EXPERT REVIEWS

Plasma high mobility group box 1 protein reflects fibrosis in pediatric nonalcoholic fatty liver disease

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Anna Alisi*[1,2‡], Valerio Nobili[1,2‡], Sara Ceccarelli[1,2], Nadia Panera[1,2], Cristiano De Stefanis[1,2], Rita De Vito[3], Roberta Vitali[4], Giorgio Bedogni[5], Clara Balsano[6], Salvatore Cucchiara[7] and Laura Stronati[4]

[1]*Hepato-Metabolic Disease Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy*
[2]*Liver Research Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy*
[3]*Pathology Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy*
[4]*Department of Radiobiology and Human Health, ENEA, Rome, Italy*
[5]*Epidemiology Unit, Liver Research Center, Trieste, Italy*
[6]*Institute of Biology, Molecular Medicine and Nanobiotechnology – CNR, Rome, Italy*
[7]*Pediatric Gastroenterology and Liver Unit, Department of Pediatrics, Sapienza University of Rome, Rome, Italy*
**Author for correspondence:*
Tel.: +39 066 859 2186
Fax: +39 066 859 2904
anna.alisi@opbg.net

[‡]*Authors contributed equally*

Non-alcoholic fatty liver disease (NAFLD) affects 3–12% of the general pediatric population. HMGB1 protein is presently considered a potent inflammatory mediator in several liver diseases, even if its role in NAFLD is still unknown in clinical studies. Here we investigated the relationships between circulating HMGB1, TGF-β and MCP-1 and liver damage in pediatric NAFLD. HMGB1, TGF-β and MCP-1 plasma levels were measured in 110 obese children with biopsy-proven NAFLD and 40 age-matched obese controls. HMGB1, TGF-β and MCP-1, ALT, AST and cholesterol plasma levels were significantly higher in NAFLD than in control children. A significant association between increased levels of HMGB1, TGF-β and MCP-1 and high degrees of fibrosis was found. In this study, we showed for the first time that circulating levels of HMGB1 were raised in children with NAFLD and strongly correlated with fibrosis and systemic inflammation.

KEYWORDS: high mobility group box 1 • liver fibrosis • monocyte chemoattractant protein-1 • non-alcoholic fatty liver disease • TGF β

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases throughout the world. About 20–30% of the general population and 3–12% of the children worldwide are estimated to have NAFLD [1,2]. Unhealthy lifestyle, obesity and other metabolic syndrome features, such as insulin resistance and hyperlipidemia, are considered the most important risk factors for pediatric NAFLD. Therefore, recent studies suggest that NAFLD prevalence is increasing especially in obese children (ranging from 70 to 90%), becoming a serious public health concern [3].

As its adult counterpart, pediatric NAFLD is currently conceived as a spectrum of different diseases, ranging from simple steatosis (i.e., fatty liver, NAFL), to nonalcoholic steatohepatitis (NASH), characterized by steatosis and inflammation, and fibrosis [4,5]. However, NAFL development and it progression to NASH is not still fully pathogenetically clarified both in adults and children [2]. It is known that visceral fat accumulation and insulin resistance increase intrahepatic free fatty acids by promoting *de novo* lipogenesis and by reducing the export and enhancing the uptake of lipids [6]. Once NAFL is established, circulating factors, including adipocytokines and gut-derived portal products, may lead to NASH by means of oxidative stress, apoptosis, inflammation and fibrogenesis with the recruitment of different liver-resident cells [2,7–9].

Damage-associated molecular patterns (DAMPs), also known as alarmins, are endogenous molecules released by stressed cells in response to tissue injury. DAMPs may cause liver necro-inflammation and fibrosis in NAFLD [10,11]. DAMPs, which are normally sequestered inside cells and therefore hidden from recognition by the immune system, may be released into the extracellular environment by cells and trigger sterile inflammation (SI) in the absence of pathogens [12]. SI may occur in tissues after injury of different etiologies. In the liver, SI is particularly relevant because it is a major component of the pathology of a wide range of diseases, such as alcoholic steatohepatitis,

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drug-induced liver injury, ischemia/reperfusion and NASH [13–16]. Collectively, these diseases are responsible for the majority of liver pathology in the industrialized world and lack a specific therapy [14].

