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Decoding clinical and molecular pathways of liver dysfunction in Psoriatic Arthritis: Impact of cumulative methotrexate doses

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ABSTRACT

Background: The occurrence of liver abnormalities in Psoriatic Arthritis (PsA) has gained significant recognition. Identifying key factors at the clinical and molecular level can help to detect high-risk patients for non-alcoholic fatty liver disease in PsA.
Objectives: to investigate the influence of PsA and cumulative doses of methotrexate on liver function through comprehensive *in vivo* and *in vitro* investigations.
Methods: A cross-sectional study involving 387 subjects was conducted, 200 patients with PsA, 87 NAFLD-non-PsA patients, and 100 healthy donors (HDs), age and sex-matched. Additionally, a retrospective longitudinal study was carried out, including 83 PsA patients since initiation with methotrexate. Detailed clinical, and laboratory parameters along with liver disease risk were analyzed. *In vitro,* experiments with hepatocyte cell line (HEPG2) were conducted.
Results: PsA patients present increased liver disease risk associated with the presence of cardiometabolic comorbidities, inflammatory markers, onychopathy, and psoriasis. The treatment with PsA serum on hepatocytes encompassed inflammatory, fibrotic, cell stress, and apoptotic processes. At the molecular level, methotrexate impacts liver biology, although the cumulative doses did not affect those alterations, causing any potential damage to liver function at the clinical level. Finally, anti-PDE-4 or anti-JAK decreased the inflammatory profile induced by PsA serum on hepatocytes.
Conclusion: 1)This study identifies the complex link between liver disease risk, comorbidities, and disease-specific features in PsA patients. 2)Methotrexate dose in PsA patients had no significant effect on liver parameters,

Abbreviations: PsA, psoriatic arthritis; NAFLD, non-alcoholic fatty liver disease; anti-PDE-4, phosphodiesterase 4 inhibitors; anti-JAK, jakus kinase inhibitors; HDs, healthy donors; HEPG2, hepatocyte cell line; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HT, hypertension; T2DM, type 2 diabetes mellitus; IR, insulin resistance; DAPSA, Disease Activity for Psoriatic Arthritis; BMI, body mass index; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides; Apo-A, apolipoprotein A; Apo-B, apolipoprotein B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; LDH, lactate dehydrogenase; C3, complement components 3; FIB-4, fibrosis-4 score; APRI, AST-Platelet ratio index; TyG, triglyceride and glucose index; HSI, hepatic steatosis index; HOMA-IR, the homeostatic model assessment index-insulin resistance; MEM, minimum essential medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; ROC, receiver-operating characteristics curve; PsO, psoriasis; EMA, European Medicine Agency; RA, rheumatoid arthritis; CAP, Controlled Attenuation Parameter; JAK, Janus Kinase; TNF- α, tumour necrosis factor alpha; IL-6, interleukin-6.

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confirmed by hepatocyte *in vitro* studies. 3)Anti-PDE-4 and anti-JAK therapies show promise in reducing PsA serum-induced hepatocyte activation, potentially aiding liver complication management.

1. Introduction

Psoriatic arthritis (PsA) is a chronic inflammatory joint disease that affects either the peripheral or axial skeleton, characterized by the presence of psoriatic cutaneous plaques that can result in progressive bone destruction, leading to joint dysfunction and a decreased quality of life [1–4]. Additionally, PsA patients are at increased risk for a range of cardiometabolic complications, representing a leading cause of morbidity and mortality in this disease [5]. Thus, recent research has confirmed that patients with PsA are more likely to experience metabolic comorbidities, such as obesity, metabolic syndrome, hypertension (HT), type 2 diabetes mellitus (T2DM), dyslipidemia, and insulin resistance (IR), pointing out the possible dysfunction of adipose tissue in this disease [6,7]. Furthermore, levels of adipocytokines and cardiovascular disease-related molecules were found to be abnormal and correlated with disease activity and the presence of metabolic comorbidities [8].

Numerous investigations have reported a higher prevalence of non-alcoholic fatty liver disease (NAFLD) in patients with PsA in comparison to the general population [9–11]. NAFLD is associated with an increased risk for cardiovascular disease and often co-exists with specific metabolic alterations including obesity, IR, or high triglyceride levels. Thus, knowing the factors (at the clinical and molecular level) involved in the development of NAFLD in PsA could help to identify patients at higher risk, allowing patient stratification and early detection and management of liver disease.

The treatment strategy for PsA can have implications for liver function. Methotrexate, currently regarded as the gold standard therapy for PsA, has been extensively investigated for its potential hepatotoxic effects [12]. However, the research findings on this matter remain controversial [13,14]. Other therapeutic options, such as Apremilast, an anti-PDE-4 agent, have emerged as a promising approach for managing both inflammation and metabolic abnormalities in PsA [8,15]. Similarly, Janus Kinase (JAK) inhibitors have shown encouraging results in treating PsA [16]. However, the impact of these treatments on liver abnormalities in PsA is currently unknown, as there is a lack of available data.

In this study, we investigated the influence of PsA and cumulative doses of methotrexate on liver function, employing *in vivo* and *in vitro* approaches. Our research encompassed two distinct clinical studies: a cross-sectional study that compared PsA patients with individuals diagnosed with NAFLD and healthy donors (HDs), and a longitudinal study involving a retrospective cohort of PsA patients who had been receiving methotrexate treatment since its initiation. In addition, we conducted *in vitro* experiments using a hepatocyte cell line to elucidate the complex mechanisms underlying hepatocyte dysfunction in PsA. Furthermore, we evaluated the *in vitro* effects of methotrexate, anti-PDE-4, and anti-JAK treatments on the hepatocyte functionality, profoundly impacted by the disease.

2. Materials and methods

2.1. Patients

A cross-sectional study was performed on 387 subjects, 200 patients with PsA, 87 patients with NAFLD but not exhibiting any rheumatic disease (NAFLD-non-PsA), and 100 HDs matched for age and sex. PsA patients were recruited according to CASPAR criteria [17] at the Rheumatology Department of the Reina Sofia Hospital in Cordoba, Spain. Disease activity was assessed using the Disease Activity for Psoriatic Arthritis (DAPSA) score for joint involvement in PsA patients. None of the patients had a history of previous liver disease (viral, autoimmune, toxic or metabolic) or alcohol consumption (defined as >30 g of alcohol per day in men and more than 20 g per day in women).

The cumulative doses of methotrexate were retrospectively determined in a longitudinal study performed on 95 PsA patients treated with methotrexate since its initiation until the day of blood extraction by calculating the total milligrams per week administered over the treatment period. The mean cumulative doses of methotrexate were 1.98 ± 1.24 gr (minimum=0.04 gr; maximum=5.46 gr), while the mean duration of treatment was 6.16 ± 3.01 years.

Likewise, HDs with no history of immune-mediated inflammatory or liver diseases were recruited from the Hospital Universitario Reina Sofía de Córdoba.

A cohort of patients with NAFLD and no immune-mediated inflammatory disorder with similar values of T2DM, body mass index (BMI), age and sex as the PsA cohort was prospectively selected following an abdominal ultrasound study, due to previous evidence of altered liver function test, requested by a liver specialist physician according to clinical practice at the Hospital General de Tomelloso (Tomelloso, Spain).

All subjects recruited gave written informed consent, previously approved by the hospital ethics committee. The study was conducted following the principles outlined in the Declaration of Helsinki. Non-alcoholic fatty liver disease (NAFLD) was diagnosed after abdominal ultrasonography, excluding those with a history of causes secondary to autoimmune or viral hepatitis, Wilson's disease, haemochromatosis, alpha-1-antitrypsin deficiency, total parenteral nutrition, or previous or current heavy alcohol consumption. Abdominal ultrasound was performed with a Philips DH11 XE greyscale ultrasound with a 5 MHz convex transducer and interpreted by experienced hepatologists. Metrological data such as weight and height were collected to calculate the BMI of all participants. A complete clinical history, physical examination, and biochemical analysis were performed (Supplementary Table 1).

