

Increased Circulating Products of Lipid Peroxidation in Patients with Alcoholic Liver Disease

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F₂-isoprostanes (F₂-IP) and 4-hydroxynonenal (4-HNE), peroxidation products of polyunsaturated fatty acids (PUFA), are considered the most reliable indicators of endogenous lipid peroxidation in vivo. To determine to what extent these are also altered in patients with alcoholic liver disease, plasma free and esterified F₂-IP as well as 4-HNE were measured by GC/MS in 49 fasting subjects who underwent diagnostic percutaneous needle biopsies of the liver. Compared to patients with mild steatosis and no fibrosis, free F₂-IP and 4-HNE were strikingly increased in individuals with alcoholic hepatitis. There was also a significant but lesser rise of 4-HNE in patients with perivenular fibrosis. An increase of F₂-IP was also found in subjects with transition to, or complete, alcoholic cirrhosis, with a comparable trend for 4-HNE. By contrast, in patients who were drinking heavily up to 48 hr before admission, F₂-IP were not abnormal, but they increased later ($p < 0.005$). Contrasting with plasma free F₂-IP, esterified F₂-IP were not significantly changed with fibrosis. Thus, whereas circulating esterified F₂-IP were unchanged in patients with alcoholic liver disease, there was an increase in free F₂-IP as well as 4-HNE during recovery from intoxication. The increase was not a result of accompanying hepatitis C but a function of the stage of alcoholic liver injury, possibly reflecting enhanced lipid peroxidation as well as interference with biliary excretion and/or hepatic esterification.

Alcohol, Liver Disease, 4-Hydroxynonenal, F₂-Isoprostanes, Lipid Peroxidation.

PEROXIDATION OF LIPIDS leads to cell damage, and byproducts of lipid peroxidation have been shown to promote collagen production, thereby promoting fibrosis.^{1,2} Lipid peroxidation has been implicated in the development of alcoholic liver disease, but its role remains the subject of controversy. Some authors reported an increase of various parameters of lipid peroxidation after alcohol intake,³⁻⁶ whereas others showed an opposite effect of drinking alcohol⁷ or only slightly increased malondialdehyde (MDA) levels in the blood and liver of patients with chronic liver disease, and no difference between patients with alcoholic and nonalcoholic liver disease.⁸ These reports may be contradictory, in part, because of the paucity of appropriate and reliable methods for the measurement of in vivo lipid peroxidation. Commonly, MDA has been

used for the estimation of lipid peroxidation but, in the presence of amino acids, sugars, and/or bile salts, plasma MDA levels can be considerably overestimated.^{9,10} Diene conjugates are another nonspecific parameter of lipid peroxidation, especially in patients with alcoholic liver disease.¹¹⁻¹³ Furthermore, levels of short chain alkanes in breath depend on the molar yield of the reaction and the oxygen pressure.⁹

F₂-isoprostanes (F₂-IP), have been shown to be reliable markers of in vivo lipid peroxidation in experimental animals¹⁴⁻¹⁶ and in humans,¹⁷⁻¹⁹ but no comparable data are available for patients with alcoholic liver injury. F₂-IP are initially formed from arachidonate (contained in phospholipids) and then released into the circulation. F₂-IP are mainly the product of nonenzymatic reactions initiated and catalyzed by free radicals. They have been found in virtually all organs and tissues investigated, and the liver is the main source of circulating F₂-IP.²⁰ 4-HNE is another product of lipid peroxidation deriving from ω -6 PUFA, mainly linoleic and arachidonic acids, which are abundant in PUFA of mammalian cells. The GC/MS method of 4-HNE determination, as an O-pentafluorobenzyl (PFB) derivative, is considered the most suitable one,²¹ and the formation of the O-PFB oxime before 4-HNE extraction has the advantage that Schiff bases of 4-HNE with protein and lipid amino groups are cleaved, providing a better yield of 4-HNE.²² Thus, 4-HNE represents a broadly based index of lipid peroxidation, but as yet there are few clinical studies of this use of 4-HNE.

The present study addresses whether there is a relationship between increased plasma F₂-IP and 4-HNE in alcoholics at the stage of liver injury immediately after a bout of intoxication, as well as subsequently.

