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Original article

Clinical features and immune mechanisms directly linked to the altered liver function in patients with rheumatoid arthritis

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ABSTRACT

Background: The aim of this study was to explore the impact of arthritis on liver function using different approaches *in vivo* and *in vitro*.

Methods: A cross-sectional study was performed on 330 non-obese/non-T2DM subjects: 180 RA patients, 50 NAFLD non-RA patients, and 100 healthy donors (HDs). A longitudinal study was conducted on 50 RA patients treated with methotrexate for six months. Clinical and laboratory parameters and markers of liver disease were collected. Mechanistic studies were carried out in both the CIA mouse model and hepatocytes treated with anti-citrullinated protein antibodies (ACPAs).

Results: RA patients have an increased risk of suffering from liver disease independent of obesity or T2DM. This risk was associated with factors such as insulin resistance, autoantibodies, inflammation, and component C3. Methotrexate treatment for six months was associated with liver abnormalities in those newly-diagnosed patients having CV risk factors. ACPAs induced a defective hepatocyte function, promoting IR and inflammation. The induction of arthritis in mice caused the infiltration of immune cells in the liver and increased inflammatory, apoptotic, and fibrotic processes.

Conclusion: RA patients may experience mild to moderate liver inflammation due to the infiltration of T, B cells, and macrophages, and the action of ACPAs. This is independent of obesity or diabetes and linked to systemic inflammation, and disease activity levels. The negative effects of methotrexate on liver function could be restricted to the concomitant presence of cardiovascular risk factors.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that mainly affects the synovial membrane, cartilage, and bone tissue, and is associated with progressive disability, systemic complications, and increased mortality [1]. Antibodies to citrullinated protein antigens (ACPAs) are biomarkers in the diagnosis of RA and active effectors in the development of this disease. The presence of these antibodies is related to the appearance of cardiovascular comorbidities independently of traditional risk factors [2]. In this sense, ACPAs have been described as direct inducers of oxidative stress and inflammation, which could increase an atherogenic profile and be detrimental to the cardiovascular system [3]. In fact, cardiovascular and cardiometabolic comorbidities, including dyslipidemia, increased body mass index, type 2 diabetes mellitus (T2DM), and non-alcoholic fatty liver disease (NAFLD) are strongly associated with RA [4,5]. However, up to date, there is no clear evidence of the possible deleterious effects of ACPAs and/or RA on liver function.

Liver dysfunction has been described in numerous studies of RA patients. Specifically, RA patients show abnormal elevations of blood alkaline phosphatase and levels of γ-glutamyltransferase [5–7]. Moreover, liver biopsies from patients with RA show steatosis and nodular regenerative hyperplasia which could lead to liver dysfunction and progression of NAFLD [5,7]. Although these anomalies appear as asymptomatic alterations, they are at potential risk of developing major complications. It should be noted that several studies have been performed to analyze the potential effects of methotrexate [8] on liver function and the development of NAFLD [9,10].

In this study, we explored the impact of arthritis on liver function using different approaches *in vivo* and *in vitro*.

We conducted a cross-sectional study comparing RA patients with a NAFLD cohort without RA, as well as a longitudinal study of RA patients undergoing six months of methotrexate treatment. Additionally, we used a mouse model of collagen-induced arthritis (CIA) and HepG2 hepatocyte cell line experiments to assess RA's impact on NAFLD development.

2. Patients and methods

2.1. Cross-sectional study

A cross-sectional study was carried out on 330 subjects, including 180 patients with RA, 50 non-RA patients with NAFLD, and 100 healthy donors (HDs). Clinical and laboratory parameters were recorded (table I). RA patients fulfilled a total score of 6 or greater according to the 2010 criteria [11]. The HDs and RA patients were recruited at the Rheumatology department in the Reina Sofia Hospital (Cordoba, Spain) after approval from the ethics committee of the Reina Sofia. In addition, non-RA subjects with NAFLD were prospectively selected from among those who were referred for an abdominal ultrasound study between November 2021 and February 2022, due to abnormal liver function tests, requested by a liver specialist physician according to clinical practice in the General Hospital of Tomelloso (Tomelloso, Spain). All subjects provided written informed consent. NAFLD was defined as liver steatosis on abdominal ultrasound after excluding a history of known secondary causes of hepatic steatosis or fibrosis. For NAFLD diagnosis, gray-scale abdominal sonographies were performed by experienced hepatologists [12]. To avoid the potential effects of obesity and type 2 diabetes mellitus (T2DM) on hepatic alterations, these comorbidities were excluded.

2.2. Longitudinal study

Fifty RA patients were included in a longitudinal study to analyze the effect of methotrexate on the liver disease biomarkers and hepatic steatosis and fibrosis indexes and the impact of cardiovascular comorbidities and hepatic steatosis index in the therapeutical response. The participants were recruited at the Rheumatology department in the Reina Sofia Hospital (Cordoba, Spain) and the Experimental and Observational Rheumatology and Rheumatology Unit, Health Research Institute of Santiago de Compostela (IDIS) – University Clinical Hospital of Santiago de Compostela (Spain) after approval from the ethics committee. Those patients were evaluated clinically and analytically at baseline and after six months of treatment. After 8 h of fasting, blood samples were collected.

2.3. Assessment of liver disease risk

The levels of hepatic enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), and lactate dehydrogenase (LDH) were evaluated. In addition, levels of albumin and platelet counts were also analyzed. Four indexes to determine the liver disease risk were evaluated:

Fibrosis-4 (FIB-4) score estimates fibrosis: age (years), ALT (U/L), AST (U/L), and platelet count (10^9/L). FIB4= Age (years)x AST(U/L)/ [*platelets* (10^9/L)x$ALT^{1/2}$(U/L)]. Values less than 1.3 show a low risk of advanced liver fibrosis, values between 1.3 and 2.67 moderate risk, and FIB-4 values great than 2.67 indicate a high risk of advanced liver fibrosis [13].

AST-to-platelet ratio index (APRI) for detecting hepatic fibrosis= [(AST (U/L) / upper limit of the normal AST range) x 100] / Platelet count [14].

Triglycerides and glucose (TyG) index is a method of detecting insulin resistance [15] and hepatic steatosis [16] = ln [fasting triglycerides (mg/dL) x fasting glucose (mg/dL)] / 2.

Hepatic steatosis index (HSI)= 8 x ALT (U/L) /AST (U/L) + BMI (+2 if T2DM, +2 if female) [17,18].

