CORRESPONDENCE

Fibrosis in Liver as a Predictive Marker for Hepatitis C Virus Therapy

To the Editor:

We read with great interest the article by Petta et al.[1] The compound 25-hydroxyvitamin D3 (25[OH]D3) was reported as an independent predictor of cardiovascular disease (by a decreased expression of profibrotic markers, and an increased expression of antifibrotic markers) despite the fact that its real pathological pathway is still not clear.[2] Incubation of the multipotent mesenchymal cell with 25(OH)D3 also resulted in antiproliferative and antiapoptotic processes.[2] Therefore, the lower levels of 25(OH)D3 in liver with greater fibrosis is understandable. Lower cholesterol and lower 25(OH)D3 levels, along with greater steatosis, were found to be risk factors affecting sustained virological response (SVR) as seen in recent studies. The stage of fibrosis was found to be a risk factor for SVR not only in hepatitis C virus (HCV) alone, but also in patients coinfected with human immunodeficiency virus and HCV, in contrast to the results of the current article.[3,4] Moreover, age, sex, and body mass index were also described as predictors for SVR in patients infected with HCV,[5] in contrast to the current study. These challenging results could be related in the methodologic differences between the present study and recent studies, or mistakes could have happened during the sampling and/or analyzing periods. For example, SVR was reached in the half the male patients, whereas it was reached in just one-third of the females, results which are also different from the recent data. The patients in the study may also be infected with an unknown subgroup of HCV, which could explain these patients' characteristics.

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Reply:

We thank Dr. Altınbaş and colleagues for their interest in our recent article.[1] They pointed out that in our study population fibrosis was not a driver of low sustained virological response (SVR) as in other studies. We are fully aware that advanced fibrosis and cirrhosis in particular strongly affect the likelihood of SVR, as shown also in a large-scale study.[2] We have in fact included in our study a cohort of consecutive patients with a histological diagnosis of genotype 1 chronic hepatitis C and a low prevalence of cirrhosis (less than 10%) because at our center patients with clinical evidence of cirrhosis are excluded from biopsy according to current guidelines.[3] Hence, the small number of subjects with cirrhosis in our study does not allow us to point out the inverse relationship between fibrosis and SVR. In contrast, the absence of a major factor of unresponsiveness, such as severe fibrosis, allowed us to explore in this highly homogeneous cohort of patients with genotype 1 chronic hepatitis C the impact on SVR of metabolic factors, such as steatosis, and vitamin D, a promising mediator of liver damage and immune response modulation.

The lack of an association in our study between SVR and age, sex, and body mass index, also underlined by Altınbaş and colleagues,[1] is likely due to differences in demographic, anthropometric, biochemical, and histological features. Accordingly, all these negative predictors have not been identified together in phase III trials of pegylated interferon and ribavirin,[4-6] probably because of heterogeneity in the enrolled populations.

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Is Oil Red-O Staining and Digital Image Analysis the Gold Standard for Quantifying Steatosis in the Liver?

To the Editor:

We read with great interest the randomized placebo-controlled trial by Abdelmalek et al.,[1] in which the effects of betaine were studied in nonalcoholic fatty liver disease (NAFLD). The authors used the widely employed NAFLD activity score (NAS)[2] as part of the inclusion criteria to their study and as the "gold-standard" endpoint to define treatment response. One of the major outcomes identified in the trial was change in the steatosis grade.

The NAS is a well-validated, pragmatic, semiquantitative scoring system for determining the severity of NAFLD.[3] The degree of steatosis contributes up to 3 of the 8 points to this score, and hepatocyte ballooning (which may be confused with small-droplet steatosis) contributes a further 2 points. Given the large contribution of steatosis to the overall score, it is important to correctly identify steatosis in a liver biopsy.

During the study of both human tissue and tissue from mouse models of NAFLD, we have found that accurately determining the amount of steatosis on hematoxylin and eosin (H&E) staining, especially when the fat droplets are small, is extremely challenging. Consequently, we have explored an alternative approach adopting Oil red-O (ORO) as the "gold standard" histochemical stain for specifically identifying lipids.[4] Using steatotic murine liver tissue from C57BL/6 mice, we compared the assessment of steatosis in H&E-stained sections by two expert liver pathologists with digital image analysis (DIA) quantification of ORO-stained sections. Hepatic triglyceride levels were quantified in the same tissues (triglyceride quantification kit, ab65336; Abcam Inc., Cambridge, MA).

