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HCV eradication with DAAs differently affects HIV males and females: A whole miRNA sequencing characterization

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ABSTRACT

Gender-specific consequences after HCV eradication are unexplored. MicroRNAs (miRNAs) play a crucial role in the immune response against viral infections. However, few have highlighted miRNA role in sex-biased disease or therapy response. We aim to assess gender differences reflected in the miRNA expression of HIV/HCV-coinfected patients who achieve sustained virological response (SVR) with direct acting antivirals (DAAs). We conducted a prospective study of miRNA expression in PBMCs from 28 chronic HIV/HCV-coinfected patients (HIV/HCV) at baseline and after achieving SVR with DAAs. Sixteen HIV-monoinfected patients (HIV) and 36 healthy controls (HC) were used as controls. Identification of significant differentially expressed (SDE) miRNAs was performed with generalized linear model and mixed GLMs. We also explored putative dysregulated biological pathways. At baseline, the HIV/HCV patients showed differences in the miRNA profile concerning the HIV group (165 and 102 SDE miRNAs for males and females, respectively). Gender-stratified analysis of HIV/HCV group at baseline versus at SVR achievement showed higher differences in males (80 SDE miRNAs) than in females (55 SDE miRNAs). After SVR, HIV/HCV group showed similar values to HIV individuals, especially in females (1 SDE miRNA). However, ten miRNAs in males remained dysregulated, which were mainly involved in cancer, fatty acid, and inflammatory pathways. Taken together, our results show gender-biased dysregulation in the miRNA expression profile of PBMCs after HCV eradication with DAAs. These differences were normalized in females, while miRNA profile and their target-related pathways in males lack of normalization, which may be related to a high-risk of developing liver-related complications.

Abbreviations: AI, atherogenic index; AIP, atherogenic index for plasma; ALP, alkaline phosphatase; BMI, body mass index; C14MC, Chromosome 14 MiRNA Cluster; CHC, Chronic Hepatitis C; CPM, count per million; DAAs, Direct Acting Antivirals; ECM, extracellular matrix; FC, Fold Change; FDR, False Discovery Rate; GGT, gamma-glutamyltransferase; GLM, Generalized Linear Model; GLMM, Generalized Linear Mixed-effect Model; GOT, glutamate oxaloacetate transaminase; GPT, glutamic-pyruvic transaminase; HCV, Hepatitis C Virus; HDL, high density lipoprotein; HIV, Human Immunodeficiency Virus; KEGG, Kyoto Encyclopedia of Genes and Genomes; LCI, lipoprotein combine index; LDL, low density lipoprotein; MiRNAs, microRNAs; PBMCs, Peripheral Blood Mononuclear Cell; PCA, Principal Component Analysis; SDE, Significantly Differential Expressed; SVR, Sustained Virological Response; TC, total cholesterol; TG, triglycerides; TMM, Trimmed Mean of M-values.

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1. Introduction

HIV/HCV-coinfected patients take longer to spontaneously clarify HCV than HCV-monoinfected patients [1], and many other factors such as gender, could be influencing. Several studies postulate that gender may affect the development of chronic hepatitis C (CHC) and the response to antiviral therapy. For instance, females show higher viral clearance rates compared to males, while significant gender differences in HCV related malignancies, such as liver disease progression, have been identified to be higher in males [2]. These differences may be attributed to a stronger immune response against HCV infection in females [3]. However, there is a lack of studies concerning sex-differences within HIV/HCV coinfection.

Some studies have addressed a potential role of microRNAs (miRNAs) in maintaining immunological homeostasis in various biological and pathological processes [4]. These small non-coding RNAs play an essential role in the regulation of gene expression [5], modulating immune response to infectious diseases [6], among others. Thus, host miRNAs may show an antiviral role by repressing viral mRNAs, or can be hijacking to enhance viral replication [7]. Additionally, as a high proportion of miRNAs are encoded in the X chromosome compared with the autosomes, these miRNAs could be related to the pathogenesis of different diseases, between males and females, where females usually show immunological advantages [8]. Cui et al. explored the human sex-biased miRNAs [9], identifying that men miRNAs tend to be clustered in the human genome and shown higher expression tissue specificity and lower disease spectrum width. However, the contribution of miRNAs to gender-specific immunity against viral infections has not been previously explored.

Direct acting antivirals (DAAs) has dramatically improved cure rates of HCV, and has led to fewer side-effects [10]. However, a worsening of liver-related diseases after the sustained virological response (SVR) with DAAs may occurs, such as liver-related tumor development [11], especially in HIV/HCV coinfected patients [12]. The implications of human immunological stress in the process of HCV elimination through DAAs is still unclear. The immune system recovery after DAAs therapy may be different by gender, although no study has addressed gender different responses to date, which may explain potential unknown effects of DAA therapy.

Peripheral blood transcriptome host crucial information on host immune response against pathogens. Additionally, although HCV is predominantly hepatotropic, peripheral blood mononuclear cells (PBMCs) may constitute a critical extrahepatic reservoir for HCV replication [13], where the miRNA profile can suffer dysregulation due to HCV infection [14] or even coinfection with HIV [15], as both viruses co-localized in coinfected patients. Thus, HIV/HCV coinfection leaves a fingerprint in PBMCs where miRNAs are deregulated [15].

Our study assesses whether the HCV eradication with DAAs impact on the miRNA profile of PBMCs, and if the remained deregulation differently impacts on gender. We will analyse the miRNA expression profile changes of HIV/HCV-coinfected patients before (HIV/HCV^{+}b) and after (HIV/HCV^{-}f) achieving SVR by gender, and its normalization compared to an HIV control group.

2. Materials and methods

Multicenter prospective observational study on 44 patients recruited from 2016 to 2017 from three Public Spanish Hospitals from Madrid Autonomous Community: Hospital Universitario La Paz, Hospital Universitario Infanta Leonor and Hospital Universitario La Princesa. Samples were processed at National Center for Microbiology, Institute of Health Carlos III (ISCIII), Madrid (Spain). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the ISCIII review committee (CEI PI 81_2017-V3), and written informed consent was obtained from all patients involved.

2.1. Patient groups

Forty-four HIV-infected patients, all of them of Caucasian origin, were enrolled. All patients were undetectable for HIV-RNA and had CD4+ lymphocytes T-cells counts $\geq$ 500 cell/mm^3 in the last year before sample collection. Groups: 1) HIV/HCV-group (n = 28): HIV-infected patients with chronic hepatitis C (positive PCR for at least six months and positive HCV antibodies), before treatment (HIV/HCV^{+}b) and 48 weeks after SVR with DAAs (HIV/HCV^{-}f); 2) HIV-group (n = 16): HIV-monoinfected patients (negative antibody and PCR for HCV) (Fig. 1); 3) healthy controls (HC) (n = 32) that were never infected with either HCV and HIV (antibody and PCR negative).

Patients satisfied the following exclusion criteria: pregnancy, individuals less than 18 years of age, previously treated for HCV infection, advanced liver fibrosis (F $\geq$ 3), clinical evidence of hepatic decompensation, active drug or alcohol addiction, alcohol-induced liver injury, hepatitis B viral active infection, opportunistic infections, and other concomitant diseases such as diabetes, nephropathies, autoimmune disease, hemochromatosis, cryoglobulinemia, primary biliary cirrhosis, Wilson's disease, α1-antitrypsin deficiency, and neoplasia.