High-mobility group box 1 (HMGB1), which is presently considered a DAMP prototype, is a 30-kDa protein, implicated in bending DNA to facilitate gene transcription, DNA replication and repair [17–20]. To exert these activities, HMGB1 must transit from the nucleus, through the cytoplasm, to the outside of the cell. HMGB1, following a number of post-translational modifications, is actively secreted and forms highly inflammatory complexes with single-stranded DNA, lipopolysaccharide (LPS), IL-1β and nucleosomes, which interact with toll-like receptors (TLR) 9, 4 and 2 and IL-1 receptor [21,22].

HMGB1 also induces the recruitment of inflammatory cells [23–25], contributing both to dendritic cell maturation [26,27] and proliferation of activated T cells [28]. For all these reasons, HMGB1 is presently considered to be a potent inflammatory mediator involved in several inflammatory and autoimmune disorders, such as sepsis, rheumatoid arthritis, lupus erythematosus, myositis, diabetes and inflammatory bowel disease [29–33].

In recent investigations, increased plasma levels of HMGB1 have been associated with unfavorable outcomes in patients with diseases characterized by low-grade inflammation, such as Type 1 diabetes and ischemic stroke [34,35]. Importantly, an increase of plasma HMGB1 was recently reported in patients with acute liver failure or acetaminophen-induced acute liver injury, which suggests a possible relationship between HMGB1 with hepatocellular injury and liver damage [36,37]. However, the role of HMGB1 in NAFLD and its correlation with systemic inflammation and liver damage is unknown as yet in both children and adults. Therefore, here we point to investigate whether a potential pathogenetically explainable relationship exists between circulating HMGB1 and NAFLD in children.

In the present study, we analyzed, in particular, the presence of extracellular HMGB1 in the bloodstream of obese pediatric patients with NAFLD compared with obese controls without NAFLD. We also tested whether plasma HMGB1 is associated with fibrosis as detected by liver biopsy and biochemical markers in NAFLD patients. Besides HMGB1, circulating levels of monocyte chemoattractant protein-1 (MCP-1) and TGF-β were also analyzed.

Patients & methods

Study subjects

We performed a cross-sectional study of 110 consecutive overweight/obese children with biopsy-proven NAFLD and 40 overweight/obese children with normal liver at ultrasonography and normal liver enzymes. All children were visited as outpatients at the Liver Unit of Bambino Gesù Children's Hospital between March 2011 and December 2012. Inclusion criteria were complete abstinence from alcohol; absence of serological markers of hepatitis B and C; absence of drugs known to induce fatty liver and; ceruloplasmin, antitransglutaminase antibodies, antinuclear antibodies, antimitochondrial antibodies and antismooth muscle antibodies within normal limits. Secondary causes of fatty liver disease were ruled out. The study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Ethical Committee of the Bambino Gesù Children's Hospital. Written informed consent was obtained from the parents or legal guardians of the children.

Anthropometry

Body weight and height were measured following standard procedures [38]. BMI was calculated as weight (kg)/height $(m)^2$ and transformed into standard deviations scores using Italian reference values [39].

Laboratory measurements

Alanine transaminase, aspartate transaminase, gamma-glutamyltransferase, glucose, triglycerides and total cholesterol were measured on fasting venous blood samples using standard laboratory methods. The homeostatic model assessment was used as surrogate measure of insulin resistance [40].

Enzyme-linked immunosorbent assay

Part of the blood was centrifuged at 3000 RPM for 12 min and plasma was stored at -80°C pending further analysis. Plasma samples were thawed only once and used to perform specific sandwich ELISA to measure the HMGB1 (IBL International GmbH, Hamburg, Germany), TGF-β (Biovendor Laboratory Medicine Inc., Brno, Czeck Republic), MCP-1 (Listarfish, Cernusco, Milan, Italy), hyaluronic acid (HA) (Biogenic SA, Maurin, France), cytokeratin-18 fragment (CK18) (Peviva, Grünwald, Germany) and LPS (Hycult Biotechnology, Uden, The Netherlands).