We found that, although ORO DIA assessment correlates well with the total liver triglyceride concentration and is therefore an accurate reflection of liver steatosis (Pearson correlation R = 0.706, $P = 0.001$), assessment by the expert pathologists showed poor correlation (R = −0.422).

In samples with macrovesicular steatosis, we found that liver pathologists overestimated the amount of steatosis present on H&E stain as compared with ORO DIA (71.8% versus 46.7%, $P < 0.01$). In 67% of cases, the NAS steatosis score decreased from 3 to 2 when using ORO DIA.

We conclude that the ORO DIA technique provides a more accurate quantification of microvesicular and macrovesicular steatosis.

The NAS score is a useful and pragmatic tool in clinical practice and for patient selection for trial entry, but when the score is used as an outcome measure in a clinical trial setting, its performance is less robust. When, as Abdelmalek et al.[1] report, steatosis changes are the major study outcome, it is therefore difficult to know whether inaccurate scoring of H&E-stained sections by expert pathologists confounds these observations. ORO DIA is the most reliable way to accurately assess and quantify histological liver steatosis.

We accept that ORO staining is not practical for everyday use. It is optimally performed on frozen tissue because processing removes the lipids, hence the stain is not routinely performed on liver biopsies. However, its use may be of merit in clinical trials. Indeed, we have been able to successfully perform ORO staining on frozen sections of formalin-fixed human liver biopsies prior to processing, making wider adoption of this technique viable.

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Reply:

The suggestion by Levene et al. to further investigate new approaches to the quantification of the histological features of nonalcoholic fatty liver disease (NAFLD) for the purpose of inclusion in clinical studies and the assessment of the response to treatment interventions is interesting and appropriate. The authors have correctly pointed out the limitations of the NAFLD activity score (NAS) scoring system. In our study, a treatment response to betaine (if one existed) may have been missed with standard techniques used to interpret liver histology.[1] The NAS scoring system, although well validated, is a semiquantitative score system that may not accurately quantify the amount of steatosis on a hematoxylin and eosin stain, especially when fat droplets are small and/or hepatocyte ballooning is present. More importantly, it remains unknown whether a change in the NAS score reported in such treatment trials influences the natural history of NAFLD and/or alters the risk of NAFLD-related morbidity or mortality.

In the validation study by Kleiner et al.,[2] agreement on scoring and diagnostic categorization [nonalcoholic steatohepatitis (NASH), borderline, or not NASH] was evaluated with weighted kappa statistics. Inter-rater agreement on adult cases was 0.84 for fibrosis, 0.79 for steatosis, 0.56 for ballooning, and 0.45 for lobular inflammation. The validation of the NAS scoring system demonstrated reasonable inter-rater agreement among experienced hepatopathologists that was similar to the agreement found in other studies of variability in fatty liver disease.[3,4] The NAS scoring system is simple, can readily be used by practicing pathologists, and requires only routine

histochemical stains (hematoxylin and eosin and Masson tri-chrome stains). As proposed by Levene et al., other special stains, such as Oil Red O and digital image analysis, can increase the diagnostic accuracy for identifying lipids and pro-vide a more accurate quantification of microvesicular and mac-rovesicular steatosis. Studies in animal models of NASH have often used Oil Red O staining to quantify hepatic steatosis and have demonstrated good control with triglyceride stores, and this suggests that this approach has merit. However, the exten-sion of this application to routine clinical practice has several handicaps.[5] Of greatest importance are the poor tissue detail of the frozen section and the added burden of storing and process-ing frozen tissue. Although this is feasible, it is not routinely done now. Nonetheless, the addition of special staining to better define and quantitate the histological features of NASH (e.g., Oil Red O and cytokeratin 8/18) is worthy of consideration for defining treatment endpoints in future clinical studies of NASH.

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Comments on AASLD Practice Guidelines for Alcoholic Liver Disease

To the Editor:

We read with great interest the article by O'Shea et al. in the January issue of HEPATOLOGY, regarding the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines on alcoholic liver disease.[1] The article is well written and informative. However, we would like to bring some points to your kind atten-tion which may be of interest to the physicians, gastroenterologists, and hepatologists.