2.2. Clinical records

Clinical and epidemiological data were obtained from medical records, as previously described [16].

2.3. High throughput sequencing of small RNA

Blood samples were collected in EDTA tubes, and PBMCs were isolated within the first 4 h after the extraction. Small RNA library synthesis and sequencing was carried out as previously described [14].

2.4. Data processing pipeline

Bioinformatics analysis was carried out as previously described [14]. Briefly, raw data were filtered for reads with ambiguous base calls. A quality control of the remaining sequences was performed by using FastQC (v0.11.3) [17]. Adapter sequences, as well as low-quality base calls (q < 20), were trimmed with cutadapt (v. 1.18). Adapter trimmed reads were processed with miRDeep2 (v. 0.0.7) [18]. Only the alignments with zero mismatches in the seed region and those that did not map to more than five different loci in the genome were retained. miRBase v21 was used as reference.

2.5. Statistical analysis

For the descriptive study of clinical and demographic data, continuous variables were summarized as median and interquartile range and categorical variables as frequency and percentage. Significant differences between categorical data were calculated using the chi-squared test. The Mann-Whitney *U* test was used to compare continuous variables among independent groups. The McNemar test and the Wilcoxon test were used for categorical and continuous variables to compare dependent groups.

Expression analysis was carried out with the R statistical software v4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) as follows:

2.5.1. Data preprocessing

First, the *filterByExpr* edgeR function [19] were used for each comparison. Trimmed Mean of M-values (TMM) was used to normalize the expression matrix and count per million (CPM) approaches for library size normalization.

2.5.2. Data exploration analysis

We conducted a Principal Component Analysis (PCA) of normalized

count matrix.

2.5.3. Significant differentially expressed (SDE) miRNA analysis

Firstly, we selected the most suitable method for the differential expression analysis in our paired samples approach. A more detailed information is available at Supplementary Material 1.1.

We explored the SDE miRNAs by a generalized linear mixed model (GLMMs) for paired samples (HIV/HCV^{+}b vs. HIV/HCV^{-}f), and a generalized linear model (GLMs) for non-paired comparisons (HIV/HCV^{+}b vs. HIV and HIV/HCV^{-}f vs. HIV). SDE miRNAs were identified by a statistically significant p-value < 0.05 adjusted by false discovery rate (FDR) using Benjamin-Hochberg correction, and a fold change (FC) $>$ 1.5. Model formulas and additional information are shown in the Supplementary Material 1.2.

2.6. MiRNA-based target prediction and pathway enrichment analysis of the target genes

The resulting SDE miRNAs were candidates for further *in silico* target KEGG pathway with the web-based computational tool DIANA miRPathv3 [20], as previously described [14,21]. Enrichment p-values (Fischer's exact test with hypergeometric distribution) were corrected for the false discovery rate (FDR) ($p \leq 0.05$).

3. Results

The raw sequencing data have been deposited in the ArrayExpress repository (EMBL-EBI) under the accession number E-MTAB-10566 for HIV patient́s samples and E-MTAB-8023 for HC individuals.

3.1. Clinical characteristics of each group of patients

The statistical analysis of clinical and epidemiological data for all patients (Table 1) and patients stratified by gender (Supplementary Table 1) showed significant differences in the HIV transmission route. The HIV group were uniquely infected by the sexual route ($p < 0.001$), while patients in the HIV/HCV group by sexual and parenteral infection routes.

Metabolic characteristics of all patients was shown in Table 2. Stratification by gender (Supplementary Table 2) showed differences in the lipid and biochemical parameters. Regarding the lipid profile, both genders at baseline (HIV/HCV^{+}b) presented significantly higher levels of lipoprotein combine index (LCI) compared with themselves after SVR (HIV/HCV^{-}f) ($p_{females} = 0.005$ and $p_{males} = 0.001$). Regarding liver biochemical parameters, glutamate oxaloacetate transaminase (GOT), glutamic-pyruvic transaminase (GPT), and gamma-glutamyltransferase (GGT) were significantly higher in both females and males at baseline (HIV/HCV^{+}b) relative to themselves after SVR (HIV/HCV^{-}f) and to the HIV group. No significant differences were detected after SVR with respect to the HIV group. However, with respect to the HC group, HIV/HCV^{-}f showed significant differences for weight, total cholesterol (TC) and high density lipoprotein (HDL), showing higher values for all.

Within the HIV/HCV group, triglycerides (TG), low-density lipoprotein to high-density lipoprotein ratio (LDL/HDL), atherogenic index (AI), and atherogenic index for plasma (AIP) were significantly higher in males rather than females before starting HCV treatment. After SVR, only the LDL/HDL ratio and the AI were similar among genders. No significant differences between males and females were identified in liver biochemical parameters.

Table 1
Clinical and epidemiological characteristics of patients enrolled in the study regarding HIV and HCV infection.

Patients characteristic	HIV	HIV/HCV	p-value
No. (44)	16	28	
Male sex	8 (50.00%)	17 (60.71%)	0.443
Time of HIV infection (months)	219.83 (89.13; 287.97)	277.26 (166.46; 338.10)	0.055
Transmission route			< 0.001 *
IDU	0 (0%)	13 (46.40%)	
Sexual	13 (81.03%)	9 (32.10%)	
HIV clinical status			0.352
A	10 (62.50%)	12 (42.90%)	
B	3 (18.80%)	7 (25.00%)	
C	2 (12.50%)	7 (25.00%)	
HCV genotype			–
1a	–	8 (28.60%)	
1b	–	7 (25.00%)	
2 and 4	–	2 (7.10%)	
3	–	1 (3.60%)	
IFNL4 (*IL28B*) SNP			0.118
CC (*favourable*)	7 (43.75%)	7 (25.00%)	
CT	9 (56.25%)	15 (53.60%)	
TT	0 (0%)	5 (17.90%)	

Values are expressed as absolute numbers (%) and median (percentile 25; percentile 75). P-values were estimated by Mann–Whitney *U* test for continuous variables and Chi-square test for categorical variables. Abbreviations: HIV: Human Immunodeficiency Virus; HCV: Hepatitis C Virus; IDU: Intravenous Drug Users.

* Statistically significant differences between HIV and HIV/HCV groups ($P < 0.05$).