2.2. Samples and data collection

2.2.1. Blood sample collection and isolation of serum and plasma

Fasting peripheral venous blood samples were collected from patients with PsA, NAFLD, and HDs for subsequent plasma and serum isolation. Laboratory parameters such as 1) lipid profile (total cholesterol [TC], high-density lipoprotein [HDL], low-density lipoprotein [LDL], triglycerides [TG], glucose, insulin, apolipoprotein A [Apo-A] and apolipoprotein B [Apo-B]) were recorded; 2) classical liver disease biomarkers (alanine aminotransferase [ALT], aspartate aminotransferase [AST] and gamma-glutamyl transpeptidase [GGT], lactate dehydrogenase [LDH] and albumin); 3) inflammatory markers (C-reactive protein [CRP] and erythrocyte sedimentation rate [ESR]), platelets and complement components 3 (C3).

2.2.2. Assessment of the potential presence of liver disease

Several indexes to examine hepatic steatosis or fibrosis were evaluated:

Fibrosis-4 score (FIB-4): the FIB-4 score helps to estimate the amount of scarring in the liver using four variables: age (years), AST (U/L), platelet count (10^9/L), and ALT (U/L). Values below 1.3 show a low risk of advanced liver fibrosis, values between 1.3 and 2.67 suggest a moderate risk, and FIB-4 values above 2.67 indicate a high risk of advanced liver fibrosis [18].

The AST-Platelet Ratio Index (APRI): the APRI score is a tool for detecting hepatic fibrosis that includes AST levels and platelet count: APRI= [(AST(U/L)/upper limit of normal AST range) x 100]/Platelet

count [19].

Triglyceride and glucose index (TyG): the TyG index is a method to detect insulin resistance [20] and hepatic steatosis [21] whose equation is TyG=ln [fasting triglycerides (mg/dL) x fasting glucose (mg/dL)]/2.

Hepatic Steatosis Index (HSI): the HSI is a screening tool to manage NAFLD that includes the patient's sex, BMI, AST, ALT, and the presence of T2DM. The equation is: HSI = 8 x ALT (U/L) /AST (U/L) + BMI (+2 if T2DM, +2 if female) [22,23].

2.2.3. Assessment of cardiovascular risk

HOMA-IR: to assess IR, the homeostatic model assessment index-insulin resistance (HOMA-IR) was used, whose equation makes use of glucose and insulin levels: [insulin concentration (mU/L) × glucose concentration (mg/dl)]/40 [24,25].

T2DM: patients with T2DM were identified by rapid blood glucose levels > 126 mg/dl, haemoglobin A1c level > 6.5 %, or anti-diabetic treatment.

BMI: normal weight (BMI ranges from 18.5 to 24.9 kg/m^2), overweight (BMI ranges from 25 to 29.9 kg/m^2), and obese (BMI >30 kg/m^2) using weight (kg) and height (m^2) [26].

2.2.4. In vitro experiments with HEPG2 cell line

In vitro experiments were performed on the HEPG2 hepatocyte cell line. Cells were cultured in Minimum Essential Medium (MEM) (ThermoFisher, Walthman, United States) at 10 % Fetal Bovine Serum (FBS), 1 % sodium pyruvate (Merk KGaA, Darmstadt, Germany) and 1 % ZellShield (Minerva Biolabs GmbH, Berlin, Germany) at 37 % and 5 % CO2. Cells were seeded in 12-well plates at a density of 100.000 cells per well in 1 mL of MEM medium with 10 % FBS. These cells were treated with serum from HDs or PsA patients alone or in combination with methotrexate (50 μM), anti-JAK (Tofacitinib-250 nM), or anti-PDE-4 (Roflumilast-1 nM) for 24 h. On the other hand, for the *in vitro* experiment with cumulative doses, hepatocytes were treated with methotrexate for 72 h with a final concentration of 50 μM, 100 μM, and 150 μM. Cells were collected for analysis of mRNA and proteome profiles.

We used STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) to identify the functional enrichment of altered molecular pathways induced by the serum of PsA patients in hepatocytes.

2.2.5. Inflammatory-related proteins measurement

An inflammatory panel of 92 proteins was measured using a proximity extension assay method [27], 96 Olink Target Inflammation panel (Supplementary Table 2) (Cobiomic Bioscience S.L, Córdoba, Spain).

2.2.6. RNA extraction and gene expression

Total RNA from HEPG2 cells was extracted with TRI reagent (Sigma) following the manufacturer's recommendations, and transcribed into cDNA. Gene expression was assessed by real-time PCR using the Light Cycler thermal cycler system (Roche Diagnostics, Indianapolis, Indiana, USA). Expression of genes of interest was corrected for the geometric average of β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-microglobulin and 36B4 using the BestKeeper tool [28]. Expression of genes related to fibrogenesis, lipid accumulation, insulin signaling, endoplasmic reticulum oxidative stress, and inflammation were analyzed. The sequences of the genes analyzed are included in supplementary Table 3.

2.3. Statistical analysis

The normal distribution of each variable was analyzed. To compare two independent groups, we used parametric (Student's unpaired t-test) or non-parametric test (Mann-Whitney rank sum text). To analyze qualitative data, Chi-squared tests were performed. For multiple comparisons, one-way analysis of variance (ANOVA) test or Kruskal-Wallis test was used. In addition, logistic regression models and receiver operating curves were performed to analyze the specificity and sensibility of the different models. GraphPad Prism version 9.0.1 were used for all the analysis.

3. Results

3.1. Subclinical liver alterations in PsA

Clinical and laboratory characteristics of PsA patients, NAFLD-non-PsA patients, and HDs are summarized in Supplementary Table 1. PsA patients exhibited moderate disease activity (DAPSA: 14.60 ± 10.26 (mean ± standard deviation) and a mean duration of the disease of 10 years. In addition, PsA patients presented significantly higher levels of BMI, acute phase reactants, insulin, TG, ALT, platelet count, and C3 compared to HDs. Furthermore, the levels of these modified variables did not demonstrate any association with the administered treatments, except for the treatment involving statins (Supplementary Table 4). Overall, NAFLD-non-PsA patients had significantly higher levels of glucose, TG, AST, ALT, and GGT and significantly lower levels of CRP compared to PsA patients, even matched for BMI and the presence of T2DM. No changes were observed in ALP, LDH, platelet count, and C3 between disease groups. As expected, NAFLD patients showed significantly higher values of BMI, glucose, TG, ALT, AST, ALP, GGT, LDH, platelet count, albumin, and C3 compared to HDs. These results showed a subclinical liver alteration in PsA compared to HDs, highlighting the relevance of the intrinsic disease components in PsA compared to NAFLD-non-PsA.

3.2. Increased hepatic steatosis risk in PsA patients is associated with clinical characteristics and cardiometabolic comorbidities

We conducted a study using a carefully matched cohort of non-PsA individuals with NAFLD. The matching was done based on age, sex, body mass index (BMI), and the presence of type 2 diabetes (T2DM) since these variables are used in the calculation of the HSI index. Our main objective was to evaluate the accuracy and determine the appropriate cut-off values of the HSI index for identifying NAFLD patients within this cohort of non-PsA individuals (matched with our PsA cohort) compared with a control group of healthy individuals who do not have NAFLD.