MATERIALS AND METHODS

Materials

Pentafluorobenzylbromide, O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride, N,N-diisopropylethylamine, bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorsilane (TMS), undecane, N,N-dimethylformamide, and acetone were purchased from Sigma Chemical Co. (St. Louis, MO) and other organic solvents from J.T. Baker Co. (Philipsburg, NJ). Deuterated [²H₄] prostaglandin F_{2 α} and 8-epi-prostaglandin F_{2 α} were obtained from Cayman Chemical (Ann Arbor, MI), and deuterated 4-HNE ([²H]₃-4-HNE) was kindly provided by Dr. F.J.G.M. van Kuijk.

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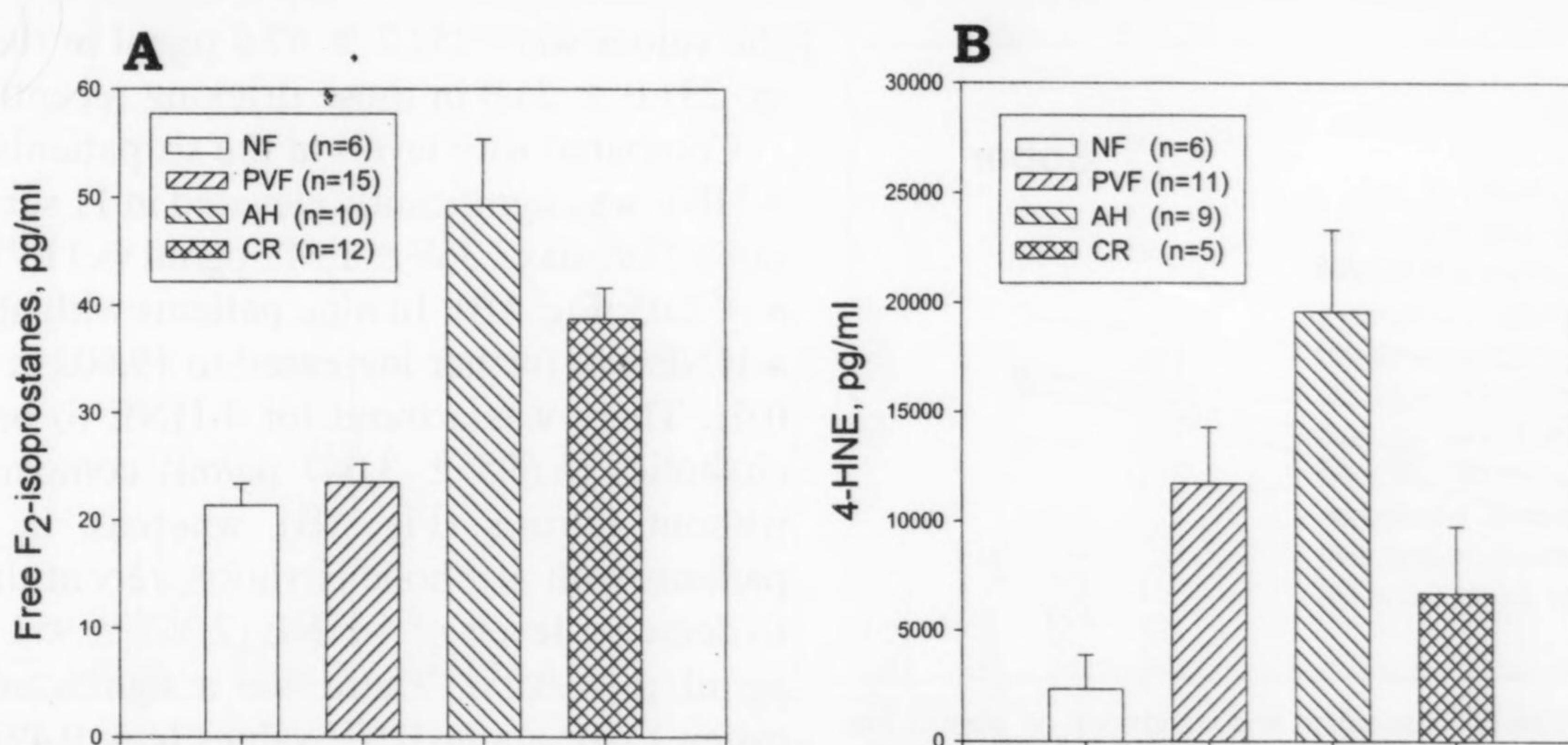


