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**Noninvasive assessment of liver disease severity with liver fat score and CK-18 in NAFLD:
Prognostic value of liver fat equation goes beyond hepatic fat estimation.**

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/66264> since

Published version:

DOI:10.1002/hep.23255

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with respect to wild-type animals fed water, and the enrichment with fructose exclusively caused the restoration of the significantly increased levels of these two parameters. These results clearly suggest that the presence of TLR-4 is essential to explain liver damage, body weight gain, and ALT impairment due to the fructose intake.

Furthermore, the authors found that plasma endotoxin levels were significantly increased both in wild-type and mutant mice fed chronically with a 30% fructose solution, in comparison to water-fed controls.

The role of fructose in NAFLD development was not entirely unknown to researchers. In particular, a recent work⁴ demonstrates that patients with NAFLD have a significantly greater consumption of fructose than controls, and an increased hepatic expression of fructokinase messenger RNA. Although the role of TLR-4 in carbohydrate-dependent NAFLD has been only recently suggested by Thuy and colleagues,⁵ they have pinpointed one of the potential mechanisms through which fructose could participate in NAFLD development and progression in humans: a carbohydrate-rich diet may produce ethanol when intestinal stasis favors bacterial overgrowth in the upper parts of the gastrointestinal tract. The increased portal endotoxemia could initiate TLR signaling and induce necroinflammation, which characterizes steatohepatitis, the most advanced form of NAFLD. Accordingly, this study also highlighted significant correlations between hepatic expression of TLR-4, plasminogen activator inhibitor 1, and endotoxin, even though they are still unable to explain the molecular signaling pathways.

Interestingly, another recent study investigated the potential importance of Kupffer cells and TLR-4 in the pathogenic mechanisms underlying nonalcoholic steatohepatitis induced by a methionine-deficient and choline-deficient diet.⁶

Unfortunately, the study by Spruss et al. does not provide additional clues to the mechanisms by which fructose intake, endotoxemia, and the resulting activation of TLR-4 signaling might promote NAFLD. On the other hand, the experimental results in this work allow the exclusion of the involvement of some important TLR-4-dependent proinflammatory inducing transcriptional factors (i.e., IRF3 and IF37), suggesting that fructose feeding may lead to NAFLD through an insulin-independent *de novo* lipogenesis and/or an endotoxin-dependent activation of Kupffer cells. In this last hypothesis, an interaction network which involves TLR-4, Myd88, c-Jun N-terminal kinase, and nuclear factor κ B might induce tumor necrosis factor- α production and release, oxidative stress, and insulin resistance.⁷

We believe that although Spruss et al. present a well-conducted study, the precise role of TLR-4-dependent pathways in NAFLD requires further experimentation. In fact, it is possible that new additional signaling proteins of innate immunity, as yet uncovered, may be involved in the necroinflammatory process and in the progression to steatohepatitis and fibrosis.

ANNA ALISI, PH.D.

NADIA PANERA

VALERIO NOBILI, M.D.

Liver Unit

*Bambino Gesù Children's Hospital and Research Institute
Rome, Italy*

References

1. Spruss A, Kanuri G, Wagnerberger S, Haub S, Bischoff SC, Bergheim I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. *HEPATOLOGY* 2009;50:1094-1104.
2. Bray GA, Nielsen SJ, Popkin BM. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. *Am J Clin Nutr* 2004;79:537-543.
3. Bergheim I, Weber S, Vos M, Krämer S, Volynets V, Kaserouni S, et al. Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin. *J Hepatol* 2008;48:983-992.
4. Ouyang X, Cirillo P, Sautin Y, McCall S, Bruchette JL, Diehl AM, et al. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. *J Hepatol* 2008;48:993-999.
5. Thuy S, Ladurner R, Volynets V, Wagner S, Strahl S, Königsrainer A, et al. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. *J Nutr* 2008;138:1452-1455.
6. Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol* 2007;47:571-579.
7. Valenti L, Ludovica Fracanzani A, Fargion S. The immunopathogenesis of alcoholic and nonalcoholic steatohepatitis: two triggers for one disease? *Semin Immunopathol* 2009; doi: 10.1007/s00281-009-0152-9

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23241

Potential conflict of interest: Nothing to report.