2.4. CIA mouse model

All animal experiments were carried out in accordance with the ARRIVE guidelines and with the UK Animals (Scientific Procedures) Act, 1986, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Twenty-five DBA1/J male mice (7–8 weeks old) were used in this study. Five mice were used as healthy controls, and 20 mice were injected subcutaneously with collagen/complete Freund's adjuvant emulsion (100 μg per mouse); on day 21, mice were boosted with a mixture of collagen solution and incomplete Freund's adjuvant emulsion (100 μg per mouse). Between days 22 and 42, macroscopic signs of arthritis were scored three times weekly, where each paw received a score: 0 = no visible effects of arthritis; 1 = edema and/or erythema of one digit; 2 = edema and/or erythema of two digits; 3 = edema and/or erythema of more than two digits; and 4 = severe arthritis of entire paw and digits. The arthritic index (AI) was calculated by the addition of individual paw scores (up to a maximum of 16). Next, mice were killed at day 42, and liver tissue and plasma were isolated and frozen at −80 °C.

2.5. Histology and immunohistochemistry of liver tissue

Freshly dissected mouse livers were fixed with 4% paraformaldehyde and embedded in paraffin using standard processing. Cryosections (3 μm) were stained with H&E. For liver morphology evaluation, we used Masson's trichrome and reticulin stains in an automated staining system (Roche, VENTANA BenchMark, Switzerland). Immunohistochemical staining was performed using the following primary antibodies: CD3 (1:100, clone SK7, Dako®), CD20 (1:50, clone L26, Dako®), CD68 (1:100, clone PG-M1, Dako®), BCL2 (1:50, clone 124, Dako®) and BCL6 (1:100, clone 1+PG-B6p, Dako®). CD3, CD20, and CD68 were evaluated as the presence of positive cells in 10 high magnification fields (40x), establishing a distribution percentage in the different fields observed

(0–25%, 25–50%, 50–75, and 75–100%). *Bcl2* and *Bcl6* were evaluated as positive nuclei in 10 high-power fields (40x).

2.6. Isolation of enriched IgGs-ACPAs

IgG-ACPAs antibodies from the pooled sera of 12 RA patients (characterized by high titers of ACPAs >300 U/ml) and IgG-NHS (normal human serum) antibodies from the pooled sera of 12 HDs were purified using HiTrap ® Protein G HP kit (GE Healthcare, Uppsala, Sweden). To assess the ACPAs activity of these isolated antibodies, 1:100 diluted samples were tested using ACPAs ELISA kit according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany).

2.7. In vitro experiments with HEPG2 cell line

The HEPG2 cell line (ATCC, Manassas, USA) was cultured in minimum essential medium (MEM) (ThermoFisher Scientific, Walthman, United States) at 10% FBS, 1% sodium pyruvate (Merk KGaA, Darmstadt, Alemania), and 1% ZellShield (Minerva Biolabs GmbH, Berlin, Germany) at 37% and 5% CO_2. Cells were treated with purified IgG-enriched ACPAs and IgG-NHS at 500μg/mL for 48 h. Supernatant and cells were collected to perform mRNA and proteomic analyses.

2.8. Complement component 3 quantification

Levels of complement C3 were quantified through enzyme-linked immunosorbent assay (ELISA), following the manufacturer's recommendation (Cusabio, Houston, USA).

2.9. Leptin and TNF-α plasma levels

Plasma levels of tumor necrosis factor-alpha (TNF-α) and leptin were quantified through ELISA following the manufacturer's recommendation (Bionova, Diaclone).

2.10. Inflammatory proteome profile

An inflammation panel of 92 proteins was measured in the supernatant of *in vitro* experiments, using the proximity extension assay method, Olink Target 96 Inflammation panel (Cobiomic Bioscience, Cordoba, Spain).

2.11. RT-PCR

Total RNA was extracted using TRI Reagent (Sigma) following the manufacturer's recommendations. Gene expression was assessed by real-time PCR using Light Cycler Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA). Expression of genes of interest was corrected by the geometrical average of β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2 microglobulin, and 36B4 using the BestKeeper tool [19].

2.12. Statistical analysis

Normal distribution of all variables was analyzed. To compare two independent groups, Student's unpaired *t*-test or Mann-Whitney rank sum test were used. In addition, a paired *t*-test was performed between the baseline and 6 months of treatment with methotrexate. Besides, receiver operating characteristic (ROC) curves were used to determine the sensitivity, specificity, and cut-off values. For multiple comparisons, one-way ANOVA test or Kruskall-Wallis test were performed.

Table 1
Cross-sectional study. Clinical and laboratory characteristics of RA, NAFLD patients and healthy donors.

	Healthy donors	RA patients	NAFLD patients
Clinical parameters			
Female/Male (n/n)	58/42 (100)	122/58 (180)	23/27 (50)
Age (years)	49.01 ± 8.61	51.22 ± 10.53	49.82 ± 9.61
Disease duration (years)	–	8.92 ± 8.29	–
DAS-28	–	4.06 ± 1.59	–
BMI (kg/m^2)	24.26 ± 2.73	24.90 ± 2.67	25.77 ± 2.38
Inflammatory profile			
ESR (mm/h)	7.31 ± 5.87	21.43 ± 17.70[a]	11.95 ± 9.73[b]
CRP (mg/L)	0.88 ± 1.54	12.30 ± 24.72[a]	0.51 ± 1.04[b]
Lipid profile			
Total Cholesterol (mg/dL)	200.23 ± 32.91	197.56 ± 38.49	207.07 ± 46.31
HDL-Cholesterol (mg/dL)	60.06 ± 14.98	58.16 ± 20.22	51.56 ± 20.49[a]
LDL-Cholesterol (mg/dL)	123.78 ± 27.79	119.65 ± 30.77	123.35 ± 45.10
Triglycerides (mg/dL)	101.86 ± 48.55	98.85 ± 43.80	175.14 ± 108.36[a,b]
Liver biomarkers			
AST (U/L)	20.53 ± 6.00	22.30 ± 9.79	29.92 ± 21.98[a]
ALT (U/L)	18.88 ± 8.58	22.38 ± 15.22	39.60 ± 28.07[a]
ALP (U/L)	68.07 ± 18.91	74.25 ± 23.45[a]	82.94 ± 24.43[a,b]
GGT (U/L)	30.24 ± 28.36	27.30 ± 20.56	76.20 ± 87.60[a,b]
LDH (U/L)	182.74 ± 40.60	197.00 ± 43.07	255.36 ± 99.50[a]
Platelets (10^3/μL)	235.95 ± 70.21	279.51 ± 98.96[a]	246.58 ± 70.04[b]
Albumin (g/dL)	4.52 ± 0.25	4.09 ± 0.29[a]	4.62 ± 0.32[b]
C3 (mg/dL)	114.95 ± 23.27	134.87 ± 31.74[a]	Not determined
Treatments			
NSAIDs (%)	–	66.66	–
Corticosteroids (%)	–	51.11	–
Methotrexate (%)	–	56.11	–
Leflunomide (%)	–	31.11	–
Hydroxychloroquine (%)	–	21.11	–
Statins (%)	–	4.44	2.00

Data are represented by mean ± SD. RA: rheumatoid arthritis; NAFLD: non-alcoholic liver disease; DAS: disease activity score; BMI: body mass index; ESR: erythrocyte sedimentation rate; CRP: c-reactive protein HDL: high density lipoprotein; LDL: low density lipoproteins; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; NSAIDs: non-steroidal anti-inflammatory drugs.