Liver histology

We performed liver biopsies with an 18G needle following a standard protocol. Histological samples were excised and fixed in 10% buffered formalin pending further analysis. The presence of collagen, an index of fibrosis, was examined in Masson's trichrome-stained preparations. Histological assessment of fibrosis was performed by an experienced pathologist (RDV) following the criteria put forth by the NAFLD Clinical Research Network [41]. Briefly, steatosis was graded: 0 = <5% steatosis; 1 = 5–33%; 2 = 33–66% and 3 = >66%. Lobular inflammation was scored based on the number of inflammatory foci per 200 × field: 0 = no inflammatory foci; 1 = <2 foci; 2 = 2–4 foci and 3 = >4 foci. Ballooning was scored as 0 = none; 1 = few balloon cells and 2 = prominent ballooning. Fibrosis was classified as 0 = no fibrosis; 1 = periportal or perisinusoidal (1A = mild, zone 3, perisinusoidal; 1B = moderate, zone 3, perisinusoidal; 1C = portal/periportal); 2 = perisinusoidal and portal/periportal; 3 = bridging fibrosis and 4 = cirrhosis. The pathologist also calculated

NAFLD activity score, even if the diagnosis of NASH was not based on it [41].

Statistical analysis

Continuous variables are reported as 50th, 25th and 75th percentiles because of non-Gaussian distributions. Categorical variables are reported as counts or percentages. Between-group comparisons of medians were performed using robust median regression [42]. The outcome variable was the continuous measurement of interest and the predictor was the discrete group. To this purpose, NAFLD was coded as 0 = no and 1 = yes; inflammation as 0 = degree 0, 1 = degree 1, and ≥ 2 = degrees 2+3; fibrosis as 0 = stage 0, 1 = stage 1 and 2 = stages 2+3. Bonferroni's correction was applied when the comparison involved more than two groups, that is, when evaluating inflammation and fibrosis. Robust median regression was also used to study the association between HMGB1, TGF- β, MCP-1, HA and LPS in children with NAFLD. For this analysis, all variables were log-transformed using natural logarithms and degree 2 fractional polynomials were used to test the linearity of their associations [43].

Results

Patient characteristics

The anthropometrical and biochemical measurements of the 110 NAFLD and 40 control children are given in Table 1. In total, 55% of the control and 58% of the NAFLD children were male. Median plasma levels of alanine transaminase, aspartate transaminase, cholesterol, HMGB1, TGF-β, MCP-1, HA, CK18 and LPS were higher in NAFLD than in control children ($p < 0.001$ for all). All the measurements were similar in males and females ($p > 0.05$ for all, data not shown).

The histological features of the children with NAFLD are given in Table 2. Fifty-seven of the children had NASH, 87% inflammation of any degree and 66% fibrosis of any stage. Because there was just one case of degree 3 inflammation and seven cases of stage 3 fibrosis, classes 2 and 3 of these outcomes were pooled for further analysis.

Table 1. Measurements in controls and nonalcoholic fatty liver disease subjects.

	Controls (n = 40; males = 22)			NAFLD (n = 110; males = 64)		
	P_{50}	P_{25}	P_{75}	P_{50}	P_{25}	P_{75}
Age (years)	10	7	13	11	8	12
Weight (kg)	51.5	45.0	61.0	52.5	40.0	64.0
Height (m)	1.41	1.35	1.50	1.46	1.30	1.53
BMI (kg/m^2)	25.4	24.0	27.1	25.6	22.3	27.8
BMI (SDS)	1.93	1.32	2.38	1.83	1.49	2.33
ALT (U/l)	28	23	32	67*	49	87
AST (U/l)	27	24	29	48*	40	64
GGT (U/l)	22	19	24	23	18	38
Triglycerides (mg/dl)	84	80	90	90	75	128
Cholesterol (mg/dl)	126	121	130	149*	129	184
Glucose (mg/dl)	80	77	82	81	75	87
Insulin (mcU/ml)	11	9	13	10	7	18
HOMA	2.2	1.7	2.6	2.1	1.4	3.6
HMGB1 (ng/ml)	1.1	0.9	1.3	3.7*	2.7	5.4
TGF-β (ng/ml)	1.1	0.8	1.4	9.6*	8.7	10.8
MCP-1 (ng/ml)	102	90	114	170*	152	237
HA (ng/mL)	17.8	14.4	19.6	37.4*	25.7	41.2
CK18 (U/l)	128	114	144	336*	182	380
LPS (EU/ml)	2.5	2.0	3.1	4.5*	3.5	15.6

