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Biomarkers for Assessing Ethanol Consumption and the Development of Alcoholic Liver Disease

Immune Responses against Ethanol Metabolites, Cytokine Profiles and Markers of Fibrogenesis

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ACADEMIC DISSERTATION
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To my family

Yhteenveto

Alkoholin suurkulutus ja siitä aiheutuvat terveyshaitat ovat huomattava ongelma useimmissa länsimaissa. Alkoholin aiheuttamien sairauksien taustalla olevat mekanismit ovat kuitenkin edelleen epäselviä. Tarjolla ei myöskään ole tarpeeksi tarkkoja menetelmiä alkoholin aiheuttamien terveysongelmien toteamiseen.

Tässä tutkimuksessa selvitettiin etanolin aineenvaihduntatuotteiden aiheuttamien immuunivasteiden, alkoholinkulutuksen biomarkkereiden, sytokiinien ja fibroosimuodostuksen yhteyksiä ja diagnostista käyttökelpoisuutta alkoholin suurkuluttajilla. Tutkimushenkilöinä oli maksasairaita alkoholisteja, alkoholisteja, joilla ei ole maksasairautta, alkoholin kohtuukäyttäjiä ja täysraittiita. Heiltä määritettiin perinteisten laboratoriokokeiden (MCV, GGT, CDT, AST, ALT) lisäksi autoimmuunivasteita asetaldehydin muokkaamia proteiineja vastaan, sytokiiniprofiileja, fibrogeneesin osoittimia (PIIINP, PINP ja hyaluronihappo) sekä kollageenin hajoamista kuvaavia osoittimia (β-CrossLaps ja ICTP).

Anti-asetaldehydiaddukti-IgA-tasot olivat alkoholisteilla merkittävästi korkeammat kuin kohtuujuojilla tai täysraittiilla. Kohtuujuojilla arvot olivat suuremmat kuin täysraittiilla. Keskimääräisen alkoholikulutuksen (grammaa päivässä) viimeisen kuukauden ajalta havaittiin korreloivan merkitsevästi anti-asetaldehydiaddukti-IgA-tason kanssa ($r = 0.67$, $p < 0.001$). Alkoholin suurkuluttajilla asetaldehydiadduktеja vastaan kehittyvien autovasta-aineiden ja punasolujen makrosytoosiin välillä havaittiin olevan yhteys. Niistä potilaista, joilla oli kohonneet MCV-arvot, 94 %:lla oli myös koholla olevat anti-asetaldehydiaddukti-IgA-arvot ja 64 %:lla koholla olevat anti-asetaldehydiaddukti-IgM-arvot. Niillä alkoholisteilla, joilla MCV-arvot olivat normaalit, kohonneita anti-asetaldehydiaddukti-IgA-tasoja esiintyi vähemmän (63 %).

Maksasairailla alkoholisteilla IgA-tasot sekä asetaldehydiadduktеja että kudostransglutaminaasia (tTG) vastaan olivat merkittävästi korkeammat kuin terveillä kontrolleilla tai alkoholisteilla, joilla ei ole maksasairautta. Nelikenttätestissä näiden kahden vasta-aineen kohoamisella havaittiin myös merkittävä yhteys. Anti-tTG-IgA korreloi positiivisesti pro-inflammatoristen sytokiinien (IL-2, IL-6, IL-8, TNF-α) kanssa ja negatiivisesti anti-inflammatoristen sytokiinien (IL-1, TGF-β) kanssa. Vahva positiivinen korrelaatio havaittiin anti-tTG-IgA:n ja fibroosiosoittimien (PIIINP ja hyaluronihappo) välillä.

Seerumin β-CrossLaps-tasot (β-CTx) olivat maksasairailla alkoholisteilla merkittävästi matalammat kuin terveillä kontrolleilla tai alkoholisteilla, joilla ei ole maksasairautta. PINP ja PIIINP, jotka kuvaavat tyypin I ja III kollageenien synteesiä, olivat maksasairailla alkoholisteilla merkittävästi koholla. Terveiden

kontrollien arvot eivät merkittävästi eronneet niiden alkoholistien arvoista, joilla ei ole maksasairautta. Seerumin β-CTx-tasot korreloivat negatiivisesti seerumin PIIINP-tasojen ja pro-inflammatoristen sytokiinien (IL-2, IL-6, IL-8, TNF-α) kanssa ja positiivisesti anti-inflammatoristen sytokiinien (IL-1, TGF-β) kanssa, kun taas seerumin PIIINP-tasot korreloivat positiivisesti pro-inflammatoristen ja negatiivisesti anti-inflammatoristen sytokiinien kanssa. PIIINP:n ja β-CTx:n suhteen laskemisella saavutettiin erinomainen herkkyys (94 %) ja tarkkuus (98 %) maksasairaiden alkoholistien tunnistamisessa.

Tutkimusten tulokset viittaavat siihen, että anti-asetaldehydiaddukti-IgA voisi tarjota uuden kliinisesti merkittävän osoittimen alkoholin suurkulutuksen toteamiseen. Spesifisillä immuunivasteilla etanolin aineenvaihduntatuotteille saattaa olla sekä diagnostisia että patogeenisiä sovellutuksia alkoholin aiheuttamien sairauksien tutkimisessa. Vahva yhteys anti-tTG- ja anti-asetaldehydiaddukti-IgA-tasojen välillä viittaa siihen, että sairauksissa, joissa sisäisten suojamekanismien toiminta on häiriintynyt alkoholi- ja asetaldehydialtistuksen seurauksena, saattaa esiintyä samankaltainen limakalvovaste kyseisille antigeeneille. Tulokset viittaavat myös siihen, että maksasairailla alkoholisteilla tasapaino sekä pro-inflammatoristen ja anti-inflammatoristen sytokiinien välillä että kollageenin synteesin ja hajoamisen välillä on häiriintynyt. Nämä löydökset pitäisi ottaa huomioon, kun kehitetään uusia menetelmiä erottelemaan yksilöt, joilla on suurentunut riski kehittää fibroosi ja kirroosi.

Abstract

Although excessive ethanol consumption is a significant cause of health problems in most Western countries, the primary mechanisms of ethanol-induced diseases have remained largely unknown. There has also been a lack of specific methods for assessing ethanol-induced health problems.

This study sets out to explore the associations and diagnostic usefulness of immune responses induced by ethanol metabolites, biomarkers of ethanol consumption, cytokines and fibrogenesis in alcohol consumers. Conventional laboratory markers of excessive ethanol consumption (MCV, GGT, CDT, AST, ALT), autoimmune responses to proteins modified by acetaldehyde (the first metabolite of ethanol), cytokine profiles and markers of fibrogenesis (carboxyterminal propeptides of type I and type III procollagens, PINP and PIIINP and hyaluronic acid), and markers of collagen degradation (aminoterminal telopeptides of type I collagen, β-CrossLaps and ICTP) were measured in alcoholics with or without liver disease, moderate drinkers and abstainers.

Anti-acetaldehyde adduct IgA levels were significantly higher in the alcoholics than in the moderate drinkers or abstainers. The values in the moderate drinkers were also higher than those in the abstainers. Anti-acetaldehyde adduct IgAs correlated significantly with the amount of ethanol consumed during the month preceding blood sampling ($r = 0.67$, $p < 0.001$). Induction of autoantibodies against acetaldehyde adducts was also found to be associated with red cell macrocytosis induced by heavy ethanol intake. Anti-acetaldehyde adduct IgA and IgM against acetaldehyde-induced protein modifications were elevated in 94 % and 64 % of the patients with high MCV, respectively, the former being significantly less frequent in the alcoholics with normal MCV (63 %).

The patients with alcoholic liver disease (ALD) had significantly higher levels of IgAs, not only against acetaldehyde adducts but also against tissue transglutaminase (tTG), than did the healthy controls or alcoholics without liver disease. There was also a significant co-occurrence between these two antibodies in the alcohol consumers. Anti-tTG IgA showed significant positive correlations with proinflammatory cytokines (IL-2, IL-6, IL-8, TNF-α) and negative correlations with anti-inflammatory cytokines (IL-1, TGF-β). Highly positive correlations occurred between anti-tTG IgA and markers of fibrogenesis (PIIINP, hyaluronic acid).

Serum β-CrossLaps (β-CTx) in ALD patients were at a significantly lower level than in the healthy controls or the alcoholics without liver disease, while PINP and PIIINP, reflecting type I and type III collagen synthesis, respectively,

were significantly increased. The alcoholics without liver disease showed values which were not significantly different from those of the healthy controls. Serum β-CTx correlated negatively with serum PIIINP and pro-inflammatory cytokines (IL-2, IL-6, IL-8, TNF-α), and positively with anti-inflammatory cytokines (IL-1, TGF-β), whereas serum PIIINP correlated positively with pro-inflammatory cytokines and negatively with anti-inflammatory cytokines. Calculation of the PIIINP/β-CTx ratio was found to yield an excellent sensitivity (94 %) and specificity (98 %) in differentiating alcoholics with liver disease.

The present data suggest that anti-acetaldehyde adduct IgAs may provide a new clinically useful marker of excessive ethanol consumption. Specific immune responses to ethanol metabolites may prove to have both diagnostic and pathogenic implications in ethanol-related diseases. The close association between anti-tTG and anti-acetaldehyde adduct IgAs may indicate that, under conditions in which the integrity of the intestinal barriers has been disrupted upon ethanol intake and acetaldehyde exposure, there may be common mucosal responses to such antigens. The results also suggest that patients with alcoholic liver disease have a disturbed balance between pro-inflammatory and anti-inflammatory cytokines and between collagen synthesis and degradation, which may facilitate fibrogenesis in ALD. These findings also have implications for the development of non-invasive tools for discriminating individuals at risk for fibrogenesis.

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Heidi Koivisto

Contents

Abbreviations

ADH	Alcohol dehydrogenase
ALD	Alcoholic liver disease
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
ANOVA	One-way analysis of variance
ApoJ	Apolipoprotein J
AST	Aspartate aminotransferase
AUC	Area under the curve
CCLI	Combined clinical and laboratory index
CDT	Carbohydrate-deficient transferrin
%CDT	CDT as a percentage of total transferrin
CMI	Combined morphological index
EtG	Ethyl glucuronide
EtOH	Ethanol
EtS	Ethyl sulphate
FAE	Fetal alcohol effects
FAEE	Fatty acid ethyl ester
FAS	Fetal alcohol syndrome
GGT	Gamma-glutamyl transferase
HA	Hyaluronic acid
HIAA	Hydroxyindole-acetic acid
HTOL	Hydroxytryptophol
ICTP	Carboxyterminal telopeptide of type I collagen
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MCV	Mean corpuscular volume
MEOS	Microsomal ethanol oxidizing system
NK cell	Natural killer cell
NORIP	Nordic Reference Interval Project
PEth	Phosphatidylethanol
PICP	Carboxyterminal propeptide of type I collagen
PIIINP	Aminoterminal propeptide of type III collagen
PINP	Aminoterminal propeptide of type I collagen
ROC	Receiver-operating characteristic
ROS	Reactive oxygen species
SA	Sialic acid
SD	Standard deviation
SIJ	Sialic acid index of apolipoprotein
TGF-β	Transforming growth factor-β

TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor- α
tTG	Tissue transglutaminase
YKL-40	Human cartilage glycoprotein 39
β-CTx	β-CrossLaps

List of original publications

I Hietala J, Koivisto H, Latvala J, Anttila P, Niemelä O (2006): IgAs against acetaldehyde-modified red cell protein as a marker of ethanol consumption in male alcoholic subjects, moderate drinkers, and abstainers. Alcohol Clin Exp Res 30:1693–1698.

II Koivisto H, Hietala J, Anttila P, Parkkila S, Niemelä O (2006): Long-term ethanol consumption and macrocytosis: diagnostic and pathogenetic implications. J Lab Clin Med 147:191–196.

III Koivisto H, Hietala J, Anttila P, Niemelä O (2007): Co-occurrence of IgA antibodies against ethanol metabolites and tissue transglutaminase in alcohol consumers: correlation with proinflammatory cytokines and markers of fibrogenesis. Submitted for publication.

IV Koivisto H, Hietala J, Niemelä O (2007): An inverse relationship between markers of fibrogenesis and collagen degradation in patients with or without liver disease. Am J Gastroenterol, in press.

The original articles are referred to in the text by the above Roman numerals.

1. Introduction

Excessive ethanol consumption and consequent medical disorders are major problems in most Western countries (Lieber 1995, Room et al. 2005). In Finland, the lowering of alcohol taxes has recently led to a rapid increase in ethanol consumption (WHO 2004, Stakes 2005), so that while the total ethanol consumption per capita in 2003 was about 9 liters of 100 % alcohol per year it is estimated to reach 11 liters during 2006 (Stakes 2005). Recent national statistics also show that alcohol-related causes have simultaneously risen to be the most common cause of death among 15–65-years-old men in Finland and the second most common among the same age group of women.

In order to prevent alcohol-related health problems, reliable methods for detecting excessive ethanol consumption in its early phase are needed. Since patients´ own reports about their drinking habits are often unreliable (Midanik 1982, Watson et al. 1984), there is a need for more specific and sensitive laboratory methods, but more information about the mechanism and pathogenesis of alcohol-related tissue injuries is needed to develop such methods.

Virtually all tissues in the body are affected by excessive ethanol consumption. A well known complication of alcohol abuse is the deposition of collagen in the liver, eventually leading to fibrosis, the accumulation of fibrous scar tissue. Fibrosis showing a pericellular distribution is known to be an early feature of alcoholic liver disease (ALD), but the mechanisms responsible for the progression from mild fibrosis to cirrhosis are poorly known. Recent studies have indicated that ethanol metabolites and associated oxidative stress may play a key role in alcohol-induced tissue damage (Greenwel et al. 2000, Seitz et al. 2005, Purohit and Brenner 2006). Ethanol metabolites may also act as neoantigens which stimulate immune responses (Israel et al. 1986, Niemelä et al. 1987, Lieber 1995, Lieber 2004). Several studies have suggested that cytokines, such as interleukin (IL) -1, IL-6, IL-8, and tumor necrosis factor α (TNF-α), which have been considered crucial mediators of inflammation, could have a role in the development of alcoholic liver disease (McClain and Cohen 1989, Khoruts et al. 1991, Hill et al. 1993, McClain et al. 1998, Neuman et al. 2001). Increased cytokine activities have been shown to correlate with the severity of liver disease and it has been suggested that they may be of prognostic value (Hill et al. 1992, Rodríguez-Rodríguez et al. 1995, Fujimoto et al. 2000, Neuman et al. 2001). Especially high levels of TNF-α and IL-8 in ALD patients seem to indicate decreased long-term survival (Bird et al. 1990, Felver et al. 1990, Bird 1994, Huang et al. 1996).

The present work was designed to investigate the effects of excessive ethanol consumption on specific antibody and cytokine responses to ethanol metabolites,

and their association with ethanol-induced biochemical abnormalities and liver damage. It was also aimed at developing new approaches for assessing ethanol consumption and ethanol-induced fibrogenesis.

2. Review of the literature

2.1. Main features of ethanol metabolism

When ethanol is consumed it is readily absorbed from the gastrointestinal tract into the blood. Most of the ingested alcohol is rapidly metabolized, but the small amount that remains unmetabolized permits alcohol concentrations to be measured in the breath and urine. The first step in alcohol metabolism is the oxidation of ethanol to acetaldehyde, the three major routes for this being the alcohol dehydrogenase (ADH) pathway, the microsomal ethanol oxidizing system (MEOS) and the catalase pathway. The resulting acetaldehyde is then further oxidized to acetate and finally to CO_2 and water through the citric acid cycle.

Approximately 95 % of the ethanol ingested is metabolized by ADH and CYP2E1 in MEOS in the liver, and apart from the stomach, extrahepatic metabolism of ethanol is minor (Oneta et al. 1998). It has been shown, however, that the gastrointestinal microflora can also metabolize ethanol, as the microbes of the large bowel participate in the metabolism of exogenous ethanol (Salaspuro 1996). Due to the low intestinal capacity for acetaldehyde removal, high concentrations of acetaldehyde may accumulate in the gastrointestinal tract.

2.1.1 First-pass metabolism of ethanol

The fact that oral alcohol consumption results in a lower blood ethanol concentration than the same dose administered intravenously has been taken as evidence of what is called the first-pass metabolism of ethanol, which takes place in both the stomach and the liver. In addition to the class I and II ADH isoenzymes, which are also found in the liver, the stomach contains a class IV ADH isoform (Moreno et al. 1994). Gastric ADH activity has been shown to be influenced by various factors such as age, gender, medication, concentration of the alcoholic beverage and gastric morphology e.g. gastric mucosal atrophy (Frezza et al. 1990, Roine et al. 1991, Seitz et al. 1992, Roine et al. 1993, Pozzato et al. 1995, Amir et al. 1996, Pedrosa et al. 1996, Baraona et al. 2001).

2.1.2 The alcohol dehydrogenase (ADH) pathway

Alcohol dehydrogenase (ADH), which converts alcohols to their corresponding aldehydes using NAD+ as a cofactor, is the principal enzyme responsible for ethanol metabolism in humans. It is usually saturated and working at maximal speed, which is approximately 0.1 g/kg/h for both sexes. ADH is expressed in tissues in

the form of several isoenzymes that have different kinetic properties and substrate preferences (Ehrig et al. 1990). ADH isoenzymes are divided into five classes based on their enzymatic characteristics. Class I ADHs contain isoenzymes which have high catalytic activity for the oxidation of short-chain aliphatic alcohols and are strongly inactivated by pyrazole (Zorzano and Herrera 1990) while class II ADH isoenzymes exhibit a high activity for oxidation of long-chain aliphatic and aromatic alcohols and are less sensitive to pyrazole (Li et al. 1977). Both classes are involved in ethanol oxidation in the liver. ADH isoenzymes which have virtually no ethanol oxidation activity but have high activity for the oxidation of long-chain alcohols and are completely insensitive to pyrazole are classified into class III (Beisswenger et al. 1985, Valkonen and Goldman 1988). Class IV ADH isoenzymes are some of the most ethanol active ADHs and are suggested to be most important forms of gastric ADH participating in first-pass metabolism (Jörnvall 1994, Jelski et al. 2002). Finally, the most recently identified ADHs are those of class V, which are found in both the adult and fetal liver (Yasunami et al. 1991, Zhi et al. 2000). The fetal liver contains significantly higher levels than the adult liver, however, suggesting that class V ADHs are predominantly fetal alcohol dehydrogenases (Estonius et al. 1996).

2.1.3 The microsomal ethanol oxidation system (MEOS)

Chronic ethanol consumption leads to increased activity of the microsomal ethanol oxidation system (MEOS), with an associated rise in microsomal cytochromes P450 (Lieber and DeCarli 1968, Lieber 1988). It has also been shown that even a single acute dose of ethanol can produce a relatively rapid increase in aniline hydroxylase activity and in microsomal ethanol oxidation (Ariyoshi et al. 1970, Petersen et al. 1982). The three P450 isoenzymes, CYP2E1, CYP1A2 and CYP3A4, all metabolize ethanol (Salmela et al. 1998). The mean CYP2E1-dependent ethanol oxidation is twice that of CYP1A2 or CYP3A4 and thus it plays a major role in the MEOS. However, since the combined activity of CYP1A2 and CYP3A4 is comparable to that of CYP2E1, these P450s can also contribute significantly to MEOS (Asai et al. 1996, Salmela et al. 1998).

Both the content and catalytic activity of CYP2E1 will increase upon chronic ethanol consumption, and thus acetaldehyde production by the MEOS will be enhanced. It has been shown by immunoblot analysis that the CYP2E1 content of human liver microsomes is 4 times higher in drinking alcoholics than in non-drinkers (Tsutsumi et al. 1989). The presence and inducibility of CYP2E1 in Kupffer cells suggests that under conditions in which CYP2E1 is induced, the metabolites generated by the Kupffer cells may contribute to toxicity and lipid peroxidation in these cells and induce hepatic injury (Koop et al. 1991). Although CYP2E1 is the key enzyme for MEOS, it has been suggested that CYP1A2 and CYP3A4 are also induced by chronic ethanol consumption and may contribute to the pathogenesis of alcoholic liver damage (Niemelä et al. 1998, Salmela et al. 1998, Niemelä et al. 1999).

2.1.4 The catalase pathway

Minor amounts of ethanol may also be oxidized through the catalase pathway. Human catalase is a heme-containing peroxisomal enzyme that breaks down hydrogen peroxide into water and oxygen (Keilin and Hartree 1945). Since hydrogen peroxide production is very low under normal conditions, catalase probably does not have any major role in ethanol oxidation. Its contribution may be enhanced, however, if a significant amount of hydrogen peroxide becomes available through β-oxidation of fatty acids in the peroxisomes (Handler and Thurman 1985). It was reported a few years ago that Kupffer cells may play a key role in the activation of catalase-dependent alcohol metabolism by participating in regulation of the hydrogen peroxidase supply (Bradford et al. 1999).

2.1.5 Acetaldehyde oxidation

Acetaldehyde, the product of ethanol oxidation, is highly toxic and is rapidly metabolized to acetate, mainly by mitochondrial aldehyde dehydrogenase (ALDH) (Eriksson et al. 2001, Crabb et al. 2004). The resulting acetate is transported to peripheral tissues, mostly the muscles, and converted to CO_2 and water.

Approximately 50 % of orientals lack ALDH2 activity (Yin 1994), which means that even a small amount of alcohol causes what is known as an antabuse reaction, with a rapid facial flush, frequently associated with tachycardia, headache and nausea. Thus the lack of ALDH activity may offer some resistance to the development of alcoholism (Wall and Ehlers 1995, Higuchi et al. 1996), but in alcoholics it causes alcoholic liver disease to develop at lower levels of ethanol intake than in normal individuals (Enomoto et al. 1991, Yin 1994).

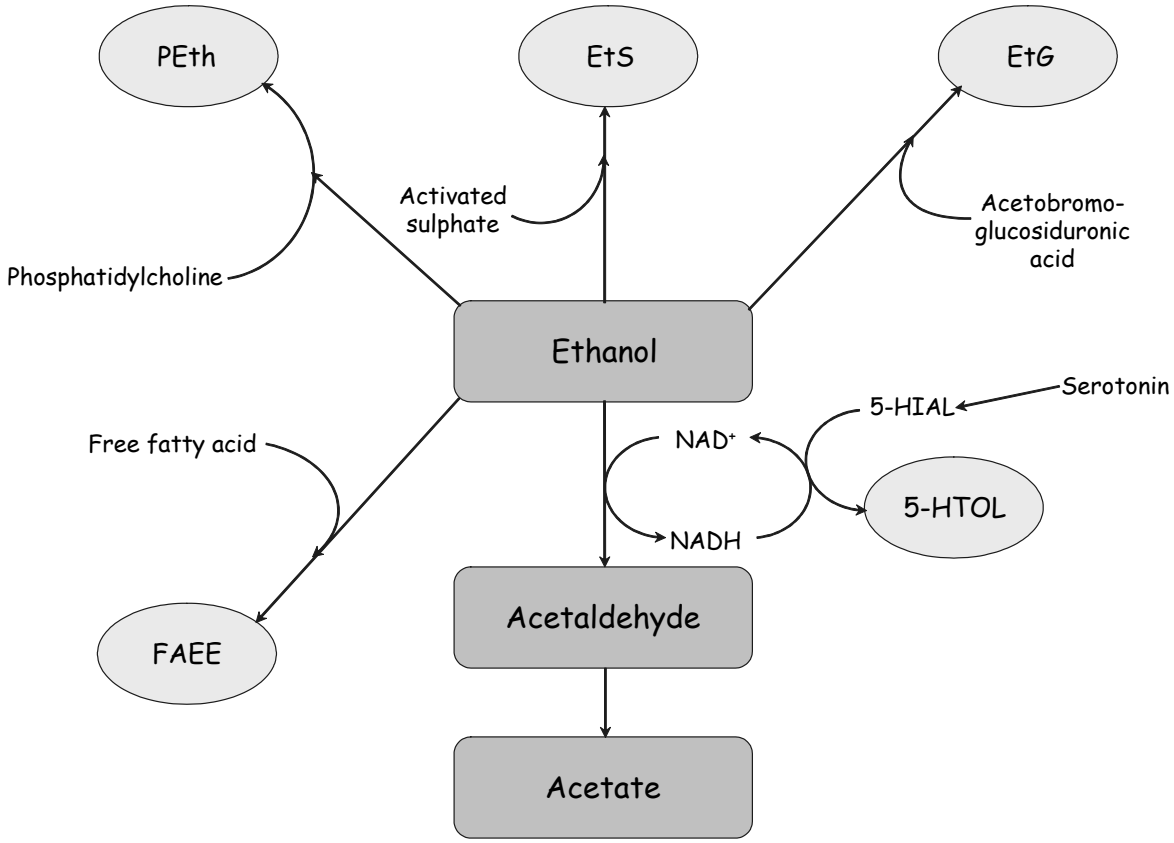

Figure 1. Products of ethanol metabolism.
EtG, ethyl glucuronide; EtS, ethyl sulphate; FAEE, fatty acid ethyl ester; 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HTOL, 5-hydroxytryptophol; PEth, phosphatidylethanol.

2.1.6 Factors influencing ethanol absorption and metabolism

A number of factors influence the absorption and metabolism of alcohol, including genetic and environmental factors, ethanol concentration, gender, age, medication, the presence of food in the gastrointestinal tract and the type of food. Lifestyle factors such as daily alcohol consumption and smoking have been shown to increase the rate of ethanol elimination from the blood (Kopun and Propping 1977). On the other hand, smoking has also been shown to slow down gastric emptying, thus delaying alcohol absorption (Johnson et al. 1991).

Women absorb and metabolize alcohol differently from men and have a higher blood alcohol concentration after consuming the same amount of ethanol. The difference is believed to be caused by their smaller amount of body water and by the fact that they have lower ADH activity in the stomach, causing a larger proportion of the ingested alcohol to reach the bloodstream (Frezza et al. 1990, Thomasson 1995, Ely et al. 1999, Baraona et al. 2001). Although there are some studies showing that ethanol elimination rates in women may be higher than in men, the differences are in general believed to be small (Kwo et al. 1998, Mumen-

thaler et al. 1999). The menstrual cycle has been shown to have an influence on ethanol elimination as elimination times are significantly shorter and disappearance rates faster during the mid-luteal phase than in the early follicular and ovulatory phases (Sutker et al. 1987).

Gastric class IV ADH isoenzymes require a relatively high ethanol concentration for optimal activity. Thus consumption of a concentrated solution of ethanol results in higher first-pass metabolism than does a diluted solution, and consequently in lower blood alcohol levels when subjects are tested in a fed state (Roine et al. 1991, Lieber et al. 1994).

It has been shown that advanced age results in decreased first-pass metabolism, although this is more likely to be associated with abnormal gastric morphology (Brown et al. 1995), since the first-pass metabolism has been shown to be increased in elderly persons (approximately 70 years of age) with normal gastric morphology as compared with young people (Oneta et al. 2001). The gender-related differences in first-pass metabolism have been shown to equal out or even be reversed at ages of 60–80 years. It has been suggested that this is most likely because of gastric mucosal atrophy, which occurs more frequently in men than in women (Pozzato et al. 1995, Parlesak et al. 2002).

Ethanol and drugs can affect each other's absorption and metabolism. Ethanol may either increase drug absorption, or in high concentrations delay it, whereas several drugs, e.g. salicylic acid and H2-receptor antagonists, have been shown to affect ethanol metabolism, mostly by affecting gastric ADH activity or gastric emptying (Linnoila et al. 1979, Ciraulo and Barnhill 1986, Palmer et al. 1991, Edelbroek et al. 1993, Gugler 1994).

The rate of alcohol absorption depends on how quickly the stomach empties its contents into the intestine (Holt 1981, Jones et al. 1997, Oneta et al. 1998). It has been shown that absorption is clearly slower when alcohol is consumed after a meal than on an empty stomach (Wedel et al. 1991). In addition to this, food intake, regardless of the composition of the food, has been shown to increase alcohol elimination from blood (Ramchandani et al. 2001).

2.2. Ethanol and health

2.2.1. Ethanol-related general health problems

Ethanol is a commonly consumed addictive substance, the lower limits for the harmful consumption of which are currently agreed to be 280 g per week for men and 190 g for women, equal to approximately 24 and 16 standard drinks respectively. It should be noted, however, that as vulnerability to alcohol dependence varies greatly among individuals, it is difficult to lay down any definite consump-

tion limits for the risk of developing dependence. Two persons exposed to alcohol at the same intensity and frequency may not have the same outcome, for many reasons, including genetic factors and clinical history (Enoch 2003, Fromme et al. 2004).

Virtually all tissues in the body are affected by excessive ethanol consumption, with chronic ethanol consumption producing different health problems from acute consumption (Lieber 1995). Those associated with acute ethanol intake are mostly intoxication, trauma and embolic strokes (Hillbom et al. 1999, Cunningham et al. 2002, Savola et al. 2004). Studies of trauma patients have shown that the risk of injury is greater in persons under the influence of alcohol (Cunningham et al. 2002), and that the relative risk of head injury starts to increase sharply above the blood alcohol level of 1.5 ‰ (Savola et al. 2005). Chronic ethanol consumption may cause various problems including gout, neurological symptoms, pancreatitis, liver disease and cancers (Diehl 1998, Cho et al. 2004, Choi et al. 2004, Harper and Matsumoto 2005, McKillop and Schrum 2005, Whitcomb 2005, Boffetta and Hashibe 2006, Thomson and Marshall 2006). The effects of ethanol on blood cells and bone marrow are discussed in section 2.4.

Alcoholism is also the cause of a variety of neurological disorders. Some neurological disorders related to long-term alcohol consumption can also be caused by inadequate nutrition, including Wernicke's encephalopathy, which is caused by thiamine deficiency and characterized by the triad of ataxia, oculomotor abnormalities and global confusion (Diamond and Messing 1994). Most patients who survive Wernicke's encephalopathy will develop Korsakoff's psychosis, which is a chronic amnestic disorder (Charness 1993, Thomson and Marshall 2006). About 25 % of these patients will never recover and will require long-term care, whereas about 20 % of patients recover completely after several months (Diamond and Messing 1994, Thomson and Marshall 2006). Some alcohol-associated neurological disorders appear to involve the neurotoxicity of ethanol or its metabolites for the brain and peripheral nerves (Harper and Matsumoto 2005). Polyneuropathy, the most common neurological complication of alcoholism, is characterized by axonal degeneration and demyelination and is believed to be caused mainly by the direct neurotoxic effect of ethanol on the peripheral nerves (Diamond and Messing 1994, Koike et al. 2001, Vittadini et al. 2001).

Excessive ethanol consumption, both acute and chronic, clearly carries a risk of acute or chronic pancreatitis, respectively. The mechanism of ethanol-induced pancreatitis is still unclear, but it is believed that pancreatic acinar cells are capable of metabolizing alcohol and that the direct toxic effects of alcohol and/or its metabolites on these cells may predispose the gland to injury in the presence of an appropriate triggering factor (Apte et al. 2005). Progression of pancreatitis may lead to various other disorders, including maldigestion, diabetes and pancreatic cancer (Ekbom et al. 1994, Nakamura et al. 1994, Petersen and Forsmark 2002, Go et al. 2005). However, only a minority of alcoholics develop pancreatitis and thus alcohol consumption cannot be the sole cause, but lifestyle and possibly ge-

netic cofactors must also be involved (Apte et al. 2005, Whitcomb 2005). Excessive ethanol consumption is also associated with development of liver disease, the risk of which is suggested to increase linearly with ethanol intake (Becker et al. 1996, Bellentani et al. 1997, Lu et al. 2004). A more detailed account of alcoholic liver disease is given in section 2.5.

Heavy alcohol consumption has been shown to have strong association with development of gout (Lin et al. 2000, Wortmann 2002, Choi et al. 2004), which is the most common form of inflammatory arthritis in men. It is believed that ethanol-induced hyperuricemia is not only related to the amount of ethanol ingested but also to the type of alcohol beverage. The highest risk of developing gout is associated with beer consumption, while wine drinking does not seem to have any influence (Choi et al. 2004, Ka et al. 2005) and whisky may even be capable of reducing serum uric acid levels (Nishioka et al. 2002).

Chronic alcohol consumption has been identified on the basis of epidemiological data as a significant risk factor for various cancers, including upper gastrointestinal and colorectal cancer (Kabat et al. 1993, Cho et al. 2004, McKillop and Schrum 2005, Wakai et al. 2005, Layke and Lopez 2006). Acetaldehyde is believed to be the most carcinogenic and mutagenic agent in alcohol-associated cancer (McKillop and Schrum 2005, Boffetta and Hashibe 2006). Gastrointestinal bacteria and various ADH isoenzymes metabolize ethanol to acetaldehyde, which can disturb DNA synthesis and repair, and may thus contribute to tumor development. Oxidative stress due to the induction of CYP2E1 is considered to be another key factor in alcohol-induced carcinogenesis (Pöschl and Seitz 2004, Boffetta and Hashibe 2006).

It has been suggested during the last few decades that alcohol may also have some beneficial health effects when consumed in small amounts (Doll 1997, Gall 2001, Agarwal 2002, Ellison 2002). It has been shown that small amounts of ethanol decreases the risk of coronary heart disease (Wannamethee and Shaper 2002) and suggested that this is at least partly due to the beneficial effects of ethanol on lipid metabolism (Sillanaukee et al. 1993, Gall 2001, Baer et al. 2002). Red wine has been reported to have greater cardioprotective effects than are associated solely with ethanol or other types of alcoholic beverages. These effects have been connected to several polyphenolic antioxidants, including resveratrol (Wu et al. 2001). It remains controversial, however, whether patients with a high risk of cardiovascular disease should be advised to drink small amounts of alcohol. While some studies have supported the idea (Ellison 2002), others have severe doubts about the overall benefits even of moderate drinking (Friedman and Klatsky 1993, Goldberg 2003). Especially among teenagers and young adults, the risks associated with alcohol consumption seem to outweigh the benefits that may evolve later in life (Friedman and Klatsky 1993, Doll 1997).

2.2.2. Gender-dependent adverse consequences of ethanol intake

The limits of harmful ethanol consumption are lower for women than for men. Due to their smaller size and smaller amount of body water, women have a lower distribution volume of ethanol, and thus their blood ethanol levels become higher than in men after consuming the same amount of ethanol (Sherlock 1995, Schenker 1997, Bradley et al. 1998). The lower level of alcohol metabolism in the stomach (Frezza et al. 1990, Thomasson 1995, Ely et al. 1999, Baraona et al. 2001) also makes the acute effects of ethanol last longer. A combination of these factors may make women more vulnerable to alcohol-induced damage to the liver and other tissues (Schenker 1997), and it has also been shown that the risk of breast cancer increases significantly even with moderate ethanol consumption (Martin-Moreno et al. 1993, Longnecker 1994, Schatzkin and Longnecker 1994, Bradley et al. 1998, Petri et al. 2004, Boffetta and Hashibe 2006).

Fetal alcohol syndrome (FAS) is a set of specific birth defects caused by maternal alcohol consumption during pregnancy. It is characterized mainly by physical and mental retardation, craniofacial abnormalities and minor joint abnormalities. FAS was first described in 1973 (Jones and Smith 1973) and it is nowadays considered to be the most common non-hereditary cause of mental retardation. The term "fetal alcohol effects" (FAE) is used when a child exhibits some, but not all, of the characteristics of FAS (Jacobson and Jacobson 2002). It is not currently known what amount of ethanol, if any, may be safely consumed during pregnancy, but it has been shown that even 1–2 daily drinks, or 5 or more drinks on any single occasion, may cause fetal damage (Streissguth et al. 1990, Larroque et al. 1995, Larroque and Kaminski 1998, Stoler et al. 1998, Sood et al. 2001, Linneberg et al. 2004, Nulman et al. 2004).

2.3. Biomarkers for assessing ethanol consumption and ethanol-induced diseases

2.3.1. Blood ethanol concentration

An ethanol concentration in the blood, breath or urine is a specific marker of recent alcohol consumption, and when combined with clinical observations it may also give information on the long-term alcohol consumption. High blood ethanol levels may be a sign of tolerance, so that levels exceeding 150 mg/l (1.5 ‰) without obvious evidence of intoxication or 300 mg/l (3 ‰) on any given occasion is an indication of tolerance and alcoholism. However, the short half-life of ethanol limits its usefulness as a tool for diagnosing chronic drinking problems (Rosman and Lieber 1992).

2.3.2. Mean corpuscular volume (MCV)

Red blood cells are the most common type of blood cells, and their principal role is to deliver oxygen from the lungs to other body tissues. An elevated mean corpuscular volume (MCV) is the most typical morphological blood cell abnormality associated with excessive ethanol consumption (Wu et al. 1974). It is so common that it has been widely used for the detection of alcohol abuse in clinical screening procedures. The specificity of MCV as an alcohol marker is limited in patients with liver diseases, vitamin B_{12} or folate deficiency, a variety of hematological diseases, hypothyroidism or reticulocytosis (Maruyama et al. 2001, Niemelä 2007). Due to the long normalization time (2 to 4 months), it may take several months for changes in drinking to be reflected in MCV levels, limiting the usefulness of this measure for the follow-up of abstinence. MCV also has limited value as a single marker of alcohol abuse because of its poor sensitivity (Meerkerk et al. 1999, Reynaud et al. 2000). However, several authors have reported MCV to be more sensitive in women than in men (Morgan et al. 1981, Sillanaukee et al. 1998), so that despite its limitations, it may be the best of the traditional markers for analyzing female patients (Mundle et al. 2000).

2.3.3. Gamma-glutamyl transferase (GGT)

Gamma-glutamyl transferase (GGT), a membrane-bound glycoprotein enzyme which catalyses the transfer of the γ-glutamyl moiety of glutathione to various peptide acceptors, is one of the most common laboratory tests used for detecting alcohol abuse. However, it is not specific to excessive ethanol consumption as it can also be induced by liver disease (Whitfield 2001). Serum GGT values may also be increased by smoking, obesity, and certain drugs and diseases such as biliary tract disease, severe heart and kidney diseases, trauma and hyperthyroidism (Cushman 1992, Sillanaukee et al. 2000b, Whitfield 2001, Puukka et al. 2006). It has also been shown that serum GGT levels tend to increase with age independently of alcohol consumption (Sillanaukee et al. 2000b), and it has been suggested that GGT may be of limited value in the case of patients who are less than 20 to 30 years old (Chan et al. 1989, Nyström et al. 1993, Sillanaukee et al. 1998, Conigrave et al. 2002).