As expected, HSI was significantly elevated in NAFLD-non-PsA with respect to HDs (Fig. 1A). Next, to determine the importance of HSI for distinguishing NAFLD patients from HDs, receiver-operating characteristics (ROC) curve analysis was carried out. The results indicated that a cut-off value of HSI > 35.68 identifies patients with NAFLD with high specificity and sensitivity (AUC=0.865; $p < 0.0001$) (Fig. 1B). After obtaining this HSI cut-off value in a NAFLD-non-PsA cohort of similar BMI and diabetes rates of our cohort of PsA patients, it was used to identify potential PsA patients with steatosis. Thus, 66 % of PsA patients presented a significantly higher probability of having steatosis compared to 27 % of HDs (Fig. 1C). Clinical and analytical characteristics of the PsA groups with different liver disease risks are displayed in Table 1. There were no significant variations in the treatments administered across the different groups. Of note, the PsA patients with probability of having NAFLD (HSI>35.68) displayed higher levels of BMI, glucose, insulin, HOMA-IR, TG, AST, ALT, GGT, and C3 levels compared to those with low probability of having NAFLD (HSI<35.68) and consequently significant higher prevalence of obesity, insulin resistance, HT and T2DM (Fig. 1D). Parallelly, levels of acute phase reactants (CRP and ESR) were significantly elevated in the PsA-NAFLD likelihood group (Fig. 1E). Interestingly, the presence of onychopathy was significantly associated with PsA patients with high possibility of having liver disease (Fig. 1F), and the body surface area affected by psoriasis (PsO) was significantly increased in this group (Fig. 1G). These results suggest the strong association among different factors in the development of NAFLD in PsA patients including metabolic alterations, systemic inflammation, and clinical manifestations.

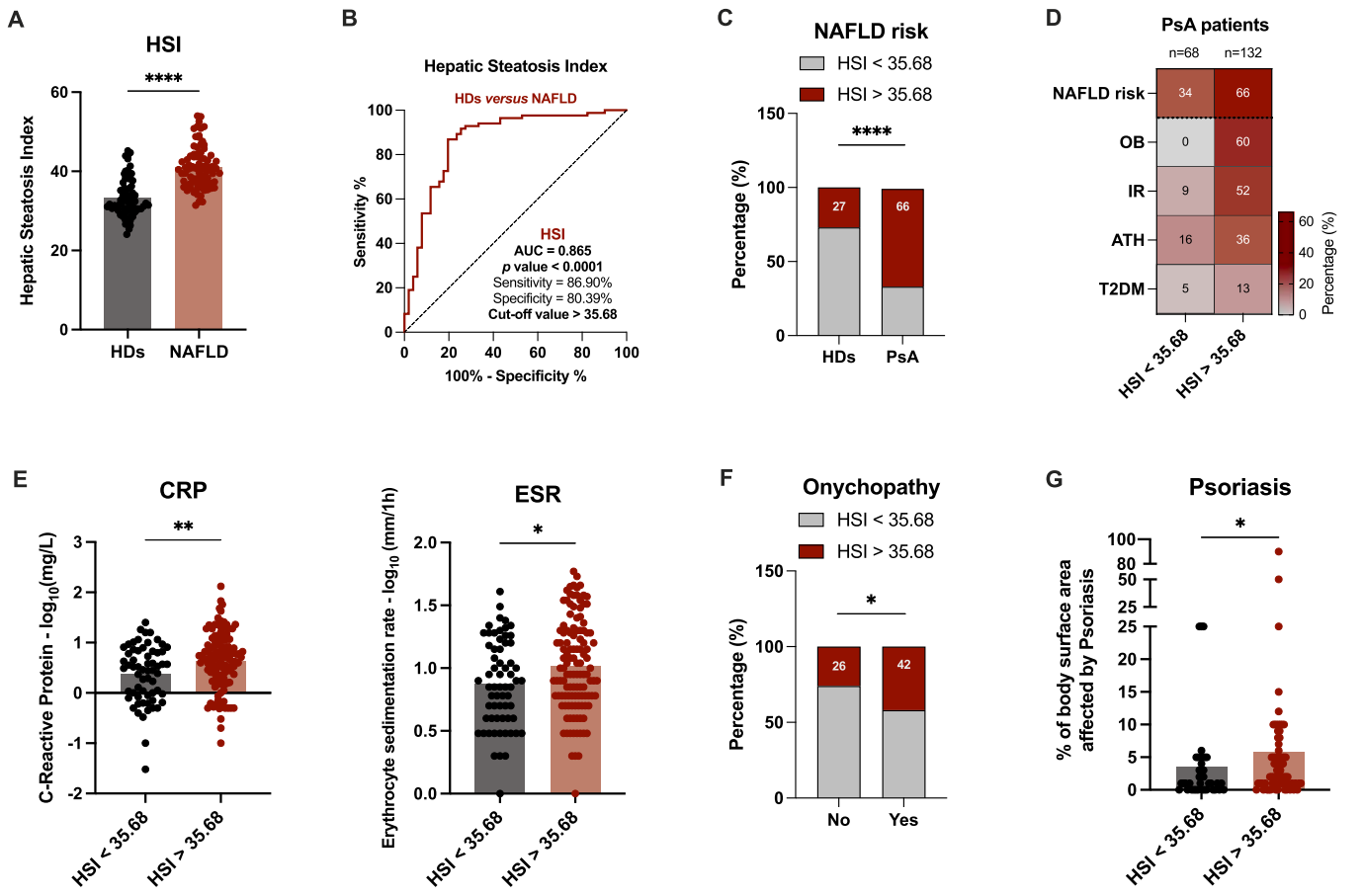


Fig. 1. Hepatic steatosis risk in PsA patients: association studies with clinical features and cardiometabolic complications. (A) Levels of HSI in NAFLD compared to HDs. (B) ROC curve of HSI to differentiate NAFLD respect to HDs. (C) Percentage of PsA patients with hepatic steatosis risk compared to HDs. (D) Prevalence of cardiometabolic comorbidities in PsA patients with HSI > 35.68 (with NAFLD risk) compared to PsA patients with HSI < 35.68 (without NAFLD risk). (E) Levels of CRP and ESR in PsA patients depending on NAFLD risk. (F) Percentage of PsA patients with onychopathy among groups of NAFLD (G) Percentage of psoriasis between groups of NAFLD risk. Quantitative data are presented as scatter dot plot with bars and individual values. Qualitative data are presented as a percentage (method to compute the *p* value: Chi-square test). Results from ROC curve are presented as percentage (method of Wilson/Brown). HDs: Healthy Donors; NAFLD: Non-alcoholic Fatty Liver Disease; HSI: Hepatic Steatosis Risk; OB: Obesity; IR: Insulin Resistance; ATH: Arterial Hypertension; T2DM: Type 2 Diabetes Mellitus; CRP: C-reactive protein; ESR: Erythrocyte Sedimentation Rate. (*) indicates significant differences with a *p* value < 0.05. (**) indicates significant differences with a *p* value < 0.01. (***) indicates differences with a *p* value < 0.001. (****) indicates significant differences with a *p* value < 0.0001.

3.3. In vitro effect of PsA serum on hepatocytes

After observing the increased probability of having liver disease in PsA patients compared to HDs, we next analyzed the effects of PsA serum in a cell line of hepatocytes (HEPG-2) to assess the direct impact of systemic inflammatory mediators in hepatocyte biology. Clinical characteristics of PsA patients and HDs included in the *in vitro* study are displayed in supplementary Table 5. These patients displayed moderate-to-high disease activity according to DAPSA score and significantly elevated levels of CRP and ESR compared to HDs. Additionally, an assessment of the inflammatory proteome was conducted on a serum pool obtained from both PsA patients and HDs to molecularly identify the dysregulated proteins associated with inflammation in these samples (Fig. 2A). Twenty-two inflammatory proteins were significantly altered in the serum of PsA patients compared to HDs (Fig. 2A).

Next, the levels of 26 proteins directly associated with cell stress were evaluated in the hepatocytes. Thus, the treatment with PsA serum promoted an increase in the levels of 22 out of 26 proteins compared to HDs serum (Fig. 2B).

Likewise, we also analyzed the levels of inflammatory-related proteins in extracts of hepatocyte cells after 24 h of PsA serum treatment. Thus, the treatment with PsA serum on hepatocytes significantly altered the expression of 24 inflammatory proteins compared to the treatment with HDs serum (Fig. 2C). Of note, the expression of genes associated with molecular pathways that are related to liver disease was evaluated. Consequently, the treatment with PsA serum promoted the upregulation of genes specifically related to inflammatory response, immune system, and apoptotic processes (Fig. 2D).