*$p < 0.001$ versus controls (median regression).
ALT: Alanine transaminase; AST: Aspartate transaminase; CK18: Cytokeratin-18; GGT: Gamma-glutamyl-transferase; HA: Hyaluronic acid; HMGB1: High-mobility group box 1; HOMA: Homeostatic model assessment; LPS: Lipopolysaccharide; MCP-1: Monocyte chemoattractant protein-1; NAFLD: Nonalcoholic fatty liver disease; SDA: Standard deviations scores.

Association of HMGB1, TGF-β & MCP-1 with inflammation & fibrosis in children with NAFLD

As a recent experimental study demonstrated that HMGB1 may switch hepatic stellate cells (HSCs) toward a proinflammatory and profibrogenic phenotype by increasing the expression of MCP-1 and by enhancing the effect of TGF-β [44], we evaluated the association between the circulating levels of these molecules and inflammation and fibrosis.

Figure 1 shows the association between HMGB1, TGF-β, MCP-1 and inflammation and fibrosis in children with NAFLD. A median band plot is superimposed to a scatterplot to contrast the medians corresponding to the different degrees of inflammation and stages of fibrosis. Between-group comparisons were performed using median regression with Bonferroni's correction for multiple comparisons. The median level of HMGB1 was similar in degrees 0 (3.6 ng/ml), 1 (3.9 ng/ml) and ≥2 (3.1 ng/ml) of inflammation ($p > 0.01$ for all comparisons). The median level of TGF-β increased from degree 0 (6.7 ng/ml) to degree 1 (9.6 ng/ml) and to degree ≥2 (12.2 ng/ml) of inflammation ($p < 0.01$ for all comparisons). No between-group difference was observed for MCP-1 (160, 170 and 180 ng/ml, respectively, $p > 0.01$ for all comparisons). In children with NAFLD, the median level of HMGB1 was similar in stages 0 (2.9 ng/ml) and 1 (3.8 ng/ml, $p > 0.01$) of fibrosis but higher in stage ≥2 than in stages 0 and 1 (6.9 ng/ml) ($p < 0.01$ for both). The median level of TGF-β was similar in stages 0 (8.9 ng/ml) and 1 (9.7 ng/ml, $p > 0.01$) of fibrosis and higher in stages ≥2 (11.9 ng/ml) than in stages 0 and 1 ($p < 0.01$ for both). The median level of MCP-1 was similar in stages 0 (160 pg/ml) and 1 (170 pg/ml, $p > 0.01$) but higher in stages ≥2 (280 pg/ml) than in stages 0 and 1 ($p < 0.01$ for both comparisons).

Table 2. Histological features of children with nonalcoholic fatty liver disease.

	n	%
Steatosis		
1	38	34.5
2	53	48.2
3	19	17.3
Inflammation		
0	14	12.7
1	78	70.9
2	17	15.5
3	1	0.9
Ballooning		
0	58	52.7
1	32	29.1
2	20	18.2
NAS		
1	6	5.5
2	21	19.1
3	32	29.1
4	23	20.9
5	18	16.4
6	8	7.3
7	2	1.8
NASH		
0	47	42.7
1	63	57.3
Fibrosis		
0	37	33.6
1	55	50.0
2	11	10.0
3	7	6.4

NAS: NAFLD activity score.

