



# Acute Phase Inflammatory Proteins, Gut Microbiota Dysbiosis and Severity of Nonalcoholic Fatty Liver Disease in Patients with Type 2 Diabetes Mellitus

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**Abstract.** *Since obesity and type 2 diabetes mellitus (DM) became almost pandemic, incidence and prevalence of associated nonalcoholic fatty liver disease (NAFLD) rose accordingly. Aim of the study was assessing whether there is a link between circulating C-reactive protein(CRP), gut microbiota dysbiosis and severity of liver disease in patients with NAFLD and confirmed type 2 DM. 50 patients diagnosed with type 2 DM were consecutively enrolled in this cross-sectional, pilot study, being evenly divided in two groups, as matched pairs, on the basis of presence or absence of NAFLD confirmed by abdominal and CT exam, after ruling out a lot of diseases and conditions. Patients underwent measurements of waist circumference, blood pressure (BP), body mass index (BMI) and thoroughly physical examination. Complete blood count (CBC), liver tests, CRP, fasting plasma glucose (FPG), low density lipoprotein(LDL) and high-density lipoprotein(HDL) cholesterol, triglycerides, creatinin and uric acid, micro proteinuria as well as stool microbiology were run, using standardized methods. Fatty liver index (FLI) was also calculated. Fibromax (BioPredictive) was performed in NAFLD (+) patients. NAFLD (+) group displayed significant differences of age and BMI, as well as lab biochemistry: ALT, GGT, LDL-cholesterol and triglycerides, CRP, microprotenuria and stool microbiology.. Strong positive correlation of CRP with gut dysbiosis (DB) severity, microproteinuria, as well as with nonalcoholic steatohepatitis (NASH) score, were observed. DB range correlated positively in NAFLD (+) group with age, BMI, BP, dyspepsia as well as with NASH scores. In conclusion patients with NAFLD and associated type 2 DM displayed a specific profile with significant differences of age, BMI, FLI, diabetic treatment, biochemistry and gut microbiological charts. Strong positive correlations between CRP, gut DB and NASH score suggest the possibility of link between inflammation, gut microbiota DB and severity of liver disease.*

**Keywords:** CRP, gut microbiota dysbiosis, NAFLD, type 2 DM

## 1.Introduction

Nonalcoholic fatty liver disease (NAFLD) is a complex entity characterized by  $\geq 5\%$  accumulation of fat in liver cells, in the absence of excessive alcohol consumption or any other secondary causes, ranging from simple steatosis to inflammation and hepatocytes necrosis known as NASH (nonalcoholic steatohepatitis), or end stage liver disease (NASH-induced cirrhosis): fibrosis and architectural liver changes [1,2]. Since obesity and type 2 DM became almost pandemic, incidence and prevalence of NAFLD rose accordingly. It is estimated that in Asia and USA, between 2005 and 2010, the prevalence of NAFLD increased exponentially [3].

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The real burden of NAFLD remains yet obscure, since the diagnosis is probably highly underrated and not always accurate, based mostly on imagistic approaches and seldom as a result of liver biopsy, not to mention numerous associated health conditions that multiply its morbidity and mortality. Since NAFLD rarely has characteristic symptoms or signs, diagnostic is frequently incidental. Two situations drive the clinical diagnostic: either increase of transaminases and GGT or imagistic features consistent with liver steatosis. Given the liver biopsy is not systematically used as a diagnostic method, noninvasively approach is lately preferred, especially for screening NAFLD. Liver ultrasound, CT or MRI are currently accepted as reliable methods to detect fatty infiltration. However, there is a difference of diagnostic sensitivity, when it comes to method, which consecutively reflects in large variation of epidemiological data reporting NAFLD. For several reasons ultrasonography is considered the method of choice for screening medium to severe fatty liver. [4-6] If diagnosis is established in end stage liver disease, patients will display, as all patients with liver cirrhosis, no matter etiology, liver stigmata signs along with portal hypertension and severe alterations of biochemical liver tests.

So called primary NAFLD, often associated with central obesity, dyslipidemia, insulin resistance and diabetes mellitus, became nowadays more and more serious. That results in important problems of public health, especially in developed countries, not only related to its increasing incidence, but also due to its potential of evolution toward severe hepatic lesions including liver fibrosis, cirrhosis and its complications, such as portal vein thrombosis, and hepatocellular carcinoma [7,8].