[a] Significant differences respect to HDs, $p < 0.05$

[b] Significant differences respect to RA, $p < 0.05$.

Correlation studies were assessed by Pearson correlation coefficients and a linear regression model was constructed.

3. Results

3.1. Subclinical liver alterations in non-obese/non-T2DM RA patients

The clinical features of the three non-obese/non-diabetic cohorts: RA patients ($n = 180$), NAFLD patients ($n = 50$), and HDs ($n = 100$) are summarized in Table 1. Acute phase reactants and platelet counts were significantly elevated in RA patients compared to NAFLD patients. Accordingly, NAFLD patients had significantly high levels of triglycerides, ALP, and GGT compared to RA patients. Of note, non-obese RA patients display significantly higher levels of CRP, ESR, ALP, platelet counts, and C3 and reduced levels of albumin compared to the HDs. NAFLD group had prominent variations in liver disease biomarkers in parallel with an altered lipid profile compared to RA and HDs. RA patients showed a significantly altered inflammatory profile and other related liver disease biomarkers, such as ALP, albumin, platelet counts, and complement C3.

Linear regression analysis showed that treatments were not related to the changes in the liver disease biomarkers. Only changes in albumin levels were independently associated with methotrexate therapy (Supplemental table I).

3.2. Increased hepatic steatosis risk in non-obese/non-T2DM RA patients: association with insulin resistance, inflammation, and C3 levels

We used a cohort of NAFLD non-RA with no presence of T2DM, carefully matched with our cohort of RA patients in terms of BMI, age, and sex. Our primary objective was to assess the accuracy and cut-off values of four liver disease indexes (TyG, APRI, FIB-4, and HSI) for identifying NAFLD patients.

Among them, only the levels of HSI were significantly increased in non-obese/non-T2DM RA patients compared to HDs (Supplemental figure 1 and Fig. 1A). NAFLD patients had significantly higher levels of

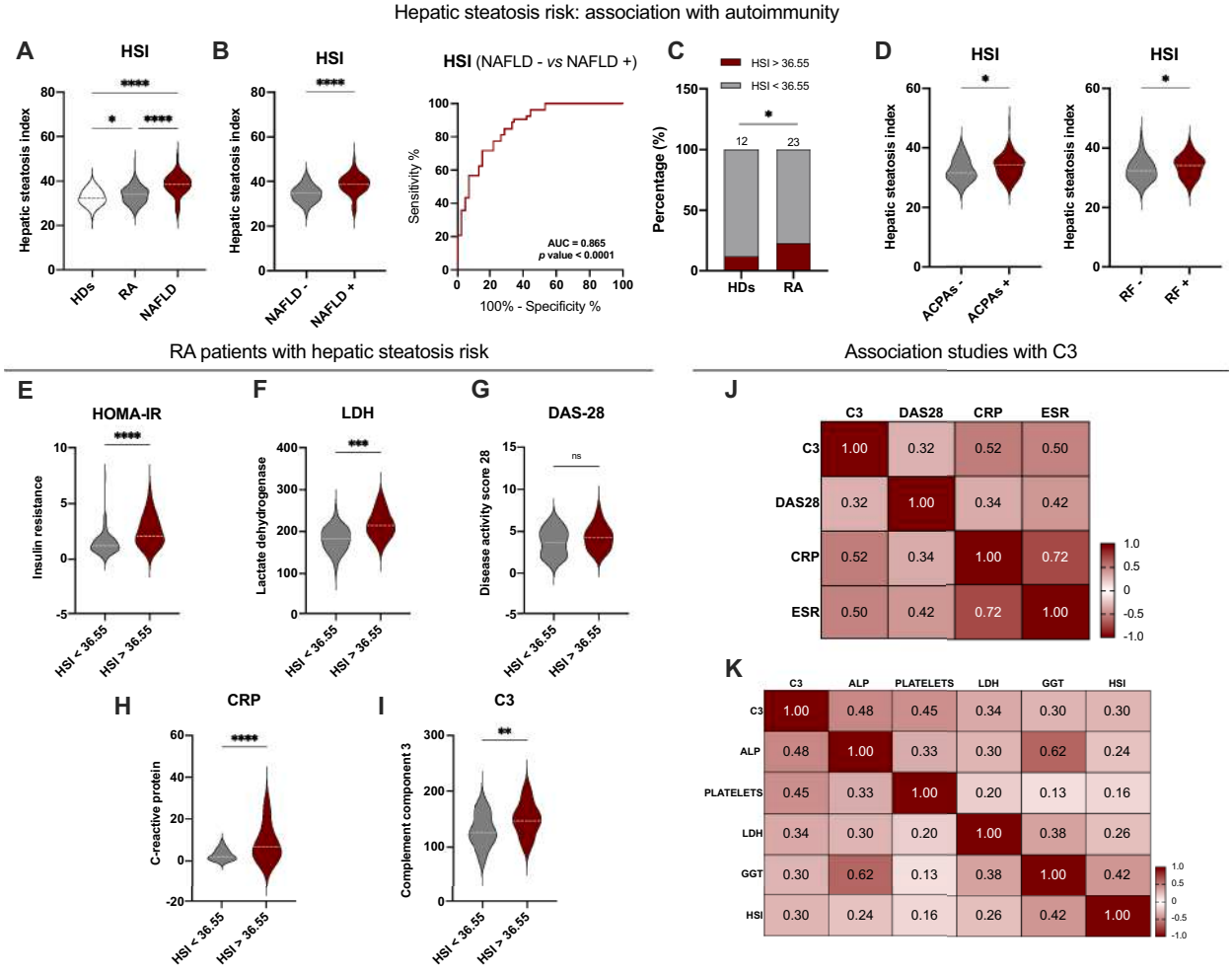

Fig. 1. Hepatic steatosis risk in RA patients: association studies with insulin resistance state and disease activity. (A) Levels of HSI in HDs, RA and NAFLD patients. (B) Levels of HSI in NAFLD patients with respect to HDs and ROC curve analyses to discriminate between the two groups. (C) Percentage of RA patients and HDs with liver steatosis risk. (D) Association studies of HSI with autoimmunity. (E) Comparative analysis of HOMA-IR between RA patients with low and high NAFLD risk. (F) Comparative analysis of LDH between RA patients with low and high NAFLD risk. (G) Comparative analysis of DAS-28 levels between RA patients with low and high NAFLD risk. (H) Comparative analysis of CRP levels between RA patients with low and high NAFLD risk. (I) Comparative analysis of component C3 levels between RA patients with low and high NAFLD risk. (J) Correlation matrix between component C3 levels and acute phase reactants and disease activity. (K) Correlation matrix between component C3 levels and liver disease biomarkers and HSI. RA: Rheumatoid Arthritis; HSI: Hepatic Steatosis Index; HDs: healthy donors; NAFLD: non-alcoholic fatty liver disease; ROC: receiver operating characteristic; HOMA-IR: homeostatic model assessment- insulin resistance; LDH: lactate dehydrogenase; DAS-28: disease activity score 28; CRP: c-reactive protein; C3: complement component 3; ALP: alkaline phosphatase; GGT: γ-glutamil transferase. Violin plots: lines represent the median value. *Significant differences: $p < 0.05$; **Significant differences: $p < 0.01$; ***Significant differences: $p < 0.001$; ****Significant differences: $p < 0.0001$.

