Monitoring oxidative damage in patients with liver cirrhosis and different daily alcohol intake

P Clot, M Tabone, S Aricò, E Albano

Abstract
This study looked at the possible association between alcohol abuse and free radical mediated oxidative injury by examining the presence of oxidative damage, as monitored by erythrocyte malonaldehyde and plasma lipid hydroperoxides, in patients with liver cirrhosis and different lifetime daily alcohol intake. All patients with an alcohol intake above 100 g/day (ALC) showed concentrations of malonaldehyde and lipid hydroperoxide on average four to fivefold higher than cirrhotic patients with alcohol intake below 100 g/day (NAC) or healthy controls. Further subgrouping of ALC patients showed that those with alcohol intake ranging between 100 and 200 g/day (ALC1) had malonaldehyde and lipid hydroperoxide concentrations significantly lower than those with an intake higher than 200 g/day (ALC2). These differences were not related to the extent of liver injury or to the liver derangement as assessed by Child's classification. The increase in lipid peroxidation markers in ALC cirrhotic patients was associated with a decrease in, respectively, plasma α-tocopherol and erythrocyte glutathione concentrations. Significant differences were also seen between ALC1 and ALC2 groups in plasma α-tocopherol, but not in erythrocyte glutathione concentrations. The concentrations of α-tocopherol and glutathione in the blood of NAC patients were in contrast not substantially different from those of healthy controls. The close association between oxidative damage and alcohol abuse suggested that free radical intermediates produced during ethanol metabolism might be responsible for causing oxidative damage.

In recent years the finding that reactive oxygen species and hydroxethyl radicals1-3 are produced during the hepatic metabolism of ethanol has given new emphasis to the possible participation of free radical mediated damage in the pathogenesis of tissue injury resulting from alcohol abuse. Consistently, several authors have reported that either acute or chronic alcohol intoxication of rats is associated with the appearance of lipid peroxidation, as measured by conjugated diene absorbance and malonaldehyde accumulation in the liver as well as by the breath exhalation of pentane and ethane.4-8 The occurrence of oxidative injury during ethanol intoxication is further supported by several reports concerning the decrease in the hepatic content of antioxidants, such as glutathione and α-tocopherol.7,8 These findings are not limited to rodents, as stimulation of lipid peroxidation and glutathione depletion have also been seen in baboons treated with alcohol.9

The presence of oxidative damage in relation to alcoholic intoxication has also been investigated in clinical studies performed on alcoholic patients. These studies, however, have given contradictory results. For instance, lipoperoxide concentrations, measured by the thiobarbituric acid method in liver and in serum were found to be higher in heavy drinkers than in non-drinkers.10-13 A significant increase in the conjugated diene content has also been detected in the lipids extracted from hepatic biopsy specimens of alcoholic patients at different stages of liver disease. These patients also presented a decrease in the hepatic concentrations of glutathione and α-tocopherol that was unrelated with their nutritional state.11-13 Other authors have raised doubts, however, about the specificity of the association between oxidative damage and ethanol intoxication, as an increase in liver or serum thiobarbituric acid reactive substances has been seen in patients suffering from alcoholic as well as non-alcoholic liver disease.14,15

A further problem in the interpretation of these results is that subsequent studies have shown that the analytical methods most often used for the measurement of lipid peroxidation in alcoholic patients were unpecific or unsuitable for the detection of oxidative damage in humans.16

The above criticisms have prompted us to reinvestigate the problem by using different and more appropriate analytical procedures. Moreover, in the light of the recent experimental findings concerning the formation of free radicals during ethanol metabolism, we have investigated whether the detection of oxidative damage in patients with alcoholic cirrhosis might be specifically related to alcohol consumption.

Methods
We enrolled 72 consecutive patients with liver cirrhosis admitted to the Division of Gastroenterology of a general district hospital in Turin, Italy, during a six month period. All admissions occurred either from the emergency ward of our hospital, where all liver patients undergo gastroenterological consultation, or from our outpatient clinic. We excluded from the study 25 patients because of
Figure 1: Erythrocyte malondialdehyde and plasma lipid hydroperoxide content in healthy controls and cirrhotic patients with a lifetime daily alcohol intake above 100 g/day (ALC) or below 100 g/day (NAC). The values represent mean (SEM). *p>0.0005 v controls; †p>0.0001 v controls; ‡p>0.0005 v NAC.