In order to explore the potential connections among all the molecules examined in our *in vitro* study and gain a deeper insight into the molecular pathways affected in the context of PsA within hepatocytes, we conducted a functional enrichment analysis using the STRING platform. Notably, we observed enrichment in pathways and biological functions related to pro-inflammatory and pro-fibrotic mediators, as well as the response and regulation of cellular stress (specifically, oxidative stress), inflammatory responses, the regulation of apoptotic processes, responses to chemical stimuli, and signal transduction (Supplementary figure 1). These findings suggest that the PsA context may induce liver abnormalities, directly impacting hepatocytes and leading to the disruption of pathways associated with liver dysfunction.

These findings conclusively revealed the pathogenic impact of PsA serum on hepatocytes, triggering the alteration of molecular pathways directly implicated in liver dysfunction. These pathways encompassed inflammatory, fibrotic, cell stress, and apoptotic processes. These results strongly suggest the potential development of liver abnormalities within the context of PsA.

Table 1
Clinical and analytical variables of PsA patients with liver disease risk according hepatic steatosis index.

	PsA patients	
	Low probability of having hepatic steatosis	**High probability of having hepatic steatosis**
Hepatic Steatosis Index (HSI)	**<35.68**	**>35.68**
Size population (n)	68	132
Female/Male (n/n)	27/41	53/79
Age (years)	51.78 ± 12.72	52.05 ± 10.81
Disease duration (years)	12.68 ± 11.30	9.89 ± 8.80
DAPSA	12.35 ± 8.51	14.55 ± 10.34
BMI (kg/m^2)	24.66 ± 2.31	31.42 ± 4.71*
HOMA-IR	1.81 ± 1.43	4.44 ± 6.14*
Metabolic profile		
Glucose (mg/dL)	86.77 ± 12.26	98.45 ± 32.69*
Insulin (mU/L)	8.30 ± 5.48	15.64 ± 13.93*
Total-Cholesterol (mg/dL)	196.25 ± 42.29	202.77 ± 36.19
HDL-Cholesterol (mg/dL)	52.92 ± 15.36	53.79 ± 20.28
LDL-Cholesterol (mg/dL)	122.55 ± 34.90	122.25 ± 30.70
Triglycerides (mg/dL)	106.68 ± 50.39	139.38 ± 69.85*
Liver disease biomarkers		
AST (U/L)	23.59 ± 6.94	25.14 ± 10.13*
ALT (U/L)	21.20 ± 10.40	29.77 ± 17.30*
ALP (U/L)	74.17 ± 23.26	77.28 ± 24.46
GGT (U/L)	26.44 ± 19.49	34.41 ± 29.25*
LDH (U/L)	206.35 ± 33.18	211.50 ± 41.23
Platelets (10^3/μL)	251.88 ± 62.31	250.52 ± 70.19
Albumin (g/dL)	4.55 ± 0.32	4.44 ± 0.27
C3 (mg/dL)	139.65 ± 25.28	160.56 ± 29.54*
Treatments		
NSAIDs (%)	73	72
Corticosteroids (%)	20	15
Methotrexate (%)	46	45
Leflunomide (%)	17	14
Biologicals (%)	6	11

PsA patients were classified according to the levels of HSI, using a cut-off value of 35.68 obtained from the NAFLD-non-PsA cohort of similar BMI and diabetes rates of the PsA cohort. This index identify potential presence of steatosis in PsA patients.
Data are represented by mean ± SD. MTX: methotrexate; PsA: psoriatic arthritis; DAPSA: disease activity in psoriatic arthritis 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; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; LDH: lactate dehydrogenase; C3: complement component 3; NSAIDs: non-steroidal anti-inflammatory drugs. *Significant differences respect to < 35.68, $p < 0.05$.

3.4. *In vivo effect of cumulative doses of methotrexate on liver function*

First, linear regression analysis performed in the cross-sectional PsA cohort revealed that hepatic variables altered in PsA compared to HDs were not influenced by the treatment with methotrexate (Supplementary Table 4). Additionally, to better understand the potential impact of cumulative doses of methotrexate on liver disease risk we retrospectively analyzed doses since the beginning of the treatment in 83 PsA patients. According to previous studies [27–30] and the indication of European Medicine Agency (EMA), PsA patients were divided into two groups depending on the risk of hepatotoxicity (cut-off value of 1.5 g of cumulative dose of methotrexate). The frequency distribution of cumulative doses of methotrexate with respect to the number of PsA patients is displayed in Fig. 3A. Thus, PsA patients with a potential risk of hepatotoxicity (>1.5 gr) were older and presented longer disease duration but had significantly lower levels of c-reactive protein with respect to the group with lower risk (<1.5 gr). Likewise, the group with higher hepatotoxic potential (>1.5 g) had fewer swollen and painful joints compared to the lower hepatotoxic risk group. On the other hand, metabolic profile and liver biomarkers were not significantly different between the two groups of cumulative doses (Table 2). Besides, PsA patients with more than 1.5 g of cumulative doses of methotrexate did not show significant differences in HSI, TyG and APRI indexes (Fig. 3B-D). On the contrary, levels of FIB-4 were significantly elevated compared to patients with less than 1.5 g of methotrexate (Fig. 3E). Furthermore, FIB-4 significantly correlated with cumulative doses of methotrexate (Fig. 3F), suggesting its association with higher liver fibrosis risk. However, the analysis of different components of FIB-4 revealed that this association was influenced by the age variable and not due to the levels of AST, ALT, or platelets (Fig. 3G-H). These results suggest that cumulative doses of methotrexate have no potential impact on liver function in our cohort of PsA patients.

3.5. *In vitro hepatocyte response to cumulative doses with methotrexate treatment in the absence of inflammatory stimuli*

To explore the molecular effects of cumulative doses of methotrexate on the hepatocyte, we performed *in vitro* experiments with low methotrexate cumulative doses (50 μM), intermediate methotrexate cumulative doses (100 μM), or high methotrexate cumulative doses (150 μM) for 72 h in a cell line of hepatocytes (Fig. 4A).

