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### Article

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# Alcohol-induced generation of lipid peroxidation products in humans

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To address the hypothesis that elevated blood alcohol increases systemic oxidant stress, we measured urinary excretion of isoprostanes (iPs), free radical-catalyzed products of arachidonic acid. Ten healthy volunteers received acute doses of alcohol (Everclear-R) or placebo under randomized, controlled, double-blind conditions. Urinary iPF<sub>2α</sub>-III increased in a time- and dosage-dependent manner after dosing with alcohol, with the peak urinary iPF<sub>2α</sub>-III excretion correlating with the rise in blood alcohol. To determine whether oxidant stress was associated with alcohol-induced liver disease (ALD), we then studied the excretion of iPs in individuals with a documented history of alcohol-induced hepatitis or alcohol-induced chronic liver disease (AC). Both urinary iPF<sub>2α</sub>-III and urinary iPF<sub>2α</sub>-VI were markedly increased in patients with acute alcoholic hepatitis. In general, urinary iPF<sub>2α</sub>-III was significantly elevated in cirrhotic patients, relative to controls, but its excretion was more pronounced when cirrhosis was induced by alcohol than by hepatitis C. Excretion of iPF<sub>2α</sub>-VI, as well as 4-hydroxynonenal and the iPF<sub>2α</sub>-III metabolite, 2,3-dinor-5,6-dihydro-iPF<sub>2α</sub>-III, was also increased in AC. Vitamin C, but not aspirin, reduced urinary iPs in AC. Thus, vasoactive iPs, which serve as indices of oxidant stress, are elevated in the urine in both acute and chronic ALD. Increased generation of iPs by alcohol in healthy volunteers is consistent with the hypothesis that oxidant stress precedes and contributes to the evolution of ALD.

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## Introduction

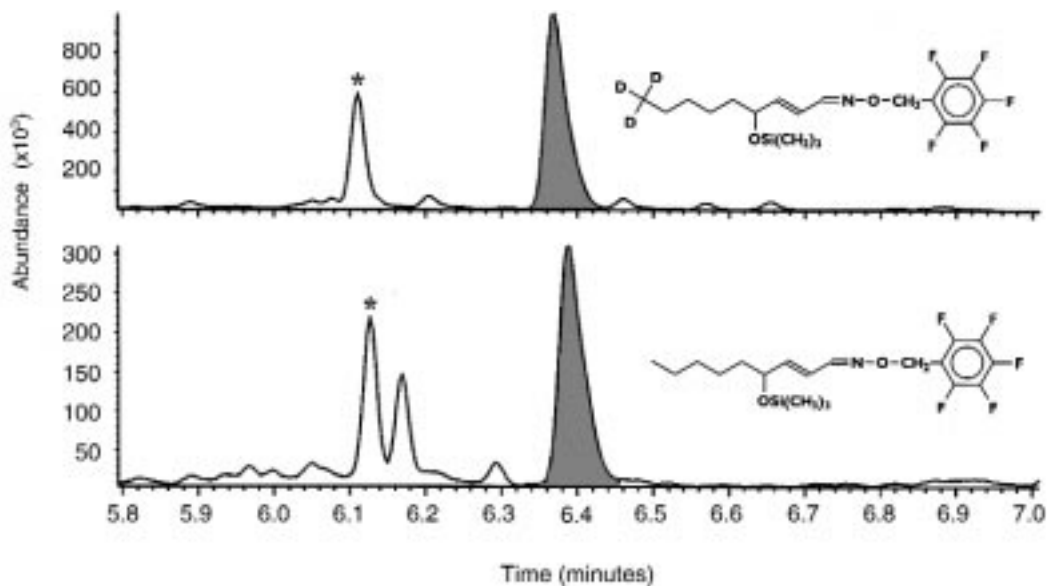
Alcohol-associated cirrhosis is estimated to account for almost half of the serious liver disease in the Western world (1). Although light to moderate ingestion of alcohol may reduce the risk of coronary heart disease (2), intake of more than 30 g of alcohol per day has been associated with increased mortality due to breast cancer in women (3), and with cirrhosis in both sexes (1–5). The mechanisms involved in alcohol-induced liver disease (ALD) are poorly understood (6), although prevalence of the disease appears to follow a dose-response curve in relation to lifetime intake (4, 5).

The potential role that oxidant injury plays in the pathogenesis of ALD has received considerable scrutiny (6–9). Alcohol ingestion, through induction of the CYP4502E1 isozyme, may result in increased generation of reactive oxygen intermediates (ROIs), which have the potential to peroxidize lipids (10). The degree of lipid peroxidation may be modulated by endogenous antioxidant defenses or by xenobiotics, such as cigarette smoking or dietary fats (11). Thus, genetic and environmental factors may contribute to the variable expression of ALD as a function of alcohol intake in different populations. Evidence consistent with a causal relationship between lipid peroxidation and morphologically assessed liver damage has recently been reported in a rat model of ALD (11). Lipid peroxidation was determined indirectly by measurement of conjugated dienes and protein adduct formation (12).

Isoprostanes (iPs) are free radical-catalyzed products of arachidonic acid (13–16). Quantitation of iPs in biological fluids appears to reflect oxidant stress *in vivo*. We have developed specific methods for measurement of the distinct iPs iPF<sub>2α</sub>-III (formerly known as 8-*iso*-PGF<sub>2α</sub>; refs. 16 and 17) and iPF<sub>2α</sub>-VI (formerly known as IPF<sub>2α</sub>-1; ref. 18). Urinary excretion of both compounds is increased in syndromes of oxidant stress (19–21), and depressed by antioxidants *in vitro* (18) and *in vivo* (21, 22). Urinary iPs may be used to select effective antioxidant doses of vitamins *in vivo* (22). We now report that excretion of both iPs is increased in patients with acute and chronic ALD. Furthermore, the concomitant increase in excretion of an iP metabolite and an independent index of lipid peroxidation, 4-hydroxynonenal (4-HNE; ref. 23), implies that this reflects increased generation, rather than altered disposition, of the iPs in liver disease. Vitamin C, but not aspirin, suppresses iP generation in cirrhosis. Furthermore, alcohol ingestion dose dependently increases excretion of iPF<sub>2α</sub>-III in healthy volunteers. This is consistent with the possibility that alcohol-induced oxidant stress might precede liver damage and may contribute to the evolution of ALD.