Blood samples were taken using EDTA as the anticoagulant agent and immediately refrigerated in ice. The plasma was separated by centrifugation at 4°C and the leucocyte layer was immediately removed by aspiration. The remaining erythrocyte was washed in 5 ml ice cold phosphate buffered saline pH 7.4 and sedimented for five minutes at 1500 rpm. Aliquots (0-5 ml) of the packed erythrocyte were added to 1 ml 50% trichloroacetic acid and the supernatant was used for measurement of free malondialdehyde and of reduced glutathione.

Free malondialdehyde was routinely estimated by reacting for 10 minutes in a boiling bath of 0-8 ml of protein free supernatant with equal volume of 0-67% thiobarbituric acid solution in water as previously described.19

The effective measurement of malondialdehyde in the erythrocyte extracts was confirmed by high performance liquid chromatography (HPLC) analysis according to Esterbauer et al.20 Briefly 0-2 ml of packed erythrocytes were thoroughly mixed with the same volume of ice cold acetone/1 litre of Eppendorf tubes. After 20 minutes of extraction in ice the samples were centrifuged for one minute at 13 000 rpm and 20 l of supernatant analysed by HPLC using an Lichrospher 100-NH₂ column (Merck, Darmstadt, Germany) and acetone/30 mM TRIS buffer, pH 7-4 (1:9 vol/vol) as eluent. The effluent was monitored at 270 nm wavelength and the malondialdehyde peak in the chromatogram was identified by comparison with that of free malondialdehyde standard freshly prepared by the hydrolysis of malondialdehyde-bisdiacetal in 1% sulphuric acid solution.

Plasma lipid hydroperoxides, mostly phospholipid hydroperoxides, were estimated by measuring the polymerised adducts of 4-hydroxy-2-nonenal and methylglyoxal with thiobarbituric acid and the values were expressed as stable 1,1,3,3-tetramethylinl-propan-2-one (23830). Briefly, plasma samples were mixed with 100 ml sodium dodecylsulphate (SDS) solution (1%) and ethanolic sodium acetate (pH 5-7) to stop lipid peroxidation and the mixture was boiled for 15 minutes and centrifuged at 15 000 rpm for 15 minutes. The supernatant was mixed with a volume of phosphate buffer and placed in a spectrophotometer at 532 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 1 nmol 4-hydroxy-2-nonenal per min at 31°C in the presence of 80 nmol 2,3-dimethylmaleimide and 0.1 U catalase.

The content of a-tocopherol was measured by HPLC analysis according to Burton et al.23 Plasma aliquots (1 ml) were mixed with 1 ml 100 mM sodium dodecylsulphate solution in water, 2 ml absolute ethanol, and 1 ml n-heptane and shaken vigorously for one minute. After 15 minutes of extraction in the dark the heptane phase was separated by centrifugation and 50 l aliquots were used for the HLPC determination using Lichrosorb CN 250 RT column (Merck, Darmstadt, Germany) and a fluorescence detector operating at 296 nm excitation and 325 nm emission wavelengths.

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Results
Previous studies in our laboratory have shown that malonildialdehyde can be measured in the erythrocyte by the reaction with thiobarbituric acid. Thus, this parameter has been used to monitor 47 patients with liver cirrhosis and different mean lifetime daily alcohol intake.

As Figure 1 shows, in all 30 patients with cirrhosis associated with high alcohol intake (ALC) (mean lifetime daily alcohol intake above 100 g) we have found a relevant increase in erythrocyte malonildialdehyde, which was on average about fourfold higher than healthy controls. Cirrhotic patients (N=17) with mean lifetime daily alcohol intake below 100 g (NAC) also showed a moderate, but significant, rise compared with the controls in erythrocyte malonildialdehyde (Fig 1). The mean value of malonildialdehyde in NAC was, however, about 2–5-fold lower compared with ALC patients.