Association of HMGB1 & TGF- β, MCP-1, HA, CK18 & LPS in children with NAFLD

The association of TGF-β and MCP-1 with liver damage in NAFLD and their relationship with LPS suggests a close association with HMGB1. Several biomarkers are associated with fibrosis severity in NAFLD. Among these, HA and CK18 fragments are useful to stratify fibrosis in pediatric NAFLD [45,46]. Therefore, we evaluated the association of HMGB1 with these biomarkers of liver fibrosis.

Figure 2 shows the association between HMGB1 and TGF-β, MCP-1, HA, CK18 and LPS. All variables were transformed using natural logarithms to reduce skewness of distributions. Superimposed to the scatterplots are the lines (and 95% confidence intervals) estimated from median regression models. No regression line is depicted for the $\log_e$HMGB1-CK18 as it was characterized by two separated clusters of points. Such distribution is explained by the bimodal distribution of $\log_e$CK18 as the distribution of $\log_e$HMGB1 was close to Gaussian. Log_eHMGB1 was linearly and significantly associated with $\log_e$TGF-β ($p < 0.01$), $\log_e$MCP-1 ($p < 0.001$), $\log_e$HA ($p < 0.001$) and $\log_e$LPS ($p < 0.001$).

Discussion

NAFLD pathogenesis remains an unsolved issue in both children and adults. It is now accepted that the development of steatosis and its progression to NASH are produced by the interaction of multiple factors and organs [2,47]. Indeed, extrahepatic tissues and organs (adipose tissue and the gut), under the pressure of triggering factors, such as genes and diet, may contribute to the production and the release of circulating endotoxins, adipokines and proinflammatory cytokines, which cooperate with insulin resistance and free fatty acids accumulation causing oxidative stress, hepatocellular damage and profibrotic activation of HSCs [48,49].

The activation of TLR4 signaling, mediated by gut-derived LPS, may be one of the most important proinflammatory and profibrogenic pathways that trigger NASH and fibrosis. In this scenario, LPS, as exemplary pathogen-associated molecular pattern, cooperates with several liver-derived DAMPs, including HA from HSCs and HMGB1 from apoptotic hepatocytes, to induce inflammation and fibrogenesis [9]. These signals, in turn, may cause a low-grade systemic inflammation characterized by the release of several circulating cytokines, growth factors and metabolites that could explain not only the increased hepatic tissue damage in the site of SI but also the severe multiorgan damage featuring the cardiometabolic syndrome [11,50,51]. The identification of circulating factors involved in this response could be of great help to understand the possible connection between them and liver damage in NAFLD, making more easy the identification of new therapeutic targets as well as the implementation of early diagnostic tools.

In this study, we pointed to investigate the possible correlation between plasma levels of HMGB1 with systemic inflammation and liver damage in children with NAFLD. Our results showed that plasma levels of HMGB1, LPS and HA are higher in overweight/obese pediatric NAFLD patients than in age-matched overweight/obese controls without NAFLD. Although our findings need replication in larger and external samples, they are in agreement with a previous studies [45,52,53]. Li *et al.* showed that, in the early stages of NAFLD in mice, hepatocellular expression and