Fig. 1. Effect of alcoholic liver disease on free plasma F₂-isoprostanes and 4-hydroxynonenal. (A) Compared with levels in six patients without fibrosis and only mild steatosis (NF), free F₂-IP were strikingly increased in 10 individuals with alcoholic hepatitis (AH; $p < 0.001$). A lesser increase was found in 12 subjects with alcoholic cirrhosis (CR; $p < 0.01$) and no difference in 15 patients with perivenular fibrosis (PVF). The difference between alcoholic hepatitis (AH) and cirrhosis (CR) groups was significant ($p < 0.05$). (B) Compared with levels in six patients without fibrosis and only mild steatosis (NF), 4-HNE were strikingly increased in nine individuals with alcoholic hepatitis (AH; $p < 0.01$). A lesser increase was found in 11 subjects with perivenular fibrosis ($p < 0.05$) and a similar trend in patients with alcoholic cirrhosis (CR; $p > 0.05$).

Patients

The study was conducted in 49 male patients with various stages of alcoholic liver disease. Patients were sober for at least 8 days. Diagnoses confirmed by liver biopsy were no fibrosis ($n = 6$), perivenular fibrosis ($n = 15$), alcoholic hepatitis ($n = 10$), cirrhosis ($n = 12$). Their alcohol intake exceeded 80 g/day for at least 2 years, and the liver histology was consistent with the diagnosis of alcoholic liver disease, e.g., steatosis, perivenular fibrosis, or alcoholic hepatitis. The latter was characterized by polymorphonuclear inflammation, with or without Mallory bodies. Most of the patients had some degree of fibrosis (with or without nodular transformation, characteristic of cirrhosis). The patients with cirrhosis did not have associated alcoholic hepatitis. Nine subjects had, in addition, hepatitis C viral (HCV) infection, but the alcoholic liver lesions predominated. HCV antibodies were detected by ELISA (second generation assay).

Venous blood was collected in the fasting state. In six patients (two with perivenular fibrosis, two with alcoholic hepatitis, and two with cirrhosis) blood was also taken at the time of admission for detoxification and again 1 or 2 weeks later (at the time of biopsy). In addition, four patients with cirrhosis were studied only at admission while breath alcohol was still detectable. All patients were smokers. The study was approved by the Institutional Review Board.

Measurement of F₂-IP and 4-HNE

Venous blood was collected into Vacutainer R-tubes containing heparin, and plasma was stored at -80°C. For analysis of both F₂-IP and 4-HNE, we used an HP 5890 gas chromatograph and VG TRIO-1000 mass spectrometer, interfaced with a LAB BASE computer system. F₂-IP were measured by the method of Morrow and Roberts²³ with slight modifications. F₂-IP were chromatographed as pentafluorobenzyl esters trimethylsilyl ethers on a DB 1701 fused silica capillary column, 15-m long, inner diameter 0.25 mm, film thickness 0.15 μm (J&W Scientific, Folsom, CA). The injection port temperature was 250°C; the gas chromatograph oven temperature was programmed from 190 to 300°C at 20°C/min. The interface temperature of the mass spectrometer was 275°C, and that of the ion source was 200°C. F₂-IP were monitored at amu 569. Deuterated prostaglandin F_{2α} ([²H₄]PGF_{2α}) was used as an internal standard (amu 573). The peak of 8-*epi*-prostaglandin F_{2α} (8-*epi*-PGF_{2α}), which eluted 0.12 to 0.14 min before the peak of the internal standard [²H₄]PGF_{2α}, was used for quantification of F₂-IP. The levels of free and esterified F₂-IP found in individuals without fibrosis were comparable to those of control groups in the studies of Morrow et al.^{16, 17}

4-HNE was quantified according to the method of van Kuijk²⁴ with minor modifications. Separation of O-pentafluorobenzyl oxime trimethylsilyl ether derivatives of 4-HNE was performed on a DB 5 ms capillary column, 15-m long, inner diameter 0.25 mm, film thickness 0.25 μm (J&W Scientific, Folsom, CA). The injection port temperature was 270°C; the gas chromatograph oven temperature was programmed from 60° to 205°C at 25°C/min. 4-HNE was monitored at amu 152; while deuterated [²H₃]4-HNE, used as an internal standard, was monitored at amu 155. The *syn*-peak of 4-HNE was eluted 0.01 to 0.02 min before the peak of the internal standard. For both F₂-IP and 4-HNE, the filament emission current was 175 to 250 μA, and results were expressed in picograms of F₂-IP or 4-HNE per milliliter of plasma.