## Methods

**Study design.** The first study was a randomized, placebo-controlled trial to determine the dose-response relationship between alcohol consumption and urinary



**Figure 1**

Quantitation of 4-HNE in human urine. Five milliliters of urine was spiked with 5 ng of trideuterated 4-HNE. The upper trace ( $m/z$  286) represents the internal standard; the lower trace ( $m/z$  283) represents endogenous 4-HNE. Each ion trace shows 2 peaks (filled peak and asterisk-marked peak), which originate from the *syn* and *anti* isomers generated when the pentafluorobenzoyloxime derivative is formed.

iPF<sub>2α</sub>-III excretion. All clinical studies were approved by the Institutional Review Board and the General Clinical Research Center (GCRC) Advisory Committee, as appropriate. All patients who participated in these studies consented to do so and were compensated for incurred expenses. Ten volunteers (5 males and 5 females), between the ages of 21 and 60 years (mean: 38.0 ± 11.5 years), were studied. The sample size was based on the known coefficient of variation of the methods (maximally 4.5%) and the desire to detect a dose-dependent increase of at least 50% at the peak from baseline values, where  $\alpha = 0.05$  and the power (1- $\beta$ ) of the comparison is 0.85. A similar basis was used for sample size calculation in all subsequent studies.

Individuals with a history of liver disease, alcoholism, or intake of any medication — specifically nonsteroidal anti-inflammatory drugs (NSAIDs), anovulant contraceptives, or vitamins — in the preceding 2 months were excluded. Other exclusion criteria included pregnancy, tobacco smoking, and a history of myocardial infarction in the preceding 3 months. Volunteers desisted from all drug and alcohol intake from the time of screening (14 days before dosing) to the conclusion of the study, except as mandated by the trial design.

Volunteers were randomized under double-blind, placebo-controlled conditions for the order in which they drank 240 mL of a nonalcoholic solution of lemonade (Wylers' Lemonade) or 0.2, 0.3, 0.4, 0.6, or 0.9 g/kg body wt of a 98% solution of alcohol (Everclear-R), made up to 240 mL with the lemonade solution. Before starting the study, a complete dietary history was obtained, using a quantitative food-frequency questionnaire. In an attempt to standardize this variable, subjects were instructed to comply with the US Dietary Goals and Guidelines for Healthy Adults. Dietary compliance was monitored by interview during each GCRC admission and by random

24-hour recalls of dietary intake, as assessed by telephone interview during the outpatient periods. During each of the 4 admissions, subjects received the same standardized diet. Meals were administered at 5 hours, 12 hours, and 24 hours after dosing. The diet provided 1.2 × basal energy expenditure (BEE) kcal per day for each study participant, which is based on the Harris-Benedict equation (24). Multiplication by a factor of 1.2 was to account for sedentary activity. Foods were prepared ahead of time and weighed to the nearest 0.1 g before serving. All foods were prepared by trained technicians in the GCRC metabolic kitchen. Participants were encouraged to consume all foods offered. However, if there were residual foods, they were weighed to the nearest 0.1 g and recorded on each subject's daily intake chart.

The 6 study days were each separated by 2-week "washout" periods. The volunteers were admitted to the GCRC, and fasting and dosing occurred over a 15-minute period at 0800 hours on each study day. Blood-alcohol levels were measured at baseline, at 20, 40, and 60 minutes, and at 2, 3, 4, 6, 12, and 24 hours after dosing. Sequential urine collections were performed for iPF<sub>2α</sub>-III during the 2 hours before dosing (-2 to 0 hours), and from 0 to 6, 6 to 12, and 12 to 24 hours after dosing, in timed aliquots on each study day. Urine was aliquoted, spiked with internal standards, and stored for analysis by stable-isotope dilution gas chromatography/mass spectrometry (GC/MS).

The second study addressed the hypothesis that patients with established ALD have increased ROI generation *in vivo*, as assessed by urinary excretion of iPF<sub>2α</sub>-III and iPF<sub>2α</sub>-VI, when compared with patients with non-alcohol-related liver disease, and with age- and sex-matched controls. Fifteen healthy age- and sex-matched controls (8 males and 7 females) were recruited through advertisements placed in local papers. Nineteen patients

between the ages of 38 and 64 years (mean:  $52.0 \pm 6.2$  years), with either biopsy-proven cirrhosis or end-stage liver failure compatible with cirrhosis, were recruited from the Hepatology and Liver Transplant Clinic at the Hospital of the University of Pennsylvania. Six of the patients had a known diagnosis of ALD, 8 had a history of serologically confirmed hepatitis C (HCV) cirrhosis, and 5 had combined ALD and HCV cirrhosis (Table 1). The severity of liver disease was comparable among the 3 patient groups, as demonstrated by Child-Pugh class (ref. 25; Table 1). Patients were excluded if they had a known diagnosis of hepatocellular carcinoma, spontaneous bacterial peritonitis, other infective processes, or a history of gastrointestinal hemorrhage within the previous month. Patients with cholestatic liver disease and hemochromatosis were excluded. Study subjects were also excluded if they had a history of established renal or pulmonary disease, or a myocardial infarct within the last 3 months. No patient gave a history of a myocardial infarct occurring in the year preceding the study. All patients and volunteers recruited were nonsmokers. Patients received a standard therapeutic regimen of a loop diuretic and a potassium-sparing diuretic. All subjects discontinued vitamin and iron supplements and abstained from taking NSAIDs and alcohol for a minimum period of 4 weeks before being studied. Eleven patients had a history of alcohol dependence according to psychiatric interview, including a history of excess alcohol intake for more than 10 years ( $n = 8$ ), a history of prior rehabilitation attempts ( $n = 11$ ), attendance at Alcoholics Anonymous ( $n = 10$ ), and convictions for driving while impaired by alcohol ( $n = 7$ ). In addition, each of these patients had a positive score on the CAGE screening questionnaire for covert alcoholism, whereas all patients with non-alcohol-related liver disease and all volunteers had negative scores (26). All 11 patients gave a history of abstinence from alcohol at the time of the study, with a range of 4 months to 4 years, and a median interval of 2 years.