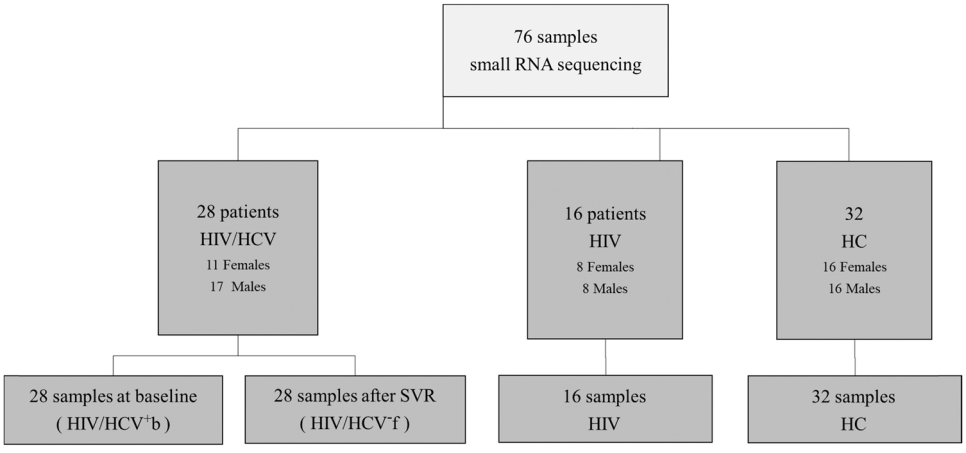


Fig. 1. Organigram of patients involved in this study. Patients were grouped in accordance with their viral status: HIV/HCV, HIV or HC. For each patient in the HIV/HCV group, we took 28 samples at baseline and 28 samples after SVR.

Table 2
Clinical, epidemiological and metabolic characteristics of all group of patients enrolled in the study.

Patient characteristics	HIV	HC	HIV/HCV		p-value[a]	p-value[b]	p-value[c]	p-value[d]
			HIV/HCV^{+}b	HIV/HCV^{-}f				
No. (44)	16	32	28	28				
Clinical Characteristics								
Age	49 (41; 58)	49.00 (43.75–55.00)	50 (44; 54)	51 (45.50; 55)	0.845	< 0.001*	0.816	0.791
CD4 + T cells	788 (716; 1038)	x	720,40 (521.50; 1078.45)	843 (574; 1152)	0.231	0.290	0.812	–
CD4 + T cells (%)	37 (33; 44)	x	33 (26; 43)	37 (30; 39.91)	0.139	0.311	0.294	–
Metabolic Characteristics								
Weight	66 (63.50; 75.20)	75.80 (64.60–88.00)	63 (58; 69)	63.45 (56.40; 76.40)	0.223	0.896	0.247	0.024*
BMI	25.28 (23.41; 26.84)	25.22 (22.78–28.58)	22.58 (21.15; 25.72)	22.52 (20.45; 25.44)	0.077	0.968	0.074	0.083
Glu	90.50 (87; 98.50)	90.50 (80.75–93.75)	89 (86; 100)	89 (84; 98)	0.980	0.069	0.546	0.549
Lipid profile:								
TC	179 (169; 207)	207.50 (186.50–225.25)	186 (166; 206)	180 (155.50; 201)	0.940	0.981	0.442	0.005*
LDL	108 (97; 132)	–	104 (92; 130)	110 (93; 132)	0.642	0.214	0.883	–
HDL	48 (43.50; 54)	67.50 (50.75–78.75)	53 (40; 59)	48.5 (37; 62)	0.725	0.502	0.979	0.033*
TG	114 (75; 174.50)	85.00 (70.00–134.50)	126 (79; 180)	106.5 (69; 154)	0.821	0.048*	0.468	0.596
LDL/HDL	2.35 (2.07; 2.58)	–	2.23 (1.68; 2.85)	2.27 (1.67; 2.94)	0.841	0.530	0.894	–
AI	3.99 (3.37; 4.25)	3.00 (2.58–3.82)	3.83 (3.12; 4.43)	4.16 (3.08; 4.57)	0.702	0.778	0.736	0.351
AIP	0.41 (0.17; 0.53)	0.18 (− 0.12 to 0.87)	0.42 (0.12; 0.55)	0.3 (0.04; 0.61)	0.980	0.088	0.476	0.329
LCI	50.07 (28.36; 97.29)	–	49.03 (23.77; 75.73)	46.70 (22.15; 78.95)	0.782	< 0.001*	0.454	–
Biochemical parameters of liver function:								
GOT	21.5 (19.50; 24)	18.50 (15.00–20.00)	36 (29.50; 44.5)	21.50 (18; 26)	< 0.001*	< 0.001*	0.726	0.234
GPT	21 (15.50; 25.50)	15.00 (12.25–20.75)	45.5 (33.50; 58.50)	18 (11; 21)	< 0.001*	< 0.001*	0.085	0.347
GGT	20.50 (16; 25)	19.00 (12.00–28.00)	45 (33; 107)	18 (13; 25)	< 0.001*	< 0.001*	0.468	0.521
ALP	68 (53.50; 78.50)	–	85 (63; 96)	82 (61; 93)	0.040*	0.317	0.063	–
Albumin	3.80 (1.75; 4.35)	–	4.40 (4.20; 4.50)	4.25 (4.20; 4.40)	0.140	0.705	0.333	–

Values are expressed as absolute numbers (%) and median (percentile 25; percentile 75). p-values were estimated by Mann–Whitney *U* test for continuous variables and Chi-square test for categorical variables: **a** comparison between HIV and HIV/HCV^{+}b groups; **b** comparison between the HIV/HCV group before and after SVR (HIV/HCV^{+}b and HIV/HCV^{-}f); **c** comparison between HIV and HIV/HCV^{-}f group; **d** comparison between HC and HIV/HCV^{-}f group. Abbreviations: BMI, body mass index; Glu, glucose (mg/dL); TC, total cholesterol (mg/dL); LDL, low density lipoprotein (mg/dL); TG, triglycerides (mg/dL); HDL, high density lipoprotein (mg/dL); AI, atherogenic index (TC/HDL); AIP, atherogenic index (log(TG/HDL)); LCI, lipoprotein combine index defined as the ratio of TC * TG * LDL/HDL × 10^{3}; GOT, glutamate oxaloacetate transaminase (mg/dL); GPT, glutamic-pyruvic transaminase (mg/dL); GGT, gamma-glutamyltransferase (mg/dL); ALP, alkaline phosphatase. Age (years); Weight (kg); CD4 + T cells (cels/mm^{3}).* Statistically significant differences ($P < 0.05$).

3.2. MiRNA expression profile analysis and in silico target pathway prediction

On average, 10 million reads per sample were obtained. 2888 known miRNAs were identified, and 1634 remained for subsequent analysis after filtering.

The PCA showed that, despite the distribution of HIV/HCV patients at baseline (HIV/HCV^{+}b) were slightly different after HCV eradication (HIV/HCV^{-}f), and this later group showed a closer distribution to the HIV group. (Supplementary Fig. S1).

Different miRNA expression profile pattern was identified between males and females with the interaction plot (Supplementary Fig. S2), therefore we performed the subsequent analysis with all patients and stratifying by sex. Below we will focus on highlighting the top dysregulated miRNAs due to the high number of differentially expressed miRNAs found in our analysis.