Data are given as means ± standard error of the mean (M ± SEM). The significance of the differences was assessed by one-way ANOVA with *posthoc* Student-Newman-Keuls multiple groups comparison tests or nonparametric one-way ANOVA with Dunn's test for nonGaussian distribution when appropriate. For comparisons between two groups, the paired and unpaired two-tailed Student's *t* or nonparametric Mann-Whitney tests were applied.²⁵ Correlation was estimated with the Spearman's coefficient of correlation. *P* values of 0.05 or less were considered significant.

The plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamylaminotransferase (GGT), bilirubin, and HCV antibodies were measured by the Bronx VA clinical laboratory. Normal ranges were 0 to 45 U/liter for ALT, 0 to 41 U/liter for AST, 6 to 65 U/liter for GGT, and 0.2 to 1.2 mg/100 ml for bilirubin. There were not sufficient blood samples available to carry out all tests in all subjects.

RESULTS

As shown in Fig. 1A, free F₂-IP were significantly elevated in subjects with alcoholic hepatitis and cirrhosis compared with patients without fibrosis; the results were 21.3 ± 2.0 vs. 49.3 ± 6.1 ($p < 0.001$) and 38.8 ± 2.9 pg/ml ($p < 0.01$), respectively. F₂-IP in the alcoholic hepatitis group were significantly increased compared with those for the cirrhotics ($p < 0.05$). Levels of F₂-IP in patients without fibrosis or with only perivenular fibrosis were comparable (21.3 ± 2.0 and 23.5 ± 1.7 pg/ml, $p > 0.05$). Plasma esterified F₂-IP were not significantly different among the groups; the results were 197.2 ± 16.8 ($n = 6$) in patients