Subjects were admitted to the GCRC for 3 consecutive days. Each subject received a standardized diet throughout the study period. In addition, the research dietician obtained a comprehensive dietary history from each participant, and subjects were instructed to follow a standard diet for 2 weeks before the study. The subjects' usual dietary intake was estimated by a quantitative food-frequency score, which has been previously validated (24). Baseline measurements of hepatic and renal function included aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, bilirubin, prothrombin time, creatinine, sodium, and urea. In addition, blood was drawn on admission for vitamin E and C levels. Three consecutive 24-hour urine collections were obtained for  $iPF_{2\alpha}$ -III. Given that little information is available on the metabolism of  $iPF_{2\alpha}$ -III and that the liver might be a site of metabolite formation, we developed methods to analyze a putative 2,3-dinor-5,6-dihydro- $iPF_{2\alpha}$ -III metabolite of  $iPF_{2\alpha}$ -III (27, 28) in a subset ( $n = 20$ ) of study subjects to determine if an increment in  $iPF_{2\alpha}$ -III might reflect reduced metabolism, rather than increased generation, of the compound. Urinary  $iPF_{2\alpha}$ -VI was also measured in patients with ALD, and in age- and sex-matched controls.

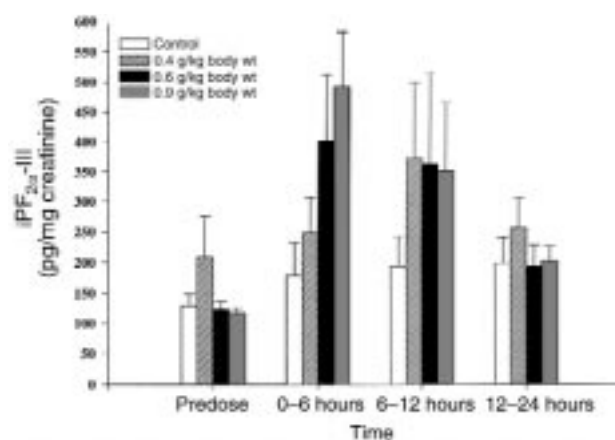
**Table 1**  
Child Pugh classification of patient population

Group	Class A	Class B	Class C	Total number of patients (sex)
ALD	0	2	4	6 (4 male, 2 female)
HCV cirrhosis	1	3	4	8 (5 male, 3 female)
Combined disease	0	3	2	5 (3 male, 2 female)

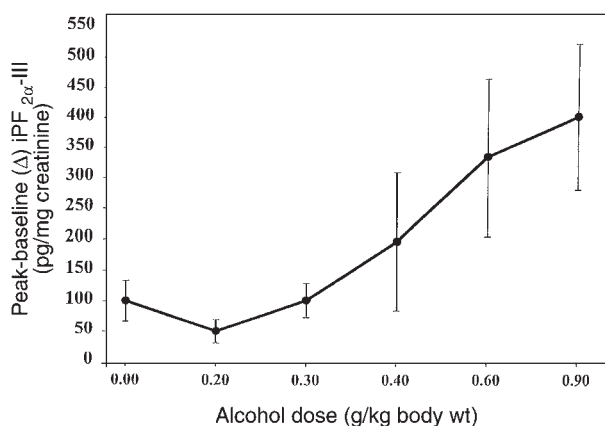
An additional cohort of 16 patients (13 males) with chronic ALD, aged  $49.9 \pm 13.0$  years, were compared with a control group of 12 healthy volunteers (6 males) aged  $40.3 \pm 19.9$  years ( $P = NS$ ). Entry and exclusion criteria were as described above. A 24-hour urine sample was collected for analysis of 4-HNE and  $iPF_{2\alpha}$ -VI.

A third study involved assessment of  $iP$  generation in patients with acute alcoholic hepatitis. Patients were admitted directly to the hepatology service from the emergency room. Baseline measurements of hepatic and renal function included AST, ALT, albumin, bilirubin, prothrombin time, creatinine, sodium, and urea. In addition, vitamin E and C levels were obtained. Three of the 5 patients had a Maddrey score (a discriminant function) of greater than 32, indicating a poor prognosis (29). In addition to routine medical care, 3 consecutive 24-hour urine collections were obtained for  $iPF_{2\alpha}$ -III and  $iPF_{2\alpha}$ -VI in a manner identical to that in the preceding study.

A fourth study addressed the effect of the antioxidant vitamin C on  $iP$  excretion in patients with established liver disease. Given that  $iPF_{2\alpha}$ -III, but not  $iPF_{2\alpha}$ -VI, may be formed as a minor product of either of the cyclooxygenase (COX) isoforms (17, 18, 30), we also assessed the effects of aspirin. Patients were again recruited from the hepatology and liver-transplant clinics. Fifteen patients were studied: 5 with ALD, 5 with HCV cirrhosis, and 5 with combined disease. Samples for measurement of



**Figure 2**  
Time- and dose-dependent increase in excretion of  $iPF_{2\alpha}$ -III in healthy volunteers administered alcohol. Acute oral doses (240 mL) of alcohol or a control solution were administered under randomized, double-blind, controlled conditions. Urinary  $iPF_{2\alpha}$ -III increased significantly ( $P < 0.001$ ) as a function of dose. Alcohol also increased urinary  $iPF_{2\alpha}$ -III in a time-dependent manner, with the 3 highest dose groups ( $P < 0.01$ ) peaking in the fraction collected 0-6 hours after dosing.