2.3.4. Carbohydrate-deficient transferrin (CDT)

Transferrin is a glycoprotein which carries iron through the bloodstream to the bone marrow, liver and spleen. The first reports on carbohydrate-deficient transferrin (CDT) were published about 30 years ago (Stibler and Kjellin 1976, Stibler et al. 1978), since when it has become one of the most widely used markers of excessive ethanol consumption (Stibler 1991, Allen et al. 1994, Bortolotti et al. 2006, Niemelä 2007). CDT includes transferrin isoforms which have fewer sialic acid moieties than normal transferrin (Stibler 1991). Excessive ethanol consump-

tion has been shown to increase the proportions of the asialotransferrin and disialotransferrin isoforms, which together with monosialotransferrin are generally regarded as CDT. Asialotransferrin is totally absent in abstainers and individuals consuming moderate amounts of alcohol (Legros et al. 2002, 2003). Trisialotransferrin, on the other hand, has been shown to reduce the diagnostic accuracy of CDT and thus is not usually included in CDT measurements (Helander et al. 2001a, Arndt et al. 2002, Legros et al. 2002, 2003). Currently there is no uniform opinion on the amount and pattern of ethanol consumption that is needed to increase CDT levels. Levels have been reported to be elevated following a daily ethanol consumption of 40–80 g for 2–3 weeks (Stibler 1991, Helander 1999, Schellenberg et al. 2005), but it has also been suggested that higher amounts may be needed to increase levels in the general population (Salmela et al. 1994, Lesch et al. 1996a, Oslin et al. 1998). The half-life of CDT has been estimated to be approximately two weeks (Behrens et al. 1988, Lesch et al. 1996b).

One of the postulated advantages of CDT relative to traditional markers of ethanol consumption is that the values do not seem to be influenced by the presence of liver disease (Stibler 1991, Nalpas et al. 1997). The other advantage of CDT is its high specificity (Stibler 1991, Arndt 2001). False positives have been reported in rare cases of genetic transferrin variants (Stibler et al. 1988, Helander et al. 2001a) and in some other conditions, including rheumatoid arthritis and chronic pulmonary disease (Bell et al. 1994, Fleming et al. 2004). The sensitivity of CDT has varied greatly depending on the population (Anton and Moak 1994, Bell et al. 1994, Huseby et al. 1997, Anton et al. 2001, Anttila et al. 2003b, Hietala et al. 2006) and measurements seem to be more accurate in men than in women (Behrens et al. 1988, Oslin et al. 1998, Allen et al. 2000, Anton et al. 2001). It has been suggested that women express higher CDT levels under natural conditions but may produce less CDT in response to heavy drinking (Stibler et al. 1988, Anton and Moak 1994).

There have been two kinds of CDT tests in routine use. The CDTect method expresses CDT as the total concentration of CDT in serum (U/l) and measures asialotransferrin, monosialotransferrin and minor amounts of disialotransferrin. More recently developed %CDT method expresses CDT concentrations as percentages of total transferrin and measures asialotransferrin, monosialotransferrin and all of the disialotransferrin (Helander et al. 2001b). These two methods are well comparable for detection of excessive ethanol consumption (Anton et al. 2001), but %CDT may show an improved performance when diagnosing women, since it takes into account the natural variability in serum transferrin concentration (Helander 1999, Anton et al. 2001).

2.3.5. Aminotransferases

Aspartate aminotransferase (AST) is an enzyme that catalyzes the conversion of alanine to pyruvate, while alanine aminotransferase catalyzes the conversion of

aspartic acid to oxaloacetate. Serum concentrations of AST and ALT are commonly used for detecting excessive alcohol consumption, although they are more directly related to liver status (Sharpe 2001). More specific information on alcoholic liver disease (ALD) may be obtained from the AST/ALT ratio (Niemelä 2007), which is often above two in ALD, while it is usually below one in patients with non-alcoholic liver disease (Rosman and Lieber 1994, Nyblom et al. 2004). The levels of aminotransferases have been reported to normalize in 2 to 3 weeks, depending on the original level (Niemelä 2002, Chrostek et al. 2006).

2.3.6. Potential new markers

Acetaldehyde adducts, formed when acetaldehyde reacts with proteins and cellular constituents (Gaines et al. 1977, Stevens et al. 1981, Tuma and Sorrell 1987, Niemelä 2001), have been found to persist in the blood for 1 to 3 weeks after ethanol ingestion (Niemelä and Israel 1992). Thus acetaldehyde adducts have been suggested as potential markers of excessive ethanol consumption (Niemelä and Israel 1992, Sillanaukee et al. 1992, Lin et al. 1993, Hazelett et al. 1998, Niemelä 2007). It has also been reported that social drinkers have elevated levels of adducts after occasional drinking bouts as compared with abstainers (Niemelä and Israel 1992). A potential problem in these analyses, however, may result from endogenous acetaldehyde formation, especially in view of the fact that in some studies acetaldehyde adducts have been shown to be increased in non-alcoholic liver disease (Rosman and Lieber 1992). Acetaldehyde adducts have also been found in patients with diabetes and uremia, although the presence of at least some of these adducts could be explained by moderate or heavy alcohol consumption (Niemelä and Israel 1992).

The ratio of the serotonin metabolites, 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindole-3-acetic acid (5-HIAA), in urine has been shown to increase after heavy ethanol consumption (Helander et al. 1992, Voltaire et al. 1992), and it has been suggested that this ratio may be more sensitive as an indicator of recent ethanol consumption than the measurement of plasma ethanol (Helander et al. 1996, Helander and Eriksson 2002). The main advantage when compared to plasma ethanol is that the ratio remains elevated for 6–20 hours after the disappearance of ethanol (Helander et al. 1993, 1996, Helander and Eriksson 2002).

Urine concentrations of ethyl glucuronide (EtG), a metabolite of ethanol, have been reported to increase after ethanol intake (Dahl et al. 2002, Sarkola et al. 2003), and it has been suggested to be a highly sensitive and specific marker of recent alcohol consumption (Wurst and Metzger 2002). EtG has been found in the serum, urine, tissues and even hair, and may have significant applications in both clinical and forensic medicine (Wurst et al. 1999, Wurst et al. 2003, Bergström et al. 2003). Use of the EtG/creatinine ratio in urine as a marker of ethanol consumption has also been suggested. While EtG concentrations may be lowered by drink-

ing large amounts of water, the EtG/creatinine ratio remains unaffected (Dahl et al. 2002).

Phosphatidylethanol (PEth) is a unique phospholipid that is formed via the action of phospholipase D only in the presence of ethanol (Gustavsson 1995). PEth has been found in the blood of alcoholics (Hansson et al. 1997) and it may be detected for more than two weeks after the last dose of ethanol (Hansson et al. 1997, Gunnarsson et al. 1998). Blood PEth has not been found until after a substantial consumption of ethanol (Varga et al. 1998). This marker seems to have a high specificity and sensitivity and prolonged detectability (Hansson et al. 1997, Aradottir et al. 2006).

Fatty acid ethyl esters (FAEEs), which are products of non-oxidative ethanol metabolism, were first found in organs that had been damaged by excessive ethanol consumption, and it was suggested that they may mediate organ damage (Laposata and Lange 1986, Laposata 1997). Later they were also found in the blood and hair (Doyle et al. 1994, Auwärter et al. 2001). Blood FAEEs are detectable for at least 24 h after the last dose of alcohol (Doyle et al. 1996, Borucki et al. 2004) and may be used as a marker of recent excessive ethanol consumption, while FAEEs in the hair remain detectable for at least 2 months (Pragst et al. 2001) and may thus serve as a long-term marker.

It has been shown that serum and saliva levels of sialic acid (SA), a carbohydrate which is predominantly found in the terminal position of oligosaccharide chains on the surface of cells and molecules (Traving and Schauer 1998), are elevated in alcoholics (Pönniö et al. 1999a, Sillanaukee et al. 1999b, Romppanen et al. 2002). Serum SA levels also seem to decrease during abstinence, indicating that they may be useful in the follow-up of treatment for alcohol dependence (Pönniö et al. 1999a, Sillanaukee et al. 1999b). However, SA concentrations also seem to increase in a number of other conditions, including inflammatory and cardiovascular diseases, diabetes and cancer, and also with ageing, pregnancy and smoking (Stefenelli et al. 1985, Sillanaukee et al. 1999a, Pönniö et al. 1999b). These factors limit the clinical potential of SA. The plasma sialic acid index of apolipoprotein J (SIJ) has also been suggested as a marker of ethanol consumption (Ghosh et al. 2001), since the loss of SA from apolipoprotein J (ApoJ) seems to correlate positively with both the amount and duration of ethanol consumption (Ghosh et al. 2001).

Other postulated markers include circulating levels of hepatic lysosomal glycosidase β-hexosaminidase, urinary dolichols, erythrocyte ALDH, urinary salsolinol and urinary ethyl sulphate (EtS). EtS is a direct ethanol metabolite that appears to have quite similar characteristics to EtG (Wurst et al. 2006). Since EtS and EtG are formed via different pathways, they could be used side by side to increase sensitivity. Hepatic lysosomal glycosidase β-hexosaminidase has been shown to increase in alcoholics (Isaksson et al. 1985, Kärkkäinen et al. 1990, Hultberg et al. 1991), but its clinical usefulness is limited by the fact that levels

are also elevated in several other conditions, including cholestasis, cirrhosis and diabetes (Rosman and Lieber 1992, Javors and Johnson 2003). Urinary dolichols are long-chain 2,3-dihydroxy-polyprenols which serve as lipid carriers in the biosynthesis of glycoproteins. They have been shown to increase in chronic alcohol abuse (Pullarkat and Raguthu 1985, Roine et al. 1987), but their use as a marker is limited by their short half-life and lack of specificity and sensitivity (Roine et al. 1989, Stetter et al. 1991, Roine et al. 1992). Mitochondrial AST (mAST) (Nalpas et al. 1986), erythrocyte ALDH (Agarwal et al. 1983), urinary salsolinol (Collins et al. 1979, Haber et al. 1995) and urinary alanine aminopeptidase (Taracha et al. 2004) have also been proposed as markers of excessive ethanol consumption, but more research is needed to establish their clinical usefulness.

2.3.7. Marker combinations

Although a variety of biochemical markers are available for detecting excessive ethanol consumption, none of them has so far provided perfect diagnostic accuracy (Allen et al. 1994, Salaspuro 1999, Arndt 2001, Conigrave et al. 2002). Over the past 20 years many researchers have suggested that diagnostic accuracy could be improved by combining two or more markers (Monteiro and Masur 1985, Sillanaukee et al. 1998, Anton et al. 2001, Sillanaukee and Olsson 2001, Conigrave et al. 2002, Hock et al. 2005, Hietala et al. 2006). The conventional approach is to see whether either of the markers is elevated. This frequently improves sensitivity, but at the same time reduces specificity (Rosman and Lieber 1992, Anton and Moak 1994, Salaspuro 1999, Anton et al. 2001, Schwan et al. 2004).

Researchers have also introduced the idea of combining markers by means of mathematical equations (Hartz et al. 1997, Harasymiw and Bean 2001, Sillanaukee and Olsson 2001, Hietala et al. 2006) as such combinations may give increased sensitivity without any loss of specificity (Sillanaukee et al. 2000a, Sillanaukee and Olsson 2001, Chen et al. 2003, Anttila et al. 2003a, Niemelä 2007). Sillanaukee and Olsson introduced the most promising approach by combining CDT and GGT by means of the equation 0.8 x ln(GGT) + 1.3 x ln(CDT) (Sillanaukee and Olsson 2001). Chen and coworkers (2003) suggested that the integration of clinical information into the results obtained from this combined marker could increase the accuracy of the diagnosis. A further improvement for the combination of CDT and GGT was achieved by replacing absolute CDT with %CDT (Anttila et al. 2003a, Hietala et al. 2006).

When considering the use of marker combinations for the detection of excessive alcohol consumption, the increased costs should also be taken into account. Increasing the number of markers tested will always increase the costs, but on the other hand, the long-term effects of improved early detection and treatment may make the use of marker combinations cost-effective.

2.4. Effects of ethanol on blood cells

Excessive ethanol consumption has been shown to affect both blood cells and their bone marrow progenitors (Ballard 1989, Niemelä and Parkkila 2004). The most typical abnormality associated with heavy drinking is elevated MCV (macrocytosis) (Morgan et al. 1981, Wymer and Becker 1990, Seppä et al. 1991). Other abnormalities in the erythropoietic cell lineage, including erythroblast vacuolization in bone marrow, have also been reported (Heidemann et al. 1981, Lindenbaum 1987). Anemia, folate deficiency and megaloblastic alterations in bone marrow are common in alcoholics with liver disease (Lindenbaum 1987, Halsted et al. 2002a, Halsted 2004, Latvala et al. 2004).

Megakaryocytes, the precursors of platelets, are typically increased in the bone marrow of alcoholics (Lindenbaum 1987, Latvala et al. 2004). A low platelet count in circulation is a common finding after excessive ethanol consumption, while an elevated platelet count may occur within a week of alcohol withdrawal. It has been postulated that alcohol intoxication itself is the cause of thrombocytopenia, but it is likely that the mechanism is a combination of ineffective thrombocytopoiesis and a shortened platelet life span (Lindenbaum 1987).

2.4.1 Acetaldehyde-protein adducts in the circulation

Acetaldehyde is highly reactive and may bind to proteins and cellular constituents and form stable adducts. Acetaldehyde can bind to reactive amino acid residues in several target proteins (Stevens et al. 1981, Israel et al. 1986, Tuma and Sorrell 1987, Behrens et al. 1990, Latvala et al. 2001a, 2001b). Previous studies have shown the formation of acetaldehyde adducts with hemoglobin and albumin (Stevens et al. 1981, Latvala et al. 2001a), and with number of other proteins, including erythrocyte membrane proteins (Gaines et al. 1977, Latvala et al. 2001b), lipoproteins (Wehr et al. 1993, Latvala et al. 2001a) and collagens (Behrens et al. 1990).

Acetaldehyde adducts can be either stable or unstable (Tuma and Sorrell 1987). Unstable Schiff base adducts are formed by the binding of acetaldehyde to lysine residues, which are then further stabilized by reduction. Free ε-aminolysine groups are important targets for adduct formation (Tuma et al. 1991), but the binding reactions may also involve other amino acids, including valine and tyrosine (Stevens et al. 1981). Spontaneous stabilization of linkages has been shown to occur between acetaldehyde and the α-amino groups of hemoglobin in reactions yielding stable imidazolidinone derivates (Fowles et al. 1996).

2.5. Main features of alcoholic liver disease

It is widely accepted that alcohol consumption increases the risk of liver disease. The liver is the primary site for ethanol metabolism and therefore vulnerable to a variety of harmful effects of excessive ethanol consumption. Up to half of all end-stage liver-related deaths are linked to alcohol (Brunt 2002). Histologically, ALD involves a wide spectrum of morphological abnormalities, including fatty liver, alcoholic hepatitis, fibrosis and cirrhosis (Diehl 1998, Niemelä 2002, Mann et al. 2003). These injuries usually develop sequentially, but in some cases they may also coexist (Niemelä 2002, Mann et al. 2003). The most important factor in both the short and long-term survival of patients with alcoholic liver disease (ALD) is abstinence from alcohol.

ALD is often associated with vitamin, mineral and protein deficiencies. This is due to a decreased intake, malabsorption or increased loss of essential nutrients (Marsano and McClain 1991, Lieber 2003, Leevy and Moroianu 2005). Thiamine (B1) and folate deficiencies appear to be among the most common and most important causes of tissue damage in alcoholics (Butterworth 1995, Thomson 2000, Halsted et al. 2002b), while hepatic vitamin A deficiency plays an important role in hepatic fibrosis. A decrease in vitamin A storage in the hepatic stellate cells may be associated with their activation to myofibroblast-like cells, which then can synthesize collagen (Lieber 2003, Leevy and Moroianu 2005). Selenium is essential for the activity of glutathione peroxidase, which provides protection against alcohol-induced liver damage, and selenium supplementation may be of benefit for patients with alcoholic cirrhosis (Van Gossum and Neve 1995, Leevy and Moroianu 2005). It has been demonstrated, however, that ALD can occur despite adequate nutrition, indicating that the direct toxicity of ethanol and its metabolites contributes to the liver injury in ALD (Lieber et al. 1965, Lieber 2004). Concepts of liver injury caused by alcohol-related oxidative stress (lipid peroxidation) and iron overload, immune responses (cytokines), fatty acid metabolites, acetaldehyde production (protein-acetaldehyde adduct formation) and mitochondrial dysfunction (Sherlock 1995, Hill et al. 1998, Day 2000, Lieber 2004, Kohgo et al. 2005) have all been proposed, and it is most likely that several mechanisms operate simultaneously to initiate liver injury (Figure 2). The immunology of ALD is discussed further in section 2.7.1

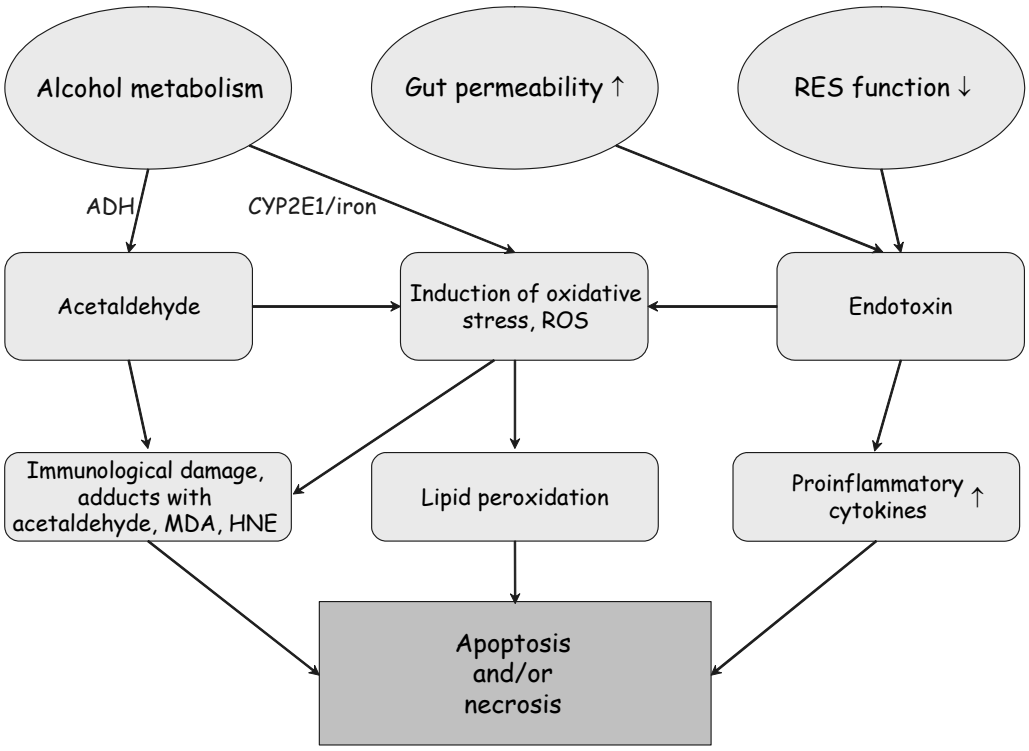

Figure 2. Possible mechanisms of alcohol-related hepatocyte injury. Increased gut permeability, stimulation of oxidative stress and immunological cascades induced by excessive ethanol intake generate interaction triads which result in apoptosis and necrosis of hepatic cells (Day 2000).
ADH, alcohol dehydrogenase; RES, reticuloendothelial system; ROS, reactive oxygen species.

However, not all individuals with problem drinking and alcohol dependence develop ALD. The wide range of individual vulnerability to ALD could in part be explained by the genetic polymorphism of various metabolic and enzymatic pathways that modulate ethanol metabolism (Vidali et al. 2003, Sass and Shaikh 2006). Such metabolic pathways generate oxygen species that are potent inducers of lipid peroxidation, which in turn causes hepatocyte death by apoptosis or necrosis (Sass and Shaikh 2006). It is now also recognized that in addition to the direct hepatotoxic effects of ethanol and genetically based differences in alcohol-metabolizing enzymes, there are other genetic and lifestyle factors that may predispose certain subjects to the development of liver diseases. Female gender has been noted as a risk factor for development of liver disease in alcoholic patients, even when alcohol consumption is lower than that of men (Becker et al. 1996, Schenker 1997, Day 2000, Brunt 2002). The course of liver disease is also more aggressive in women and carries a poorer prognosis (Pares et al. 1986, Schenker 1997, Younossi 1998). Additional factors include obesity, genetically determined polymorphisms for cytokines and their receptors, immune mechanisms, patterns

of alcohol consumption, coexistent viral infection and the use of drugs (Sherlock 1995, Day 2000, Maddrey 2000, Järveläinen et al. 2001, Brunt 2002, Diehl 2004). Cigarette smoking has been shown to increase the rate of progression of fibrosis in ALD (Klatsky and Armstrong 1992, Corrao et al. 1994), whereas coffee consumption has been shown to have a protective effect against cirrhosis (Klatsky and Armstrong 1992, Corrao et al. 1994, Tverdal and Skurtveit 2003).

2.5.1. Fatty liver

Alcoholic fatty liver (steatosis) is the earliest and most common histological consequence of alcohol consumption. It may appear even after a few days of excessive alcohol consumption and is usually reversible (Lieber 1995, Niemelä 2002). Alcoholic fatty liver is characterized by the accumulation of neutral lipids in the hepatocytes, leading to microvesicular and macrovesicular steatosis and balloon cell degeneration (Diehl 1998, Lefkowitch 2005, Tsukamoto 2005). This accumulation of lipids is believed to be caused by intracellular changes in the redox stage of the hepatocytes. Excessive reducing equivalents generated by the oxidation of ethanol favor a net accumulation of triglycerides in the hepatocytes (Diehl 1997, Stewart et al. 2001). In addition, ethanol alters the activity of the enzymes that regulate the biosynthesis and intermediary metabolism of cholesterol, fatty acids and triglycerides (Lieber 1995, Diehl 1998).

While patients with alcohol-induced steatosis often have hepatomegaly, they usually lack any clinical symptoms of liver disease (Diehl 1998, Niemelä 2002, Mann et al. 2003). Simple steatosis is rarely fatal, and with complete abstinence the histological changes can return to normal within 2 weeks (Diehl 1997, Younossi 1998). However, if heavy ethanol consumption continues, steatosis may lead to more progressive forms of liver disease, such as alcoholic hepatitis or cirrhosis. The rare steatosis-related deaths that have been reported seem to be associated more with extensive microvesicular rather than macrovesicular steatosis (Diehl 1998).

2.5.2. Hepatitis

Alcoholic hepatitis, typically presenting in the form of steatohepatitis, is a clinically severe condition characterized by steatosis, necrosis of hepatocytes, intracytoplasmic Mallory bodies, a neutrophilic inflammatory response and perivenular fibrosis (Niemelä 2002, Mann et al. 2003, Lefkowitch 2005, Sass and Shaikh 2006). The steatosis is mainly macrovesicular and is most prominent in the centrilobular regions (Lefkowitch 2005, Sass and Shaikh 2006). Patients with alcoholic hepatitis may be asymptomatic, have only hepatomegaly and perhaps some abdominal pain, or have a full-blown picture with tender hepatomegaly, jaundice, fever, malaise, anorexia, nausea and vomiting, liver failure and bleeding (Niemelä 2002, Mann et al. 2003, Sass and Shaikh 2006).

Alcoholic hepatitis is considered to be a precirrhotic condition, since continued heavy drinking usually leads to cirrhosis in 40–80 % of such patients (Diehl 1997, Mann et al. 2003, Leevy and Elbeshbeshy 2005). Mortality among patients hospitalized for alcoholic hepatitis varies widely, the short-term mortality (< 3 months) ranging from 15 % to 55 % for those who have mild and severe alcoholic hepatitis, respectively (Younossi 1998, Mann et al. 2003, Sass and Shaikh 2006). As with other forms of ALD, the most important factor for both short and long-term survival is abstinence from alcohol. However, in some cases alcoholic hepatitis will progress to cirrhosis despite complete abstinence from alcohol, indicating that immunological mechanisms may play a role in the progression of the disease (Pares et al. 1986, Viitala et al. 1997, Younossi 1998, Leevy and Elbeshbeshy 2005). Patients with alcoholic hepatitis are also often malnourished because of factors such as poor diet, anorexia and encephalopathy. Decreased intestinal absorption and hepatic storage of nutrients may also contribute to the condition. It is thus vital to maintain a nutrient balance and meet the patient's energy requirements adequately through nutritional support (Mendenhall et al. 1993, Stickel et al. 2003a, Sass and Shaikh 2006).

2.5.3. Fibrosis and cirrhosis

Excessive deposition of collagen leading to cirrhosis is a major complication of alcohol abuse. Fibrosis with a pericellular distribution can be seen as an early feature of ALD. Continuing inflammation usually leads to progressive fibrosis, and in some patients eventually to cirrhosis, which is the most serious form of ALD and a cause of many deaths and serious illnesses. Patients with cirrhosis present distinct systemic effects of altered metabolism, changes in hormone levels, protein abnormalities and defective coagulation (Niemelä 2002, Mann et al. 2003). About 10–20 % of alcoholics develop cirrhosis (Diehl 1997, Younossi 1998, Mann et al. 2003, Phillips et al. 2003), but many survive it. The 5-year survival rate is about 90 % for those who stop drinking but less than 70 % for those who do not. The corresponding figures for late-stage cirrhosis, however, are only about 60 % and 35 % (Diehl 1997, Mann et al. 2003). The most common causes of death from cirrhosis are hepatic failure, gastrointestinal bleeding, infection, hepatoma and renal failure (Younossi 1998).

Various studies have shown that the development of cirrhosis is closely associated with the amount of alcohol consumed and the duration of consumption (Bellentani et al. 1997, Mann et al. 2003). There are also important gender differences in the associated mortality risk and rates (Younossi 1998, Mann et al. 2003). Cirrhosis mortality rates are about twice as high in men as in women. This is due to the fact that men typically drink more than women. However, as alcohol consumption increases, the risk of cirrhosis seems to be increasing more rapidly for women (Mann et al. 2003). Mortality from cirrhosis may also vary depending on the type of alcoholic beverage consumed, and it has been proposed that it may be

more closely associated with spirits than with other alcoholic beverages (Gruenewald and Ponicki 1995, Roizen et al. 1999, Kerr et al. 2000).

2.6. Non-invasive biomarkers of fibrosis

A liver biopsy is considered to be the gold standard for assessing hepatic fibrosis, but it is a costly, invasive method with possible complications. Therefore extensive research efforts over the past decades have been focused on the development of non-invasive collagen-type specific applications for monitoring changes in human connective tissue metabolism, and thus possibly avoid the need for invasive biopsy examination.

2.6.1. Type I collagen-derived peptides

The methods that address the metabolism of type I collagens are assays for its carboxyterminal (PICP) and aminoterminal (PINP) propeptides and cross-linked carboxyterminal (ICTP and β-CTx) telopeptides (Okabe et al. 2001, Niemelä 2002, Garnero et al. 2003). Serum PICP measurements have been developed primarily for monitoring collagen metabolism in bone, since most of the organic matrix in bone consists of type I collagen (Melkko et al. 1990, Risteli et al. 1991). Although serum PICP levels also seem to be increased in ALD patients, the changes are usually rather small, and thus it is not a very valuable marker for assessing fibrogenesis in ALD (Niemelä et al. 1992). Serum PINP levels are also elevated in ALD patients (Schytte et al. 1999). It seems to be a more sensitive marker of type I collagen metabolism than PICP, since type I collagen is frequently deposited in the tissue as pC-collagen (collagen retaining its carboxyterminal end) (Niemelä 2002). The assays the type I collagen degradation fragments ICTP and β-CTx were originally developed to monitor collagen metabolism in bone. They originate from mature type I collagen fibers and reflect primarily collagen degradation (Okabe et al. 2001, Garnero et al. 2003, Reichel et al. 2004). However, it appears that both ICTP and β-CTx also show significant changes in the presence of liver diseases (Ricard-Blum et al. 1996, Guañabens et al. 1998). ICTP values have been shown to be elevated in ALD patients, and it has been suggested that this may be related to the clinical severity of the disease (Møller et al. 1999).

2.6.2. The aminoterminal propeptide of type III procollagen

The aminoterminal propeptide of type III procollagen (PIIINP) is the cleavage product obtained during the conversion of procollagen III into collagen III and it has been a widely used marker of fibrogenesis in a variety of hepatic diseases (Niemelä et al. 1983, Torres-Salinas et al. 1986, McCullough et al. 1987, Kamal et al. 2006). It has been shown that serum PIIINP levels are elevated in patients

with alcoholic liver disease (ALD) and may help to identify patients with increased or progressive collagen synthesis (Torres-Salinas et al. 1986, Niemelä et al. 1990, Rosman and Lieber 1994, Pemberton et al. 2005). Changes in serum PIIINP may also serve as indicators of the prognosis in patient follow-up (Niemelä et al. 1990, Trinchet et al. 1992, Teare et al. 1993, Nøjgaard et al. 2003).

2.6.3. Type IV collagen and laminin

Type IV collagen and laminin are major components of basement membranes. Type IV collagen has been suggested to undergo changes early in the pathogenesis of liver disease (Niemelä 2001). The aminoterminal 7S domain of type IV collagen has been shown to be elevated in patients with ALD and to correlate with the clinical severity of liver disease (Niemelä et al. 1990, Tsutsumi et al. 1996). Serum levels of the triple-helix (TH) domain of type IV collagen have been shown to increase in parallel with the progression of hepatic fibrosis. It has also been suggested as a marker for distinguishing ALD from non-alcoholic liver disease (Tsutsumi et al. 1996). Serum TH domain levels also show a good correlation with the hepatic type IV collagen content (Tsutsumi et al. 1996). Laminin, a glycoprotein synthesized by Ito cells, has been considered as a marker of fibrosis. Increased serum laminin concentration is associated with hepatic fibrosis (Kropf et al. 1988, Tsutsumi et al. 1996) and seems to increase in parallel with progression of hepatic fibrosis (Tsutsumi et al. 1996). It also shows an association with hepatic inflammation and necrosis (Niemelä et al. 1990).

2.6.4. Hyaluronic acid

Serum concentrations of hyaluronic acid (HA), a mucopolysaccharide primarily synthesized by fibroblasts and hepatic stellate cells (Gressner and Schafer 1989, Phillips et al. 2003) have been shown to increase in various liver diseases, especially ALD (Laurent and Fraser 1992, Phillips et al. 2003). Thus serum HA measurements have been suggested as a non-invasive method for assessing liver fibrosis in alcoholics (Phillips et al. 2003) and has been shown to be a good predictor of the presence of even moderate liver fibrosis in ALD patients (Stickel et al. 2001, 2003b). In addition, it has been suggested that HA concentrations may also be associated with active drinking by ALD patients and not only with hepatic fibrosis (Tsutsumi et al. 1997, Urashima et al. 1999).

2.6.5. Tissue inhibitors of metalloproteinases

One of the factors contributing to the development of alcoholic cirrhosis may be the decrease in the activity of collagenase, which is responsible for collagen degradation. The tissue inhibitors of metalloproteinases (TIMPs) secreted by hepatic stellate cells are capable of reducing the activity of metalloproteinases and thus

decreasing the degradation of the extracellular matrix (Iredale et al. 1992, Siegmund and Brenner 2005). It has been shown that serum TIMPs are elevated in alcoholics in precirrhotic and cirrhotic states and that the concentrations increase in parallel with the severity of ALD (Li et al. 1994, Campbell et al. 2001). Serum levels of TIMPs may therefore also serve as a useful marker of liver fibrosis in alcoholics. TIMP levels also correlate with fibrosis scores in liver biopsies (Murawaki et al. 1997).

2.6.6. Human cartilage glycoprotein 39

Human cartilage glycoprotein 39 (YKL-40), a member of the glycosyl hydrolase family 18 (Hakala et al. 1993, De Ceuninck et al. 2001), is a growth factor for connective tissue cells and endothelial cells (Recklies et al. 2002). YKL-40 is secreted by articular chondrocytes and synovial cells (Hakala et al. 1993, De Ceuninck et al. 2001, Fusetti et al. 2003), and is also expressed in the monocyte-derived macrophages of the peripheral blood in association with their differentiation from monocytes to macrophages (Kirkpatrick et al. 1997, Fusetti et al. 2003). Serum YKL-40 levels have been shown to be elevated in patients with various degrees of ALD (Johansen et al. 2000, Nøjgaard et al. 2003), and high levels of YKL-40 may also be closely associated with short-term survival in ALD patients (Nøjgaard et al. 2003).

2.7. Immune and cytokine responses in alcoholics

Chronic ethanol consumption can lead to various metabolic alterations which may affect immune reactivity, immune cell growth and the toxicity of other chemicals (Cook 1998). Although recent studies on ethanol and immunity have focused mostly on the immune mechanisms involved in ALD, these mechanisms may also contribute to alcohol-related cardiac, lung, endocrine and neurological damage (Wilke et al. 1996, Ware and Matthay 2000, Schoppet and Maisch 2001, Chase et al. 2005, Crews et al. 2006). Ethanol is known to disturb the functioning of certain cells in the immune system such as B and T cells, macrophages, monocytes and dendritic cells, which may lead to increased vulnerability to infections (Cook et al. 1996, Cook 1998, Szabo 1999, Neuman 2003, Szabo et al. 2004, Chase et al. 2005). It has further been suggested that the imbalanced production of Th1 and Th2 cytokines may contribute to alcoholic pathologies (Laso et al. 1999, Crews et al. 2006).

Chronic alcoholics often have greatly increased serum immunoglobulin (Ig) levels (van de Wiel et al. 1988, Israel et al. 1988, Koskinas et al. 1992, Viitala et al. 1997, Cook 1998, Szabo 1999), and although these are normally associated with the development of specific immunity, the affected alcoholic patients are often immunodeficient (Crews et al. 2006). The consequences of this immunode-

ficiency include increased vulnerability to pneumonia, tuberculosis and other infectious diseases (Cook 1998).

2.7.1. Immune responses to ethanol metabolites

As described earlier, acetaldehyde is a highly reactive metabolite of ethanol which can form stable condensates with various proteins and cellular constituents. Acetaldehyde-protein adducts may also act as neoantigens which stimulate immune responses (Israel et al. 1986, Niemelä et al. 1987, Lieber 1995, Lieber 2004). Thus the development of autoimmunity as a result of chronic alcohol consumption may be largely or in part due to the protein alterations caused by these condensates (Cook 1998). Previous studies have demonstrated the production of class IgA, G and M antibodies against acetaldehyde adducts after chronic ethanol consumption (Niemelä et al. 1987, Hoerner et al. 1988, Koskinas et al. 1992, Worrall et al. 1996, Viitala et al. 1997, 2000). Of these, class IgA appears to be most specifically linked to ALD. The antibody response generated appears to be specific to acetaldehyde-modified epitopes and does not depend on the carrier protein (Niemelä et al. 1987, Israel et al. 1988). Other reactive metabolites produced as a result of ethanol intake, such as malondialdehyde and 4-hydroxynonenal, are also capable of generating protein adducts and associated immune responses (Niemelä 1999, Stewart et al. 2004).

Acetaldehyde adducts in the liver have been intensively studied and their association with alcohol-induced liver damage has been well established (Holstege et al. 1994, Klassen et al. 1995, Paradis et al. 1996, Li et al. 1997a, Niemelä 1999, Niemelä 2001). Adducts have also been found in the muscles, brain, gastrointestinal tract, pancreas and erythrocyte cell membranes after heavy ethanol intake (Salmela et al. 1997, Biewald et al. 1998, Rintala et al. 2000, Worrall et al. 2000, Niemelä 2001, Worrall et al. 2001, Niemelä and Parkkila 2004). The pathogenic significance of such immune responses is, however, currently unclear. It has been suggested that antibody responses against acetaldehyde adducts may contribute to the pathogenesis of alcoholic liver disease (Niemelä et al. 1987, Holstege et al. 1994), but on the other hand, antibodies may serve as neutralizing factors by binding acetaldehyde adducts and removing them from the circulation (Israel et al. 1988, Latvala et al. 2005).

2.7.2. The liver as an immunological organ

The liver has a number of important functions in innate and adaptive immunity. About 30 % of the total blood passes through the liver in every minute (Racanelli and Rehermann 2006). Oxygen-rich blood is supplied via the hepatic arteries while protein and antigen-rich blood enters the hepatic parenchyma mainly via terminal portal vessels, then passes through a network of liver sinusoids and leaves the parenchyma via the central hepatic veins. In addition to parenchymal

hepatocytes, the liver contains various non-parenchymal cells (Sheth and Bankey 2001). Hepatocytes constitute about two thirds of the total cell population in the liver and are capable of producing acute-phase proteins that provide non-specific help in pathogen clearance and immune defence (Weiler-Normann and Rehermann 2004). Non-parenchymal cells, such as Kupffer cells, sinusoidal endothelial cells, stellate cells, dendritic cells and liver-associated lymphocytes play a role in immunological surveillance within the hepatic sinusoids.

Kupffer cells represent the largest group of fixed macrophages in the body, and account for about 20 % of the non-parenchymal cells in the liver. They are located in the hepatic sinusoids, predominantly in the periportal area, which allows them to phagocytose and eliminate pathogens entering the liver (Racanelli and Rehermann 2006). They are also capable of temporarily impeding blood flow and thereby extending the duration of cell-cell contact. Another critical role of Kupffer cells in immune function is the production of an enormous spectrum of immunomodulatory cytokines, eicosanoids, and reactive oxygen and nitrogen species (Sheth and Bankey 2001).

Lymphocytes constitute about 25 % of non-parenchymal cells in the liver. Their population in the liver is selectively enriched in natural killer and natural killer T cells, which play critical roles in first-line immune defence against invading pathogens, modulation of liver injury and recruitment of circulating lymphocytes (Racanelli and Rehermann 2006). They are predominantly located around the portal tracts, but can also be found throughout the parenchyma.

Liver sinusoidal endothelial cells account about 50 % of the non-parenchymal hepatic cells. They form a monolayer that separates hepatocytes from the passing blood (Weiler-Normann and Rehermann 2004). Sinusoidal endothelial cells express molecules that promote antigen uptake by endocytosis and/or phagocytosis, and molecules necessary for antigen presentation, such as MHC class I and II, CD80 and CD86 (Sheth and Bankey 2001, Racanelli and Rehermann 2006).

Stellate cells are located between the sinusoidal endothelial cell monolayer and the hepatocytes, in a small space termed the Space of Dissé (Weiler-Normann and Rehermann 2004). In the normal liver they store vitamin A, control the turnover of extracellular matrix, and regulate the contractility of sinusoids. Acute hepatocyte damage activates the transformation of quiescent stellate cells to myofibroblast-like cells, which play a key role in the development of inflammatory fibrotic response (Kmiec 2001).

Dendritic cells are derived from bone marrow and are the most specialized antigen-presenting cells in the liver. In the healthy liver they are located around the central veins and portal tract and are predominantly immature cells. Once activated, they increase their capacity to migrate via the Space of Dissé to the lymph nodes and to present the processed antigens to lymphocytes (Weiler-Normann and Rehermann 2004, Racanelli and Rehermann 2006).