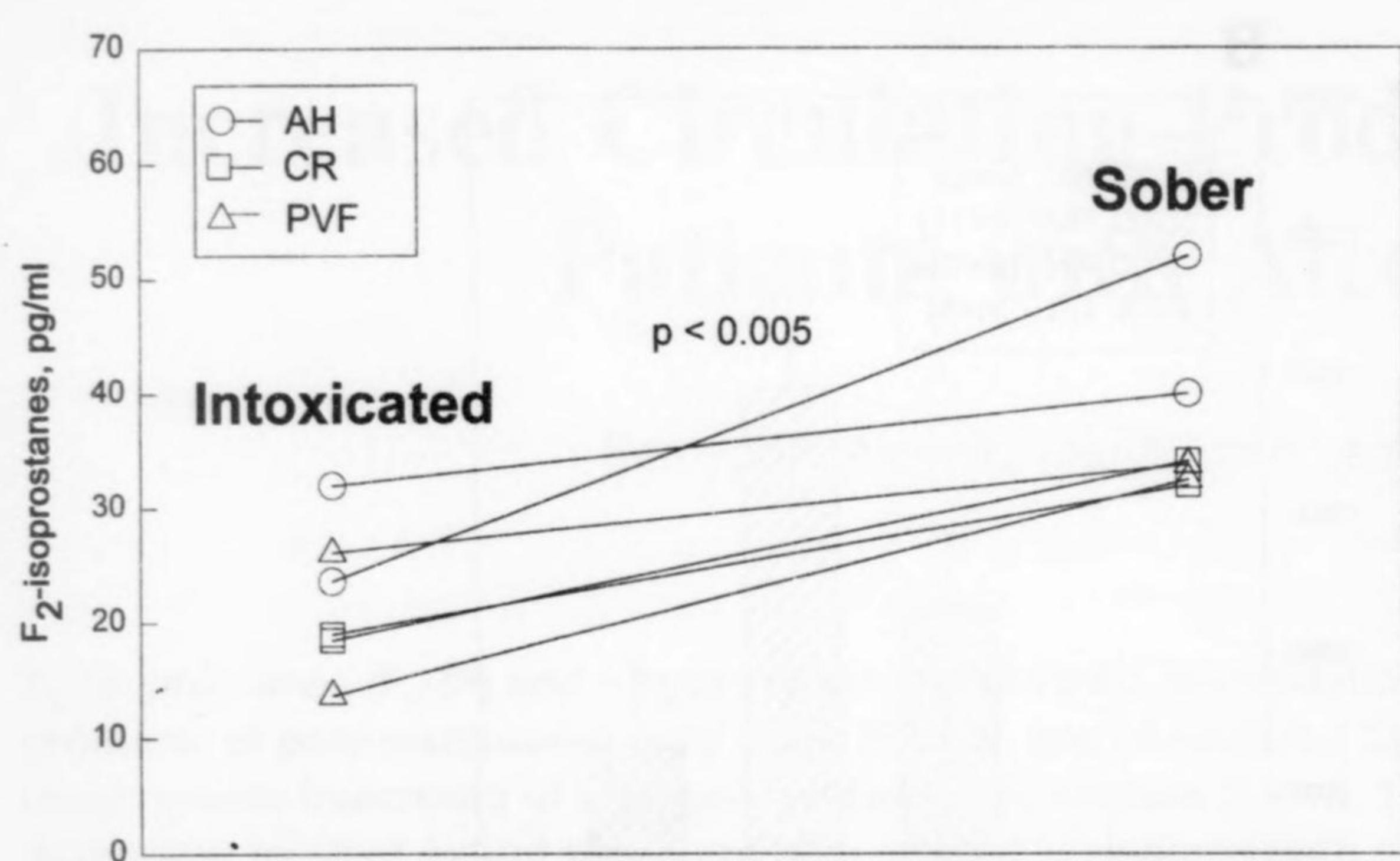


Fig. 2. Effect of acute ethanol intoxication and withdrawal on plasma free F_2 -isoprostanes. When free F_2 -IP were measured in the same subject while intoxicated and subsequently when sober, there was an almost two-fold increase ($p < 0.005$), as illustrated for two cases each of alcoholic hepatitis (AH), cirrhosis (CR) or perivenular fibrosis (PVF).

