

Original Research Article

Extracellular vesicles derived microRNAs as non-invasive markers of liver fibrosis in chronically infected HCV patients: a pilot study



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ABSTRACT

Extracellular vesicles (EVs) are an increasingly promising tool for liquid biopsy in liver diseases. Hepatitis C Virus (HCV) infection, alone or together with Human Immunodeficiency Virus (HIV) infection significantly impacts on the microRNA (miRNA) EVs content resembling chronic hepatitis C (CHC) progression. The objective of the study was to delve into the intricate EVs-miRNA profiles in CHC patients with different liver fibrosis stages, aiming to pinpoint non-invasive markers capable of distinguishing significant fibrosis.

Plasma EV-miRNAs from 50 CHC patients (HCV+ and HCV+/HIV+) stratified in no significant ($F < 2$) and significant ($F \geq 2$) fibrosis, were massively sequenced. General linear models (GLM) were used to identify significantly differential expressed (SDE) miRNAs according to liver fibrosis stages ($F \geq 2$ and $F < 2$). Dysregulated biological pathways were subsequently analyzed *in silico* for the following groups: i) all patients; ii) HCV+; and iii) HCV+/HIV+. Multiple-ordered logistic regression analysis was performed to develop a score to identify $F \geq 2$ cases. The diagnostic potential of both the SDE miRNAs and the developed score was assessed using ROC curve analysis.

Abbreviations: ALP, alkaline phosphatase; APRI, AST to Platelet Ratio Index; ART, antiretroviral treatment; AUROC, area under ROC; Cryo-EM, Cryo-electron microscopy; CHC, chronic hepatitis C; ECM, extracellular matrix; EVs, extracellular vesicles; FC, fold change; FDR, false discovery rate; FIB-4, fibrosis-4 score; GLM, generalized linear models; GPT, glutamic pyruvic transaminase; GOT, glutamic oxaloacetic transaminase; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; NTA, nanoparticle tracking analysis; PLSDA, Partial least squares discriminant analysis; ROC, receiver operating characteristic curves; SDE, significant differentially expressed; Se, sensibility; Sp, specificity; TEM, transmission electron microscopy.

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With respect to all CHC patients, two SDE miRNAs (hsa-miR-122-5p and hsa-miR-92a-3p) were identified which regulate genes related to cytoskeleton organization. Regarding their diagnostic performance to discriminate $F \geq 2$, both miRNAs individually demonstrated acceptable diagnostic values. However, their combined use in a new score enhanced their diagnostic performance (AUROC = 0.833).

In the HCV+ subgroup, 8 SDE miRNAs (hsa-miR-122-5p, hsa-miR-320c, hsa-miR-3615, hsa-miR-320a-3p, hsa-miR-374b-5p, hsa-let-7a-3p, hsa-miR-199a-5p, hsa-miR-142-5p), which regulate macrophage activity and cell growth/death regulation, were recognized. Among them, hsa-miR-3615 displayed the highest diagnostic performance to discriminate $F \geq 2$ (AUROC = 0.936).

With respect to HCV+/HIV+, 18 SDE miRNAs (hsa-miR-4508, hsa-miR-122-5p, hsa-miR-451a, hsa-miR-1290, hsa-miR-1246, hsa-miR-107, hsa-miR-15b-5p, hsa-miR-194-5p, hsa-miR-22-5p, hsa-miR-20b-5p, hsa-miR-142-5p, hsa-miR-328-3p, hsa-miR-335-3p, hsa-miR-125a-5p, hsa-miR-423-3p, hsa-let-7d-3p, hsa-miR-128-3p, hsa-miR-10a-5p) were recognized that regulate RNA silencing processes. In this case, hsa-miR-423-3p and hsa-miR-128-3p showed outstanding diagnostic performances (AUROC > 0.900).

Distinct EVs-miRNA profiles were identified in patients with varying liver fibrosis stages, both in the overall CHC cohort and within HCV+ and HCV+/HIV+ subgroups. These specific miRNA signatures would allow the elucidation of potential mechanisms involved in clinical evolution and identification of specific biomarkers of unfavorable progression, plausible to be used in a diagnostic panel. Furthermore, the developed score demonstrates the ability to discriminate within the CHC group those individuals with significant fibrosis regardless of their HIV infection status.

1. Introduction

Hepatitis C virus (HCV) infection is a persistent public health challenge that still causes chronic hepatitis (CHC) in approximately 70 % of the people exposed to the virus [1]. Its asymptomatic behavior in most patients leads to a lack of early diagnosis and, therefore, a high risk of developing hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) that ultimately makes CHC one of the main causes of liver transplantation worldwide [1]. Moreover, it is well known that human immunodeficiency virus (HIV) infection accelerates HCV-related liver disease anticipating cirrhosis development and death [2]. HCV and HIV coinfection is a common condition since both viruses share transmission routes, thus it is estimated that 6.2 % of the people living with HIV have serological evidence of HCV exposure [1].

The mechanisms leading to liver cell injury, inflammation, steatosis, and fibrosis are under constant revision, but it is widely accepted that both HCV and the immune system play a major role in damage progression [3]. Hepatic fibrosis is an extremely complex process that involves a large number of cellular and molecular events. Different signaling pathways which initiate hepatic fibrosis are triggered in response to inflammatory stimuli caused by hepatocyte damage, leading to the activation of quiescent hepatic stellate cells (HSCs) and an increase in extracellular matrix (ECM) deposition [4].

The progression of liver fibrosis is determined by the imbalance between ECM deposition and degradation [4]. During long-term HCV infection irreversible liver damage occurs, becoming evident when patients efficiently treated with direct-acting antiviral therapies manage to cure the infection but do not ameliorate all immune activation and fibrotic mediators. Therefore, some patients do not achieve liver disease cure, especially those with advanced liver injury [5]. Then, understanding the mechanisms of chronic liver disease and developing noninvasive methods to accurately predict disease stage and progression remains a high priority medical need.