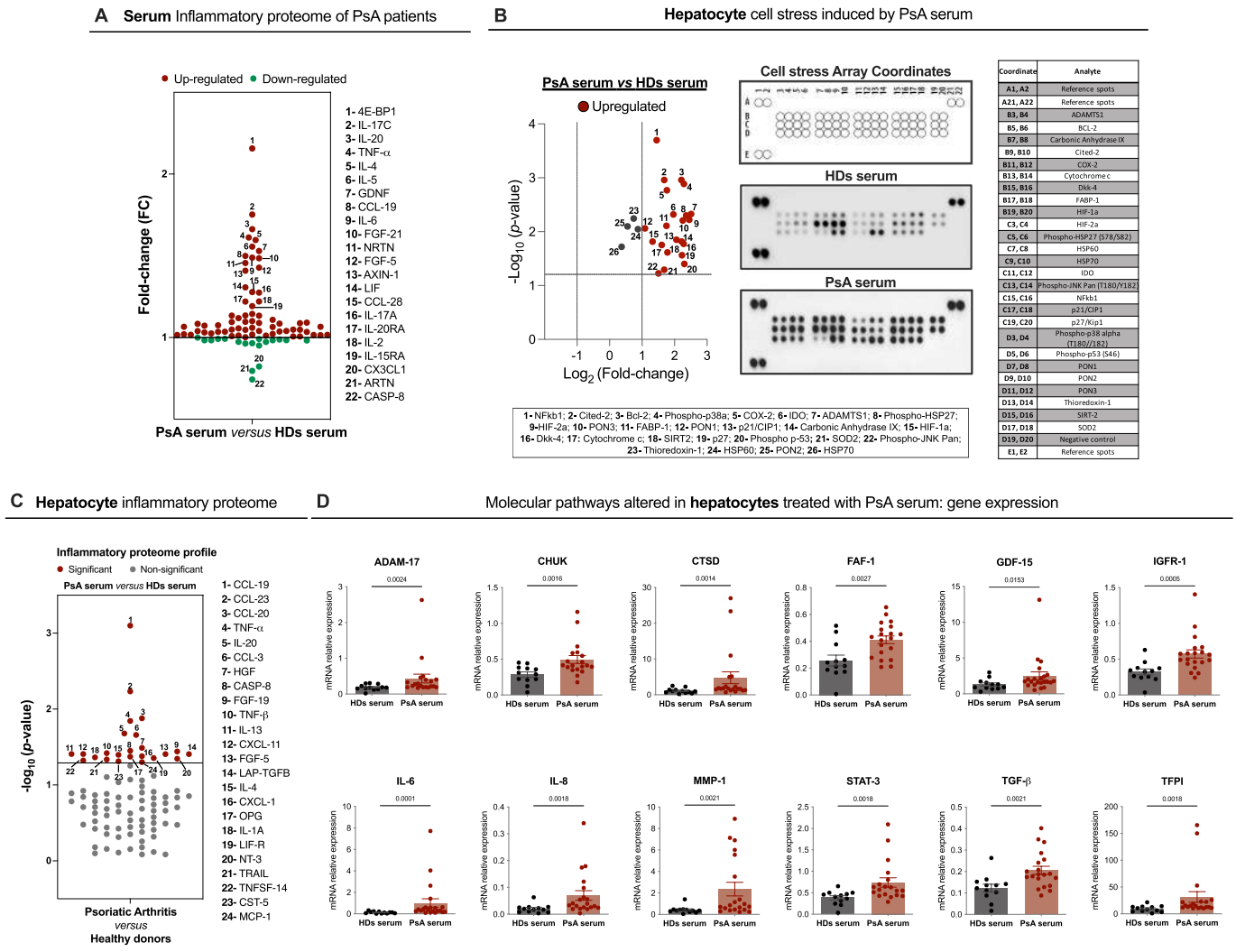
Next, an inflammatory proteome profile, containing the analysis of 92 inflammatory proteins, was carried out. Thus, levels of 47 inflammatory proteins were detected in the HEPG2 hepatocytes. The treatment with the lower doses of methotrexate resulted in significant alterations in 8 proteins compared to untreated cells, 4 upregulated proteins (CSF-1, CTS-5, CASP-8 and TGF-α) (Fig. 4B) and 4 downregulated proteins (CD-40, STAMBP, VEGF-A and LIF-R) (Fig. 4C). However, these alterations were not significantly affected upon higher cumulative doses of methotrexate (Fig. 4D).

These findings revealed that methotrexate in absence of inflammatory stimulation impacts the hepatocyte at an inflammatory level, modifying the expression of several proteins. Besides, these protein alterations induced by methotrexate are not contingent upon the cumulative dosage of the treatment.

3.6. *In vitro effects of methotrexate, anti-PDE-4, and anti-JAK treatments on Psoriatic Arthritis serum-induced hepatocyte activation*

Subsequently, following the observation of the pathogenic effects of PsA serum and methotrexate separately on hepatocytes, we next analyzed the effect of PsA serum simultaneously treated with methotrexate. Additionally, our aim was to test the effects of novel intracellular signaling pathways targeted therapies used in the treatment of PsA such as anti-PDE-4, or anti-JAK, in hepatocytes. The experimental design for the *in vitro* study is depicted in Fig. 4E.

Under inflammatory stimuli (hepatocytes treated with PsA serum), treatment with methotrexate significantly upregulated the levels of CTS-5, IL-1α and CSP-8 and downregulated LIF-R proteins (Fig. 4F) which were previously altered by PsA serum in hepatocytes. Besides, anti-PDE-4 treatment significantly reduced CTS-5, CCL-3, IL-13, LIF-R, and IL-1α hepatocyte inflammatory-related proteins (Fig. 4G) while anti-JAK treatment resulted in the downregulation of CCL-3, TNF-β, FGF-19 and IL-1α (Fig. 4H). Finally, while the treatment with methotrexate significantly augmented the global levels of these inflammatory proteins under inflammatory stimuli, both anti-PDE-4 and anti-JAK treatments markedly reduced the overall inflammatory proteome profile in hepatocytes induced by PsA serum (Fig. 4I). These findings suggest that novel therapies like anti-PDE-4 and anti-JAK have the potential to restore hepatocyte damage induced by PsA serum. These results pave the way to carry out clinical studies focused on the potential beneficial effect of anti-JAK or anti-PDE4 treatments on the liver in PsA patients.



Coordinate	Analyte
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A21, A22	Reference spots
B3, B4	ADAMTS1
B5, B6	BCL-2
B7, B8	Carbonic Anhydrase IX
B9, B10	Cited-2
B11, B12	COX-2
B13, B14	Cytochrome c
B15, B16	Dkk-4
B17, B18	FABP-1
B19, B20	HIF-1a
C3, C4	HIF-2a
C5, C6	Phospho-HSP27 (S78/S82)
C7, C8	HSP60
C9, C10	HSP70
C11, C12	IDO
C13, C14	Phospho-JNK Pan (T180/Y182)
C15, C16	NFkb1
C17, C18	p21/CIP1
C19, C20	p27/Kip1
D3, D4	Phospho-p38 alpha (T180//182)
D5, D6	Phospho-p53 (S46)
D7, D8	PON1
D9, D10	PON2
D11, D12	PON3
D13, D14	Thioredoxin-1
D15, D16	SIRT-2
D17, D18	SOD2
D19, D20	Negative control
E1, E2	Reference spots

Fig. 2. Pathogenic impact of PsA serum on hepatocytes. (A) Serum levels of inflammatory molecules altered in PsA patients compared to HDs used for the *in vitro* experiments. (B) Levels of cell stress-related proteins altered in hepatocytes treated with PsA serum compared to HDs serum. (C) Inflammatory proteins altered in hepatocytes treated with PsA serum compared to HDs serum. (D) Expression of genes related to liver dysfunction in hepatocytes treated with PsA serum compared to HDs serum. Fold-change is represented by mean protein from PsA serum / mean protein from HDs serum. Volcano plot represent points depending on the fold change and *p*-value of the cell stress proteins altered in hepatocytes by PsA serum with respect to HDs serum. Gene expression is presented as a scatter dot plot with bars and individual values by mean ± SEM. Abbreviations of inflammatory proteins are presented in Supplemental Figure II. Abbreviations of genes are presented in Supplemental Figure III. Abbreviations for cell stress proteins: NFkb1: Nuclear factor kappa B subunit 1; Cited-2: Cbp/P300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2; Bcl-2: Bcl-2 apoptosis regulator; COX-2: Mitochondrially encoded cytochrome C oxidase II; IDO: Indoleamine 2,3-dioxygenase 1; ADAMTS1: ADAM metallopeptidase with thrombospondin type 2 motif 1; HSP: Heat shock protein; HIF: Hypoxia-inducible factor; PON: Paraoxonase; FABP-1: Fatty acid binding protein 1; p21/CIP1: Cyclin dependent kinase inhibitor 1 A; Dkk-4: Dickkopf WNT signaling pathway inhibitor 4; SIRT2: Sirtuin 2; SOD2: Superoxide dismutase 2; JNK: Jun N-terminal kinase.

4. Discussion

Due to the recent acknowledgment of liver involvement in PsA, there is limited literature regarding the presence of NAFLD and the contributing factors to its development.

The present study aimed to evaluate the potential high prevalence of liver disease development in PsA, and to define the clinical factors that are related, using a cohort of PsA patients and NAFLD-non-PsA patients. Additionally, we assessed the potential impact of methotrexate treatment on liver function, including experimental *in vitro* studies on hepatocytes and a retrospective PsA cohort with data on retrospective cumulative doses of the medication. We also explored the effect of PsA inflammatory burden on hepatocytes and the *in vitro* impact of the common treatments currently used in PsA on the hepatocyte inflammatory profile.