3.2.1. MiRNA profile analysis of HIV/HCV group at baseline

Before treatment, the HIV/HCV group (HIV/HCV^{+}b) showed 154 SDE miRNAs with respect to the HIV group **(**Supplementary Table S3**)**, 70 upregulated and 84 downregulated (Fig. 2**a**). Among them, the hsa-miR-3196 has shown the strongest upregulation in HIV/HCV patients (logFC = 2.57). On the other hand, 37 miRNAs were SDE, being the hsa-miR-144–3p and the hsa-miR-1307–5p (logFC = − 2.14 and − 2.06, respectively) the most downregulated miRNAs (Fig. 3**a**). The target genes regulated by these miRNAs belong to fatty acid metabolism and pathways related to cancer as the main routes targeted by these SDE miRNAs. Some pathways were uniquely represented in this comparison such as cell cycle, hepatitis B, steroid biosynthesis and protein processing endoplasmic reticulum (Fig. 3**b, blue area**).

3.2.1.1. By gender. We explored differences by gender, and we identified a higher SDE miRNAs for males (165), than females (102) (Fig. 2**b)**. 63 miRNAs were commonly deregulated for both genders (Supplementary Tables S4 **and** S5).

Males also showed the higher differences, most of them being uniquely for this gender. Among the downregulated miRNAs, males showed the highest disruption with 56 miRNAs highly downregulated in HIV/HCV males, none of them in common with females. 11 miRNAs shown a deep downregulation (logFC < − 2; FC < 0.25) in HIV/HCV males: hsa-miR-136–5p, hsa-miR-369-3p, hsa-miR-539-3p, hsa-miR-136-3p, hsa-miR-376a-3p, hsa-miR-299-3p, hsa-miR-539-5p, hsa-miR-654-3p, hsa-miR-337-5p, hsa-miR-656-3p, hsa-miR-190a-5p. On the other hand the most upregulated miRNA with the HCV active infection was the has-miR-3196 (logFC = 2.38) (Fig. 4**a-left**). Regarding females, the most upregulated miRNAs, which were also specific for HIV/HCV women, were the hsa-miR-6718-5p, hsa-miR-542-5p and hsa-miR-4767, although none of them achieved a logFC < − 2. On the contrary, the most downregulated miRNAs for females were the hsa-miR-144-3p, hsa-miR-1307-5p, hsa-miR-605-3p and hsa-miR-145-3p (Fig. 4**b-left**).

We analyzed the enriched pathways for each gender, and common pathways were identified (Fig. 4**c-left**). SDE miRNAs were mainly involved in routes related to fatty acid metabolism and viral carcinogenesis in both genders, while pathways related to cancer the cell cycle, and the p53 signaling pathway were unique for females.

3.2.2. MiRNA profile evolution after HCV eradication with DAAs

For all patients in the HIV/HCV group, we identified 82 SDE miRNAs after SVR with DAAs (HIV/HCV^{-}f) with respect to baseline (HIV/HCV^{+}b), 57 were upregulated and 25 were downregulated; among them, the well-known hsa-miR-122-5p which has a relevant role during HCV replication (Fig. 2**a and** Supplementary Table S6). The most upregulated miRNAs after HCV eradication were the hsa-miR-144-3p and the hsa-miR-96-5p (logFC = 3.16 and 2.78 respectively), as they

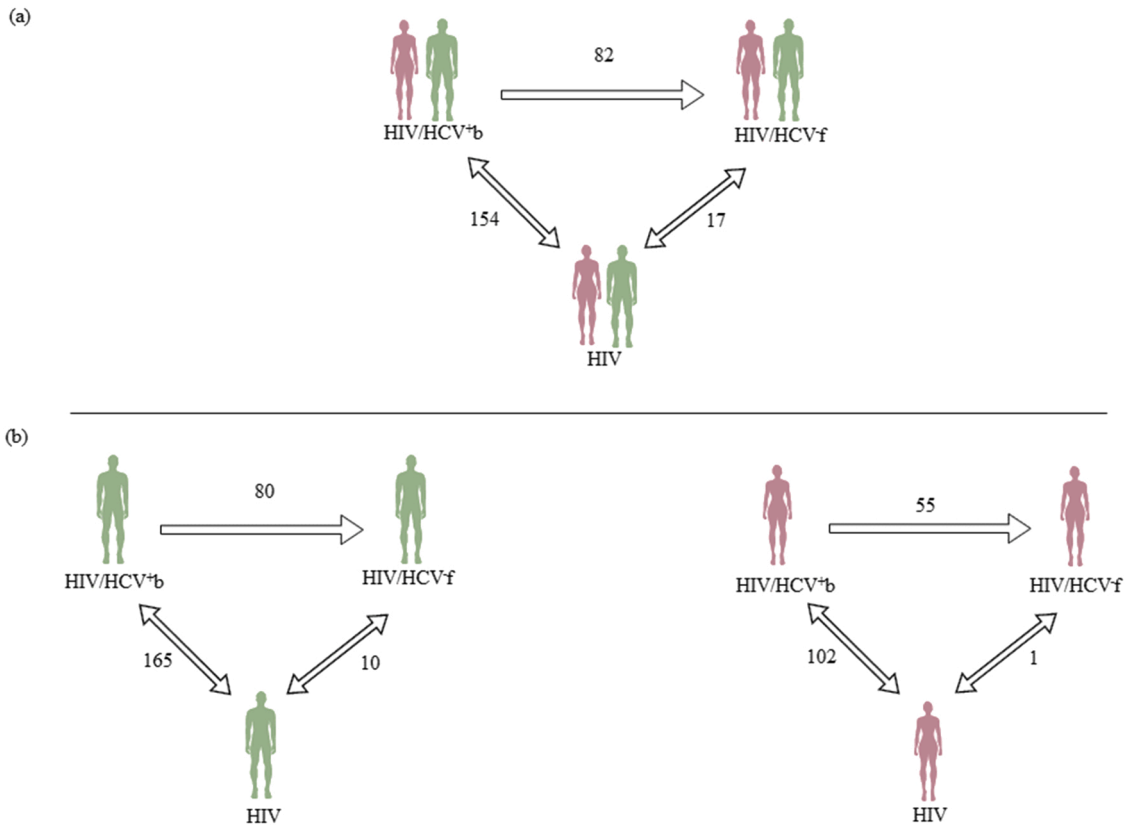


Fig. 2. Overview of SDE miRNAs observed in the differential expression analysis when the miRNA profile from each group of patients involved in this study was compared. The arrow indicates the comparison between two groups (single arrows point to longitudinal comparison and double arrows do so for independent group comparisons), and numbers represents the number of SDE miRNAs identified in each comparison. Females are coloured dark-pink and males in green. (a) Differential expression analysis for all patients; (b) Differential expression stratified by gender.

seem to normalize their expression, similarly to the expression level observed for HIV patients. On the other hand, the most downregulated miRNAs were the hsa-miR-10394-3p (logFC = − 2.68) and the hsa-miR-3196 (logFC = − 2.13) (Fig. 3**a**).

All the 82 SDE miRNAs are targeting genes related to some specific functional routes, highlighting fatty acid metabolism and several cancer-related pathways (Fig. 3**b**).

3.2.2.1. By gender. When analyzing each gender separately, again we observed higher differences between men. After SVR, 80 miRNAs were SDE, while 55 miRNAs were SDE for females (Fig. 2**b**).