In this context, extracellular vesicles (EVs) recently emerged as intercellular communication mediators since they regulate several physiological and pathological processes [6]. EVs are particles between 50 and 2000 nm diameter, stable in most biological fluids, released to the extracellular space by all kinds of cells. Since they carry a variety of bioactive molecules (ie. protein, lipid, DNA, mRNA and mainly microRNA, among others) known as cargo, they influence the behavior of the target cells. The EV-cargo is modified due to physiological conditions, being specific during different chronic hepatitis stages [7]. In this context, EVs modifications could be linked to long-term liver disease progression, indicating their great potential for the diagnosis and prognosis of liver diseases. Thus, liquid biopsies have emerged as new

diagnostic strategies which could represent a personalized therapeutic opportunity. However, although exosome studies have made great progress in recent years, further efforts are required to explore them in liver diseases in clinical practice [7].

Among EV-cargo, microRNAs (miRNA; 18–22 nt long) have gained great significance since they are fine-tuning regulators of gene expression that act by binding specific mRNAs inducing its degradation or protein translation suppression. This mechanism modulates a variety of biological processes such as organ development, cell proliferation, differentiation and liver fibrosis progression, among others [8]. It has been described that miRNAs have the potential to modulate fibrogenic signaling pathways in HSCs; with both pro- and anti-fibrotic outcomes [9]. As circulating miRNAs reflect the conditions of an abnormal liver, they may function as non-invasive biomarkers for the early detection, prognosis, and evaluation of liver progression. Consequently, they are emerging as a new potential tool in precision medicine for liver disease management. Nevertheless, in order to validate this utility, further studies are required [8,10]. Circulating miRNAs stored in exosomes have sufficient stability which could be a great advantage for this purpose. Different studies showed that the miRNA expression profile within EVs may be unique in different liver diseases such as HCC and CHC and could therefore be used as novel serological biomarkers [6]. In this sense, non-invasive fibrosis biomarkers are especially important given that those currently used in clinical practice have limited diagnostic accuracy in cases with moderate damage. Therefore, we performed a comprehensive evaluation of the plasma-derived EV-miRNA profile of CHC patients (HCV+ and HCV+/HIV+) to assess the specific miRNA signature linked to fibrosis stages in each group of patients. We further elucidate their potential biological function and assessed the diagnostic accuracy of specific miRNAs as liver fibrosis markers.

2. Material and methods

2.1. Patients

A total of 50 CHC patients (21 HCV+ and 29 HCV+/HIV+) were analyzed. HCV+ cases were recruited from Italian's Hospital of Buenos Aires and Ramos Mejía Hospital from Argentina, and HCV+/HIV+ cases from La Paz University Hospital, Infanta Leonor University Hospital, La Princesa University Hospital, Puerta de Hierro and 12 de Octubre Hospital from Spain.

All cases were naive of treatment for HCV. All HCV+/HIV+ co-infected cases received suppressive antiretroviral treatment (ART) for at least one year. They maintained undetectable levels of HIV with CD4⁺ T-cells counts ≥ 500 cells/mm³ since at least one year before sample

collection. Viral load was similar in cases with HCV+ and HCV+/HIV+ [median (25th percentile; 75th percentile): HCV group: 2.5E06 IU/mL (8.8E05; 6.4E06); HCV+/HIV+ group: 4.4E06 IU/mL (6.6E05; 1.1E07); $p = 0.548$].

Patient's exclusion criteria were: individuals below 18 years old, pregnancy, previous HCV treatment, clinical evidence of hepatic decompensation, other cause of liver disease [autoimmune hepatitis (AIH), non-alcoholic fatty liver disease (NAFLD), alcohol-related liver disease (ARLD)], hepatitis B virus co-infection, alcohol consumption (men > 30 g/day; women > 20 g/day), opportunistic infections, and other concomitant diseases such as diabetes, metabolic disorders, cancer history, autoimmune disease, among others.

Clinical and epidemiological data were obtained from medical records. Liver fibrosis was assessed at the time of blood sample collection according to the METAVIR index by histological observation of liver biopsies or transient elastography according to sample availability and medical criteria [11]. Cases were categorized as significant (≥ 2) and no significant (< 2) fibrosis.

Informed written consent was obtained from each patient to ensure privacy and confidentiality. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was reviewed and approved by the Ethics Committees of all institutions: *Comité de Ética de la Investigación* (CEI) of Hospital Italiano de Buenos Aires (Ref: PI-5721), Hospital General de Agudos J M Ramos Mejía (Ref: PI-6172), Institute of Health Carlos III (Ref: CEI PI81_2017-v3), CEI Hospital La Paz (Ref: PI-3005), CEI Hospital La Princesa (Ref: PI15CIII/00031), and CEI Hospital Infanta Leonor (Ref: 09/2016).

2.2. Extracellular vesicle isolation and characterization

Plasma fraction was separated from whole EDTA peripheral blood and stored pre-clarified at -80°C . EVs isolation was performed using the ExoRNeasy Serum/Plasma Midi kit (QIAGEN) according to the manufacturer's instructions with custom modifications. All samples were processed in parallel at the National Center for Microbiology (Madrid, Spain). EV characterization was performed by the gold standard method of cryoelectron microscopy (cryoEM) complemented with nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) as previously described [12].

2.3. High throughput sequencing and bioinformatics analysis

Total RNA enriched in small RNAs was purified from the isolated EVs with the ExoRNeasy Serum/Plasma Midi kit (QIAGEN). Purity and integrity evaluation, small RNA library preparation and sequencing were performed by Bioanalyzer at the Centre for Genomics Regulation (Barcelona, Spain). Small RNA library was constructed with NEBNext Multiplex Small RNA Library Prep Kit (NewEngland BioLabs). Sequencing was performed on the Illumina HiSeq2500 platform, single-end-sequencing, 50 nts (1×50) to get roughly 10 million reads per sample.

Raw data was analyzed using a specific bioinformatic pipeline for the identification of known and novel miRNAs detailed in Supplementary Information (SI) 1. Specific analysis on RNA sequencing revealed that a minimum of 12 individuals per group is required to achieve a minimum 1.5-fold change with an average power exceeding 80 % with an FDR control [13], therefore sample size of the present study is sufficiently large to reliably detect the required 1.5-fold expression differences.