the four indexes compared to RA patients and HDs (Supplemental figure 1 and Fig. 1A).

ROC curve analysis was conducted to compare NAFLD patients with a group of healthy individuals (HDs) consisting of 50 participants, all of whom had NAFLD confirmed by ultrasound. As illustrated in Supplemental figure 1 and Fig. 1B, the ROC curves of the four indexes under study were examined, and the results revealed that the HSI index demonstrated the highest efficiency in distinguishing between NAFLD and HDs, with the area under the curve of 0.865 (p-value < 0.0001) and a cut-off value of 36.55. Applying this cut-off value to the cohort of HDs and RA patients, we could observe that 23% of RA patients exhibited significantly higher HSI above the cut-off displaying a positive predictive value for NAFLD compared to HDs (12%) (fold-change = 1.91) (Fig. 1C).

Of note, levels of HSI were significantly associated with the presence of autoimmunity (levels of ACPAs and Rheumatoid factor) (Fig. 1D), suggesting a potential impact of the autoimmune component in liver function.

Significantly elevated levels of HOMA-IR, LDH, CRP, and component C3 were observed in the RA-NAFLD risk group (HSI<36.55) (Fig. 1E, F, G, and I). Disease activity score (DAS-28) was also elevated in the RA-NAFLD risk group, although did not reach statistical significance.

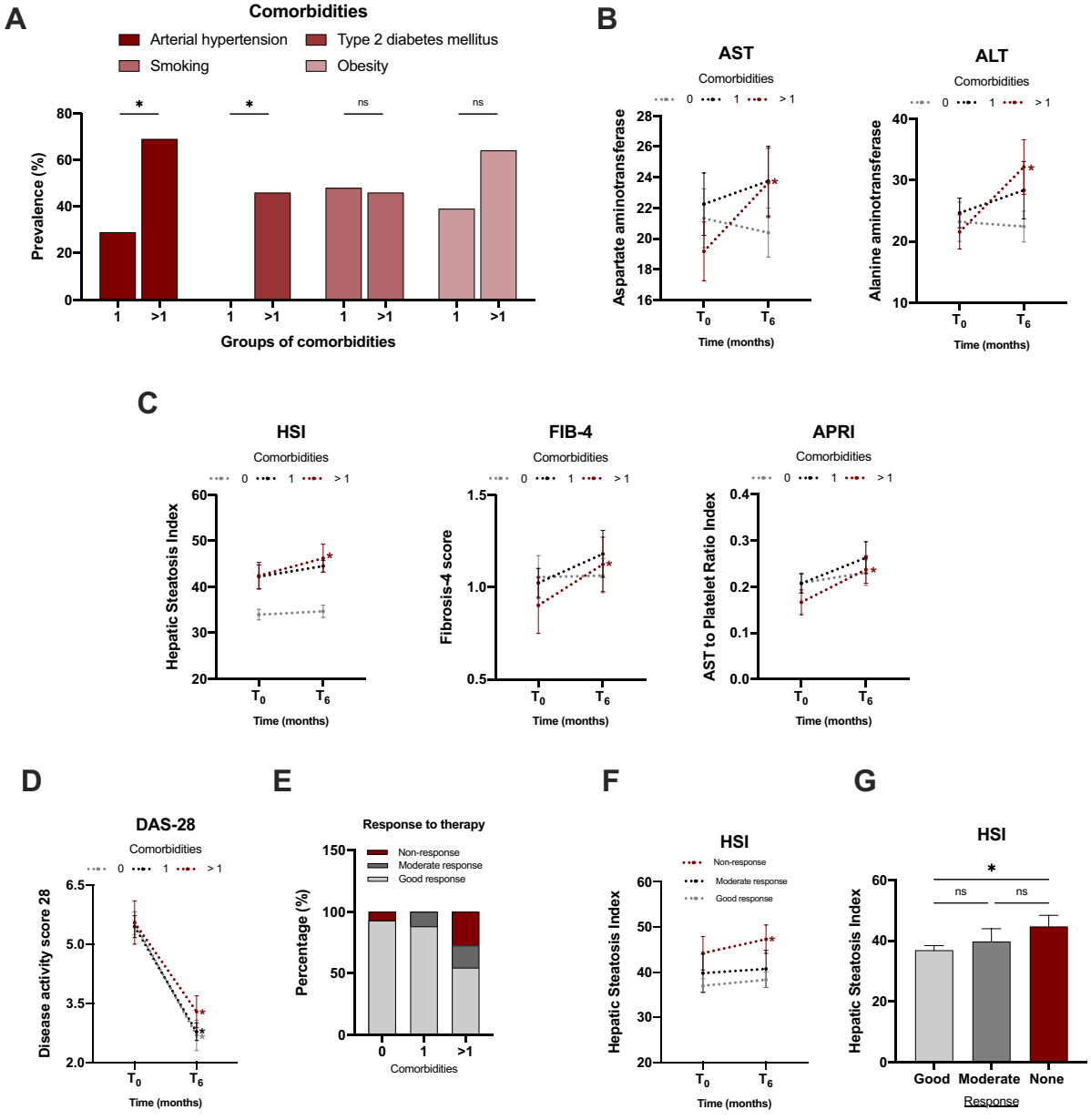

Fig. 2. Effect of methotrexate treatment in RA patients depending on the presence of CV risk factors (0, 1 or more than 1 comorbidities). (A) Prevalence of CV risk factors depending on the group (1 or more than 1). (B) Effect of methotrexate on the levels of transaminases in the three groups of comorbidities after 6 months. (C) Effect of methotrexate on the hepatic steatosis and fibrosis indexes in the three groups of comorbidities after 6 months. (D) Effect of methotrexate on the levels of DAS-28 in the three groups of comorbidities after 6 months. (E) Percentage of RA patients with non-response, moderate and good response in the three groups of comorbidities. (F) Effect of methotrexate on the levels of HSI depending on the group of response to therapy. (G) Levels of basal HSI in the groups of RA patients based on the response to therapy. RA: rheumatoid arthritis; AHT: arterial hypertension; T2DM: type 2 diabetes mellitus; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; HSI: hepatic steatosis index; FIB-4: fibrosis-4 score; APRI: AST to platelet ratio index; DAS-28: disease activity score 28. Error bars represent standard deviation of the mean. *Significant differences: $p < 0.05$; ns: non-significant differences.

