This review includes a description of short-term and long-term markers of ethanol intake and their clinical utility. The major portion of this report is a summary of studies on fatty acid ethyl ester, a new marker for monitoring both acute and chronic ethanol intake. With the markers described in the review, algorithms to assess recent ethanol intake, chronic ethanol intake, and end organ damage are included to provide a practical approach to the evaluation of the patient.

INDEXING TERMS: alcohol • fatty acids • lipids • pancreatic disorders • liver disorders • cirrhosis • alcoholism • ethanol abuse • addiction

In the context of ethanol abuse, trait markers are those that identify a genetic predisposition to ethanol abuse or to development of complications from excess ethanol intake. State markers reflect the likelihood of acute and chronic ethanol intake. Specifically, the trait markers are used to address two major questions: individual predisposition to developing alcoholism and individual susceptibility to developing alcoholic cirrhosis. The state markers are valuable in addressing three separate questions: recent intake of ethanol, evidence of chronic ethanol intake, and evidence of end organ damage as a result of ethanol intake. Clinically useful markers, particularly those detectable in blood and urine, are needed to provide answers to these five questions. Several review articles provide an overview of the state and trait markers of ethanol intake [1–3].

Trait Markers

IS THIS INDIVIDUAL PREDISPOSED TO DEVELOP ALCOHOLISM?

Several markers have been under investigation to address this question, particularly for those whose risk is higher because of a family history of alcoholism [4]. Substantial attention has been given to the presence of the A1 allele of the D2 dopamine receptor [5]. The role of the D2 dopamine receptor allele as a trait marker for development of alcoholism has been widely publicized [5–7] and seriously challenged [8–10]. Platelet monoamine oxidase B has also been proposed as a marker to identify those predisposed to alcohol abuse. It has been reported that a low monoamine oxidase B activity in platelets is a marker for predisposition to alcoholism [11–14]. Subjects with low platelet monoamine oxidase B activities typically have sensation-seeking traits and an inability to abstain from ethanol [13, 14]. Use of this assay is not widespread. Finally, a low value for lymphocyte and platelet adenylyl cyclase has also been proposed as a marker to identify those predisposed to alcohol abuse [15, 16]. In a recent study, no difference was found in newly identified tetranucleotide polymorphisms in the human adenylyl cyclase type 7 gene between alcoholics and nonalcoholic controls [17]. The assay for adenylyl cyclase has not been popularized as a test for assessing inheritance for alcoholism.

DOES THIS INDIVIDUAL HAVE A HIGH SUSCEPTIBILITY TO DEVELOP ALCOHOLIC CIRRHOSIS?

Even less accepted than the genetic markers for predisposition to alcoholism are the proposed markers for determining susceptibility to cirrhosis by alcohol abusers. Several HLA antigens (B8, BW40, B13, A2, DR3, and DR2) have been proposed as markers to address this question [18]. In addition, the collagen α(1) gene polymorphism [19], the alcohol dehydrogenase 3*1 gene [20, 21], and the aldehyde dehydrogenase 2*2 allele [22] have been evaluated in preliminary studies as markers for susceptibility to cirrhosis.

State Markers

HAS THERE BEEN RECENT INTAKE OF ETHANOL?

Blood ethanol is a widely accepted marker for recent ethanol intake (within the last 4–6 h). However, the rapid elimination of ethanol from the blood nearly always makes it impossible to assess ethanol ingestion beyond
the most recent 6–8 h. Alcohol consumption can lead to increased concentrations of 5-hydroxytryptophol and decreased production of 5-hydroxyindole-3-acetic acid [23, 24]. Urinary 5-hydroxytryptophol/5-hydroxyindole-3-acetic acid ratios increase in a dose-dependent fashion with consumption of alcohol. The ratio remains increased for 5–15 h after blood ethanol is no longer detectable [24]. Serum fatty acid ethyl ester (FAEE) has recently emerged as a potential marker to assess intake of ethanol [25, 26] because it is detectable in the blood both when ethanol is present and long after ethanol has been removed from the circulation. FAEEs are described in detail below.