As many studies recently reported, various factors consistent with “multiple hit theory” could at some point trigger liver injury, resulting in inflammation and hepatocytes necrosis and consecutively could promote liver fibrosis. These factors could be genetic, insulin resistance, dietary habits with high carbohydrates and fats, hormones imbalance and , gut microbiota dysbiosis (DB).[9] Gut microbiota DB seems to play an important part in NAFLD pathogenesis and could intervene in modulation the energy metabolism, resulting in fat accumulation as droplets of triglycerides, as well as in development of insulin resistance, increase of FFA production, as well as decrease of choline production and increase of many hepatotoxic compounds [10].

Aim of the study was assessing whether there is a link between inflammation, gut microbiota DB and severity of liver disease in patients with NAFLD and type 2 DM.

## 2. Patients and methods

### 2.1 Study design and methodology

**2.1.1 Inclusion criteria:** 50 patients diagnosed with type 2 diabetes mellitus were consecutively enrolled in this cross-sectional, pilot study, being evenly divided in two groups, as matched pairs, on the basis of presence or absence of NAFLD confirmed by abdominal ultrasonography and CT exam. Study was approved by the Ethics Committee of Scientific Research of the University of Medicine and Pharmacy “Victor Babes” from Timisoara, Romania and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before the beginning of the study.

**2.1.2 Exclusion criteria:** heavy consumption of alcohol: more than 40gr/day in men and more than 30gr/day in women, in past 10 years, significant exposure to industrial toxic substances or to medication with liver toxicity, hemochromatosis,  $\alpha_1$  antitripsine deficiency, Wilson disease, autoimmune hepatitis, infection with viral B or viral C hepatitis, thyroid diseases, various organ failure (cardio-pulmonary, kidney or liver), oncologic conditions, long parenteral nutrition, associated inflammatory bowel disease, celiac disease or gluten sensitivity, various infectious diseases, malnutrition syndromes, as well as recent treatment with antibiotics or probiotics.

**2.2 Protocol of examination and laboratory work-up** Patients underwent measurements of waist circumference and blood pressure (BP), BMI assessment, as well as thoroughly clinical examination. Laboratory work up: complete blood count (CBC), routine liver tests including HBs antigen, Delta



antigen, anti HCV antibodies, plasma iron and copper, alpha 1 antitripsine, antinuclear antibodies (ANA), antimitochondrial antibodies (AMA), as well as high sensitive C-reactive protein (CRP), fasting plasma glucose (FPG), cholesterol low-density lipoprotein(LDL) and high-density lipoprotein(HDL), triglycerides, creatinin and uric acid, microproteinuria, urine and stool microbiology were run, using standardized methods After. identifying different types of stool species by matrix-assisted laser desorbtion ionization-time of flight –mass spectrometry( MALDI-TOF-MS) method, the severity of gut microbiota DB, i, was semiquantitative scored as: 0=absent, 1=mild, 2=medium, 3=severe [11].

**2.3 C-reactive protein (CRP)** is a nonglycosilated plasma protein, with an annular shape and pentameric structure, consisting of five conformational protein subunits, with high resistance to proteolysis, that migrates near the gamma zone on electrophoresis. It is synthesized by many immune cells with various locations, predominantly in the liver, as a reaction of proinflammatory interleukins and cytokines. Occasionally could be produced de novo in extrahepatic sites. Because its levels rise and fall depending on the amount of inflammation or tissue lesions in the body, CRP is also known as an acute phase reactant. To determine CRP, venous blood samples were collected in the morning, in fasting patients, within vacutainers and processed after centrifugation, using immunoturbidimetry assay. The cutoffs were 0.5 mg/dL [12].

#### 2.4 Non invasive assessment of NAFLD

**2.4.1.** Abdominal helical CT, as well as real-time high resolution ultrasonography were used to confirm or rule out fatty liver disease in study participants. Lipid accumulation determines decrease of liver density, which becomes less dense when compared to spleen. It is considered that a decrease more than 10 Hounsfield units (HU) on a native CT is consistent with diagnosis of liver steatosis, while using contrast the difference between liver and spleen augments to more than 20 HU. Abdominal ultrasonography was performed in all study participants, with semiquantitative assessment of steatosis, when observed, as follows: mild, moderate and severe [13].