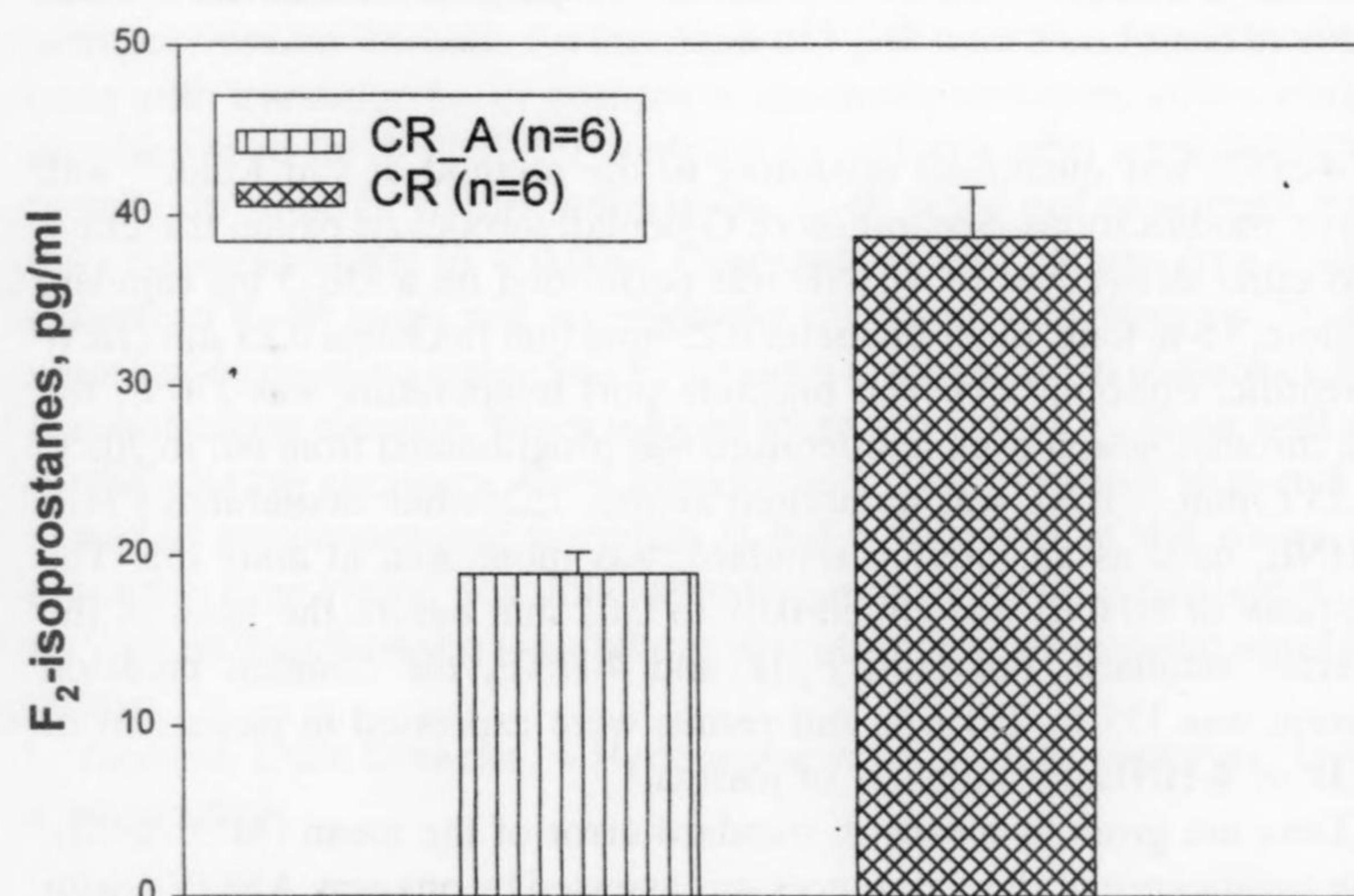


Fig. 3. Effect of recent drinking on plasma free F_2 -isoprostanes. In six patients with cirrhosis who were drinking heavily up to 24 to 48 hr before admission (cirrhosis with recent drinking history group: CR_A), free F_2 -IP were much lower than in the group of six subjects with comparable liver cirrhosis (CR) studied after periods of abstinence of at least 48 hr ($p < 0.001$).

without fibrosis, 190.6 ± 25.7 ($n = 3$) in those with perivenular fibrosis, 205.3 ± 26.5 ($n = 7$) in the alcoholic hepatitis group, and 231.0 ± 24.0 pg/ml ($n = 6$) in the cirrhotics.

In six individuals for whom sequential measurements were taken (two with alcoholic hepatitis, two with cirrhosis, and two with perivenular fibrosis), there was an increase of F_2 -IP upon sobering up (Student's paired t test, $p < 0.005$; Fig. 2). In addition, in six individuals, circulating F_2 -IP were measured after recent drinking up to the day of admission to the hospital (for detoxification). Levels of free F_2 -IP were 18.9 ± 1.3 vs. 38.8 ± 2.9 pg/ml in six individuals with comparable liver disease studied after abstinence ($p < 0.001$; Fig. 3).

Free F_2 -IP were also measured in two groups of nine subjects with or without HCV infection (matched for age and stage of fibrosis). There was no difference in F_2 -IP between the two groups (25.5 ± 3.2 and 23.2 ± 3.0 pg/ml). Esterified F_2 -IP in cirrhotics were not altered by drinking;

the values were 252.7 ± 47.6 pg/ml in the sober cirrhotics vs. 231.0 ± 24.0 in those drinking recently.

Compared with levels in the six patients without fibrosis, 4-HNE was significantly elevated in 11 subjects with perivenular fibrosis ($2,368 \pm 1,312$ pg/ml vs. $11,715 \pm 2,563$ pg/ml, $p < 0.05$; Fig. 1B). In nine patients with alcoholic hepatitis, 4-HNE was further increased to $19,602 \pm 3,715$ pg/ml, $p < 0.01$. There was a trend for 4-HNE to be elevated in five cirrhotics ($6,695 \pm 3,047$ pg/ml) compared with patients without fibrosis (Fig. 1B), whereas, in three additional patients with alcoholic cirrhosis, recent drinking appeared to decrease levels of 4-HNE ($2,367 \pm 953$ vs. $6,695 \pm 3,047$ pg/ml, $p > 0.05$). There was a significant correlation between F_2 -IP and 4-HNE values ($r = 0.4935$, $p < 0.005$).

Serum GGT was generally abnormal, but the liver tests did not differ significantly among the groups (Fig. 4A), except for serum bilirubin, which was found to be significantly elevated in patients with cirrhosis (Fig. 4B).

The patients' ages were, respectively, 46.3 ± 1.5 , 48.6 ± 2.2 , 52.1 ± 2.3 , 58.1 ± 2.4 , and 50.8 ± 3.6 years in subjects with no fibrosis, perivenular fibrosis, alcoholic hepatitis, and cirrhosis, with and without recent drinking history. Age was comparable, except for the cirrhotics group, whose ages were significantly higher than those of patients with no fibrosis or perivenular fibrosis.