2.4. Statistical analysis

For the descriptive, significant differences between categorical data were calculated using the chi-squared test and Fisher's exact test. Student t-test and Mann-Whitney U test were used to analyze parametric and non-parametric continuous variables among independent groups, respectively.

Differential expression analysis according to fibrosis severity ($F < 2$ vs $F \geq 2$) was analyzed using a generalized linear model (negative binomial distribution family) (bnGLM) adjusted by liver fibrosis and sex. Subsequently significant differentially expressed (SDE) miRNAs were analyzed independently for HCV+ and HCV+/HIV+ subgroups.

Finally, the diagnostic value to assess significant fibrosis of SDE miRNAs for each comparison was evaluated by the receiver operating characteristic curves (ROC) using pROC (v 1.18.2). To identify the adjusted variables to be included in the new fibrosis severity score, a stepwise forward analysis was performed, and the Akaike information criterion (AIC) was used to determine the best-fitted model (SI1).

The miRNA-based target prediction and pathway enrichment analysis of the target genes were performed in detail in SI1.

Statistical software R (v4.2.2) (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

3. Results

3.1. Clinical characteristics of patients

The clinical and epidemiological characteristics of all studied CHC patients organized by fibrosis severity are summarized in Table 1. There were no significant differences in sex, age or body mass index (BMI). Predictably, the $F \geq 2$ group showed significantly higher GOT, GPT, and ALP levels, and significantly lower platelet count ($p = 0.0005$, $p = 0.023$, $p = 0.014$ and $p = 0.0001$, respectively). SI2 shows a more detailed analysis according to disease etiology. HCV+ patients with $F \geq 2$ demonstrated a significantly lower platelet count ($p = 0.016$) while HCV+/HIV+ patients with $F \geq 2$ had significant higher GOT levels ($p =$

Table 1
Clinical and epidemiological characteristics of CHC patients according to fibrosis severity.

Variables	$F < 2$	$F \geq 2$	P value
n	32	18	
HCV monoinfection	10 (31.2 %)	11 (61.1 %)	0.072
Sex (% female)	19 (59.4 %)	6 (33.3 %)	0.140
Age (years)	50 (45.2; 53)	53.5 (46.5; 61.2)	0.225
BMI (kg/m^2)	23.2 (20.6; 26.7)	26.1 (22; 27.3)	0.371
Risk of infection			
Parenteral	16	7	0.382
Sexual	9	1	
Unknown	7	10	
Time of HCV infection (months)	234.0 (57.0; 337.0)	341 (327.2; 373.5)	0.067
Time of HIV infection (months)	262.2 (93.1; 326.4)	345.1 (200; 365.9)	0.146
HCV Genotype			
1	19	17	0.009*
2, 3 and 4	13	1	
HCV viral load (UI/mL)	1.7E6 (3.3E5; 9.1E6)	4.5E6 (1.4E6; 1.3E7)	0.080
TC (mg/dL)	190 (173.3; 202.8)	187 (144; 201)	0.331
GOT (UI/mL)	37 (26.7; 56.2)	69 (51; 119)	0.0005*
GPT (UI/mL)	43.5 (32; 72.5)	70 (50.7; 153.8)	0.023*
GGT (UI/mL)	53.5 (33; 128.8)	68.5 (25.7; 111.5)	0.940
ALP (UI/mL)	86.5 (73; 101)	110 (92; 145)	0.014*
Platelets ($103/\mu\text{L}$)	212.5 (187.7; 230.5)	144 (90.1; 213.7)	0.0001*
CD4 ⁺ T cel (cel/mm^3)	712.8 (528; 1114)	734.5 (579.9; 1112)	>0.999

Statistics: values expressed as cases (%) or median (percentile 25; percentile 75). Abbreviations: HCV: Hepatitis C Virus; HIV: Human Immunodeficiency Virus; BMI: Body Mass Index; TC: total cholesterol; GOT: Glutamic oxaloacetic transaminase; GPT: glutamic pyruvic transaminase; GGT: gamma-glutamyl trans-ferase; ALP: alkaline phosphatase. * Statistically significant differences between $F < 2$ and $F \geq 2$ groups ($p < 0.05$). Note that the $F \geq 2$ group is conformed by 3 F2, 11 F3 and 4 F4 cases.

0.043). In addition, no female cases with significant fibrosis were observed in the HCV+/HIV+ group. Notably, no significant correlations were observed between HCV viral load and biochemical parameters (total cholesterol, transaminases, ALP, platelet count).

3.2. EV characterization

TEM and cryoEM image analysis revealed the presence of spherical particles with a mean diameter of ~130 nm, ranging from 63 to 230 nm. No significant differences in particle size or concentration were observed between $F < 2$ and $F \geq 2$ groups according to NTA analysis (SI3).

3.3. EV-derived miRNA SDE analysis

A total of 536 known miRNAs and 10 putative *de novo* miRNAs were identified. Detailed information about *de novo* miRNAs identification is shown in SI4. Fig. 1a summarizes the SDE miRNAs identified for each comparison. Partial least squares discriminant analysis (PLS-DA) showed differences in the miRNA expression profile between fibrosis severity groups in the whole cohort of CHC patients, and also in each subgroup of infection (Fig. 1b).

3.3.1. miRNA expression profile according to fibrosis stage

Two miRNAs were differentially expressed between $F < 2$ and $F \geq 2$ groups in CHC patients. The well-known hsa-miR-122-5p was significantly upregulated in those cases with $F \geq 2$, meanwhile, hsa-miR-92a-3p was downregulated (Fig. 2a; SI5, 6, and 7). The miRNA-gene interaction analysis demonstrated that these miRNAs putatively regulate the expression of 67 genes (Fig. 2b; SI8) implicated in 3 pathways related to the cytoskeleton organization and in the regulation of cellular component biogenesis (SI9).