**2.4.2.** Fibromax, developed by BioPredictive®, consistent with 5 different noninvasive tests, from which: FibroTest®, SteatoTest® and NashTest® were actually used in this study, in the evaluation of NAFLD (+) group. This complex test is based on validated mathematical application, that uses some biochemical blood variables, such as: alpha-2 macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, gamaglutamiltranspeptidase( GGT), alanin - aminotransferase (ALT), aspartat – aminotransferase (AST), fasting plasma glucose (FPG), cholesterol and triglycerides, as well as some clinical data such as: age, gender, weight and height, in order to determine the severity of liver disease [14].

**2.4.3.** Fatty liver index (FLI), as instrument of prediction of fatty liver risk was calculated using a mathematical formula based on measurements of waist circumference (cm), BMI (kg/m<sup>2</sup>), level of GGT (U/l) and triglycerides (mg/dl).The FLI formula that calculates the likelihood of fatty liver disease was:  $FLI = (e^{0.953 \cdot \log e(\text{triglycerides})} + 0.139 \cdot \text{BMI} + 0.718 \cdot \log e(\text{GGT}) + 0.053 \cdot \text{waist circumference} - 15.745) / (1 + e^{0.953 \cdot \log e(\text{triglycerides})} + 0.139 \cdot \text{BMI} + 0.718 \cdot \log e(\text{GGT}) + 0.053 \cdot \text{waist circumference} - 15.745) \times 100$ . The values range between 0 and 100. A FLI<30, basically rules out a fatty liver (FL), whereas a FLI>60 represents a high risk for FL and mandates further exams [15].

**2.5 Assessment of DM** According to American Diabetes Association (ADA) criteria, a FPG of 126 mg% or higher, or a 2 hour plasma glucose level of 200mg% during 75g oral glucose tolerance test, are consistent with the diagnosis of DM [16].

**2.6 Statistical analysis** Graph Pad Prism 8.3.1 software (Graph Pad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Given exploratory, pilot study, no sample size calculation was needed. Quantitative variables were expressed as mean values (MV) ± standard deviation (SD). Chi-squared test was used to compare the two of groups, in cases of qualitative variables expressed as percentages. The unpaired t test was calculated,  $p \leq 0.05$  was considered statistically significant, with confidence interval CI= 95%. Nonparametric Pearson’s correlation test was also performed in order to establish the r coefficient, drawing the direction and magnitude of possible links between variables.

### 3. Results and discussions

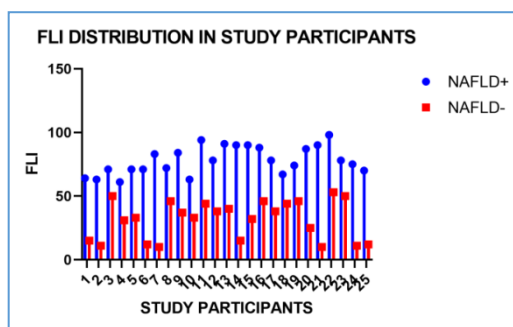
As depicted in Table 1, analysis of demographical and clinical characteristics in study participants showed significant statistically differences related to age ( $p=0.0294$ ), BMI ( $p=0.0084$ ), FLI ( $p=0.0001$ ) and therapy of DM. Thus, NAFLD(+) patients needed insulin in significantly higher percentage to compensate DM ( $p<0.0001$ ), while those without NAFLD were treated mostly with oral antidiabetics and no insulin ( $p<0.0001$ ). In respect to treatment of dyslipidemia, significant differences related to statins were seen in favor of NAFLD positive group ( $p=0.0035$ ).