There was no difference in the treatments, sex, or age between both groups of NAFLD risk (data not shown).

Interestingly, component C3 in RA patients significantly correlated with disease activity and inflammatory markers (Fig. 1J) in parallel with classical liver disease biomarkers, such as ALP, platelet counts, LDH and GGT, and the hepatic steatosis index (Fig. 1K), pointing out the potential role of the component C3 as liver abnormality biomarker in RA.

3.3. The influence of methotrexate treatment on RA patients

We analyzed the effect of methotrexate in newly-diagnosed RA patients after 6 months of treatment. Taking into account the importance of cardiovascular comorbidities in the development of liver disease, RA patients from a prospective cohort were classified into 3 groups depending on the prevalence of arterial hypertension (ATH), obesity, type 2 diabetes mellitus (T2DM), and smoking habit: none comorbidities (0) ($n = 17$), with one comorbidity (1) ($n = 21$) and more than 1 comorbidity (>1) ($n = 13$) (Supplemental table II). The prevalence of comorbidities and smoking habits in each group of RA patients treated with methotrexate are displayed in Fig. 2A. Interestingly, patients without comorbidities did not show significant alterations in classical liver disease biomarkers, AST and ALT and indexes to evaluate the risk for hepatic steatosis or fibrosis (HSI, APRI, and FIB-4) after 6 months of treatment (Fig. 2B-C). On the other hand, RA patients with more than 1 comorbidity showed significantly increased levels of transaminases, HSI, APRI, and FIB-4 after the treatment (Fig. 2B-C). These changes in liver markers were not influenced by the dosage of methotrexate administered to the patients. The observed dosages across the groups were relatively consistent: 16.2 ± 4.15 mg in patients without comorbidities, 16.07 ± 4.45 mg in patients with one comorbidity, and 16.36 ± 6.36 mg in patients with more than one comorbidity.

These results suggest the potential deleterious effect of methotrexate in the liver occurred in patients with cardiometabolic risk.

Disease activity was significantly reduced in the three groups independently of cardiometabolic profile (Fig. 2D), however, RA patients with more than 1 comorbidity showed lower reduction of DAS-28 (mean difference of −2.255) compared to the other groups which showed a mean difference of −2.670 in patients with one comorbidity and a mean difference of −2.846 for patients with none comorbidity.

Then, according to the EULAR response criteria, RA patients were divided into good, moderate, or non-responders. Thus, RA patients with no comorbidities showed the highest rate of good response (92.9%), followed by RA patients with one comorbidity (88.2%) and RA patients with more than one comorbidity (54.5%) (Fig. 2E). Of note, RA non-responder patients showed significantly higher levels of HSI after 6 months of treatment (Fig. 2F). The dose of methotrexate was not statistically different between both groups: responder and non-responder. Finally, we also observed a significant increase of HSI at baseline in non-responder patients compared to good responders (Fig. 2G) suggesting its potential role as a marker of therapeutic response.

3.4. Pathogenic effects of IgG-ACPAs on hepatocytes

Given the pathogenic role of ACPAs in RA [20] and after observing the potential impact of the autoimmune component in liver function, we next aimed to analyze the effects of enriched IgG-ACPAs in a cell line of hepatocytes (HepG2). After 48 h of treatment, IgG-ACPAs clearly altered the expression of genes related to glucose and lipid metabolism, inflammation and fibrosis, and apoptotic processes (Fig. 3A). Also, the expression of 27 apoptosis-related proteins was significantly altered after the IgG-ACPAs treatment compared to IgG-NHS (Fig. 3B-D).

To further explore the effects of IgG-ACPAs inducing inflammation in hepatocytes, the levels of 92- inflammatory proteins were analyzed in the supernatant of the HepG2 hepatocytes after the treatment with IgGs-ACPAs for 48 h. Thus, IgG-ACPAs promoted a significant increase in the levels of 51 out of 92 inflammation-related proteins compared to IgG-NHS (Fig. 3E, F). Finally, complement C3 supernatant levels were also significantly increased after the treatment with IgG-ACPAs compared to IgG-NHS (Fig. 3G).

3.5. Effect of arthritis on the liver function: mouse model of arthritis

The CIA mouse model showed an elevation in the plasma levels of complement C3, TNF-α, leptin, and a significant reduction of plasma levels of adiponectin (Supplemental figure 2A), in parallel with a systemic insulin resistance state (Supplemental figure 2B) [21]. Besides, the mRNA expression profile revealed an alteration in the genes involved in glucose and lipid metabolisms (AKT, IRS-2, GLUT-2, and LPL) (Fig. 4A). In parallel, we found overexpression of genes related to the presence of B cells (CD-20) and M1 macrophage polarization state (pro-inflammatory state) (TNF-α and CD-11c) and the downregulation of CD-209 (M2 macrophage polarization state (anti-inflammatory)) (Fig. 4B). The presence of immune cells was confirmed by immunohistochemistry revealing a chronic mild to moderate portal and intralobular inflammation (Fig. 4C). Besides, an increase in the expression of genes related to apoptosis and cell stress was observed in the liver of CIA mice (Fig. 4D). Parallelly, a mouse-apoptosis-related proteins array was performed. Thus, 11 out of the 21 proteins included in the analyses were significantly altered in CIA mice compared to the control group (Fig. 4E). The evidence of apoptosis in this tissue was also confirmed due to the increased presence of positive cells for BCL-2/6 in the hepatic tissue of CIA (Fig. 4F). Also, genes related to fibrotic processes were significantly increased in the CIA liver (Fig. 4G). Next, steatosis and fibrosis histology analyses were performed. Thus, the assessment of liver fat in CIA mice revealed marked hepatocellular fat accumulation in CIA mice (17%) (Fig. 4H, I). We then analyzed the presence of fibrosis. CIA mice presented an F1 stage (75%) and F2 stage (25%) compared to the control group with no fibrosis (F0) suggesting the presence of minimal scarring or scarring extend outside the liver area (Fig. 4H, J). These results demonstrated the profound alterations induced by arthritis in the liver promoting the extravasation of immune cells which in turn generate inflammation, and insulin resistance alongside increased apoptotic and fibrotic processes in this tissue.