Noninvasive Assessment of Liver Disease Severity With Liver Fat Score and CK-18 In NAFLD: Prognostic Value of Liver Fat Equation Goes Beyond Hepatic Fat Estimation

To the Editor:

We read with interest the article by Kotronen et al.,¹ reporting on liver fat score and liver fat equation—new noninvasive, easy-to-calculate indexes that estimate the presence and severity of hepatic fat accumulation. We assessed the ability of these two indexes and of plasma cytokeratin-18 fragments (CK-18) to predict the presence of nonalcoholic fatty liver disease (NAFLD) and of nonalcoholic steatohepatitis (NASH),^{1,2} respectively, and their relations to validated predictors of incident cardiovascular disease and diabetes.^{3,4}

To this purpose, 125 subjects (40 nondiabetic patients with biopsy-proven NAFLD and 85 healthy controls) underwent an oral fat tolerance test,⁵ with measurement of postprandial plasma lipid responses, and a standard oral glucose tolerance test (OGTT), whose results were elaborated by Minimal Model analysis to assess whole-body, hepatic, and muscle insulin sensitivity and indexes of pancreatic β -cell function

(namely, CP-genic index [CGI] and Adaptation Index [AI]), as previously described.⁵⁻⁷ Finally, circulating markers of inflammation (C-reactive protein), endothelial dysfunction (E-selectin and intercellular adhesion molecule-1 [ICAM-1]) and oxidative stress (nitrotyrosine and oxidized low-density lipoproteins) were measured.

Results are shown in Table 1. NASH group showed higher postprandial lipemia and oxidative stress than either steatosis or controls. Patients with NASH had also more severe whole-body insulin resistance, hepatic insulin resistance, and pancreatic β -cell dysfunction and higher plasma C-reactive protein, E-selectin, ICAM-1, and nitrotyrosine levels than steatosis and control groups.

Liver fat equation correlated with the degree of histological steatosis in both NASH and steatosis groups (in both groups: $r_s > 0.66$, $P < 0.003$). The area under the receiver operating characteristic curve (AU-ROC) of liver fat score for predicting NAFLD was 0.86 (95% confidence interval [CI]: 0.82-0.91). A cutoff of -0.640 individuated