In Table 2, the authors described how to calculate the quantity of alcohol in a standard drink. This is an important piece of infor-mation when taking a history of alcohol intake from the patients. However, what constitutes one drink should also be described as patients describe their history of alcohol intake as the amount (in milliliters) of wine, beer, or hard liquor. It is defined that 12 oun-ces of beer (360 mL), 4 ounces of wine (120 mL), and 1.5 ounces of hard liquor (45 mL) constitutes one drink.[2]

Antioxidants have been used in the treatment of alcoholic liver disease, based on data from animal models as well as in patients with alcoholic liver disease.[3,4] The authors did discuss the current status of vitamin E supplementation. However, another powerful antioxidant, *N*-acetylcysteine (NAC), has been studied. A random-ized controlled study reported in abstract form at the AASLD 2009 meeting showed benefit of NAC in the treatment of severe acute alcoholic hepatitis (AH). Patients with AH treated with a combination of steroids and NAC (n = 85) compared to patients with AH treated with steroids alone (n = 89) had a lower mortal-ity at month 2 (15% versus 33%; $P = 0.007$) and lower complica-tion rate at month 6 (19% versus 42%; $P = 0.001$).[5] If these results are confirmed in subsequent studies from other centers, a combination of steroids and NAC may be a potential option to improve the outcome of patients with severe AH.

While discussing the role of liver transplantation (LT) in AH, the authors did point out the requirement of 6 months of absti-nence from alcohol to be eligible for LT. However, in an acute set-ting such as AH, this may not be possible and 30%-40% of patients with nonresponse to steroids (Lille score ≥0.45) succumb to their illness.[6] Louvet et al., in a case-control study reported at the AASLD 2009 meeting in patients with nonresponse to steroids at 1 week showed improved survival at 6 months after LT (n = 18) as compared to matched controls (n = 18) (83% ± 9% versus 44% ± 12%; $P = 0.009$).[7] All the patients in the LT group underwent transplantation within 9 (range 5-13) days of classifying them as nonresponders to steroids. At 1 year, none of the trans-planted patients relapsed for alcohol intake. Although we are far from making any firm recommendations on LT in patients with AH, this study is stimulating and challenges the current require-ment of 6 months of abstinence.

Furthermore, the bibliography list has 276 references in the ar-ticle. However, we could see 262 references cited in the text. If this observation is correct, the authors may like to submit correction as an erratum.

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Reply:

We thank Dr. Singal for his interest in our practice guidelines on alcoholic liver disease.[1] Dr. Singal raises three issues to which we will take this opportunity to respond in turn.

First, we agree that it is important for a physician to know the quantity of alcohol consumed by his or her patient because of alcohol's beneficial effects at moderate doses[2,3] versus its adverse effects at higher doses.[1] We also agree with Dr. Singal that providing a definition of what constitutes a drink (1 can of beer, 360 mL; 4-oz glass of wine, 120 mL; and 1.5 oz of hard liquor, 45 mL) may be helpful. However, these descriptions are neither precise nor universally accepted for a number of reasons. As described in Table 2 of our practice guidelines on alcoholic liver disease,[1] what constitutes a drink varies by country. There are eight different formulas for calculating the amount of alcohol in a drink.[4] The patient typically does not know how many ounces of wine or hard liquor is poured into his or her drink, although providing pictures of the size of the drinking glass may help. The percentage of alcohol may vary by as much as 2.5% in commercial wines and by 1.5% in commercial beers. In addition, what volume of alcohol constitutes a standard drink of port or a liqueur? Finally, there is disagreement even among experts. Dr. Singal uses a reference that describes a standard glass of wine to be 4 oz, whereas the National Institute on Alcohol Abuse and Alcoholism and the US Department of Health and Human Services define a standard glass of wine to be 5 oz. Consequently, we chose to use the amount of alcohol rather than a descriptive term to define a standard drink.[5,6]

The next issue concerns the fact that our guidelines on alcoholic liver disease[2] do not include information on the potential benefits of N-acetylcysteine and liver transplantation in patients with severe alcoholic hepatitis. Although these treatments may eventually be proven efficacious, they have been published in abstract form. Abstracts are not usually included in guidelines because abstracts do not undergo rigorous peer review prior to publication, and many are never published in manuscript form.[7,8]

Finally, as for Dr. Singal's concern about the difference between the number of references in the bibliography (276) and the number of references that he identified in the text (262), references 263 and 264 are cited in Table 2, reference 265 is cited in Table 3, and references 266 to 276 are cited in Table 7.