2.7.3. Immunology of alcoholic liver disease

Most researchers currently agree that activation of the innate immune system is a key event in the initiation and progression of ALD beyond the fatty liver stage (Thurman 1998, McClain et al. 2002, Nagy 2003, Hines and Wheeler 2004, Bode and Bode 2005). Liver function tests and morphological examinations have revealed that alcoholic hepatitis is characterized by extensive hepatocyte death and infiltration by immune system cells, sometimes in large numbers (Cook 1998, Stewart et al. 2001). There are also clinically observable features which suggest that the immune system causes at least part of the liver damage in patients with alcoholic hepatitis. First, alcoholic hepatitis often continues to worsen for one or more weeks after the withdrawal of alcohol, suggesting that the damage is not caused only by the presence of alcohol (Klassen et al. 1995, Leevy and Elbeshbeshy 2005). Secondly, patients who recover from alcoholic hepatitis and resume drinking typically suffer new episodes of a greater severity precipitated by lesser amounts of alcohol than before (Klassen et al. 1995). This indicates the possible involvement of an autoimmune process in which immunity to some components of the patient's own liver has developed and is increased by resuming drinking (Cook 1998). The involvement of the immune system in alcoholic cirrhosis is currently under study, but there are some results that indicate that fibrogenesis and cirrhosis in alcoholic patients are associated with marked alterations of the immune system (Laso et al. 1996, Crews et al. 2006). Antibodies against the acetaldehyde-derived adducts may also contribute to the liver cell damage and subsequent progression of fibrosis (Niemelä et al. 1987, Israel et al. 1988, Koskinas et al. 1992, Niemelä 1999).

Lymphocytes have an important role in regulating the immune/inflammatory response to alcohol (Leevy and Elbeshbeshy 2005). Despite the fact that B cell lymphocytes in ALD patients produce abnormally large amounts of immunoglobulins, their number is often significantly reduced (Cook et al. 1996, Cook 1998). It has also been shown that some ALD patients, especially those with cirrhosis, have greatly reduced number and activity of natural killer (NK) cells (Charpentier et al. 1984, Ledesma et al. 1990, Cook et al. 1997). Normal human NK cells are found to be slightly stimulated by overnight exposure to alcohol if their activity is measured after alcohol removal (Li et al. 1997b). This indicates that NK cell loss in ALD patients may be an indirect consequence of other immune alterations resulting from chronic ethanol exposure rather than a direct effect of ethanol (Li et al. 1997b, Cook 1998).

Neutrophils are white blood cells that form one of the first lines of defence against infectious agents or exogenous substances that penetrate the body's physical barriers. Alcoholic hepatitis often involves an increase in the numbers of these cells in the peripheral blood, and microscopic examination of the damaged liver shows infiltration by them (Bautista 2002). As these cells typically release powerful enzymes that damage tissue, the abnormal attraction of neutrophils into the liver is one possible mechanism that may contribute to the liver damage that oc-

curs in ALD (Cook 1998, Bautista 2002). Monocytes and macrophages are white blood cells that circulate normally in the peripheral blood but also have fixed tissue counterparts residing in many tissues (Beutler 2004). They are capable of engulfing and killing microbes and play an important role in the production of cytokines (McClain et al. 1997, Cook 1998, Beutler 2004), which modulate immune responses and participate in cell growth and differentiation. Defective phagocytic function has been reported in the monocytes of patients with alcoholic cirrhosis (Silvain et al. 1995).

Cytokines are multifunctional proteins that play a critical role in cellular communication and activation. They are classified as pro-inflammatory (Th1) or anti-inflammatory (Th2), depending on their effects on the immune system. Pro-inflammatory cytokines, like reactive oxygen species (ROS), can act locally to recruit neutrophils and other immune cells to the site of activation, or they can mediate a systematic immune response (McClain et al. 1997, Bode and Bode 2005). The liver tissue produces only small amounts of cytokines under normal circumstances, but when the liver suffers damage the production of cytokines increases and mediates the regeneration of the liver tissue (Neuman 2003). In alcoholics, increased intestinal permeability may lead to increased blood levels of bacterial lipopolysaccharide (LPS), a well-known, powerful activator of many immune system cells (Cook 1998, Nagy 2003). The resulting endotoxemia activates monocyte/macrophage-type cells, especially the liver Kupffer cells, to produce pro-inflammatory cytokines, which promote inflammatory responses in the liver (Wheeler 2003, Bode and Bode 2005, Leevy and Elbeshbeshy 2005). While under typical conditions cytokine levels return to normal once the inflammation is under control (Neuman 2003), it has been suggested that the cytokine balance in alcoholics may be shifted towards persistent inflammation (Nagy 2003, Crews et al. 2006). The continuous inflammation in the liver may eventually lead to the formation of scar tissue (Neuman 2003). The Kupffer cells are also the major source of ROS in the liver, and excessive levels of ROS within the cell together with a lack of antioxidants can lead to oxidative stress, which is detrimental to the cell (Wheeler 2003).

An important intermediary in the pathogenesis of ALD appears to be the excess release of pro-inflammatory cytokines, especially TNF-α (Bird et al. 1990, Iimuro et al. 1997, McClain et al. 1998, McClain et al. 1999, Neuman 2003). As TNF-α causes cells to undergo apoptosis, it appears that excessive secretion of this cytokine by Kupffer cells contributes to the death of liver cells. Consistent with this concept, patients admitted with acute alcoholic hepatitis have a poorer outcome if they have markedly elevated serum concentrations of TNF-α and cytokines induced by TNF-α, including interleukins 6 (IL-6) and 8 (IL-8) (Felver et al. 1990, Bird 1994, Huang et al. 1996, Fujimoto et al. 2000). Increased IL-8 levels are also of interest in the case of alcoholics because IL-8, which can stimulate an increase in neutrophil numbers, metabolism and chemotaxis (Joshi-Barve et al. 2003), is elevated in patients with alcoholic hepatitis, and its elevation may be one mechanism for the increased infiltration of the liver by neutrophils (Hill et al.

1993, Bird 1994, Huang et al. 1996, Fujimoto et al. 2000). The important role of cytokines indicates that anticytokine agents may be useful for interrupting the pathogenesis of alcohol-induced liver injury (McClain et al. 1993, Iimuro et al. 1997, McClain et al. 1999, Bergheim et al. 2005).

3. Aims of the present research

Although a variety of biochemical methods are available for assessing excessive ethanol consumption and ethanol-induced diseases, they are currently inefficient, and more accurate methods are needed. In order to develop new approaches for the assessment of alcoholic patients, more information is also needed on the sequence of events by which excessive ethanol consumption leads to tissue injury, such as liver fibrosis.

The aims of the present work were as follows:

1. To develop methods for assessing immune responses against protein adducts with acetaldehyde and to examine the association between ethanol consumption, biomarkers of alcohol abuse and such immune responses.
2. To investigate the relationships between immune responses to acetaldehyde adducts, cytokine profiles, fibrogenesis and tissue transglutaminase in individuals with different levels of alcohol consumption and associated liver damage.

4. Materials and methods

4.1. Patients and control subjects

Paper I: This study included 40 male heavy drinkers with an alcohol consumption of 40–540 g/day during the 4 weeks prior to sampling. All heavy drinkers had been admitted for detoxification and nineteen of them volunteered for a follow-up, which was carried out with supervised abstinence and repeated sampling during hospitalization over a period of 8 ± 3 days. Abstinence was controlled by repeated analyses of ethanol in their breath. The healthy non-alcoholic controls comprised 41 male volunteers, who were either abstainers (n = 16) or moderate whose daily ethanol consumption did not exceed 40 g (n = 25). None of the controls had any previous history of alcohol abuse.

Paper II: A total of 105 heavy drinkers (92 men, 13 women) who had been admitted for detoxification were examined. Their mean alcohol consumption during the 4 weeks prior to sampling had been 147 g/day and they all met the DSM-IV criteria for alcohol dependence, including pathological alcohol use, social impairment and the presence of tolerance and withdrawal symptoms. The healthy controls were 86 volunteers (54 men, 32 women) who were either abstainers (n = 24) or moderate drinkers (n = 62) whose mean daily ethanol consumption was < 40 g/day. For comparing reference intervals, data from the 1999–2003 survey on 845 apparently healthy individuals representing either moderate drinkers or abstainers from the NORIP (Nordic Reference Interval Project) for common hematological and clinical chemical parameters were used. Persons who had clinical or laboratory evidence of current or recent illnesses or infections, were pregnant, had donated blood during the past five months or had used any prescription drugs during the preceding week were excluded. Smoking had not been allowed within the hour prior to sampling.

Paper III: There were 164 subjects, who represented a wide range of alcohol consumption, from abstainers to heavy drinkers with a mean ethanol consumption of 130 g/day. The 89 heavy drinkers were all patients who had been admitted for detoxification and included 44 patients with alcoholic liver disease, the severity of which was assessed using a previously established combined clinical and laboratory index (CCLI) and a combined morphological index (CMI) (Orrego et al. 1983, 1987, Niemelä et al. 1990). The liver histology ranged from mild fibrosis and fatty change to cirrhosis with a wide distribution of morphological abnormalities related to alcoholic hepatitis. The remaining 75 subjects formed a reference population of healthy volunteers who were either abstainers (n = 35) or moderate drinkers (n = 40) with a mean daily ethanol consumption that did not exceed 40 g.

Paper IV: This study was conducted with 84 male alcoholic patients, including 52 patients who showed clinical, laboratory and morphological evidence of alcoholic liver disease, the severity of which was assessed using the CCLI and CMI indices. In addition, there were 32 patients who were heavy drinkers with mean alcohol consumption of 130 g/day during the 4 weeks prior to sampling and had been admitted for detoxification but did not have any clinical or laboratory signs of significant liver disease. Due to ethical considerations, these patients were, however, not biopsied. The healthy non-alcoholic controls were 20 male volunteers who were either abstainers (n = 5) or moderate drinkers with a mean daily ethanol consumption between 1 and 36 g in the past month (n = 15). None of these individuals had any history of alcohol abuse.

All the alcoholic patients had a well-documented history of continuous ethanol consumption or binge drinking. The extent of alcohol consumption in these patients was assessed by means of detailed personal interviews using a time-line follow-back technique. The mean duration of abstinence prior to sampling was 2 ± 2 days. The blood sampling was performed by trained laboratory personnel. The samples were taken between 8 a.m. and noon, and liver enzymes and blood cell counts were analyzed on the same day. The serum samples separated by centrifugation were stored at –70 °C for approximately 1–5 years until used for additional analyses. Such conditions of storage have no effects on the measurement of the parameters studied in this work (Risteli and Risteli 1987, Kenis et al. 2002, Qvist et al. 2004). All the participants gave their informed consent and the research was approved by Seinäjoki Central Hospital ethics committee and carried out according to the provisions of the Declaration of Helsinki.

4.2. Assays for antibodies against acetaldehyde adducts

Human erythrocyte protein was prepared from EDTA blood from an abstainer. The cells were separated by centrifugation and washed three times with PBS (7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), lysed with polyoxyethylene ether, 0.1 % V/V in borate buffer (Hemolysis Reagent, DIAMAT^{TM} Analyzer System, Bio-Rad), and incubated for 35 minutes at 37 °C to remove unstable Schiff bases. The hemolysate was diluted with PBS to a hemoglobin protein concentration of 12 mg/ml and stored frozen at –70 °C in aliquots prior to use. The hemolysate with a hemoglobin concentration of 12 mg/ml was mixed with acetaldehyde (in PBS) to an acetaldehyde concentration of 10 mM. After incubation in tightly sealed containers overnight (18 hours, 4 ºC), the protein adducts were reduced by the addition of sodium cyanoborohydride (10 mM) and mixing for 5 hours at 4 ºC. The protein solutions were dialyzed twice against PBS (4 ºC) and stored in aliquots for individual use at –70 ºC. Samples representing unmodified protein were prepared and treated similarly to those of the modified protein except for the addition of acetaldehyde.

For measurement of the antibody titers the microtiter plates (Nunc-Immuno Plate, Maxisorb™, InterMed, Denmark) were coated with acetaldehyde-modified hemoglobin or corresponding unmodified proteins in PBS (3 µg protein in 100 µl/well) and incubated for 1½ hour at 37 °C. Non-specific binding was blocked by incubation with 0.2 % gelatin in PBS (150 µl/well) for 1 hour at 37 °C. The sample sera were diluted (1:40) in PBS which contained 0.04 % Tween-20. The serum dilutions were allowed to react with the coated proteins for 1 hour at 37 °C followed by extensive washing with PBS-Tween. Antibody-antigen complexes were labeled using alkaline phosphatase-linked goat anti-human immunoglobulin IgA, IgG or IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove) diluted in PBS-Tween which contained 8 mM $MgCl_2$ and a small amount of dithiothreitol (DTT). The plates were incubated overnight at 4 °C and then washed, after which 100 µl of p-nitrophenylphosphate solution was added as a color reaction substrate (Alkaline Phosphatase Substrate Kit, Bio-Rad Laboratories, Hercules, CA). The color reaction was stopped by adding 100 µl NaOH (0.4 M) when one of the samples reached 0.8 OD units. The optical densities were read at 405 nm with an Anthos HTII microplate reader (Anthos Labtec Instruments, Salzburg, Austria). The anti-acetaldehyde adduct IgA values ($OD_{405\ nm}$) obtained in a reaction with the sample and the unconjugated protein (background) were subtracted from the corresponding values measured from the reaction between the sample and the acetaldehyde-protein-conjugate. The results were corrected to the same levels by reference to previous measurements using four standard samples. The anti-acetaldehyde adduct IgA results are expressed as units/liter (U/l), corresponding to $OD_{405\ nm} \times 10^3$. Although the intra- and interassay variations for this method have not yet been fully established, preliminary experiments have indicated 5 % intra-assay variation and 8 % interassay variation.

4.3. Biomarkers of alcohol abuse and liver status

Serum carbohydrate-deficient transferrin (CDT) was measured with a turbidimetric immunoassay (TIA) after ion exchange chromatography (Axis-Shield, Oslo, Norway), which separates the transferrin variants with 0–2 sialic acid residues. The measurements were performed using a Behring Nephelometer II (Dade Behring, Behring Diagnostics GmbH, Marburg, Germany) according to the manufacturer's instructions. The blood cell counts were analyzed with Coulter Hematology Analyzer systems. Serum gamma-glutamyl transferase (GGT) was measured using an enzymatic colorimetric assay according to the IFCC (International Federation of Clinical Chemistry). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using pyridoxal-5'-phosphate activated methods according to the IFCC. Albumin and bilirubin were measured using immunoturbidimetric method and a diazo method, respectively. Alkaline phosphatase was measured according to the recommended IFCC reference method. These analyses were carried out with Cobas Integra 800 analyzer (Roche Diagnostics, Basel, Switzerland). All the assays were performed in an accredited laboratory (SFS-EN ISO/IEC 17025) at Seinäjoki Central Hospital, Finland.

4.4. Markers of fibrogenesis and cytokines

Serum concentrations of the aminoterminal propeptide of type III procollagen (PIIINP) were measured by a radioimmunological assay (Orion Diagnostica, Espoo, Finland), serum hyaluronic acid (HA) concentrations were determined using an enzyme-linked binding protein assay (Corgenix, Tejon St. Westminster, USA) and the concentration of serum β-CrossLaps (β-CTx) and the aminoterminal propeptide of type I procollagen (PINP) were determined using electrochemiluminescence immunoassay kits (Roche Diagnostics, Espoo, Finland). Serum concentrations of the carboxyterminal telopeptide of type I collagen (ICTP) were determined using quantitative enzyme immunoassay kits (Orion Diagnostica, Espoo, Finland) and the concentrations of cytokines (IL-1, IL-2, IL-6, IL-8, IL-10, TNF-α and TGF-β) were determined using Quantikine ELISA kits (R&D Systems, Abington Science Park, U.K.) according to the manufacturer's instructions.

4.5. Tissue transglutaminase antibodies

Antibodies against tissue transglutaminase were measured using the Pharmacia Celikey Tissue Transglutaminase IgA Antibody Assay (Pharmacia Diagnostics, Sweden) according to the manufacturer's instructions and the results were expressed in units/liter (U/l). Serum total IgA was measured by a nephelometric method using a BN ProSpec analyzer (Dade Behring, Behring Diagnostics GmbH, Marburg, Germany).

4.6. Statistical methods

The t-test was used for comparisons between two groups following a Gaussian distribution, and the Mann-Whitney test for non-Gaussian populations. Differences between three or more groups were compared using a one-way analysis of variance (ANOVA) when the data followed a Gaussian distribution and passed the homogeneity of variance test. The Kruskal-Wallis test was used if a Gaussian distribution with equal variances was not obtained.

Correlations were calculated using Pearson product-moment correlation coefficients for continuous non-skewed parameters or Spearman's rank correlations for non-continuous or skewed parameters, as required. Incidences were compared with Fisher's exact test. A p-value of less than 0.05 was considered statistically significant.

Cut-offs were either determined with ROC analyses or calculated using the mean + 2SD if the data came from a Gaussian distribution and the non-parametric method if the data came from a skewed distribution (Horn and Pesce 2003).

The statistical analyses were carried out using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) and SPSS for Windows 13.0 (SPSS Inc., Chicago, IL, USA) statistical softwares. The ROC analyses were performed with Analyse-it software (Analyse-it Software Ltd, Leeds, U.K.).

5. Results

5.1. Antibodies against acetaldehyde adducts in alcoholics

Measurements of antibodies against acetaldehyde adducts by the ELISA technique indicated that the titers of IgAs against such adducts in alcoholics were significantly higher than those in the moderate drinkers or abstainers ($p < 0.001$ for both comparisons). The alcoholics with liver disease showed higher values than those without liver disease or the healthy controls ($p < 0.001$ for both comparisons) and the difference between the latter two groups was also significant ($p < 0.05$). In addition, the values for the moderate drinkers were significantly higher than those for the abstainers ($p < 0.05$).

The diagnostic sensitivity and specificity of the anti-acetaldehyde adduct IgA measurements as a marker of alcohol consumption were subsequently compared with those of the traditional markers by contrasting the data obtained for the heavy drinkers with the data obtained for the abstainers and/or moderate drinkers. In the comparisons with the abstainers only, the sensitivity of anti-acetaldehyde adduct IgAs was 73 % with a specificity of 94 %, which exceeded the corresponding values found for CDT, GGT, MCV and AST. When both moderate drinkers and abstainers were included in the reference population, the sensitivity of the anti-acetaldehyde adduct IgA assay was 65 % with a specificity of 88 %, showing essentially similar analytical characteristics to those for CDT, although still remaining higher than those for GGT, MCV or AST.

The anti-acetaldehyde adduct IgAs correlated significantly with all the conventional markers of alcohol abuse and also highly significantly with actual amount of recent ethanol consumption ($r = 0.67$, $p < 0.001$). In the comparisons with the major cytokines, anti-acetaldehyde adduct IgAs were found to correlate significantly with serum IL-6 ($r = 0.33$, $p < 0.05$) and TNF-α ($r = 0.31$, $p < 0.05$) levels, but not with IL-2, IL-8, IL-10 or TGF-β1.

In the follow-up of heavy drinkers, the IgA titers decreased significantly ($p < 0.01$) during a period of supervised abstinence lasting 8 ± 3 days, and after this time the levels were still higher than those for the moderate drinkers or abstainers, the difference with respect to the latter group remaining statistically significant ($p < 0.001$). The rate of anti-acetaldehyde adduct IgA disappearance was estimated to be about 3 % per day, and the mean normalization time to be about four weeks.

5.2. Red blood cell abnormalities and immune responses against acetaldehyde adducts

The occurrence of typical red blood cell abnormalities as a result of excessive ethanol consumption is assessed and compared with the immune responses to ethanol metabolites in paper II. The alcohol abusers were found to present with abnormalities both in blood cell counts and morphology, of which elevated MCV was found to be the most typical finding. Although the highest MCV values occurred in the alcoholic patients, the values also tended to be higher in the moderate drinkers than those in the abstainers. A significant correlation was found to exist between MCV levels and the amount of recent ethanol consumption during the month preceding blood sampling ($r = 0.48$, $p < 0.001$). The follow-up of apparently healthy subjects with careful records of their monthly and yearly ethanol consumption also indicated parallel changes in MCV values and the intensity of alcohol drinking.

The reference intervals (mean ± 2SD) for MCV calculated from the present material of moderate drinkers (82–98) were found to be in accordance with the data obtained from the NORIP (Nordic Reference Interval Project) but different from the normal limits based on the data from abstainers (82–96).

Measurements of IgA, IgG and IgM antibodies against acetaldehyde adducts in the alcoholic patients indicated elevated levels in 94 %, 21 % and 64 % of cases with elevated MCV and in 63 %, 19 % and 56 % of those with normal MCV, respectively (Figure 3). The difference in the incidences was found to be significant for IgAs ($p < 0.05$). Statistically significant correlations also occurred between MCV and anti-acetaldehyde adduct IgA and IgM in the men, whereas these correlations did not reach statistical significance in the women.

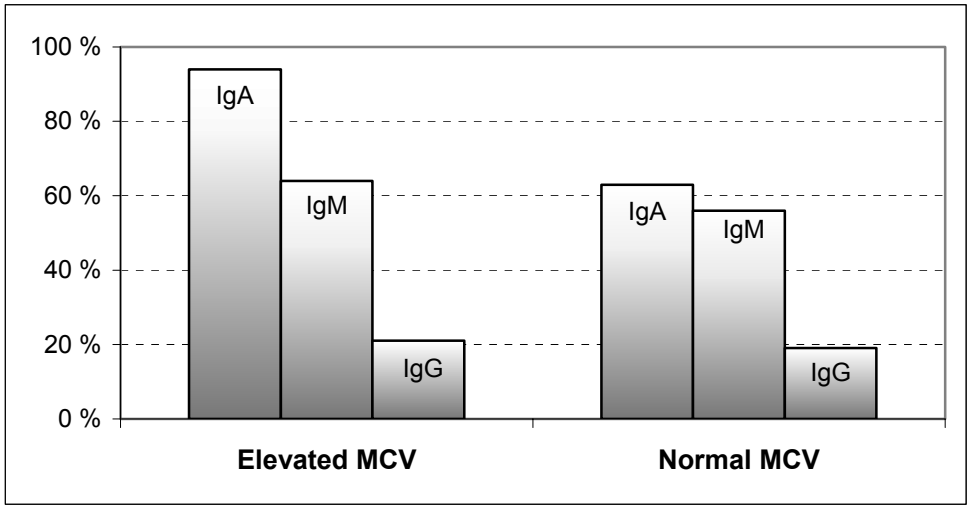

Figure 3. Occurrence of elevated anti-acetaldehyde adduct IgA, IgM and IgG antibodies against acetaldehyde adducts in alcoholic patients with elevated or normal MCV values.

5.3 Comparison of IgA antibodies against acetaldehyde adducts and tissue transglutaminase

In order to gain further insight into the role of IgA responses against acetaldehyde-derived modifications, the titers against acetaldehyde adducts were compared with IgAs against tissue transglutaminase (tTG), which have previously been found to be characteristic of patients with celiac disease. The Anti-tTG IgA titers in the alcoholic patients were also found to be significantly higher than those in the healthy controls ($p < 0.001$). When the alcoholic patients were further divided according to the presence or absence of liver disease, the ALD patients showed significantly higher values than the alcoholics without liver disease ($p < 0.001$). The difference between the alcoholics without liver disease and the healthy controls did not, however, reach the level of significance.

When the population was further divided by gender (Figure 4) anti-tTG IgA levels in both genders were significantly higher in the ALD patients than in the alcoholics without liver disease or the healthy controls ($p < 0.001$ for all comparisons). Anti-acetaldehyde adduct IgA levels in both genders were significantly higher in the ALD patients than in the controls ($p < 0.01$) and also higher than in the alcoholics without liver disease in the case of the men ($p < 0.05$).

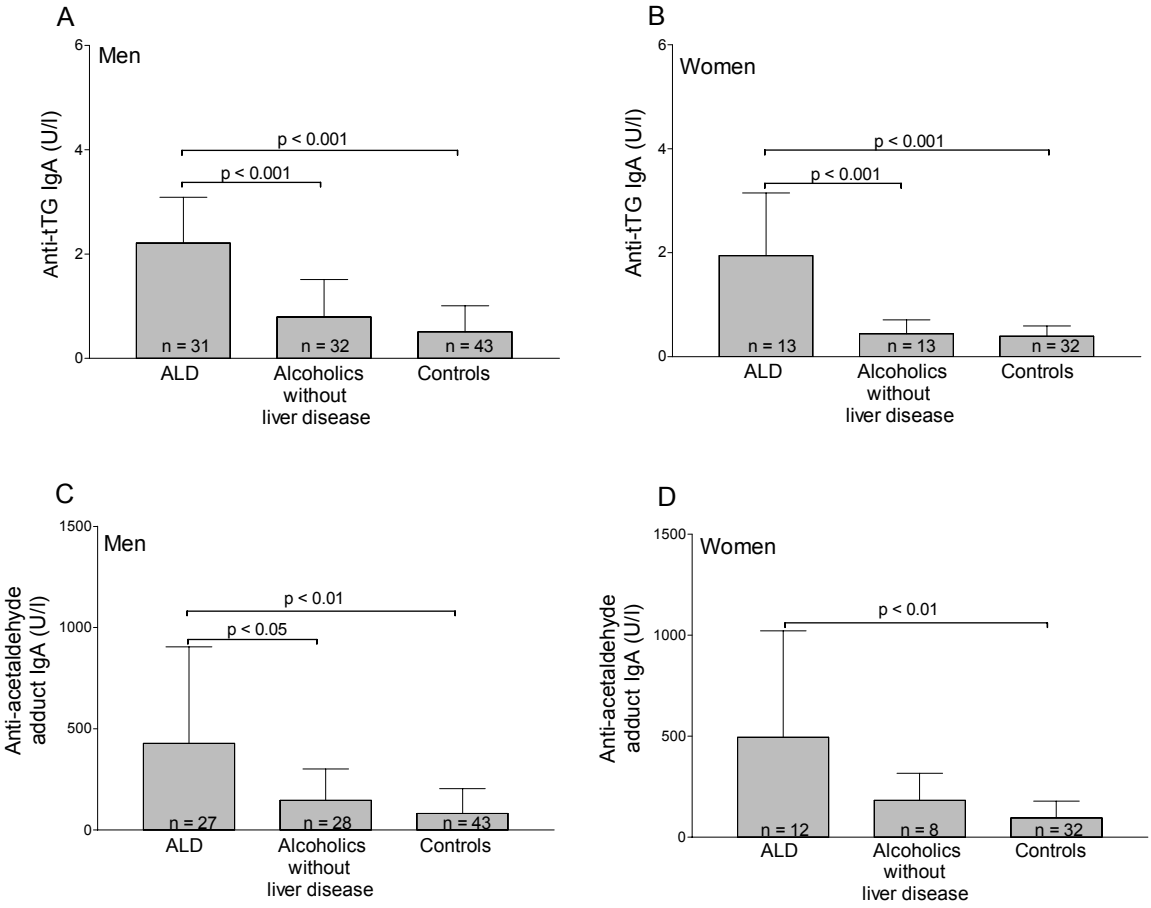

Figure 4. Anti-tTG IgA (A–B) and anti-acetaldehyde adduct IgA (C–D) antibodies in patients with alcoholic liver disease (ALD), heavy drinkers without liver disease and healthy controls. Anti-tTG IgA titers in both genders and anti-acetaldehyde adduct IgA titers in men were significantly higher in the ALD patients than in the heavy drinkers without liver disease or the healthy controls. Anti-acetaldehyde adduct IgA titers in women were significantly higher in ALD patients than in healthy controls. The data are expressed as mean ± SD.

Anti-tTG IgAs correlated significantly with anti-acetaldehyde adduct IgAs ($r = 0.46$, $p < 0.001$) and this correlation was also significant in the subgroups consisting of either moderate drinkers and abstainers ($r = 0.49$, $p < 0.001$) or heavy drinkers only ($r = 0.33$, $p < 0.01$). When cross-tabulated, there was also a significant co-occurrence between the elevations in anti-tTG IgAs and anti-acetaldehyde adduct IgAs ($p < 0.001$).

Significant positive correlations emerged between anti-tTG IgA and the pro-inflammatory cytokines IL-2 ($r = 0.42$, $p < 0.001$), IL-6 ($r = 0.54$, $p < 0.001$), IL-8 ($r = 0.68$, $p < 0.001$) and TNF-α ($r = 0.64$, $p < 0.001$) and negative correlations between anti-tTG IgA and the anti-inflammatory cytokines TGF-β ($r = –0.37$, $p < 0.01$) and IL-1 ($r = –0.43$, $p < 0.001$). Anti-tTG IgA also correlated with the biomarkers reflecting ethanol consumption and liver damage, including GGT (r =

0.48, $p < 0.001$), CDT ($r = 0.41$, $p < 0.001$), MCV ($r = 0.21$, $p < 0.05$), AST ($r = 0.37$, $p < 0.001$) and ALT ($r = 0.24$, $p < 0.05$). Interestingly, the serum aminoterminal propeptide of type III procollagen (PIIINP; $r = 0.69$, $p < 0.001$) and hyaluronic acid (HA; $r = 0.63$, $p < 0.001$), markers of fibrogenesis, were found to correlate highly significantly with anti-tTG IgA. On the other hand, an inverse relationship was observed between anti-tTG IgA and a marker of collagen degradation derived from the carboxyterminal telopeptide of type I collagen (β-CrossLaps; $r = 0.52$, $p < 0.001$).

5.4. Cytokines and markers of fibrogenesis in alcoholics

Alcoholic liver disease was found to be significantly associated with both the collagen and cytokine markers. Serum PIIINP and PINP levels were both significantly higher in the ALD patients than in the alcoholics without liver disease or the healthy non-alcoholic controls. In contrast, mean serum β-CrossLaps (β-CTx) concentrations in the corresponding comparisons were significantly lower among the ALD patients ($p < 0.001$ for both comparisons). β-CTx values were found to be abnormally low in 79 % of the ALD patients, but only in 13 % of the alcoholics without liver disease and in none of the healthy controls. Serum PIIINP values were in turn increased in 85 % of the ALD patients and in 6 % of the alcoholics without liver disease. Calculations of the PIIINP/β-CTx and PINP/β-CTx ratios were found to further potentiate the differences between the ALD patients and the other study groups. ROC analyses of the PIIINP/β-CTx ratio yielded an excellent sensitivity (94 %) and specificity (98 %) for differentiating the alcoholics with liver disease. The sensitivity for this ratio was also higher than the corresponding sensitivity for the PINP/β-CTx ratio (87 % for a specificity of 98 %).

Serum β-CTx correlated negatively with PIIINP ($r = –0.42$, $p < 0.001$) and the pro-inflammatory cytokines IL-2 ($r = –0.26$, $p < 0.01$), IL-6 ($r = –0.22$, $p < 0.05$), IL-8 ($r = –0.23$, $p < 0.05$) and TNF-α ($r = –0.48$, $p < 0.001$), whereas positive correlations emerged between β-CTx and the anti-inflammatory cytokines IL-1 ($r = 0.35$, $p < 0.001$) and TGF-β ($r = 0.39$, $p < 0.001$). Serum PIIINP showed statistically significant positive correlations with PINP ($r = 0.63$, $p < 0.001$) and the pro-inflammatory cytokines IL-2 ($r = 0.33$, $p < 0.001$), IL-6 ($r = 0.44$, $p < 0.001$), IL-8 ($r = 0.55$, $p < 0.001$) and TNF-α ($r = 0.67$, $p < 0.001$), and negative correlations with the anti-inflammatory cytokines IL-1 ($r = –0.31$, $p < 0.01$) and TGF-β ($r = –0.41$, $p < 0.001$). When the markers of collagen metabolism were further compared with the histological and clinical indices of liver disease severity and the grading of fibrosis in the biopsy sample, significant correlations emerged between PIIINP and fibrosis ($r = 0.63$, $p < 0.001$), CMI ($r = 0.50$, $p < 0.01$) and CCLI ($r = 0.42$, $p < 0.05$), whereas there were no significant correlations between these parameters and PINP or β-CTx levels.

6. Discussion

6.1. Immune responses against ethanol metabolites

The present results indicate that a humoral immune response consisting of IgA isotype antibodies against acetaldehyde adducts is highly typical of alcoholic patients. Since IgAs among moderate drinkers may also exceed the levels observed in abstainers, it appears that the generation of such immune responses is an early event in the physiological consequences of ethanol ingestion. Previous studies in patients with alcoholic liver disease have demonstrated an increase in serum total IgAs and the appearance of IgA deposits in liver and kidney tissue (van de Wiel et al. 1988, Tuma and Klassen 1992, Amore et al. 1994) and more recent evidence has suggested that the generation of excess IgAs in such patients could in fact be antigen-driven (Worrall et al. 1991, Koskinas et al. 1992, Viitala et al. 1997, Latvala et al. 2005). Such specific antibodies could originate from intestinally induced B-cells under conditions where the gastrointestinal tract, which is rich in enzymes capable of metabolizing ethanol to acetaldehyde, is repeatedly exposed to ethanol (Seitz et al. 1994, Salaspuro 1996, Visapää et al. 1998, Latvala et al. 2005). This view is also consistent with the close correlations between specific IgA antibody levels and recent ethanol ingestion and with the presence of anti-acetaldehyde adduct IgAs in individuals reporting moderate drinking. The positive correlation found between anti-acetaldehyde adduct IgAs and the pro-inflammatory cytokines TNF-α and IL-6 indicate that there is also an early-phase inflammatory response to ethanol-derived neo-antigens. The induction of the immune responses and the activation of cytokine cascades upon continuing heavy drinking may also play an important role in the sequence of events leading to liver pathology. Since acetaldehyde modification of proteins and cellular constituents has been shown previously to disturb protein function *in vivo*, the generation of IgAs to ethanol metabolites could also play a role in the destruction of such protein modifications.

The present data further indicate that the assessment of IgA antibodies to acetaldehyde-modified protein epitopes may provide a clinically useful tool for diagnosing excessive ethanol consumption. A high sensitivity and specificity emerged for the adduct-specific IgA measurements in differentiating between alcoholics and non-alcoholics. Worrall and coworkers (1996, 1998) previously investigated anti-acetaldehyde adduct IgA responses as markers of alcohol abuse using acetaldehyde-modified bovine serum albumin as a test antigen. Despite the difference in the method and in the nature of protein adducts in these and the present studies it should be noted that these investigators also found a significant correlation ($r = 0.44$) between ethanol intake and anti-acetaldehyde adduct IgAs among moderate drinkers and alcoholics. The present data show that the diagnostic sensitivity of

anti-acetaldehyde adduct IgAs exceeds that of the conventional markers, especially when abstainers are taken as the reference individuals. On the other hand, when moderate drinkers are included in the reference population the diagnostic sensitivity decreases, indicating that reference limits for alcohol markers should perhaps preferably be based on abstainers. This approach would obviously lead to some positive values in individuals with moderate drinking habits, but it would provide us with a new tool for raising the issue of possible hazardous drinking practices at an earlier stage.

Paper I was focused on men only, because sex may be a significant confounding factor in studies of immunological responses (Makkonen et al. 2001, Kovacs and Messingham 2002). The specific mechanisms underlying these phenomena have remained unclear, but sex hormones appear to play a key role in the regulation of immune responses. Although study III included women, the population was too small for any profound conclusions to be reached. However, women generally show stronger immune responses and higher serum immunoglobulin levels, an thus future studies in larger populations consisting of women only appear to be warranted in order to evaluate the diagnostic potential and pathogenic significance of IgA responses and to determine whether separate reference intervals are needed for men and women, as noted previously for several markers reflecting ethanol intake (Niemelä 2007).

Current follow-up studies indicate that serum anti-acetaldehyde adduct IgA levels normalize at an average rate of 3 % per day, the mean time required for normalization being 29 days. Since the average half-life of a single IgA molecule is 5–6 days, the relatively long appearance of anti-acetaldehyde adduct IgAs in serum may be explained by the fact that acetaldehyde adducts in the erythrocytes of alcoholics may persist in the circulation for 1 to 3 weeks after the last dose of ethanol (Niemelä and Israel 1992). The consistent decrease in anti-acetaldehyde adduct IgAs following abstinence indicates, however, that IgA titers could also be used for monitoring treatment in recovering alcoholics.

Recent evidence has suggested that further improvement in the clinical accuracy of alcohol markers may be obtained through the development of marker combinations. Such combinations have usually consisted of CDT, GGT and MCV. For instance, recent studies have indicated the highest diagnostic accuracy to a mathematically formulated combination of GGT and CDT (Sillanaukee and Olsson 2001, Anttila et al. 2003a, Hietala et al. 2006). The present work shows that the combination of anti-acetaldehyde adduct IgAs and CDT also provides improved sensitivity relative to the parent components, without sacrificing specificity. The diagnostic performance also seems to exceed that found for the combination of GGT with CDT. It should be noted, however, that careful standardization of the IgA-based assay will be needed before the method is taken into routine clinical and multilaboratory use. This could be achieved using internationally standardized antigen preparations and test protocols including standard color reac-

tion times and expression of the assay results using international reference samples.

6.2. Red blood cell abnormalities and immune responses against acetaldehyde adducts

The data presented in paper II indicate a dose-dependent response of erythrocyte cell volume to the intensity of ethanol intake. While alcoholic patients have long been known to have characteristically high MCV values, the data further indicate that non-alcoholic individuals reporting moderate levels of drinking (< 40 g/day) show values that at the population level may lead to alterations in the characteristics of an average red blood cell.

The present findings may open new perspectives for the use of MCV for the biomonitoring of long-term alcohol abuse. Although MCV lacks specificity especially in hospitalized patients, it appears that a careful follow-up of blood cell indices even when occurring within the reference limits could be used to provide indicators of changes in drinking practices, which are of special value in subjects without any obvious clinical signs of ethanol dependence. It has been noted previously that elevated MCV values occur in 4 % of the adult populations and are possibly related to alcohol consumption in 65 % of cases (Wymer and Becker 1990, Savage et al. 2000). The usefulness of MCV measurements has previously been emphasized under certain selected conditions, including assays on pregnant women for screening the of fetal alcohol effects (Sarkola et al. 2000). The possible effects of ethanol on blood cell counts should obviously also be considered carefully when establishing reference values for use in hospital laboratories as any deviation in this respect may impair clinicians' abilities to observe the important early signals of excessive drinking practices and lead to the underdetection of ethanol abuse unless separate reference values based on abstainers can be made available.

The correlation between the alterations in red blood cell indices and ethanol drinking is consistent with a direct hematotoxic role of ethanol and its metabolites (Eriksson 2001, Latvala et al. 2001b). Ethanol can permeate cell membranes, alter the structural order of lipids and interfere with cell structure and metabolism, thus possibly affecting erythrocyte stability (Beauge et al. 1988). High concentrations of acetaldehyde have been found previously to occur inside the erythrocytes of alcoholics (Peterson et al. 1988, Hernández-Muñoz et al. 1989, Magnani et al. 1990, Sillanaukee et al. 1992). Studies using human cell lines (Wickramasinghe and Malik 1986) and more recently a survey of Japanese heavy drinkers with the aldehyde dehydrogenase-2 genotype have supported a direct role for acetaldehyde in increasing the mean cell volume of erythrocytes (Yokoyama et al. 2003a, Yokoyama et al. 2003b). Interestingly, it has also been suggested that high MCV values in such individuals may serve as predictors of esophageal squamous cell carcinoma (Yokoyama et al. 2003a). High MCV values and anemia have in turn

been shown to be characteristic of patients with alcoholic liver disease (Maruyama et al. 2001).