**Figure 3**  
The dose-related increment in iPF<sub>2α</sub>-III excretion in healthy volunteers. Alcohol significantly increased the maximal (vs. control) urinary iPF<sub>2α</sub>-III ( $P < 0.01$ ) in a dose-dependent manner. Subsequent to ANOVA, paired comparisons indicated significant increases at the 2 highest doses.

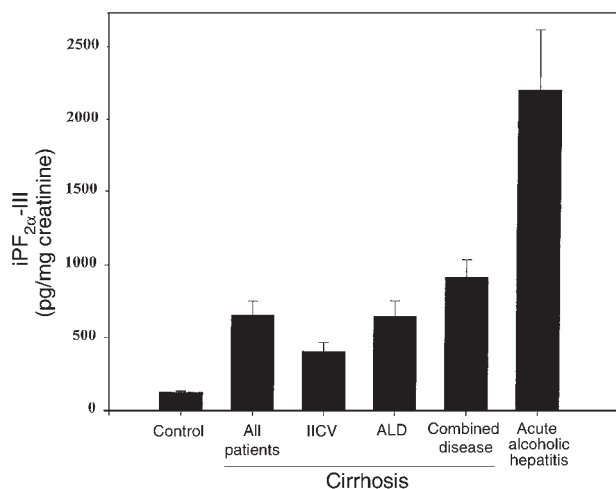
endogenous vitamin levels and urinary iPs were obtained at baseline and after taking 2.5 g of vitamin C per day for 10 days. Final samples were obtained after a 10-day washout period. The volunteers were then given 325 mg of aspirin. Urinary iP measurement was repeated in a 24-hour sample after aspirin ingestion. In this study, we also compared measurement of thiobarbituric acid-reactive substances (TBARS), an *ex vivo* assay used to reflect ROI generation (23) with iP production.

**Measurement of iPF<sub>2α</sub>-III.** Urinary iPF<sub>2α</sub>-III was assayed by GC/MS as described previously (17). Briefly, urine was aliquoted, spiked with [<sup>18</sup>O<sub>2</sub>]iPF<sub>2α</sub>-III, and extracted from the aqueous matrix by solid-phase extraction techniques. The samples were purified by TLC. Native and labeled iPF<sub>2α</sub>-III were derivatized as their pentafluorobenzyl ester, trimethylsilyl ethers. They were then analyzed by GC/MS in the negative-ion, chemical-ionization mode. Quantification values were expressed as the ratio of the area under the peak of the ion representing the endogenous compound to that of the internal standard. Urinary creatinine was determined by a standard automated colorimetric assay, using a Beckman Synchron CX System (Beckman Instruments Inc., Arlington Heights, Illinois, USA).

2,3-dinor-5,6-dihydro-iPF<sub>2α</sub>-III was obtained as a gift from Cayman Chemical Co. (Ann Arbor, Michigan, USA). The <sup>18</sup>O<sub>2</sub> compound was made using the method of Pickett and Murphy (31). Briefly, internal standard was added to urine that was acidified with formic acid. The lipid was extracted by solid-phase extraction on a RapidTrace SPE Workstation (Zymark Corp., Hopkinton, Massachusetts, USA). The sample was applied to the column and rinsed with pH 7.0 buffer followed by 1 mL of ethyl acetate, then dried under N<sub>2</sub> and redissolved in 30 μL of ethyl acetate. The metabolite was separated from other eicosanoids by TLC (Whatman Inc., Clifton, New Jersey, USA), using a mobile phase of 20% methanol and 80% ethyl acetate. Again, the compound was derivatized as its pentafluorobenzyl ester, trimethylsilyl ether, and analyzed on an MD800 GC/MS (Finnigan Corp., San Jose, California, USA). Urinary iPF<sub>2α</sub>-VI was similarly meas-

ured, as described previously, taking advantage of the pH-dependent lactonization of this class of iPs (18).

**Analysis of urinary 4-HNE.** The method for measurement of 4-HNE in urine was adapted from one previously developed by van Kuijk et al. (32) to measure 4-hydroxyalkenals in oxidized LDL. Briefly, 5-mL urine samples were spiked with 5 ng of d<sub>3</sub>-HNE (kindly provided by F.J. van Kuijk, University of Texas Medical Branch, Galveston, Texas, USA), mixed well, and allowed to equilibrate for 15 minutes at room temperature. Then 2 mg of *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride was added to each sample, and the samples were allowed to stand for 30 minutes at room temperature. 4-HNE was extracted using reverse-phase solid-phase extraction cartridges (C18 EC, 500 mg; International Sorbent Technology Ltd., Mid Glamorgan, United Kingdom) under the following conditions. The cartridge was conditioned with 5 mL of ethanol and washed with 1.5 mL of H<sub>2</sub>O. A sample was loaded onto the cartridge, which was washed with 3 mL of 60% ethanol. The cartridge was dried for 10 minutes, and the sample was eluted with 3 mL of ethyl acetate. The sample was then dried under a stream of N<sub>2</sub> and dissolved in 1 mL of hexane. A second extraction used straight-phase solid-phase extraction cartridges (100 mg silica conditioned with 1 mL hexane). The sample was loaded onto the cartridge, which was washed with 1 mL of hexane. The sample was eluted with 1 mL of 30% ethyl acetate in hexane, dried, and dissolved in 15 μL of dodecane. One microliter of the sample was used for GC/MS analysis. The mass spectrometer was operated in the negative-ion, electron-capture ionization mode, using ammonia as the moderating gas. Ions monitored were of mass-to-charge ( $m/z$ ) ratio 283 and 286 for 4-HNE and d<sub>3</sub>-HNE, respectively. A representative chromatogram obtained from human urine is depicted in Figure 1. Increasing amounts of d<sub>3</sub>-HNE (0.3, 0.625, 1.25, 2.5, 5, and 10 ng) were added to 5-mL control urine samples,



**Figure 4**  
Significant increases in urinary excretion of iPF<sub>2α</sub>-III in patients with cirrhosis due to alcoholism (ALD), viral hepatitis (HCV), or a combination of the 2 etiologies. Levels were significantly elevated in cirrhosis compared with controls. Excretion of iPF<sub>2α</sub>-III was most marked in acute alcoholic hepatitis.