As thiobarbituric acid reactive substances other than malonildialdehyde can be present in biological samples we have verified whether the values obtained by the thiobarbituric acid assay resulted from the presence of malonildialdehyde in the erythrocytes. The simultaneous analysis of 20 different samples using the thiobarbituric acid reaction and a direct HPLC assay for malonildialdehyde showed a close correlation between the values measured by the two tests (correlation coefficient r=0.97) (Fig 2).

Recently, a new assay for measuring lipid hydroperoxides in the phospholipid fraction of the plasma has been proposed. The use of this assay confirmed that in ALC patients not only erythrocyte malonildialdehyde, but also plasma lipid hydroperoxides were dramatically increased, being on average fivefold higher than in the controls (Fig 1). Conversely, no significant difference in plasma lipid hydroperoxides was seen between NAC and healthy controls (Fig 1). These differences were not related to the changes of plasma lipids induced by alcohol because in the ALC cirrhotic patients triglyceride and cholesterol concentrations were only moderately increased (data not shown). Furthermore, when corrected for plasma phospholipids, lipid hydroperoxides values mean (SEM), healthy controls and ALC subjects were 0-11 (0-021) and 0-53 (0-16) nmol/mg of phospholipid, respectively. A good correlation (r=0.71) was also seen between accumulation of malonildialdehyde in the red cells and lipid hydroperoxide content of the plasma (Fig 2).

Interestingly, by further subgrouping the ALC patients according to their estimated mean lifetime daily alcohol intake, we have seen that the concentrations of malonildialdehyde in the erythrocytes and those of plasma lipid hydroperoxides increased with alcohol consumption and were more than double in ALC2 (daily alcohol intake above 200 g) than ALC1 (daily alcohol intake below 100 and 200 g) (Table I). Moreover, the indices of lipid peroxidation in the two alcoholic groups were significantly different not only from the control values, but also from those in NAC patients (Table I).

Serum bilirubin, triglyceride, cholesterol, and albumin concentrations and aspartate aminotransferase (AST) and γ glutamyltransferase (GGT) activities were determined by standard laboratory procedures.

The statistical significance of the differences between the groups was analysed by Student’s t test for unpaired data.

TABLE 1. Erythrocyte malonildialdehyde and plasma lipid hydroperoxide content in cirrhotic patients with different daily alcohol intake

<table>
<thead>
<tr>
<th>Malondialdehyde (nmol/ml packed cells)</th>
<th>Lipid hydroperoxide (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALC1 (n=10)</td>
<td>2.3 (0.2)*†</td>
</tr>
<tr>
<td>ALC2 (n=20)</td>
<td>5.4 (0.6)*†</td>
</tr>
</tbody>
</table>

Patients with cirrhosis (ALC) were grouped according to the daily alcohol consumption estimated according to Corrao et al17 as ALC1 (consuming on average 100–200 g ethanol/day) and ALC2 (consuming more than 200 g ethanol/day). Controls subjects were all drinking less than 100 g ethanol/day. The results are mean (SEM). *p<0.001 compared with controls; †p<0.02 compared with NAC; ‡p<0.05 compared with ALC1.
The association between the increase in malondialdehyde and lipid hydroperoxides and alcohol intake did not result from differences in the extents of liver injury, because serum AST and GGT activities were similar in the two groups (Table II). Moreover, when the ALC patients were grouped according to the criteria suggested by Child,23 no significant differences were evident between the mean values of malondialdehyde and lipid hydroperoxides (Table III). These results show that the markers of lipid peroxidation detected in the blood of ALC patients were related to alcohol consumption rather than to the derangement of liver functions.

As a decrease in the antioxidant content of both liver and blood values has often been reported in alcoholic patients, we have investigated whether the changes in blood antioxidants are specifically related to alcohol intake and to the presence of oxidative damage. In all the ALC patients the content of reduced glutathione of the erythrocyte was decreased by about 50% of the normal values, while it was practically unchanged in NAC subjects (Fig 3). Similarly, the measurement of plasma α-tocopherol concentration showed an appreciable decrease in cirrhotic patients with alcohol abuse (ALC groups), but not in the patients with moderate alcohol intake (NAC group) (Fig 3). The decrease in α-tocopherol among drinkers was more evident in the ALC2 group, where it accounted for about 43% of the control values, compared with a 28% loss in ALC1 group. Such a difference among ALC1 and ALC2 groups was statistically significant (Fig 3). Conversely, erythrocyte glutathione did not show appreciable variations among ALC1 and ALC2 groups (Fig 3).

