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Original article

Hepatic mir-122-3p, mir-140-5p and mir-148b-5p expressions are correlated with cytokeratin-18 serum levels in MAFLD

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ABSTRACT

Introduction and objectives: Metabolic-associated fatty liver disease (MAFLD) is defined by steatosis in more than 5% of hepatocytes without other liver diseases. Patients with this disease can progress to multiple stages like liver fibrosis, cirrhosis, and hepatocellular carcinoma. miRNAs are single-stranded molecules that regulate metabolic homeostasis; their differential expression postulates them as potential circulating biomarkers for MAFLD. Previous research reported that hsa-miR-140-5p, hsa-miR-148-5p, and hsa-miR-122-3p have a differential expression in patients with MAFLD. This study aimed to investigate the correlation between liver hsa-miR-140-5p, hsa-miR-148-5p, and hsa-miR-122-3p and serum biomarkers CK-18, APOB, IL-6, IL-32, and TNF-α in patients with MAFLD compared with control patients.
Materials and methods: A cross-sectional study was carried out with 16 patients of both sexes, aged between 18-60 years, to determine the association between the levels of hsa-miR-140-5p, hsa-miR-148-5p, and hsa-miR-122-3p with MAFLD in liver biopsies of patients who underwent laparoscopic cholecystectomy.
Results: Twelve patients presented MAFLD, four without hepatic steatosis. Circulating levels of CK-18 showed a significant difference in patients with MAFLD, and a strong correlation was found between hsa-miR-122-3p, hsa-miR-140-5p, and hsa-miR-148b-5p versus the CAP value.
Conclusion: There is a correlation between elevated tissue expression of hsa-miR-122-3p, hsa-miR-140-5p, and hsa-miR-148b-3p with plasma levels of CK-18 in patients with simple steatosis compared with patients without the disease.

1. Introduction

Metabolic (dysfunction) associated fatty liver disease (MAFLD) is defined by steatosis in more than 5% of hepatocytes in the absence of other liver diseases, and is also the novel term for hepatic steatosis. MAFLD's current diagnosis includes evidence of hepatic steatosis, plus one of the following three criteria: overweight/obesity, metabolic dysregulation, or type 2 diabetes mellitus (T2DM) [1]. Its progression is conformed by metabolic-associated steatohepatitis (MASH), characterized by steatosis, inflammation, and fibrosis, with a final and irreversible stage such as cirrhosis and hepatocellular carcinoma [2]. The metabolic dysregulation is characterized by macrovesicular hepatic steatosis, obesity or overweight, and insulin resistance

Abbreviations: ALP, Alkaline Phosphatase; ALT, Alanine Aminotransferase; APOB, Apolipoprotein B; AST, Aspartate Aminotransferase; AUDIT, Alcohol Use Disorders Identification Test; BMI, Body Mass Index; CAP, Controlled Attenuation Parameter; CK-18, Cytokeratin 18; GGT, Gamma-Glutamyl Transpeptidase; hsa-miR, Homosapiens-microRNA; IL-6, Interleukin 6; IL-32, Interleukin 32; LDH, Lactate Dehydrogenase; MAFLD, Metabolic (dysfunction) Associated Fatty Liver Disease; MASH, Metabolic Associated Steatohepatitis; NAS, Non-alcoholic Fatty Liver Disease Activity Score; T2DM, Type 2 Diabetes Mellitus; TC, Total Cholesterol; TG, Triglycerides; TNF-α, Tumor Necrosis Factor-α

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[3]. The overall prevalence of MAFLD is 38.77% (95% CI 32.94-44.95), where 5.37% (95% CI 4.36-6.59) are lean individuals [4]. Among overweight/obese adults, the estimated global prevalence is 50.7% (95% CI 46.9-54.4); this shows how MAFLD affects more than a third of the world's population [5]. Metabolic complications such as diabetes and hypertension are significant associated factors [4].

Insulin resistance induces *de novo* hepatic lipogenesis and alters the inhibition of adipose tissue lipolysis, creating a lipotoxic environment in the liver [6]. The subsequent stage is the secretion of adipokines and inflammatory cytokines by adipose tissue, such as Apolipoprotein B (APOB), interleukin 6 (IL-6), interleukin 32 (IL-32), and tumor necrosis factor-α (TNF-α) which have also been proposed as potential biomarkers of the disease [7], as well as simultaneous predisposed genetic and epigenetic factors that modify the progression of MAFLD. Another potential biomarker is cytokeratin 18 (CK-18), a cytoskeleton protein in large amounts in the liver. It is an intermediate filament protein that represents 5% of hepatic proteins. This molecule has been reported to have a positive correlation with serum ALT levels, and with the controlled attenuation parameter of transient elastography in patients with MAFLD [8,7] CK-18 is the major intermediate filament protein in hepatocytes and is released at the initiation of cell death, this association has made circulating CK-18 a candidate marker to detect MASH and fibrosis as part of a multimarker model [9].