Again, the most upregulated miRNAs for males were the hsa-miR-144-3p (logFC = 3.53) and hsa-miR-96-5p (logFC = 2.79) (Fig. 4**a-middle).** A total of 25 SDE miRNAs were downregulated, among them we identified the well-known hsa-miR-122-5p, whose expression was drastically reduced after HCV eradication. We observed similar results for females, as hsa-miR-144-3p, hsa-miR-96-5p, hsa-miR-409-5p, and hsa-miR-1307-5p were the most upregulated miRNAs (logFC > 2). Regarding downregulated miRNAs, after HCV eradication females showed a deep decrease in hsa-miR-3196 and hsa-miR-4510 (Fig. 4**b-middle**).

Among all these SD miRNAs, 31 miRNA were common for both genders, while 49 miRNAs were exclusively dysregulated for males and 24 for females, showing a clear gender bias in the response to HCV eradication with DAAs (Supplementary Tables S7 **and** S8**)**.

The enriched pathway analysis of SDE miRNAs for each gender showed that males and females mainly shared pathways related to fatty acid biosynthesis and cancer (glioma and viral carcinogenesis pathways). The fatty acid metabolism pathway was exclusive for males and the cell cycle, colorectal cancer, and chronic myeloid leukemia, among others, were only found for females (Fig. 4**c-middle**).

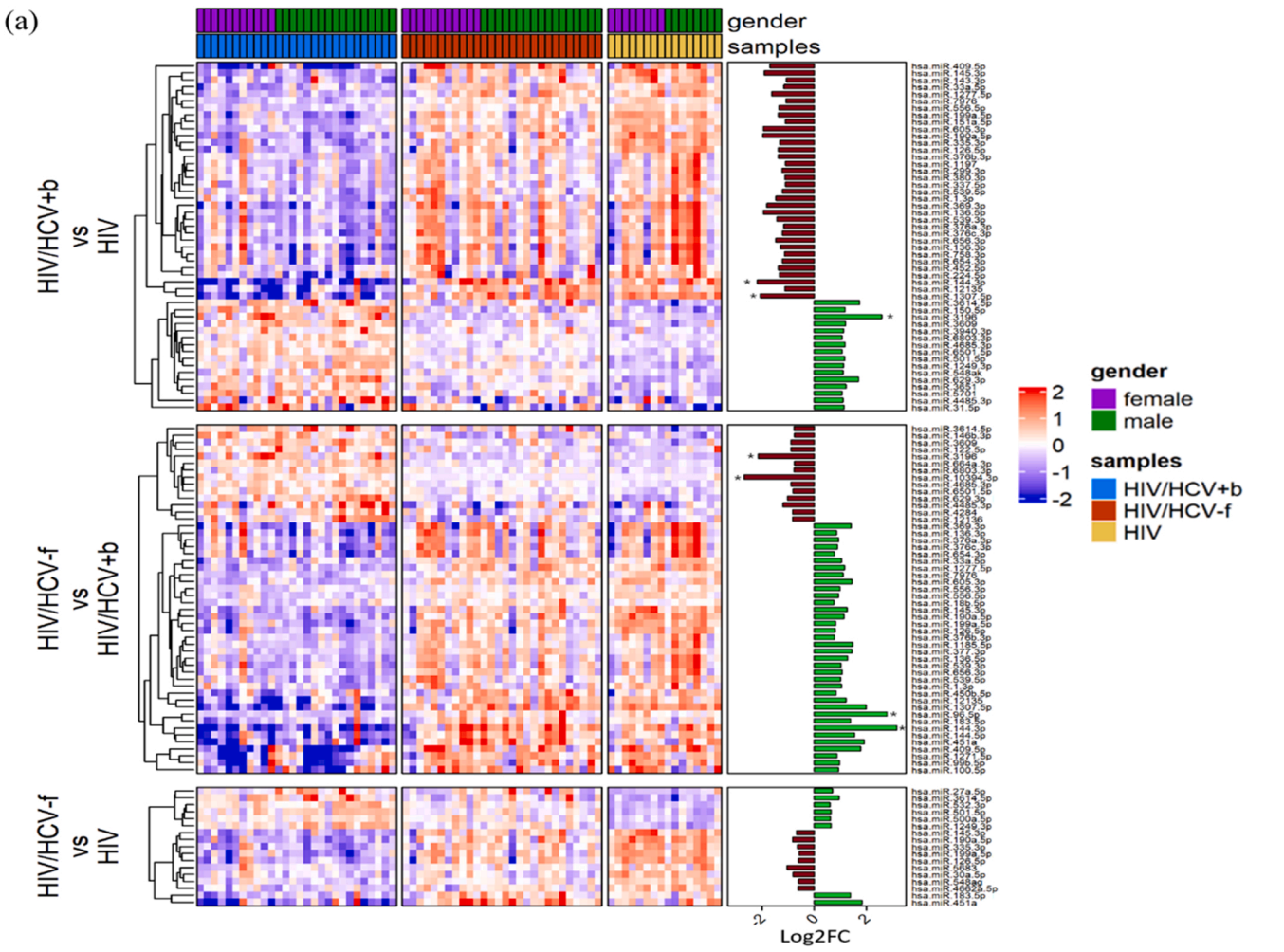
3.2.3. MiRNA normalization after SVR

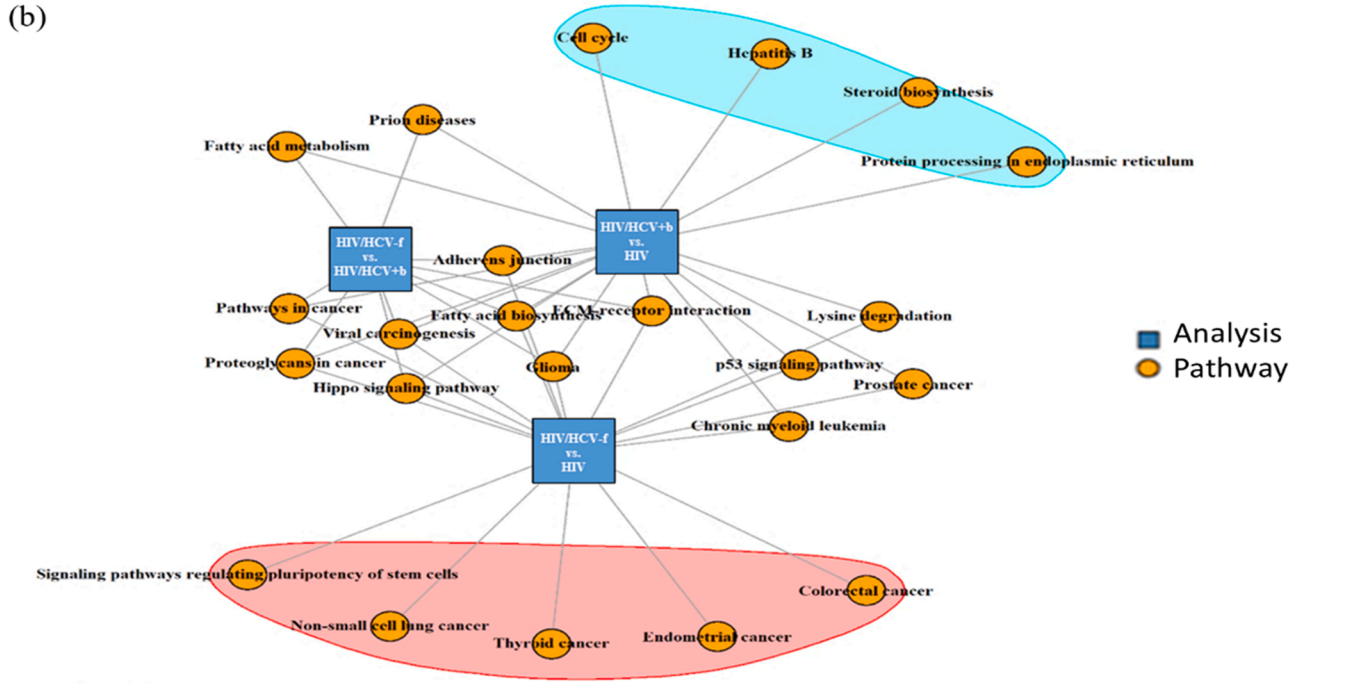
HIV/HCV group after SVR with DAAs (HIV/HCV$^-$f) was compared with the HIV control group in order to evaluate miRNA profile normalization. We observed a drastic reduction in the SDE miRNAs for all patients, as only 17 miRNAs were observed. Eight miRNAs were upregulated and nine downregulated in HIV/HCV$^-$f patients (Fig. 2**a and** Supplementary Table S9). Although significant (FC < 1.5 and FDR < 0.05), the observed differences were lower than the previous comparisons, showing a tendency to normalize the miRNA expression profile after SVR (Fig. 3**a**).