As seen with the markers of lipid peroxidation, the differences in the concentrations of α-tocopherol were not any more evident when the patients with cirrhosis of grade A or B were compared with those with cirrhosis of grade C (Table III). Furthermore, an inverse linear correlation (r = 0.68) was evident between plasma lipid hydroperoxide and α-tocopherol concentrations (Fig 4). These findings show that the decrease in plasma α-tocopherol was related to the intake of alcohol and was strictly associated with the development of lipid peroxidation.

**Discussion**

Previous studies concerning the presence of lipid peroxidation in blood and liver specimens from alcoholic patients10–15 were performed using analytical tests that have been subsequently proved to be prone to artefacts or unsuitable for use in humans.16 For instance, much evidence has been obtained using the estimation of thiobarbituric acid reactive substances that are generated by the decompositions of lipid hydroperoxides present in the plasma. Human plasma contains many substances, however, including sugars, amino acids, and bile pigments, which by reacting with thiobarbituric acid form chromogens, which can lead to an overestimation of the effective content of peroxidation products.18 Indeed the malondialdehyde values in healthy controls reported by Suematsu et al10 and Tanner et al12 are at least twice those detected using a more specific HPLC analysis of the malondialdehyde-thiobarbituric acid adducts.20 A further problem in the interpretation of previous studies relies on the fact that most of the malondialdehyde measured is generated by decomposition of lipid hydroperoxides during the acid heating stage of the test. This process might be greatly modified by the antioxidant content of the samples. Thus, a decrease in the concentration of antioxidants in the tissues can substantially increase the formation of thiobarbituric acid reactive substances during the assay.16 This should be taken into account because the concentrations of α-tocopherol, the main lipid soluble chain breaking antioxidant, are reduced in the liver as well as in the plasma of alcoholic patients.12,27

Caution should be also applied to the interpretation of the results concerning an increase

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**TABLE II** Biochemical parameters concerning liver functions in healthy controls and cirrhotic patients with high (ALC) and low (NAC) daily alcohol consumption

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number</th>
<th>Bilirubin (mg/dl)</th>
<th>Albumin (g/l)</th>
<th>AST (IU)</th>
<th>GGT (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>24</td>
<td>0.9 (0.01)</td>
<td>4.1 (0.04)</td>
<td>29.0 (0.2)</td>
<td>29.0 (0.3)</td>
</tr>
<tr>
<td>NAC</td>
<td>15</td>
<td>2.3 (0.6)</td>
<td>3.9 (0.2)</td>
<td>95.0 (17.6)</td>
<td>93.0 (19.1)</td>
</tr>
<tr>
<td>ALC1</td>
<td>10</td>
<td>3.1 (1.3)</td>
<td>3.8 (0.3)</td>
<td>67.5 (15.0)</td>
<td>274.0 (62.6)</td>
</tr>
<tr>
<td>ALC2</td>
<td>20</td>
<td>3.3 (0.6)</td>
<td>3.3 (0.2)</td>
<td>64.7 (17.6)</td>
<td>235.0 (19.1)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SEM). Cirrhotic patients were grouped according to the estimated daily alcohol consumption as ALC1 (consuming on average 100–200 g ethanol/day) and ALC2 (consuming more than 200 g ethanol/day). Controls and NAC subjects were all drinking less than 100 g ethanol/day.