Table 1. Baseline Characteristics of Patients with NAFLD and Controls

| Characteristic | Controls (n = 85) | Steatosis (n = 17) | NASH (n = 23) | P Value |
|--|-------------------|--------------------|-----------------|---------|
| Age (years) | 49 ± 4 | 48 ± 3 | 46 ± 4 | 0.712 |
| Sex (%males) | 67 | 70 | 71 | 0.549 |
| BMI (kg/m ²) | 25.1 ± 1.7 | 24.9 ± 1.6 | 25.3 ± 1.5 | 0.656 |
| Systolic BP (mmHg) | 129 ± 6 | 130 ± 7 | 131 ± 9 | 0.978 |
| Diastolic BP (mmHg) | 81 ± 4 | 84 ± 6 | 88 ± 6* | 0.034 |
| Waist (cm) | 88 ± 2 | 90 ± 4 | 91 ± 4 | 0.478 |
| Tg (mg/dL) | 89 ± 21 | 98 ± 35 | 111 ± 39* | 0.112 |
| LDL-C (mg/dL) | 99 ± 7 | 124 ± 8 | 150 ± 7* | 0.056 |
| HDL-C (mg/dL) | 59 ± 2 | 57 ± 2 | 49 ± 2* | 0.013 |
| Total C (mg/dL) | 156 ± 9 | 205 ± 9 | 241 ± 10† | 0.058 |
| Glucose (mg/dL) | 92 ± 3 | 96 ± 3 | 97 ± 4 | 0.541 |
| Insulin (μU/mL) | 4.2 ± 1.6 | 14.3 ± 2.9† | 22.7 ± 5.6† | 0.009 |
| AST (U/L) | 13 ± 3 | 56 ± 3† | 42 ± 3† | 0.123 |
| ALT (U/L) | 16 ± 5 | 127 ± 8† | 116 ± 8† | 0.245 |
| ICAM-1 (mg/mL) | 190.5 ± 7.1 | 220.4 ± 7.2* | 264.4 ± 8.7† | 0.031 |
| E-selectin (mg/mL) | 18.9 ± 2.0 | 23.1 ± 2.3† | 38.2 ± 2.5† | 0.001 |
| C-reactive protein | 1.1 ± 0.7 | 1.6 ± 1.0* | 2.4 ± 1.1† | 0.028 |
| CK-18 (IU/L) (IU/L)fragments | 98 ± 6 | 136 ± 11 | 253 ± 12† | 0.030 |
| Nitrotyrosine | 3.1 ± 3.7 | 9.9 ± 4.1* | 27.4 ± 7.3† | 0.002 |
| Met sy (%) | 21 | 36 | 47† | 0.052 |
| Histological steatosis (% hepatocytes) | - | 16 ± 7 | 33 ± 9 | 0.032 |
| Necroinflammatory grade | - | - | 2.1 ± 0.3 | - |
| Fibrosis stage | - | - | 1.9 ± 0.4 | - |
| Liver fat score | 2.428 ± 0.112 | 1.563 ± 0.290† | 5.114 ± 0.931† | 0.039 |
| Liver fat (%) | 1.5 ± 1 | 13 ± 3† | 30 ± 6† | 0.025 |
| OGTT-derived indexes of glucose homeostasis | | | | |
| OGIS (mL · minute ⁻¹ · m ⁻²) | 459.1 ± 16.5 | 400 ± 11.9* | 369 ± 10.2† | 0.032 |
| Hepatic extraction (%) | 78 ± 4 | 81 ± 5 | 70 ± 4* | 0.030 |
| Cpgenic Index (CGI) (ng _{C-pep} · g ⁻¹ _{glucose}) | 614 ± 36.1 | 540 ± 29.5 | 402 ± 19.3† | 0.009 |
| Adaptation Index (ng _{C-pep} · g ⁻¹ _{glucose} · mL ⁻¹ · m ⁻²) (ng _{C-pep} · g ⁻¹ _{glucose} · mL ⁻¹ · m ⁻²) | 281826 ± 15391 | 216703 ± 12678 | 148401 ± 10681† | 0.007 |
| Muscle insulin sensitivity | 0.032 ± 0.009 | 0.016 ± 0.008* | 0.015 ± 0.006* | 0.267 |
| Hepatic insulin resistance | 738 ± 96 | 1109 ± 112† | 1812 ± 183† | 0.011 |
| Oral Fat Load | | | | |
| IAUC Tg (mg/dL × hour) | 124 ± 28 | 344 ± 49 | 398 ± 56 | 0.034 |
| Fasting FFA (μmol/L) | 0.47 ± 0.13 | 1.09 ± 0.22 | 1.96 ± 0.29 | 0.112 |
| IAUC FFA (μmol/L × hour) | 0.8 ± 0.3 | 2.5 ± 0.5 | 4.9 ± 0.9 | 0.009 |
| Fasting oxidizedLDL (uA 234 nm/uA 200 nm × 100) | 6.42 ± 1.63 | 6.44 ± 1.23 | 7.94 ± 2.59 | 0.356 |
| IAUC oxidized LDL (uA 234 nm/uA 200 nm × 100 × hour) | 1.29 ± 0.51 | 4.29 ± 1.25 | 8.02 ± 1.93 | 0.001 |

Data are presented as mean ± standard error of the mean. Differences between groups were analyzed by analysis of variance for normal variables; otherwise, the Mann-Whitney test was used for nonparametric variables. Normality was evaluated by Shapiro-Wilk test. Fisher or chi-squared test were used to compare categorical variables, as appropriate. Differences were considered statistically significant at $P < 0.05$.

BP, blood pressure; FFA, free fatty acids; HDL-C, high-density lipoprotein cholesterol; IAUC, incremental area under the curve; ICAM-1, intercellular adhesion molecule-1; LDL-C, low-density lipoprotein cholesterol; met sy, metabolic syndrome; OGIS, oral glucose insulin sensitivity index; Tg, triglyceride; total C, total cholesterol.