As a result, our novel study affirmed the association of the potential presence of liver steatosis with metabolic comorbidities and intrinsic factors of the disease such as inflammation, onychopathy, or PsO in 200 patients with PsA. In addition, shedding some light on the controversial data on the effect of methotrexate treatment on the liver, our clinical and experimental data showed that the cumulative doses of methotrexate do not significantly induce liver alterations. Finally, the studies performed on hepatocytes revealed the potential beneficial effect of Apremilast or anti-JAKs on the liver inflammation induced in the context of PsA.

In comparison to age and sex-matched healthy donors, our PsA patient cohort exhibited significantly elevated serum levels of ALT, platelet count, and C3. While these changes may not necessarily indicate liver disease, they may signal the onset of liver dysfunction that warrants careful monitoring. Additionally, our linear regression analyses indicated that these anomalies were not linked to methotrexate treatment. In this sense, new insights about the evaluation and monitoring of the risk of liver disease in inflammatory arthritis have been described [6]. We

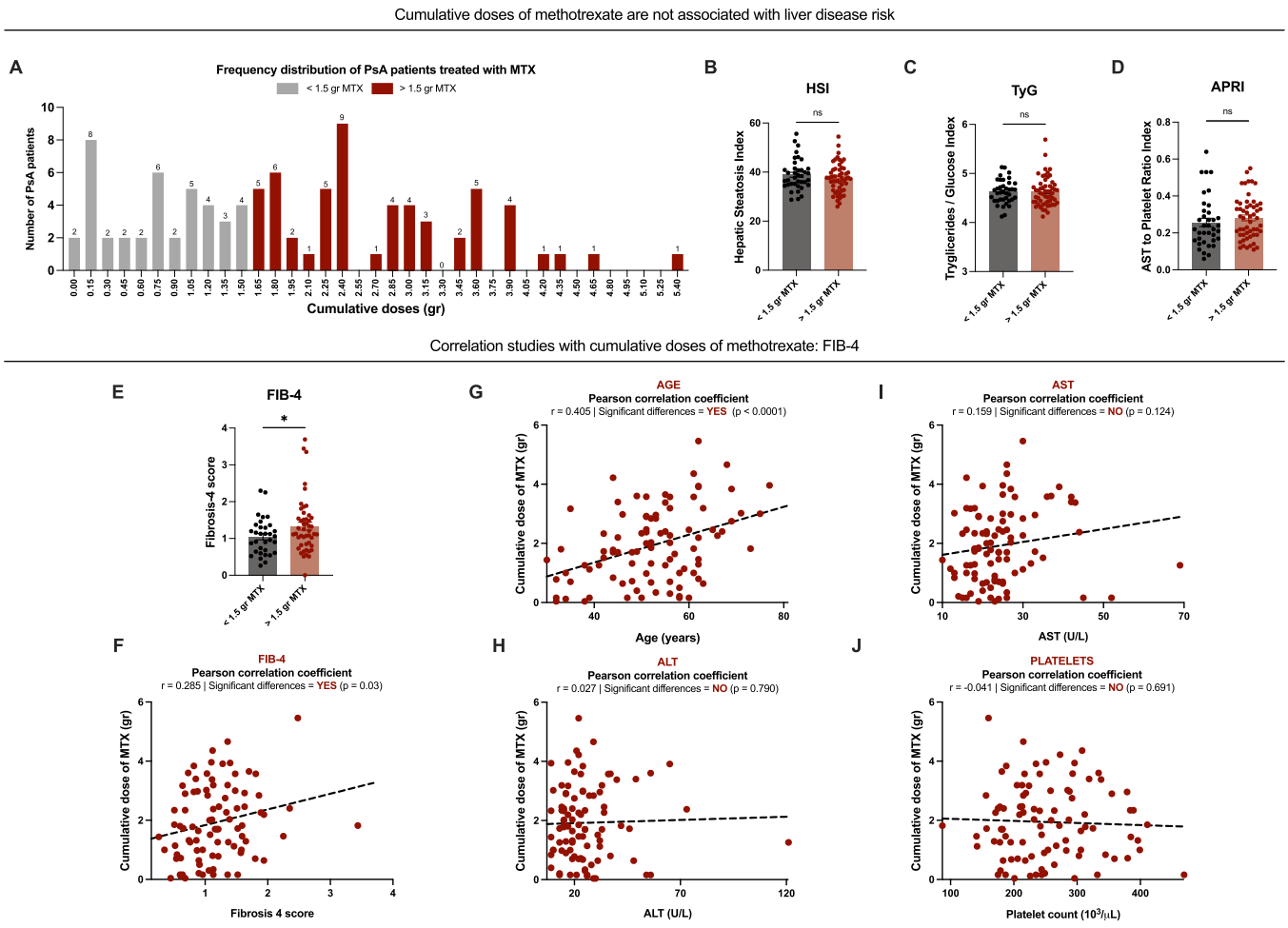

Fig. 3. ***Effects of*** **cumulative doses of methotrexate in liver parameters in PsA patients.** (A) Frequency distribution of PsA patients treated with different doses of methotrexate. (B) Levels of HSI in the two groups of patients with potential risk of hepatotoxicity. (C) Levels of TyG index in the two groups of patients with potential risk of hepatotoxicity. (D) Levels of APRI in the two groups of patients with potential risk of hepatotoxicity. (E) Levels of FIB-4 in the two groups of patients with potential risk of hepatotoxicity. (F) Correlation of cumulative dose of methotrexate with FIB-4. (G) Correlation of cumulative dose of methotrexate with age. (H) Correlation of cumulative dose of methotrexate with ALT. (I) Correlation of cumulative dose of methotrexate with AST. (J) Correlation of cumulative dose of methotrexate with platelets count. The number of PsA patients was divided into groups based on cumulative doses of methotrexate represented by bars in a frequency distribution plot. Liver disease indexes (HSI, TyG, APRI, and FIB-4) are presented as scatter dot plots with bars and individual values by mean ± SEM. Pearson correlation coefficient was used to determine the association of FIB-4 components to cumulative doses of methotrexate. PsA: Psoriatic arthritis; MTX: methotrexate; HSI: Hepatic steatosis index; TyG: triglyceride and glucose index; APRI: Aspartate to platelet ratio index; FIB-4: Fibrosis-4 score; AST: Aspartate aminotransferase and ALT: Alanine aminotransferase. (*) indicates significant differences with a p-value < 0.05.

utilized a cut-off value of HSI (obtained from a BMI and T2DM-matched NAFLD-non-PsA cohort) to identify PsA patients with high probability of having hepatic steatosis. The results showed that HSI, with an AUC of 0.865 and a cut-off value of 35.68, could discriminate NAFLD patients from HDs. This score was created to compare a sizable cohort of HDs and NAFLD patients and determine the likelihood of hepatic steatosis [22]. Our study confirms the accuracy of this score in identifying patients with steatosis. We previously reported that rheumatoid arthritis (RA) patients had significantly higher levels of HSI than HDs, showing an increased risk of NAFLD even after controlling for cardiovascular comorbidities such as obesity and T2DM [31] which are associated with the development of liver dysfunction [9]. However, the use of HSI in PsA has not been the subject of any prior research. With this cut-off value, 66 % of PsA patients had higher likelihood of having liver steatosis compared to 27 % of HDs. This is in accordance with previous studies which reported that these patients have a prevalence among 30–65 % of NAFLD [9,10,32].

After identifying the groups at risk for liver steatosis, association studies were conducted. Our results indicate that PsA patients with high possibility of having NAFLD, accounting for 65 % of the cohort, exhibited significantly higher rates of IR, obesity, HT, and T2DM when compared to those without NAFLD risk. This finding is in line with previous studies, including the work of *Ortolan et al.*, which suggest that the increased prevalence of cardiometabolic comorbidities is a contributing factor to the development of NAFLD in patients with PsA [9].