**Table 1.** Baseline demographical and clinical characteristics in study participants

	NAFLD(+)	NAFLD(-)	p
Age(years)*	72.28±8.88	66.32±9.86	<b>0.0294</b>
Gender females/males	8/17	13/12	0.1561
Residence urban/rural	15/10	13/12	0.5727
BMI(kg/m <sup>2</sup> )*	31.03±7.37	26.6±3.48	<b>0.0084</b>
SBP(mmHg)	143.00±27.16	144.00±26.18	0.8951
fatigue	40%	32%	0.5597
dyspepsia	48%	32%	0.2530
FLI (units)*	77.52±10.56	32.08±14.61	<b>0.0001</b>
Cardio-vascular conditions	100%	100%	1
Treatment –only diet	0%	8%	0.1538
Oral antidiabetics*	36%	92%	<b>&lt;0.0001</b>
Insulin therapy*	64%	0%	<b>&lt;0.0001</b>
Statins*	84%	44%	<b>0.0035</b>
Statins +fibrates	16%	12%	0.6866

legend: BMI= body mass index, SBP= systolic blood pressure, FLI=fatty liver index,\*; bold =statistically significant

As illustrated in Table 1 and Figure 1, FLI calculated in all study participants, showed in NAFLD (+) group a highly statistically significant difference (77.52 U±10.56 vs. 32.08 U±14.61;  $p=0.0001$ ).



**Figure 1.** FLI distribution: NAFLD (+) vs. NAFLD (-) participants

Analysis of patients biological charts revealed statistically significant differences in favor of first group, respectively NAFLD (+), regarding transaminases-ALT ( $p=0.023$ ), GGT ( $p=0.0064$ ), plasma

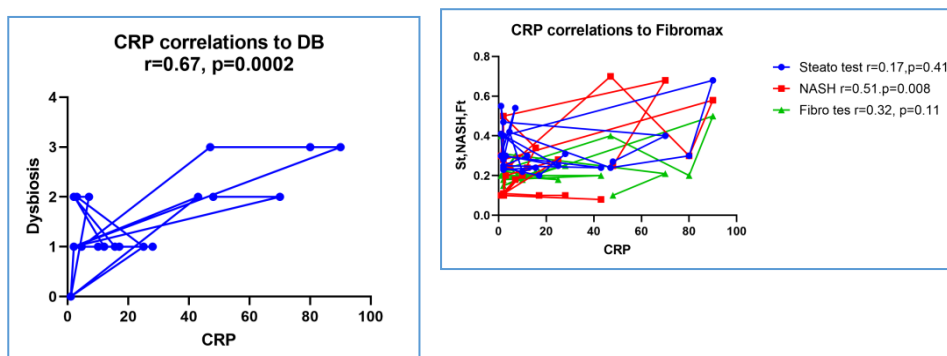
lipids: LDL (P=0.0309) and triglycerides (p=0.0506), CRP level (p=0.0416), as well as micro-proteinuria (p=0.0007) and DB range (p<0.0001). These data are displayed in Table 2.

**Table 2.** Biological characteristics in study participants

	NAFLD(+)	NAFLD(-)	p
Hb(/dl)	12.74±1.73	12.29±2.23	0.4288
L/mm <sup>3</sup>	8.68x 10 <sup>3</sup> ±2.10x10 <sup>3</sup>	9.58 x10 <sup>3</sup> ±2.79x10 <sup>3</sup>	0.2049
Plt/ mm <sup>3</sup>	238.04x10 <sup>3</sup> ±68.3x10 <sup>3</sup>	225.36x10 <sup>3</sup> ±90.86x10 <sup>3</sup>	0.5795
ALT(iu/l) *	45.2±40.82	25.16±12.4	<b>0.023</b>
GGT(u/l) *	51.52±33.2	31.56±11.07	<b>0.0064</b>
LDL cholesterol(mg/dl) *	107.2±35.81	86.8±28.64	<b>0.0309</b>
HDL coletserol(mg/dl)	41.56±13.03	40.68±8.42	0.7779
Triglycerides(mg/dl) *	184.96±166.01	119.76±31.31	<b>0.0506</b>
Creatinin(mg/dl)	1.44±0.79	1.24±0.56	0.3113
FPG(mg/dl)	171.96±50.62	142.96±44.93	0.0373
Uric acid(mg/dl)	6.75±1.78	6.8±1.65	0.9220
CRP(mg/dl) *	21.47±27.17	9.7±7.0	<b>0.0416</b>
Microproteinuria*	80%	32%	<b>0.0007</b>
Gut DB*	88%	28%	<b>&lt;0.0001</b>