Acetaldehyde is highly reactive and capable of forming stable adducts with proteins and cell membrane constituents (Stevens et al. 1981, Peterson et al. 1988, Niemelä and Israel 1992, Tuma and Klassen 1992, Latvala et al. 2001b). As a consequence, erythrocytes may become more vulnerable to damage, hemolysis, and a shortened biological half-life *in vivo*. The present data show for the first time that alcoholics with high MCV frequently have circulating antibodies which recognize acetaldehyde-modified epitopes in proteins and also correlate to some extent with the degree of macrocytosis. This observation is in line with the view that immunological mechanisms may also be involved in the generation of ethanol-induced changes in red blood cell indices in some alcoholics. However, there may be significant gender differences in the driving of the immune responses, which appear to warrant further investigations. Future studies also appear to be warranted in order to address the possibility that MCV changes, when measured together with immune parameters directed against ethanol-induced membrane alterations, could further improve the diagnostic or prognostic value of such measurements.

6.3 Comparison of anti-acetaldehyde adduct and anti-tissue transglutaminase IgAs in alcoholic liver disease

The findings detailed in paper III indicate that excessive ethanol consumption is associated with mounting of IgA immune responses not only to ethanol-derived neo-antigens but also to tissue transglutaminase (tTG), which has been specifically linked with celiac disease during the past few years (Dieterich et al. 1997, Sulkanen et al. 1998, Koop et al. 2000, Carroccio et al. 2002). The antibody titers were highest in the alcoholics with liver disease, which is in accordance with the view that liver disease per se may be associated with the occurrence of elevated tTG antibodies (Vecchi et al. 2003, Lo Iacono et al. 2005, Villalta et al. 2005). The association may also be influenced by the severity of liver disease, as suggested here by the correlation between antibody levels and the combined clinical and laboratory index (CCLI) of liver disease severity.

It appears that excessive ethanol intake even in the absence of liver disease could lead to a breakdown of immune tolerance and initiation of autoimmune responses against both ethanol metabolites and tissue transglutaminase. Alcohol abuse may create mucosal lesions and the increased gut permeability may subsequently lead to the exposure of new antigens regarded as foreign by the mucosal immune system. However, it remains to be established in future studies to what extent such immune responses could also contribute to the pathogenesis of gastrointestinal symptoms occurring as a result of excessive ethanol consumption. Nevertheless, the generation of IgAs against both tTG and acetaldehyde-derived neo-

antigens appears to be an early event in the sequence leading from excessive ethanol intake to tissue injury.

The parallel reactions to these antigens could also open up new perspectives on the pathogenesis of ethanol-induced diseases. Tissue transglutaminase is a widely distributed enzyme which seems to show multiple functions *in vivo* through the generation of protein modifications (Griffin et al. 2002). Its most characteristic enzymatic function is the formation of an epsilon (gamma-glutamyl) lysine isopeptide linkage. Studies of patients with celiac disease have indicated that tTG forms high molecular weight complexes with gliadin and leads to novel cross-linking patterns with extracellular matrix proteins. It has been suggested that this may favor gliadin toxicity and perpetuate intestinal inflammation and associated autoimmune phenomena (Hansson et al. 1999, Gianfrani et al. 2005, Dieterich et al. 2006). Indeed, pro-inflammatory cytokine responses were also found here to be associated with IgA responses in heavy drinkers, suggesting that such immunological mechanisms could also contribute to disease progression in alcoholic patients. Acetaldehyde has previously been shown to bind readily to proteins and to generate lysine-derived modifications (Israel et al. 1986, Tuma et al. 1987, Bao et al. 1999, Niemelä 2001, Freeman et al. 2005). Accelerated protein cross-linking may be expected to take place following modifications generated at high concentrations of acetaldehyde (Niemelä 2001). We cannot at this time rule out the possibility that the antigens recognized by these antibodies could also share common conformational or sequential epitopes and it remains to be established whether the protein modifications induced by the above pathways also have additive toxic effects in tissues and lead to the generation of cytotoxic proteinatious aggregates in alcohol-induced diseases, or in conditions such as neurodegenerative diseases (Wodzinska 2005) or diabetes (Bao et al. 1999).

In the light of recent findings indicating that elevated anti-tTG antibodies may also be indicative of a higher all-cause mortality risk at the population level (Metzger et al. 2006), future studies to examine the interactions between such immune responses, ethanol intake and their links to inflammation and fibrogenesis appear to be warranted. The present findings should also be considered when assessing the clinical use of tTG antibodies as screening tools for celiac disease.

6.4. Fibrogenesis, collagen degradation and cytokines in alcoholic liver disease

Morbidity and mortality related to liver cirrhosis have been continuously growing in most Western countries (Lieber 1995, Room et al. 2005). The current prevalence of liver cirrhosis is known to be particularly high in the countries that have the highest per capita consumption of alcohol. Since not all chronic alcoholics develop cirrhosis, extensive research efforts have been devoted to examining the molecular and cellular mechanisms regulating the deposition of hepatic extracellular matrix proteins. Susceptibility to ALD may also be influenced by genetic

factors such as functional polymorphisms in the ADH and ALDH genes (Wilfred de Alwis and Day 2007). Due to the limitations of liver biopsies for assessing disease activity and liver fibrosis, the need for new, non-invasive modalities for differentiating those at risk of progressive fibrosis has been widely acknowledged (Imbert-Bismut et al. 2001, Bedossa et al. 2003, Friedman 2003, Callewaert et al. 2004, Ghany and Doo 2005).

The present results indicate that serum β-CTx, a marker of collagen degradation, and PINP and PIIINP, markers of the synthesis of collagen types I and III, respectively, follow inverse kinetics in alcoholic patients with and without liver disease, and suggests a disturbed balance between collagen synthesis and matrix degradation in cases of liver disease. It should be noted that although β-CTx shows significant circadian variation even during the sampling period used in these studies (Qvist et al. 2002), the differences in its serum levels were so significant that this variation does not have any influence on the results. Significant alterations in both the amount and composition of the extracellular matrix components are known to be associated with liver fibrosis, and there may be aberrant accumulation of the various collagen types (Rojkind et al. 1979, Maher and McGuire 1990). In advanced cirrhosis the liver may contain six times the normal amount of collagen, the majority of this consisting of types I and III (Rojkind et al. 1979). The increased rates of synthesis of these collagens in alcoholic liver disease have been acknowledged in several previous studies involving type-specific methods (McCullough et al. 1987, Niemelä et al. 1990, Trinchet et al. 1992, Rosman and Lieber 1992). However, although specific fragments of C-terminal collagen telopeptides (CTx) have recently been found to be useful for assessing collagen degradation (Okabe et al. 2001), such measurements have so far been applied exclusively to diseases affecting bone metabolism (Rosen et al. 2000, Garnero et al. 2003). The present data indicate that this cross-linked isomerized fragment of type I collagen in serum is markedly decreased in ALD patients and shows an inverse relationship with the peptides reflecting enhanced synthesis of collagen types I and III. The fibrous collagens may thus be deposited at a faster rate than that at which the collagenolytic activity removing the accumulated scar matrix is functioning, which would be in accordance with previous findings of decreased collagenase and matrix metalloproteinase activities in cirrhotic patients (Maruyama et al. 1982, Iredale 2003). There may also be overexpression of their inhibitors, such as TIMPs, further aggravating the failure of collagen removal to keep pace with the enhanced collagen synthesis (Maruyama et al. 1982, Li et al. 1994, Friedman 2003, Iredale 2003).

The present findings also open up the possibility that assays of these collagen markers, particularly the PIIINP/β-CTx ratio, could provide a simple and cost-efficient tool for improving the clinical discrimination of persons with a risk of liver disease and severe fibrosis. Such an approach could perhaps be more suitable for routine clinical use than the recently introduced models involving large combinations of fibrotic markers together with mathematically formulated equations for interpreting the data (Imbert-Bismut et al. 2001). It should also be noted that

although β-CTx is a degradation fragment of type I collagen, the analyses of the PINP/β-CTx ratios indicated a lower sensitivity (87 %) for this ratio in discriminating the ALD patients than for the corresponding ratio PIIINP/β-CTx calculated from the type III collagen peptide data (94 %). There was also a lack of correlation between type I collagen-derived fragments and the indices of liver disease severity, especially those reflecting inflammation (CCLI, CMI), whereas PIIINP showed close positive correlations with this parameters. This is likely to be due to differences in the metabolic kinetics of type I and type III collagens in the different phases of liver disease and in their tissue specificities. While type I collagen is the major fibrous collagen of bone, it also occurs in soft connective tissue together with type III collagen (Rojkind et al. 1979). Type III collagen is the more pliable collagen of organs like liver and its half-life is shorter than that of type I collagen. In the early phase of the fibroproliferative reaction, fibroblastic cells synthesize an extracellular matrix, which contains large amounts of type III collagen, whereas type I collagen may predominate in advanced liver cirrhosis (Rojkind et al. 1979). Measurements of type III collagen-derived fragments may therefore be more sensitive in reflecting the changes occurring during the inflammatory phases and in the early phase of hepatic fibrogenesis (Niemelä et al. 1990). There may also be collagen type-specific changes in the removal of collagen peptides from the circulation (Melkko et al. 1994).

Cytokines are important mediators of inflammation and seem to play a specific role in the development and progression of alcoholic liver disease (McClain and Cohen 1989, Khoruts et al. 1991, Hill et al. 1993, Neuman et al. 2001, Latvala et al. 2005, Crews et al. 2006). Paper III indicated close correlations between autoimmune responses and interleukin-8 and TNF-α, which have been previously linked with decreased long-term survival in alcoholics (Bird et al. 1990, Felver et al. 1990, Huang et al. 1996, McClain et al. 2005, Świątkowska-Stodulska et al. 2006). TNF-α production is closely associated with Kupffer cell activation, inflammation and necrosis (Iimuro et al. 1997, Yin et al. 1999, Colantoni et al. 2003), whereas interleukin-8 is a neutrophil chemoattractant and activator (Joshi-Barve et al. 2003). The findings reported in paper IV further indicate that hepatic fibrogenesis may be facilitated by a switch between the pro-inflammatory Th1 cytokines and anti-inflammatory Th2 cytokines. Markers of collagen synthesis were found to correlate positively with IL-8 and TNF-α, and it should also be noted that although enhanced TGF-β expression has previously been linked with the development of fibrosis in alcoholics (Chen et al. 2002), the present data indicate a negative correlation between collagen synthesis markers and TGF-β levels. Interestingly, anti-tTG IgA antibodies also showed highly significant correlations with markers of fibrogenesis, indicating that such immune responses may also be involved in the stimulation of extracellular matrix production. By virtue of its cross-linking activity, tissue transglutaminase also seems to play a central role in regulating extracellular matrix remodeling (Stephens et al. 2004).

Thus there seem to be both collagen and cytokine markers that are significantly associated with ALD status in alcoholic patients. Although the possibility

of minor overlapping with individuals classified as heavy drinkers without liver disease (who were not biopsied, due to ethical considerations) and patients with mild degrees of liver disease cannot be ruled out at this time, the data showing a positive relationship between markers of collagen synthesis and pro-inflammatory cytokines (IL-8 and TNF-α) and a negative relationship between these markers and a marker of collagen degradation (β-CTx) and anti-inflammatory cytokines (IL-1, TGF-β) may prove to be of value when studying the pathogenesis of ALD in humans and when developing more specific clinical markers for the early identification of patients with a risk of progression to cirrhosis.

6.5 Possible limitations of this study

It should be noted that this investigation included apparently healthy controls and alcoholics with or without liver disease, while in clinical practice the hospital population contains patients with various other medical conditions. Conditions such as diabetes, celiac disease and non-alcoholic liver disease may have an influence on the specificities of the parameters used. Future studies should also address the specificities of the methods in larger materials including hospitalized patients, among whom a lower degree of specificity may be expected. The alcohol consumption data are based on individuals' own reports and thus the possibility of having some excessive drinkers among the control population cannot be excluded. Also, since the permitted alcohol intake for women was similar to that for men, some females with excessive ethanol consumption may have been included in the population of moderate drinkers.

It should also be noted that most of the methods used here are currently in routine use and thus quite well characterized in terms of variation and accuracy as well as clinical usefulness. The anti-acetaldehyde adduct IgA assay, however, has so far been used for research purposes only and has not been fully standardized for routine use. We cannot exclude some additional variation in results due to this unstandardized method, and therefore future work aimed at standardization of the assay is warranted.

7. Conclusions

I Specific IgA responses against acetaldehyde-modified epitopes are characteristic of alcoholic patients and may provide a tool for detecting heavy drinking. Assays for such immune responses could also be important in studies on the pathogenesis of ethanol-induced tissue injury and in the assessment of abnormalities in red blood cell morphology in alcoholics.

II The correlation between anti-acetaldehyde adduct IgAs and anti-tissue transglutaminase IgAs may indicate common mucosal responses to these antigens under conditions where the integrity of intestinal barriers has been disrupted upon excessive ethanol intake. The data also indicate distinct positive relationships between anti-adduct IgAs and cytokine profiles in alcoholic patients. In addition, the positive relationships between markers of collagen synthesis and proinflammatory cytokines and a negative relationship between these markers and a marker of collagen degradation and anti-inflammatory cytokines indicate that such inverse relationships may be important in the pathogenesis of ALD and may also help in the development of more specific clinical markers for assessing the risk of fibrogenesis.

References

Agarwal DP (2002): Cardioprotective effects of light-moderate consumption of alcohol: a review of putative mechanisms. Alcohol Alcohol 37:409-415.

Agarwal DP, Tobar-Rojas L, Harada S and Goedde HW (1983): Comparative study of erythrocyte aldehyde dehydrogenase in alcoholics and control subjects. Pharmacol Biochem Behav 18 Suppl 1:89-95.

Allen JP, Litten RZ, Anton RF and Cross GM (1994): Carbohydrate-deficient transferrin as a measure of immoderate drinking: remaining issues. Alcohol Clin Exp Res 18:799-812.

Allen JP, Litten RZ, Fertig JB and Sillanaukee P (2000): Carbohydrate-deficient transferrin, gamma-glutamyltransferase, and macrocytic volume as biomarkers of alcohol problems in women. Alcohol Clin Exp Res 24:492-496.

Amir I, Anwar N, Baraona E and Lieber CS (1996): Ranitidine increases the bioavailability of imbibed alcohol by accelerating gastric emptying. Life Sci 58:511-518.

Amore A, Coppo R, Roccatello D, Piccoli G, Mazzucco G, Gomez-Chiarri M, Lamm ME and Emancipator SN (1994): Experimental IgA nephropathy secondary to hepatocellular injury induced by dietary deficiencies and heavy alcohol intake. Lab Invest 70:68-77.

Anton RF and Moak DH (1994): Carbohydrate-deficient transferrin and gamma-glutamyltransferase as markers of heavy alcohol consumption: gender differences. Alcohol Clin Exp Res 18:747-754.

Anton RF, Dominick C, Bigelow M and Westby C (2001): Comparison of Bio-Rad %CDT TIA and CDTect as laboratory markers of heavy alcohol use and their relationships with gamma-glutamyltransferase. Clin Chem 47:1769-1775.

Anttila P, Järvi K, Latvala J, Blake JE and Niemelä O (2003a): A new modified gamma-%CDT method improves the detection of problem drinking: studies in alcoholics with or without liver disease. Clin Chim Acta 338:45-51.

Anttila P, Järvi K, Latvala J, Blake JE and Niemelä O (2003b): Diagnostic characteristics of different carbohydrate-deficient transferrin methods in the detection of problem drinking: effects of liver disease and alcohol consumption. Alcohol Alcohol 38:415-420.

Apte MV, Pirola RC and Wilson JS (2005): Molecular mechanisms of alcoholic pancreatitis. Dig Dis 23:232-240.

Aradottir S, Asanovska G, Gjerss S, Hansson P and Alling C (2006): PHosphatidylethanol (PEth) concentrations in blood are correlated to reported alcohol intake in alcohol-dependent patients. Alcohol Alcohol 41:431-437.

Ariyoshi T, Takabatake E and Remmer H (1970): Drug metabolism in ethanol-induced fatty liver. Life Sci 9:361-369.

Arndt T (2001): Carbohydrate-deficient transferrin as a marker of chronic alcohol abuse: a critical review of preanalysis, analysis, and interpretation. Clin Chem 47:13-27.

Arndt T, Korzec A, Bär M and Kropf J (2002): Further arguments against including trisialo-Fe2-transferrin in carbohydrate-deficient transferrin (CDT): a study on male alcoholics and hazardous drinkers. Med Sci Monit 8:CR411-CR418.

Asai H, Imaoka S, Kuroki T, Monna T and Funae Y (1996): Microsomal ethanol oxidizing system activity by human hepatic cytochrome P450s. J Pharmacol Exp Ther 277:1004-1009.

Auwärter V, Sporkert F, Hartwig S, Pragst F, Vater H and Diefenbacher A (2001): Fatty acid ethyl esters in hair as markers of alcohol consumption. Segmental hair analysis of alcoholics, social drinkers, and teetotalers. Clin Chem 47:2114-2123.

Baer DJ, Judd JT, Clevidence BA, Muesing RA, Campbell WS, Brown ED and Taylor PR (2002): Moderate alcohol consumption lowers risk factors for cardiovascular disease in postmenopausal women fed a controlled diet. Am J Clin Nutr 75:593-599.

Ballard HS (1989): Hematological complications of alcoholism. Alcohol Clin Exp Res 13:706-720.

Bao F, Yu L, Babu S, Wang T, Hoffenberg EJ, Rewers M and Eisenbarth GS (1999): One third of HLA DQ2 homozygous patients with type 1 diabetes express celiac disease-associated transglutaminase autoantibodies. J Autoimmun 13:143-148.

Baraona E, Abittan CS, Dohmen K, Moretti M, Pozzato G, Chayes ZW, Schaefer C and Lieber CS (2001): Gender differences in pharmacokinetics of alcohol. Alcohol Clin Exp Res 25:502-507.

Bautista AP (2002): Neutrophilic infiltration in alcoholic hepatitis. Alcohol 27:17-21.

Beauge F, Gallay J, Stibler H and Borg S (1988): Alcohol abuse increases the lipid structural order in human erythrocyte membranes. A steady-state and time-resolved anisotropy study. Biochem Pharmacol 37:3823-3828.

Becker U, Deis A, Sørensen TI, Grønbaek M, Borch-Johnsen K, Müller CF, Schnohr P and Jensen G (1996): Prediction of risk of liver disease by alcohol intake, sex, and age: a prospective population study. Hepatology 23:1025-1029.

Bedossa P, Dargère D and Paradis V (2003): Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology 38:1449-1457.

Behrens UJ, Worner TM and Lieber CS (1988): Changes in carbohydrate-deficient transferrin levels after alcohol withdrawal. Alcohol Clin Exp Res 12:539-544.

Behrens UJ, Ma XL, Bychenok S, Baraona E and Lieber CS (1990): Acetaldehyde-collagen adducts in CCl4-induced liver injury in rats. Biochem Biophys Res Commun 173:111-119.

Beisswenger TB, Holmquist B and Vallee BL (1985): chi-ADH is the sole alcohol dehydrogenase isozyme of mammalian brains: implications and inferences. Proc Natl Acad Sci U S A 82:8369-8373.

Bell H, Tallaksen CM, Try K and Haug E (1994): Carbohydrate-deficient transferrin and other markers of high alcohol consumption: a study of 502 patients admitted consecutively to a medical department. Alcohol Clin Exp Res 18:1103-1108.

Bellentani S, Saccoccio G, Costa G, Tiribelli C, Manenti F, Sodde M, Saveria CL, Sasso F, Pozzato G, Cristianini G and Brandi G (1997): Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group. Gut 41:845-850.

Bergheim I, McClain CJ and Arteel GE (2005): Treatment of alcoholic liver disease. Dig Dis 23:275-284.

Bergström J, Helander A and Jones AW (2003): Ethyl glucuronide concentrations in two successive urinary voids from drinking drivers: relationship to creatinine content and blood and urine ethanol concentrations. Forensic Sci Int 133:86-94.

Beutler B (2004): Innate immunity: an overview. Mol Immunol 40:845-859.

Biewald J, Nilius R and Langner J (1998): Occurrence of acetaldehyde protein adducts formed in various organs of chronically ethanol fed rats: An immunohistochemical study. Int J Mol Med 2:389-396.

Bird G (1994): Interleukin-8 in alcoholic liver disease. Acta Gastroenterol Belg 57:255-259.

Bird GL, Sheron N, Goka AK, Alexander GJ and Williams RS (1990): Increased plasma tumor necrosis factor in severe alcoholic hepatitis. Ann Intern Med 112:917-920.

Bode C and Bode JC (2005): Activation of the innate immune system and alcoholic liver disease: effects of ethanol per se or enhanced intestinal translocation of bacterial toxins induced by ethanol? Alcohol Clin Exp Res 29:166S-171S.

Boffetta P and Hashibe M (2006): Alcohol and cancer. Lancet Oncol 7:149-156.

Bortolotti F, De Paoli G and Tagliaro F (2006): Carbohydrate-deficient transferrin (CDT) as a marker of alcohol abuse: a critical review of the literature 2001-2005. J Chromatogr B Analyt Technol Biomed Life Sci 841:96-109.

Borucki K, Kunstmann S, Dierkes J, Westphal S, Diekmann S, Bogerts B and Luley C (2004): In heavy drinkers fatty acid ethyl esters in the serum are increased for 44 hr after ethanol consumption. Alcohol Clin Exp Res 28:1102-1106.

Bradford BU, Enomoto N, Ikejima K, Rose ML, Bojes HK, Forman DT and Thurman RG (1999): Peroxisomes are involved in the swift increase in alcohol metabolism. J Pharmacol Exp Ther 288:254-259.

Bradley KA, Badrinath S, Bush K, Boyd-Wickizer J and Anawalt B (1998): Medical risks for women who drink alcohol. J Gen Intern Med 13:627-639.

Brown AS, Fiatarone JR, Wood P, Bennett MK, Kelly PJ, Rawlins MD, Day CP and James OF (1995): The effect of gastritis on human gastric alcohol dehydrogenase activity and ethanol metabolism. Aliment Pharmacol Ther 9:57-61.

Brunt EM (2002): Alcoholic and nonalcoholic steatohepatitis. Clin Liver Dis 6:399-420, vii.

Butterworth RF (1995): Pathophysiology of alcoholic brain damage: synergistic effects of ethanol, thiamine deficiency and alcoholic liver disease. Metab Brain Dis 10:1-8.

Callewaert N, Van Vlierberghe H, Van Hecke A, Laroy W, Delanghe J and Contreras R (2004): Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. Nat Med 10:429-434.

Campbell S, Timms PM, Maxwell PR, Doherty EM, Rahman MZ, Lean ME and Danesh BJ (2001): Effect of alcohol withdrawal on liver transaminase levels and markers of liver fibrosis. J Gastroenterol Hepatol 16:1254-1259.

Carroccio A, Vitale G, Di Prima L, Chifari N, Napoli S, La Russa C, Gulotta G, Averna MR, Montalto G, Mansueto S and Notarbartolo A (2002): Comparison of anti-transglutaminase ELISAs and an anti-endomysial antibody assay in the diagnosis of celiac disease: a prospective study. Clin Chem 48:1546-1550.

Chan AW, Leong FW, Schanley DL, Welte JW, Wieczorek W, Rej R and Whitney RB (1989): Transferrin and mitochondrial aspartate aminotransferase in young adult alcoholics. Drug Alcohol Depend 23:13-18.

Charness ME (1993): Brain lesions in alcoholics. Alcohol Clin Exp Res 17:2-11.

Charpentier B, Franco D, Paci L, Charra M, Martin B, Vuitton D and Fries D (1984): Deficient natural killer cell activity in alcoholic cirrhosis. Clin Exp Immunol 58:107-115.

Chase V, Neild R, Sadler CW and Batey RG (2005): The medical complications of alcohol use: understanding mechanisms to improve management. Drug Alcohol Rev 24:253-265.

Chen WX, Li YM, Yu CH, Cai WM, Zheng M and Chen F (2002): Quantitative analysis of transforming growth factor beta 1 mRNA in patients with alcoholic liver disease. World J Gastroenterol 8:379-381.

Chen J, Conigrave KM, Macaskill P, Whitfield JB and Irwig L (2003): Combining carbohydrate-deficient transferrin and gamma-glutamyltransferase to increase diagnostic accuracy for problem drinking. Alcohol Alcohol 38:574-582.

Cho E, Smith-Warner SA, Ritz J, van den Brandt PA, Colditz GA, Folsom AR, Freudenheim JL, Giovannucci E, Goldbohm RA, Graham S, Holmberg L, Kim DH, Malila N, Miller AB, Pietinen P, Rohan TE, Sellers TA, Speizer FE, Willett WC, Wolk A and Hunter DJ (2004): Alcohol intake and colorectal cancer: a pooled analysis of 8 cohort studies. Ann Intern Med 140:603-613.

Choi HK, Atkinson K, Karlson EW, Willett W and Curhan G (2004): Alcohol intake and risk of incident gout in men: a prospective study. Lancet 363:1277-1281.

Chrostek L, Cylwik B, Szmitkowski M and Korcz W (2006): The diagnostic accuracy of carbohydrate-deficient transferrin, sialic acid and commonly used markers of alcohol abuse during abstinence. Clin Chim Acta 364:167-171.

Ciraulo DA and Barnhill J (1986): Pharmacokinetic mechanisms of ethanol-psychotropic drug interactions. NIDA Res Monogr 68:73-88.

Colantoni A, Idilman R, De Maria N, La Paglia N, Belmonte J, Wezeman F, Emanuele N, Van Thiel DH, Kovacs EJ and Emanuele MA (2003): Hepatic apoptosis and proliferation in male and female rats fed alcohol: role of cytokines. Alcohol Clin Exp Res 27:1184-1189.

Collins MA, Nijm WP, Borge GF, Teas G and Goldfarb C (1979): Dopamine-related tetrahydroisoquinolines: significant urinary excretion by alcoholics after alcohol consumption. Science 206:1184-1186.

Conigrave KM, Degenhardt LJ, Whitfield JB, Saunders JB, Helander A and Tabakoff B (2002): CDT, GGT, and AST as markers of alcohol use: the WHO/ISBRA collaborative project. Alcohol Clin Exp Res 26:332-339.

Cook RT (1998): Alcohol abuse, alcoholism, and damage to the immune system--a review. Alcohol Clin Exp Res 22:1927-1942.

Cook RT, Waldschmidt TJ, Cook BL, LaBrecque DR and McLatchie K (1996): Loss of the CD5+ and CD45RAhi B cell subsets in alcoholics. Clin Exp Immunol 103:304-310.

Cook RT, Li F, Vandersteen D, Ballas ZK, Cook BL and LaBrecque DR (1997): Ethanol and natural killer cells. I. Activity and immunophenotype in alcoholic humans. Alcohol Clin Exp Res 21:974-980.

Corrao G, Lepore AR, Torchio P, Valenti M, Galatola G, D'Amicis A, Arico S and di Orio F (1994): The effect of drinking coffee and smoking cigarettes on the risk of cirrhosis associated with alcohol consumption. A case-control study. Provincial Group for the Study of Chronic Liver Disease. Eur J Epidemiol 10:657-664.

Crabb DW, Matsumoto M, Chang D and You M (2004): Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. Proc Nutr Soc 63:49-63.

Crews FT, Bechara R, Brown LA, Guidot DM, Mandrekar P, Oak S, Qin L, Szabo G, Wheeler M and Zou J (2006): Cytokines and alcohol. Alcohol Clin Exp Res 30:720-730.

Cunningham RM, Maio RF, Hill EM and Zink BJ (2002): The effects of alcohol on head injury in the motor vehicle crash victim. Alcohol Alcohol 37:236-240.

Cushman P (1992): Blood and liver markers in the estimation of alcohol consumption. In: Measuring Alcohol Cunsumption. Psychosocial and Biochemical Methods, pp. 135-147. Eds. RZ Litten and JP Allen, The Humana Press Inc., Totowa, New Jersey.

Dahl H, Stephanson N, Beck O and Helander A (2002): Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide. J Anal Toxicol 26:201-204.

Day CP (2000): Who gets alcoholic liver disease: nature or nurture? J R Coll Physicians Lond 34:557-562.

De Ceuninck F, Gaufillier S, Bonnaud A, Sabatini M, Lesur C and Pastoureau P (2001): YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes. Biochem Biophys Res Commun 285:926-931.

Diamond I and Messing RO (1994): Neurologic effects of alcoholism. West J Med 161:279-287.

Diehl AM (1997): Alcoholic liver disease: natural history. Liver Transpl Surg 3:206-211.

Diehl AM (1998): Alcoholic liver disease. Clin Liver Dis 2:103-118.

Diehl AM (2004): Obesity and alcoholic liver disease. Alcohol 34:81-87.

Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO and Schuppan D (1997): Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat Med 3:797-801.

Dieterich W, Esslinger B, Trapp D, Hahn E, Huff T, Seilmeier W, Wieser H and Schuppan D (2006): Cross linking to tissue transglutaminase and collagen favours gliadin toxicity in coeliac disease. Gut 55:478-484.

Doll R (1997): One for the heart. BMJ 315:1664-1668.

Doyle KM, Bird DA, Al-Salihi S, Hallaq Y, Cluette-Brown JE, Goss KA and Laposata M (1994): Fatty acid ethyl esters are present in human serum after ethanol ingestion. J Lipid Res 35:428-437.

Doyle KM, Cluette-Brown JE, Dube DM, Bernhardt TG, Morse CR and Laposata M (1996): Fatty acid ethyl esters in the blood as markers for ethanol intake. JAMA 276:1152-1156.

Edelbroek MA, Horowitz M, Wishart JM and Akkermans LM (1993): Effects of erythromycin on gastric emptying, alcohol absorption and small intestinal transit in normal subjects. J Nucl Med 34:582-588.

Ehrig T, Bosron WF and Li TK (1990): Alcohol and aldehyde dehydrogenase. Alcohol Alcohol 25:105-116.

Ekbom A, McLaughlin JK, Karlsson BM, Nyren O, Gridley G, Adami HO and Fraumeni JF, Jr. (1994): Pancreatitis and pancreatic cancer: a population-based study. J Natl Cancer Inst 86:625-627.

Ellison RC (2002): Balancing the risks and benefits of moderate drinking. Ann N Y Acad Sci 957:1-6.

Ely M, Hardy R, Longford NT and Wadsworth ME (1999): Gender differences in the relationship between alcohol consumption and drink problems are largely accounted for by body water. Alcohol Alcohol 34:894-902.

Enoch MA (2003): Pharmacogenomics of alcohol response and addiction. Am J Pharmacogenomics 3:217-232.

Enomoto N, Takase S, Takada N and Takada A (1991): Alcoholic liver disease in heterozygotes of mutant and normal aldehyde dehydrogenase-2 genes. Hepatology 13:1071-1075.

Eriksson CJP (2001): The role of acetaldehyde in the actions of alcohol (update 2000). Alcohol Clin Exp Res 25:15S-32S.

Eriksson CJP, Fukunaga T, Sarkola T, Chen WJ, Chen CC, Ju JM, Cheng AT, Yamamoto H, Kohlenberg-Müller K, Kimura M, Murayama M, Matsushita S, Kashima H, Higuchi S, Carr L, Viljoen D, Brooke L, Stewart T, Foroud T, Su J, Li TK and Whitfield JB (2001): Functional relevance of human adh polymorphism. Alcohol Clin Exp Res 25:157S-163S.

Estonius M, Svensson S and Höög JO (1996): Alcohol dehydrogenase in human tissues: localisation of transcripts coding for five classes of the enzyme. FEBS Lett 397:338-342.

Felver ME, Mezey E, McGuire M, Mitchell MC, Herlong HF, Veech GA and Veech RL (1990): Plasma tumor necrosis factor alpha predicts decreased long-term survival in severe alcoholic hepatitis. Alcohol Clin Exp Res 14:255-259.

Fleming MF, Anton RF and Spies CD (2004): A review of genetic, biological, pharmacological, and clinical factors that affect carbohydrate-deficient transferrin levels. Alcohol Clin Exp Res 28:1347-1355.

Fowles LF, Beck E, Worrall S, Shanley BC and de Jersey J (1996): The formation and stability of imidazolidinone adducts from acetaldehyde and model peptides. A kinetic study with implications for protein modification in alcohol abuse. Biochem Pharmacol 51:1259-1267.

Freeman TL, Tuma DJ, Thiele GM, Klassen LW, Worrall S, Niemelä O, Parkkila S, Emery PW and Preedy VR (2005): Recent advances in alcohol-induced adduct formation. Alcohol Clin Exp Res 29:1310-1316.

Frezza M, di Padova C, Pozzato G, Terpin M, Baraona E and Lieber CS (1990): High blood alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. N Engl J Med 322:95-99.

Friedman SL (2003): Liver fibrosis -- from bench to bedside. J Hepatol 38 Suppl 1:S38-S53.

Friedman GD and Klatsky AL (1993): Is alcohol good for your health? N Engl J Med 329:1882-1883.

Fromme K, de Wit H, Hutchison KE, Ray L, Corbin WR, Cook TA, Wall TL and Goldman D (2004): Biological and behavioral markers of alcohol sensitivity. Alcohol Clin Exp Res 28:247-256.

Fujimoto M, Uemura M, Nakatani Y, Tsujita S, Hoppo K, Tamagawa T, Kitano H, Kikukawa M, Ann T, Ishii Y, Kojima H, Sakurai S, Tanaka R, Namisaki T, Noguchi R, Higashino T, Kikuchi E, Nishimura K, Takaya A and Fukui H (2000): Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: relation to severity of liver disturbance. Alcohol Clin Exp Res 24:48S-54S.

Fusetti F, Pijning T, Kalk KH, Bos E and Dijkstra BW (2003): Crystal structure and carbohydrate-binding properties of the human cartilage glycoprotein-39. J Biol Chem 278:37753-37760.

Gaines KC, Salhany JM, Tuma DJ and Sorrell MF (1977): Reaction of acetaldehyde with human erythrocyte membrane proteins. FEBS Lett 75:115-119.

Gall N (2001): Is wine good for your heart? A critical review. Postgrad Med J 77:172-176.

Garnero P, Ferreras M, Karsdal MA, Nicamhlaoibh R, Risteli J, Borel O, Qvist P, Delmas PD, Foged NT and Delaisse JM (2003): The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. J Bone Miner Res 18:859-867.

Ghany MG and Doo E (2005): Assessment of liver fibrosis: palpate, poke or pulse? Hepatology 42:759-761.

Ghosh P, Hale EA and Lakshman MR (2001): Plasma sialic-acid index of apolipoprotein J (SIJ): a new alcohol intake marker. Alcohol 25:173-179.

Gianfrani C, Auricchio S and Troncone R (2005): Adaptive and innate immune responses in celiac disease. Immunol Lett 99:141-145.

Go VL, Gukovskaya A and Pandol SJ (2005): Alcohol and pancreatic cancer. Alcohol 35:205-211.

Goldberg IJ (2003): To drink or not to drink? N Engl J Med 348:163-164.

Greenwel P, Domínguez-Rosales JA, Mavi G, Rivas-Estilla AM and Rojkind M (2000): Hydrogen peroxide: a link between acetaldehyde-elicited alpha1(I) collagen gene up-regulation and oxidative stress in mouse hepatic stellate cells. Hepatology 31:109-116.

Gressner AM and Schafer S (1989): Comparison of sulphated glycosaminoglycan and hyaluronate synthesis and secretion in cultured hepatocytes, fat storing cells, and Kupffer cells. J Clin Chem Clin Biochem 27:141-149.

Griffin M, Casadio R and Bergamini CM (2002): Transglutaminases: nature's biological glues. Biochem J 368:377-396.

Gruenewald PJ and Ponicki WR (1995): The relationship of alcohol sales to cirrhosis mortality. J Stud Alcohol 56:635-641.

Guañabens N, Parés A, Alvarez L, Martínez de Osaba MJ, Monegal A, Peris P, Ballesta AM and Rodés J (1998): Collagen-related markers of bone turnover reflect the severity of liver fibrosis in patients with primary biliary cirrhosis. J Bone Miner Res 13:731-738.

Gugler R (1994): H2-antagonists and alcohol. Do they interact? Drug Saf 10:271-280.

Gunnarsson T, Karlsson A, Hansson P, Johnson G, Alling C and Odham G (1998): Determination of phosphatidylethanol in blood from alcoholic males using high-performance liquid chromatography and evaporative light scattering or electrospray mass spectrometric detection. J Chromatogr B Biomed Sci Appl 705:243-249.

Gustavsson L (1995): ESBRA 1994 Award Lecture. Phosphatidylethanol formation: specific effects of ethanol mediated via phospholipase D. Alcohol Alcohol 30:391-406.

Haber H, Putscher I, Georgi M and Melzig MF (1995): Influence of ethanol on the salsolinol excretion in healthy subjects. Alcohol 12:299-303.

Hakala BE, White C and Recklies AD (1993): Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J Biol Chem 268:25803-25810.

Halsted CH (2004): Nutrition and alcoholic liver disease. Semin Liver Dis 24:289-304.

Halsted CH, Villanueva JA and Devlin AM (2002a): Folate deficiency, methionine metabolism, and alcoholic liver disease. Alcohol 27:169-172.

Halsted CH, Villanueva JA, Devlin AM, Niemela O, Parkkila S, Garrow TA, Wallock LM, Shigenaga MK, Melnyk S and James SJ (2002b): Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. Proc Natl Acad Sci U S A 99:10072-10077.

Handler JA and Thurman RG (1985): Fatty acid-dependent ethanol metabolism. Biochem Biophys Res Commun 133:44-51.

Hansson P, Caron M, Johnson G, Gustavsson L and Alling C (1997): Blood phosphatidylethanol as a marker of alcohol abuse: levels in alcoholic males during withdrawal. Alcohol Clin Exp Res 21:108-110.

Hansson T, Dannæus A and Klareskog L (1999): Cytokine-producing cells in peripheral blood of children with coeliac disease secrete cytokines with a type 1 profile. Clin Exp Immunol 116:246-250.

Harasymiw JW and Bean P (2001): Identification of heavy drinkers by using the early detection of alcohol consumption score. Alcohol Clin Exp Res 25:228-235.

Harper C and Matsumoto I (2005): Ethanol and brain damage. Curr Opin Pharmacol 5:73-78.

Hartz AJ, Guse C and Kajdacsy-Balla A (1997): Identification of heavy drinkers using a combination of laboratory tests. J Clin Epidemiol 50:1357-1368.

Hazelett SE, Liebelt RA, Brown WJ, Androulakakis V, Jarjoura D and Truitt EB, Jr. (1998): Evaluation of acetaldehyde-modified hemoglobin and other markers of chronic heavy alcohol use: effects of gender and hemoglobin concentration. Alcohol Clin Exp Res 22:1813-1819.

Heidemann E, Nerke O and Waller HD (1981): [Alcohol induced changes in hemopoiesis (author's transl)]. Klin Wochenschr 59:1303-1312.