In our study, we observed that the PsA-NAFLD risk group had elevated levels of TG, C3, acute phase reactants, onychopathy, and body surface area affected by psoriasis compared to PsA patients without NAFLD risk. Of note, PsO and NAFLD share potentially similar pathophysiological mechanisms [33], and their coexistence may affect disease severity [34]. Furthermore, NAFLD has been linked to psoriatic lesions in PsA patients [35], and C3 has been identified as a potential liver disease biomarker in NAFLD [35,36]. In previous studies, C3 has been recognized as a potential NAFLD biomarker in RA patients [37], but not in PsA patients. Our findings suggest that clinical features of PsA disease, such as inflammation, the body surface area affected by PsO, the presence of onychopathy, or metabolic dysfunction, may be key factors in the development of NAFLD.

The pro-inflammatory state directly associated with PsA may have an

Table 2
Association study of cumulative doses of methotrexate treatment in patients with PsA.

	PsA patients	
	Low risk of hepatotoxicity	**High risk of hepatotoxicity**
Cumulative dose of Methotrexate (gr)	**<1.5**	**>1.5**
Mean dose of Methotrexate (gr)	0.72 ± 0.47	2.80 ± 0.85*
Size population (n)	34	49
Female/Male (n/n)	14/20	19/31
Age (years)	51.15 ± 11.62	56.50 ± 11.06*
Disease duration (years)	5.64 ± 4.51	11.84 ± 8.54*
DAPSA	16.14 ± 12.40	12.69 ± 8.74
Swollen joint (n)	1.46 ± 1.83	0.70 ± 1.30*
Tender joint (n)	3.18 ± 3.43	1.84 ± 1.95*
BMI (kg/m^2)	29.51 ± 5.15	28.73 ± 4.73
HOMA-IR	4.11 ± 5.58	2.87 ± 2.76
Inflammatory profile		
ESR (mm/h)	14.88 ± 14.99	12.02 ± 9.20
CRP (mg/dL)	9.20 ± 8.36	5.90 ± 6.58*
Metabolic profile		
Glucose (mg/dL)	88.54 ± 16.17	96.20 ± 31.98
Insulin (mU/L)	17.69 ± 20.90	12.52 ± 11.65
Total-Cholesterol (mg/dL)	198.00 ± 32.26	202.02 ± 37.26
HDL-Cholesterol (mg/dL)	55.03 ± 30.89	52.88 ± 14.91
LDL-Cholesterol (mg/dL)	121.19 ± 28.99	122.54 ± 33.43
Triglycerides (mg/dL)	130.53 ± 54.71	129.22 ± 64.31
Liver biomarkers		
AST (U/L)	24.74 ± 14.05	26.02 ± 7.77
ALT (U/L)	26.03 ± 20.52	26.29 ± 13.33
ALP (U/L)	73.71 ± 16.32	74.27 ± 17.62
GGT (U/L)	27.74 ± 14.53	26.98 ± 16.28
LDH (U/L)	211.00 ± 39.04	217.69 ± 43.32
Platelets (10^3/μL)	262.25 ± 83.19	248.22 ± 74.04
Albumin (g/dL)	4.40 ± 0.32	4.43 ± 0.25
C3 (mg/dL)	151.10 ± 31.69	151.95 ± 27.42
Treatments		
NSAIDs (n)	28	34
Corticosteroids (n)	11	8
Leflunomide (n)	0	2
Biologicals (n)	2	1

Retrospective doses of methotrexate recorded since the beginning of the treatment in 83 PsA patients. According to previous studies (26–29) and the indication of European Medicine Agency (EMA), PsA patients were divided into two groups depending on the risk of hepatotoxicity, cut-off value of 1.5 g of cumulative dose of methotrexate.
Data are represented by mean ± SD. PsA: psoriatic arthritis; DAPSA: disease activity in psoriatic arthritis 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; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; LDH: lactate dehydrogenase; C3: complement component 3; NSAIDs: non-steroidal anti-inflammatory drugs. *Significant differences respect to < 1.5gr, $p < 0.05$.

impact on metabolic organs, such as the liver and adipose tissue. Previously, we reported on the impact of PsA on adipocytes, suggesting that adipose tissue is altered in the context of PsA [12]. In the present study, we further investigated the impact of PsA on liver function. We found that PsA serum from patients with high disease activity, characterized by increased acute phase reactants and high levels of inflammatory molecules, profoundly impacted hepatocyte biology. This was evidenced by alterations in the expression of genes directly associated with liver dysfunction, as well as increased cellular stress and inflammatory proteome when compared to HDs serum. These findings provide further evidence about the impact of the inflammation associated with PsA on liver dysfunction.

On the other hand, the potential hepatotoxicity of methotrexate has been extensively studied in patients with inflammatory arthritis, with controversial results. Some studies have suggested that either low doses of methotrexate or long-term treatment might be associated with NAFLD/NASH or liver stiffness, respectively, in RA patients [13,14]. On the contrary, a study performed in both, RA and PsA, suggested that methotrexate with doses lower than 25 mg per week showed no hepatotoxicity [38]. Moreover, a recent meta-analysis involving a large number of studies reported no significant association between methotrexate cumulative dose and hepatotoxicity [39]. However, out of the 35 studies included in the analysis, only two were performed on PsA [40,41]. Supporting these findings, our study demonstrated that PsA patients with a risk of hepatoxicity defined by EMA (>1.5 gr of methotrexate) [29,30,42], and mean cumulative dose of 2.8 gr, did not show a significant increase in liver function test and biochemical scores indicating the risk of fatty liver or fibrosis. However, there was a notable association with the FIB-4 score when comparing groups with low and high cumulative doses. Nevertheless, it is important to note that this association with FIB-4 was clearly influenced by the age variable included in this index.

Simultaneously, we investigated the molecular effects of methotrexate in the inflammatory pattern of hepatocytes. Unstimulated cells were exposed to increasing doses of methotrexate for 72 h. Although no significant differences were found in the entire proteome compared to untreated cells, specific molecules such as CSF-1, CTS-5, CASP-8, TGF-α, CD-40, STAMBP, VEGF-A, and LIF-R showed significant alterations independent of the dosage of methotrexate. On the other hand, methotrexate was administered in combination with PsA serum to gain a deeper understanding of its potential impact on hepatocytes within a PsA context. The combined treatment altered the levels of proteins that PsA serum alone modified, indicating that methotrexate may have a deeper impact on hepatocytes under inflammatory conditions.

These results provide, for the first time, a comprehensive description of a specific altered proteomic profile at the hepatocyte level following treatment with methotrexate under inflammatory stimuli and at various cumulative doses. Notably, it is important to highlight that increasing the dosage of this treatment did not affect the changes observed at low doses, which might resemble the results found in the *in vivo* data.

In the setting of anti-PDE-4 treatment, prior studies have demonstrated its remarkable efficacy in mitigating liver injury in rats [43,44]. Furthermore, PDE-4 has emerged as a promising therapeutic target for alcoholic liver disease [45], and recent evidence has showcased the consequential impact of PDE4D overexpression in the liver of mice, leading to the onset of NAFLD and hypertension [46]. On the other hand, the potential of Tofacitinib in providing protection against immune-mediated liver injury in mice with hepatitis was previously explored. This study demonstrates that Tofacitinib treatment effectively mitigates inflammation in a mouse model of hepatitis by suppressing the expression of pro-inflammatory cytokines. Additionally, it alleviates liver fibrosis in the context of autoimmune hepatitis [47]. In addition, a recent study revealed that Tofacitinib diminished acute hepatitis in mice by reducing aminotransferase levels, ameliorating histological abnormalities in the liver, and lowering plasma levels of TNF-α and IL-6 [48]. However, there is a lack of studies that have examined the impact of anti-JAK or anti-PDE-4 on liver function or its involvement in liver-related dysfunction, particularly within the context of PsA. In this regard, we present pioneering evidence that highlights the potential advantages of Tofacitinib or Apremilast in the liver alterations linked to PsA. Our research demonstrated that both, anti-JAK or anti-PDE-4 have the ability to restore hepatocyte dysfunction induced by PsA in hepatocytes, as evidenced by the remarkable reduction observed in the entire inflammatory proteome profile, paving the way to future clinical studies.