**IS THERE EVIDENCE OF CHRONIC ETHANOL INTAKE?**

Obtaining evidence for chronic ethanol intake is often problematic because individuals with chronic alcoholism may abstain long enough before presenting to a physician to have a blood ethanol of zero at the time of the visit. Acetaldehyde adducts to proteins, such as hemoglobin [27], albumin [28], and lipoproteins [29], have been proposed as markers of chronic ethanol ingestion. FAEE is also potentially useful in this setting. An additional marker, carbohydrate-deficient transferrin (CDT), has emerged to identify individuals who have been ingesting large amounts of ethanol for prolonged periods but are not acutely intoxicated at the time of evaluation [1–3, 30].

**IS THERE EVIDENCE OF END ORGAN DAMAGE AS A RESULT OF CHRONIC ETHANOL INTAKE?**

The most common target organs for ethanol-induced end organ damage are the liver and the pancreas. For that reason, calibrated liver and pancreatic function tests are useful in the evaluation of end organ damage from ethanol abuse. One particular liver function test, the assay for γ-glutamyltransferase, has long been used as a marker of liver injury following ethanol intake [3, 31]. This serum enzyme becomes increased more readily than other liver enzymes after an episode of ethanol abuse. In addition, aspartate aminotransferase and alanine aminotransferase have been used in assessment of liver injury. However, the specificity of all of these markers for liver dysfunction as a result of ethanol abuse is low [3]. Injury to the pancreas is assessed with assays for pancreatic amylase and pancreatic lipase, but these tests are also not reflective of pancreatic dysfunction specifically as a result of ethanol abuse [3]. Independent of liver and pancreatic function, an increase of the mean corpuscular volume of red blood cells has also been used as a marker of chronic ethanol intake [3, 31]. An increased mean corpuscular volume reflects dysfunctional production of red blood cells. Markers of injury to other organs may be valuable in a patient with signs and symptoms related to dysfunction of organs other than the liver and the pancreas, such as serum creatine kinase isoenzyme MB for damage to the heart. As with the markers originating from the liver and the pancreas, however, these markers also cannot specifically implicate alcohol as the cause for the dysfunction.

**CDT: Emerging Marker for Chronic Ethanol Intake**

Serum transferrin, which has a molecular mass of 80 kDa, is synthesized in the liver. The most important known function of transferrin is to transport and deliver iron. It has two N-linked carbohydrate units that are added to the amino acid chain posttranslationally. The biological half-life of serum transferrin is 6–12 days.

Chronic alcohol intake interferes with the metabolism of several glycoconjugates, of which transferrin is one. Regular high amounts of alcohol consumption result in the appearance of isofoms of serum transferrin that are deficient in their carbohydrate moiety [30]. These isoforms are less negatively charged, and thus they have higher isoelectric points than normal transferrin. Because of this, these isoforms can be detected by separation methods on the basis of charge. However, immunoassay is the most commonly used methodology for determination of CDT [32–35].

Daily alcohol intake of more than 60 g of ethanol (~4.5 drinks in the US) for at least 1 week, in most cases, will result in an increased concentration of CDT in the plasma. During alcohol abstinence, the values normalize with a mean half-life for the CDT of 14–17 days [30]. The mechanism for generation of CDT in alcohol abuse may be an acetaldehyde-mediated inhibition of glycosyltransferase. However, this has not yet been established. There have been several recent studies on CDT as a marker for ethanol intake [32–35]. The results of these investigations are that CDT is not a discriminating marker for detection of as much as 80 g of ethanol ingested daily for 3 weeks by healthy subjects [32], that the diagnostic detection limit of CDT as a marker for chronic ethanol intake is not sufficient to permit its use as a screening test in the general population [33], that an increased CDT cannot be regarded as a reliable indicator for chronic alcohol abuse in patients with liver disease because such patients may have an increased CDT on the basis of liver disease alone [34], and that changes in blood CDT concentrations of 20–30% may be the most sensitive indicator of a change in ethanol intake [35].

**FAEE—An Overview**

FAEEs are esterification products of ethanol and fatty acids. As shown in Fig. 1, ethanol can be metabolized by oxidative and nonoxidative pathways [36]. In the oxidative pathway, ethanol can be converted to acetaldehyde through the action of alcohol dehydrogenase, the microsomal ethanol-oxidizing system, or catalase. Acetaldehyde is then subsequently metabolized to acetate through the action of aldehyde dehydrogenase. In one of the nonoxidative pathways of ethanol metabolism, ethanol can be inserted as the head group of a phospholipid to form phosphatidylethanol. This transformation occurs through the action of phospholipase D on phosphatidylcholine in the presence of ethanol. The nonoxidative ethanol pathway that is the focus of this review is the pathway leading to the synthesis of FAEE. This is an
enzyme-mediated esterification of fatty acid or fatty acyl-CoA and ethanol.