Helander A (1999): Absolute or relative measurement of carbohydrate-deficient transferrin in serum? Experiences with three immunological assays. Clin Chem 45:131-135.

Helander A and Eriksson CJP (2002): Laboratory tests for acute alcohol consumption: results of the WHO/ISBRA Study on State and Trait Markers of Alcohol Use and Dependence. Alcohol Clin Exp Res 26:1070-1077.

Helander A, Wikström T, Lowenmo C, Jacobsson G and Beck O (1992): Urinary excretion of 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol after oral loading with serotonin. Life Sci 50:1207-1213.

Helander A, Beck O, Jacobsson G, Lowenmo C and Wikström T (1993): Time course of ethanol-induced changes in serotonin metabolism. Life Sci 53:847-855.

Helander A, Beck O and Jones AW (1996): Laboratory testing for recent alcohol consumption: comparison of ethanol, methanol, and 5-hydroxytryptophol. Clin Chem 42:618-624.

Helander A, Eriksson G, Stibler H and Jeppsson JO (2001a): Interference of transferrin isoform types with carbohydrate-deficient transferrin quantification in the identification of alcohol abuse. Clin Chem 47:1225-1233.

Helander A, Fors M and Zakrisson B (2001b): Study of Axis-Shield new %CDT immunoassay for quantification of carbohydrate-deficient transferrin (CDT) in serum. Alcohol Alcohol 36:406-412.

Hernández-Muñoz R, Baraona E, Blacksberg I and Lieber CS (1989): Characterization of the increased binding of acetaldehyde to red blood cells in alcoholics. Alcohol Clin Exp Res 13:654-659.

Hietala J, Koivisto H, Anttila P and Niemelä O (2006): Comparison of the combined marker GGT-CDT and the conventional laboratory markers of alcohol abuse in heavy drinkers, moderate drinkers and abstainers. Alcohol Alcohol 41:528-533.

Higuchi S, Matsushita S, Muramatsu T, Murayama M and Hayashida M (1996): Alcohol and aldehyde dehydrogenase genotypes and drinking behavior in Japanese. Alcohol Clin Exp Res 20:493-497.

Hill DB, Marsano L, Cohen D, Allen J, Shedlofsky S and McClain CJ (1992): Increased plasma interleukin-6 concentrations in alcoholic hepatitis. J Lab Clin Med 119:547-552.

Hill DB, Marsano LS and McClain CJ (1993): Increased plasma interleukin-8 concentrations in alcoholic hepatitis. Hepatology 18:576-580.

Hill DB, Deaciuc IV, Nanji AA and McClain CJ (1998): Mechanisms of hepatic injury in alcoholic liver disease. Clin Liver Dis 2:703-721.

Hillbom M, Numminen H and Juvela S (1999): Recent heavy drinking of alcohol and embolic stroke. Stroke 30:2307-2312.

Hines IN and Wheeler MD (2004): Recent advances in alcoholic liver disease III. Role of the innate immune response in alcoholic hepatitis. Am J Physiol Gastrointest Liver Physiol 287:G310-G314.

Hock B, Schwarz M, Domke I, Grunert VP, Wuertemberger M, Schiemann U, Horster S, Limmer C, Stecker G and Soyka M (2005): Validity of carbohydrate-deficient transferrin (%CDT), gamma-glutamyltransferase (gamma-GT) and mean corpuscular erythrocyte volume (MCV) as biomarkers for chronic alcohol abuse: a study in patients with alcohol dependence and liver disorders of non-alcoholic and alcoholic origin. Addiction 100:1477-1486.

Hoerner M, Behrens UJ, Worner TM, Blacksberg I, Braly LF, Schaffner F and Lieber CS (1988): The role of alcoholism and liver disease in the appearance of serum antibodies against acetaldehyde adducts. Hepatology 8:569-574.

Holstege A, Bedossa P, Poynard T, Kollinger M, Chaput JC, Houglum K and Chojkier M (1994): Acetaldehyde-modified epitopes in liver biopsy specimens of alcoholic and nonalcoholic patients: localization and association with progression of liver fibrosis. Hepatology 19:367-374.

Holt S (1981): Observations on the relation between alcohol absorption and the rate of gastric emptying. Can Med Assoc J 124:267-77, 297.

Horn PS and Pesce AJ (2003): Reference intervals: an update. Clin Chim Acta 334:5-23.

Huang YS, Chan CY, Wu JC, Pai CH, Chao Y and Lee SD (1996): Serum levels of interleukin-8 in alcoholic liver disease: relationship with disease stage, biochemical parameters and survival. J Hepatol 24:377-384.

Hultberg B, Isaksson A, Berglund M and Moberg AL (1991): Serum beta-hexosaminidase isoenzyme: a sensitive marker for alcohol abuse. Alcohol Clin Exp Res 15:549-552.

Huseby NE, Nilssen O, Erfurth A, Wetterling T and Kanitz RD (1997): Carbohydrate-deficient transferrin and alcohol dependency: variation in response to alcohol intake among different groups of patients. Alcohol Clin Exp Res 21:201-205.

Iimuro Y, Gallucci RM, Luster MI, Kono H and Thurman RG (1997): Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. Hepatology 26:1530-1537.

Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y and Poynard T (2001): Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet 357:1069-1075.

Iredale JP (2003): Cirrhosis: new research provides a basis for rational and targeted treatments. BMJ 327:143-147.

Iredale JP, Murphy G, Hembry RM, Friedman SL and Arthur MJ (1992): Human hepatic lipocytes synthesize tissue inhibitor of metalloproteinases-1. Implications for regulation of matrix degradation in liver. J Clin Invest 90:282-287.

Isaksson A, Blanche C, Hultberg B and Joelsson B (1985): Influence of ethanol on the human serum level of beta-hexosaminidase. Enzyme 33:162-166.

Israel Y, Hurwitz E, Niemelä O and Arnon R (1986): Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde-protein adducts. Proc Natl Acad Sci U S A 83:7923-7927.

Israel Y, Orrego H and Niemelä O (1988): Immune responses to alcohol metabolites: pathogenic and diagnostic implications. Semin Liver Dis 8:81-90.

Jacobson JL and Jacobson SW (2002): Effects of prenatal alcohol exposure on child development. Alcohol Res Health 26:282-286.

Järveläinen HA, Orpana A, Perola M, Savolainen VT, Karhunen PJ and Lindros KO (2001): Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease. Hepatology 33:1148-1153.

Javors MA and Johnson BA (2003): Current status of carbohydrate deficient transferrin, total serum sialic acid, sialic acid index of apolipoprotein J and serum beta-hexosaminidase as markers for alcohol consumption. Addiction 98 Suppl 2:45-50.

Jelski W, Chrostek L, Szmitkowski M and Laszewicz W (2002): Activity of class I, II, III, and IV alcohol dehydrogenase isoenzymes in human gastric mucosa. Dig Dis Sci 47:1554-1557.

Johansen JS, Christoffersen P, Møller S, Price PA, Henriksen JH, Garbarsch C and Bendtsen F (2000): Serum YKL-40 is increased in patients with hepatic fibrosis. J Hepatol 32:911-920.

Johnson RD, Horowitz M, Maddox AF, Wishart JM and Shearman DJ (1991): Cigarette smoking and rate of gastric emptying: effect on alcohol absorption. BMJ 302:20-23.

Jones KL and Smith DW (1973): Recognition of the fetal alcohol syndrome in early infancy. Lancet 2:999-1001.

Jones AW, Jönsson KA and Kechagias S (1997): Effect of high-fat, high-protein, and high-carbohydrate meals on the pharmacokinetics of a small dose of ethanol. Br J Clin Pharmacol 44:521-526.

Jörnvall H (1994): The alcohol dehydrogenase system. EXS 71:221-229.

Joshi-Barve S, Barve SS, Butt W, Klein J and McClain CJ (2003): Inhibition of proteasome function leads to NF-kappaB-independent IL-8 expression in human hepatocytes. Hepatology 38:1178-1187.

Ka T, Moriwaki Y, Takahashi S, Yamamoto A, Tsutsumi Z, Inokuchi T and Yamamoto T (2005): Effects of long-term beer ingestion on plasma concentrations and urinary excretion of purine bases. Horm Metab Res 37:641-645.

Kabat GC, Ng SK and Wynder EL (1993): Tobacco, alcohol intake, and diet in relation to adenocarcinoma of the esophagus and gastric cardia. Cancer Causes Control 4:123-132.

Kamal SM, Turner B, He Q, Rasenack J, Bianchi L, Al Tawil A, Nooman A, Massoud M, Koziel MJ and Afdhal NH (2006): Progression of fibrosis in hepatitis C with and without schistosomiasis: correlation with serum markers of fibrosis. Hepatology 43:771-779.

Kärkkäinen P, Poikolainen K and Salaspuro M (1990): Serum beta-hexosaminidase as a marker of heavy drinking. Alcohol Clin Exp Res 14:187-190.

Keilin D and Hartree EF (1945): Properties of catalase. Catalysis of coupled oxidation of alcohols. Biochem J 39:293-301.

Kenis G, Teunissen C, De Jongh R, Bosmans E, Steinbusch H and Maes M (2002): Stability of interleukin 6, soluble interleukin 6 receptor, inteleukin 10 and CC16 in human serum. Cytokine 19:228-235.

Kerr WC, Fillmore KM and Marvy P (2000): Beverage-specific alcohol consumption and cirrhosis mortality in a group of English-speaking beer-drinking countries. Addiction 95:339-346.

Khoruts A, Stahnke L, McClain CJ, Logan G and Allen JI (1991): Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. Hepatology 13:267-276.

Kirkpatrick RB, Emery JG, Connor JR, Dodds R, Lysko PG and Rosenberg M (1997): Induction and expression of human cartilage glycoprotein 39 in rheumatoid inflammatory and peripheral blood monocyte-derived macrophages. Exp Cell Res 237:46-54.

Klassen LW, Tuma D and Sorrell MF (1995): Immune mechanisms of alcohol-induced liver disease. Hepatology 22:355-357.

Klatsky AL and Armstrong MA (1992): Alcohol, smoking, coffee, and cirrhosis. Am J Epidemiol 136:1248-1257.

Kmiec Z (2001): Cooperation of liver cells in health and disease. Adv Anat Embryol Cell Biol 161:1–151.

Kohgo Y, Ohtake T, Ikuta K, Suzuki Y, Hosoki Y, Saito H and Kato J (2005): Iron accumulation in alcoholic liver diseases. Alcohol Clin Exp Res 29:189S-193S.

Koike H, Mori K, Misu K, Hattori N, Ito H, Hirayama M and Sobue G (2001): Painful alcoholic polyneuropathy with predominant small-fiber loss and normal thiamine status. Neurology 56:1727-1732.

Koop DR, Chernosky A and Brass EP (1991): Identification and induction of cytochrome P450 2E1 in rat Kupffer cells. J Pharmacol Exp Ther 258:1072-1076.

Koop I, Ilchmann R, Izzi L, Adragna A, Koop H and Barthelmes H (2000): Detection of autoantibodies against tissue transglutaminase in patients with celiac disease and dermatitis herpetiformis. Am J Gastroenterol 95:2009-2014.

Kopun M and Propping P (1977): The kinetics of ethanol absorption and elimination in twins and supplementary repetitive experiments in singleton subjects. Eur J Clin Pharmacol 11:337-344.

Koskinas J, Kenna JG, Bird GL, Alexander GJ and Williams R (1992): Immunoglobulin A antibody to a 200-kilodalton cytosolic acetaldehyde adduct in alcoholic hepatitis. Gastroenterology 103:1860-1867.

Kovacs EJ and Messingham KA (2002): Influence of alcohol and gender on immune response. Alcohol Res Health 26:257-263.

Kropf J, Gressner AM and Negwer A (1988): Efficacy of serum laminin measurement for diagnosis of fibrotic liver diseases. Clin Chem 34:2026-2030.

Kwo PY, Ramchandani VA, O'Connor S, Amann D, Carr LG, Sandrasegaran K, Kopecky KK and Li TK (1998): Gender differences in alcohol metabolism: rela-

tionship to liver volume and effect of adjusting for body mass. Gastroenterology 115:1552-1557.

Laposata M (1997): Fatty acid ethyl esters: short-term and long-term serum markers of ethanol intake. Clin Chem 43:1527-1534.

Laposata EA and Lange LG (1986): Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. Science 231:497-499.

Larroque B and Kaminski M (1998): Prenatal alcohol exposure and development at preschool age: main results of a French study. Alcohol Clin Exp Res 22:295-303.

Larroque B, Kaminski M, Dehaene P, Subtil D, Delfosse MJ and Querleu D (1995): Moderate prenatal alcohol exposure and psychomotor development at preschool age. Am J Public Health 85:1654-1661.

Laso FJ, Madruga JI, López A, Ciudad J, Alvarez-Mon M, San Miguel J and Orfao A (1996): Distribution of peripheral blood lymphoid subsets in alcoholic liver cirrhosis: influence of ethanol intake. Alcohol Clin Exp Res 20:1564-1568.

Laso FJ, Iglesias-Osma C, Ciudad J, López A, Pastor I and Orfao A (1999): Chronic alcoholism is associated with an imbalanced production of Th-1/Th-2 cytokines by peripheral blood T cells. Alcohol Clin Exp Res 23:1306-1311.

Latvala J, Melkko J, Parkkila S, Järvi K, Makkonen K and Niemelä O (2001a): Assays for acetaldehyde-derived adducts in blood proteins based on antibodies against acetaldehyde/lipoprotein condensates. Alcohol Clin Exp Res 25:1648-1653.

Latvala J, Parkkila S, Melkko J and Niemelä O (2001b): Acetaldehyde adducts in blood and bone marrow of patients with ethanol-induced erythrocyte abnormalities. Mol Med 7:401-405.

Latvala J, Parkkila S and Niemelä O (2004): Excess alcohol consumption is common in patients with cytopenia: studies in blood and bone marrow cells. Alcohol Clin Exp Res 28:619-624.

Latvala J, Hietala J, Koivisto H, Järvi K, Anttila P and Niemelä O (2005): Immune Responses to Ethanol Metabolites and Cytokine Profiles Differentiate Alcoholics with or without Liver Disease. Am J Gastroenterol 100:1303-1310.

Laurent TC and Fraser JR (1992): Hyaluronan. FASEB J 6:2397-2404.

Layke JC and Lopez PP (2006): Esophageal cancer: a review and update. Am Fam Physician 73:2187-2194.

Ledesma F, Echevarria S, Casafont F, Lozano JL and Pons-Romero F (1990): Natural killer cell activity in alcoholic cirrhosis: influence of nutrition. Eur J Clin Nutr 44:733-740.

Leevy CB and Elbeshbeshy HA (2005): Immunology of alcoholic liver disease. Clin Liver Dis 9:55-66.

Leevy CM and Moroianu SA (2005): Nutritional aspects of alcoholic liver disease. Clin Liver Dis 9:67-81.

Lefkowitch JH (2005): Morphology of alcoholic liver disease. Clin Liver Dis 9:37-53.

Legros FJ, Nuyens V, Minet E, Emonts P, Boudjeltia KZ, Courbe A, Ruelle JL, Colicis J, de L'Escaille F and Henry JP (2002): Carbohydrate-deficient transferrin isoforms measured by capillary zone electrophoresis for detection of alcohol abuse. Clin Chem 48:2177-2186.

Legros FJ, Nuyens V, Baudoux M, Zouaoui BK, Ruelle JL, Colicis J, Cantraine F and Henry JP (2003): Use of capillary zone electrophoresis for differentiating excessive from moderate alcohol consumption. Clin Chem 49:440-449.

Lesch OM, Walter H, Antal J, Heggli DE, Kovacz A, Leitner A, Neumeister A, Stumpf I, Sundrehagen E and Kasper S (1996a): Carbohydrate-deficient transferrin as a marker of alcohol intake: a study with healthy subjects. Alcohol Alcohol 31:265-271.

Lesch OM, Walter H, Antal J, Kanitz RD, Kovacz A, Leitner A, Marx B, Neumeister A, Saletu M, Semler B, Stumpf I and Mader R (1996b): Alcohol dependence: is carbohydrate-deficient transferrin a marker for alcohol intake? Alcohol Alcohol 31:257-264.

Li TK, Bosron WF, Dafeldecker WP, Lange LG and Vallee BL (1977): Isolation of pi-alcohol dehydrogenase of human liver: is it a determinant of alcoholism? Proc Natl Acad Sci U S A 74:4378-4381.

Li J, Rosman AS, Leo MA, Nagai Y and Lieber CS (1994): Tissue inhibitor of metalloproteinase is increased in the serum of precirrhotic and cirrhotic alcoholic patients and can serve as a marker of fibrosis. Hepatology 19:1418-1423.

Li CJ, Nanji AA, Siakotos AN and Lin RC (1997a): Acetaldehyde-modified and 4-hydroxynonenal-modified proteins in the livers of rats with alcoholic liver disease. Hepatology 26:650-657.

Li F, Cook RT, Alber C, Rasmussen W, Stapleton JT and Ballas ZK (1997b): Ethanol and natural killer cells. II. Stimulation of human natural killer activity by ethanol in vitro. Alcohol Clin Exp Res 21:981-987.

Lieber CS (1988): The microsomal ethanol oxidizing system: its role in ethanol and xenobiotic metabolism. Biochem Soc Trans 16:232-239.

Lieber CS (1995): Medical disorders of alcoholism. N Engl J Med 333:1058-1065.

Lieber CS (2003): Relationships between nutrition, alcohol use, and liver disease. Alcohol Res Health 27:220-231.

Lieber CS (2004): Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. Alcohol 34:9-19.

Lieber CS and DeCarli LM (1968): Ethanol oxidation by hepatic microsomes: adaptive increase after ethanol feeding. Science 162:917-918.

Lieber CS, Gentry RT and Baraona E (1994): First pass metabolism of ethanol. Alcohol Alcohol Suppl 2:163-169.

Lieber CS, Jones DP and DeCarli LM (1965): Effects of prolonged ethanol intake: production of fatty liver despite adequate diets. J Clin Invest 44:1009-1021.

Lin RC, Shahidi S, Kelly TJ, Lumeng C and Lumeng L (1993): Measurement of hemoglobin-acetaldehyde adduct in alcoholic patients. Alcohol Clin Exp Res 17:669-674.

Lin KC, Lin HY and Chou P (2000): The interaction between uric acid level and other risk factors on the development of gout among asymptomatic hyperuricemic men in a prospective study. J Rheumatol 27:1501-1505.

Lindenbaum J (1987): Hematologic complications of alcohol abuse. Semin Liver Dis 7:169-181.

Linneberg A, Petersen J, Grønbæk M and Benn CS (2004): Alcohol during pregnancy and atopic dermatitis in the offspring. Clin Exp Allergy 34:1678-1683.

Linnoila M, Mattila MJ and Kitchell BS (1979): Drug interactions with alcohol. Drugs 18:299-311.

Lo Iacono O, Petta S, Venezia G, Di Marco V, Tarantino G, Barbaria F, Mineo C, De Lisi S, Almasio PL and Craxì A (2005): Anti-tissue transglutaminase antibodies in patients with abnormal liver tests: is it always coeliac disease? Am J Gastroenterol 100:2472-2477.

Longnecker MP (1994): Alcoholic beverage consumption in relation to risk of breast cancer: meta-analysis and review. Cancer Causes Control 5:73-82.

Lu XL, Luo JY, Tao M, Gen Y, Zhao P, Zhao HL, Zhang XD and Dong N (2004): Risk factors for alcoholic liver disease in China. World J Gastroenterol 10:2423-2426.

Maddrey WC (2000): Alcohol-induced liver disease. Clin Liver Dis 4:115-31, vii.

Magnani M, Laguerre M, Rossi L, Bianchi M, Ninfali P, Mangani F and Ropars C (1990): In vivo accelerated acetaldehyde metabolism using acetaldehyde dehydrogenase-loaded erythrocytes. Alcohol Alcohol 25:627-637.

Maher JJ and McGuire RF (1990): Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. J Clin Invest 86:1641-1648.

Makkonen K, Viitala KI, Parkkila S and Niemelä O (2001): Serum IgG and IgE antibodies against mold-derived antigens in patients with symptoms of hypersensitivity. Clin Chim Acta 305:89-98.

Mann RE, Smart RG and Govoni R (2003): The epidemiology of alcoholic liver disease. Alcohol Res Health 27:209-219.

Marsano L and McClain CJ (1991): Nutrition and alcoholic liver disease. JPEN J Parenter Enteral Nutr 15:337-344.

Martin-Moreno JM, Boyle P, Gorgojo L, Willett WC, Gonzalez J, Villar F and Maisonneuve P (1993): Alcoholic beverage consumption and risk of breast cancer in Spain. Cancer Causes Control 4:345-353.

Maruyama K, Feinman L, Fainsilber Z, Nakano M, Okazaki I and Lieber CS (1982): Mammalian collagenase increases in early alcoholic liver disease and decreases with cirrhosis. Life Sci 30:1379-1384.

Maruyama S, Hirayama C, Yamamoto S, Koda M, Udagawa A, Kadowaki Y, Inoue M, Sagayama A and Umeki K (2001): Red blood cell status in alcoholic and non-alcoholic liver disease. J Lab Clin Med 138:332-337.

McClain CJ and Cohen DA (1989): Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. Hepatology 9:349-351.

McClain C, Hill D, Schmidt J and Diehl AM (1993): Cytokines and alcoholic liver disease. Semin Liver Dis 13:170-182.

McClain CJ, Shedlofsky S, Barve S and Hill DB (1997): Cytokines and alcoholic liver disease. Alcohol Health Res World 21:317-320.

McClain CJ, Barve S, Barve S, Deaciuc I and Hill DB (1998): Tumor necrosis factor and alcoholic liver disease. Alcohol Clin Exp Res 22:248S-252S.

McClain CJ, Barve S, Deaciuc I, Kugelmas M and Hill D (1999): Cytokines in alcoholic liver disease. Semin Liver Dis 19:205-219.

McClain CJ, Hill DB, Song Z, Deaciuc I and Barve S (2002): Monocyte activation in alcoholic liver disease. Alcohol 27:53-61.

McClain C, Barve S, Joshi-Barve S, Song Z, Deaciuc I, Chen T and Hill D (2005): Dysregulated cytokine metabolism, altered hepatic methionine metabolism and proteasome dysfunction in alcoholic liver disease. Alcohol Clin Exp Res 29:180S-188S.

McCullough AJ, Stassen WN, Wiesner RH and Czaja AJ (1987): Serum type III procollagen peptide concentrations in severe chronic active hepatitis: relationship to cirrhosis and disease activity. Hepatology 7:49-54.

McKillop IH and Schrum LW (2005): Alcohol and liver cancer. Alcohol 35:195-203.

Meerkerk GJ, Njoo KH, Bongers IM, Trienekens P and van Oers JA (1999): Comparing the diagnostic accuracy of carbohydrate-deficient transferrin, gamma-glutamyltransferase, and mean cell volume in a general practice population. Alcohol Clin Exp Res 23:1052-1059.

Melkko J, Niemi S, Risteli L and Risteli J (1990): Radioimmunoassay of the carboxyterminal propeptide of human type I procollagen. Clin Chem 36:1328-1332.

Melkko J, Hellevik T, Risteli L, Risteli J and Smedsrød B (1994): Clearence of NH_2-terminal propeptides of types I and III procollagen is a physiological function of the scavenger receptor in liver endothelial cells. J Exp Med 179:405–412.

Mendenhall CL, Moritz TE, Roselle GA, Morgan TR, Nemchausky BA, Tamburro CH, Schiff ER, McClain CJ, Marsano LS, Allen JI and . (1993): A study of oral nutritional support with oxandrolone in malnourished patients with alcoholic hepatitis: results of a Department of Veterans Affairs cooperative study. Hepatology 17:564-576.

Metzger MH, Heier M, Mäki M, Bravi E, Schneider A, Löwel H, Illig T, Schuppan D and Wichmann HE (2006): Mortality excess in individuals with elevated IgA anti-transglutaminase antibodies: the KORA/MONICA Augsburg cohort study 1989-1998. Eur J Epidemiol 21:359-365.

Midanik L (1982): Over-reports of recent alcohol consumption in a clinical population: a validity study. Drug Alcohol Depend 9:101-110.

Møller S, Hansen M, Hillingsø J, Jensen JE and Henriksen JH (1999): Elevated carboxy terminal cross linked telopeptide of type I collagen in alcoholic cirrhosis: relation to liver and kidney function and bone metabolism. Gut 44:417-423.

Monteiro MG and Masur J (1985): Diagnostic of alcoholism: how useful is the combination of gamma glutamyl transferase with different biochemical markers? Drug Alcohol Depend 16:31-37.

Moreno A, Pares A, Ortiz J, Enriquez J and Pares X (1994): Alcohol dehydrogenase from human stomach: variability in normal mucosa and effect of age, gender, ADH3 phenotype and gastric region. Alcohol Alcohol 29:663-671.

Morgan MY, Camilo ME, Luck W, Sherlock S and Hoffbrand AV (1981): Macrocytosis in alcohol-related liver disease: its value for screening. Clin Lab Haematol 3:35-44.

Mumenthaler MS, Taylor JL, O'Hara R and Yesavage JA (1999): Gender differences in moderate drinking effects. Alcohol Res Health 23:55-64.

Mundle G, Munkes J, Ackermann K and Mann K (2000): Sex differences of carbohydrate-deficient transferrin, gamma-glutamyltransferase, and mean corpuscular volume in alcohol-dependent patients. Alcohol Clin Exp Res 24:1400-1405.

Murawaki Y, Ikuta Y, Idobe Y, Kitamura Y and Kawasaki H (1997): Tissue inhibitor of metalloproteinase-1 in the liver of patients with chronic liver disease. J Hepatol 26:1213-1219.

Nagy LE (2003): Recent insights into the role of the innate immune system in the development of alcoholic liver disease. Exp Biol Med (Maywood) 228:882-890.

Nakamura T, Takebe K, Yamada N, Arai Y, Tando Y, Terada A, Ishii M, Kikuchi H, Machida K and Imamura K (1994): Bile acid malabsorption as a cause of hypocholesterolemia seen in patients with chronic pancreatitis. Int J Pancreatol 16:165-169.

Nalpas B, Hispard E, Thépot V, Pot S, Dally S and Berthelot P (1997): A comparative study between carbohydrate-deficient transferrin and gamma-glutamyltransferase for the diagnosis of excessive drinking in a liver unit. J Hepatol 27:1003-1008.

Nalpas B, Vassault A, Charpin S, Lacour B and Berthelot P (1986): Serum mitochondrial aspartate aminotransferase as a marker of chronic alcoholism: diagnostic value and interpretation in a liver unit. Hepatology 6:608-614.

Neuman MG (2003): Cytokines--central factors in alcoholic liver disease. Alcohol Res Health 27:307-316.

Neuman MG, Brenner DA, Rehermann B, Taieb J, Chollet-Martin S, Cohard M, Garaud JJ, Poynard T, Katz GG, Cameron RG, Shear NH, Gao B, Takamatsu M, Yamauchi M, Ohata M, Saito S, Maeyama S, Uchikoshi T, Toda G, Kumagi T, Akbar SM, Abe M, Michitaka K, Horiike N and Onji M (2001): Mechanisms of alcoholic liver disease: cytokines. Alcohol Clin Exp Res 25:251S-253S.

Niemelä O (1999): Aldehyde-protein adducts in the liver as a result of ethanol-induced oxidative stress. Front Biosci 4:D506-D513.

Niemelä O (2001): Distribution of ethanol-induced protein adducts in vivo: relationship to tissue injury. Free Radic Biol Med 31:1533-1538.

Niemelä O (2002): Serum Diagnosis of Alcoholic Liver Disease and Markers of Ethanol Intake. In: Ethanol and the Liver, pp. 411-449. Eds. DIN Sherman, V Preedy and RR Watson, Taylor and Francis, New York and London.

Niemelä O (2007): Biomarkers in alcoholism. Clin Chim Acta 377:39-49.

Niemelä O and Israel Y (1992): Hemoglobin-acetaldehyde adducts in human alcohol abusers. Lab Invest 67:246-252.

Niemelä O and Parkkila S (2004): Alcoholic macrocytosis--is there a role for acetaldehyde and adducts? Addict Biol 9:3-10.

Niemelä O, Risteli L, Sotaniemi EA and Risteli J (1983): Aminoterminal propeptide of type III procollagen in serum in alcoholic liver disease. Gastroenterology 85:254-259.

Niemelä O, Klajner F, Orrego H, Vidins E, Blendis L and Israel Y (1987): Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. Hepatology 7:1210-1214.

Niemelä O, Risteli J, Blake JE, Risteli L, Compton KV and Orrego H (1990): Markers of fibrogenesis and basement membrane formation in alcoholic liver disease. Relation to severity, presence of hepatitis, and alcohol intake. Gastroenterology 98:1612-1619.

Niemelä O, Blake JE and Orrego H (1992): Serum type I collagen propeptide and severity of alcoholic liver disease. Alcohol Clin Exp Res 16:1064-1067.

Niemelä O, Parkkila S, Pasanen M, Iimuro Y, Bradford B, Thurman RG (1998): Early alcoholic liver injury: formation of protein adducts with acetaldehyde and lipid peroxidation products, and expression of CYP2E1 and CYP3A. Alcohol Clin Exp Res 22:2118-2124.

Niemelä O, Parkkila S, Pasanen M, Viitalal K, Villenueva JA and Halsted CS (1999): Induction of cytochrome P450 enzymes and generation of protein-aldehyde adducts are associated with sex-dependent sensitivity to alcohol-induced liver disease in micropigs. Hepatology 30:1011-1017.

Nishioka K, Sumida T, Iwatani M, Kusumoto A, Ishikura Y, Hatanaka H, Yomo H, Kohda H, Ashikari T, Shibano Y and Suwa Y (2002): Influence of moderate drinking on purine and carbohydrate metabolism. Alcohol Clin Exp Res 26:20S-25S.

Nøjgaard C, Johansen JS, Christensen E, Skovgaard LT, Price PA and Becker U (2003): Serum levels of YKL-40 and PIIINP as prognostic markers in patients with alcoholic liver disease. J Hepatol 39:179-186.

Nulman I, Rovet J, Kennedy D, Wasson C, Gladstone J, Fried S and Koren G (2004): Binge alcohol consumption by non-alcohol-dependent women during pregnancy affects child behaviour, but not general intellectual functioning; a prospective controlled study. Arch Womens Ment Health 7:173-181.

Nyblom H, Berggren U, Balldin J and Olsson R (2004): High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. Alcohol Alcohol 39:336-339.

Nyström M, Peräsalo J, Pikkarainen J and Salaspuro M (1993): Conventional laboratory tests as indicators of heavy drinking in young university students. Scand J Prim Health Care 11:44-49.

Okabe R, Nakatsuka K, Inaba M, Miki T, Naka H, Masaki H, Moriguchi A and Nishizawa Y (2001): Clinical evaluation of the Elecsys beta-CrossLaps serum assay, a new assay for degradation products of type I collagen C-tlopeptides. Clin Chem 47:1410-1414.

Oneta CM, Simanowski UA, Martinez M, Allali-Hassani A, Parés X, Homann N, Conradt C, Waldherr R, Fiehn W, Coutelle C and Seitz HK (1998): First pass metabolism of ethanol is strikingly influenced by the speed of gastric emptying. Gut 43:612-619.

Oneta CM, Pedrosa M, Ruttimann S, Russell RM and Seitz HK (2001): Age and bioavailability of alcohol. Z Gastroenterol 39:783-788.

Orrego H, Israel Y, Blake JE and Medline A (1983): Assessment of prognostic factors in alcoholic liver disease: toward a global quantitative expression of severity. Hepatology 3:896-905.

Orrego H, Blake JE, Blendis LM and Medline A (1987): Prognosis of alcoholic cirrhosis in the presence and absence of alcoholic hepatitis. Gastroenterology 92:208-214.

Oslin DW, Pettinati HM, Luck G, Semwanga A, Cnaan A and O'Brien CP (1998): Clinical correlations with carbohydrate-deficient transferrin levels in women with alcoholism. Alcohol Clin Exp Res 22:1981-1985.

Palmer RH, Frank WO, Nambi P, Wetherington JD and Fox MJ (1991): Effects of various concomitant medications on gastric alcohol dehydrogenase and the first-pass metabolism of ethanol. Am J Gastroenterol 86:1749-1755.

Paradis V, Scoazec JY, Köllinger M, Holstege A, Moreau A, Feldmann G and Bedossa P (1996): Cellular and subcellular localization of acetaldehyde-protein adducts in liver biopsies from alcoholic patients. J Histochem Cytochem 44:1051-1057.

Pares A, Caballeria J, Bruguera M, Torres M and Rodes J (1986): Histological course of alcoholic hepatitis. Influence of abstinence, sex and extent of hepatic damage. J Hepatol 2:33-42.

Parlesak A, Billinger MH, Bode C and Bode JC (2002): Gastric alcohol dehydrogenase activity in man: influence of gender, age, alcohol consumption and smoking in a caucasian population. Alcohol Alcohol 37:388-393.

Pedrosa MC, Russell RM, Saltzman JR, Golner BB, Dallal GE, Sepe TE, Oates E, Egerer G and Seitz HK (1996): Gastric emptying and first-pass metabolism of ethanol in elderly subjects with and without atrophic gastritis. Scand J Gastroenterol 31:671-677.

Pemberton PW, Smith A and Warnes TW (2005): Non-invasive monitoring of oxidant stress in alcoholic liver disease. Scand J Gastroenterol 40:1102-1108.

Petersen JM and Forsmark CE (2002): Chronic pancreatitis and maldigestion. Semin Gastrointest Dis 13:191-199.

Petersen DR, Atkinson N and Hjelle JJ (1982): Increase in hepatic microsomal ethanol oxidation by a single dose of ethanol. J Pharmacol Exp Ther 221:275-281.

Peterson CM, Jovanovic-Peterson L and Schmid-Formby F (1988): Rapid association of acetaldehyde with hemoglobin in human volunteers after low dose ethanol. Alcohol 5:371-374.

Petri AL, Tjonneland A, Gamborg M, Johansen D, Hoidrup S, Sorensen TI and Gronbaek M (2004): Alcohol intake, type of beverage, and risk of breast cancer in pre- and postmenopausal women. Alcohol Clin Exp Res 28:1084-1090.

Phillips MG, Preedy VR and Hughes RD (2003): Assessment of prognosis in alcoholic liver disease: can serum hyaluronate replace liver biopsy? Eur J Gastroenterol Hepatol 15:941-944.

Pönniö M, Alho H, Heinälä P, Nikkari ST and Sillanaukee P (1999a): Serum and saliva levels of sialic acid are elevated in alcoholics. Alcohol Clin Exp Res 23:1060-1064.

Pönniö M, Alho H, Nikkari ST, Olsson U, Rydberg U and Sillanaukee P (1999b): Serum sialic acid in a random sample of the general population. Clin Chem 45:1842-1849.

Pöschl G and Seitz HK (2004): Alcohol and cancer. Alcohol Alcohol 39:155-165.

Pozzato G, Moretti M, Franzin F, Croce LS, Lacchin T, Benedetti G, Sablich R, Stebel M and Campanacci L (1995): Ethanol metabolism and aging: the role of "first pass metabolism" and gastric alcohol dehydrogenase activity. J Gerontol A Biol Sci Med Sci 50:B135-B141.

Pragst F, Auwaerter V, Sporkert F and Spiegel K (2001): Analysis of fatty acid ethyl esters in hair as possible markers of chronically elevated alcohol consumption by headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS). Forensic Sci Int 121:76-88.

Pullarkat RK and Raguthu S (1985): Elevated urinary dolichol levels in chronic alcoholics. Alcohol Clin Exp Res 9:28-30.

Purohit V and Brenner DA (2006): Mechanisms of alcohol-induced hepatic fibrosis: a summary of the Ron Thurman Symposium. Hepatology 43:872-878.

Puukka K, Hietala J, Koivisto H, Anttila P, Bloigu R and Niemela O (2006): Additive effects of moderate drinking and obesity on serum gamma-glutamyl transferase activity. Am J Clin Nutr 83:1351-1354.

Qvist P, Christgau S, Pedersen BJ, Schlemmer A and Christiansen C (2002): Circadian variation in the serum concentration of C-terminal telopeptide if type I collagen (serum CTx): effects of gender, age, menopausal status, posture, daylight, serum cortisol, and fasting. Bone 31:57-61.

Qvist P, Munk M, Hoyle N and Christiansen C (2004): Serum and plasma fragments of C-telopeptides of type I collagen (CTX) are stable during storage at low temperatures for 3 years. Clin Chim Acta 350:167-173.

Racanelli V and Rehermann B (2006): The liver as an immunological organ. Hepatology 43:S54-S62.

Ramchandani VA, Kwo PY and Li TK (2001): Effect of food and food composition on alcohol elimination rates in healthy men and women. J Clin Pharmacol 41:1345-1350.

Recklies AD, White C and Ling H (2002): The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. Biochem J 365:119-126.

Reichel H, Roth HJ and Schmidt-Gayk H (2004): Evaluation of serum beta-carboxy-terminal cross-linking telopeptide of type I collagen as marker of bone resorption in chronic hemodialysis patients. Nephron Clin Pract 98:c112-c118.

Reynaud M, Schellenberg F, Loisequx-Meunier MN, Schwan R, Maradeix B, Planche F and Gillet C (2000): Objective diagnosis of alcohol abuse: compared values of carbohydrate-deficient transferrin (CDT), gamma-glutamyl transferase (GGT), and mean corpuscular volume (MCV). Alcohol Clin Exp Res 24:1414-1419.

Ricard-Blum S, Chossegros P, Guerret S, Trepo C, Grimaud JA and Chevallier M (1996): The carboxy-terminal cross-linked telopeptide of type I collagen (ICTP) is a potential serum marker of ongoing liver fibrosis. Clin Chim Acta 248:187-195.

Rintala J, Jaatinen P, Parkkila S, Sarviharju M, Kiianmaa K, Hervonen A and Niemelä O (2000): Evidence of acetaldehyde-protein adduct formation in rat brain after lifelong consumption of ethanol. Alcohol Alcohol 35:458-463.

Risteli L and Risteli J (1987): Analysis of extracellular matrix proteins in biological fluids. Method Enzymol 145:391-411.

Risteli J, Melkko J, Niemi S and Risteli L (1991): Use of a marker of collagen formation in osteoporosis studies. Calcif Tissue Int 49 Suppl:S24-S25.

Rodríguez-Rodríguez E, González-Reimers E, Santolaria-Fernández F, Milena-Abril A, Rodríguez-Moreno F, Oramas-Rodríguez J and Martínez-Riera A (1995): Cytokine levels in acute alcoholic hepatitis: a sequential study. Drug Alcohol Depend 39:23-27.

Roine RP, Turpeinen U, Ylikahri R and Salaspuro M (1987): Urinary dolichol--a new marker of alcoholism. Alcohol Clin Exp Res 11:525-527.

Roine RP, Nykänen I, Ylikahri R, Heikkilä J, Suokas A and Salaspuro M (1989): Effect of alcohol on blood dolichol concentration. Alcohol Clin Exp Res 13:519-522.