Liver biopsy is the gold standard [10]; however, due to the high prevalence of the disease, this method cannot be considered optimal for screening the general population, which has generated significant interest in developing and researching non-invasive methods to identify the disease and its late progression stages. MicroRNAs have been proposed as diagnostic and prognostic biomarkers involved in adaptation to changes in the liver microenvironment, which could improve clinical intervention strategies in patients with MAFLD [11,12]. Previous data reported the hsa-miR-140-5p, hsa-miR-148-5p, and hsa-miR-122-3p have a differential expression in patients with MAFLD compared with control subjects without MAFLD [13]. The miR122-3p is the most abundant and studied hepatic microRNAs; it represents 70% of the total hepatic miRNAs [11,14–16], while little is known about miR-140-5p and miR-148-5p and their liver functions and results as novel non-coding microRNAs proposed as biological markers. In this study, we aimed to investigate the association between the levels of hsa-miR-140-5p, hsa-miR-148-5p, and hsa-miR-122-3p in liver biopsies in patients with MAFLD, which can be crucial to be candidate biomarkers for diagnosis.

2. Methods

A cross-sectional study was carried out with 16 patients of both sexes, aged between 18-60, years to determine the association between the levels of hsa-miR-140-5p, hsa-miR-148-5p, and hsa-miR-122-3p with MAFLD in liver biopsies of patients who underwent laparoscopic cholecystectomy. Once they agreed to participate, informed consent was signed to obtain a liver biopsy of the right lobe (approximately 0.5-1.0 cm^3), and a blood sample was obtained before surgery. The presence of other liver diseases, such as viral, autoimmune, toxicological, or drug-induced, was ruled out, and alcohol intake was not greater than 20g per day through the Alcohol Use Disorders Identification Test (AUDIT).

2.1. Clinical evaluation

Anthropometric variables such as gender, age (years), weight (Kg), and height (m) were obtained from the patient's clinical record. Laboratory data were obtained: fasting glucose, triglycerides (TG), total cholesterol (TC), alanine, and aspartate aminotransferase (ALT-AST), gamma-glutamyltranspeptidase (GGT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and albumin.

2.2. Hepatic vibration-controlled transient elastography

Transient elastography was performed using Fibroscan® Touch 502 (Echosense, France) after a minimum of 4 hours of fasting with the M or XL probe. The patient was placed supine with the right arm adducted and the hand under the head; the rest of the limbs extended. Next, an imaginary line was drawn between the xiphoid apophysis and the mid-clavicular line, on which an optimal intercostal space was sought, and the transducer was placed. The study was completed with the following characteristics: at least ten valid measurements, 60% success (valid measurements/invalid measurements), and the interquartile/median range was 30%. The median Controlled Attenuation Parameter (CAP) in decibels per meter (dB/m), the median liver stiffness in kilopascals (kPa), and the interquartile range were obtained. Steatosis degree according to the CAP was determined, cutoff values were S0: <232 dB/m, S1: 232−256 dB/m, S2: 257−290 dB/m, S3: ≥290 dB/m, and hepatic fibrosis degree was determined according to LSM, were F2≥7.0 kPa, F3: ≥8.7 kPa, F4: ≥10.3 kPa.

2.3. Liver histology

Liver biopsies were formalin-fixed, paraffin-embedded, and examined using hematoxylin-eosin staining. Two expert pathologists independently diagnosed the biopsy specimens blindly, without knowing the patient's clinical data. Any difference in observations was rectified by consensus. The Kleiner score [17] includes three semi-quantitative parameters, which are added to obtain a score of 0-8 points: steatosis according to the hepatocytes percentage lipid droplets (grade 0: <5%, 1: 5−33%, 2: 33−66%, and 3: >66%), lobular inflammation measured in foci per field (grade 0: none, 1: <2, 2: 2-4, 3: >4), hepatocytes ballooning (grade 0: none, 1: few cells, 2: many cells). Liver fibrosis was also assessed (grade 1: perisinusoidal or periportal, 1A: mild perisinusoidal fibrosis in zone 3, 1B: moderate perisinusoidal fibrosis in zone 3, 1C: only portal/periportal fibrosis, 2: perisinusoidal fibrosis in zone 3, with portal/periportal fibrosis, 3: fibrosis bridges, 4: cirrhosis).