DISCUSSION

This study revealed an increase of lipid peroxidation in patients with alcoholic liver disease, as determined by circulating free F_2 -IP and 4-HNE, whereas, esterified F_2 -IP were similar in all groups. The rise was greatest in patients with alcoholic hepatitis (Fig 1A, B). In addition, there was a significant increase of 4-HNE (but not of F_2 -IP) in patients with perivenular fibrosis. This greater sensitivity of 4-HNE may reflect the fact that it has several PUFA as precursors, including the abundant linoleic acid, whereas F_2 -IP has only one precursor, arachidonic acid.

Whereas we found here that the lipid peroxidation is related to the stage of alcoholic liver disease, Letteron et al.⁵ and Clot et al.⁶ concluded that increased lipid peroxidation in alcoholics depends on the amount of alcohol consumed rather than the severity of liver disease. However, they used other parameters of lipid peroxidation, that is, exhaled ethane and MDA in plasma and erythrocytes, respectively. These may respond differently from the free F_2 -IP and 4-HNE measured here. Indeed, whereas we found F_2 -IP to be higher in patients with alcoholic hepatitis than in those with cirrhosis, Letteron et al.⁵ found the opposite for exhaled ethane. Furthermore, Clot et al.⁶ did not assess patients with early fibrosis.

The rise in F_2 -IP (Fig. 2) was not observed immediately after intoxication, suggesting that time was needed for liver injury to result in peroxidation and/or that the presence of alcohol in some way traps the peroxidation products formed. The latter interpretation is consistent with the