Within the HCV+ subgroup, 8 SDE miRNAs were identified; 4 upregulated (hsa-miR-122-5p, hsa-miR-320c, hsa-miR-3615 and hsa-miR-320a-3p), and 4 downregulated (hsa-miR-374b-5p, hsa-let-7a-3p, hsa-miR-199a-5p and hsa-miR-142-5p) in the $F \geq 2$ group (Fig. 2a; SI5, 6, and 7). Together, these SDE miRNAs regulate the expression of 89 genes (Fig. 2b; SI8) significantly linked to 168 pathways mainly related to macrophage activity and the regulation of cell growth/death (SI9).

Regarding the HCV+/HIV+ subgroup, 18 known SDE miRNAs were identified, 11 upregulated (hsa-miR-4508, hsa-miR-122-5p, hsa-miR-451a, hsa-miR-1290, hsa-miR-1246, hsa-miR-107, hsa-miR-15b-5p, hsa-miR-194-5p, hsa-miR-22-5p, hsa-miR-20b-5p and hsa-miR-142-5p) and 7 downregulated (hsa-miR-328-3p, hsa-miR-335-3p, hsa-miR-125a-5p, hsa-miR-423-3p, hsa-let-7d-3p, hsa-miR-128-3p and hsa-miR-10a-5p) in

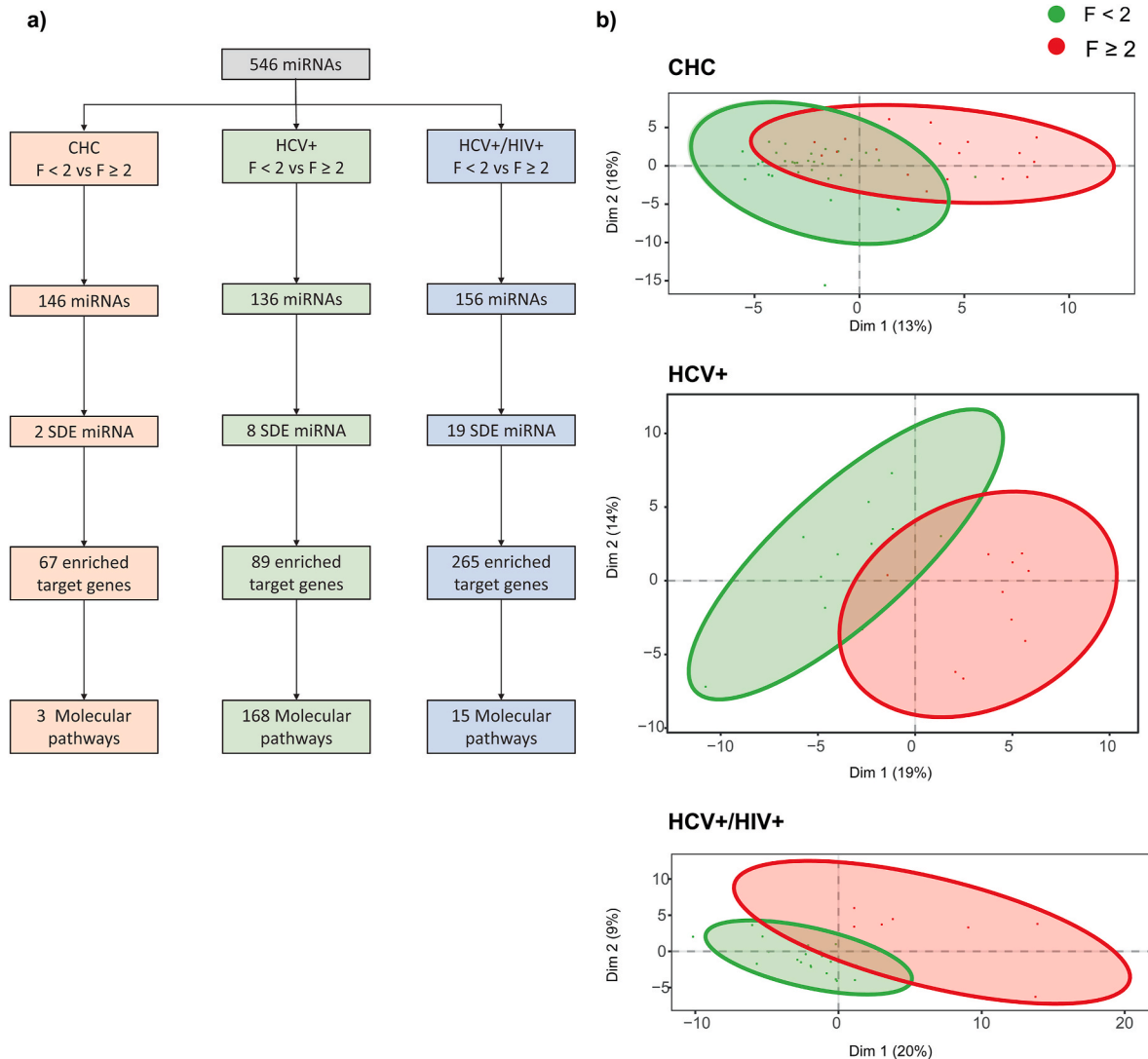


Fig. 1. Analysis of EV-microRNA cargo in each group of patients. **a)** Schematic representation of RNA-seq analysis and results. **b)** Exploratory analysis. Multivariate analysis was performed by supervised partial least squares discriminant analysis (PLS-DA) from normalized log-transformed and scaled miRNA count per million (cpm) data.