2.4. RNA extraction

Sixteen liver samples were selected based on their histology (4 without MAFLD and 12 MAFLD) to determine the level expression of the miR-140-5p, miR-148-5p, and miR-122-3p. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and chloroform. After that, RNA quality was determined using the Agilent 2100 bioanalyzer (Agilent Technologies, China).

2.5. Quantitative Real-Time PCR

A reverse transcription assay was performed using TaqMan microRNA reagents (Applied Biosystems, USA), adding the small nucleolar RNA RNU-48 as an internal control. Total RNA was isolated from 16 independent samples (4 without MAFLD and 12 MAFLD). The Taqman Universal MasterMix NO UNG kit and the Taqman probes for each microRNA hsa-miR-122-3p, hsa-miR-140-5p, and hsa-miR-148-5p (Applied Biosystems, USA) were used. The assay was performed in triplicate, and the relative amount of change of each microRNA was calculated using equation 2 −(ΔCT), where ΔCT= Ct (miRNA MAFLD - CtRNU48-MAFLD) - Ct (miRNA control − CtRNU48-control). The rate of change and the p-value were calculated.

2.6. Determination of circulating levels of CK-18, APO-B, IL-6, IL-32, TNF-a

An ELISA assay was used to measure circulating levels of human CK-18, APO-B, IL-6, IL-32, and TNF-α by Quantikine Immunoassays

(R&D systems, Minneapolis, MN), following the manufacturer's instructions.

2.7. Statistical analysis

Statistical analysis was performed using SPSS for Macintosh v20.0 (IBM, USA). Results are presented as median ± interquartile range or percentages. Due to the small sample size, the continuous variables were analyzed by a non-parametric U test to compare two groups, and the chi-square test was used to compare categorical variables. Spearman and Pearson's correlation was determined to analyze the relationship between expression levels of microRNAs and MAFLD. Statistical significance was defined as $p \leq 0.05$.

2.8. Ethical declarations

The ethics committee of Medica Sur Hospital approved this study (2017-EXT-238). The patients signed the informed consent to obtain the liver biopsy and store the information in the database that was used in the research, which follows the basic principles of human research in the Helsinki Declaration (Helsinki Finland 1975, last amendment at the 52nd General Assembly in Fortaleza, Brazil, October 2013). The collected data were treated confidentially, with an attached privacy notice to the informed consent.

3. Results

Table 1 describes the patient's anthropometric and biochemical characteristics, where 87.5% were women and 12.5% were men. The mean age was 49.4±10.6 years, the mean weight was 70.9±14.6 Kg, and the BMI 29±4Kg/m^2. The BMI, CAP, and liver stiffness had a statistical difference between the groups. Sixteen liver samples were selected by histopathological evaluation; four patients did not present microscopic abnormalities with a Kleiner score equal to 0, forming the control group (Figure 1A), and twelve patients with a score of 1-4 formed the MAFLD group (Figure 1B). Patients with a NAS score ≥5 were discarded for this study. The histological characteristics are presented in Table 2.

Circulating levels of CK-18, APO-B, IL-6, IL-32, and TNF-α were evaluated; however, only CK-18 showed a significant difference ($p \leq 0.001$) when comparing patients without MAFLD (104.1pg/mL) against patients with MAFLD (230.2pg/mL). (Figure 1C) No differences were found between the groups in the remaining markers explored. On the other hand, the change rates ($2^{-\Delta\Delta CT}$) of hsa-miR-122-3p, hsa-miR-140-5p, and hsa-miR-148-5p were determined by qRT-PCR. When the $2^{-\Delta\Delta CT}$ was compared between groups, only hsa-miR-148a-5p showed a statistical difference; however, hsa-miR-122-3p and hsa-miR-140-5p had a higher fold change, showing the same behavioral trend as previous microarray differential expression data. [13] (Figure 1D-F)