CGI (Cpgenic index) = $\Delta C - \text{peptide}_{30'} / \Delta \text{glucose}_{30'}$ during the OGTT; Adaptation Index was computed by multiplying CGI × OGIS. Hepatic extraction is the percent secreted insulin extracted by the liver.

* $P < 0.05$ versus controls; † $P < 0.01$ versus controls.

NAFLD with a sensitivity, specificity, and positive and negative likelihood ratio of 0.93, 0.80, 4.63, and 0.09, respectively.

The AUROC of CK-18 for NASH was 0.83 (95% CI: 0.80-0.90). A cutoff of 246 IU/L for CK-18 individuated NASH with a sensitivity, specificity, and positive and negative likelihood ratio of 0.78, 0.88, 6.65, and 0.25, respectively.

On multiple regression analysis, liver fat equation independently correlated with hepatic insulin resistance ($\beta = 0.52$; 95% CI: 0.48-0.56, $P = 0.005$) and with indexes of pancreatic β -cell function (for CGI: $\beta = -0.43$; 95% CI: 0.48-0.56, $P = 0.01$; for AI: $\beta = -0.46$; 95% CI: 0.42-0.51, $P = 0.009$) in the whole sample. Liver fat equation also independently predicted plasma C-reactive protein ($\beta =$

0.40; 95% CI: 0.37-0.44, $P = 0.02$), nitrotyrosine ($\beta = 0.41$; 95% CI: 0.38-0.46, $P = 0.02$), E-selectin ($\beta = 0.49$; 95% CI: 0.45-0.54, $P = 0.006$), and postprandial triglyceride ($\beta = 0.42$; 95% CI: 0.39-0.46, $P = 0.02$) and oxidized low-density lipoprotein ($\beta = 0.40$; 95% CI: 0.38-0.45, $P = 0.03$) responses to the fat load.

Histological steatosis, inflammatory grade, and fibrosis stage were not independently related to the abovementioned parameters.

In conclusion, liver fat equation and CK-18 accurately individuated the presence and severity of liver fat infiltration and the presence of NASH in our cohort of nondiabetic subjects. Most importantly, liver fat equation was tightly related to validated predictors of increased cardiometabolic risk in both healthy and NAFLD subjects. The clini-

cal significance of liver fat equation may thus go beyond hepatic fat content estimation and this easy-to-calculate index may aid in predicting individual cardio-metabolic risk of patients with NAFLD. Our cross-sectional findings warrant prospective confirmation in independent large cohorts.

GIOVANNI MUSSO, M.D.¹
ROBERTO GAMBINO, PH.D.²
MARILENA DURAZZO, PH.D.²
MAURIZIO CASSADER, PH.D.²
¹Gradenigo Hospital, Turin, Italy
²Department of Internal Medicine,
University of Turin, Italy

References

1. Kotronen A, Peltonen M, Hakkarainen A, Sevastianova K, Bergholm R, Johansson LM, Lundbom N, Rissanen A, Ridderstråle M, Groop L, Orholm-Melander M, Yki-Järvinen H. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology* 2009;137:865-872.
2. Feldstein AE, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarker for nonalco-

- holic steatohepatitis: A multicenter validation study. *HEPATOLOGY* 2009; doi:10.1002/hep.23050.
3. Abdul-Ghani MA, Williams K, DeFronzo RA, Stern M. What is the best predictor of future type 2 diabetes? *Diabetes Care* 2007;30:1544-1548.
4. Adiels M, Olofsson SO, Taskinen MR, Boren J. Diabetic dyslipidaemia. *Curr Opin Lipidol* 2006;17:238-246.
5. Musso G, Gambino R, Pacini G, Pagano G, Durazzo M, Cassader M. Transcription factor 7-like 2 polymorphism modulates glucose and lipid homeostasis, adipokine profile, and hepatocyte apoptosis in NASH. *HEPATOLOGY* 2009;49:426-435.
6. Tura A, Kautzky-Willer A, Pacini G. Insulinogenic indices from insulin and C-peptide: comparison of beta-cell function from OGTT and IVGTT. *Diabetes Res Clin Pract* 2006;72:298-301.
7. Ahrén B, Pacini G. Importance of quantifying insulin secretion in relation to insulin sensitivity to accurately assess beta cell function in clinical studies. *Eur J Endocrinol* 2004;150:97-104.