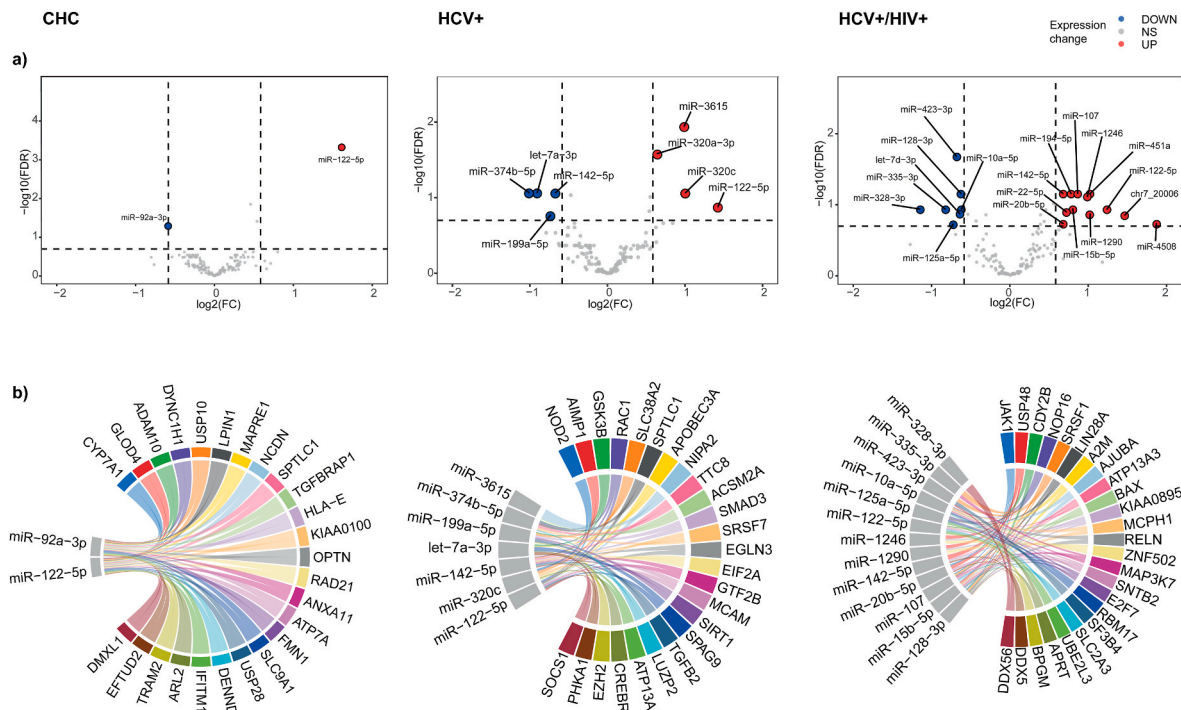


Fig. 2. Analysis of EV-microRNA cargo in the CHC group and in HCV+ and HCV+/HIV+ subgroups. **a)** Volcano plot showing SDE miRNAs in $F \geq 2$ patients. FDR, false discovery rate; $\log_2 \text{FC}$, $\log_2$ fold change. Red dots show miRNAs with a $\text{FDR} \leq 0.2$ and a $\log_2 \text{FC} \geq 0.585$ (equivalent to $\text{FC} \geq 1.5$); blue dots show miRNAs with a $\text{FDR} \leq 0.2$ and a $\log_2 \text{FC} < -0.585$; grey dots show miRNAs without statistical significance between fibrosis severity groups. **b)** Chord diagram of the top 25 target genes. Each targeted gene is represented by one different color, together with their corresponding interaction, while SDE miRNAs are shown on the left.

$F \geq 2$ cases (Fig. 2a; SI5, 6, and 7). These miRNAs regulate the expression of over 265 genes (Fig. 2b; SI8), which were involved in 15 pathways, mostly associated with gene silencing by RNA (SI9). It is worth mentioning that, among the 10 putative *de novo* miRNAs found in this analysis, hsa-chr7_20006, showed to be significantly upregulated in HCV+/HIV+ patients with $F \geq 2$ (SI6).

The hsa-miR-122-5p was commonly identified when comparing groups (SI7) and it was significantly upregulated in $F \geq 2$ cases (SI6).

3.4. SDE miRNAs diagnostic performance

The diagnostic value of the SDE miRNAs was assessed for the purpose of discrimination significant fibrosis (Table 2). Except for 3 miRNAs (hsa-miR-122-5p in HCV+, and hsa-miR-4508 and hsa-chr7_20006 in HCV+/HIV+), all SDE miRNAs showed an AUROC ≥ 0.700 .

In CHC patients, hsa-miR-92a-3p and hsa-miR-122-5p displayed acceptable diagnostic values (AUROC 0.767 and 0.746, respectively) for discriminating $F \geq 2$ cases. The hsa-miR-3615 in the HCV+ subgroup and both hsa-miR-423-3p and hsa-miR-128-3p in the HCV+/HIV+ subgroup showed an outstanding performance (Fig. 3). Interestingly, despite being the only common miRNA upregulated in the three groups, hsa-miR-122-5p showed only excellent performance in the HCV+/HIV+ subgroup (AUROC: 0.831, Table 2).

As described above, considering that in the CHC group, the diagnostic performance of the 2 SDE miRNAs was only acceptable, a multiple logistic regression analysis was performed in order to improve their power by developing a new score to predict significant fibrosis. A training dataset was formed and applied to create this new score, in which both hsa-miR-92a-3p and hsa-miR-122-5p counts were selected as independent variables. After regression analysis, the diagnostic value for the proposed new score was tested and the results are shown in SI10. Interestingly, the proposed new score significantly improved the diagnostic performance of both miRNAs independently, showing an

Table 2
Diagnostic accuracy of the SDE miRNAs to determine $F \geq 2$ cases.