It has long been known that ethanol abuse leads to end organ damage in the liver and pancreas and, to a smaller extent, in the heart and brain. Acetaldehyde has been proposed as a mediator of this organ damage. However, acetaldehyde has been shown to be generated primarily in the liver with little or no synthesis in the pancreas [37–40]. For this reason an ethanol metabolite other than acetaldehyde has been sought to account for the toxicity of ethanol. A 1986 autopsy study involving subjects acutely intoxicated at the time of death demonstrated that the organs most frequently damaged by ethanol abuse, the pancreas and liver, have the highest concentrations of both FAEE and FAEE synthase, the enzyme responsible for FAEE synthesis [41]. An enzyme now known as FAEE synthase has been purified from several different organs [42–44], but it is not clear whether this enzyme is responsible for the bulk of FAEE synthesis. Carboxylester lipase, which has the ability to liberate fatty acids from complex lipids to which they are esterified, has FAEE synthase capability [45]. This observation has raised the possibility that hydrolysis of a fatty acid from a phospholipid or a triglyceride molecule in the presence of ethanol can lead to formation of FAEEs.

To evaluate the biochemical mechanism for FAEE synthesis, secretion, and degradation, and to evaluate the toxic effects of FAEE, it was first necessary to develop a system for solubilization of the highly nonpolar FAEE in aqueous medium. We had found in a clinical study that FAEEs appear in the serum after ethanol ingestion bound to albumin and in the core of lipoproteins with other neutral lipids [25]. With this finding in mind, a method was developed for the solubilization of FAEE in isolated LDL particles [46]. In this method, LDL are isolated, and the core lipids are removed with heptane. FAEEs, which can be synthesized from triglyceride incubated with 0.5 mol/L KOH in ethanol and subsequently purified by solid-phase extraction [47], are added to the core of the delipidated LDL particle. This results in the accumulation of FAEEs into the core of the water-soluble LDL particle.

**SYNTHESIS AND SECRETION OF FAEEs**

We have demonstrated that a human hepatoma cell line (HepG2 cells) exposed to ethanol will synthesize and secrete FAEEs. In these studies, radiolabeled fatty acid is added to HepG2 cells for 12 h, and then the cells are exposed to ethanol for an additional 10 h. The culture medium and cell monolayer are harvested, the lipids are extracted from each, and the FAEEs are isolated from all other lipids and quantitated. We have shown that FAEE synthesis and secretion are linearly correlated to the ethanol concentration in the culture medium of HepG2 cells. We have also demonstrated that secretion is highly dependent on the presence of a carrier for FAEE in the medium. In the absence of any carrier in the medium for the FAEEs, secretion of FAEEs into the medium is very limited. FAEEs secreted into the HepG2 cell culture medium are associated with lipoproteins, most predominantly an HDL secreted by the cells. The secretion of FAEE from HepG2 cells can be interrupted by cycloheximide, brefeldin, and monensin— inhibitors of protein synthesis and various stages of vesicular transport [A. Kabakibi and M. Laposata, unpublished observations].

**TOXICITY OF FAEEs**

There have been several reports suggesting that FAEEs are toxic metabolites of ethanol. In 1983, FAEEs in emulsions were shown to cause uncoupling of oxidative phosphorylation in mitochondria [48]. In 1986, as noted earlier, an autopsy study demonstrated the presence of FAEEs selectively in the organs damaged by ethanol abuse. However, no causal association of FAEE for toxicity was shown in this investigation [41]. In 1988, FAEEs in emulsions were found to produce changes in membrane fluidity in synaptosomal membranes [49]. In 1993, FAEEs in emulsions were found to increase rat pancreatic lysosomal fragility [50]. In none of these studies, however, were FAEEs shown to be toxic for intact cells, and there was little acceptance after these reports of the suggestion that FAEEs are cytotoxic.