The main limitation of this study lies in the lack of Fibroscan and Controlled Attenuation Parameter (CAP) data in patients with PsA, which hinders the direct confirmation of findings related to the presence of hepatic steatosis and fibrosis. Both Fibroscan and CAP are essential tools for a more precise and objective assessment of the liver's condition in terms of fat and fibrosis. It is important to highlight that the inclusion

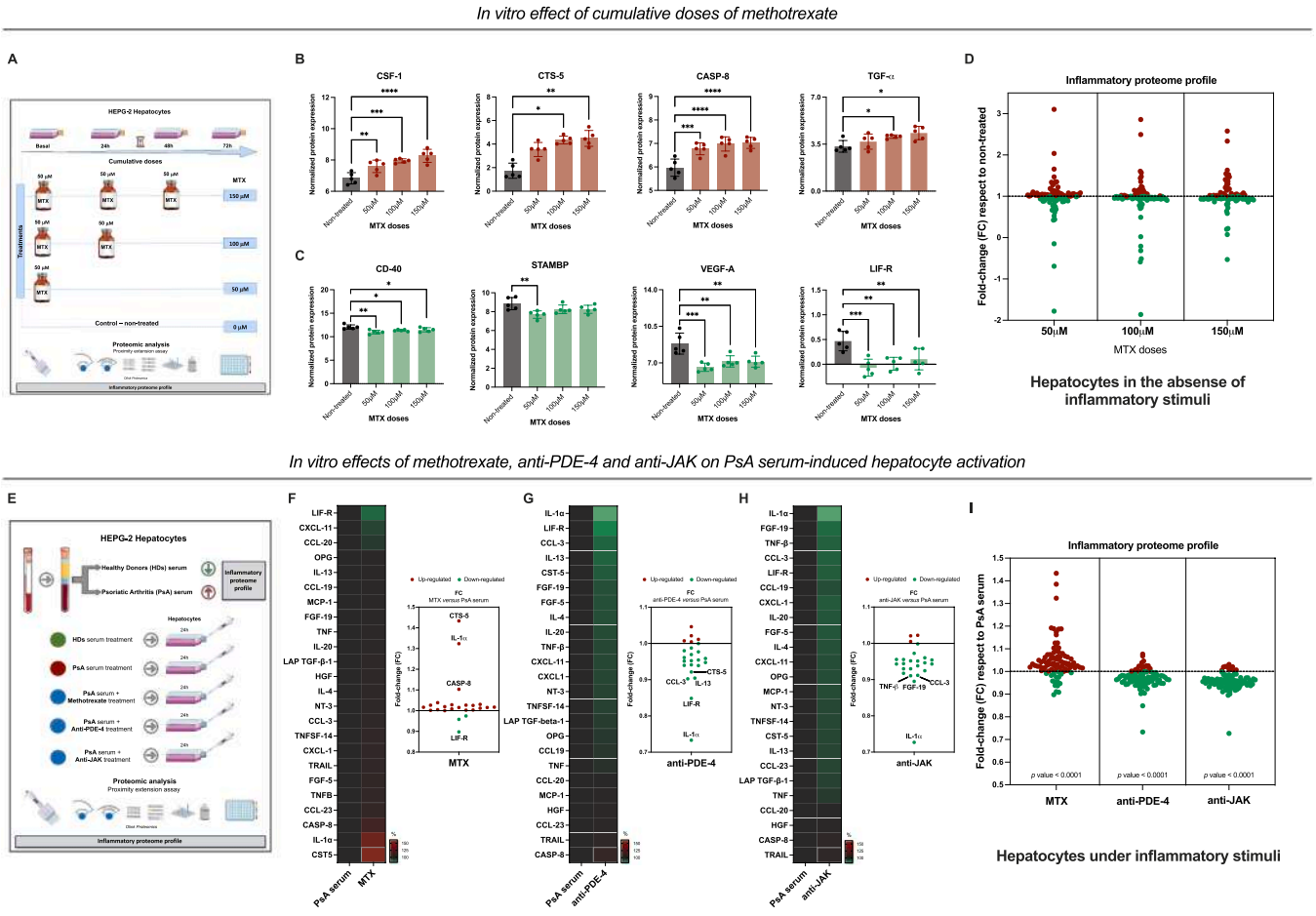

Fig. 4. Hepatocyte response to cumulative methotrexate treatment in the absence of inflammatory stimuli. Effect of methotrexate, anti-PDE-4, and anti-JAK treatments on the PsA serum-activated hepatocytes. (A) Study design of the *in vitro* experiment with cumulative doses of methotrexate. (B) Inflammatory-related proteins altered by cumulative doses of methotrexate. (D) Fold-change of global inflammatory proteome among cumulative doses of methotrexate respect to non-treated cells in the absence of inflammation. (E) Study design of hepatocytes treated with methotrexate, anti-PDE-4 and anti-JAK under inflammatory conditions (hepatocytes treated with PsA serum). (F) Effect of methotrexate on the inflammatory-related proteins altered by PsA serum: heatmap and fold change plot. (G) Effect of anti-PDE-4 on the inflammatory-related proteins altered by PsA serum: heatmap and fold change plot. (H) Effect of anti-JAK on the inflammatory-related proteins altered by PsA serum: heatmap and fold change plot. (I) Fold-change of global inflammatory proteome of different treatments respect to PsA serum-treated cells. Proteins altered by cumulative doses of methotrexate (CSF-1, CTS-5, CASP-8, TGF-α, CD-40, STAMBP, VEGF-A and LIF-R) are presented as scatter dot plot with bars and individual values by mean ± SEM. Fold-change is represented by mean protein/protein among the different *in vitro* conditions. Heatmap represent the percentage of the altered inflammatory-related proteins respect to PsA serum treatment. Abbreviations of inflammatory proteins are presented in Supplemental figure II. MTX: Methotrexate; PDE-4: Phosphodiesterase 4; JAK: Janus kinase. (*) indicates significant differences with a p value < 0.05. (**) indicates significant differences with a p value < 0.01. (***) indicates differences with a p value < 0.001. (****) indicates significant differences with a p value < 0.0001.

of these measurements in future investigations would allow for a more comprehensive and reliable analysis of the association between both medical conditions.

5. Conclusions

Our findings show the elevated susceptibility of PsA patients to liver disease, which manifests not only an association with cardiometabolic complications such as IR, T2DM, obesity, or hypertension but also related to the presence of intrinsic disease factors such as psoriasis, onychopathy, and the burden of inflammation. Remarkably, these alterations occur independently of methotrexate treatment within our cohort of PsA patients. Despite the ongoing debate surrounding the potential impact of methotrexate on liver function, our analysis reveals no discernible changes influenced by cumulative doses of this administered treatment. Consequently, PsA patients experiencing liver dysfunction might stand to gain significant benefits from therapeutic approaches such as anti-JAK or anti-PDE-4 treatments, offering promising prospects for advancing the management of this condition.

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CRediT authorship contribution statement

MRP, LCL, MD-LM, CPS, CLM, POB, AB, MD-G, NHS, AJL, PN, Ch-LP, AEC, ECE, IAR and NB (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, MRP, LCL, IAR and NB (2) drafting the article or revising it critically for important intellectual content, MRP, LCL, MD-LM, CPS, CLM, POB, AB, MD-G, NHS,

AJL, PN, Ch-LP, AEC, ECE, IAR and NB (3) final approval of the version to be submitted.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest. C.P-S and N.B are co-founders of Cobiomic Bioscience S.L.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115779.

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