miRNA	AUROC	Cut-off	95 % CI	Sp (%)	Se (%)
CHC					
hsa-miR-92a-3p	0.767	<8680.8	0.63–0.88	68.8	88.9
hsa-miR-122-5p	0.746	>32127.6	0.60–0.86	65.6	77.8
HCV+					
hsa-miR-3615	0.936	>391.7	0.83–1.00	100	81.8
hsa-miR-374b-5p	0.891	<156.3	0.74–1.00	90	81.8
hsa-let-7a-3p	0.873	<332.2	0.71–1.00	90	81.8
hsa-miR-142-5p	0.873	<7074.5	0.70–1.00	90	81.8
hsa-miR-320a-3p	0.855	>13060.2	0.68–1.00	80	90.9
hsa-miR-320c	0.855	>200.6	0.67–1.00	80	81.8
hsa-miR-199a-5p	0.791	<1399.2	0.59–0.99	70	81.8
hsa-miR-122-5p	0.618	>114722.8	0.35–0.89	100	45.4
HCV+/HIV+					
hsa-miR-423-3p	0.967	<2522.1	0.91–1.00	86.4	100
hsa-miR-128-3p	0.916	<2457.2	0.81–1.00	81.8	100
hsa-miR-194-5p	0.896	>548.2	0.77–1.00	81.8	100
hsa-miR-10a-5p	0.883	<10404.1	0.74–1.00	90.9	71.4
hsa-miR-328-3p	0.883	<259.7	0.76–1.00	72.7	100
hsa-miR-22-5p	0.877	>252.8	0.74–1.00	86.4	85.7
hsa-miR-125a-5p	0.870	<3262.3	0.73–1.00	72.7	100
hsa-let-7d-3p	0.864	<513.3	0.72–1.00	63.6	100
hsa-miR-335-3p	0.857	<110.2	0.69–1.00	81.8	85.7
hsa-miR-451a	0.831	>93619.8	0.68–0.98	59.1	100
hsa-miR-122-5p	0.831	>32498.5	0.65–1.00	81.8	85.7
hsa-miR-1246	0.805	>364.2	0.55–1.00	77.3	85.7
hsa-miR-15b-5p	0.779	>233.3	0.57–0.99	90.9	57.1
hsa-miR-107	0.766	>645.1	0.55–0.98	63.6	85.7
hsa-miR-20b-5p	0.727	>282.3	0.50–0.95	86.4	57.1
hsa-miR-142-5p	0.721	>6925.4	0.51–0.93	68.2	85.7
hsa-miR-1290	0.708	>88.4	0.49–0.93	45.4	100
hsa-miR-4508	0.565	>47.4	0.28–0.85	100	28.6
hsa-chr7_20006	0.500	>5193.9	0.19–0.81	95.4	28.6

AUROC: area under the receiver operating characteristic curve, Cut-off expressed as normalized counts, Sp: Specificity, Se: Sensitivity.

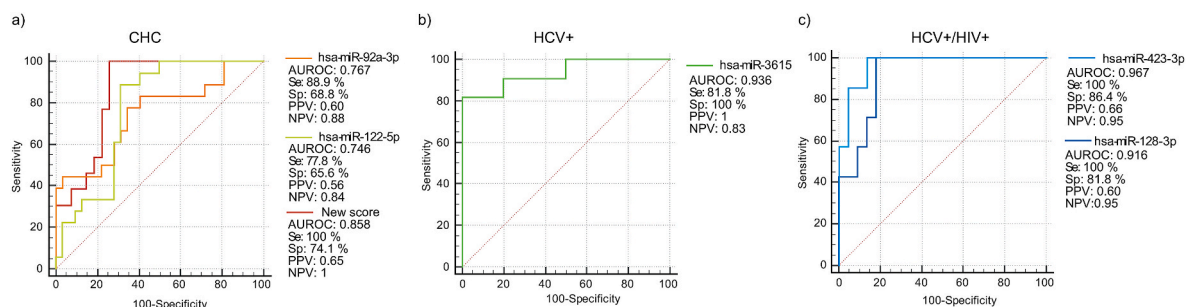


Fig. 3. Receiver operating characteristic curves analysis for discriminating $F \geq 2$ cases. **a)** ROC curves of SDE miRNAs and the proposed new score in CHC patients. **b)** ROC curve of hsa-miR-3615 for HCV+ patients. **c)** ROC curves of hsa-miR-423-3p and hsa-miR-128-3p for HCV+/HIV+ patients. AUROC: area under the receiver operator characteristics curves; Se: Sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predicted value.

excellent AUROC (0.858, Se 74.1 %, Sp 100 %) (Table 2, Fig. 3 and SI10). According to the cutoff value, the majority of cases of the test set were correctly classified (60 %), and, of those misclassified, only one (10 %) was a false negative.

Finally, in order to compare the performance of the proposed score with other indexes widely used in clinical hepatology, APRI and FIB-4 were calculated for each patient in the training set according to data availability (SI11). Although both indexes displayed excellent diagnostic performances (AUROC-APRI = 0.815 and AUROC-FIB-4 = 0.833), similar to the one of the proposed score, the last one correctly classified the majority of cases unclassified by the APRI and FIB-4 scores (11 of 15 and 13 of 14 indeterminate cases, respectively) (SI11).

4. Discussion

This study provides new insight into the EVs-miRNA cargo in HCV chronically infected patients with different liver fibrosis stages. Moreover, we proposed a new score based on involved SDE miRNAs' counts to accurately identify significant fibrosis cases in CHC patients.

MicroRNAs are considered key regulators of gene expression in physiological and pathological conditions and constitute probably the most well studied molecules of EVs cargo. They are emerging as potential biomarkers not only in hepatology, but also in different medical areas [10,14]. In light of these findings, a noteworthy opportunity arises for harnessing miRNAs as non-invasive tools capable of monitoring fibrosis severity throughout the follow-up of patients with CHC. The need for a non-invasive marker to detect liver damage is a current issue given that even though liver biopsy is the gold standard method, it is indeed an invasive and risky procedure that cannot be used to periodically monitor disease progression. Transient elastography is widely accepted and used to measure liver stiffness; however, the equipment is not accessible and available at low-income health centers. Although many non-invasive diagnostic strategies are currently under study or being applied, most of them show low accuracy to discriminate among intermediate stages of fibrosis [15–17]. Therefore, developing noninvasive tests that can accurately predict the initial disease stage and progression over time represents a high priority and growing medical need. Increasing evidence from experimental liver injury models and studies in patients with various liver diseases highlights the potential of EVs as a novel liquid biopsy tool for the liver-related pathologies [18]. This approach may open a new era of precision medicine, allowing for the accurate, non-invasive identification of disease-specific and liver injury-specific signatures. Under pathological conditions, cells increase EV production, leading to a higher number of circulating EVs. Chronic liver disease is associated with systemic inflammation, which raises not only liver-derived EV levels but also EVs from general inflammatory cells [19,20]. Therefore, peripheral miRNA profiles reflect not only liver contributions but also those from cells involved in disease pathogenesis. While liver fibrosis is a local process, systemic factors influence fibrogenesis, making the content of EVs promising biomarkers for liver

injury.