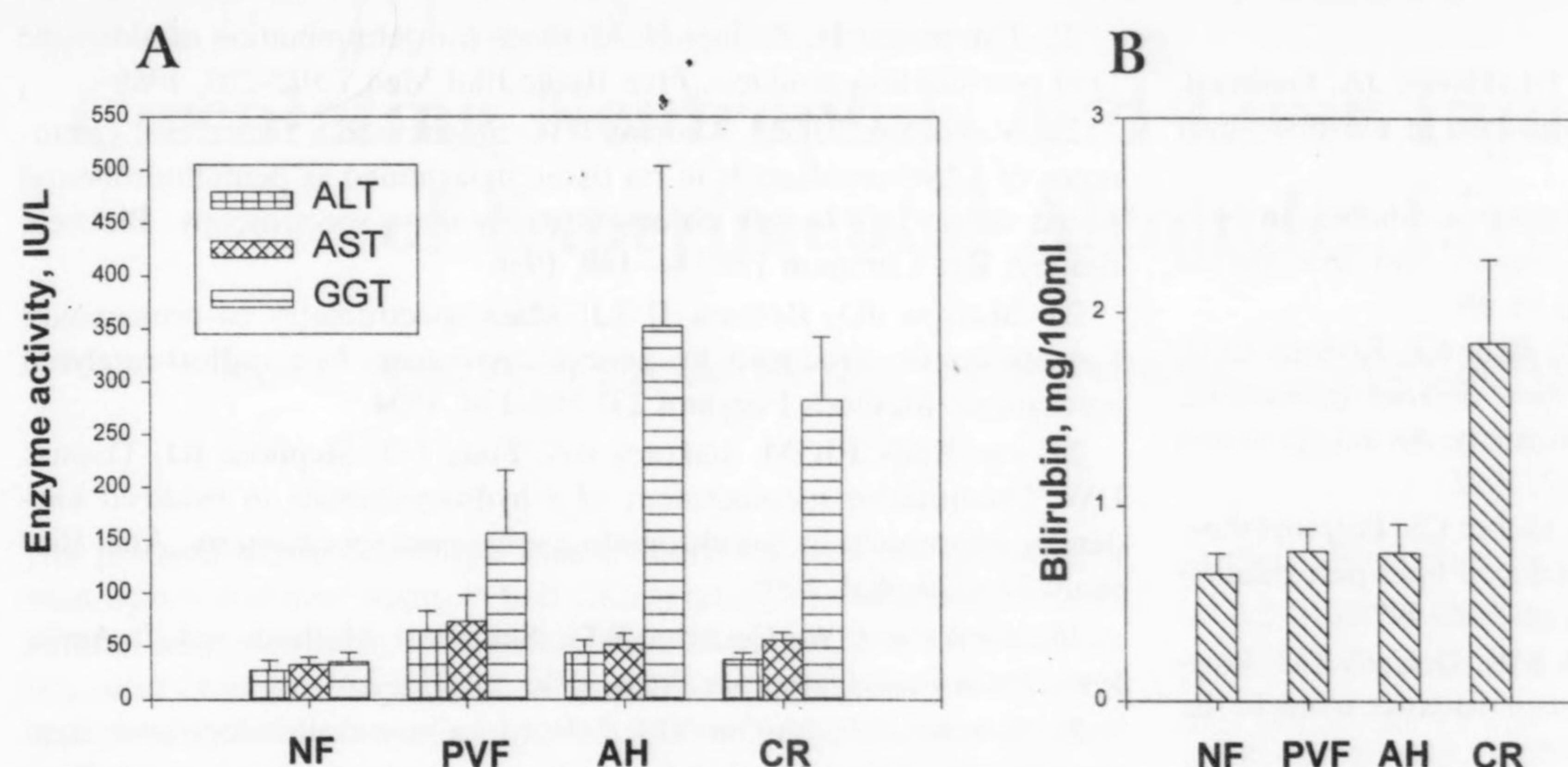


Fig. 4. Effect of alcoholic liver disease on serum ALT, AST, GGT and bilirubin. (A) Serum aminotransferases were generally abnormal (reference ranges 0 to 45, 0 to 41, 6 to 65 U/liter respectively). (B) Serum bilirubin was abnormal only in the group with cirrhosis, $p < 0.05$ compared with individuals with no fibrosis (NF), and $p < 0.01$ compared with the perivenular fibrosis (PVF) or alcoholic hepatitis (AH) groups. Reference range for serum bilirubin is 0.2 to 1.2 mg/100 ml.

observation of Koterov and Shilina,²⁶ who found a reduction of MDA in mouse blood and liver after acute ethanol administration (3 g/kg). This could result from the fact that ethanol not only serves as a substrate for the production of OH⁻ radical in the reaction catalyzed by P450 2E1,^{27,28} but it can also trap free radical species, as shown before in vivo and in vitro rodent experiments.²⁹ Once the alcohol disappears, this trapping effect should vanish, with unmasking of the increased of F₂-IP and 4-HNE resulting from enhanced hepatic liver peroxidation associated with alcohol-induced liver injury. This hypothesis is consistent with the clinical observation that many patients with alcoholic liver disease deteriorate strikingly after admission to the hospital and alcohol withdrawal. Alternatively, since the F₂-IP formed in the liver are subsequently released into either the circulation or bile,¹⁸ the increase of F₂-IP in patients with severe liver disease could reflect, in part, decreased biliary excretion secondary to the liver injury.

Hepatitis C infection is common in patients with alcoholic liver disease and, in these patients, portal and/or lobular inflammation is strongly associated with antibodies to the hepatitis C virus.³⁰ The elevation of circulating F₂-IP could reflect liver injury caused by hepatitis C rather than by the alcoholic liver disease. However, our data show that hepatitis C associated with alcoholic liver disease did not appear to aggravate the rise in circulating F₂-IP. Furthermore, Letteron et al.⁵ reported that ethane exhalation increased several fold in alcoholics but not in patients with various nonalcoholic liver diseases. Moreover, Clot et al.⁶ found no difference in plasma lipid hydroperoxides between healthy controls and cirrhotics consuming less than 100 g/day of alcohol. In addition, recently, we found that in baboons with alcoholic liver disease who were no longer drinking alcohol, hepatic F₂-IP and 4-HNE were not significantly different from those in controls.¹⁶ Thus, the increase in circulating F₂-IP observed here appears to be the consequence of alcohol-induced liver damage and does not simply reflect the degree of liver injury.

In conclusion, this study revealed a rise of free plasma F₂-IP and 4-HNE in patients with alcoholic liver disease,

which appears to reflect two concomitant and partially opposing processes: an increase, associated with (and most likely caused by) liver injury, best illustrated by the subjects with alcoholic hepatitis, and a decrease due to an acute effect of alcohol, probably secondary to the trapping of free radicals. Consequently, upon alcohol withdrawal, one might contemplate treatment with antioxidants, in order to continue the trapping of radicals without the untoward effects of alcohol.

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