Statistical significance:

- ALC1 or ALC2 vs controls: <0.001
- ALC1 vs ALC2: NS
- ALC1 vs ALC2 + NAC: NS
- ALC1 or ALC2 vs NAC: NS

**TABLE III** Variations in erythrocyte malondialdehyde and glutathione content and plasma lipid hydroperoxide and α-tocopherol values in patients with cirrhosis and high alcohol intake (ALC) grouped according to Child's criteria

<table>
<thead>
<tr>
<th>Grade</th>
<th>A-B (n=15)</th>
<th>Grade C (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde (nmol/ml packed cells)</td>
<td>3.4 (0.4)</td>
<td>4.1 (0.6)</td>
</tr>
<tr>
<td>Lipid hydroperoxide (nmol/ml plasma)</td>
<td>7.7 (1.5)</td>
<td>11.3 (2.0)</td>
</tr>
<tr>
<td>Glutathione (nmol/ml packed cells)</td>
<td>0.8 (0.05)</td>
<td>0.73 (0.1)</td>
</tr>
<tr>
<td>α-tocopherol (nmol/ml plasma)</td>
<td>16.4 (2.2)</td>
<td>17.8 (1.5)</td>
</tr>
</tbody>
</table>

Data shown as mean (SEM).
in conjugated dienes among drinkers.\textsuperscript{11,13} Recent researches show that in human tissues the main compound containing conjugated diene bonds is represented by octadeca-9\textup{(cis)}-11\textup{(trans)}-dienoic acid, an isomer of linoleic acid\textsuperscript{28} which, despite having the diene configuration, does not originate from lipid peroxidation.\textsuperscript{29} The lack of specificity of this index is suggested by the increase in the plasma concentrations of octadeca-9\textup{(cis)}-11\textup{(trans)}-dienoic acid in alcoholic patients\textsuperscript{13,30} as well as in patients suffering from a number of diseases unrelated to alcohol consumption.\textsuperscript{28}

Researches performed in our laboratory have shown that a number of carbonyl products derived from the peroxidative degradation of unsaturated fatty acids can be readily detected in human erythrocytes.\textsuperscript{19} Thus, the thiobarbituric acid test performed in the acid extract of erythrocytes can be regarded as a reliable measure for lipid peroxidation in vivo, providing care is taken to perform the reaction in the protein free supernatant.

The determination in vivo of lipid peroxidation has also been attempted by using an assay for plasma lipid hydroperoxides based on the reaction of organic hydroperoxides with a leuco-derivative of methylen blue (10-methyl-carbamoyl-3,7-dimethylamino-10-phenothiazine).\textsuperscript{20} With this method we have found that lipid hydroperoxides concentrations in the plasma of healthy controls are on average 2 nmol/ml (range 0.4-3.2 nmol/ml). These values are lower than those detected by the same method by Taheishi \textit{et al.}\textsuperscript{31} in Japanese controls (7-65 (2-77)), but are consistent with the concentrations of plasma lipid hydroperoxide ranging from 0.5 to 4 nmol/ml found by other authors using different analytical procedures.\textsuperscript{32,33}

The results obtained by the use of the above assays of lipid peroxidation have shown that peroxidation products are actually increased in both the erythrocytes and the plasma of patients with cirrhosis associated to alcohol abuse. It is interesting to note that there is a good linear correlation ($r=0.71$) between the values of erythrocyte malonilialdehyde and those of plasma lipid hydroperoxides in the same subjects, showing that the two tests are actually measuring the same phenomenon. The presence of lipid peroxidation seems specific for the alcohol associated disease, as patients with cirrhosis and mean lifetime daily alcohol intake below 100 g show only a small increase in erythrocyte malonilialdehyde, without changes in plasma lipid hydroperoxide content. The relation between the stimulation of lipid peroxidation and the amount of alcohol consumed is further strengthened by the finding that among alcohol abusers there are significant differences in malonilialdehyde and lipid hydroperoxides in relation to the daily ethanol intake. These findings are consistent with a recent report by Lett\texter{\textsc{ron}} \textit{et al.}\textsuperscript{34} who have found that the exhalation of ethane, another end product of lipid peroxidation, is increased fivefold compared with controls in 89 alcohol abusers, but not in 52 patients with non-alcoholic liver diseases. Furthermore, in the above study ethane exhalation is weakly, but significantly, correlated with the daily ethanol intake before hospital admission.\textsuperscript{34}

It has been proposed that the presence of lipid peroxidation products in the blood might reflect the extent of liver damage rather than the presence of a pro-oxidant stimulus.\textsuperscript{15} In this study, however, the indices of liver injury are essentially the same in all groups of patients irrespective of the alcohol intake and the differences among alcoholic patients are no more appreciable when the same patients are grouped according to the Child’s criteria for the estimation of liver derangement.\textsuperscript{53} Thus, ethanol consumption is seen as a main factor associated with the detection of lipid peroxidation.