Our results pointed out that patients with CHC present a differential profile of plasma derived EVs-miRNAs according to their liver fibrosis status. In addition, two specific miRNA profiles that correlate with fibrosis stages were also found for HCV+ and HCV+/HIV+ subgroups. These findings could ultimately suggest that different mechanisms are being regulated in each infectious situation. It is known that altered miRNA expression patterns would compromise the progression of fibrosis by potentially targeting genes encoding for ECM proteins as well as proteins involved in inflammatory processes [9,21]. This fact was evident in the CHC group since a restricted number of miRNAs (hsa-miR-122-5p and hsa-miR-92a-3p) related to the cytoskeleton organization and cellular component biogenesis regulation were differentially expressed between $F < 2$ and $F \geq 2$ groups. In turn, significant fibrosis in the context of HCV monoinfection displayed an EVs-miRNA fingerprint characterized by the deregulation of 8 miRNAs, 4 upregulated and 4 downregulated, in $F \geq 2$ cases. Interestingly, among their top 25 target genes, some of them (*NOD2*, *AIMP1*, *GSK3B*, *APOBEC3A*, *TGFB2* and *SOC1*) are related to inflammation and/or immune response. It is worth mentioning that the SDE miRNAs regulate molecular pathways which are involved in macrophage cytokine production and macrophage apoptotic processes, as well as cell autophagy/growth, cell adhesion, cell-matrix adhesion, and fat cell differentiation pathways. These observations are particularly significant given the role of cell growth/death as HSCs activator during fibrogenesis, the importance of cellular-matrix and cell-cell interaction in the ECM deposition, and the particular role by both hepatic (Kupffer cells) and peripheral monocyte/macrophages in HCV pathogenesis [22,23]. It is well known that during the chronic stage of HCV infection, Kupffer cells have a dual role: they contribute to fibrosis by activating HSCs and producing profibrotic cytokines such as TGF- β and PDGF; however, they also express matrix metalloproteases (*MMP-9*, -12, etc) that promote the ECM degradation and favor fibrosis resolution [23]. In HCV/HIV coinfection, the EVs-miRNA profile showed the highest number of SDE miRNAs, including some known for regulating HSC activation, such as hsa-miR-122-5p, hsa-miR-15b-5p, hsa-miR-194-5p and hsa-miR-335 [9, 24,25]. Note that the *in silico* analysis of the experimentally validated miRNA-target interactions revealed that while some SDE miRNAs regulate genes related to inflammation and ECM deposition (*JAK1*, *A2M*, *RELN*), most of them are involved in RNA-mediated gene silencing processes (i.e. *LIN28A*, *AJUBA*, *RBM17*, *SF3B4*, *DDX5*, *DDX56*). This suggests that unlike the HCV cohort, SDE miRNAs in the coinfection context may be involved in less specific processes, reflecting the complex interplay between both viral infections.

Several studies have described specific miRNA profiles in chronic liver disease of various etiologies (autoimmune hepatitis (AIH), non-alcoholic fatty liver disease (NAFLD), alcohol-related liver disease (ARLD), among others). Most of them have identified the hsa-miR-122 as the only miRNA consistently altered [26]. In line with this, the hsa-miR-122-5p was the only common miRNA in all our comparisons,

showing upregulation in $F \geq 2$ cases. Remarkably, this is the most abundant miRNA in the liver (comprises 70 % of the total miRNAs) and regulates metabolic pathways, including cholesterol biosynthesis, fatty acid synthesis, and oxidation [27]. Moreover, hsa-miR-122-5p is a crucial host factor for HCV replication since it facilitates viral genome translation [28]. Upregulation of hsa-miR-122-5p has also been observed in cells, like macrophages, infected with HIV-1 although its connection with HIV-1 genome is not yet clear [29,30]. Elevated serum miR-122 levels have consistently been reported in patients with liver diseases compared to healthy controls [31,32]. Importantly, miR-122 shows a strong positive correlation with alanine aminotransferase (ALT), a widely recognized marker of liver function [31,32]. Distinct serum miR-122 profiles have also been observed in patients with hepatitis B and HCC, emphasizing its potential to distinguish between closely related liver conditions [33,34]. Therefore, serum hsa-miR-122-5p has been directly linked to liver damage, suggesting its utility as a novel biomarker for various liver pathologies; however, the role and clinical relevance of plasma derived-EV hsa-miR-122-5p remain poorly understood [35]. On the other hand, hsa-miR-92a-3p belongs to the hsa-miR-17-92 cluster, and it has been described as a biomarker for many human cancers [36]. Particularly in HCC, miR-92a-3p mediates malignant progression by activating the PI3K/AKT/mTOR signaling pathway [37]. Our results showed its downregulation in significant fibrosis, which has been also described in HCC cells, promoting the up-regulation of cancer-associated pathways [38]. Additionally, hsa-miR-92a-3p as part of the content in EVs has also been identified as a predictor of primary and metastatic colorectal cancer [39]. Therefore, the lower count of this miRNA detected in CHC patients with significant fibrosis may indicate a higher risk of developing HCC in the future.