Roine RP, Gentry RT, Lim RT, Jr., Baraona E and Lieber CS (1991): Effect of concentration of ingested ethanol on blood alcohol levels. Alcohol Clin Exp Res 15:734-738.

Roine R, Nykanen I and Salaspuro M (1992): Effect of alcohol on urinary and blood dolichols. Biochem Cell Biol 70:404-407.

Roine RP, Gentry RT, Lim RT, Jr., Helkkonen E, Salaspuro M and Lieber CS (1993): Comparison of blood alcohol concentrations after beer and whiskey. Alcohol Clin Exp Res 17:709-711.

Roizen R, Kerr WC and Fillmore KM (1999): Cirrhosis mortality and per capita consumption of distilled spirits, United States, 1949-94: trend analysis. BMJ 319:666-670.

Rojkind M, Giambrone MA and Biempica L (1979): Collagen types in normal and cirrhotic liver. Gastroenterology 76:710-719.

Romppanen J, Punnonen K, Anttila P, Jakobsson T, Blake J and Niemelä O (2002): Serum sialic acid as a marker of alcohol consumption: effect of liver disease and heavy drinking. Alcohol Clin Exp Res 26:1234-1238.

Room R, Babor T and Rehm J (2005): Alcohol and public health. Lancet 365:519-530.

Rosen HN, Moses AC, Garber J, Iloputaife ID, Ross DS, Lee SL and Greenspan SL (2000): Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. Calcif Tissue Int 66:100-103.

Rosman AS and Lieber CS (1992): On overview of current and emerging markers of alcoholism. In: Measuring Alcohol Consumption. Psychosocial and Biochemical Methods, pp. 99-134. Eds. RZ Litten and JP Allen, The Humana Press Inc., Totowa, New Jersey.

Rosman AS and Lieber CS (1994): Diagnostic utility of laboratory tests in alcoholic liver disease. Clin Chem 40:1641-1651.

Salaspuro M (1996): Bacteriocolonic pathway for ethanol oxidation: characteristics and implications. Ann Med 28:195-200.

Salaspuro M (1999): Carbohydrate-deficient transferrin as compared to other markers of alcoholism: a systematic review. Alcohol 19:261-271.

Salmela KS, Laitinen K, Nyström M and Salaspuro M (1994): Carbohydrate-deficient transferrin during 3 weeks' heavy alcohol consumption. Alcohol Clin Exp Res 18:228-230.

Salmela KS, Sillanaukee P, Itälä L, Väkeväinen S, Salaspuro M and Roine RP (1997): Binding of acetaldehyde to rat gastric mucosa during ethanol oxidation. J Lab Clin Med 129:627-633.

Salmela KS, Kessova IG, Tsyrlov IB and Lieber CS (1998): Respective roles of human cytochrome P-4502E1, 1A2, and 3A4 in the hepatic microsomal ethanol oxidizing system. Alcohol Clin Exp Res 22:2125-2132.

Sarkola T, Eriksson CJP, Niemelä O, Sillanaukee P and Halmesmäki E (2000): Mean cell volume and gamma-glutamyl transferase are superior to carbohydrate-deficient transferrin and hemoglobin-acetaldehyde adducts in the follow-up of pregnant women with alcohol abuse. Acta Obstet Gynecol Scand 79:359-366.

Sarkola T, Dahl H, Eriksson CJP and Helander A (2003): Urinary ethyl glucuronide and 5-hydroxytryptophol levels during repeated ethanol ingestion in healthy human subjects. Alcohol Alcohol 38:347-351.

Sass DA and Shaikh OS (2006): Alcoholic hepatitis. Clin Liver Dis 10:219-237, vii.

Savage DG, Ogundipe A, Allen RH, Stabler SP and Lindenbaum J (2000): Etiology and diagnostic evaluation of macrocytosis. Am J Med Sci 319:343-352.

Savola O, Niemelä O and Hillbom M (2004): Blood alcohol is the best indicator of hazardous alcohol drinking in young adults and working-age patients with trauma. Alcohol Alcohol 39:340-345.

Savola O, Niemelä O and Hillbom M (2005): Alcohol intake and the pattern of trauma in young adults and working aged people admitted after trauma. Alcohol Alcohol 40:269-273.

Schatzkin A and Longnecker MP (1994): Alcohol and breast cancer. Where are we now and where do we go from here? Cancer 74:1101-1110.

Schellenberg F, Schwan R, Mennetrey L, Loiseaux MN, Pagès JC and Reynaud M (2005): Dose-effect relation between daily ethanol intake in the range 0-70 grams and %CDT value: validation of a cut-off value. Alcohol Alcohol 40:531-534.

Schenker S (1997): Medical consequences of alcohol abuse: is gender a factor? Alcohol Clin Exp Res 21:179-181.

Schoppet M and Maisch B (2001): Alcohol and the heart. Herz 26:345-352.

Schwan R, Loiseaux MN, Schellenberg F, Albuisson E, Favre JD, Rigaud A, Llorca PM, Gillet C and Reynaud M (2004): Multicenter validation study of the %CDT TIA kit in alcohol abuse and alcohol dependence. Alcohol Clin Exp Res 28:1331-1337.

Schytte S, Hansen M, Møller S, Junker P, Henriksen JH, Hillingsø J and Teisner B (1999): Hepatic and renal extraction of circulating type I procollagen amino-propeptide in patients with normal liver function and in patients with alcoholic cirrhosis. Scand J Clin Lab Invest 59:627-633.

Seitz HK, Simanowski UA, Egerer G, Waldherr R and Oertl U (1992): Human gastric alcohol dehydrogenase: in vitro characteristics and effect of cimetidine. Digestion 51:80-85.

Seitz HK, Gartner U, Egerer G and Simanowski UA (1994): Ethanol metabolism in the gastrointestinal tract and its possible consequences. Alcohol Alcohol Suppl 2:157-162.

Seitz HK, Maurer B and Stickel F (2005): Alcohol consumption and cancer of the gastrointestinal tract. Dig Dis 23:297-303.

Seppä K, Laippala P and Saarni M (1991): Macrocytosis as a consequence of alcohol abuse among patients in general practice. Alcohol Clin Exp Res 15:871-876.

Sharpe PC (2001): Biochemical detection and monitoring of alcohol abuse and abstinence. Ann Clin Biochem 38:652-664.

Sherlock S (1995): Alcoholic liver disease. Lancet 345:227-229.

Sheth K and Bankey P (2001): The liver as an immune organ. Curr Opin Crit Care 7:99-104.

Siegmund SV and Brenner DA (2005): Molecular pathogenesis of alcohol-induced hepatic fibrosis. Alcohol Clin Exp Res 29:102S-109S.

Sillanaukee P and Olsson U (2001): Improved diagnostic classification of alcohol abusers by combining carbohydrate-deficient transferrin and gamma-glutamyltransferase. Clin Chem 47:681-685.

Sillanaukee P, Seppä K, Koivula T, Israel Y and Niemelä O (1992): Acetaldehyde-modified hemoglobin as a marker of alcohol consumption: comparison of two new methods. J Lab Clin Med 120:42-47.

Sillanaukee P, Koivula T, Jokela H, Myllyharju H and Seppä K (1993): Relationship of alcohol consumption to changes in HDL-subfractions. Eur J Clin Invest 23:486-491.

Sillanaukee P, Aalto M and Seppä K (1998): Carbohydrate-deficient transferrin and conventional alcohol markers as indicators for brief intervention among heavy drinkers in primary health care. Alcohol Clin Exp Res 22:892-896.

Sillanaukee P, Pönniö M and Jääskeläinen IP (1999a): Occurrence of sialic acids in healthy humans and different disorders. Eur J Clin Invest 29:413-425.

Sillanaukee P, Pönniö M and Seppä K (1999b): Sialic acid: new potential marker of alcohol abuse. Alcohol Clin Exp Res 23:1039-1043.

Sillanaukee P, Massot N, Jousilahti P, Vartiainen E, Poikolainen K, Olsson U and Alho H (2000a): Enhanced clinical utility of gamma-CDT in a general population. Alcohol Clin Exp Res 24:1202-1206.

Sillanaukee P, Massot N, Jousilahti P, Vartiainen E, Sundvall J, Olsson U, Poikolainen K, Pönniö M, Allen JP and Alho H (2000b): Dose response of laboratory markers to alcohol consumption in a general population. Am J Epidemiol 152:747-751.

Silvain C, Patry C, Launay P, Lehuen A and Monteiro RC (1995): Altered expression of monocyte IgA Fc receptors is associated with defective endocytosis in patients with alcoholic cirrhosis. Potential role for IFN-gamma. J Immunol 155:1606-1618.

Sood B, Delaney-Black V, Covington C, Nordstrom-Klee B, Ager J, Templin T, Janisse J, Martier S and Sokol RJ (2001): Prenatal alcohol exposure and childhood behavior at age 6 to 7 years: I. dose-response effect. Pediatrics 108:E34.

Stakes – National Research and Development Centre for Welfare and Health (2005): Yearbook of Alcohol and Drug Statistics 2005. Gummerus Kirjapaino Oy, Vaajakoski.

Stefenelli N, Klotz H, Engel A and Bauer P (1985): Serum sialic acid in malignant tumors, bacterial infections, and chronic liver diseases. J Cancer Res Clin Oncol 109:55-59.

Stephens P, Grenard P, Aeschlimann P, Langley M, Blain E, Errington R, Kipling D, Thomas D and Aeschlimann D (2004): Crosslinking and G-protein functions of transglutaminase 2 contribute differentially to fibroblast wound healing responses. J Cell Sci 117:3389-3403.

Stetter F, Gaertner HJ, Wiatr G, Mann K and Breyer-Pfaff U (1991): Urinary dolichol--a doubtful marker of alcoholism. Alcohol Clin Exp Res 15:938-941.

Stevens VJ, Fantl WJ, Newman CB, Sims RV, Cerami A and Peterson CM (1981): Acetaldehyde adducts with hemoglobin. J Clin Invest 67:361-369.

Stewart S, Jones D and Day CP (2001): Alcoholic liver disease: new insights into mechanisms and preventative strategies. Trends Mol Med 7:408-413.

Stewart SF, Vidali M, Day CP, Albano E and Jones DE (2004): Oxidative stress as a trigger for cellular immune responses in patients with alcoholic liver disease. Hepatology 39:197-203.

Stibler H (1991): Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed. Clin Chem 37:2029-2037.

Stibler H and Kjellin KG (1976): Isoelectric focusing and electrophoresis of the CSF proteins in tremor of different origins. J Neurol Sci 30:269-285.

Stibler H, Allgulander C, Borg S and Kjellin KG (1978): Abnormal microheterogeneity of transferrin in serum and cerebrospinal fluid in alcoholism. Acta Med Scand 204:49-56.

Stibler H, Borg S and Beckman G (1988): Transferrin phenotype and level of carbohydrate-deficient transferrin in healthy individuals. Alcohol Clin Exp Res 12:450-453.

Stickel F, Urbaschek R, Schuppan D, Poeschl G, Oesterling C, Conradt C, McCuskey RS, Simanowski UA and Seitz HK (2001): Serum collagen type VI and XIV and hyaluronic acid as early indicators for altered connective tissue turnover in alcoholic liver disease. Dig Dis Sci 46:2025-2032.

Stickel F, Hoehn B, Schuppan D and Seitz HK (2003a): Review article: Nutritional therapy in alcoholic liver disease. Aliment Pharmacol Ther 18:357-373.

Stickel F, Poeschl G, Schuppan D, Conradt C, Strenge-Hesse A, Fuchs FS, Hofmann WJ and Seitz HK (2003b): Serum hyaluronate correlates with histological progression in alcoholic liver disease. Eur J Gastroenterol Hepatol 15:945-950.

Stoler JM, Huntington KS, Peterson CM, Peterson KP, Daniel P, Aboagye KK, Lieberman E, Ryan L and Holmes LB (1998): The prenatal detection of significant alcohol exposure with maternal blood markers. J Pediatr 133:346-352.

Streissguth AP, Barr HM and Sampson PD (1990): Moderate prenatal alcohol exposure: effects on child IQ and learning problems at age 7 1/2 years. Alcohol Clin Exp Res 14:662-669.

Sulkanen S, Halttunen T, Laurila K, Kolho KL, Korponay-Szabó IR, Sarnesto A, Savilahti E, Collin P and Mäki M (1998): Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology 115:1322-1328.

Sutker PB, Goist KC, Jr. and King AR (1987): Acute alcohol intoxication in women: relationship to dose and menstrual cycle phase. Alcohol Clin Exp Res 11:74-79.

Świątkowska-Stodulska R, Bąkowska A and Drobińska-Jurowiecka A (2006): Interleukin-8 in the blood serum of patients with alcoholic liver disease. Med Sci Monit 12:CR215-CR220.

Szabo G (1999): Consequences of alcohol consumption on host defence. Alcohol Alcohol 34:830-841.

Szabo G, Catalano D, White B and Mandrekar P (2004): Acute alcohol consumption inhibits accessory cell function of monocytes and dendritic cells. Alcohol Clin Exp Res 28:824-828.

Taracha E, Habrat B, Lehner M, Wislowska A, Woronowicz BT, Bogulas M, Charewicz J, Markuszewski C and Plaźnik A (2004): Alanine aminopeptidase activity in urine: a new marker of chronic alcohol abuse? Alcohol Clin Exp Res 28:729-735.

Teare JP, Sherman D, Greenfield SM, Simpson J, Bray G, Catterall AP, Murray-Lyon IM, Peters TJ, Williams R and Thompson RP (1993): Comparison of serum procollagen III peptide concentrations and PGA index for assessment of hepatic fibrosis. Lancet 342:895-898.

Thomasson HR (1995): Gender differences in alcohol metabolism. Physiological responses to ethanol. Recent Dev Alcohol 12:163-179.

Thomson AD (2000): Mechanisms of vitamin deficiency in chronic alcohol misusers and the development of the Wernicke-Korsakoff syndrome. Alcohol Alcohol Suppl 35:2-7.

Thomson AD and Marshall EJ (2006): The natural history and pathophysiology of Wernicke's Encephalopathy and Korsakoff's Psychosis. Alcohol Alcohol 41:151-158.

Thurman RG (1998): II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. Am J Physiol 275:G605-G611.

Torres-Salinas M, Pares A, Caballeria J, Jimenez W, Heredia D, Bruguera M and Rodes J (1986): Serum procollagen type III peptide as a marker of hepatic fibrogenesis in alcoholic hepatitis. Gastroenterology 90:1241-1246.

Traving C and Schauer R (1998): Structure, function and metabolism of sialic acids. Cell Mol Life Sci 54:1330-1349.

Trinchet JC, Hartmann DJ, Pateron D, Munz-Gotheil C, Callard P, Ville G and Beaugrand M (1992): Serum type I collagen and N-terminal peptide of type III procollagen in patients with alcoholic liver disease: relationship to liver histology. Alcohol Clin Exp Res 16:342-346.

Tsukamoto H (2005): Fat paradox in liver disease. Keio J Med 54:190-192.

Tsutsumi M, Lasker JM, Shimizu M, Rosman AS and Lieber CS (1989): The intralobular distribution of ethanol-inducible P450IIE1 in rat and human liver. Hepatology 10:437-446.

Tsutsumi M, Takase S, Urashima S, Ueshima Y, Kawahara H and Takada A (1996): Serum markers for hepatic fibrosis in alcoholic liver disease: which is the best marker, type III procollagen, type IV collagen, laminin, tissue inhibitor of metalloproteinase, or prolyl hydroxylase? Alcohol Clin Exp Res 20:1512-1517.

Tsutsumi M, Urashima S, Takase S, Ueshima Y, Tsuchishima M, Shimanaka K and Kawahara H (1997): Characteristics of serum hyaluronate concentrations in patients with alcoholic liver disease. Alcohol Clin Exp Res 21:1716-1721.

Tuma DJ and Klassen LW (1992): Immune responses to acetaldehyde-protein adducts: role in alcoholic liver disease. Gastroenterology 103:1969-1973.

Tuma DJ and Sorrell MF (1987): Functional consequences of acetaldehyde binding to proteins. Alcohol Alcohol Suppl 1:61-66.

Tuma DJ, Newman MR, Donohue TM, Jr. and Sorrell MF (1987): Covalent binding of acetaldehyde to proteins: participation of lysine residues. Alcohol Clin Exp Res 11:579-584.

Tuma DJ, Hoffman T and Sorrell MF (1991): The chemistry of acetaldehyde-protein adducts. Alcohol Alcohol Suppl 1:271-276.

Tverdal A and Skurtveit S (2003): Coffee intake and mortality from liver cirrhosis. Ann Epidemiol 13:419-423.

Urashima S, Tsutsumi M, Shimanaka K, Ueshima Y, Tsuchishima M, Itoh T, Kawahara H and Takase S (1999): Histochemical study of hyaluronate in alcoholic liver disease. Alcohol Clin Exp Res 23:56S-60S.

Valkonen KH and Goldman D (1988): Purification and characterization of three forms of class III alcohol dehydrogenase. Electrophoresis 9:132-135.

van de Wiel A, van Hattum J, Schuurman HJ and Kater L (1988): Immunoglobulin A in the diagnosis of alcoholic liver disease. Gastroenterology 94:457-462.

Van Gossum A and Neve J (1995): Low selenium status in alcoholic cirrhosis is correlated with aminopyrine breath test. Preliminary effects of selenium supplementation. Biol Trace Elem Res 47:201-207.

Varga A, Hansson P, Lundqvist C and Alling C (1998): Phosphatidylethanol in blood as a marker of ethanol consumption in healthy volunteers: comparison with other markers. Alcohol Clin Exp Res 22:1832-1837.

Vecchi M, Folli C, Donato MF, Formenti S, Arosio E and de Franchis R (2003): High rate of positive anti-tissue transglutaminase antibodies in chronic liver disease. Role of liver decompensation and of the antigen source. Scand J Gastroenterol 38:50-54.

Vidali M, Stewart SF, Rolla R, Daly AK, Chen Y, Mottaran E, Jones DE, Leathart JB, Day CP and Albano E (2003): Genetic and epigenetic factors in autoimmune reactions toward cytochrome P4502E1 in alcoholic liver disease. Hepatology 37:410-419.

Viitala K, Israel Y, Blake JE and Niemelä O (1997): Serum IgA, IgG, and IgM antibodies directed against acetaldehyde-derived epitopes: relationship to liver disease severity and alcohol consumption. Hepatology 25:1418-1424.

Viitala K, Makkonen K, Israel Y, Lehtimäki T, Jaakkola O, Koivula T, Blake JE and Niemelä O (2000): Autoimmune responses against oxidant stress and acetaldehyde-derived epitopes in human alcohol consumers. Alcohol Clin Exp Res 24:1103-1109.

Villalta D, Crovatto M, Stella S, Tonutti E, Tozzoli R and Bizzaro N (2005): False positive reactions for IgA and IgG anti-tissue transglutaminase antibodies in liver cirrhosis are common and method-dependent. Clin Chim Acta 356:102-109.

Visapää JP, Jokelainen K, Nosova T and Salaspuro M (1998): Inhibition of intracolonic acetaldehyde production and alcoholic fermentation in rats by ciprofloxacin. Alcohol Clin Exp Res 22:1161-1164.

Vittadini G, Buonocore M, Colli G, Terzi M, Fonte R and Biscaldi G (2001): Alcoholic polyneuropathy: a clinical and epidemiological study. Alcohol Alcohol 36:393-400.

Voltaire A, Beck O and Borg S (1992): Urinary 5-hydroxytryptophol: a possible marker of recent alcohol consumption. Alcohol Clin Exp Res 16:281-285.

Wakai K, Kojima M, Tamakoshi K, Watanabe Y, Hayakawa N, Suzuki K, Hashimoto S, Kawado M, Tokudome S, Suzuki S, Ozasa K, Toyoshima H, Ito Y and Tamakoshi A (2005): Alcohol consumption and colorectal cancer risk: findings from the JACC Study. J Epidemiol 15 Suppl 2:S173-S179.

Wall TL and Ehlers CL (1995): Acute effects of alcohol on P300 in Asians with different ALDH2 genotypes. Alcohol Clin Exp Res 19:617-622.

Wannamethee SG and Shaper AG (2002): Taking up regular drinking in middle age: effect on major coronary heart disease events and mortality. Heart 87:32-36.

Ware LB and Matthay MA (2000): The acute respiratory distress syndrome. N Engl J Med 342:1334-1349.

Watson CG, Tillerskjor C, Hoodecheck-Schow EA, Pucel J and Jacobs L (1984): Do alcoholics give valid self-reports? J Stud Alcohol 45:344-348.

Wedel M, Pieters JE, Pikaar NA and Ockhuizen T (1991): Application of a three-compartment model to a study of the effects of sex, alcohol dose and concentration, exercise and food consumption on the pharmacokinetics of ethanol in healthy volunteers. Alcohol Alcohol 26:329-336.

Wehr H, Rodo M, Lieber CS and Baraona E (1993): Acetaldehyde adducts and autoantibodies against VLDL and LDL in alcoholics. J Lipid Res 34:1237-1244.

Weiler-Normann C and Rehermann B (2004): The liver as an immunological organ. J Gastroenterol Hepatol 19:S279-S283.

Wheeler MD (2003): Endotoxin and Kupffer cell activation in alcoholic liver disease. Alcohol Res Health 27:300-306.

Whitcomb DC (2005): Genetic polymorphisms in alcoholic pancreatitis. Dig Dis 23:247-254.

Whitfield JB (2001): Gamma glutamyl transferase. Crit Rev Clin Lab Sci 38:263-355.

WHO – World Health Organization (2004): Global Status Report on Alcohol 2004.

Wickramasinghe SN and Malik F (1986): Acetaldehyde causes a prolongation of the doubling time and an increase in the modal volume of cells in culture. Alcohol Clin Exp Res 10:350-354.

Wilfred de Alwis NM and Day CP (2007): Genetics of alcoholic liver disease and nonoalcoholic Fatty liver disease. Semin Liver Dis 27:44-54.

Wilke A, Kaiser A, Ferency I and Maisch B (1996): [Alcohol and myocarditis]. Herz 21:248-257.

Wodzinska JM (2005): Transglutaminases as targets for pharmacological inhibition. Mini Rev Med Chem 5:279-292.

Worrall S, de Jersey J, Shanley BC and Wilce PA (1991): Antibodies against acetaldehyde-modified epitopes: an elevated IgA response in alcoholics. Eur J Clin Invest 21:90-95.

Worrall S, de Jersey J, Wilce PA, Seppä K, Hurme L and Sillanaukee P (1996): Relationship between alcohol intake and immunoglobulin a immunoreactivity with acetaldehyde-modified bovine serum albumin. Alcohol Clin Exp Res 20:836-840.

Worrall S, de Jersey J, Wilce PA, Seppä K, Hurme L and Sillanaukee P (1998): Comparison of carbohydrate-deficient transferrin, immunoglobulin A antibodies reactive with acetaldehyde-modified protein and acetaldehyde-modified albumin with conventional markers of alcohol consumption. Alcohol Clin Exp Res 22:1921-1926.

Worrall S, Richardson PJ and Preedy VR (2000): Experimental heart muscle damage in alcohol feeding is associated with increased amounts of reduced- and unreduced-acetaldehyde and malondialdehyde-acetaldehyde protein adducts. Addict Biol 5:421-427.

Worrall S, Niemelä O, Parkkila S, Peters TJ and Preedy VR (2001): Protein adducts in type I and type II fibre predominant muscles of the ethanol-fed rat: preferential localisation in the sarcolemmal and subsarcolemmal region. Eur J Clin Invest 31:723-730.

Wortmann RL (2002): Gout and hyperuricemia. Curr Opin Rheumatol 14:281-286.

Wu A, Chanarin I and Levi AJ (1974): Macrocytosis of chronic alcoholism. Lancet 1:829-831.

Wu JM, Wang ZR, Hsieh TC, Bruder JL, Zou JG and Huang YZ (2001): Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (Review). Int J Mol Med 8:3-17.

Wurst FM and Metzger J (2002): The ethanol conjugate ethyl glucuronide is a useful marker of recent alcohol consumption. Alcohol Clin Exp Res 26:1114-1119.

Wurst FM, Kempter C, Seidl S and Alt A (1999): Ethyl glucuronide--a marker of alcohol consumption and a relapse marker with clinical and forensic implications. Alcohol Alcohol 34:71-77.

Wurst FM, Skipper GE and Weinmann W (2003): Ethyl glucuronide--the direct ethanol metabolite on the threshold from science to routine use. Addiction 98 Suppl 2:51-61.

Wurst FM, Dresen S, Allen JP, Wiesbeck G, Graf M and Weinmann W (2006): Ethyl sulphate: a direct ethanol metabolite reflecting recent alcohol consumption. Addiction 101:204-211.

Wymer A and Becker DM (1990): Recognition and evaluation of red blood cell macrocytosis in the primary care setting. J Gen Intern Med 5:192-197.

Yasunami M, Chen CS and Yoshida A (1991): A human alcohol dehydrogenase gene (ADH6) encoding an additional class of isozyme. Proc Natl Acad Sci U S A 88:7610-7614.

Yin SJ (1994): Alcohol dehydrogenase: enzymology and metabolism. Alcohol Alcohol Suppl 2:113-119.

Yin M, Wheeler MD, Kono H, Bradford BU, Gallucci RM, Luster MI and Thurman RG (1999): Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. Gastroenterology 117:942-952.

Yokoyama A, Yokoyama T, Muramatsu T, Omori T, Matsushita S, Higuchi S, Maruyama K and Ishii H (2003a): Macrocytosis, a new predictor for esophageal squamous cell carcinoma in Japanese alcoholic men. Carcinogenesis 24:1773-1778.

Yokoyama M, Yokoyama A, Yokoyama T, Hamana G, Funazu K, Kondo S, Yamashita T, Yoshimizu H and Nakamura H (2003b): Mean corpuscular volume and the aldehyde dehydrogenase-2 genotype in male Japanese workers. Alcohol Clin Exp Res 27:1395-1401.

Younossi ZM (1998): Epidemiology of alcohol-induced liver disease. Clin Liver Dis 2:661-671.

Zhi X, Chan EM and Edenberg HJ (2000): Tissue-specific regulatory elements in the human alcohol dehydrogenase 6 gene. DNA Cell Biol 19:487-497.

Zorzano A and Herrera E (1990): Differences in kinetic characteristics and in sensitivity to inhibitors between human and rat liver alcohol dehydrogenase and aldehyde dehydrogenase. Gen Pharmacol 21:697-702.

IgAs Against Acetaldehyde-Modified Red Cell Protein as a Marker of Ethanol Consumption in Male Alcoholic Subjects, Moderate Drinkers, and Abstainers

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Background: Alcohol abuse has been shown to result in the production of antibodies against acetaldehyde-modified epitopes in proteins. However, as yet, only limited information has been available on the clinical usefulness of such responses as markers of hazardous drinking.

Methods: We developed an ELISA to measure specific IgAs against acetaldehyde-protein adducts. This method was evaluated in cross-sectional and follow-up studies on male heavy drinkers with a current ethanol consumption of 40 to 540 g/d ($n = 40$), moderate drinkers consuming 1 to 40 g/d ($n = 25$), and abstainers ($n = 16$). The clinical assessments included detailed interviews on the amounts and patterns of ethanol consumption and various biochemical markers of alcohol abuse and liver function.

Results: The mean antiadduct IgAs (198 ± 28 U/L) in the alcohol abusers were significantly higher than those in the moderate drinkers (58 ± 11 U/L, $p < 0.001$) or abstainers (28 ± 8 U/L, $p < 0.001$). The values of moderate drinkers were also higher than those in abstainers ($p < 0.05$). The amount of ethanol consumed during the period of 1 month preceding blood sampling correlated strongly with antiadduct IgAs ($r = 0.67$, $p < 0.001$). The sensitivity (73%) and specificity (94%) of this marker were found to exceed those of the conventional laboratory markers of alcohol abuse in comparisons contrasting heavy drinkers with abstainers although not in comparisons contrasting heavy drinkers with moderate drinkers. During abstinence, antiadduct IgAs disappeared with a mean rate of 3% per day. In additional analyses of possible marker combinations, antiadduct IgAs, together with CDT, were found to provide the highest sensitivity and specificity.

Conclusions: Measurements of antiadduct IgAs may provide a new clinically useful marker of alcohol abuse, providing a close relationship between marker levels and the actual amounts of recent ethanol ingestion.

Key Words: Ethanol, Marker, Acetaldehyde, Immune Responses.

EXCESSIVE ALCOHOL CONSUMPTION has been shown to induce the production of circulating antibodies, which recognize sequential and conformational epitopes generated in covalent binding reactions between proteins and ethanol metabolites (Israel et al., 1986; Koskinas et al., 1992; Niemelä, 2001; Viitala et al., 1997; Worrall et al., 1991; Xu et al., 1998). Previously, studies in alcoholic patients with liver disease have indicated that in such patients, there is often an increase in serum total IgAs coinciding with abnormal IgA tissue deposition (Amore et al 1994; van de Wiel et al 1988). Other studies have further shown high levels of IgA antibodies directed against acetaldehyde-derived neoantigens in alcoholic subjects, suggesting antigen-driven IgA responses (Latvala et al., 2005; Viitala et al., 1997; Worrall et al., 1991). Worrall et al. (1996, 1998) have previously suggested the possibility of using antiadduct IgAs also as markers of ethanol consumption. However, as yet, only a few studies have been available on clinical evaluations of this approach.

The present study was set out to gain further insight on the diagnostic utility of measuring antiadduct IgAs by comparing the IgA marker with several traditional markers of ethanol consumption in male subjects with a wide range of alcohol consumption.

MATERIALS AND METHODS

Patients and Control Subjects

The study population included 40 male heavy drinkers (mean age 45 ± 11 years), who showed a well-documented history of excessive ethanol consumption. All of these patients had been admitted for detoxification, but were devoid of clinical and laboratory signs of significant liver disease. They showed a history of continuous ethanol consumption or binge drinking, the mean recent consumption

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being 40 to 540 g/d from the period of 4 weeks before sampling. The documentation of alcohol abuse was based on detailed personal interviews using a time-line follow-back technique. The mean duration of abstinence before sampling was 2 ± 2 days. Nineteen alcoholic subjects (mean age 42 ± 12 years) volunteered for a follow-up, which was carried out with supervised abstinence and repeated sampling during hospitalization over a period of 8 ± 3 days. Blood alcohol concentrations during this time were controlled by repeated ethanol analyses from breath air.

Reference individuals were healthy male volunteers who were either abstainers ($n = 16$, age 54 ± 15 years) or moderate drinkers ($n = 25$, age 45 ± 12 years), whose daily ethanol consumption did not exceed 40 g. All of these subjects were devoid of any history or clinical evidence of alcohol abuse, recent illnesses, or immunological disorders.

All serum samples were stored at $-70\,^{\circ}C$ until analysis. All participants of the study gave their informed consent, and the study was carried out according to the provisions of the Declaration of Helsinki.

Antiadduct IgA Analyses

Preparation of Acetaldehyde-Modified Red Cell Protein. For preparation of the test antigen, human erythrocyte protein fraction was first purified from EDTA blood of an abstainer (Latvala et al., 2005). The cells were separated by centrifugation and washed 3 times with phosphate-buffered saline (PBS, 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The washed cells were lysed with polyoxyethylene ether, 0.1% v/v in borate buffer (Hemolysis Reagent, DIAMAT^{TM} Analyzer system, Bio-Rad Laboratories, Hercules, CA), and incubated for 35 minutes at $37\,^{\circ}C$ to remove unstable Schiff bases. The hemolyzate containing 12 mg of red cell protein/mL (in PBS) was stored frozen in aliquots at $-70\,^{\circ}C$.

For acetaldehyde labeling, the hemolyzate was mixed with acetaldehyde (in PBS) to obtain a final acetaldehyde concentration of 10 mM. After incubation for 18 hours at $4\,^{\circ}C$ in tightly sealed containers, the protein adducts generated were reduced by addition of sodium cyanoborohydride (10 mM) and gentle mixing for 5 hours at $4\,^{\circ}C$. Protein solutions were then dialyzed exhaustively against PBS at $4\,^{\circ}C$ and stored in aliquots for single use at $-70\,^{\circ}C$. The unmodified proteins were obtained by hemolyzing and treating EDTA blood of an abstainer without acetaldehyde.

Measurements of the Antibody Titers. Microtiter plates (Nunc-Immuno Plate, Maxisorb^{TM}, InterMed, Denmark) were coated with acetaldehyde-modified red cell protein, or corresponding unmodified proteins in PBS (3 μg protein in 100 μL/well) and incubated for $1\frac{1}{2}$ hours at $+37\,^{\circ}C$. Nonspecific binding was blocked by incubation (1 hour, $+37\,^{\circ}C$) with 0.2% gelatin in PBS (150 μL/well). The samples were diluted (1:40) in PBS, which contained 0.04% Tween-20. The serum dilutions were allowed to react with the coated proteins (1 hour, $+37\,^{\circ}C$), followed by extensive washing with PBS-Tween. Antibody–antigen complexes were detected using alkaline phosphatase–linked goat anti-human immunoglobulin IgA (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The immunoglobulins were diluted in PBS-Tween, containing 8 mM $MgCl_2$ and a small amount of dithiothreitol (DTT). The plates were incubated overnight at $+4\,^{\circ}C$ and washed with PBS-Tween and 100 μL of *p*-nitrophenylphosphate solution was added as a color reaction substrate (alkaline phosphatase substrate kit, Bio-Rad). This reaction was stopped by adding 100 μL NaOH (0.4 M), and the optical densities were read at 405 nm by an Anthos HTII microplate reader (Anthos Labtec Instruments, Salzburg, Austria).

Other Measurements

Serum carbohydrate-deficient transferrin (CDT) was measured by a turbidimetric immunoassay (TIA) after ion-exchange chromatography (Axis-Shield, Oslo, Norway), which separates transferrin variants with 0 to 2 sialic acid residues. The measurements were carried out on a Behring Nephelometer II (Dade Behring, Behring Diagnostics GmbH, Marburg, Germany) according to the instructions of the manufacturer. Serum γ-glutamyl transferase (GGT), aspartate aminotransferase (AST), and mean corpuscular volume (MCV) of erythrocytes were measured by standard clinical chemical methods in an accredited (SFS-EN 45001, ISO/IEC Guide 25) laboratory of EP Central Hospital, Seinäjoki, Finland. Cytokines IL-2, IL-6, IL-8, IL-10, TNF-α, and TGF-β1 were measured using Quantikine high-sensitivity ELISA kits (R&D Systems, Abington Science Park, UK).

Calculations and Statistical Methods

The data for the study variables are expressed as mean $\pm$ SEM. In the assays for the antiadduct IgAs, the values (OD_{405}) obtained in a reaction with the sample and the unconjugated protein (background) were subtracted from the corresponding values measured from the reaction between the sample and the acetaldehyde-protein conjugate. The antiadduct IgA values are expressed as U/L, corresponding to $OD_{405} \times 10^3$. The combination marker IgA-CDT was calculated with an equation $\log CDT + (IgA/10^3)$. The combination of GGT and CDT (GGT-CDT) was counted using a previously established equation $GGT\text{-}CDT = 0.8 \times \ln(GGT) + 1.3 \times \ln(\%CDT)$ (Anttila et al., 2003). The differences between the groups of heavy drinkers, moderate drinkers, and abstainers were analyzed with an ANOVA or a Kruskal–Wallis test, as required. A paired *t*-test was used for comparing alcoholic subjects before and after abstinence, and the difference between moderate drinkers and abstainers was further examined with an unpaired *t*-test or a Mann–Whitney test, as required. Correlations were calculated using the Pearson product–moment correlation coefficients for continuous nonskewed parameters or Spearman's rank-correlations for noncontinuous or skewed variables. A *p*-value < 0.05 was considered statistically significant.

RESULTS

The mean antiadduct IgA levels (198 ± 28 U/L) in the alcohol abusers were significantly higher than those in the moderate drinkers (58 ± 11 U/L, $p < 0.001$) or abstainers (28 ± 8 U/L, $p < 0.001$) (Fig. 1). When comparing only moderate drinkers and abstainers with each other, the values of moderate drinkers were also significantly higher than those in abstainers ($p < 0.05$).

The diagnostic sensitivity and specificity of antiadduct IgA measurements were subsequently compared with those of the traditional markers of alcohol abuse by contrasting the data obtained from the heavy drinkers with the data obtained from the abstainers and/or moderate drinkers (Table 1). In comparison with the abstainers only, the sensitivity of antiadduct IgAs was 73% for a specificity of 94%, which exceeded the corresponding figures found for CDT, GGT, MCV, and AST. When both moderate drinkers and abstainers were included in the reference population, the sensitivity of antiadduct IgA assay was 65% for a specificity of 88%, showing essentially similar analytical characteristics to those of CDT although remaining higher than those of GGT, MCV, or AST (Table 1). The antiadduct IgAs correlated significantly to all the conventional markers of alcohol abuse (Table 2) and also highly significantly to the actual amount of recent ethanol consumption ($r = 0.67$, $p < 0.001$). In comparison

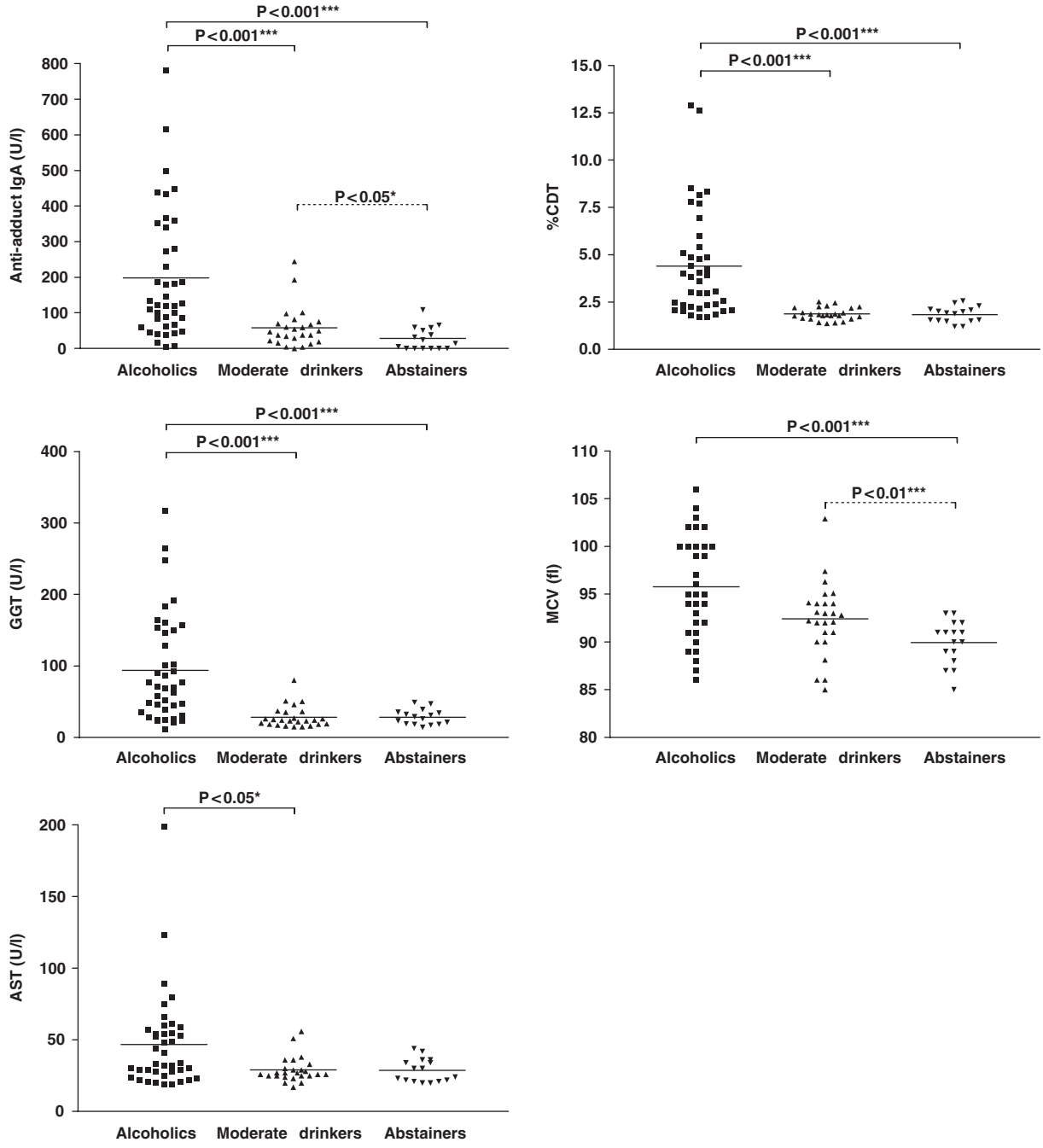

Fig. 1. Markers of ethanol consumption in heavy drinkers, moderate drinkers, and abstainers. Antiadduct IgA, carbohydrate-deficient transferrin, and γ-glutamyl transferase values in heavy drinkers are all significantly higher than those in both moderate drinkers and abstainers ($p<0.001$ for all comparisons). Mean corpuscular volume (MCV) was significantly higher ($p<0.001$) in alcoholic subjects than in abstainers, and aspartate aminotransferase was significantly higher ($p<0.05$) in alcoholic subjects than in moderate drinkers. Significant differences between moderate drinkers and abstainers were found in antiadduct IgA and in MCV values (dotted lines).

with the major cytokines, antiadduct IgAs were found to correlate significantly with serum IL-6 ($r = 0.33$, $p<0.05$) and TNF-α ($r = 0.31$, $p<0.05$) levels, whereas not with IL-2, IL-8, IL-10, or TGF-β1.