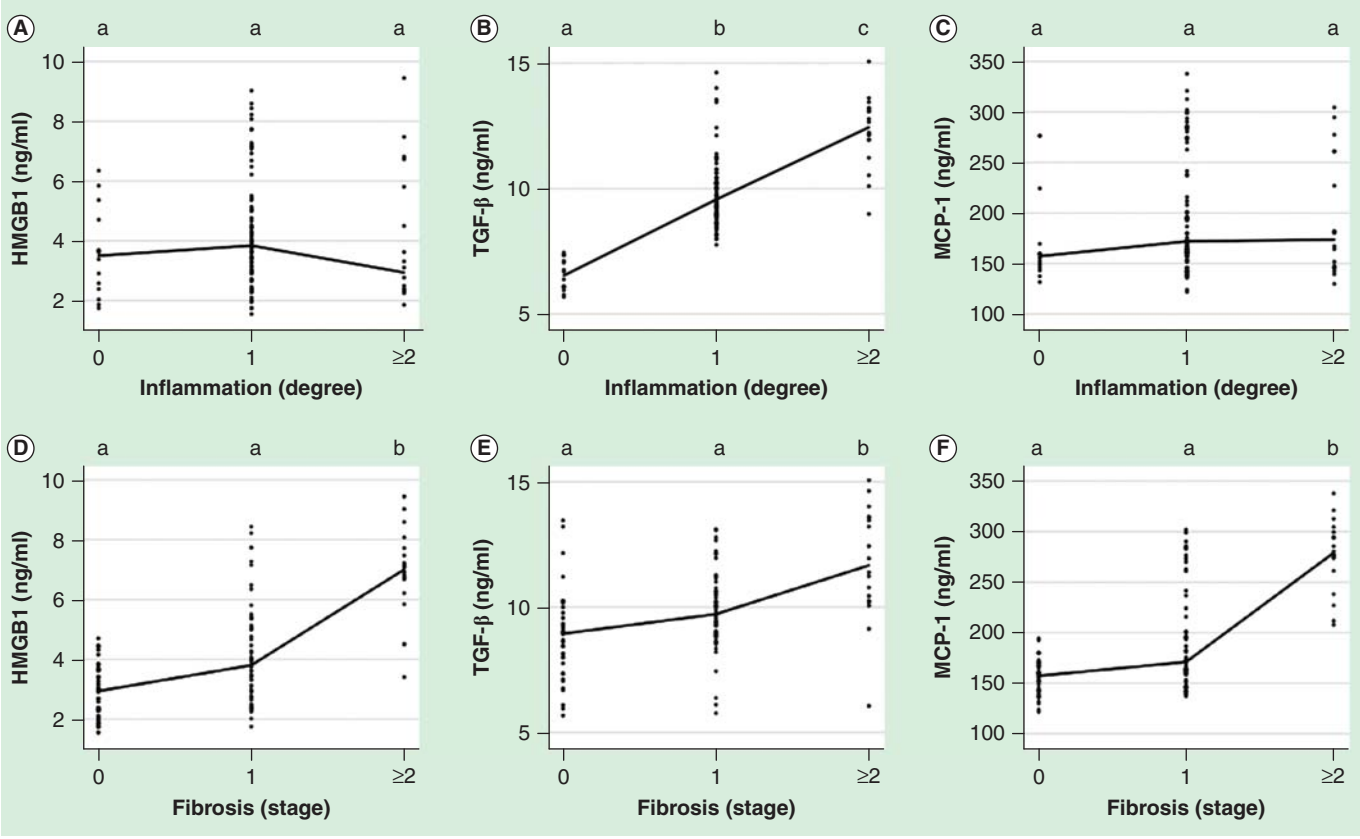

Figure 1. Association of high-mobility group box 1, TGF-β and monocyte chemoattractant protein-1 with inflammation and fibrosis grades. A median band plot is superimposed to a scatterplot to contrast the medians corresponding to the different degrees of inflammation **(A–C)** and stages of fibrosis **(D–F)**. Between-group comparisons were performed using median regression with Bonferroni's correction for multiple comparisons.
[a,b,c]Groups not sharing the same letter are significantly different at $p < 0.01$ (median regression).
HMGB1: High-mobility group box 1; MCP-1: Monocyte chemoattractant protein-1.

release of HMGB1 are increased so that this protein could trigger the TLR4-mediated inflammatory response due to free fatty acid infusion [52]. Moreover, it has been reported that in children with NAFLD LPS serum levels are greater than in controls, and HA circulating levels strongly correlate with fibrosis [45,53].