4. Discussion

It is well recognized direct association between NAFLD and obesity or diabetes [22,23], and it should be noted that NAFLD-associated metabolic comorbidities are increased in RA. We aimed to analyze the direct influence of arthritis on hepatic function avoiding these confounder metabolic factors. Thus, obesity and diabetes comorbidities were excluded from the cross-sectional study.

Several serological indexes have been developed to test liver damage risk [24]. Among the four indexes analyzed in our study, only levels of HSI were increased in RA patients compared to HDs. We used the cohort of non-obese/T2DM NAFLD patients non-RA and HDs (diagnosed by ultrasound) to test the efficacy of the HSI index discriminating NAFLD patients. ROC curve analysis showed that HSI could identify patients with NAFLD with an AUC of 0.86 and a cut-off value of 36.55. HSI has recently been created to evaluate the hepatic steatosis risk [17]. In RA, there is no previous evidence of the use of HSI. Using that cut-off value, the percentage of the non-obese/T2DM RA patients with HSI levels above the cut-off value for discriminating NAFLD was significantly increased compared to HDs. Those patients had increased inflammation, levels of autoantibodies, and disease activity, which might indicate that in addition to metabolic comorbidities, clinical features of RA can be associated with liver damage. Of note, it was identified for the first time an association of autoimmunity with HSI due to the increased levels of ACPAs and RF in RA patients.

ACPAs target several cells involved in the pathogenesis of RA [25], although their effects on the hepatocytes have not previously been reported. The citrullination process is the key pathogenic mechanism in

RA [26]. It is worth mentioning that citrullinated proteins have been described in liver fibrosis [27] and in the hepatocyte cell line (HEPG2) [28]. In this sense, our *in vitro* studies showed that ACPAs antibodies can act on the hepatocyte, promoting the release of C3 and inflammatory-related proteins in parallel with deleterious effects on glucose and lipid metabolism, and inducing fibrotic and apoptotic processes. These novel data might indicate a new pathogenic mechanism of ACPAs in hepatocytes.

To our knowledge, this is the first work deeply focused on the study of liver tissue in arthritic mice. In this line, a recent study reported that