The results of the Spearman analysis show a very strong correlation between hsa-miR-122-3p and MAFLD (rho= 0.751 p= 0.001), a good correlation for hsa-miR-148b-5p (rho= 0.689 p= 0.003) and acceptable for hsa-miR-140-5p (rho= 0.564 p= 0.023). On the other hand, through Pearson's analysis, a strong correlation was found between hsa-miR-122-3p, hsa-miR-140-5p, and hsa-miR-148b-5p versus the CAP value. While hsa-miR-122-3p, hsa-miR-140-5p and hsa-miR-148b-5p show a moderate correlation with CK-18 (r= 0.536; r= 0.404 and r= 0.504 respectively); however, the correlation between CK-18 and hsa-miR-140-5p was not statistically different. (Table 3)

4. Discussion

As obesity and metabolic syndrome increase, cases of MAFLD grow in parallel to the point that it is currently the liver disease with the highest incidence worldwide [18–20], which has aroused greater interest in establishing the pathophysiological mechanisms, in addition to promoting the exploration of new tools for early diagnosis and novel biomarkers that determine the risk of progression to more severe stages of the disease.

Circulating biomarkers levels, previously described and proposed as possible diagnostic tools, are CK-18, APO-B, IL-6, IL-32, and TNF-α. Among these, a difference was found in CK-18 levels in our cohort study [7,21]. CK-18 is a protein related to apoptotic processes through caspase-3 and has been evaluated as a differentiating factor between subjects with simple steatosis and those with steatohepatitis; also a potential hepatocellular marker [22–24]. In this study, we corroborated the potential of this circulating protein fragment as a biomarker of steatosis in patients with a chronic inflammatory process.

MicroRNAs are currently considered potential molecular biomarkers to diagnose multiple diseases, including MAFLD [11,25–27] Using liver samples from patients with MAFLD, we demonstrated a strong correlation between hepatic expression levels of hsa-miR-122-3p, hsa-miR-140-5, and hsa-miR-148b-5p, and the disease. Due

Table 1
Anthropometric and biochemical characteristics

	Without MAFLD (n=4)			MAFLD (n=12)			p value
	Median	**1Q**	**3Q**	**Median**	**1Q**	**3Q**	
Age (years)	47	33.7	57.2	51.5	40.2	58.2	0.521
BMI (Kg/m^2)	23.8	22.5	24.8	31.1	30.1	32.1	**0.004**
Fastin Glucose (mg/dL)	81	76.5	125.2	99.5	91.8	127.6	0.316
Triglycerides (mg/dL)	161.5	101	267	177	95	388.2	0.9
Total cholesterol (mg/dL)	186.5	171.7	296.5	196	171.2	238.4	0.862
ALT (IU/L)	18.5	12.7	25.7	19	14	41.7	0.684
AST (IU/L)	22	16.5	33.5	20	17	27	0.953
GGT(IU/L)	15.5	12.5	100.2	31.5	17.5	276.9	0.262
LDH (IU/L)	198.5	172	228.7	163.5	154.5	188.2	0.17
ALP (IU/L)	103.5	54.7	138	76.5	58	102.5	0.521
Albumin (mg/dL)	4	3.6	4.6	4.3	4.1	4.4	0.684
CAP (dB)	119.5	118.2	120.7	299.5	290.2	311.7	**0.001**
Liver stiffness (kPa)	2.6	2.1	2.9	3.9	3.2	5.2	**0.004**

Mann-Whitney U test, differences were considered significant at $p \leq 0.05$ between groups. MAFLD, Metabolic associated fatty liver disease; **1Q**, first interquartile; **3Q**, third interquartile; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase, GGT, gamma glutamyl transpeptidase; LDH, lactate dehydrogenase, ALP, alkaline phosphatase, CAP, Controlled Attenuation Parameter