The enriched pathway analysis of the targeted genes pointed to functional routes such as fatty acid biosynthesis, cancer pathways, and adherent junctions, which remained altered after the HCV eradication. Additionally, several cancer-related pathways were predominantly targeted by the SDE miRNAs identified in this analysis, where five of them were uniquely represented in this comparison (Fig. 3**b, red area**).

However, complete normalization was not achieved, as 283 miRNAs were still SDE between HIV/HCV$^-$f and HC (Supplementary Table S10). These miRNAs belong to a broad number of pathways, being most of them related to cancer (Supplementary Table S11).

3.2.3.1. By gender. When patients were stratified by gender, ten SDE miRNAs were identified in males (hsa-miR-548j-5p, hsa-miR-11400, hsa-miR-30a-5p, hsa-miR-654-5p, hsa-miR-539-5p, hsa-miR-654-3p, hsa-miR-411-5p, hsa-miR-1185-1-3p, hsa-miR-381-3p, hsa-miR-299-3p), all of them remained significantly downregulated with respect to HIV control (Fig. 4**a-right**). In females, only the hsa-miR-30a-5p remained dysregulated, which was downregulated in both genders (Fig. 4**c-right and** Supplementary Tables S12 **and** S13). The pathway enrichment analysis showed that the dysregulated miRNAs may alter routes related to fatty acid metabolism and pathways in cancer.





(caption on next page)

Fig. 3. Heatmap and network graph for SDE miRNAs identified in the analysis of all patients. a) Heatmap of the top 50 SDE miRNAs in each comparison (HIV/HCV + b vs. HIV; HIV/HCV-f vs. HIV/HCV-f; HIV/HCV-f vs. HIV). Columns represent samples of patients within each group under study, while rows corresponds to the top 50 SDE miRNAs identified in each analysis for differential expression of all patients. The colour scale at the right illustrates the relative expression level of SDE miRNAs, with red indicating a higher expression level and blue a lower expression level. The vertical bar-plot represent the fold change in the logarithm scale of each SDE miRNA to illustrate the up (green) and down (dark-red) regulation in the HIV/HCV + b group or the HIV/HCV-f group, depending on the comparison. Those SDE miRNAs with strong up (logFC > 2; FC > 4) or down regulation (logFC < − 2; FC < 0.25) has been marked. b) Network interaction map for the in silico target pathway prediction with DIANA mirPathv3. The blue square shape represents the set of SDE miRNAs obtained from the differential expression analysis with the complete cohort of patients: HIV/HCV + b vs. HIV, HIV/HCV-f vs. HIV/HCV + b, and HIV/HCV-f vs. HIV. Orange circles indicates the target KEGG pathway prediction by these miRNAs with FDR< 0.05. The light-blue area highlights the KEGG pathways uniquely altered by SDE miRNAs in HIV/HCV + b vs. HIV, and red area do so for the KEGG pathways exclusively affected by SDE miRNAs in HIV/HCV-f vs. HIV. The interaction network map has been designed and has been plotted with the R packages network v1.16.0 and igraph v1.2.5, respectively.