**Table 2**

Endogenous antioxidants are depressed in ALD

Patient group	Vitamin C (mg/dL)	Vitamin E (mg/dL)
Healthy volunteers	1.1 ± 0.3	11.3 ± 2.1
HCV cirrhosis	0.83 ± 0.24	10.6 ± 5.9
ALD	0.74 ± 0.3 <sup>A</sup>	6.4 ± 1.75 <sup>A</sup>
Combined disease	0.68 ± 0.18 <sup>A</sup>	8.4 ± 2.7 <sup>A</sup>
Acute alcoholic hepatitis	0.36 ± 0.16 <sup>A</sup>	4.15 ± 2.2 <sup>A</sup>

<sup>A</sup>*P* < 0.05 vs. values in volunteers.

from which 4-HNE was extracted as described above, to assess the linearity of the assay.

**Other measurements.** Blood was collected from the antecubital vein, and ethanol concentration was measured using an alcohol dehydrogenase kit from Sigma Chemical Co. (St. Louis, Missouri, USA). After hexane extraction, plasma concentrations of vitamin C and serum levels of  $\alpha$ -tocopherol were measured by HPLC on a reverse-phase C18 column (33). Serum was heated with thiobarbituric acid at a pH of less than 5.0 for measurement of TBARS. The resulting malondialdehyde adduct was measured by its absorbance at 532 nm, as described previously (27).

**Statistical analysis.** Data were initially subjected to Kruskal-Wallis ANOVA, with subsequent pairwise analysis using a 2-tailed distribution-free test, as appropriate. Data are expressed as the mean  $\pm$  SEM. A Bonferroni correction was used when multiple paired comparisons were performed.

## Results

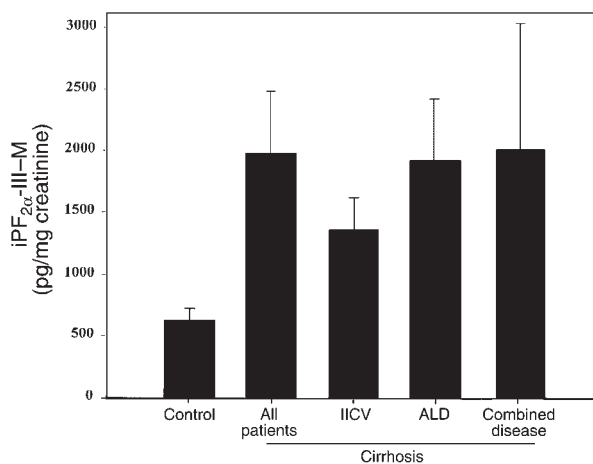
**Alcohol increases iP excretion in healthy volunteers.** No alteration in urinary iPF<sub>2 $\alpha$</sub> -III was observed after administration of the control solution. However, urinary iPF<sub>2 $\alpha$</sub> -III excretion rose from 116  $\pm$  10.1 pg/mg creatinine before dosing, to 491  $\pm$  90, 349.5  $\pm$  117.5, and 201.8  $\pm$  25.6 pg/mg creatinine at 0–6 hours, 6–12 hours, and 12–24 hours after dosing with 0.9 gm/kg alcohol, respectively (*P* < 0.001; Figure 2). The time dependence of this response reflected the acute increase in blood alcohol. Alcohol also significantly (*P* < 0.01) increased peak urinary iPF<sub>2 $\alpha$</sub> -III excretion in a dose-dependent manner. For example, the increase from baseline was 50  $\pm$  19.6, 102  $\pm$  28, 197  $\pm$  112, 335  $\pm$  130, and 401  $\pm$  120 pg/mg creatinine after dosing with 0.2, 0.3, 0.4, 0.6, and 0.9 gm/kg alcohol, respectively (Figure 3). Paired comparisons revealed significant elevations at the 2 highest doses of alcohol. The corresponding peak plasma alcohol levels were 30.2  $\pm$  5.3, 64.6  $\pm$  6.1, 81.2  $\pm$  4.2, 105.1  $\pm$  4.2, and 130.6  $\pm$  5.6 mg/dL. While mean peak plasma alcohol levels and mean peak urinary iPF<sub>2 $\alpha$</sub> -III excretion were significantly correlated (*P* < 0.001), the relationship between individual alcohol and urinary iPF<sub>2 $\alpha$</sub> -III levels across the study did not attain significance. This may reflect the discordant timing of plasma versus urinary measurements, and/or differences in the kinetics of alcohol and iP in a study of this sample size.

**Urinary iP and 4-HNE are elevated in acute and chronic ALD.** All patients with cirrhosis had elevated levels of urinary

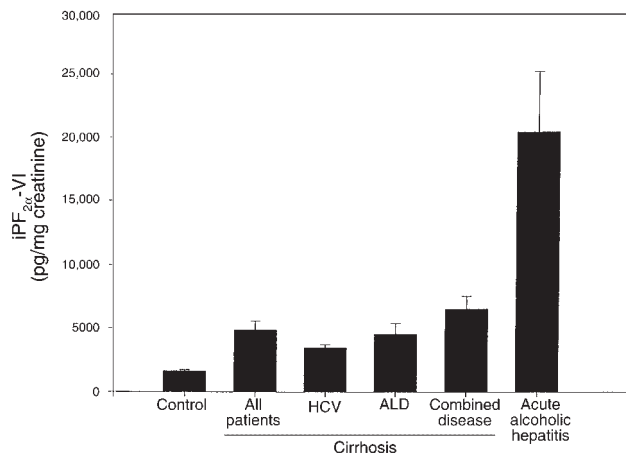
iPF<sub>2 $\alpha$</sub> -III when compared with noncirrhotic controls (664  $\pm$  95 vs. 127.2  $\pm$  7.9 pg/mg creatinine; *P* < 0.001). Urinary iPF<sub>2 $\alpha$</sub> -III excretion was significantly increased in patients with HCV cirrhosis (411.7  $\pm$  59.8 pg/mg creatinine; *P* < 0.01). However, this increase was even more marked in patients with ALD (656.9  $\pm$  105.8 pg/mg creatinine; *P* < 0.001) and in those with combined disease (921.9  $\pm$  120 pg/mg creatinine; *P* < 0.001) (Figure 4). There was no significant variability in the 3 consecutive measurements in the patients with cirrhosis.