Financial support: this work was funded in part by the Piedmont Region Funds Comitato Interministeriale per la Programmazione Economica 2008.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23255

Potential conflict of interest: Nothing to report.

Serum Markers of Hepatocyte Apoptosis: Current Terminology and Predictability in Clinical Practice

To the Editor:

We read with interest the article by Feldstein et al.,¹ in which they validated the use of cytokeratin-18 (CK-18) fragment serum levels as a noninvasive biomarker for the diagnosis of nonalcoholic steatohepatitis (NASH) in a large cohort of adults with biopsy-proven nonalcoholic fatty liver disease (NAFLD). According to their conclusions, this test can be reliably used to diagnose NASH among patients with suspected NAFLD in clinical practice. This is an important study, because it confirms the significant associations between high CK-18 fragment levels and NASH reported in previous smaller studies in other cohorts of adults^{2,3} or even children with NAFLD.⁴ However, we would like to draw your attention to two issues: terminology and predictability.

The "Keratin Nomenclature Committee" recently established the new consensus nomenclature for mammalian keratin genes and proteins,⁵ which is based on and extends the first comprehensive keratin nomenclature developed back in 1982.⁶ According to this new nomenclature, which is in agreement with the nomenclature of the Human Genome Organisation (HUGO) for both gene and protein names,⁵ it is suggested the term "keratin" be used instead of "cytokeratin", because it covers all intermediate filament-forming proteins with specific physicochemical properties produced in any vertebrate epithelia.⁵ Thus, the term "keratin-18 (K-18)" is now more appropriate than "cytokeratin-18 (CK-18)" for all manuscripts.

K-18 and keratin-8 (K-8) are the only cytoplasmic intermediate filaments of hepatocytes but are not hepatocyte-specific, because they are also expressed in most simple epithelial cells including bile duct cells.⁷ K-18 fragment serum levels, which are increasingly used as a biomarker of hepatocyte apoptosis, are also not liver-specific, because these levels may be elevated in patients with epithelial tumors.⁸ Moreover, this marker is not specific for NASH, because it is increased in several liver diseases with ongoing necroinflammation and fibrosis, such as chronic hepatitis C or B.^{9,10}

The specificity issues are substantially limited if the test is used in patients with probable NAFLD. However, even in such patients followed in specialized centers, its diagnostic accuracy does not seem to be excellent. In the study by Feldstein et al.,¹ the area under the receiver operating characteristic (AUROC) curve for NASH diagnosis was

0.83 (not excellent) with sensitivity and specificity values of 75% and 81% or 65% and 92% for cutoff values of 246 or 292 U/L, respectively. In 58 adult patients with NAFLD studied in our center, K-18 fragment levels offered an AUROC curve of 0.87 and sensitivity and specificity values of 60% and 93%, respectively, for a cutoff of 250 U/L (G. V. Papatheodoridis, unpublished data). Similar findings for variable cutoff values were previously reported by others with the best results reported in the first relevant study.^{2,3} Thus, measurement of K-18 fragment levels will probably be helpful in the noninvasive diagnosis of NAFLD, particularly in cases with rather high levels. The specificity issues should be restricted by ensuring the NAFLD diagnosis, but a decision may not be easy in a large proportion of NAFLD cases with K-18 values of <300 U/L, and particularly <200 U/L.

DINA G. TINIAKOS¹

GEORGE V. PAPATHEODORIDIS²

¹Laboratory of Histology and Embryology
and ²2nd Department of Internal Medicine,
Medical School,
University of Athens,
Greece

References

1. Feldstein AE, Wieckowska A, Lopez R, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: A multi center validation study. *HEPATOLOGY* 2009; doi:10.1002/hep.23050.
2. Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *HEPATOLOGY* 2006;44:27-33.
3. Yilmaz Y, Dolar E, Ulukaya E, Akgoz S, Keskin M, Kiyici M, et al. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. *World J Gastroenterol* 2007;13:837-844.
4. Vos MB, Barve S, Joshi-Barve S, Carew JD, Whittington PF, McClain CJ. Cytokeratin 18, a marker of cell death, is increased in children with sus-