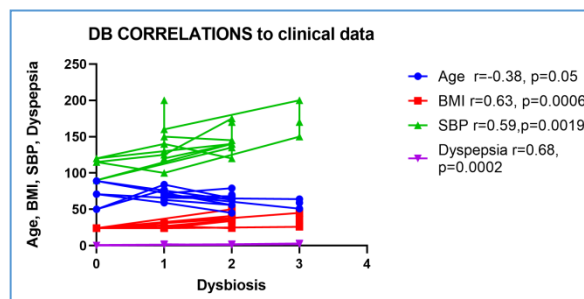
Legend: Hb= hemoglobin, L=leukocytes, Plt=platelets, FPG= fasting plasma glucose, CRP=C reactive protein, DB= dysbiosis, \*,\*: bold=statistically significant

As depicted in Figure 2A and 2B, CRP displayed strong positive correlation with gut DB ( r=0.67, p=0.0092) and NASH score (r=0.51, p=0.008).



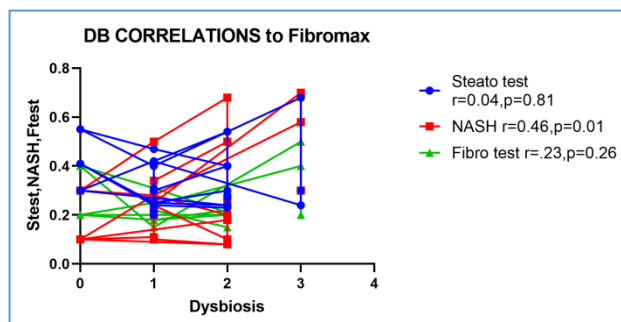
**Figure 2.** CRP correlations in NAFLD (+) group: **2A** to gut DB, **2B.** to Fibromax scores

Correlation studies applied to DB illustrated a strong link with different demographical and clinical data, as follows: age(r=0.38, p=0.05), BMI(r=0.63, p=0.0006), SBP(r=0.59, p=0.0019) and dyspepsia (r=0.68, p=0.0002), as seen in Figure 3A.



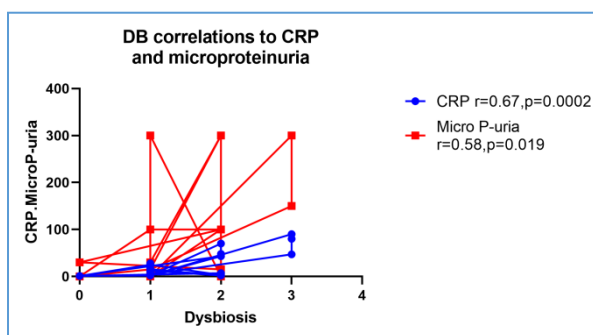
**Figure 3A.** Correlations of gut DB to demographical and clinical data in NAFLD (+) group

Related to Fibromax scores, DB positively good correlated to NASH score, respectively:  $r=0.46$ ,  $p=0.01$ . These aspects are depicted in Figure 3B.



**Figure 3B.** Correlations of gut DB to Fibromax scores in NAFLD (+) group

As seen in Figure 3C, DB also strong positively correlated to microproteinuria;  $r=0.58$ ,  $p=0.019$ .



**Figure 3C.** Correlations of gut DB to CRP and microproteinuria in NAFLD (+) group

In accordance with others, we observed that NAFLD+ group displayed significant older age by comparison to NAFLD- participants. It seems that incidence and prevalence of NAFLD increase with growing old, also resulting in more severe biochemical and histological features [17].

As others reported, we also observed that FLI showed utility and reliability in the assessment of risk for liver steatosis, while all study participants with confirmed NAFLD exhibited levels above 60 units [18].

Whether current, DZ treatment influenced results of this study, in respect to presence and severity of fatty liver, it is however not clear. Patients with the second group (NAFLD negative), were mainly treated with oral antidiabetic drugs (OAD) (metformin), while the group displaying NAFLD did not further receive OAD, patients becoming insulin necessitant. In this regard many multicenter studies and meta-analysis investigated relation between various OAD and NAFLD, but results are neither coherent nor homogeneous, and rather contradictory. However, to date, European Association for Study of the Liver (EASL) did not yet introduce any specific recommendation of drugs in the guidelines for NAFLD treatment [19]. To record, all patients in this study with NAFLD received lipid lowering agents, basically statins, in a significant higher percentage, by comparison to those without NAFLD and a few of them combination of statins with fibrates. Even NAFLD group was basically treated with antidiabetics and statins, they showed elevated transaminases and GGT level. However, there is yet no conclusive evidence that dyslipidemia treatment with any kind of agent either statins, fibrates, fish oil, etc., could significantly improve liver histology severity or outcome in NAFLD patients [20].