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Hepatitis C Virus Genotype 2/3 Patients Who Can Receive an Abbreviated Course of Peginterferon/Ribavirin: The Important Role of Initial Ribavirin Doses

To the Editor:

We read with great interest the article in a recent issue of HEPATOLOGY by Diago et al.,[1] who reported that the overall sustained virologic response (SVR) rate to peginterferon alfa-2a (40KD) (PEGIFN) plus ribavirin among patients infected with hepatitis C virus genotype 2/3 (HCV-2/3) was significantly higher in patients randomized to 24 weeks, rather than 16 weeks, of treatment (91% versus 82%; $P = 0.0006$) and among patients infected with genotype 2 (92% versus 81%; $P = 0.001$) but not genotype 3 (90% versus 84%; $P = 0.13$). In particular, the SVR rates in patients with a viral load $\leq$400,000 IU/mL randomized to 24 and 16 weeks of treatment were similar (95% versus 91%; $P = 0.20$). Assignment to 24 weeks of treatment, absence of advanced fibrosis on liver biopsy, lower HCV RNA level, and lower body weight were significant pretreatment predictors of SVR. The authors concluded the standard 24-week regimen of PEGIFN/ribavirin is significantly more effective than an abbreviated 16-week regimen in genotype 2/3 patients who achieve a rapid virologic response (RVR) (HCV RNA < 50 IU/mL at week 4). Abbreviated regimens may be considered in patients with a low baseline viral load who achieve an RVR.

The present study, which uses ribavirin 800 mg/day, showed RVR rates of 65.9% (863 of 1309 patients) in patients with HCV-2/3, which was similar to 62% reported by Lagging et al.[2] Our previous results from a randomized controlled trial of Taiwanese patients with HCV-2 showed that an RVR was achieved by 86.7% of patients[3] which seemed higher than the 64%-75.0% RVR rate for patients with HCV-2 in western countries.[4-6] The higher RVR rate in Taiwanese patients may be due to a higher initial dose of ribavirin per body weight (BW) (15.5 mg/kg/day) which makes the possibly "suboptimal" initial doses of ribavirin noteworthy.[7]

With higher initial dose of ribavirin per BW, the SVR rates in response to short-term treatment (12-16 weeks) in patients with HCV-2 with RVR were 89%-95% in studies by Dalgard et al., Mangia et al., and Andriulli et al.,[4,5,8,9] which were similar to the 92% SVR rate with 24 weeks treatment by Diago et al. In Taiwanese patients with HCV-2 who had RVR, we have shown that the very high SVR rates to 16 weeks and 24 weeks of PEGIFN treatment with weight-based ribavirin at a dose of 1000-1200 mg/day were comparable (100% versus 98%, respectively).[3] For SVR, Di Martino et al. reported in their meta-analysis study that shorter-duration therapy with fixed-dose 800 mg/day ribavirin yielded a lower SVR rate than 24 weeks of treatment, and a weight-based ribavirin regimen for a 16-week course of therapy seemed to achieve equivalent effect as a 24-week treatment duration with fixed-dose 800 mg/day ribavirin.[10] Diago et al. have reported SVR rates of 92% and 81% in patients with HCV-2 who were treated with PEGIFN and ribavirin 800 mg/day for 24 weeks and 16 weeks, respectively.[1] Ferenci et al. have shown the lower rates of SVR in patients with HCV-2 who were treated with lower initiation doses (77.8% and 55.6% with ribavirin 800 mg/day and 400 mg/day for 24 weeks, respectively).[11] In addition, the lower BW was an independent predictor of SVR by Diago et al. which might further suggest the importance of the weight-based dose of ribavirin. Taken together, a better SVR rate can be achieved when patients with HCV-2 are treated by regimens with higher initial dose of ribavirin per BW, even with shortened duration of therapy in HCV-2 patients who achieve an RVR.

Diago et al. also showed the role of lower HCV RNA level on the SVR in patients infected with HCV-2/3.[1] Our previous randomized trial for HCV-1 patients has shown that HCV RNA level, in addition to an RVR and mean weight-based exposure of ribavirin, was the significant predictor for SVR; patients with RVR and low HCV RNA level achieved similar SVR rates after 24 or 48 weeks of PEGIFN/ribavirin therapy (96% and 100%, respectively).[12] However, in patients with HCV-2 with RVR and a higher initial dose of ribavirin per BW, the HCV RNA level played a minimal role on the SVR rate and, in addition, the similar SVR rates between shortened (12-16 weeks) and standard (24 weeks) duration of therapy were observed in our study (100% versus 98%)[3] and in reports by Mangia et al. (87% versus 89%)[4] and Dalgard et al. (93% versus 97%).[5] In patients with HCV-2 who had RVR, the weight-based ribavirin regimen seemed to be able to ameliorate the deteriorated efficacy of shortened duration and covered the role of HCV RNA level. Further large-scale studies to confirm the critical role of weight-based dosing of ribavirin in abbreviated regimens for patients with HCV-2/3 who achieve RVR are necessary.