For that reason, we performed a study with HepG2 cells incubated with LDL containing FAEE in the core of a human LDL particle [51]. The HepG2 cells were incubated with the FAEEs in LDL for 12 h, and tritiated thymidine was then added for 5 h. Ethyl oleate and ethyl arachidonate substantially inhibited the proliferation of HepG2 cells, while native LDL and LDL reconstituted with cholesterol esters or triglycerides had no effect (Fig. 2). These two different FAEE species were also shown to decrease the synthesis of [35S]methionine-labeled protein by the HepG2 cells. Thus, this study demonstrated that FAEEs could be toxic for intact cells.

To determine whether FAEEs could be toxic in vivo, we performed a study in which FAEEs in reconstituted LDL particles were delivered as an intraarterial bolus followed by subsequent infusion into the circulation of rats [52]. Control animals received saline or LDL reconstituted with cholesterol esters. After periods up to 12 h,
the animals were killed, and blood and pancreas were removed for analysis. The toxicity to the pancreas was determined by assessment of edema formation, measurement of trypsinogen activation peptide for pancreatic cell injury, and histologic and electron microscopic examination of the pancreas. The increase over control values in edema formation and in trypsinogen activation peptide concentrations 3 and 6 h after infusion of FAEEs were

Fig. 2. Effect of ethyl oleate (left panel) or ethyl arachidonate (right panel) delivered in reconstituted LDL on [methyl-3H]thymidine incorporation into HepG2 cells.

HepG2 cells grown in 24-well plates were incubated with ethyl esters in reconstituted LDL for 12 h at the concentrations shown. The amount of native LDL in companion wells, serving as controls for reconstituted LDL, was adjusted to the amount of protein in reconstituted LDL. [methyl-3H]Thymidine (1 μCi/well, 2.2 mCi/L) in serum-free medium was added for 6 h. The cells were harvested into 1 mL of ice-cold phosphate-buffered saline. Radioactivity incorporated into the cells was quantitated by liquid scintillation spectrometry. Closed circles, reconstituted LDL; open circles, native LDL. Reprinted with permission from Gastroenterology [51].

Fig. 3. Time courses for serum FAEE concentration and ethanol concentration for subjects 1 through 7 over a 24-h period. Ethanol ingestion occurred during the first 1.5 h of the time course. Reprinted with permission from JAMA [26].
highly statistically significant ($P < 0.001$ for all comparisons). Ultrastructurally, the cells exposed to FAEE showed dilatation of the endoplasmic reticulum and an increased number of lipid droplets and secondary lysosomes.

All of these measurements demonstrated that only lipoprotein particles containing FAEEs produced injury to the pancreas. With evidence that FAEEs can produce a toxic effect, we investigated whether orally ingested FAEEs, used clinically to supplement patients with specific fatty acids, can be toxic in vivo. Before the availability of FAEEs, fatty acids used for therapeutic purposes were provided as triglycerides. To evaluate whether FAEE supplements are associated with organ toxicity, we first evaluated the degradation of FAEE in the gastrointestinal tract and in the blood [53]. Radiolabeled FAEEs were delivered as an oil directly into the rat stomach through a gastrostomy. Blood was collected from each rat at 5, 15, 30, 60, 90, and 120 min, after which the animal was killed and the organs were harvested. The organ distribution of total radioactivity from the radiolabeled FAEEs 2 h after delivery into the stomach, for both radiolabeled ethyl oleate and radiolabeled ethyl eicosapentaenoate, indicated that the radioactivity was largely present in the gastrointestinal tract. The highest amounts were in the stomach, duodenum, jejunum, and liver. When the radioactive lipid classes were quantitated in these organs to determine the percentage of radioactivity remaining as FAEE, only a partial hydrolysis of the FAEE was found in the stomach. In the duodenum, however, there were no residual FAEEs. This suggests that lipases in the gastrointestinal tract, primarily in the duodenum, can result in hydrolysis of the FAEEs and thereby limit any toxic effect from FAEE supplements.

Because of the likely absorption into the blood of undegraded FAEEs through the stomach, we evaluated the hydrolysis of FAEEs in LDL in the vascular compartment after intraarterial injection into the rat. We demonstrated that the degradation of FAEEs in the blood is extremely rapid, with a half-life of 58 s. This provides additional evidence that FAEEs ingested as fatty acid supplements are unlikely to produce toxic effects.