Gut microbiota DB, obesity and metabolic syndrome are close related, as confirmed by many recent studies, including ours, conducted both in animal and humans, by modulation of host nutritional and energy metabolic pathways. No less important seems to be so called metabolic endotoxemia, as a part of this complex process, related to overgrowth of some microbial gut species and reduction of local flora biodiversity, with increased ratio Firmicutes to Bacteroidetes. These aspects correlate with adiposity, insulin resistance and proinflammatory status.[21] Interestingly, metformin seems to stimulate bacteria species that produce butyrate and consecutively promote normobiosis or a healthy microbiome.[22] In accordance with these observations we have recorded significant lower rates of dysbiosis severity in patients without NAFLD, treated by OAD-metformin.

Inflammation, gut DB and NAFLD seem to be linked, as many studies support this hypothesis. The bioenergetic pathways related to mitochondria activity, immunometabolism with pivotal, biochemical reactions, performed by numerous immune cells effectors, could be reprogrammed under particular stimuli. One of these stimuli that can drive host immunological functions is represented by gut microbiota DB. DB could definitively affect the ability of adaptive, inflammatory responses and trigger proinflammatory cells with release of various interleukins and cytokines, eventually altering the host metabolism.[23] In this regard, we have noticed a positively strong correlation not only between the levels of CRP and range of DB severity, as well as NASH scores, but also directly between DB severity itself and NASH scores.

Results of the present study have also highlighted strong positive correlations between range of DB and several clinical data, such as: age, BMI and SBP. Many recent studies advocated the role of DB in the setting of cardio-vascular risk, via trimethylamine N-oxide, short chain fatty acids and secondary bile acids, that interfere various signaling and communication pathways, resulting in hypertension, atherosclerosis and coronary artery disease. Otherwise cardio-vascular comorbidities represent another important issue to manage in NAFLD population, as we also observed in present study [24].

There is an increased risk of associated microproteinuria and chronic kidney disease (CKD) in patients with NAFLD, as a recently meta-analysis concluded. [25] Our study identified significant increased incidence of microalbuminuria in NAFLD (+) group, suggesting the importance of screening for CKD in population already affected by NAFLD, not to mention the risk for cardio-vascular diseases. We also observed a strong correlation between microalbuminuria levels and DB severity.

Given data from present study and many others, inflammation is a fundamental process, implicated in many conditions, orchestrated by gut microbiota DB, that triggers other participants either local or distant, which further aggravates the initial condition.[26] Since the understanding of this basic process associates DB with inflammation, a lot of scientists embraced the idea of natural remedies, such as probiotics, in order to influence this pathophysiological pathway and the occurrence or development of many disorders. [27] In this regard, other potential natural antiinflammatory formulas could be taken into account, based on their promising preliminary results [28,29]. No less important will be to improve our possibility to predict the risk for DM and its complications, including NAFLD. [30]

**Limitations** This observational study has a few limitations. Even if it is a pilot study, though the sample size of study population is relatively small, with consecutive potential statistical draw-backs. Another limitation is related to methodology of NAFLD diagnostic. Although the diagnosis protocol was homogenous, it did include only noninvasive methods, which could trigger ambiguity, considering that liver biopsy remains the gold standard in liver diseases diagnosis. Not least, given the cross-sectional study, a lot of statistical bias could also intervene, related to sources of information bias and possible confounders.

#### 4. Conclusions

Patients with type 2 DM that developed NAFLD displayed a profile characterized by older age, higher BMI and FLI range, necessity of insulin therapy, modified biological charts that included acute inflammatory phase protein- CRP, liver tests, serum circulating lipids, microproteinuria, as well as



higher range of gut DB. Strong positive correlations observed between CRP, gut DB and NASH and also between DB, BMI and NASH suggest the possibility of link between inflammation, gut microbiota DB and severity of liver disease.

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