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Reply:

We appreciate the interest in our article[1] expressed by Dai and colleagues.

Dai et al. present the hypothesis that the use of a fixed dose of ribavirin in genotype 2 or 3 patients is suboptimal and that weight-based dosing should be preferred. Unfortunately, the studies that they cite in support of their hypothesis were primarily designed to examine the duration of treatment rather than the dose of ribavirin and thus do not necessarily support their argument.

Retrospective calculation of ribavirin dosages on a milligram per kilogram basis can be problematic. In fact, analyses based on such calculations may actually be confounded by the patient's

weight. Increased weight is a well-established negative predictor of sustained virological response in patients with chronic hepatitis C treated with peginterferon plus ribavirin.[2]

The reason for the negative impact of body weight on sustained virological response is complex and may involve insulin resistance or hepatic steatosis.[2] The question of whether higher initial dosages of ribavirin increase sustained virological response rates in genotype 2 or 3 patients regardless of baseline viral load strata can be answered only with the results of trials that have randomized patients to different dosage regimens. This is the only way to avoid confounding by body weight. Three such studies are available, none of which has reported a statistically significant differences in sustained virological response rates between genotype 2 or 3 patients randomized to two different dosage regimens of ribavirin.[3-5] Hadziyannis et al.[3] reported sustained virological response rates of 79% to 84% in genotype 2 and 3 patients randomized to peginterferon alfa-2a (40 kDa) plus ribavirin at either a fixed dosage (800 mg/day) or a weight-based dosage (1000/1200 mg/day) for either 24 or 48 weeks. Importantly, sustained virological response rates were similar in patients treated for a longer duration (48 weeks) with a fixed dose of ribavirin (800 mg/day) and in those treated for a shorter duration (24 weeks) with a weight-based dose (1000/1200 mg/day; 79% versus 81%, respectively), but relapse rates were higher with the shorter treatment duration.[3] Jacobsen et al.[4] reported similar sustained virological response rates in patients randomized to peginterferon alfa-2b (12 kDa) plus ribavirin at a fixed dose (800 mg/day; 62%) or a weight-based dosage (800-1400 mg/day; 60%) for 24 or 48 weeks. More recently, Ferenci et al.[5] reported no statistically significant difference between sustained virological response rates in genotype 2 or 3 patients randomized to a fixed ribavirin dosage of 400 or 800 mg/day. Secondary analyses of data from the trials by Hadziyannis et al. (reported by Rizzetto et al.[6]) and Ferenci et al. confirmed the overall finding of no statistically significant differences between ribavirin dosage regimens in the smaller subgroups of genotype 2 and genotype 3 patients. The best evidence shows clearly that weight-based ribavirin dosing does not significantly increase sustained virological response rates in genotype 2 or 3 patients.

The most important drawback to the use of abbreviated treatment for genotype 2 or 3 patients is relapse.[7] Patients assigned to abbreviated 12- to 16-week treatment regimens generally have 2- to 3-fold higher relapse rates in comparison with those treated for the standard 24-week duration.[8-12] This trend includes patients treated with higher weight-based ribavirin dosages,[8,10,12] patients with a rapid virological response,[8-10] and Taiwanese patients enrolled in the trial by Yu et al.[12]

Sustained virological response rates generally decrease as the baseline viral load increases. Shiffman and colleagues[11] showed that this trend is accentuated in genotype 2 or 3 patients treated with an abbreviated 16-week regimen in comparison with the standard 24-week regimen. Patients enrolled in the trial by Yu et al.[12] had a low mean baseline viral load (4.8-log), and other studies have not reported outcomes stratified by viral load. Thus, there are no data to show whether increasing the dose of ribavirin compensates for the negative effect of increasing the baseline viral load.