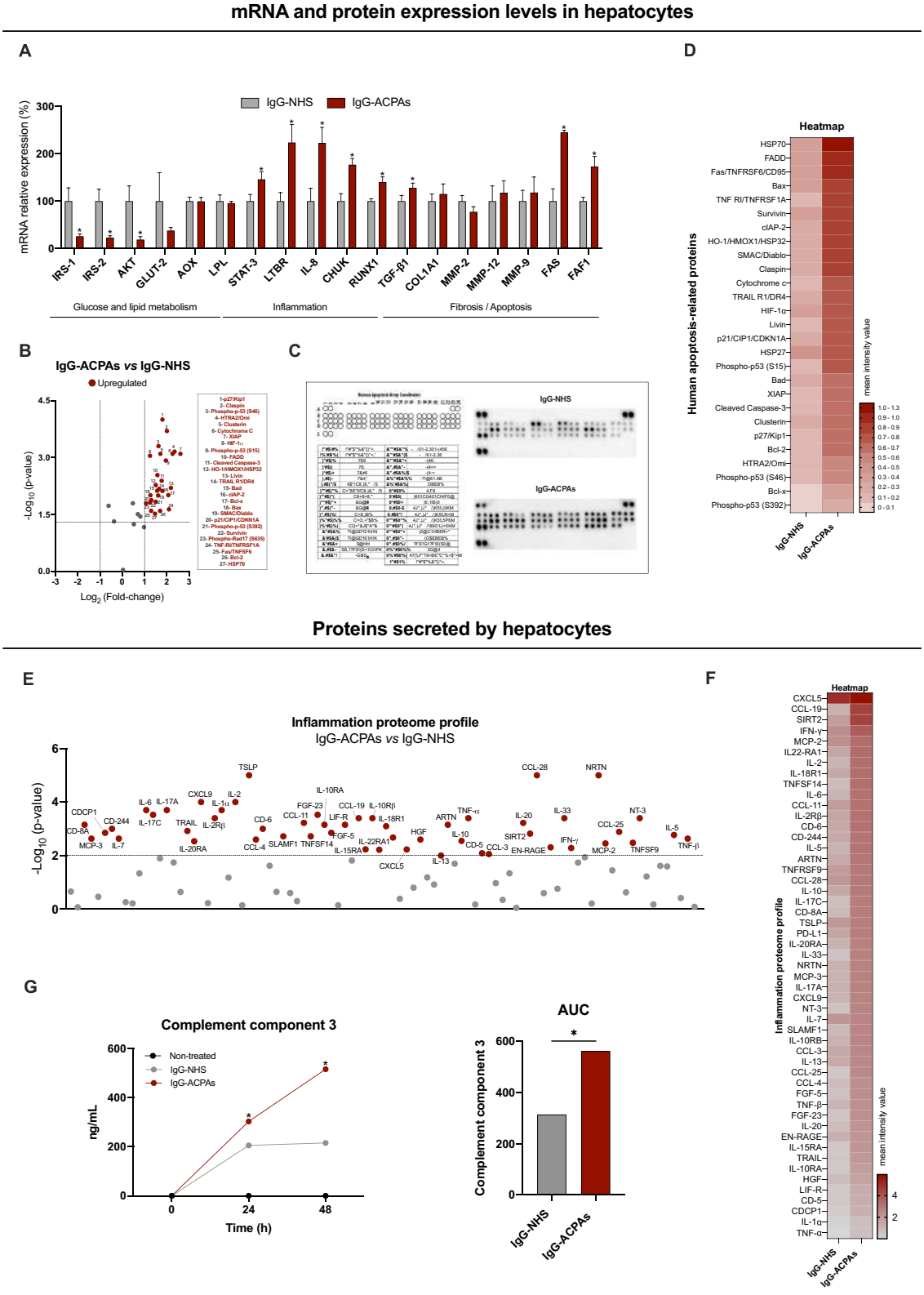

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Fig. 3. Effect of IgG-ACPAs on hepatocytes. (A) mRNA expression of genes related to glucose and lipid metabolisms, inflammation and fibrosis and apoptosis processes after the treatment with IgG-ACPAs compared to IgG-NHS. (B) Apoptosis-related proteins altered after the treatment with IgG-ACPAs compared to IgG-NHS. (C) Human apoptosis array coordinates: template of proteins analyzed. (D) Heatmap of proteins altered after the treatment with IgG-ACPAs *versus* IgG-NHS. (E) Levels of inflammatory proteins (96 target inflammation, Olink) secreted by hepatocytes after the treatment with IgG-ACPAs compared to IgG-NHS. (F) Heatmap of proteins significantly altered after the treatment with IgG-ACPAs compared to IgG-NHS. (G) Component C3 secreted by hepatocytes after the treatment with IgG-ACPAs compared to IgG-NHS 24 h −48 h and area under the curve. IgG: immunoglobulin G; ACPAs: antibodies to citrullinated protein antigens; NHS: normal human serum; C3: complement component 3. IRS-1: insulin receptor substrate 1; IRS-2: insulin receptor substrate 2; AKT: kinase protein B; GLUT-2: glucose transporter type 2; AOX: alternative oxidase; LPL: lipoprotein lipase; STAT-3: signal transducer and activator of transcription 3; LTBR: lymphotoxin beta receptor; IL-8: interleukin 8; CHUK: component of inhibitor of nuclear factor kappa B kinase complex; RUNX1: runt-related transcription factor 1; TGF-b1: TGF-β: transforming growth factor beta 1;; COL1A1: collagen type I alpha 1 chain; MMP-2: matrix metallopeptidase 2; MMP-12: matrix metallopeptidase 12; MMP-9: matrix metallopeptidase 9; FAS: fas cell surface death receptor; FAF1: fas associated factor 1; p27/Kip1: cyclin-dependent kinase inhibitor P27; HTRA2/Omi: high temperature requirement protein A2; XIAP: X-linked inhibitor of apoptosis; HIF-1α: hypoxia inducible factor 1 subunit alpha; FADD: fas associated via death domain; HO1/HMOX1/HSP32: heme oxygenase 1; TRAIL-R1/DR4: tumor necrosis factor-related apoptosis inducing ligand receptor 1; BAD: BCL-2 associated agonist of cell death; cIAP-2: baculoviral IAP repeat containing 3; BCL-x: b-cell lymphoma x, apoptosis regulator; BAX: BCL-2 associated X, apoptosis regulator; SMAC/Diablo: second mitochondria-derived activator of caspase/diablo IAP-binding mitochondrial protein; p21/CIP1/CDKN1A: cyclin dependent kinase inhibitor 1A; TNF-RI: tumor necrosis factor receptor type 1; HSP70: heat shock protein 70. C3: complement component 3; CXCL5: C-X-C motif chemokine ligand 5; CCL- C—C motif chemokine ligand; SIRT2: sirtuin; IFN-γ: interferon gamma; MCP-: monocyte chemotactic protein; IL-: interleukin; -RA1: receptor subunit alpha 1; TNFSF14: TNF superfamily member 14; Rβ: receptor beta; CD: cluster differentiation; ARTN: artemin; TNFRSF9: TNF receptor superfamily member 9; TSLP: thymic stromal lymphopoieitin; PD-L1: programmed cell death 1 ligand 1; NRTN: neurturin; NT-3: neurotrophin 3; SLAMF1: signaling lymphocytic activation molecule family member 1; FGF-: fibroblast growth factor; TNF-β: tumor necrosis factor beta; EN-RAGE: extracellular newly identified RAGE-binding protein; HGF: hepatocyte growth factor; LIF-R: leukemia inhibitory factor receptor; CDCP1: CUB domain containing protein 1. AUC: area under the curve. Error bars represent standard deviation of the mean. *Significant differences: $p < 0.05$.