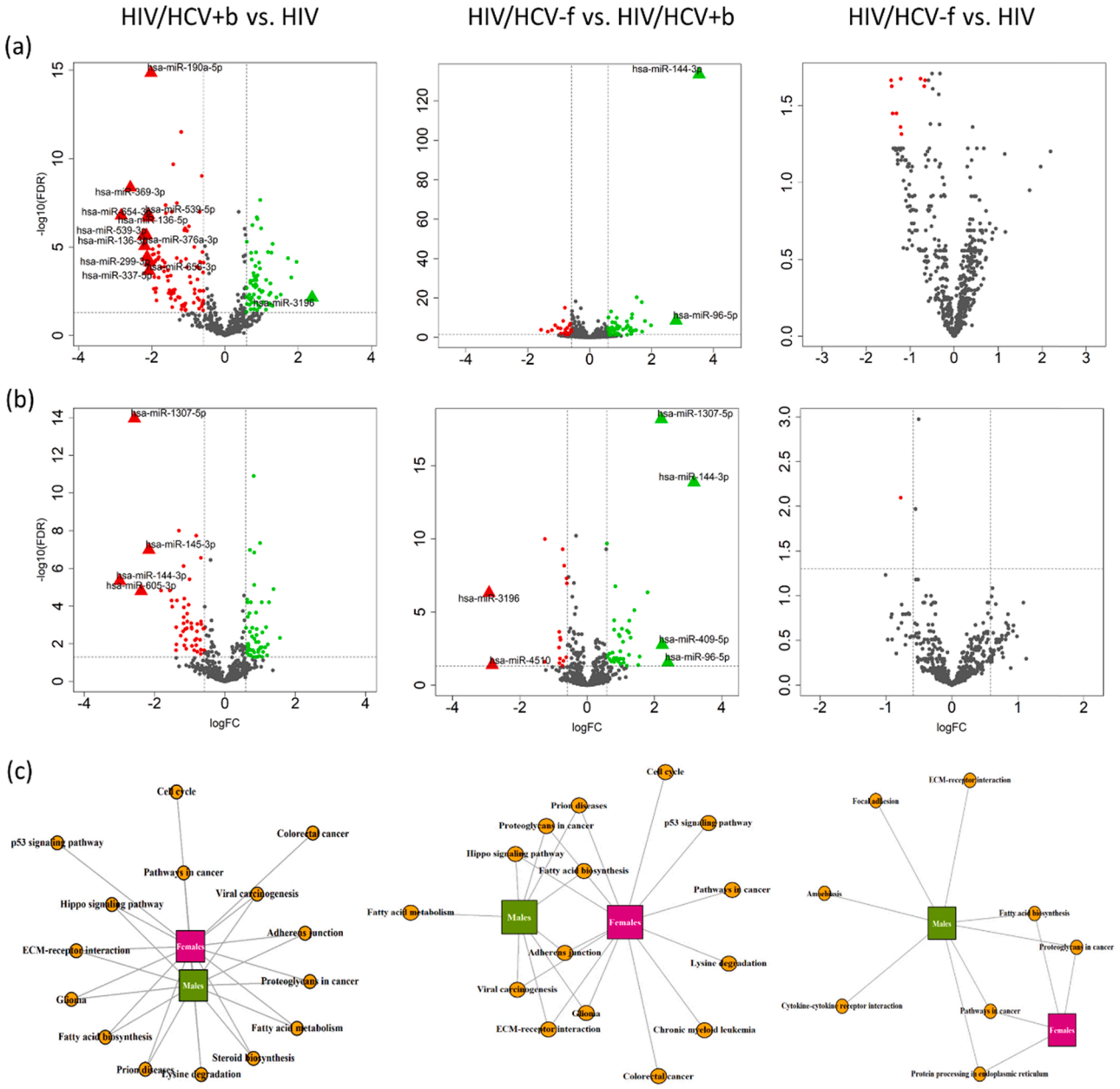


Fig. 4. : Differential miRNA expression analysis and target KEGG-pathway analysis stratified by gender. The different comparisons between HIV/HCV patients (both baseline and after SVR) and patients in the HIV control group are shown in columns. The volcano plots in the first two rows indicates the log2FC and the adjusted p-value of the miRNA expression analysis for males (a) and females (b). The coloured dots represent significant differentially expressed miRNAs (FDR < 0.05 and FC > 1.5), indicating upregulated in green and downregulated in red. Those SDE miRNAs with strong up/down regulation (LogFC > 2; FC > 4 or LogFC < − 2; FC < 0.25) are highlighted in wither triangle-shapes and labelled. (c) In the las row, the network interaction map represent significant target pathways (orange circles) for the SDE miRNAs identified in both males (green square) and females (dark-pink square).

Interestingly, routes related to fibrosis and inflammation, such as focal adhesion and cytokine-cytokine receptor interaction, were exclusive for males (Fig. 4**c-right**).

A higher number of SDE miRNAs were identified either for HIV/HCV^{-}f females (171) and for males (239) with respect to HC (Supplementary Tables S14 and S15 respectively**)**. Most of the miRNAs were common (126) among sexes, displaying similar dysregulated pathways (Supplementary Tables S16 and S17 for female and male respectively**)**. HIV/HCV^{-}f females showed five pathways exclusively dysregulated with respect to HC, such as the Jak-STAT signaling pathway, which has a central role in cytokine receptor signaling and Thyroid cancer, which is more common in women, among others. On the other hand, HIV/HCV^{-}f males showed nine exclusive pathways, some of them related with cardiomyopathy.

4. Discussion

Our study reports for the first time the evolution of miRNA profile from PBMCs of HIV/HCV coinfected patients before and after HCV eradication with DAAs. We found wide gender-related miRNA expression differences, which were also maintained in their associated functional routes (cancer-related and fatty acid metabolism pathways) before and after achieving SVR. Additionally, the miRNA profile normalization was almost achieved in females but not completely in males. Previous studies have approached the analysis of miRNAs in PBMC cells of HIV-monoinfected [22], HCV-monoinfected [23], HIV/HCV-coinfected [15], and HIV/HCV-coinfected patients after HCV spontaneous clarification [14,21]. However, few studies have analysed the effects of HCV eradication on HIV/HCV-coinfected patients with antiviral treatment, and none of them the miRNA profile of PBMCs after treatment with DAAs.

4.1. HCV chronic infection in HIV patients

The eradication of HCV reduces immune activation [24], but there are scarce data on how DAA therapy affects the regulation of the immune system in HIV/HCV coinfected patients. This population suffer from greater inflammation and fibrogenesis than HCV-monoinfected patients despite being under ART, thus residual inflammation due to HIV further hinders recovery [25]. HIV patients with an active HCV chronic infection shown high differences with HIV control patients [26]. In the same line, our results showed high dysregulation of the miRNA profile in HIV patients with an active HCV infection with respect to HIV controls.

Our results indicate that HCV produces a deep downregulation of the has-miR-144-3p in PBMCs of HIV patients, whose expression is restored after achievement of SVR. Hsa-miR-144-3p is involved in immune regulation processes [27], modulating an antiviral transcriptional network [28], and promoting pathological inflammation [29]. This miRNA has also a dual-regulation effect, as it inhibits tumorigenesis and tumor progression and also promotes the pathological progress of some cancers. However, its downregulation has been mainly identified in several types of cancers [30], such as in hepatocellular carcinoma (HCC) caused by HCV infection.

Additionally, we also found that the HCV chronic infection induces a deep downregulation of the hsa-miR-1307-5p. The function of this miRNA is still unknown, although some evidences point to a role in cell proliferation, differentiation and tumorigenesis [31]. Besides, this miRNA has an antiviral effect as it has been reported against H1N1 viruses [32]. The downregulation of hsa-miR-1307–5p upon viral infection seem to be a first mechanism of viral evasion from the innate immune response, as hsa-miR-1307–5p is also a key regulator of several genes of immune response. Therefore, the deep downregulation observed in PBMCs of HIV/HCV patients could be related with an additional HCV evasion mechanism.

All the observed differences were higher for males, and most of the miRNAs with higher downregulation were gender-specific. One of the most downregulated miRNA in males was the miR-136–5p, which is a modulator of the inflammatory response and cytokine production. It is also considered a tumor suppressor miRNA [33], as it is essential for HCC progression being its downregulation related to carcinogenesis and aggressiveness of HCC [34]. This downregulated miRNA could be contributing to the sexual dimorphism in HCV HCC, where males show a faster disease progression and a worse overall survival [35].

Females generally present immunological advantages against viral infections governed by various biological factors such as sexual hormones, which are directly involved in the immune response. The different hormonal regulation in both genders leads to immune dimorphism [36], as high levels of female hormones like oestrogen, show a protective effect by enhancing the immune response against viral infections [36]. Sexual hormones periodically oscillates [36] up to menopause, which could introduce some bias. However, all female patients in our study were over 49 years old, and therefore, their oestrogen and progesterone levels were similar to males [37]. Additionally, differences among genders could be related to the considerable amount of X-linked genes involved in the innate and adaptive immune responses and the high proportion of miRNAs (~ 10%) encoded in the X chromosome [38]. Our data showed that 18 differentially expressed miRNAs in females were located in the X chromosome while 13 in males.