Dai et al. point to the higher rates of rapid virological response and sustained virological response in Taiwanese patients versus Western patients. This phenomenon, which has been noted by others,[13] may be due in part to the lower mean body weight of patients in their trial in comparison with some of the other studies that they cite.[8,9,11] However, it is more likely that this difference is due to a recently identified genetic polymorphism that is a highly significant predictor of sustained virological response.[14] In their analysis of data from genotype 1-infected individuals, Ge and colleagues[14] found that Asian patients had the highest frequency of the advantageous allele. To our knowledge, the impact of this polymorphism in the gene for interleukin 28B on sustained virological response rates has not yet been reported in patients infected with hepatitis C virus genotype 2 or 3. It will be interesting to determine the impact of this polymorphism not only on sustained virological response rates in genotype 2 or 3 patients but also on the rate of relapse in patients with a rapid virological response assigned to abbreviated treatment regimens. Perhaps the frequency of interleukin 28B genotypes will ultimately explain the differences in virological response rates described by Dai and colleagues and direct us toward improved use of ribavirin in different patient populations.

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Adjunctive Vitamin E Treatment in Wilson Disease and Suggestions for Future Trials

To the Editor:

Wilson disease (WD) is a genetic disorder involving copper accumulation in various tissues, and oxidative stress plays a central role in its pathogenesis. We read with great interest the article by Linn et al.[1] in which they report that long-term exclusive zinc monotherapy in patients with symptomatic WD generally led to a good outcome for neurological disease, whereas the results were less satisfactory in cases of hepatic disease. However, because of (1) the significantly lower serum vitamin E levels in WD patients treated with zinc[2] and (2) the beneficial effects of vitamin E reported in WD animal models and also occasionally in WD patients, it is reasonable to assume the potential of vitamin E as an adjunctive treatment to further improve zinc treatment in WD, and rigorous trials should be conducted as suggested recently.[3] More importantly, because of the disappointing trials of vitamin E in many oxidative stress–related diseases, including chronic liver diseases,[4] Alzheimer's disease (AD),[5,6] cardiovascular diseases, and cancer,[7] we suggest that the potential factors leading to the negative trials in these diseases should be taken into consideration when future trials of vitamin E in WD are conducted.

For example, similarly to WD, both oxidative stress and excessive transition-metal ions (e.g., Cu^{2+}) have been proved to play crucial roles in the pathogenesis of AD. However, among the numerous trials of vitamin E conducted for the prevention and treatment of AD, many have shown disappointing results.[5,6] For instance, it was recently reported that vitamin E was ineffective in preventing oxidative stress, did not prevent loss of cognition in AD patients, and may even have been detrimental.[6] Moreover, the beneficial effects of vitamin E are still controversial, and many trials have failed to confirm any protective effect of vitamin E for either cardiovascular diseases or cancer.[7] Therefore, the disappointing trials of vitamin E in many other diseases should be paid full attention, and future trials of vitamin E in WD will benefit from these disappointing trials. Besides the intrinsic limitations of antioxidants and the heterogeneity of biological systems attenuating the reactive oxygen species–scavenging capacity proposed by us,[8] many other important factors have been suggested to be responsible for the disappointing trials of vitamin E.[9,10] Brewer[9] recently analyzed why vitamin E is ineffective for the treatment of AD, and the reasons, including inappropriate doses, inappropriate timing, and unbalanced monotherapy in the trials, were presumed. In addition, Steinhubl[10] provided several possibilities for the negative trials of vitamin E in atherosclerosis, such as the wrong form of vitamin E (a synthetic form instead of a natural form comprising eight different isoforms used in the trials), inadequate durations, and the wrong patients. All these aspects should be taken into account when rigorous trials of vitamin E in WD are conducted. In addition, the rational suggestions proposed by Lu[4] for the antioxidant treatment of chronic liver diseases have important implications for future trials of vitamin E in WD.

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Reply:

We have read with interest the suggestion by Shen and Ji to include vitamin E in future therapeutic trials of zinc in patients with hepatic manifestations of Wilson disease (WD). We do endorse this suggestion for any patient with acute liver failure due to WD or other diseases due to copper overload.[1] In these patients, hepatocyte apoptosis is induced by activation of the Fas pathway through oxidative damage.[2] Inhibition of this devastating effect of copper overload through the restoration of the intracellular

oxidative balance by vitamin E supplementation would probably be beneficial and outbalance potential side effects.