Urinary excretion of 2,3-dinor-5,6-dihydro-iPF<sub>2 $\alpha$</sub> -III, a putative metabolite of iPF<sub>2 $\alpha$</sub> -III (29, 30), was higher than that of parent iPF<sub>2 $\alpha$</sub> -III in cirrhosis (1,926  $\pm$  497 vs. 664  $\pm$  95 pg/mg creatinine; *P* < 0.01). Like the parent iP, excretion of the metabolite was increased in cirrhosis; this was particularly apparent in patients with a history of alcoholism (Figure 5). Thus, the increment in urinary iPF<sub>2 $\alpha$</sub> -III is likely to reflect increased generation of the iP, rather than its decreased metabolism in cirrhosis.

Evidence for increased generation of iP in cirrhosis was also supported by measurement of an iP from a second class, iPF<sub>2 $\alpha$</sub> -VI, in a subset of patients and controls. Urinary iPF<sub>2 $\alpha$</sub> -VI was also significantly elevated over control in cirrhosis (4,841  $\pm$  720 vs. 1,642  $\pm$  110 pg/mg creatinine; *P* < 0.001) (Figure 6). Excretion of iPF<sub>2 $\alpha$</sub> -III and iPF<sub>2 $\alpha$</sub> -VI was highly correlated in patients with cirrhosis (*r* = 0.90, *P* < 0.001; Figure 7). Excretion of both iPF<sub>2 $\alpha$</sub> -III and iPF<sub>2 $\alpha$</sub> -VI was even more markedly elevated (*P* < 0.001) in patients with acute alcoholic hepatitis (2,205  $\pm$  4,008 and 20,405  $\pm$  4,772 pg/mg creatinine, respectively) than in those with chronic ALD. Finally, confirmation of increased oxidant stress in patients with chronic ALD was afforded by measurement of 4-HNE, using a novel assay for this compound in urine. The relationship of measured 4-HNE with added authentic spike was linear (*r*<sup>2</sup> = 0.96, *P* < 0.0001) in human urine. The assay was also highly reproducible.

**Figure 5**

Significant increases in urinary excretion of 2,3-dinor-5,6-dihydro-iPF<sub>2 $\alpha$</sub> -III (iPF<sub>2 $\alpha$</sub> -III-M), the putative dinor-dihydro metabolite of iPF<sub>2 $\alpha$</sub> -III, in patients with cirrhosis induced by alcohol (ALD), hepatitis C (HCV), or a combination of etiologies. Like the parent iPF<sub>2 $\alpha$</sub> -III, excretion of the metabolite was increased in all forms of cirrhosis, but particularly in ALD.



**Figure 6** Significant increases in iPF<sub>2α</sub>-VI excretion in patients with cirrhosis due to alcoholism, hepatitis C virus (HVC), or a combination of the 2 etiologies. Levels were significantly elevated in cirrhosis compared with controls. iP excretion was most marked in acute alcoholic hepatitis.

Analysis of 5 samples, frozen at  $-70^{\circ}\text{C}$  and thawed for analysis on 4 successive days, gave  $m/z$  ratios for ions 283/286 of  $187.3 \pm 13.6$ ,  $175.6 \pm 6.4$ ,  $181.6 \pm 11.8$ , and  $179.25 \pm 4.8$ , none of which differed significantly from each other ( $P > 0.05$ ). Excretion of this independent marker of lipid peroxidation (23, 32) was significantly ( $P < 0.01$ ) elevated in patients with chronic ALD ( $1.19 \pm 0.3$  ng/mg creatinine) compared with controls ( $0.49 \pm 0.07$  ng/mg creatinine). We failed to observe sex-based differences in urinary iPs or 4-HNE in our patients with ALD.

Both plasma vitamin C and serum  $\alpha$ -tocopherol were significantly ( $P < 0.05$ ) lower in all patient groups with ALD when compared with controls. Levels of the endogenous vitamins were not significantly depressed in patients with HCV cirrhosis (Table 2); however, this may reflect the comparative severity, rather than the origin, of the disease in the 2 groups and/or the constraints of a small sample size. The differences were most marked in patients with acute alcoholic hepatitis. However, levels of both vitamins fell within their normal ranges for this laboratory in all patient groups (0.2–1.9 and 4.6–14.5 mg/dL plasma, respectively).