Figure 2 demonstrates the results of the follow-up of heavy drinkers. The values at the initiation (160 ± 31 U/L) decreased significantly to 131 ± 26 U/L ($p<0.01$) during a period of 8 ± 3 days of supervised abstinence. After this time, the levels were still higher than those of moderate drinkers or abstainers, the difference to the latter group remaining statistically significant ($p<0.001$). The rate of antiadduct IgA disappearance was estimated to be about 3% per day, and the mean normalization time to be about 4 weeks.

In the analyses for possible marker combinations, the highest sensitivities and specificities were obtained by

Table 1. Sensitivity and Specificity Characteristics of Various Markers of Ethanol Consumption When Either Abstainers or Moderate Drinkers Are Used as the Control Population

Source of cutoff	Cutoff	Sensitivity %	Specificity %	AUC (SE)
Antiadduct IgA				
Abstainers	74	73	94	0.902 (0.0401)
Abstainers and moderate drinkers	93	65	88	0.843 (0.0436)
Moderate drinkers	99	65	88	0.806 (0.0545)
CDT				
Abstainers	2.67	63	100	0.889 (0.0432)
Abstainers and moderate drinkers	2.54	65	98	0.897 (0.0343)
Moderate drinkers	2.46	65	96	0.902 (0.0366)
GGT				
Abstainers	50	63	100	0.846 (0.0508)
Abstainers and moderate drinkers	55	60	98	0.850 (0.0433)
Moderate drinkers	59	58	96	0.852 (0.0465)
MCV				
Abstainers	95	47	100	0.822 (0.0591)
Abstainers and moderate drinkers	98	41	98	0.738 (0.0623)
Moderate drinkers	100	19	96	0.684 (0.0713)
AST				
Abstainers	45	43	100	0.688 (0.0715)
Abstainers and moderate drinkers	46	43	95	0.689 (0.0604)
Moderate drinkers	47	43	92	0.690 (0.0656)

Cut-offs were determined as mean+2SD.

AUC, area under curve; SE, standard error of AUC; IgA, immunoglobulin A; CDT, carbohydrate-deficient transferrin; GGT, γ-glutamyl transferase; MCV, mean corpuscular volume of erythrocytes; AST, aspartate aminotransferase.

combining IgAs with CDT, which yielded a sensitivity of 90%, a specificity of 98%, and an area under the curve of 0.966 (Table 3).

DISCUSSION

The present data indicate that the assessment of IgA antibodies to acetaldehyde-modified protein epitopes may provide a clinically useful tool for diagnosing excessive ethanol consumption. As IgAs already among moderate drinkers may exceed the levels observed in teetotallers, it appears that the generation of such immune responses is an early event in the physiological consequences induced by ethanol ingestion, which should also be considered in studies on the mechanisms of ethanol-induced tissue effects.

Table 2. Correlations Between the Different Markers of Ethanol Intake

	Antiadduct IgA	CDT	GGT	MCV	AST
Antiadduct IgA	1				
CDT	0.39***	1			
GGT	0.35**	0.43***	1		
MCV	0.51***	0.28*	0.48***	1	
AST	0.23*	0.46***	0.46***	0.32**	1

*$p<0.05$; **$p<0.01$; ***$p<0.001$.

CDT, carbohydrate-deficient transferrin; GGT,γ-glutamyl transferase; MCV, mean corpuscular volume of erythrocytes; AST, aspartate aminotransferase.

Previous studies in patients with alcoholic liver disease have demonstrated an increase in serum total IgAs and the appearance of IgA deposits in liver and kidney tissue (Amore et al., 1994; Tuma and Klassen, 1992; van de Wiel et al., 1988). More recent evidence has suggested that the generation of excess IgAs in such patients could in fact be antigen-driven (Koskinas et al., 1992; Latvala et al., 2005; Viitala et al., 1997; Worrall et al., 1991). Such specific antibodies could originate from intestinally induced B-cells under conditions where the gastrointestinal tract, which is rich in enzymes capable of metabolizing ethanol to acetaldehyde, is repeatedly exposed to ethanol (Latvala et al., 2005; Salaspuro, 1996; Seitz et al., 1994; Visapää et al., 1998). This view is also consistent with the strong correlation between the specific IgA antibody levels and the amounts of recent ethanol ingestion as well as with the presence of antiadduct IgAs in individuals reporting moderate drinking. As alcohol is also known to increase intestinal permeability, this could further enhance the immune responses toward the intestinal antigens. The positive correlation found between antiadduct IgAs and the proinflammatory cytokines TNF-α and IL-6 indicates that there is also an early-phase inflammatory response to ethanol-derived neoantigens. Upon continuing heavy drinking, the induction of the immune responses and the cytokine cascades may also play an important role in the sequence of events leading to liver pathology. Acetaldehyde modification of proteins and cellular constituents has been previously shown to disturb protein function in vivo, and the generation of IgAs toward ethanol metabolites could also promote the destruction of such protein modifications.

The present data indicate a high sensitivity and specificity for the adduct-specific IgA measurements in differentiating between alcoholic subjects and nonalcoholic subjects. Worrall et al. (1996, 1998) have previously investigated antiadduct IgA responses as markers of alcohol abuse using acetaldehyde-modified bovine serum albumin as a test antigen. Despite the difference in the method and the nature of protein adducts in these and the present studies, it should be noted that Worrall and coworkers also found a significant correlation ($r = 0.44$) between ethanol intake and antiadduct IgAs among social drinkers and alcoholic subjects. Previously, the control populations in biomarker studies have usually consisted primarily of social drinkers, and the possible differences occurring between moderate drinkers and teetotallers have received less attention. The present data show that the diagnostic sensitivity of antiadduct IgAs exceeds that of the conventional markers especially when abstainers are used as the reference individuals. Conversely, inclusion of moderate drinkers in the reference population decreases the diagnostic sensitivity, indicating that future reference limits for alcohol markers should perhaps preferably be based on

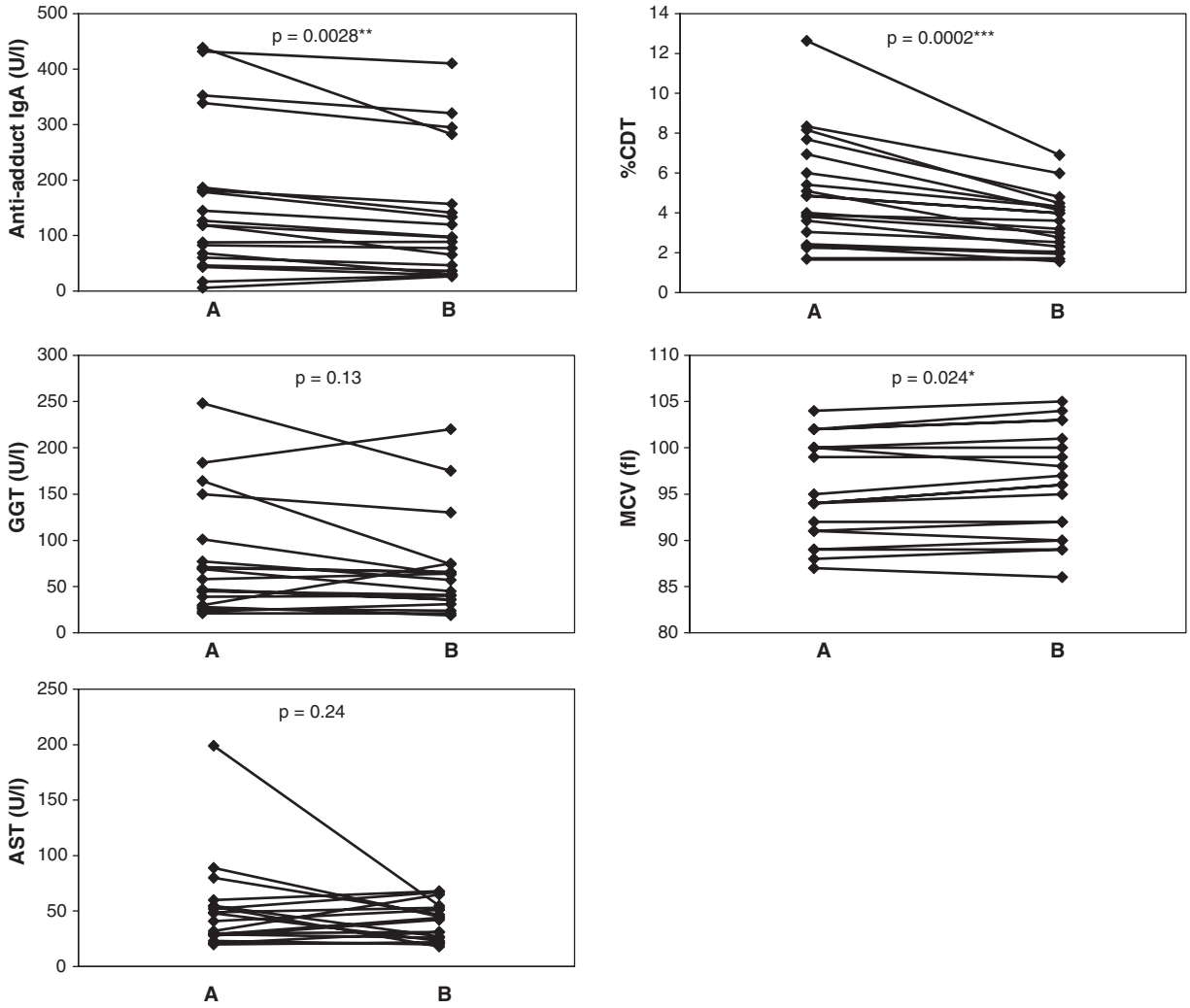

Fig. 2. Laboratory parameters of alcoholic subjects before and after 8 ± 3 days of abstinence. The decreases in antiadduct IgA ($p = 0.0028$) and carbohydrate-deficient transferrin values ($p = 0.0002$) were statistically significant. Mean corpuscular volume values showed a significant increase during abstinence ($p = 0.024$). (**A**) Alcoholic subjects before abstinence and (**B**) after abstinence.

abstainers. This approach would obviously lead to some positive values in individuals with moderate drinking habits, but provide us with a new tool for raising the issue of possible hazardous drinking practices in an earlier phase.

Current follow-up studies indicate that serum antiadduct IgA levels normalize at an average rate of 3% per day, the mean time required for normalization being 29 days. As the average half-life of a single IgA molecule is 5 to 6 days, the relatively long appearance of antiadduct IgAs in the serum may be explained by the fact that acetaldehyde adducts in erythrocytes of alcoholic subjects may persist in circulation for 1 to 3 weeks after the last dose of ethanol (Niemelä and Israel, 1992). The consistent decrease in the antiadduct IgAs upon abstinence indicates, however, that IgA titers could also be used for monitoring treatment in recovering alcoholic subjects.

Table 3. Sensitivities and Specificities of Marker Combinations

	Cutoff	Sensitivity %	Specificity %
IgA-CDT	0.412	90	98
GGT-CDT	4.20	83	98

CDT, carbohydrate-deficient transferrin.

Recent evidence has suggested that further improvement in the clinical accuracy of alcohol markers may be obtained through the development of ideal marker combinations. Such combinations have usually consisted of CDT, GGT, and MCV. Recent studies have indicated the highest diagnostic accuracy for a mathematically formulated combination of GGT and CDT (Anttila et al., 2003; Sillanaukee and Olsson, 2001). The present work shows that the combination of antiadduct IgAs and CDT also provides improved sensitivity compared with its parent components without sacrificing assay specificity. The diagnostic performance also seems to exceed that found for the combination of GGT and CDT. It should be noted, however, that before adopting the IgA-based assay into

routine clinical and multilaboratory use, a careful standardization of the method will be needed. This could be achieved by using internationally standardized antigen preparations and test protocols including standard color reaction times and the expression of the assay results using international reference samples.

In this study, we chose to study men only, because sex may be a significant confounding factor in studies on immunological responses in vivo (Kovacs and Messingham, 2002; Makkonen et al., 2001). Although the specific mechanisms underlying these phenomena have remained unclear, sex hormones appear to play a key role in the regulation of immune responses. As women generally also show stronger immune responses and higher serum immunoglobulin levels, future studies in populations consisting of women only also appear warranted to evaluate the diagnostic potential and pathogenic significance of the IgA responses.

Taken together, this work shows that measurements of specific IgAs against acetaldehyde-modified epitopes in proteins could be used as a sensitive tool for detecting heavy drinking. Assays for such immune responses could also be important in studies on the pathogenesis of ethanol-induced tissue injury.

REFERENCES

Amore A, Coppo R, Roccatello D, Piccoli G, Mazzucco G, Gomez-Chiarri M, Lamm ME, Emancipator SN (1994) Experimental IgA nephropathy secondary to hepatocellular injury induced by dietary deficiencies and heavy alcohol intake. Lab Invest 70:68–77.

Anttila P, Järvi K, Latvala J, Blake JE, Niemelä O (2003) A new modified gamma-%CDT method improves the detection of problem drinking: studies in alcoholics with or without liver disease. Clin Chim Acta 338:45–51.

Israel Y, Hurwitz E, Niemelä O, Arnon R (1986) Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde-protein adducts. Proc Natl Acad Sci U S A 83:7923–7927.

Koskinas J, Kenna JG, Bird GL, Alexander GJ, Williams R (1992) Immunoglobulin A antibody to a 200-kilodalton cytosolic acetaldehyde adduct in alcoholic hepatitis. Gastroenterology 103:1860–1867.

Kovacs EJ, Messingham KA (2002) Influence of alcohol and gender on immune response. Alcohol Res Health 26:257–263.

Latvala J, Hietala J, Koivisto H, Järvi K, Anttila P, Niemelä O (2005) Immune responses to ethanol metabolites and cytokine profiles differentiate alcoholics with or without liver disease. Am J Gastroenterol 100:1303–1310.

Makkonen K, Viitala KI, Parkkila S, Niemelä O (2001) Serum IgG and IgE antibodies against mold-derived antigens in patients with symptoms of hypersensitivity. Clin Chim Acta 305:89–98.

Niemelä O (2001) Distribution of ethanol-induced protein adducts in vivo: relationship to tissue injury. Free Radic Biol Med 31: 1533–1538.

Niemelä O, Israel Y (1992) Hemoglobin-acetaldehyde adducts in human alcohol abusers. Lab Invest 67:246–252.

Salaspuro M (1996) Bacteriocolonic pathway for ethanol oxidation: characteristics and implications. Ann Med 28:195–200.

Seitz HK, Gartner U, Egerer G, Simanowski UA (1994) Ethanol metabolism in the gastrointestinal tract and its possible consequences. Alcohol Alcohol 2 (suppl): 157–162.

Sillanaukee P, Olsson U (2001) Improved diagnostic classification of alcohol abusers by combining carbohydrate-deficient transferrin and gamma-glutamyltransferase. Clin Chem 47:681–685.

Tuma DJ, Klassen LW (1992) Immune responses to acetaldehyde-protein adducts: role in alcoholic liver disease. Gastroenterology 103:1969–1973.

van de Wiel A, van Hattum J, Schuurman HJ, Kater L (1988) Immunoglobulin A in the diagnosis of alcoholic liver disease. Gastroenterology 94:457–462.

Viitala K, Israel Y, Blake JE, Niemelä O (1997) Serum IgA, IgG, and IgM antibodies directed against acetaldehyde-derived epitopes: relationship to liver disease severity and alcohol consumption. Hepatology 25:1418–1424.

Visapää JP, Jokelainen K, Nosova T, Salaspuro M (1998) Inhibition of intracolonic acetaldehyde production and alcoholic fermentation in rats by ciprofloxacin. Alcohol Clin Exp Res 22:1161–1164.

Worrall S, de Jersey J, Shanley BC, Wilce PA (1991) Antibodies against acetaldehyde-modified epitopes: an elevated IgA response in alcoholics. Eur J Clin Invest 21:90–95.

Worrall S, de Jersey J, Wilce PA, Seppä K, Hurme L, Sillanaukee P (1996) Relationship between alcohol intake and immunoglobulin a immunoreactivity with acetaldehyde-modified bovine serum albumin. Alcohol Clin Exp Res 20:836–840.

Worrall S, de Jersey J, Wilce PA, Seppä K, Hurme L, Sillanaukee P (1998) Comparison of carbohydrate-deficient transferrin, immunoglobulin A antibodies reactive with acetaldehyde-modified protein and acetaldehyde-modified albumin with conventional markers of alcohol consumption. Alcohol Clin Exp Res 22:1921–1926.

Xu D, Thiele GM, Beckenhauer JL, Klassen LW, Sorrell MF, Tuma DJ (1998) Detection of circulating antibodies to malondialdehyde-acetaldehyde adducts in ethanol-fed rats. Gastroenterology 115:686–692.

Long-term ethanol consumption and macrocytosis: diagnostic and pathogenic implications

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Although excessive alcohol consumption is known to elevate the mean cell volume (MCV) of erythrocytes, the relationships among the intensity of ethanol exposure, the generation of abnormal red blood cell indices, and the underlying pathogenic mechanisms have remained unclear. The authors examined 105 alcoholics with a wide range of ethanol consumption (40–500 g of ethanol/day), 62 moderate drinkers (mean consumption 1–40 g/day), and 24 abstainers, who underwent detailed interviews, measurements of blood cell counts, markers of liver status, and circulating antibodies against ethanol-derived protein modifications. Follow-up information was collected from healthy volunteers with detailed records on drinking habits. Data from the NORIP project for laboratory parameters in apparently healthy moderate drinkers or abstainers (n = 845) were used for reference interval comparisons. The highest MCV ($P < 0.001$) and mean cell hemoglobin (MCH) ($P < 0.01$) occurred in the alcoholics. However, the values in the moderate drinkers also responded to ethanol intake such that the upper normal limit for MCV based on the data from moderate drinkers was 98 fl, as compared with 96 fl from abstainers. Follow-up cases with carefully registered drinking habits showed parallel changes in MCV and ethanol intake. Anti-adduct IgA and IgM against acetaldehyde-induced protein modifications were elevated in 94% and 64% of patients with high MCV, respectively, the former being significantly less frequent in the alcoholics with normal MCV (63%) ($P < 0.05$). The data indicate dose-related responses in red blood indices upon chronic ethanol consumption, which may also be reflected in reference intervals for hematological parameters in health care. Generation of immune responses against acetaldehyde-modified erythrocyte proteins may be associated with the appearance of such abnormalities. (J Lab Clin Med 2006;147:191–196)

Abbreviations: ALT = alanine aminotransferase; ANOVA = one-way analysis of variance; AST = aspartate aminotransferase; DTT = dithiothreitol; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; NORIP = Nordic Reference Interval Project; PBS = phosphate buffered saline; RBC = red blood cell; SD = standard deviation

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Although alcohol abuse is a well-known cause for a wide array of abnormalities in blood cells and circulating proteins, the dose-response relationships in such actions have remained poorly defined. Their molecular mechanisms are also unknown, although several studies have supported direct physicochemical effects of ethanol and its metabolites.[1–3] Elevated MCV, macrocytosis, is the most typical morphological abnormality associated with excessive ethanol consumption. It is so common that it has also been widely used in clinical screening procedures for the detection of alcohol abuse.[4,5]

The incidence of hazardous drinking practices and associated medical disorders have continued to grow in most Western countries.[6–8] For instance in Finland, the total ethanol consumption per capita has steadily increased over the past decades (Figure 1 upper panel), and simultaneously the percentage of persons fully abstaining from ethanol has been decreasing. Unfortunately, studies on ethanol-induced adverse effects on health and biochemical indices of disease have usually dealt with advanced alcoholics, whereas those without apparent clinical signs of hazardous drinking have received less attention.

In this work, the authors explored the relationship among ethanol consumption, red blood cell abnormalities, and immune responses against ethanol metabolites in a population of alcoholics and healthy persons representing a wide range of ethanol intake. The cross-sectional and follow-up data indicate distinct dose-related responses in erythrocyte cell volume and alcohol consumption, which may occur already at relatively low levels of ethanol intake.

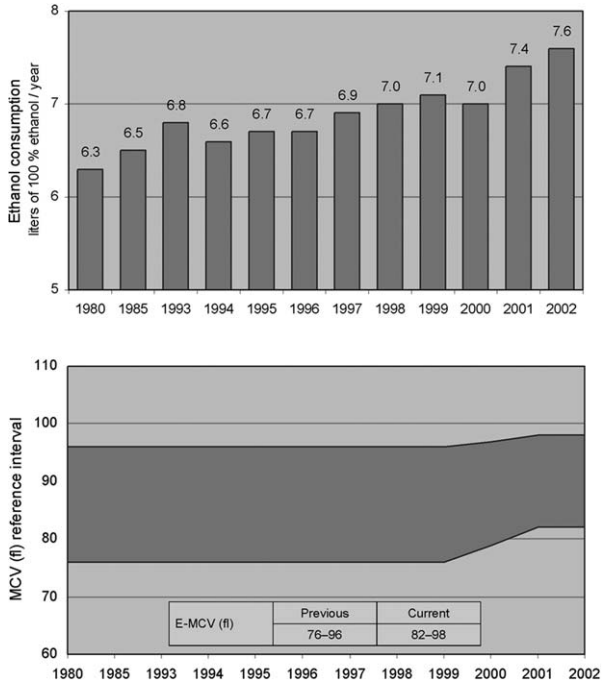

Fig 1. Upper panel: mean ethanol consumption per capita in Finland over the years 1993–2002. Lower panel: the change in MCV reference values before and after the 1999–2003 NORIP survey.

METHODS

Subjects. The population of alcoholics included 105 heavy drinkers (92 men, 13 women), who had been admitted for detoxification. All of them showed a history of continuous ethanol consumption or binge drinking, the mean consumption being from 40 to 540 g/day (mean 147 g/day) during the period of 4 weeks before sampling. All patients met the DSM-IV criteria of alcohol dependence, including pathological alcohol use, social impairment, and the presence of tolerance and withdrawal symptoms. The documentation of the amount of alcohol consumption in these patients was carried out by detailed personal interviews using a timeline follow-back technique. The mean duration of abstinence before sampling was 2 ± 2 days. Similar protocols were also carried out for 86 healthy volunteers (54 men, 32 women) who were either abstainers (n = 24) or moderate drinkers (n = 62) whose mean daily ethanol consumption was 10 ± 8 g/day, range 1–40 g/day. The main characteristics of these study groups are summarized in Table I. For comparisons, data from the 1999–2003 survey on 845 apparently healthy persons representing either moderate drinkers or abstainers from the NORIP (Nordic Reference Interval Project) for common hematological and clinical chemical parameters were used. The survey excluded persons who had clinical or laboratory evidence of current or recent illnesses or infections, who were pregnant, had donated blood during the past 5 months, or had used any prescription drugs during the preceding week. In these persons, the amount of alcohol had not been more than two drinks during the 24 hours preceding blood sampling. Smoking had not been allowed for 1 hour before sampling.

Follow-up data. For follow-up purposes, data from repeated assays for blood cell counts, blood chemistry analyses, and detailed diaries on the amounts of alcohol consumption from three healthy male volunteers from a period 1983–2003 were collected.

Ethical considerations. The procedures were approved by the institutional review board. Informed consents were obtained from the participants, and the study was carried out according to the provisions of the Declaration of Helsinki.

Analytical procedures. Blood cell counts of the study population were analyzed by Coulter Hematology Analyzer systems. Measurements of serum AST, ALT, albumin, bilirubin, erythrocyte folate, and serum vitamin B_{12} levels, which were used to exclude known causes of non-alcohol-related reasons of abnormal mean corpuscular volume, were carried out using standard clinical chemical methods in an accreditated (SFS-EN 45001, ISO/IEC Guide 25) laboratory of Seinäjoki Central Hospital, Seinäjoki, Finland.

Measurements of antibody titers against protein modifications induced by ethanol metabolites were carried out essen-

Table I. Main characteristics of the study population

	Men			Women		
	Alcoholics	Moderate drinkers	Abstainers	Alcoholics	Moderate drinkers	Abstainers
n	92	42	12	13	20	12
Age	42 ± 10	40 ± 14	46 ± 21	41 ± 8	38 ± 13	42 ± 17
Ethanol consumption g/day	147 ± 90	11 ± 9	0	152 ± 101	6 ± 6	0
range	40 – 539	3 – 40		61 – 317	1 – 22	

Table II. Red blood cell indices in alcoholics, moderate drinkers, and abstainers, and the change in reference intervals of red blood cell indices before (previous) and after (current) the 1999–2003 survey

							Reference intervals			
	Alcoholics		Moderate drinkers		Abstainers		Previous		Current	
	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women
B-Hemoglobin (g/L)	152 ± 11	136 ± 14	150 ± 9.0	136 ± 7.1	152 ± 7.4	130 ± 9.4	130–180	120–160	134–167	117–155
B-Hematocrit	0.45 ± 0.04	0.38 ± 0.02	0.43 ± 0.03	0.39 ± 0.02	0.43 ± 0.03	0.38 ± 0.03	0.40–0.54	0.36–0.47	0.39–0.50	0.35–0.46
B-RBC ($\times 10^{12}$/L)	4.7 ± 0.46	4.0 ± 0.27*	4.7 ± 0.35	4.3 ± 0.25	4.8 ± 0.34	4.33 ± 0.30	4.50–6.00	4.00–5.50	4.25–5.70	3.90–5.20
E-MCV (fl)	95 ± 4.8***	96 ± 3.4***	90 ± 3.3	90 ± 3.8	89 ± 3.0	89 ± 3.6	76–96		82–98	
E-MCH (pg)	33 ± 2.2**	33 ± 1.8**	31 ± 1.4	31 ± 1.4	31 ± 1.5	30 ± 1.7	27–32		27–33	
E-MCHC (g/L)	341 ± 8.0*	343 ± 8.6	346 ± 6.6	342 ± 5.9	347 ± 5.3	338 ± 6.9	320–360		320–355	

Abbreviations: MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cells.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$ when compared with the corresponding group of abstainers.

tially as described.[9] In brief, microtiter plates (Nunc-Immuno Plate, Maxisorb, InterMed, Denmark) were coated first with acetaldehyde-modified red cell protein, or corresponding unmodified proteins in PBS (3-μg protein in 100 μL/well) and incubated for 1.5 hours at +37°C. Nonspecific binding was blocked by incubation (1 hour, +37°C) with 0. % gelatin in PBS (150 μL/well). The sample sera were diluted (1:40) in PBS, which contained 0.04% Tween-20. The serum dilutions were allowed to react with the coated proteins (1 hour, +37°C), followed by extensive washing with PBS-Tween. Antibody-antigen complexes were labeled using alkaline-phosphatase-linked goat anti-human immunoglobulin IgA, IgG, or IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove). The immunoglobulins were diluted in PBS-Tween, which contained 8-mM $MgCl_2$ and a small amount of DTT. The plates were incubated overnight at +4°C. After incubation, plates were washed, and 100 μL of p-nitrophenylphosphate solution was added for color reaction substrate (Alkaline Phosphatase Substrate Kit, Bio-Rad Laboratories, Hercules, Calif). The color reaction was stopped by adding 100-μL NaOH (0.4 M), and the optical densities were read at 405 nm by the Anthos HTII microplate reader (Anthos Labtec Instruments, Salzburg, Austria).

Statistical methods. Values are expressed as mean ± SD. Differences between groups means were determined with one-way analysis of variance (anova) followed by Bonferroni's multiple comparisons procedure. The relationships between the different variables were analyzed using Pearson's correlation coefficient. Incidences were compared with Fisher's exact test. The analyses were carried out using Graphpadprism Statistical Software (San Diego, Calif).

RESULTS

Table II summarizes the red blood cell indices in the alcoholics, moderate drinkers, and abstainers, who underwent the interviews using the timeline follow-back method. Although the highest values of both MCV ($P < 0.001$) and MCH ($P < 0.01$) occurred in the alcoholics, the mean MCV values in the moderate drinkers also tended to be higher than those in the abstainers. A significant correlation was found to exist between MCV levels and the amount of recent alcohol intake from the period of 1 month preceding blood sampling ($r = 0.48$, $P < 0.001$) (Fig 2).

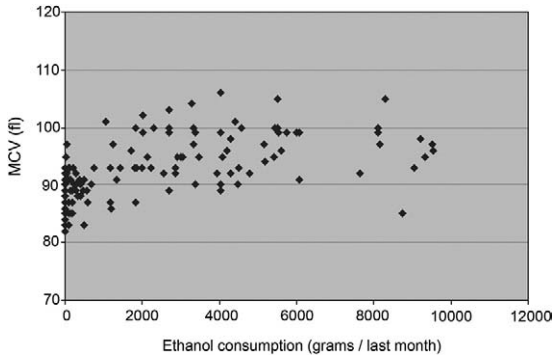

Fig 2. Correlation between MCV values and ethanol intake from the period of 4 weeks before sampling in the study population. The correlation was found to be statistically significant ($r = 0.48$), ($P < 0.0001$). The association between these parameters seemed rather linear between 0 and 4000 g of ethanol per month and to plateau thereafter.

Figure 1 demonstrates the changes in the mean amount of alcohol consumption per capita based on national statistics over the years 1993–2002 in Finland, and the change in MCV reference values, as observed before and after the 1999–2003 survey. A marked increase in both the upper and the lower normal limits of MCV occurred during this time period (Fig 2, Table II). The reference intervals (mean ± 2 SD) for MCV calculated from the current material of moderate drinkers (82–98) were found to be in accordance with the data obtained in the NORIP project (82–98) and different from the normal limits based on the data from abstainers (82–96). Use of 96 fl as a cutoff value for differentiating between the alcoholics and nonalcoholics in the current interview sample yielded a sensitivity of 44%, a specificity of 98%, a positive predictive value of 96%, and a negative predictive value of 58%.

The changes in MCV values from representative follow-ups of healthy male subjects with carefully registered amounts of monthly and yearly consumptions of ethanol are shown in Fig 3. Repeated assays of blood cell counts indicated parallel changes in MCV values and the intensity of ethanol drinking, which was more striking in a case with the higher mean levels of consumption.

Measurements of circulating IgA, IgG, and IgM antibodies against acetaldehyde-derived modifications (adducts) of proteins from the alcoholic patients indicated elevated levels in 94%, 21%, and 64% of patients with elevated MCV and in 63%, 19%, and 56% of those with normal MCV, respectively. The difference in the incidences was found to be significant for IgAs ($P < 0.05$). Statistically significant correlations also occurred between MCV and anti-adduct IgA and IgM in men,

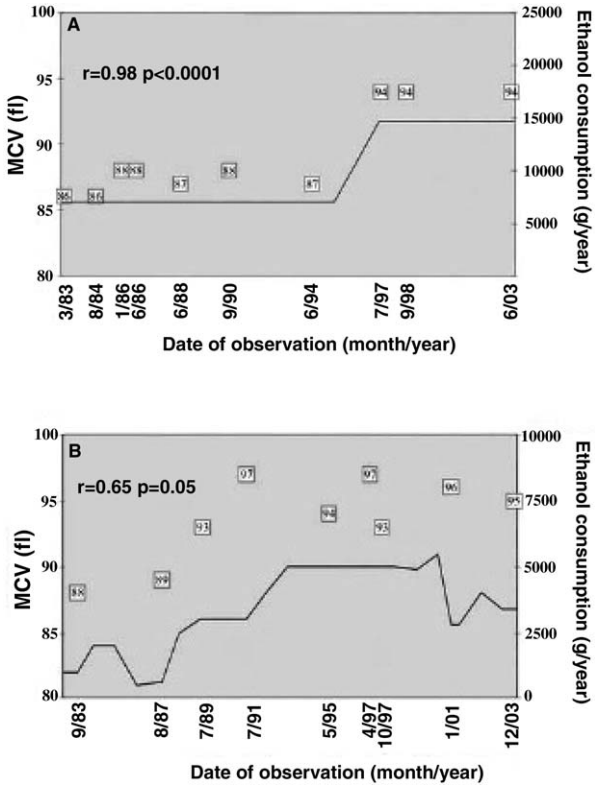

Fig 3. Follow-ups of MCV values and ethanol consumption in healthy male volunteers [age at the initiation of the follow-up: (**A**) 36 years and (**B**) 27 years] based on detailed diaries on the amounts of alcohol consumption and repeated assays for hematological parameters during 1983–2003. The changes in MCV values were found to parallel the changes in ethanol drinking habits, which was found to be more evident at higher mean levels of consumption.

Table III. Correlations between MCV and anti-adduct immunoglobulins in alcoholics

		IgA	IgG	IgM
E-MCV	Men	0.27*	0.15	0.33*
	Women	0.19	0.39	0.28

Abbreviations: IgA, anti-adduct immunoglobulin A; IgG, anti-adduct immunoglobulin G; IgM, anti-adduct immunoglobulin M; MCV, mean corpuscular volume.
*$P < 0.05$.

whereas in women the observed correlations did not reach significance (Table III).

DISCUSSION

The current findings indicate a dose-dependent response in erythrocyte cell volume to the intensity of ethanol intake. Although high MCV values have long been known to characterize alcoholic patients, the current data further indicates that non-alcoholic persons

reporting moderate levels of drinking (<40 g/day) show values that at population level may lead to alterations in the characteristics of an average red blood cell. In agreement with this view, a recent survey for laboratory reference intervals in Nordic countries in a population consisting of apparently healthy moderate drinkers and abstainers also found an increase in MCV upper normal limits.[10] Furthermore, such changes have coincided with a marked increase in ethanol consumption at population level. Similarly, the MCH seems to be sensitive to ethanol-induced elevation.

The correlation between the alterations in red blood cell indices and ethanol drinking is in accordance with a direct hematotoxic role of ethanol and its metabolites.[1,11] Ethanol can permeate cell membranes, alter the structural order of lipids, and interfere with cell structure and metabolism, thus possibly affecting erythrocyte stability.[12] Previously, high concentrations of acetaldehyde, the first metabolite of ethanol, have been found to occur inside the erythrocytes of alcoholics.[13–16] Studies in human cell lines[3] and, more recently, in Japanese heavy drinkers with aldehyde dehydrogenase-2 genotype have supported a direct role of acetaldehyde in increasing the mean cell volume of erythrocytes.[17,18] Interestingly, high MCV values in such persons have also been suggested to serve as predictors of carcinogenesis.[17] High MCV values and anemia have in turn been shown to be characteristic for patients with alcoholic liver disease.[19] Acetaldehyde is highly reactive and capable of forming stable adducts with proteins and cell membrane constituents.[1,15,20–22] As a consequence, erythrocytes may become more vulnerable to damage, hemolysis, and shortened biological half-life *in vivo*. The current data show, for the first time, that the alcoholics with high MCV frequently present with circulating antibodies, which recognize acetaldehyde-modified epitopes in proteins and to some extent correlate with the degree of macrocytosis. This observation is in line with the view that immunological mechanisms may also be involved in the generation of ethanol-induced changes in red blood cell indices in some alcoholics. However, there may be significant gender differences in driving the immune responses, which seem to warrant further investigations.

Although alcohol abuse and associated medical disorders are continuously growing problems, physicians are far too often failing to recognize hazardous drinking in clinical practice.[8,23–25] The current findings may open new perspectives for the use of MCV for biomonitoring long-term alcohol abuse. Although MCV lacks specificity in a variety of conditions, especially in hospitalized patients, it seems that a careful follow-up of blood cell indices even when occurring within reference limits could be used as indicators of changes in drinking practices, which is of special value in subjects without obvious clinical signs of ethanol dependence. Elevated MCV values have been noted to occur in 4% of adult populations, 65% of these being possibly alcohol-related.[4,25] The usefulness of MCV measurements have been emphasized in certain selected conditions, including assays on pregnant women for screening the risk for fetal alcohol effects.[26] Future studies should also address the possibility of whether MCV changes when measured together with immune parameters directed against ethanol-induced membrane alterations could further improve the diagnostic or prognostic value of such measurements. Obviously, the possible effects of ethanol on blood cell counts should also be considered carefully when establishing reference values in hospital laboratories. Any ethanol-related deviation there may impair clinicians' ability to observe the important early signals of excessive drinking practices and lead to underdetection of ethanol abuse unless separate reference values based on the values from abstainers will be made available.