We have some hesitation, however, about accepting the suggestion of potential benefits of vitamin E in patients with chronic liver damage due to WD. As Shen and Ji rightly point out, the results of vitamin E and other antioxidants in many chronic diseases are disappointing[3] and might even be detrimental,[4] as we have described for patients with cystic fibrosis, for example.[5] Before the introduction of another intervention with a questionable effect, it is necessary to first properly describe, in a substantial number of patients, the results of decoppering medications that are already available, such as trientine, zinc, and D-penicillamine. Up to January 2008, well-described evidence for efficacy in hepatic WD had been published for only 57 patients on D-penicillamine, 9 patients on zinc, and none on trientine.[6] Because prospective randomized trials are not available, a retrospective analysis of patients on any of these medications, like the analysis that we performed for exclusive zinc monotherapy,[7] will aid physicians in choosing the optimal medication for their patients with hepatic or neurological WD.

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Serum Cytokeratin-18 Fragment Level: a Noninvasive Biomarker for Not Only Nonalcoholic Steatohepatitis, but Also Alcoholic Steatohepatitis

To the Editor:

We read with great interest the article by Feldstein et al. reporting the potential usefulness of cytokeratin-18 (CK-18) fragment as a noninvasive serum biomarker for diagnosing nonalcoholic steatohepatitis.[1] We would like to draw attention to similar studies on serum CK-18 fragment in the differentiation of alcoholic steatohepatitis from healthy controls with no liver disease. Cytokeratins (CKs) are normal constituents of the epithelial cell cytoskeleton.[2] Serum CK-18 level is a clinical tool useful as a tumor marker in epithelial malignancies.[3] However, serum CK-18 level has also been found to increase in nonmalignant diseases, such as alcoholic hepatitis, which limits its specificity as a tumor marker.[4-6] Thus, CK-18 fragment level may be a noninvasive biomarker for early detection of alcoholic steatohepatitis.

We tested the serum CK-18 fragment levels in patients with alcoholic hepatitis (50), hepatocellular carcinoma (50), heavy drinkers (50), and healthy controls (50). Our results showed that serum levels of CK-18 fragment in patients with alcoholic hepatitis were higher than those of healthy controls and heavy drinkers, and even tended to be higher than those of patients with malignancy. Serum CK-18 fragment levels were median 27 U/L (range = 11-72 U/L) in controls with no liver disease, median 759 U/L (range = 152-4739 U/L) in heavy drinkers, median 1598 U/L (range = 531-4237 U/L) in patients with alcoholic hepatitis, and median 449 U/L (range = 21-17,326 U/L) in those with hepatocellular carcinoma ($P < 0.001$ for alcoholic hepatitis versus healthy controls). However, serum CK-18 fragment levels in heavy drinkers were similar or even higher than those observed in patients with advanced malignancy, which is further evidence for the belief that the diagnostic value of serum CK-18 fragment as a tumor marker

is limited by those heavy drinkers. Our results are very similar to Prof. Fayetteville's previous report.

In summary, our data highlight three points. First, we show that serum CK-18 fragment is a better noninvasive biomarker for alcoholic steatohepatitis, and is not only limited to nonalcoholic steatohepatitis. Second, the sample size is not large enough and this did not allow us to establish the performance of CK-18 fragment according to the etiology of underlying liver disease. Third, considering that the genotypes of different geographic or ethnic groups may have a significant impact on the serum CK-18 fragment levels, more multicenter cohorts of validation are still needed before this marker can be applied clinically.

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Molecular Signatures of Nonalcoholic Fatty Liver Disease: The Present and Future

To the Editor:

We read with great interest the study by Bell and coworkers[1] who identified by using label-free quantitative proteomics three different panels of serum biomarkers that can be potentially used for noninvasive diagnosis of the nonalcoholic fatty liver disease (NAFLD) spectrum. Specifically, a panel of six proteins (fibrinogen β chain, retinol binding protein 4, serum amyloid P component, lumican, transgelin 2, and CD5 antigen-like) were found to differentiate between all conditions in the spectrum of NAFLD. In addition, a group of three proteins (complement component C7, insulin-like growth factor acid labile subunit, and transgelin 2) distinguished between NAFLD (simple steatosis and nonalcoholic steatohepatitis [NASH]) versus NASH with advanced bridging fibrosis. Finally, two proteins (prothrombin fragment and paraoxonase 1) discriminated with 100% accuracy between control subjects and patients with all forms of NAFLD.[1] These interesting findings highlight some important considerations. First, part of the challenge for establishing a molecular signature for NAFLD is that the metabolic syndrome, which is commonly associated with NAFLD,[2] leads to activation of the same pathways as does NAFLD. This suggests that we need approaches to separate the effects of NAFLD from that of the metabolic syndrome *per se*. For instance, paraoxonase 1[3] and retinol binding protein 4[4] have been both previously associated with the metabolic syndrome. Second, it is noteworthy that the use of plasma is considered superior to serum because approximately 40% of signals found in serum are not found in plasma because of *ex vivo* generation during clotting.[5] Therefore, the important results by Bell et al. need to be replicated by using plasma samples. Those proteins related to the pathophysiology of NAFLD displaying stable levels in both serum and plasma should be good candidates to be tested in larger populations. Finally, an obvious prerequisite for the clinical use of proteomics-discovered biomarkers is elucidation of analytical features, standardization of analytical methods, assessment of performance characteristics, and demonstration of cost-effectiveness.[6] Proteomics offers a great opportunity for the development of novel, noninvasive assays for the diagnosis and monitoring of NAFLD without liver biopsy. Unfortunately, we remain some way from integrating any of the new NAFLD biomarkers into clinical practice. As more data like those by Bell and coworkers become available, it will be imperative that biomarkers of NAFLD with potential clinical utility are independently validated before investment is made into producing a diagnostic test.