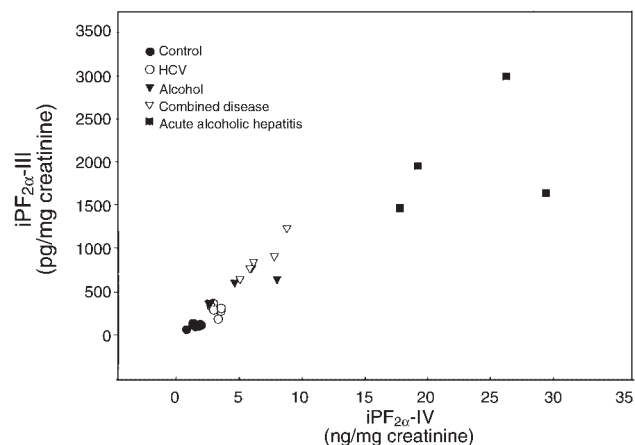
*Vitamin C suppresses iP excretion in chronic ALD.* In this study, both urinary iPF<sub>2α</sub>-III ( $764 \pm 139$  vs.  $53 \pm 92$  pg/mg creatinine;  $P < 0.01$ ) and iPF<sub>2α</sub>-VI ( $6,198 \pm 1,843$  vs.  $423 \pm 109$  pg/mg creatinine;  $P < 0.01$ ) were again elevated in all patients with established cirrhosis compared with controls. Administration of vitamin C suppressed TBARS ( $P < 0.05$ ), urinary iPF<sub>2α</sub>-III, and urinary iPF<sub>2α</sub>-VI in patients with ALD ( $P < 0.05$ ) and with combined disease ( $P < 0.05$ ). Vitamin C had no significant effect in patients with HCV cirrhosis, in whom endogenous antioxidants were not significantly depleted (Table 3). After a 10-day washout period, administration of aspirin failed to suppress urinary excretion of either iP or TBARS (Table 3). Again, endogenous levels of vitamin C (Table 3) and  $\alpha$ -tocopherol (data not shown) were lower than in controls, but fell within the normal range.

## Discussion

Although ALD is a significant cause of chronic liver disease in the developed world, it remains an enigma. It is unclear which mechanisms underlie the evolution of cirrhosis in chronic alcoholics. Putative factors include alcohol dose, sex, and comorbid conditions. Increasing lifetime alcohol dose is associated with greater prevalence of alcoholic liver disease above a threshold of 30 g of ethanol per day (5, 34). The threshold to initiate ALD may be lower, and the dose-response curve may be altered in women (4, 5). Comorbidity with viral infection, particularly chronic HCV infection, is associated with more severe liver injury in alcoholics, especially in those with a high alcohol intake (35). Consumption of diverse alcoholic beverages may be more injurious, while alcohol intake at mealtime may be less hazardous than in the fasting state (5).

Recently, there has been increasing interest in the possibility that ROIs might contribute to the evolution of ALD (7–9, 36–38). The development of hepatic disease in response to alcohol feeding in the rat is associated with an increase in lipid peroxides and in conjugated dienes in plasma (39, 40). Alcohol administration depletes the liver of the antioxidant glutathione. Administration of methionine, a precursor of glutathione, protects against alcohol-induced hepatic injury in rodents (39). Similarly, administration of polyenylphosphatidylcholine, which suppresses lipid peroxidation, attenuates alcohol-induced liver injury in baboons (41). Conversely, dietary supplementation with unsaturated fatty acids, a target for lipid peroxidation, increases susceptibility to ALD (39, 40).

Chronic alcoholics frequently consume inadequate diets, which may be deficient in antioxidant vitamins (42–44). It is possible that genetic variability in enzymes intrinsic to antioxidant defense or alcohol metabolism (11, 45, 46), or variability in consumption of antioxidants in the diet, may contribute to ethnic- and sex-based variability in the expression of ALD. Indeed, the antioxidant content of red wine has been suggested to contribute to the cardiovascular benefit associated with this form of



**Figure 7** Correlation excretion of urinary iPF<sub>2α</sub>-III and urinary iPF<sub>2α</sub>-VI in a subset of patients with cirrhosis of various etiologies (HCV, alcohol, and combined disease) and acute alcoholic hepatitis.

**Table 3**

Vitamin C suppresses iP excretion and TBARS formation in chronic ALD

Group	iPF <sub>2α</sub> -III pg/mg creatinine		iPF <sub>2α</sub> -VI pg/mg creatinine		TBARS nmol MDA	
	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10
	HCV	784.3 ± 101.0	638 ± 87	5,683 ± 1,891	5,147 ± 1,610	1,480 ± 49
ALD	805 ± 156	436 ± 90 <sup>B</sup>	6,274 ± 2,094	2,929 ± 3,513 <sup>A</sup>	2,049 ± 48	1,589 ± 52 <sup>A</sup>
Combined disease	702 ± 122	475 ± 93 <sup>A</sup>	6,639 ± 1,555	4,631 ± 1,322 <sup>A</sup>	1,323 ± 90	1,023 ± 68 <sup>A</sup>

Group	iPF <sub>2α</sub> -III pg/mg creatinine		iPF <sub>2α</sub> -VI pg/mg creatinine		TBARS nmol MDA	
	Day 20	Day 21	Day 20	Day 21	Day 20	Day 21
	HCV	796 ± 108	789 ± 115	5,237 ± 1,440	5,310 ± 1,224	1,561 ± 56
ALD	699 ± 118	700 ± 107	5,987 ± 2,169	6,071 ± 2,047	1,866 ± 76	1,841 ± 55
Combined disease	714 ± 121	719 ± 121	6,357 ± 1,098	6,291 ± 1,136	1,218 ± 86	1290 ± 67

Values are expressed as before (day 0) and after 10 days of 2,500 mg/d vitamin C (day 10); and before (day 20) and after (day 21) acute dosing with 325 mg aspirin (day 21). <sup>A</sup>*P* < 0.05, <sup>B</sup>*P* < 0.01 for comparisons between treated (day 10) and untreated (day 0) values. None of the comparisons between days 20 and 21 attained statistical significance.

alcohol consumption (36, 37, 47). While alcohol enhances cell-mediated lipoprotein oxidation *in vitro*, the same concentration of red wine suppresses this effect (47, 48). Red wine and its antioxidant polyphenols have been shown to reduce the progression of atherosclerosis in mice (49).