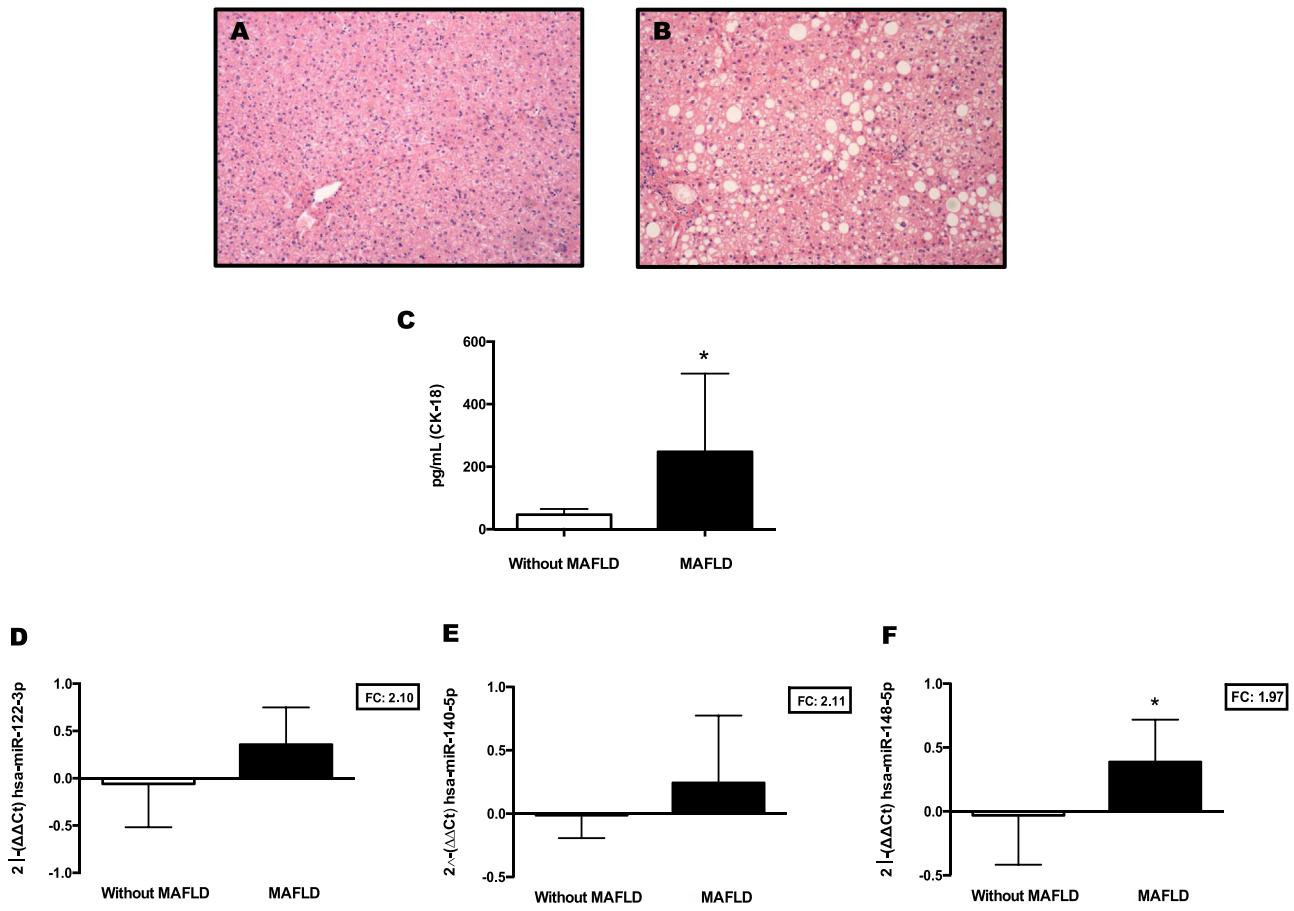

Fig. 1. Liver histology, potential MAFLD biomarkers and microRNA expression from liver biopsies. A) Liver without steatosis, B) Liver with MAFLD, representative images of sixteen hepatic tissues obtained from human biopsies, stained with H&E. Original magnification 100×. C) Cytokeratin-18 (CK-18) serum levels in patients with and without MAFLD, D) hsa-miR-122-3p change rate, E) hsa-miR-140-5p change rate, and F) hsa-miR-148-5p change rate ($2^{-\Delta\Delta CT}$) between patients with and without MAFLD. The differences (*) in the mean ± standard deviation were considered significant with a $p \leq 0.001$ between groups. Fold Change (FC).

to MAFLD complexity, there is high variability between patients and therefore in the expression levels of miR-122-3p and miR-140-5p; however, these miRNAs presented values above double when comparing the levels with the control group. Possibly, there is no statistical difference because of the reduced sample. Elevated levels of miR-122 are associated with several cellular processes such as hepatocyte lipogenesis and triglycerides and cholesterol accumulation through the repression of Sirt1 and the inactivation of the LKB1/AMPK pathway that enhances the accumulation of intrahepatic fat [28].