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Thiazolidinediones as Potent Inducers of Hepatocyte Growth Factor

To the Editor:

To date, increasing evidence indicates that the thiazolidinediones (TZDs) have benefits in certain conditions of chronic liver disease, including nonalcoholic steatohepatitis.[1,2] TZDs are selective agonists for the nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) that have potent anti-inflammatory effects on hepatic stellate cells (HSCs). For instance, exposing HSCs to TZDs resulted in reversion of most features of the activated phenotype of HSCs, reduction in the expression of
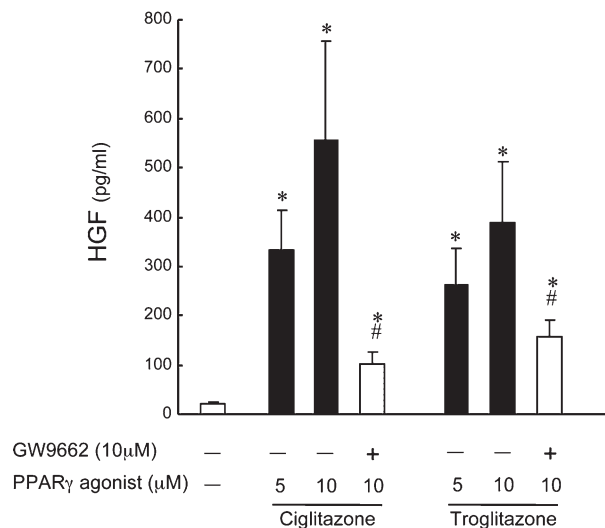
Fig. 1. Density gradient–separated peripheral blood mononuclear cells from healthy subjects (n = 4) were suspended at 1×10^6 cells/mL in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum, and stimulated with PPARγ agonists for 24 hours. The PPARγ antagonist GW9662 was added to the mononuclear cells 30 minutes prior to PPARγ agonists. The concentration of HGF in culture supernatants was measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). The bars indicate standard errors. *$P < 0.05$ versus unstimulated control. #$P < 0.05$ versus without PPARγ antagonist.

matrix proteins, and blocking of the secretion of proinflammatory chemokines.[2]

We offer an additional important mechanism for the development of a molecular target of PPARγ, i.e., PPARγ agonist-induced hepatocyte growth factor (HGF) may have an essential part in the protection from chronic liver injury. HGF has been shown to suppress liver cirrhosis, hepatocyte apoptosis, and production of transforming growth factor-β.[3] Previously, Li et al. clearly demonstrated that PPARγ agonists strongly stimulate HGF promoter and subsequent gene/protein expression in mesangial cells.[4] Indeed, we observed that peripheral blood mononuclear cells produce a significant amount of HGF in the supernatants by stimulation with

TZDs, which are blocked by a selective PPARγ antagonist (Fig. 1). This evidence suggests that, in the presence of a PPARγ agonist, both tissue and immune cells could produce HGF at an inflammatory locus and probably in blood circulation. In this context, we read with interest the article by Aoyama et al.,[5] which showed that pioglitazone treatment augumented the hepatic proliferative response in KK-A^y mice in response to partial hepatectomy. Future studies are needed to explore the connection between PPARγ and HGF, and such investigations would contribute to progress in understanding the mechanisms of the efficacy of TZDs in chronic liver disease.

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