This study uses a noninvasive approach, based on highly specific GC/MS assays, to investigate the relationship between ROI generation *in vivo* and ALD. Isoeicosanoids are a family of free radical-catalyzed products of arachidonic acid that are isomers of the enzymatically formed eicosanoids. These compounds, in contrast with conventional indices of oxidant stress, are chemically stable and may be measured specifically with GC/MS (16). Attention has focused upon the F<sub>2</sub>-iPs, which circulate in plasma and are excreted in urine. Up to 64 of these compounds exist, grouped in 4 structural classes (16, 50, 51). Nanji et al. (52) have reported elevated levels of circulating immunoreactive iPF<sub>2α</sub>-III in a rodent model of ALD. However, these investigators used a commercial immunoassay that was subsequently withdrawn because of problems with precision and quantitation. No available immunoassays have been checked for cross-reactivity with the parent F<sub>2</sub>-iPs or their potential metabolites. GC/MS-based approaches using a heterologous prostaglandin internal standard to estimate circulating total iPs have suggested an increase in lipid peroxidation in patients with chronic ALD (53). However, the complexity of these species and the potential for differences in their recovery and detection represent a limitation of this approach (16). We have favored specific analysis by GC/MS of individual iPs using homologous internal standards (16).

We have now developed assays for members of 2 iP classes, iPF<sub>2α</sub>-III and iPF<sub>2α</sub>-VI, using GC/MS. Urinary excretion of these iPs is increased in a wide variety of syndromes of oxidant stress, including cigarette smoking (19) and during reperfusion after coronary ischemia (20, 21). Excretion is also increased in patients with hypercholesterolemia (54, 55). We have immunolocalized iPF<sub>2α</sub>-III to monocytes and smooth muscle cells in human atherosclerotic plaque (56). The virtue of specific iP analysis has recently been established in a mouse model of ath-

erosclerosis in which both serum cholesterol and urinary iPs are increased. Suppression of urinary iPF<sub>2α</sub>-VI was used to identify a dosing regimen of vitamin E that suppressed lipid peroxidation while leaving serum cholesterol unchanged (22). Because atherogenesis was reduced by 40%, this approach established the functional importance of lipid peroxidation in this disease.

We previously reported that urinary iPF<sub>2α</sub>-III is elevated in patients with cirrhosis (57). However, the majority of these patients had HCV disease, and in those with a history of alcohol intake, it was poorly documented. This study extends those findings to elucidate the role of alcohol-induced oxidant stress in both healthy volunteers and in patients with ALD. Excretion of iPF<sub>2α</sub>-III was increased in patients with established cirrhosis and a history of alcohol abuse. Indeed, the increment in iP excretion was more marked than that observed in cirrhotic patients with a history of HCV. The larger increment that was noted in patients with combined etiology is consistent with the reported additive effect of alcohol and HCV infection to produce a more severe degree of injury (34). Furthermore, excretion of iPs was increased in patients with acute alcoholic liver disease and, in a dose-dependent manner, in healthy volunteers who were administered alcohol (33). Thus, oxidant stress *in vivo* was increased in patients with acute alcoholic hepatitis and in patients with cirrhosis and a history of alcohol abuse, and was provoked by alcohol administration to healthy volunteers. In addition to the increment in urinary iPF<sub>2α</sub>-III, we report that excretion of 2,3-dinor-5,6-dihydro-iPF<sub>2α</sub>-III, which may be formed as a metabolite of iPF<sub>2α</sub>-III (27), is also increased in chronic ALD. This suggests that the increment in urinary iPF<sub>2α</sub>-III reflects an increase in its generation *in vivo*, rather than reduced oxidative metabolism due to impairment of hepatic function. Finally, we have developed a method for analysis of 4-HNE in urine, using GC/MS. Pawlosky et al. (57) have reported that rhesus monkeys fed a diet for 3 years comprising 24% of total daily calories and low in vitamins C and E develop hepatic fibrosis and have elevated levels of iPF<sub>2α</sub>-III and 4-HNE in plasma. In accordance with these observations, both 4-HNE and iPF<sub>2α</sub>-VI are significantly elevated in the urine of patients with ALD.



We have previously shown that  $iPF_{2\alpha}$ -III may be formed as a minor product of both COX isoforms (17, 32). However, evidence in healthy volunteers and in syndromes of COX activation suggests that these pathways contribute trivially, if at all, to urinary concentrations of this  $iP$  (18, 19). Consistent with these observations, vitamin C, but not aspirin, suppressed urinary  $iPF_{2\alpha}$ -III, urinary  $iPF_{2\alpha}$ -VI, and circulating TBARS in patients with alcoholic cirrhosis.

The mechanisms that underlie the increase in oxidant stress in ALD are unclear. Although alcoholics often consume a diet deficient in antioxidant vitamins, the patients in this study that had chronic ALD were stabilized in the GCRC on a standardized diet for 2 weeks before participation in the study. They also had abstained from alcohol for a minimum of 4 months before being studied. Patients with acute alcoholic hepatitis gave a history of drinking within 24 hours of admission, but were studied 1–3 days after admission. Patients with ALD had higher urinary  $iPs$ , lower levels of endogenous vitamins E and C, and a significant fall in urinary  $iPs$  on vitamin C in contrast to those with HCV cirrhosis. An attempt was made to standardize the clinical severity of the groups of patients with chronic liver disease in this study. However, in patients with more severe HCV cirrhosis, in whom endogenous antioxidant defenses were depleted, supplementation with exogenous vitamin E significantly reduced their elevated urinary  $iPF_{2\alpha}$ -III (57).

Measured with precision and sensitivity in urine using GC/MS,  $iPs$  are chemically stable indices of oxidant stress. This noninvasive approach is readily applicable to clinical trials. The effect of relatively modest alcohol ingestion on urinary  $iPs$  in volunteers raises the possibility that during chronic alcohol intake, the vasoconstrictor and proliferative effects of  $iPs$  (59, 60) may contribute to the evolution of ALD. Irrespective of this possibility, these noninvasive markers of lipid peroxidation, augmented by the measurement of 4-HNE, may be used to discriminate between the oxidant effects of various forms of alcohol in humans, and to titrate the dosage of antioxidant strategies designed to modify the evolution of cirrhosis in animal models of ALD.

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