Interestingly, in the CHC hsa-miR-122-5p and hsa-miR-92a-3p independently displayed moderate accuracy to discriminate $F \geq 2$ cases; however, combining both miRNAs in the new score improved their individual discriminative abilities. While the performance of the new score did not significantly surpass those of the widely used APRI and FIB-4, it did correctly classify approximately 73 % and 93 % of the APRI and FIB-4 indeterminate cases, respectively (S110 and 11). Hence, if validated in a greater cohort, this new score could complement the classification of these challenging cases in clinical hepatology. Regarding the diagnostic accuracy of miRNAs to discriminate $F \geq 2$ in the HCV+ cohort, 6 out of 8 SDE miRNAs showed excellent performances; in fact, particularly the upregulated hsa-miR-3615 showed an outstanding performance. Interestingly, a significant upregulation of this microRNA was recently described in HCC tissues compared with adjacent non-HCC tissues, but there is no information about its participation in fibrogenesis nor its possible role as a biomarker [40]. In turn, in the HCV+/HIV+ subgroup, 12 out of the 18 SDE known miRNAs showed excellent performances. The outstanding performance of hsa-miR-423-3p and hsa-miR-128-3p observed is particularly interesting given that, although the evidence for these miRNAs in relation to fibrosis severity is scarce, previous studies indicated a possible relationship with the development of HCC, therefore they could be further investigated as potential biomarkers of liver cirrhosis and/or HCC [41,42]. Interestingly, hsa-miR-423-5p has been described as a regulator of apoptotic processes in the liver, mediating hepatocarcinogenesis, and has been identified as a possible diagnostic serum marker of steatohepatitis [41–44]. The hsa-miR-128 has also been associated with NAFLD and NAFLD-related liver damage, as its levels in plasma exosomes are higher compared to control individuals [45].

Several considerations need to be taken into account in order to interpret our results correctly. First, the limited sample size can reduce the statistical power to detect small differences between groups. However, regarding RNA sequencing a minimum of 12 individuals per group is sufficient to reliably detect 1.5-fold expression differences with an average power exceeding 80 % with an FDR control [13]. Considering the elevated costs associated with the method applied, the inclusion of 50 cases, which represents a significant number for studies of this

nature, supports its classification as a pilot study. Nevertheless, larger cohort studies are crucial to validate these findings and further elucidate virus-host interactions and disease progression. Second, while we made efforts to control for clinical and biochemical factors by balancing our populations, HCV+/HIV+ group was unbalanced according to sex due to the lack of enrollment of female cases with $F \geq 2$. Considering the significant impact of sex on the fibrogenesis process and also that there is evidence of different miRNA expression profiles between men and women [46], we included sex as a co-variable in the bnGLM in all comparisons. Additionally, there are other potential confounding factors that could be influencing our results. Although efforts have been made to control these factors, it is challenging to fully eliminate their impact, and the influence of antiretroviral therapy (ART) and other environmental stress cannot be disregarded.

One of the strengths of this study lies in the use of extracellular vesicles since it has been demonstrated that they are a very stable matrix and an increasing number of studies are focusing on miRNA-EVs as targets for potential biomarker development in several diseases. However, the majority of them are focused on cancer [47], but little is known about miRNAs' biomarker potential in infectious diseases. Moreover, this study opens the door to future discussion in the scientific community about the incorporation into the clinical practice of miRNAs as markers of significant fibrosis in the context of HCV infection. The analysis of the SDE miRNAs showed promising results, especially regarding the new score which takes into account the combination of certain miRNAs. After proper validation, miRNAs are a promising tool that might be used as complementary parameters in clinical practice to evaluate disease stage and progression. Once relevant EV-miRNAs have been identified and validated, translation into the clinic will be performed by quantitative real time PCR quantification without the need of additional massive sequencing approach. Furthermore, in the near future, advancements in methodology could make these tools more accessible, potentially improving their integration into standard healthcare settings. Extending this research to other liver diseases in the future would be valuable to determine whether these miRNAs could serve as fibrosis biomarkers regardless of the underlying etiology. In fact, extrapolating these findings to other liver diseases could help to establish a universal biomarker for liver fibrosis diagnosis and assessment, representing a significant advance in hepatology.

5. Conclusions

This study offers key insights into the molecular mechanisms of pathogenesis of HCV infection, as well as the opportunity to define a characteristic EVs-miRNA fingerprint that can be useful in clinical practice as a tool for personalized patient management and treatment. Differences in the EVs-miRNome of patients with different liver fibrosis stages were found in CHC as well as in HCV+ and HCV+/HIV+ subgroups. A combination of specific miRNAs in a single score can discriminate between individuals with significant fibrosis in CHC patients regardless of the HIV infection status.

CRedit authorship contribution statement

Victoria Cairolì: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Daniel Valle-Millares:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Pablo Ryan:** Writing – review & editing, Resources. **Lourdes Dominguez:** Writing – review & editing, Resources. **Luz Martín-Carbonero:** Writing – review & editing, Resources. **Ignacio De los Santos:** Writing – review & editing, Resources. **Elena De Matteo:** Writing – review & editing, Resources. **Beatriz Ameigeiras:** Writing – review & editing, Resources. **Marcela De Sousa:** Writing – review & editing, Resources. **Verónica Briz:** Writing – review & editing, Validation, Funding acquisition. **María V. Preciado:** Writing – review & editing,

Writing – original draft, Validation, Supervision, Funding acquisition, Conceptualization. **Amanda Fernández-Rodríguez**: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Pamela Valva**: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Ethics approval

Each individual signed an informed consent in order to assure privacy and confidentiality. The study was performed in accordance with the Declaration of Helsinki. The protocol study was revised and approved by ethic review boards from each institution involved [Comité de Ética de la Investigación (CEI) of Hospital Italiano de Buenos Aires (Ref: PI-5721), Hospital General de Agudos J M Ramos Mejía (Ref: PI-6172), Institute of Health Carlos III (Ref: CEI PI81_2017-v3), CEI Hospital La Paz (Ref: PI-3005), CEI Hospital La Princesa (Ref: PI15CIII/00031), CEI Hospital Infanta Leonor (Ref: 09/2016)].

Data availability

The datasets supporting the conclusions of this article are available in the ArrayExpress repository (EMBL-EBI) under accession number E-MTAB-11811 (EMBL-EBI; <https://www.ebi.ac.uk/>) and included within the article and its [Supplementary file \(S11 to 11\)](#).

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ncrna.2025.03.004>.

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