Elevated levels of hsa-miR-122 are associated with several cellular processes such as lipogenesis in the hepatocyte and its accumulation of intracellular triglycerides and cholesterol through the repression of Sirt1 and the inactivation of the LKB1/AMPK pathway that

Table 2
Non-alcoholic Fatty Liver Disease Activity Score (NAS) of human liver biopsies

Biopsy	Steatosis	Inflammation	Ballooning	NAS	Group
1	0	0	0	0	Without MAFLD
2	0	0	0	0	Without MAFLD
3	0	0	0	0	Without MAFLD
4	0	0	0	0	Without MAFLD
5	1	0	1	2	MAFLD
6	1	0	1	2	MAFLD
7	2	1	1	4	MAFLD
8	3	0	1	4	MAFLD
9	1	0	0	1	MAFLD
10	2	0	0	2	MAFLD
11	2	0	0	2	MAFLD
12	2	1	0	2	MAFLD
13	2	0	1	3	MAFLD
14	3	0	1	4	MAFLD
15	2	1	1	4	MAFLD
16	1	1	0	2	MAFLD

NAS, Non-alcoholic Fatty Liver Disease Activity Score; MAFLD, Metabolic (dysfunction) Associated Fatty Liver Disease

Table 3
Correlation analysis between miRNAs, MAFLD and CAP

Vs. MAFLD		
	Spearman's Rho	***p-value***
hsa-miR-122-3p	0.751	0.001
hsa-miR-140-5p	0.564	0.023
hsa-miR-148b-5p	0.689	0.003
Vs. CAP		
	Pearson's "r"	***p-value***
hsa-miR-122-3p	0.923	<0.001
hsa-miR-140-5p	0.749	0.001
hsa-miR-148b-5p	0.757	0.001
Vs. CK-18		
	Pearson's "r"	***p-value***
hsa-miR-122-3p	0.536	0.032
hsa-miR-140-5p	0.404	0.12
hsa-miR-148b-5p	0.504	0.047

Correlation analysis. The table shows the results of the Spearman correlation analysis between the MAFLD diagnosed by liver biopsy and hepatic expression levels of the 3 microRNAs, and the Pearson correlation between the CAP values versus the hepatic expression levels of the 3 microRNAs.
has-miR, Homosapiens-microRNA; CAP, Controlled Attenuation Parameter; CK-18, Cytokeratin-18

enhances intrahepatic fat accumulation. Increased levels of this microRNA are associated with progression to a pro-inflammatory state due to activation of the TLR4/MyD88 signaling pathway and increased NFκ-Bp65 [16], which could position it as a potential biomarker for liver disease.

Little has been reported about hsa-mirR-140-5p, and its relationship with MAFLD is still unknown, so it is a novel molecule in this disease. It is known from previous experiments that its overexpression is associated with negative regulation of the LDLR receptor in human hepatocytes, decreasing cholesterol uptake and causing triglyceride accumulation [29]. Another observational study found this microRNA elevated in adolescents with obesity and insulin resistance [30]. The complex mechanisms of progression to more severe stages probably involve one or more cellular pathways where hsa-miR-140-5p has interaction targets. The hsa-miR-148b-5p has been associated with various epigenetic mechanisms, B and T cell differentiation, tumorigenesis [31], and biological processes such as adipogenesis, [32] but its relationship with liver diseases has not yet been explored: So far it is known that the isoform hsa-miR-148b-3p controls the expression of *DTYMK*, a potential oncogene related to the development of HCC [33]. Our study is the first to show the potential usefulness of this molecule as a novel liver biomarker.

Our study has limitations since, in order to propose some of these microRNAs as biomarkers, it is necessary to explore them in the bloodstream, so we intend to recruit more patients to determine the expression of these microRNAs and their target genes.

5. Conclusions

In conclusion, this study describes the correlation between elevated tissue expression of hsa-miR-122-3p, hsa-miR-140-5p, and hsa-miR-148b-3p with plasma levels of CK-18 in patients with simple steatosis compared with patients without the disease. These molecules may play a vital role in the pathogenesis of the disease with non-invasive diagnostic power.

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Author's contributions

All authors contributed to the article's realization and improvement and agreed on the manuscript's content. Dr. López-Sánchez, Dr. Nuño Lámbarri and Dr. Uribe design, carried out the study and drafted the article. Dr. Montalvo-Javé was the surgeon who provided the patient's biopsies, contributed with diverse ideas, and corrected the final version of the manuscript; Dr. Domínguez-Perez, Dr. Antua-Puente, Dr. Beltrán-Anaya, Dr. Hidalgo-Miranda, Dr. Chávez-Tapia, and Dr. Uribe revised, contributed with diverse ideas, and corrected the final version of the manuscript. The definitive version has been read and approved by all authors.

Conflicts of interest

None

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