**FAEEs as Markers for Acute and Chronic Ethanol Intake**

We have recently reported that FAEEs may be useful as markers for both acute and chronic ethanol intake [26]. We performed a study in which seven subjects were given ethanol to drink at a controlled rate over 90 min. Multiple samples were then collected from the subjects for blood ethanol and serum FAEEs for up to 24 h. The results from this study indicate that the concentration of FAEE in the blood closely parallels the concentration of blood ethanol (Fig. 3). Importantly, however, the serum FAEEs in these subjects, who all achieved blood ethanol concentrations $>1.5 \text{ g/L} (1500 \text{ mg/L}, 32.5 \text{ mmol/L})$, were still detectable 24 h after ethanol ingestion (Fig. 4). Thus, this observation identifies individuals who have ingested ethanol within 24 h. Individuals who had ethanol values very slightly above baseline and $<0.10 \text{ g/L} (100 \text{ mg/L}, 2.2 \text{ mmol/L})$ and would be considered negative for ethanol.
in our clinical laboratory were all found to be positive for FAEE. This suggests that FAEE may be a more discerning marker for ethanol intake than ethanol itself. Thus, serum FAEE may evolve into both a short-term and a long-term marker of ethanol ingestion.

Algorithm to Determine Recent Intake of Ethanol
Figure 5 shows the algorithm to assess recent intake of ethanol. This algorithm begins with a test for blood ethanol. If the answer is negative, the person evaluating the patient should assess the degree of suspicion for ethanol intake within the past 24 h. If there is no suspicion, then the evaluation can be ended. However, if there is still a suspicion of ethanol intake, an assay for serum FAEE would be valuable. A negative blood ethanol with a positive FAEE is consistent with ethanol intake 4–24 h before blood collection.

If the test for blood ethanol is positive and confirming the positive blood ethanol or assessing the timing of ethanol intake is desired, an assay for serum FAEE could be performed. If the assay for serum FAEE is positive, it can be concluded that ethanol intake has occurred 0–6 h before blood collection. If the FAEE test is negative, the ethanol and FAEE tests should be repeated because a positive blood ethanol with a negative FAEE is not known to occur.

Algorithm to Assess Chronic Ethanol Intake and End Organ Damage in the Absence of Acute Intoxication
In this algorithm (Fig. 6), the first step for a patient suspected of chronic ethanol abuse is to document a negative blood ethanol to rule out ethanol intake within the past 6 h. Assuming that the result for blood ethanol is negative, the next step is to perform an FAEE assay to assess ethanol intake within the last 24 h. If FAEEs are detected, it can be concluded that substantial ethanol intake has occurred within the last 24 h, and therefore, chronic ethanol intake should be suspected. With or without detection of serum FAEE, an assay for CDT to assess chronic alcohol intake should be performed. If the CDT assay is positive, whether or not FAEEs are present, evidence of substantial chronic ethanol intake exists, and the patient should be evaluated with clinical and laboratory evaluations of the liver and pancreas. If the CDT assay is negative and the person evaluating the patient still suspects chronic ethanol intake, the CDT assay should be repeated at a later date. If the CDT result is positive at that time, the patient should be evaluated as
above for end organ damage. If the CDT assay is repeatedly negative but suspicion of ethanol abuse persists, other markers for chronic ethanol intake (many are in development) could be sought. If any are positive, this may provide evidence for chronic ethanol intake and lead to a reevaluation of the patient at a later date with FAEE and CDT assays and tests for end organ damage.

If none of the new ethanol intake markers are available and the CDT has been repeatedly negative, the next question is to ask whether FAEEs were detectable in the initial analysis. If serum FAEEs have never been detected in a patient who is CDT-negative on two occasions and has no other markers for chronic ethanol intake, there is no evidence for chronic ethanol intake. However, if FAEEs are detected with a repeatedly negative CDT, the question of whether it is the first time for FAEE detection becomes important. If the FAEEs have been detected on more than one occasion, this is strong evidence for chronic ethanol intake, even with a negative CDT test. If this is the first time for FAEE detection, it would be most prudent to repeat the evaluation from the beginning at a later date.

A clinical need for markers of ethanol intake exists. Although tests of liver function provide some evidence for excess ethanol intake, several newer markers, notably CDT and FAEE, could become widely used. Ongoing work with these new indicators of ethanol intake should provide important information regarding their clinical utility. An initial proposal for their clinical use is shown in the algorithms provided in this report (Figs. 5 and 6).

References