Previous clinical studies have shown that elevated circulating levels of HMGB1 are associated with necrosis or apoptosis and that HMGB1 is a marker of hepatocellular injury in human liver transplantation [54–56]. In the present study, we found that HMGB1 plasma levels were strongly associated with the presence of fibrosis and were able to discriminate *mild (F0-F1)* versus *severe (F2-F3) fibrosis.* These findings are mechanistically in agreement with recent experimental studies that have suggested a profibrotic function of HMGB1 in the liver [44,57,58]. In detail, these studies showed that HMGB1 may mediate the transformation of quiescent HSCs into myofibroblast-like cells with an increased production of extracellular matrix proteins and other proinflammatory and profibrogenic molecules, including TGF-β and MCP-1. Therefore, it is conceivable that, in a vicious circle, HMGB1 may act in concert with its downstream circulating factors to induce hepatocellular damage, inflammation and fibrosis in children with NAFLD. This hypothesis is supported by the fact that in our children with NAFLD also TGF-β and MCP-1 were different in mild versus severe fibrosis and both were strongly associated with HMGB1. A further evidence of the HMBG1-fibrosis relationship comes from the fact that HMGB1 was associated with CK18 and HA, two noninvasive markers of fibrosis in children [45,46]. Furthermore, circulating levels of HMGB1 were associated with LPS supporting the hypothesis that LPS may be the driving force for the release of HMGB1, HA, and proinflammatory and profibrogenic response of liver-resident cells, and a synergic action among these molecules might contribute to fibrotic damage in advanced NAFLD, when gut permeability is compromised [44]. However, neither HMGB1 nor MCP-1 plasma levels were associated with liver necro-inflammation, while there was a significant association between TGF-β and inflammation in our children with NAFLD. Such finding can be explained by hypothesizing that, while HMGB1 and other circulating factors (such as MCP-1) could be engaged only in fibrogenesis, other factors may promote the