4.2. Consequences of HCV eradication with DAAs

After SVR achievement with DAAs 82 miRNAs showed dysregulation with respect to chronic HCV infection at baseline, showing again higher differences for males. One of the most downregulated miRNAs was the well-known hsa-miR-122-5p, which were only significant for males. The hsa-miR-122-5p is a highly abundant human liver-specific miRNA that promotes HCV replication by a direct interaction with the HCV genome [39]. Various studies remarked that hsa-miR-122-5p is downregulated in PBMC or liver cells of chronic HCV monoinfected patients compared to healthy controls [14,39], due to the HCV hijacking. However, in HIV/HCV coinfected patients, HIV seems to stimulate HCV replication by enhancing the hsa-miR-122-5p expression [40]. Our results of hsa-miR-122-5p are similar to Waring et al. [41] and Santangelo et al. [42] findings, who also showed a remarkable reduction of hsa-miR-122-5p in serum and exosomes of HCV viremic patients after eliminating HCV with DAA therapy. Although our study is the first in PBMCs. The functional consequences of this reduction need to be further explored, especially in the context of HIV infection.

We have also identified several HCV-related miRNAs that interact with HCV infection within the cell, such as the hsa-miR-199a-5p and the hsa-miR-221-5p. The hsa-miR-199a-5p directly inhibits the HCV genome translation by targeting the HCV 5ŪTR IRES region [43], while the hsa-miR-221-5p accentuates interferońs anti HCV effect [44].

Additionally, for both genders the expression level of the hsa-miR-144-3p and the hsa-miR-96-5p was restored, increasing their expression more than four-fold FC. As commented above, the hsa-miR-144-3p has an antiviral function so it would be probably downregulated during the chronic HCV infection as an additional mechanism of the virus to evade immune system. The hsa-miR-96–5p plays a key role in immune system, modulating the inflammatory response, mitigating inflammation in specific cases of sepsis [45]. Thus, their upregulation after HCV eradication could be related to a decrease in the need to limit inflammation.

The analysis of the putative targeted pathways before and after SVR, showed that several cancer-related and fatty acid metabolism pathways were overrepresented. Despite some of these were shared between females and males, several routes were uniquely altered on each gender, specifically for females, were cancer-related pathways such as chronic myeloid leukaemia was identified. It is already known that HCV associated lymphoproliferative diseases are more frequently in females, which is in line with our results.

Sex differences in the physiological processes and therapeutic responses have been widely documented in the literature [9]. However, little is known about sex-biased miRNA expression changes due to infectious disease and/or antiviral treatment. Interestingly, most of the dysregulated miRNAs in males belong to the chromosome 14 miRNA cluster (C14MC), a maternally imprinted locus in the 14q32, which is one of the largest miRNA clusters in the genome [46]. The C14MC expression is regulated by differentially methylated regions, where the paternal allele is hypermethylated and therefore inactivated. The impact of viral infection in the dysregulation of this cluster is unknown. However, previous researches have elucidated that C14MC downregulation plays an essential role in carcinogenesis, proposed as a potential prognostic marker of different types of cancer [47,48]. In our study, approximately the 50% of miRNAs located in the C14MC were down-regulated in HIV/HCV males, while the HCV elimination allowed their up-regulation. These miRNAs are mainly involved in the extracellular matrix (ECM) molecular remodelling. ECM is a rich source of cytokines and growth factors that modulate host immune response to promote rapid responses to infection and injury [49]. The down-regulation of these miRNAs in HIV/HCV patients, may be promoting a favourable milieu for a higher inflammatory status and liver fibrosis development, as previously described [50]. Thus, C14MC miRNA dysregulation could be related to the higher degree of liver fibrosis [51] and potential complications for males after HCV eradication.

4.3. miRNA profile normalization after HCV eradication

After SVR both genders (HIV/HCV$^-$f) deeply reduced the differences of the miRNA expression profile with respect to the HIV group. However, sex dimorphism in the miRNA expression profile and their targeted pathways remained significant. Ten SDE miRNAs were downregulated in males, and only one of them, the hsa-miR-30a-5p, persisted downregulated in females. The hsa-miR-30a-5p belong to a miRNA family involved in organ development and clinical diseases [52], showing a key role in immune response [53], as its downregulation has been observed in several infectious diseases such as tuberculosis [53]. Similarly, among the remained miRNAs downregulated in males, the hsa-miR-548j-5p shows suppressed levels during viral infections [54] and hsa-miR-539-5p is down regulated by Tat (HIV) [55]. Therefore, the downregulation of these miRNAs could be indicating a poorer response to HIV, as a larger HIV-1 reservoir is observed in HCV patients [16]. On the other hand, the hsa-miR-299-3p, hsa-miR-654-3p and hsa-miR-654-5p are a tumor supresors miRNAs [56] which could be associated with a higher HCC in males. Further studies are needed to elucidate these issues.

Despite miRNA dysregulation, both females and males in our study normalized their transaminase levels, which are usually normalized after HCV infection, even in HIV/HCV coinfected patients [1]. However, hepatic inflammation and liver-related complications may persist despite HCV eradication [57]. Therefore, miRNA deregulation persistence after SVR may indicate potential alterations of the immune system, undetectable via classical clinical and epidemiological analysis. These results are in line with our previous data in HCV monoinfected patients [14], where the miRNA profile of HCV spontaneously clarified patients remained disrupted after CHC infection.

Additionally, our results indicate that 48 weeks after the achievement of SVR with DAAs, HIV patients lack to normalize miRNA expression profile with respect to HC, despite HIV patients without previous HCV infection on long-term suppressive antiretroviral therapy are close to normalization [58].

Finally, several considerations should be taken into account to correctly interpret our data. First, the sample size of this study could prevent us from detecting higher SDE miRNAs within each analysis. However, the GLMM properly account for the random effect in our model, which limits the false-positive rate [59] and increases the statistical power. Second, we cannot discard the existence of other potential confounding variables that could be affecting our results. To our knowledge, no prior research on gender-biased response to HCV elimination with DAAs has been published and hence, we cannot compare our results with published studies. A longitudinal analysis with a larger follow-up could help to better understand the possible complications of HCV eradication with DAA treatment in HIV/HCV-coinfected patients.

5. Conclusions

Our findings show gender-biased dysregulation in the miRNA expression profile of PBMCs after HCV eradication with DAA therapy. These differences were normalized for females, while miRNA profile and their target-related pathways remained different for males. These findings suggest a gender-biased response to HCV clarification with DAA therapy, which may have consequences on the clinical management of these patients. Thus, males should be subject to a more active monitoring after HCV elimination with DAA therapy due to the lack of complete normalization.

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CRediT authorship contribution statement

Verónica Briz, Amanda Fernández-Rodríguez: Conceptualization, Methodology, Supervision, Funding acquisition. **Alicia Gómez-Sanz, Luz Martín-Carbonero, Pablo Ryan, Ignacio De los Santos, Juan M Castro, Jesús Troya, Mario Mayoral-Muñoz, Guillermo Cuevas, Paula Martínez-Román, Jesús Sanz-Sanz, María Muñoz-Muñoz, María Á Jiménez-Sousa:** Sample collection and Clinical data acquisition. **Daniel Valle-Millares and Óscar Brochado-Kith**: Data curation. **Daniel Valle-Millares and Amanda Fernández-Rodríguez:** Formal analysis, Visualization, Writing – original draft. **Daniel Valle-Millares, Amanda Fernández-Rodríguez, Verónica Briz, Salvador Resino-García and Pablo Ryan**: Writing – review & editing.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112405.

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