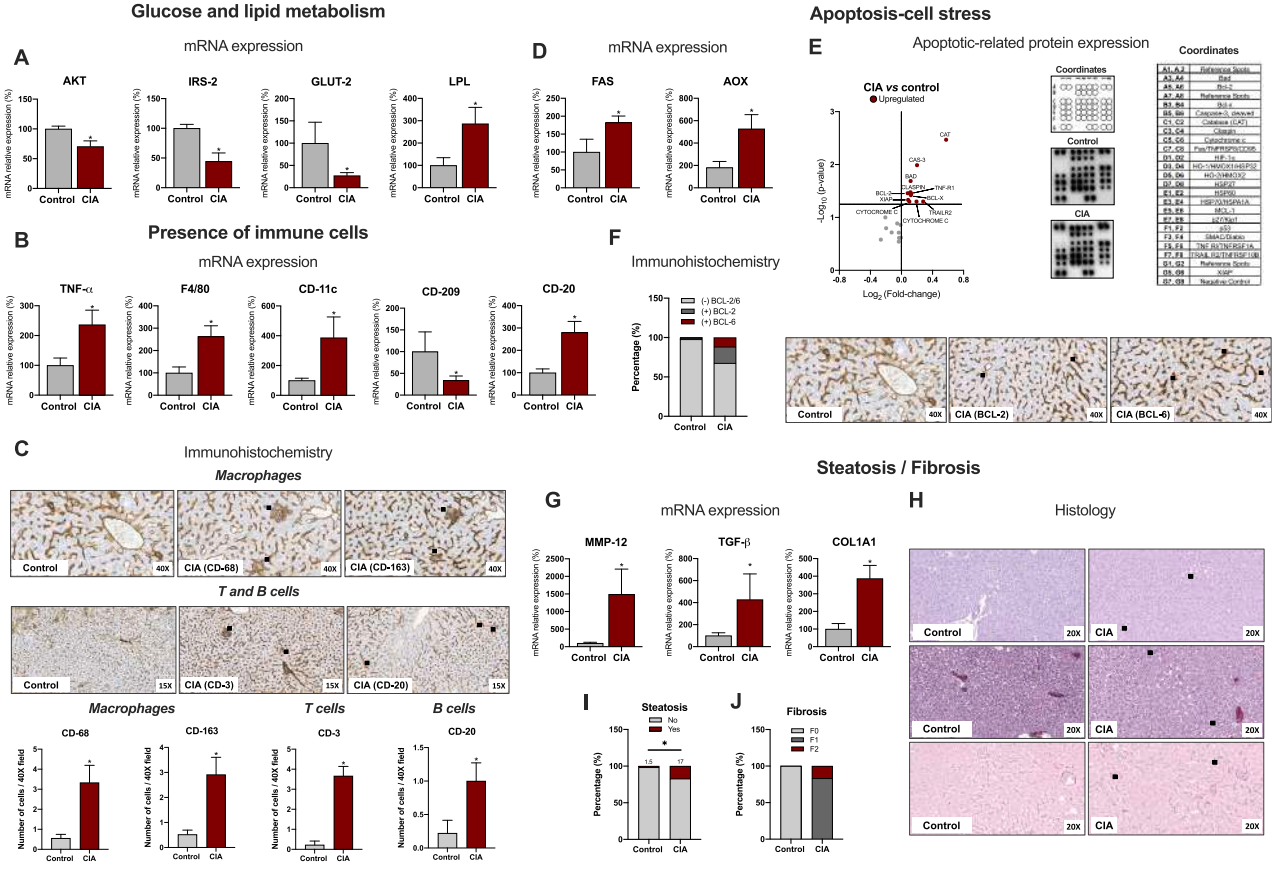

Fig. 4. Effect of arthritis on the liver function in CIA mice. (A) Expression of genes associated with glucose and lipid metabolisms in the liver of CIA mice compared to control mice. (B) Expression of genes related to the presence of immune cells in the liver of CIA mice compared to control mice. (C) Immunohistochemistry of liver: macrophages and T and B cells markers and quantification of the number of positive cells. (D) Expression of genes related to apoptosis and cell stress. (E) Volcano plot of apoptosis-related proteins, template and coordinates. (F) Immunohistochemistry of liver: markers of apoptosis and percentage of positive cells. (G) Expression of genes related to fibrosis. (H) Histology of liver: steatosis and fibrosis. (I) Quantification of hepatic steatosis. (J) Quantification of hepatic fibrosis. CIA: collagen-induced arthritis; AKT: kinase protein B; IRS-2: insulin receptor substrate 2; GLUT-2: glucose transporter type 2; LPL: lipoprotein lipase; FAS: fas cell surface death receptor; AOX: alternative oxidase; TNF-α: tumor necrosis factor alpha; F4/80: EGF-like module-containing mucin-like hormone receptor-like 1 also known as F4/80; CD-: cluster differentiation; CAT: catalase; CAS-3: caspase-3; BCL-2: B-cell lymphoma 2, apoptosis regulator; BAD: BCL-2 associated agonist of cell death; TNF-R1: tumor necrosis factor-receptor 1; XIAP: X-linked inhibitor of apoptosis; TRAIL-R2: tumor necrosis factor-related apoptosis inducing ligand receptor 2; MMP-12: matrix metallopeptidase 12; TGF-β: transforming growth factor beta 1; COL1A1: collagen type I alpha 1 chain. Error bars represent standard deviation of the mean. *Significant differences: $p < 0.05$.

CIA mice are more susceptible to suffering oxidative damage in the spleen and liver [29]. We found that arthritis directly affects liver tissue promoting inflammation, IR, steatosis, fibrosis, and apoptosis. Interestingly, we observed an increase in leptin and a reduction in adiponectin serum levels. Such changes may influence or be associated with the development of metabolic syndrome and NAFLD.

These findings align with results in non-diabetic RA patients. A positive correlation was observed between body mass index and serum leptin levels, indicating a manifestation of adiposity [30]. Moreover, in those patients, high-grade inflammation was negatively correlated with circulating adiponectin concentrations, while low adiponectin levels were associated with metabolic syndrome features [31]

While immune cell extravasation is well-documented in human liver inflammation [32], no prior research has explored immune cell presence in liver tissue during arthritis. Thus, we found a high presence of T and B cells and macrophages in the liver of CIA compared to control mice suggesting the role of lymphocytes and macrophages in the dysfunction of the liver in the context of arthritis.

Regarding liver damage in RA, the main concern is hepatic fibrosis linked to methotrexate use. A recent study by Bafna et al. [33] on 75 RA patients found that 16% showed increased liver stiffness with long-term methotrexate treatment. Another study on RA patients after 8 years of methotrexate follow-up reported only a small number of patients with elevated ALT levels above the normal limit [34]. Additionally, Darabian et al. demonstrated that liver fibrosis did not significantly correlate with cumulative methotrexate dosage [35]. However, they did identify significant correlations between Fibroscan score and BMI [35].

It is well known the interplay between CV risk factors and hepatic alterations. In fact, arterial hypertension, dyslipidemia, obesity, and insulin resistance are frequently accompanied by NAFLD [36]. Thus, due to the controversial results and the potential impact of CV risk factors on hepatic function, our strategy was to stratify RA patients to analyze the influence of methotrexate in the liver function taking into account cardiovascular comorbidities. Firstly, disease activity was significantly reduced independently of the number of comorbidities. On the contrary, the mean difference of DAS-28 was less due to the high presence of none or moderate responders in these groups in parallel with an elevated prevalence of cardiovascular comorbidities, which is in line with previous studies [37,38]. This group of RA patients with high CVD risk showed more sensitivity to suffering liver steatosis or fibrosis influenced by methotrexate treatment due to the increased levels of transaminases and hepatic steatosis (HSI) and fibrosis (FIB-4 and APRI) predictors after six months of treatment. However, RA patients without comorbidities would benefit from the treatment of methotrexate by reducing disease activity in parallel with no alterations in steatosis and fibrosis predictors. In addition, this is the first study that shows that HSI was not only elevated at baseline but also after 6 months in the non-responder group suggesting its potential as a predictor of response to therapy with methotrexate.

A key result was that RA patients with a high presence of CV risk factors would present a deleterious effect of methotrexate treatment, supporting that CV risk factors should be taken into account in clinical practice. Finally, the hepatic steatosis index could be monitored to identify potential RA patients with NAFLD and those who might not respond to methotrexate treatment.

Taken together, our research highlights the significance of the liver in RA patients, even in the absence of metabolic comorbidities, and the direct impact of ACPAs on liver biology. Through three distinct approaches - human cross-sectional and longitudinal studies, a mouse arthritis model, and *in vitro* experiments - we found that the livers of RA patients may exhibit mild to moderate inflammation due to the presence of T and B cells and macrophages, with direct effects of autoantibodies. These changes are independent of obesity or diabetes and are associated with systemic inflammatory burden, disease activity, and autoantibody levels. While these alterations may be subclinical, our study recommends monitoring liver health during clinical practice, particularly considering clinical features linked to NAFLD in RA. Additionally, our work contributes to the ongoing debate on the effects of methotrexate, suggesting its potential influence on liver function in the presence of cardiovascular risk factors.

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Ethics approval

This study involves human participants and was approved by the ethics committee of the Reina Sofia Hospital in Cordoba, Spain. Participants gave informed consent to participate in the study before taking part. The study was conducted in accordance with good clinical practice guidelines.

CRediT authorship contribution statement

I Arias-de la Rosa: Conceptualization, Formal analysis, Investigation, Writing – original draft. **M Ruiz-Ponce:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **L Cuesta-López:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **C Pérez-Sánchez:** Investigation, Methodology. **F Leiva-Cepas:** Methodology. **MD Gahete:** . **P Navarro:** Methodology. **R Ortega:** Investigation. **J Cordoba:** Supervision. **E Pérez-Pampin:** Methodology. **A González:** Investigation. **AJ Lucendo:** Writing – review & editing. **E Collantes-Estévez:** Supervision, Writing – review & editing. **Ch López-Pedrera:** Investigation. **A Escudero-Contreras:** Funding acquisition, Conceptualization, Writing – review & editing. **N Barbarroja:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

None of the authors has any conflicts of interest, financial or otherwise to disclosure.

Data availability

The lead contact will provide the data upon request for the information presented in this paper. No original code was reported in this study. For any further details required to reanalyze the data, interested parties can contact the lead author.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejim.2023.08.002.

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