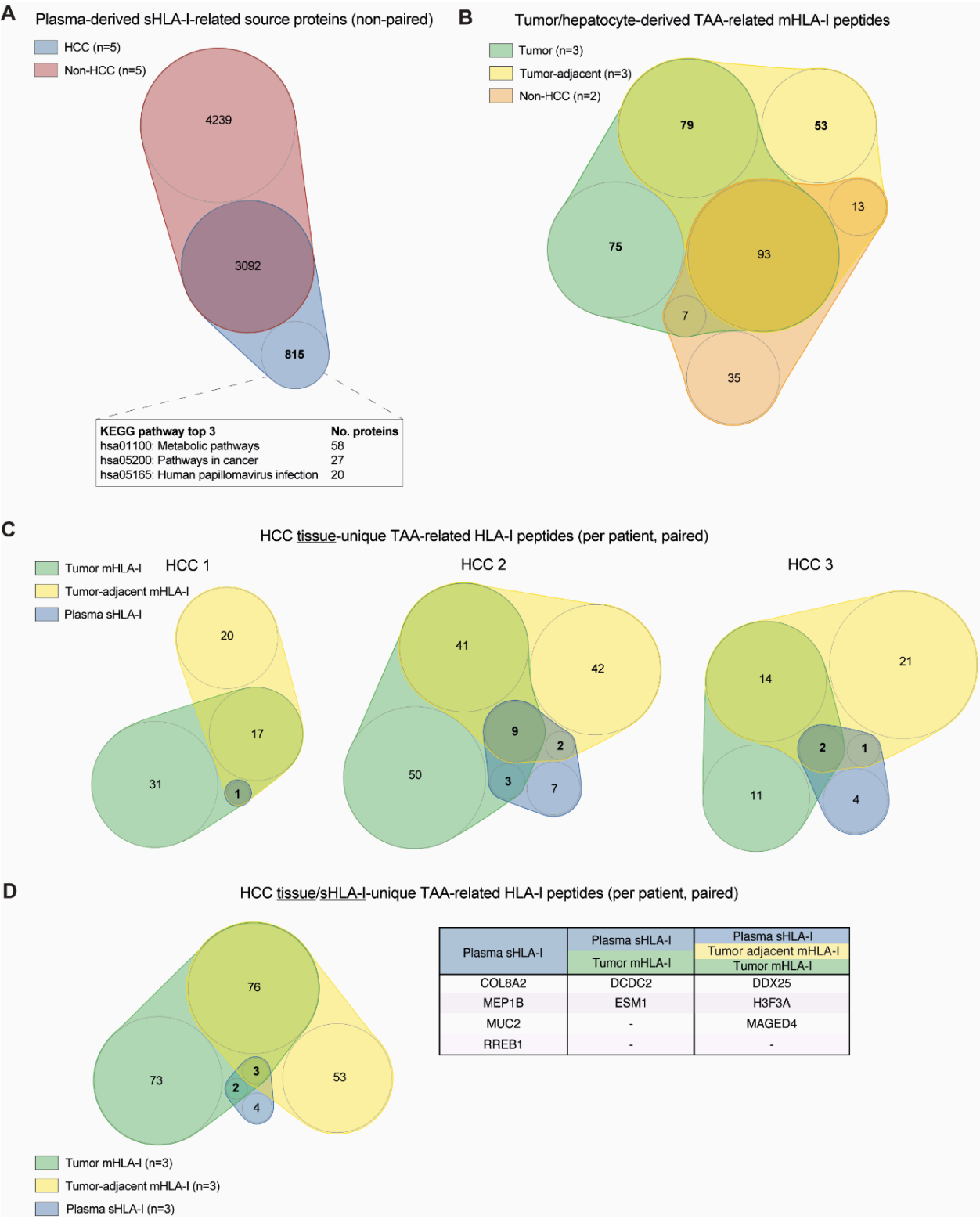


Figure 2

**Overlap in antigens carried by plasma-derived sHLA-I and tumor/hepatocyte-derived mHLA-I of HCC patients.** HLA-I peptidomes derived from isolated tissue hepatocytes/tumor cells (mHLA-I; tumor, TFL/tumor-adjacent, non-HCC) and plasma (sHLA-I) were analyzed for co-occurrence of HLA-peptide source-proteins (**Sup. Data 1**). **(A-D)** Euler diagram(s) depicting the: **(A)** source proteins associated with identified HLA binders from HCC and non-HCC patient-derived plasma in a non-paired comparison. Best covered KEGG pathways by HCC patient-unique sHLA-peptide source proteins are indicated. **(B)** Tumor/hepatocyte mHLA-I peptides associated with TAAs for tumor, tumor-adjacent, and non-HCC tissues in a non-paired comparison. **(C)** HCC tissue-unique TAA proteome from tumor/tumor-adjacent tissues and plasma in a paired comparison for HCC 1, 2, and 3. **(D)** HCC patient-unique tissue and sHLA TAA-derived mHLA-I proteome compared to the HCC-patient-restricted sHLA-I TAA proteome for the 3 paired sample-sets together. Circle size represents the absolute number of source proteins identified. Shared TAAs are depicted in the table.

## Supplementary Files

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