Studies in vitro have shown that both rat and human liver microsomes can produce hydroxyethyl radicals during ethanol metabolism and that the formation of these radical species increases with the concentration of ethanol added as well as the induction of cytochrome P-4502E1 by chronic alcohol exposure.\textsuperscript{3} \textsuperscript{35} Furthermore, reactive oxygen species are generated in the liver during chronic ethanol consumption as a result of the modification of several enzymatic functions, thus contributing to the stimulation of lipid peroxidation.\textsuperscript{1,2,7} Because the presence of hydroxyethyl free radicals and lipid derived radicals has also been detected in vivo in ethanol fed animals,\textsuperscript{4,36} it is possible that the association between the extent of lipid peroxidation and that of alcohol consumption might reflect an increased formation of hydroxyethyl radicals by the liver.

It is known that a decrease in the antioxidant defences of the liver might also influence the development of free radical mediated oxidative damage. Experimental and clinical studies have shown that chronic alcohol consumption is associated with a decrease in the hepatic content of glutathione and \textit{a}-tocopherol,\textsuperscript{7,12,13,26} which represent the main antioxidants in cytosol and cell membranes, respectively. Recent reports show that in alcoholic patients the changes in liver...
glutathione and α-tocopherol are independent from the nutritional conditions and are reflected by a parallel decrease in the blood concentrations in the two antioxidants. The reduction in erythrocyte glutathione and plasma α-tocopherol seen in cirrhotic patients with high alcohol intake is consistent with the results published by Bell et al.37 and Loguericio et al.38 Our results show, however, that the decrease in blood antioxidants is independent from the severity of liver damage.

Preliminary experiments in our laboratory have shown that the incubation of human erythrocytes and plasma with ethanol or acetaldehyde does not result in the formation of malondialdehyde or lipid hydroperoxides, or affect the content of glutathione and α-tocopherol. On the other hand, after stimulation of peroxidative reactions in isolated hepatocytes about 50% of the total amounts of carboxyl products formed is released in the extracellular space. This suggests the possibility that peroxidative damage of hepatocytes consequent to ethanol metabolism might lead to the leakage of fatty acid oxidation products such as lipid hydroperoxides, malondialdehyde, and ethane, which can be detected in the blood of the patients. The presence of blood markers of lipid peroxidation in relation to hepatic injury by ethanol is supported by a study using rats fed a high fat ethanol containing diet by intragastric infusion. In these animals the development of liver injury and fibrosis is, in fact, associated with an increase in the fluorescent adducts between plasma proteins and aldehydes derived from lipid peroxidation, including malondialdehyde. Consistently, an increase of lipid peroxidation associated with the development of hepatic fibrosis has also been seen by Kamimura et al.41 in the liver of rats fed intragastrically with ethanol and a high fat diet.

The liver is known as the main storage tissue of α-tocopherol and oxidative hepatic injury causes a decrease in the secretion of this antioxidant into the plasma well before the liver content of α-tocopherol is affected. Interestingly, the concentration of α-tocopherol in the plasma of heavy drinkers decreases with the amount of ethanol consumed and an inverse linear correlation with lipid hydroperoxide concentrations is evident. Thus, the lowering of α-tocopherol in the plasma might also reflect the pro-oxidant action of alcohol in the liver.

In conclusion, the results obtained show that the occurrence of stimulation of lipid peroxidation in patient with liver cirrhosis associated with alcohol abuse is related to the estimate of alcohol consumed. This finding suggests the possibility that free radical species produced during liver metabolism of ethanol might be responsible for causing peroxidative damage. Furthermore, the specific association between oxidative injuries and high alcohol intake gives new emphasis to their possible participation in the pathogenesis of alcoholic cirrhosis. This work has been supported by the Italian Ministry of University and Scientific and Technological Research (Projects Clot, Tabone, Ariò, Albano Carrosi Epatica e Patologia da Radicali Liberi e degli Equilibri Redox) and by the Italian National Research Council (grant no. 92.02450.CT04).

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