REFERENCES

1. Latvala J, Parkkila S, Melkko J, Niemelä O. Acetaldehyde adducts in blood and bone marrow of patients with ethanol-induced erythrocyte abnormalities. Mol Med 2001;7:401–5.
2. Meagher RC, Sieber F, Spivak JL. Suppression of hematopoietic-progenitor-cell proliferation by ethanol and acetaldehyde. N Engl J Med 1982;307:845–9.
3. Wickramasinghe SN, Malik F. Acetaldehyde causes a prolongation of the doubling time and an increase in the modal volume of cells in culture. Alcohol Clin Exp Res 1986;10:350–4.
4. Savage DG, Ogundipe A, Allen RH, Stabler SP, Lindenbaum J. Etiology and diagnostic evaluation of macrocytosis. Am J Med Sci 2000;319:343–52.
5. Wu A, Chanarin I, Levi AJ. Macrocytosis of chronic alcoholism. Lancet 1974;1:829–31.
6. Burgermeister J. French wine makers face legal action over birth defects. BMJ 2004;329:368.
7. Lieber CS. Medical disorders of alcoholism. N Engl J Med 1995;333:1058–65.
8. Room R, Babor T, Rehm J. Alcohol and public health. Lancet 2005;365:519–30.
9. Latvala J, Hietala J, Koivisto H, Järvi K, Anttila P, Niemelä O. Immune responses to ethanol metabolites and cytokine profiles differentiate alcoholics with or without liver disease. Am J Gastroenterol 2005;100:1303–10.
10. Nordin G, Mårtensson A, Swolin B, Sandberg S, Christensen NJ, Thorsteinsson V, et al. A multicentre study of reference intervals for haemoglobin, basic blood cell counts and erythrocyte indices in the adult population of the Nordic countries. Scand J Clin Lab Invest 2004;64:385–98.
11. Eriksson CJ. The role of acetaldehyde in the actions of alcohol (update 2000). Alcohol Clin Exp Res 2001;25(suppl ISBRA): 15S–32S.

12. Beauge F, Gallay J, Stibler H, Borg S. Alcohol abuse increases the lipid structural order in human erythrocyte membranes. A steady-state and time-resolved anisotropy study. Biochem Pharmacol 1988;37:3823–8.
13. Hernández-Muñoz R, Baraona E, Blacksberg I, Lieber CS. Characterization of the increased binding of acetaldehyde to red blood cells in alcoholics. Alcohol Clin Exp Res 1989;13:654–9.
14. Magnani M, Laguerre M, Rossi L, Bianchi M, Ninfali P, Mangani F, et al. In vivo accelerated acetaldehyde metabolism using acetaldehyde dehydrogenase-loaded erythrocytes. Alcohol Alcoholism 1990;25:627–37.
15. Peterson CM, Jovanovic-Peterson L, Schmid-Formby F. Rapid association of acetaldehyde with hemoglobin in human volunteers after low dose ethanol. Alcohol 1988;5:371–4.
16. Sillanaukee P, Seppä K, Koivula T, Israel Y, Niemelä O. Acetaldehyde-modified hemoglobin as a marker of alcohol consumption: comparison of two new methods. J Lab Clin Med 1992; 120:42–7.
17. Yokoyama A, Yokoyama T, Muramatsu T, Omori T, Matsushita S, Higuchi S, et al. Macrocytosis, a new predictor for esophageal squamous cell carcinoma in Japanese alcoholic men. Carcinogenesis 2003;24:1773–8.
18. Yokoyama M, Yokoyama A, Yokoyama T, Hamana G, Funazu K, Kondo S, et al. Mean corpuscular volume and the aldehyde dehydrogenase-2 genotype in male Japanese workers. Alcohol Clin Exp Res 2003;27:1395–401.
19. Maruyama S, Hirayama C, Yamamoto S, Koda M, Udagawa A, Kadowaki Y, et al. Red blood cell status in alcoholic and nonalcoholic liver disease. J Lab Clin Med 2001;138:332–7.
20. Niemelä O, Israel Y. Hemoglobin-acetaldehyde adducts in human alcohol abusers. Lab Invest 1992;67:246–52.
21. Stevens VJ, Fantl WJ, Newman CB, Sims RV, Cerami A, Peterson CM. Acetaldehyde adducts with hemoglobin. J Clin Invest 1981;67:361–9.
22. Tuma DJ, Klassen LW. Immune responses to acetaldehyde-protein adducts: role in alcoholic liver disease. Gastroenterology 1992;103:1969–73.
23. Niemelä O. Serum diagnosis of alcoholic liver disease and markers of ethanol intake. In: Sherman DIN, Preedy V, Watson RR, eds. *Ethanol and the Liver*. New York: Taylor and Francis; 2002:411–49.
24. Skinner HA, Holt S, Sheu WJ, Israel Y. Clinical versus laboratory detection of alcohol abuse: the alcohol clinical index. Br Med J (Clin Res Ed) 1986;292:1703–8.
25. Wymer A, Becker DM. Recognition and evaluation of red blood cell macrocytosis in the primary care setting. J Gen Intern Med 1990;5:192–7.
26. Sarkola T, Eriksson CJ, Niemelä O, Sillanaukee P, Halmesmäki E. Mean cell volume and gamma-glutamyl transferase are superior to carbohydrate-deficient transferrin and hemoglobin-acetaldehyde adducts in the follow-up of pregnant women with alcohol abuse. Acta Obstet Gynecol Scand 2000;79:359–66.

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An Inverse Relationship Between Markers of Fibrogenesis and Collagen Degradation in Patients With or Without Alcoholic Liver Disease

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OBJECTIVES: Excessive deposition of collagen leading to cirrhosis is a major complication of alcohol abuse. However, the mechanisms behind the accumulation of the extracellular matrix proteins are poorly understood.

METHODS: We measured serum markers of collagen degradation (β-CTx), fibrogenesis (PINP, PIIINP), and pro- and anti-inflammatory cytokines from 84 male heavy drinkers, who were either with (N = 52) or without (N = 32) clinical or histological signs of alcoholic liver disease (ALD), and from 20 healthy nonalcoholic controls.

RESULTS: Serum β-CTx levels in ALD patients were significantly lower than in healthy controls or in the alcoholics without liver disease, while PINP and PIIINP, reflecting type I and type III collagen synthesis, respectively, were significantly increased. The alcoholics without liver disease showed values, that were not significantly different from those of healthy controls. Serum β-CTx correlated negatively with serum PIIINP and proinflammatory cytokines (IL-2, IL-6, IL-8, TNF-α), and positively with anti-inflammatory cytokines (IL-1, TGF-β), whereas serum PIIINP correlated positively with these proinflammatory cytokines and negatively with the anti-inflammatory cytokines. Calculation of PIIINP/β-CTx ratio was found to yield an excellent sensitivity (94%) and specificity (98%) in differentiating the alcoholics with liver disease.

CONCLUSION: The present findings indicate a positive relationship between markers of collagen biosynthesis and proinflammatory cytokines, and a negative relationship between these markers and a marker of collagen degradation and anti-inflammatory cytokines, suggesting that a disturbed balance in these cellular responses may facilitate fibrogenesis and play a pivotal role in the pathogenesis of ALD. These findings should also be implicated in the development of noninvasive tools for discriminating individuals at risk for fibrogenesis.

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INTRODUCTION

Alcoholic liver disease (ALD) covers a wide spectrum of morphological features from minimal injury to more advanced and prognostically serious injuries including liver cirrhosis with excessive deposition of collagen (1–3). However, the mechanisms behind the disease progression from normal liver to cirrhosis in patients with excessive ethanol consumption have remained poorly defined.

Extensive research efforts over the past two decades have led to the development of noninvasive collagen-type specific applications to monitor changes in connective tissue metabolism in human tissue. Of these, serum PIIINP, the aminoterminal propeptide of type III procollagen, has been widely used as a marker of fibrogenesis in a variety of hepatic diseases (4–7). It has been shown that PIIINP levels are frequently elevated in patients with ALD and may help to identify patients with increased or progressive collagen synthesis (7–10). Changes in serum PIIINP may also serve as indicators of prognosis in patient follow-up (8, 11–13).

β-CrossLaps (β-CTx) is a carboxy-terminal cross-linking telopeptide of type I collagen, which is released into the circulation during collagen degradation (14, 15). However, although several recent studies have found β-CTx measurements useful for assessing matrix degradation in bone diseases, as yet the diagnostic usefulness of β-CTx assays in monitoring liver diseases has not been elucidated.

The purpose of this study was to investigate the relationships among serum β-CTx, PIIINP, and pro- and anti-inflammatory cytokines in alcoholic patients with or without liver disease in order to gain further insight on the pathogenesis of liver fibrosis in human alcoholics.

MATERIALS AND METHODS

Patients and Control Subjects

We studied 84 male alcoholic patients, all of whom presented with a well-documented history of excessive ethanol consumption. There were 52 patients (age 50 ± 12 yr [mean ± SD]) who showed clinical, laboratory, and morphological evidence of ALD. The severity of liver disease was assessed by previously established combined clinical and laboratory index (CCLI) and combined morphological index (CMI) (8, 16, 17). The liver histology ranged from mild fibrosis and fatty change to cirrhosis with a wide distribution of morphological abnormalities related to alcoholic hepatitis. In addition, there were 32 patients (mean age 44 ± 10 yr) who were heavy drinkers and had been consecutively admitted for detoxification, but did not have any clinical and laboratory signs of significant liver disease. They also showed, however, a history of continuous ethanol consumption or binge drinking, which had consisted of a mean of 130 g/day during the period of 4 wk prior to sampling, as assessed by detailed personal interviews using a timeline follow-back technique. Due to ethical considerations, these patients were, however, not biopsied. Healthy nonalcoholic controls were 20 male volunteers (mean age 52 ± 17 yr), who were either abstainers (N = 5) or moderate drinkers (N = 15), whose mean daily ethanol consumption from the past 1 month prior to sampling was between 1 and 36 g of ethanol per day. All of these individuals were also devoid of any previous history of alcohol abuse.

The serum samples were stored at −70°C until analysis. All participants gave their informed consent and the study was carried out according to the provisions of the Declaration of Helsinki.

Methods

The concentration of serum β-CTx and the aminoterminal propeptide type I procollagen (PINP) were determined using electrochemiluminescence immunoassay kits (Roche Diagnostics, Espoo, Finland). Serum aminoterminal propeptide of type III procollagen (PIIINP) was measured by a radioimmunological procedure (Orion Diagnostica, Espoo, Finland). The concentrations of interleukins (IL-1, IL-2, IL-6, IL-8, IL-10, TNF-α, and TGF-β) were determined using Quantikine high sensitivity ELISA kits according to the instructions of the manufacturer (R&D Systems, Abington Science Park, UK). Serum gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), alkaline phosphatase, albumin, and bilirubin concentrations were determined using standard clinical chemical methods in an accredited laboratory (SFS-EN ISO/IEC 17025) of Seinäjoki Central Hospital, Finland. Assay cutoffs were based on ROC analyses and reference limits given by the assay manufacturer.

Statistical Methods

The data are expressed as mean ± SEM. The differences among groups were determined with one-way analysis of variance (ANOVA) or with the Kruskal-Wallis test, as required. Correlations were calculated using Spearman's rank correlation. The statistical analyses were carried out using SPSS for Windows 13.0 (Chicago, IL) software. Analyse-It for Microsoft Excel software (Analyse-it Software Ltd, Leeds, UK) was used for ROC analyses.

RESULTS

ALD status in the present material was found to be significantly associated with both the collagen and cytokine markers. Mean serum β-CTx concentrations in ALD patients (130 ± 12 pg/mL) were lower than the values in the alcoholics without liver disease (340 ± 27 pg/mL) or in the healthy nonalcoholic controls (380 ± 41 pg/mL) ($P < 0.001$ for both comparisons) (Fig. 1A). In contrast, serum PIIINP (Fig. 1B) and PINP (Fig. 1C) levels in the corresponding comparisons were both significantly higher among ALD patients. β-CTx values were found to be abnormally low in 79% of the ALD patients, whereas only in 13% of the alcoholics without liver disease and in none of the healthy controls (Table 1). Serum PIIINP values in turn were increased in 85% of the ALD patients, and in 6% of the alcoholics without liver disease (Table 1). Calculation of the PIIINP/β-CTx (Fig. 1D) or PINP/β-CTx (Fig. 1E) ratios were found to further potentiate the differences between the ALD patients and the other study groups. ROC analyses on the PIIINP/β-CTx ratio (with a cutoff of 32) yielded an excellent sensitivity (94%) and specificity (98%) in differentiating the alcoholics with liver disease (AUC 0.990, 95% confidence interval 0.972–1.000). The sensitivity for this ratio was also higher than the corresponding sensitivity for the PINP/β-CTx ratio (87% for a specificity of 98%) (cutoff 254, AUC 0.978, 95% confidence interval 0.952–1.000).

Table 2 summarizes the correlations among the various markers of connective tissue metabolism, pro- and anti-inflammatory cytokines, and the indices of liver status. Figure 2 further illustrates the representative correlations by also identifying the subgroups. Serum β-CTx correlated negatively with PIIINP ($r = -0.42$, $P < 0.001$) and the proinflammatory cytokines IL-2 ($r = -0.26$, $P < 0.01$), IL-6 ($r = -0.22$, $P < 0.05$), IL-8 ($r = -0.23$, $P < 0.05$), and TNF-α ($r = -0.48$, $P < 0.001$), whereas positive correlations emerged between β-CTx and the anti-inflammatory cytokines IL-1 ($r = 0.35$, $P < 0.001$) and TGF-β ($r = 0.39$, $P < 0.001$). Serum PIIINP showed statistically significant positive correlations with PINP ($r = 0.63$, $P < 0.001$) and the proinflammatory cytokines IL-2 ($r = 0.33$, $P < 0.001$), IL-6 ($r = 0.44$, $P < 0.001$), IL-8 ($r = 0.55$, $P < 0.001$), and TNF-α ($r = 0.67$, $P < 0.001$), and negative correlations with the anti-inflammatory cytokines IL-1 ($r = -0.31$, $P < 0.01$) and TGF-β ($r = -0.41$, $P < 0.001$).

When the markers of collagen metabolism were further compared with the histological and clinical indices of liver disease severity and grading of fibrosis in the biopsy sample,

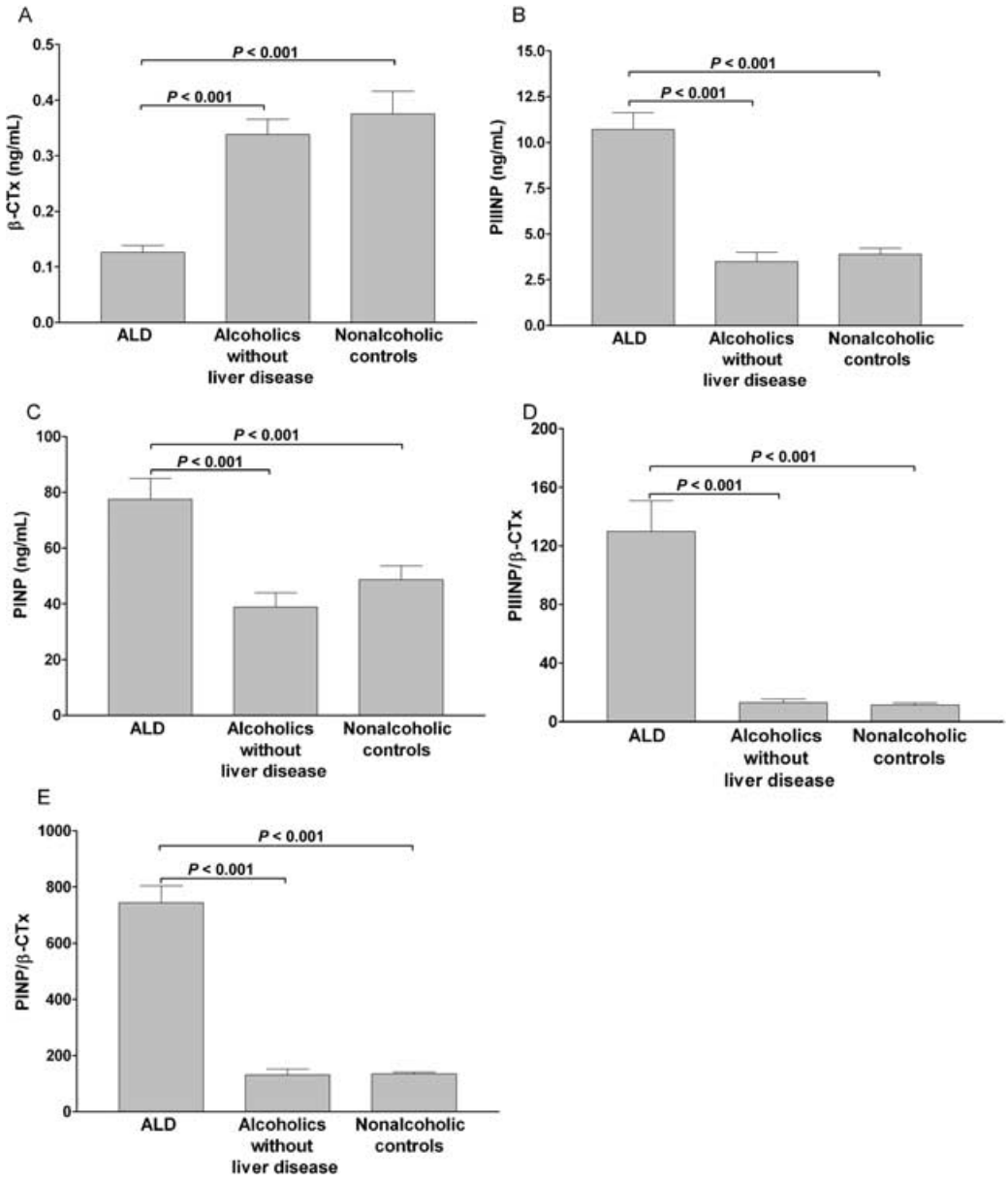

Figure 1. The concentrations of serum markers of collagen degradation (β-CTx) and fibrogenesis (PIIINP, PINP), and PIIINP/β-CTx and PINP/β-CTx ratios in patients with or without alcoholic liver disease (ALD) and in healthy controls. ALD status was associated with significantly lower levels of β-CTx when compared with the alcoholics without liver disease ($P < 0.001$) or with healthy nonalcoholic controls ($P < 0.001$). In contrast, ALD patients showed significantly higher levels of both PIIINP and PINP than the alcoholics without liver disease or the healthy controls. Calculations of the PIIINP/β-CTx or PINP/β-CTx ratio was found to further potentiate the differences between ALD patients and the other study groups. Data are expressed as mean ± SEM.

significant correlations emerged between PIIINP and fibrosis ($r = 0.63$, $P < 0.001$), CMI ($r = 0.50$, $P < 0.01$), and CCLI ($r = 0.42$, $P < 0.05$), whereas there were no significant correlations between these parameters and PINP or β-CTx levels (Table 2).

DISCUSSION

Morbidity and mortality related to excessive ethanol consumption have been continuously growing in most western countries (3, 18). Current prevalence of liver cirrhosis is known to be particularly high in countries with the highest per capita consumption of alcohol. The onset of liver fibrosis may be insidious and since not all chronic alcoholics develop cirrhosis, extensive research efforts have been devoted to examine the molecular and cellular mechanisms regulating the deposition of hepatic extracellular matrix proteins. Due to the limitations of liver biopsy in assessing disease activity and liver fibrosis, the need for new, noninvasive modalities for differentiating those at risk for progressive fibrosis has also been widely acknowledged (2, 19–22).

Table 1. Incidences of Increased or Decreased Values (%) of the Markers of Collagen Degradation (β-CTx) and Synthesis (PIIINP and PINP) in Patients With or Without ALD

		β-CTx	PIIINP	PINP
Alcoholics with liver disease	Increased	0%	85%	58%
	Decreased	79%	0%	0%
Alcoholics without liver disease	Increased	6%	6%	19%
	Decreased	13%	38%	16%

β-CTx = β-CrossLaps; PIIINP = the aminoterminal propeptide of type III procollagen; PINP = the aminoterminal propeptide of type I procollagen.

The present study indicates that serum β-CTx, a marker of collagen degradation, and PINP and PIIINP, markers of collagen types I and III synthesis, respectively, follow an inverse kinetics in alcoholic patients with and without liver disease, and suggests a disturbed balance between collagen synthesis and matrix degradation in those with liver disease. Liver fibrosis is known to be associated with significant alterations in both the amount and composition of the extracellular matrix components and there may be aberrant accumulation of the various collagen types (23, 24). In advanced cirrhosis, the liver may contain sixfold more collagen than normal, the majority of this consisting of collagen types I and III (24).

Table 2. Correlations Between the Markers of Collagen Degradation (β-CTx), Fibrogenesis (PIIINP, PINP), Cytokine Levels, and Indices of Liver Status

Markers of Collagen Metabolism	β-CTx	PIIINP	PINP
β-CTx	1		
PIIINP	–0.42***	1	
PINP	0.05	0.63***	1
Proinflammatory cytokines			
IL-2	–0.26**	0.33***	0.10
IL-6	–0.22*	0.44***	0.14
IL-8	–0.23*	0.55***	0.25*
TNF-α	–0.48***	0.67***	0.36***
Anti-inflammatory cytokines			
IL-1	0.35***	–0.31**	–0.23*
IL-10	0.17	–0.10	–0.08
TGF-β	0.39***	–0.41***	–0.16
Markers of liver status			
GGT	–0.31**	0.28***	–0.19
AST	–0.10	0.20*	–0.18
AP	–0.42***	0.50***	0.19
Albumin	0.00	–0.30**	–0.17
Bilirubin	0.33***	–0.08	–0.08
Clinical and histological data			
CCLI	0.04	0.50***	−0.03
CMI	0.33	0.50***	0.11
Fibrosis grade	0.21	0.63***	0.28

For β-CTx, PIIINP and PINP see Table 1; IL = interleukin; TNF-α = tumor necrosis factor-α; TGF-β = transforming growth factor-β; AST = aspartate aminotransferase; AP = alkaline phosphatase; GGT = gamma glutamyl transferase; CCLI = combined clinical and laboratory index; CMI = combined morphological index. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

The greater synthesis rates of these collagens in ALD have been previously acknowledged in several studies involving type-specific methods (5, 8, 10, 13). However, although specific fragments of C-terminal collagen telopeptides (CTx) have recently been found to be useful for assessing collagen degradation (14), so far such measurements have been applied exclusively for diseases affecting bone metabolism (25, 26). The present data indicate that this crosslinked isomerized fragment of type I collagen in serum is markedly decreased in ALD patients and shows an inverse relationship with the peptides reflecting enhanced synthesis of collagen types I and III. Thus, the fibrous collagens may be deposited at a faster rate than the collagenolytic activity for removing the accumulated scar matrix is functioning, which is in accordance with previous findings on lower collagenase and matrix metalloproteinase activities in cirrhotic patients (27, 28). There may also be overexpression of their inhibitors, such as tissue inhibitor of metalloproteinase aggravating the failure of collagen removal to keep pace with enhanced collagen synthesis (2, 27–29).

The present findings also open the possibility that assays of these collagen markers, particularly the PIIINP/β-CTx ratio, could provide a simple and cost-efficient tool to improve the clinical discrimination of persons at risk for liver disease and severe fibrosis. Such an approach could perhaps be more suitable for routine clinical use than the recently introduced models involving large combinations of fibrotic markers together with mathematically formulated equations for interpreting the data (22). It should also be noted that although β-CTx is a degradation fragment of type I collagen, the analyses on the PINP/β-CTx ratios indicated a lower sensitivity (87%) for this ratio in discriminating the ALD patients than the corresponding ratio calculated from the type III collagen peptide data (PIIINP/ ß-CTX) (94%). There was also a lack of correlation between type I collagen-derived fragments and the indices of liver disease severity, especially with those reflecting inflammation (CCLI, CMI), whereas PIIINP and these parameters showed strong positive correlations. This is likely to be due to differences in both the metabolic kinetics of type I and type III collagens in the different phases of liver disease and in their tissue specificities. While type I collagen is the major fibrous collagen of bone, it also occurs in soft connective tissue together with type III collagen (24). Type III collagen is the more pliable collagen of organs like liver and its half-life is shorter than that of type I collagen. In the early phase of the fibroproliferative reaction, fibroblastic cells synthesize extracellular matrix, which contains large amounts of type III collagen, whereas type I collagen may predominate in advanced liver cirrhosis (24). Therefore, measurements of type III collagen-derived fragments may be more sensitive to reflect the changes occurring during the inflammatory phases and hepatic fibrogenesis in its early phase (8).

The present findings further indicate that a switch between the proinflammatory Th1 cytokines and the anti-inflammatory Th2 cytokines may facilitate hepatic fibrogenesis. Several earlier studies have suggested that IL-1, IL-6,

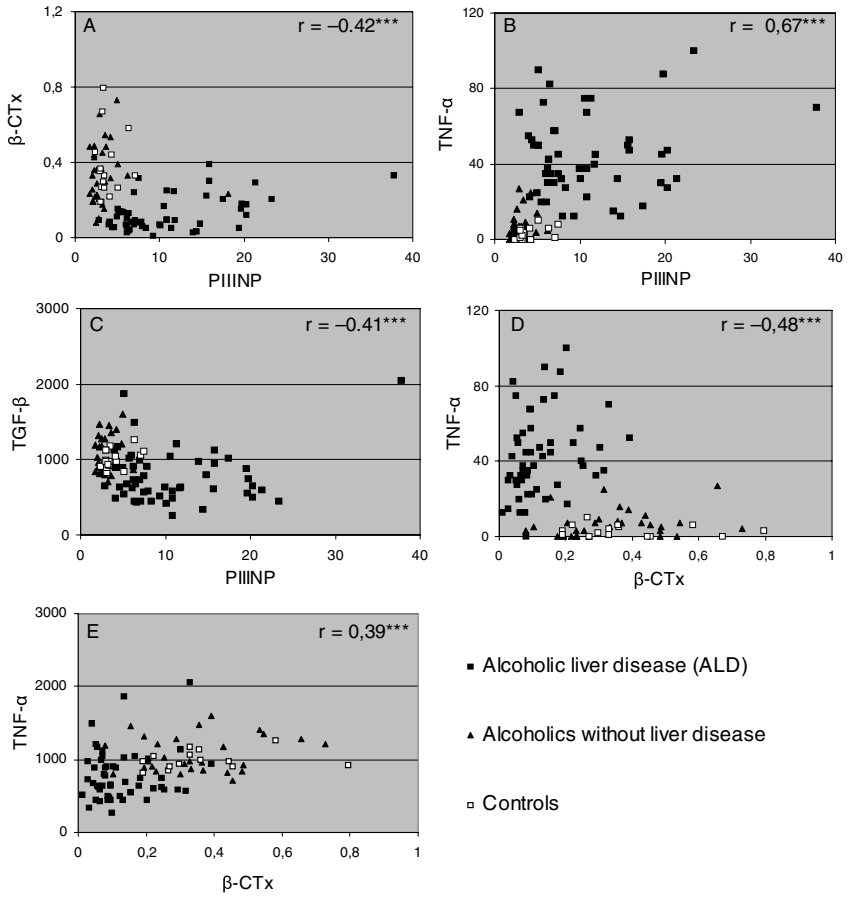

Figure 2. Representative correlations between serum markers of collagen degradation (β-CTx), fibrogenesis (PIIINP), and proinflammatory (TNF-α) and anti-inflammatory (TGF-β) cytokines. The observations from the different study subgroups in scatter plots are identified by the use of separate symbols, as indicated. ***, $P < 0.001$.

IL-8, and tumor necrosis factor-α (TNF-α) are important mediators of inflammation, which may contribute to the development and progression of ALD (30–35). Greater cytokine activities have also been suggested to correlate with the severity and prognosis of liver disease (35–38). The present data show that the markers of collagen synthesis correlate positively, particularly with the proinflammatory cytokines IL-8 and TNF-α. Interestingly, high levels of these cytokines in ALD patients have been linked with lower long-term survival (39–43). A close association among greater TNF-α production, Kupffer cell activation, inflammation, and necrosis has also been well established in experimental alcohol-induced liver injury (44–46). Interleukin-8 is a neutrophil chemoattractant and activator, and its enhanced expression coincides with lower hepatocyte survival in cultured human hepatocytes (47). On the other hand, IL-6 may be involved in liver regeneration and resistance to injury (44, 48–50). It should also be noted that although enhanced TGF-β expression has been previously linked with the development of fibrosis in alcoholics (51), the present data indicate a negative correlation between collagen synthesis markers and TGF-β levels.

Based on recent observations in patients with disturbed bone metabolism, and on the known effects of ethanol on bone mass (52–54), one could have expected elevated β-CTx values in ALD patients showing typically a high frequency of skeletal fractures. The levels of serum β-CTx were, however, significantly lower in the present group of ALD patients when compared to the healthy controls. Previously, studies in patients with primary biliary cirrhosis have also found increased greater collagen degradation-related peptides in urine, whereas serum osteocalcin, a marker of osteoblastic activity, was not influenced by the histological stage of the disease (55). The above findings suggest that collagen-related markers of bone turnover rather reflect liver collagen metabolism than bone remodeling.

Taken together, ALD status in alcoholic patients was found here to be significantly associated with both the collagen and cytokine markers. Although at this time, we cannot rule out the possibility that there may be minor overlapping with the individuals classified here as heavy drinkers without liver disease (and who due to ethical considerations were not biopsied) and patients with mild degrees of liver disease, the present material also representing a wide range of liver disease severity should be sufficient not to create any significant selection bias. The data showing a positive relationship between markers of collagen synthesis and proinflammatory

cytokines (IL-8 and TNF-α) and a negative relationship between these markers and a marker of collagen degradation (β-CTx) and anti-inflammatory cytokines (IL-1, TGF-β) should be implicated in studies on the pathogenesis of ALD in humans and in the development of more specific clinical markers for early identification of patients at risk of progression to cirrhosis.

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STUDY HIGHLIGHTS

What Is Current Knowledge

- Liver fibrosis is a major complication of alcohol abuse.
- The mechanisms behind fibrosis are poorly understood.
- New noninvasive tools for assessing fibrosis are needed.

What Is New Here

- An index of collagen degradation is decreased in alcoholic liver disease.
- Markers of collagen degradation and synthesis follow inverse kinetics.
- The approach developed here may differentiate alcoholics at risk for cirrhosis.

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REFERENCES

1. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209–18.
2. Friedman SL. Liver fibrosis—from bench to bedside. J Clin Invest 2003;38(suppl 1):S38–53.
3. Lieber CS. Medical disorders of alcoholism. J Clin Invest 1995;333:1058–65.
4. Kamal SM, Turner B, He Q, et al. Progression of fibrosis in hepatitis C with and without schistosomiasis: Correlation with serum markers of fibrosis. Hepatology 2006;43:771–9.
5. McCullough AJ, Stassen WN, Wiesner RH, et al. Serum type III procollagen peptide concentrations in severe chronic active hepatitis: Relationship to cirrhosis and disease activity. Hepatology 1987;7:49–54.
6. Niemelä O, Risteli L, Sotaniemi EA, et al. Aminoterminal propeptide of type III procollagen in serum in alcoholic liver disease. Gastroenterology 1983;85:254–9.
7. Torres-Salinas M, Pares A, Caballeria J, et al. Serum procollagen type III peptide as a marker of hepatic fibrogenesis in alcoholic hepatitis. Gastroenterology 1986;90:1241–6.
8. Niemelä O, Risteli J, Blake JE, et al. Markers of fibrogenesis and basement membrane formation in alcoholic liver disease. Relation to severity, presence of hepatitis, and alcohol intake. Gastroenterology 1990;98:1612–9.
9. Pemberton PW, Smith A, Warnes TW. Non-invasive monitoring of oxidant stress in alcoholic liver disease. Scand J Gastroenterol 2005;40:1102–8.
10. Rosman AS, Lieber CS. Diagnostic utility of laboratory tests in alcoholic liver disease. Clin Chem 1994;40:1641–51.
11. Nøjgaard C, Johansen JS, Christensen E, et al. Serum levels of YKL-40 and PIIINP as prognostic markers in patients with alcoholic liver disease. J Hepatol 2003;39:179–86.
12. Teare JP, Sherman D, Greenfield SM, et al. Comparison of serum procollagen III peptide concentrations and PGA index for assessment of hepatic fibrosis. Lancet 1993;342:895–8.
13. Trinchet JC, Hartmann DJ, Pateron D, et al. Serum type I collagen and N-terminal peptide of type III procollagen in patients with alcoholic liver disease: Relationship to liver histology. Alcohol Clin Exp Res 1992;16:342–6.
14. Okabe R, Nakatsuka K, Inaba M, et al. Clinical evaluation of the Elecsys beta-CrossLaps serum assay, a new assay for degradation products of type I collagen C-telopeptides. Clin Chem 2001;47:1410–4.
15. Reichel H, Roth HJ, Schmidt-Gayk H. Evaluation of serum beta-carboxy-terminal cross-linking telopeptide of type I collagen as marker of bone resorption in chronic hemodialysis patients. Nephron Clin Pract 2004;98:c112–8.
16. Orrego H, Israel Y, Blake JE, et al. Assessment of prognostic factors in alcoholic liver disease: Toward a global quantitative expression of severity. Hepatology 1983;3:896–905.
17. Orrego H, Blake JE, Blendis LM, et al. Prognosis of alcoholic cirrhosis in the presence and absence of alcoholic hepatitis. Gastroenterology 1987;92:208–14.
18. Room R, Babor T, Rehm J. Alcohol and public health. Lancet 2005;365:519–30.
19. Bedossa P, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology 2003;38:1449–57.
20. Callewaert N, Van Vlierberghe H, Van Hecke A, et al. Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. Nat Med 2004;10:429–34.
21. Ghany MG, Doo E. Assessment of liver fibrosis: Palpate, poke or pulse? Hepatology 2005;42:759–61.
22. Imbert-Bismut F, Ratziu V, Pieroni L, et al. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: A prospective study. Lancet 2001;357:1069–75.
23. Maher JJ, McGuire RF. Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. J Clin Invest 1990;86:1641–8.
24. Rojkind M, Giambrone MA, Biempica L. Collagen types in normal and cirrhotic liver. Gastroenterology 1979;76:710–9.
25. Garnero P, Ferreras M, Karsdal MA, et al. The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. J Bone Miner Res 2003;18:859–67.
26. Rosen HN, Moses AC, Garber J, et al. Serum CTX: A new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. Calcif Tissue Int 2000;66:100–3.
27. Iredale JP. Cirrhosis: New research provides a basis for rational and targeted treatments. J Clin Invest 2003;327:143–7.
28. Maruyama K, Feinman L, Fainsilber Z, et al. Mammalian collagenase increases in early alcoholic liver disease and decreases with cirrhosis. Life Sci 1982;30:1379–84.

29. Li J, Rosman AS, Leo MA, et al. Tissue inhibitor of metalloproteinase is increased in the serum of precirrhotic and cirrhotic alcoholic patients and can serve as a marker of fibrosis. Hepatology 1994;19:1418–23.
30. Crews FT, Bechara R, Brown LA, et al. Cytokines and alcohol. Alcohol Clin Exp Res 2006;30:720–30.
31. Hill DB, Marsano LS, McClain CJ. Increased plasma interleukin-8 concentrations in alcoholic hepatitis. Hepatology 1993;18:576–80.
32. Khoruts A, Stahnke L, McClain CJ, et al. Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. Hepatology 1991;13:267–76.
33. Latvala J, Hietala J, Koivisto H, et al. Immune responses to ethanol metabolites and cytokine profiles differentiate alcoholics with or without liver disease. Am J Gastroenterol 2005;100:1303–10.
34. McClain CJ, Cohen DA. Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. Hepatology 1989;9:349–51.
35. Neuman MG, Brenner DA, Rehermann B, et al. Mechanisms of alcoholic liver disease: Cytokines. Alcohol Clin Exp Res 2001;25:251S–3S.
36. Fujimoto M, Uemura M, Nakatani Y, et al. Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: Relation to severity of liver disturbance. Alcohol Clin Exp Res 2000;24:48S–54S.
37. Hill DB, Marsano L, Cohen D, et al. Increased plasma interleukin-6 concentrations in alcoholic hepatitis. J Lab Clin Med 1992;119:547–52.
38. Rodríguez-Rodríguez E, González-Reimers E, Santolaria-Fernández F, et al. Cytokine levels in acute alcoholic hepatitis: A sequential study. Drug Alcohol Depend 1995;39:23–7.
39. Bird GL, Sheron N, Goka AK, et al. Increased plasma tumor necrosis factor in severe alcoholic hepatitis. Ann Intern Med 1990;112:917–20.
40. Felver ME, Mezey E, McGuire M, et al. Plasma tumor necrosis factor alpha predicts decreased long-term survival in severe alcoholic hepatitis. Alcohol Clin Exp Res 1990;14:255–9.
41. Huang YS, Chan CY, Wu JC, et al. Serum levels of interleukin-8 in alcoholic liver disease: Relationship with disease stage, biochemical parameters and survival. J Hepatol 1996;24:377–84.
42. McClain C, Barve S, Joshi-Barve S, et al. Dysregulated cytokine metabolism, altered hepatic methionine metabolism and proteasome dysfunction in alcoholic liver disease. Alcohol Clin Exp Res 2005;29:180S–8S.
43. Swiatkowska-Stodulska R, Bakowska A, Drobinska-Jurowiecka A. Interleukin-8 in the blood serum of patients with alcoholic liver disease. Med Sci Monit 2006;12: CR215–20.
44. Colantoni A, Idilman R, De Maria N, et al. Hepatic apoptosis and proliferation in male and female rats fed alcohol: Role of cytokines. Alcohol Clin Exp Res 2003;27:1184–9.
45. Iimuro Y, Gallucci RM, Luster MI, et al. Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. Hepatology 1997;26:1530–7.
46. Yin M, Wheeler MD, Kono H, et al. Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. Gastroenterology 1999;117:942–52.
47. Joshi-Barve S, Barve SS, Butt W, et al. Inhibition of proteasome function leads to NF-kappaB-independent IL-8 expression in human hepatocytes. Hepatology 2003;38:1178–87.
48. Streetz KL, Luedde T, Manns MP, et al. Interleukin 6 and liver regeneration. Gut 2000;47:309–12.
49. Taub R. Hepatoprotection via the IL-6/Stat3 pathway. J Clin Invest 2003;112:978–80.
50. Zimmers TA, McKillop IH, Pierce RH, et al. Massive liver growth in mice induced by systemic interleukin 6 administration. Hepatology 2003;38:326–34.
51. Chen WX, Li YM, Yu CH, et al. Quantitative analysis of transforming growth factor beta 1 mRNA in patients with alcoholic liver disease. World J Gastroenterol 2002;8:379–81.
52. Chakkalakal DA. Alcohol-induced bone loss and deficient bone repair. J Clin Invest 2005;29:2077–90.
53. Chen CC, Wang SS, Jeng FS, et al. Metabolic bone disease of liver cirrhosis: Is it parallel to the clinical severity of cirrhosis? J Gastroenterol Hepatol 1996;11:417–21.
54. Vignesh RC, Sitta DS, Jayasudha E, et al. Effect of ethanol on human osteosarcoma cell proliferation, differentiation and mineralization. Toxicology 2006;220:63–70.
55. Guañabens N, Parés A, Alvarez L, et al. Collagen-related markers of bone turnover reflect the severity of liver fibrosis in patients with primary biliary cirrhosis. J Bone Miner Res 1998;13:731–8.

CONFLICT OF INTEREST

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