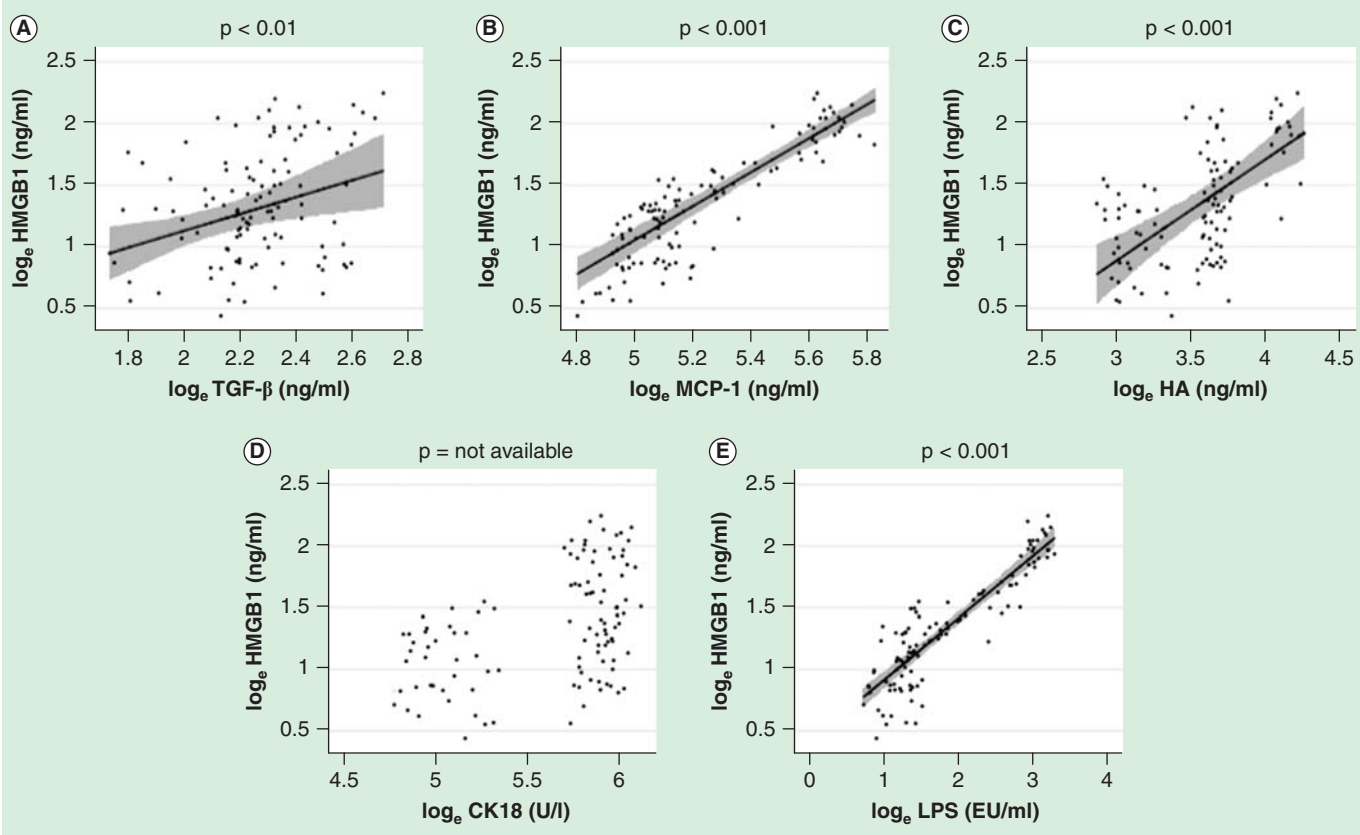

Figure 2. Association of high-mobility group box 1 with monocyte chemoattractant protein-1, TGF-β, hyaluronic acid, Cytokeratin-18 and lipopolysaccharide. The graphs represent the association of circulating levels of HMGB1 with those of MCP-1 **(A)** TGF-β; **(B)** HA; **(C)** CK18; **(D)** and LPS **(E)**. All variables were transformed using natural logarithms. Superimposed to the scatterplots are the lines and 95% confidence intervals estimated from the median regression models.
Regression lines and 95% CIs from median regression.
CK18: Cytokeratin-18; HMGB1: High-mobility group box 1; MCP-1: Monocyte chemoattractant protein-1; HA: Hyaluronic acid; LPS: Lipopolysaccharide.

necro-inflammation. Of course, our hypotheses need to be confirmed by further basic studies to better highlight the role of HMGB1 in NAFLD severity. An intriguing possibility for such studies is the use of HMGB1 inhibitors, some of which already available, as potential treatments to reduce hepatic fibrosis [59,60].

In conclusion, this is the first report that evaluates changes of HMGB1 circulating levels in NAFLD subjects, demonstrating a significant increase of this molecule in children with disease respect to control subjects. More importantly, our study suggests that HMGB1, coupled with MCP-1, is associated with fibrosis in children with NAFLD likewise to what observed in adult patients with chronic hepatitis [61]. This last finding not only reinforces the pathogenetic role of HMGB1, but also has strong clinical relevance opening the view to a possible future use of HMGB1 as a noninvasive biomarker of liver fibrosis in children with NAFLD. Further studies on larger samples of children are needed to determine whether HMGB1, alone or in combination with other biomarkers, can be used as a noninvasive tool to suspect fibrosis or monitor disease during follow-up of therapeutic interventions.

Financial & competing interests disclosure

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Key issues

- Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases in the pediatric population.
- Pathogen-associated molecular patterns, such as lipopolysaccharide, and damage-associated molecular patterns, such as high-mobility group box 1 (HMGB1), are suspected to induce low-grade inflammation and liver damage occurring in NAFLD.
- Increased plasma levels of HMGB1, were recently reported in adults with acute liver failure or acetaminophen-induced acute liver injury and with chronic hepatitis.
- We demonstrated that plasma levels of HMGB, lipopolysaccharide and hyaluronic acid are higher in overweight/obese children with NAFLD patients than in controls.
- We showed that HMGB1 plasma levels were strongly associated with the presence of fibrosis and were able to discriminate mild versus severe fibrosis in pediatric NAFLD.
- In children with NAFLD, HMGB1 plasma levels also associate with those of other noninvasive biomarkers of liver damage, including hyaluronic acid and cytokeratin-18 fragment.
- The results of this study suggest the use of HMGB1 inhibitors as potential treatments to reduce hepatic fibrosis.
- Our findings open the view to a possible future use of HMGB1 as a noninvasive biomarker of liver fibrosis in children with NAFLD.

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