JOHANNA HIETALA

Novel Use of Biomarkers and Their Combinations for Detecting Excessive Drinking

ACADEMIC DISSERTATION
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To my family
Abstract

Excessive alcohol consumption and consequent medical disorders create a major burden for modern health care. In addition to medical and social problems, excessive drinking causes considerable strains on the national economy. In order to improve the diagnosis and treatment of patients suffering from ethanol-related health problems, reliable and accurate methods for recognizing excessive alcohol consumption in its early phase need to be developed. Currently, excessive drinkers tend to escape detection, which may lead to delays in intervention.

The present study was set out to develop new approaches for detecting excessive drinking based on conventional and new laboratory tests and their combinations and to address the relationships between such markers and alcoholic liver disease. Conventional laboratory markers of excessive drinking (GGT, CDT, MCV, AST, ALT), a mathematically formulated combination of GGT and CDT, and autoimmune responses to proteins modified with acetaldehyde, the first metabolite of ethanol, were measured in alcoholics with or without liver disease, moderate drinkers and abstainers. Cytokine profiles of subjects were also studied in order to clarify the associations between alcohol intake and pathogenesis of alcoholic liver disease.

The results show that even moderate drinking may increase levels of gamma-glutamyl transferase (GGT). When GGT was combined logarithmically with carbohydrate-deficient transferrin (CDT), the diagnostic performance of the combination GGT-CDT was found to markedly exceed that of the traditional markers, reaching a sensitivity of 90% whereas the sensitivities of its parent components remained at 63% (CDT) and 58% (GGT).

Alcohol consumption was also found to induce alterations in the immune system. An association between cytokine levels and alcohol use was observed, with most evident alterations in alcoholics with liver disease. Pro-inflammatory cytokines (IL-2, IL-6, IL-8, TNF-α) were found to increase in alcoholic liver disease whereas the levels of anti-inflammatory cytokines, particularly TGF-β1 showed a slight decrease.

Acetaldehyde adducts are formed when acetaldehyde reacts with proteins and cellular constituents. Antibodies directed against acetaldehyde-modified proteins were found in the circulation of alcoholic patients. The highest anti-adduct IgA and IgG titres occurred in patients with alcoholic liver disease, while specific class IgM antibodies were most abundant in alcoholics without liver disease. The possible usefulness of anti-adduct IgA as a marker of excessive drinking was subsequently studied in alcoholics without liver disease, and the mean anti-adduct IgA levels were significantly higher than those in the moderate drinkers or abstainers (p < 0.001), while the difference between the moderate drinkers and abstainers was also significant (p < 0.05). Mean daily ethanol consumption during the previous month was found to correlate significantly with anti-adduct IgA levels. Based on these findings, anti-adduct IgA antibodies could serve as markers of alcohol con-
A combination of the anti-adduct IgA and CDT results showed improved diagnostic performance for this marker (IgA-CDT) as compared to traditional ones.

In the light of these results, it may be suggested that GGT, CDT and IgAs against acetaldehyde-derived epitopes either alone or combined by means of mathematical equations can serve as useful markers of alcohol consumption. The elevation of GGT in moderate drinkers as compared with abstainers may affect its reference ranges, since they are usually calculated from general population in which the proportion of abstainers is decreasing. The results also indicate that the antibody responses and alterations in cytokine balance may contribute to the pathogenesis and progression of alcoholic liver disease in humans.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>Apo J</td>
<td>Apolipoprotein J</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AUDIT</td>
<td>Alcohol Use Disorders Identification Test</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAGE</td>
<td>Cut down, Annoyed, Guilty, Eye-opener (acronym)</td>
</tr>
<tr>
<td>CCLl</td>
<td>Combined clinical and laboratory index</td>
</tr>
<tr>
<td>CDT</td>
<td>Carbohydrate-deficient transferrin</td>
</tr>
<tr>
<td>%CDT</td>
<td>CDT as a percentage of total transferrin</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CMI</td>
<td>Combined morphological index</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Cytochrome P450 IIE1</td>
</tr>
<tr>
<td>EOA</td>
<td>Early onset alcoholics</td>
</tr>
<tr>
<td>EtG</td>
<td>Ethyl glucuronide</td>
</tr>
<tr>
<td>EtS</td>
<td>Ethyl sulphate</td>
</tr>
<tr>
<td>FAE</td>
<td>Fetal alcohol effects</td>
</tr>
<tr>
<td>FAEEs</td>
<td>Fatty acid ethyl esters</td>
</tr>
<tr>
<td>FAS</td>
<td>Fetal alcohol syndrome</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma-glutamyl transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein -cholesterol</td>
</tr>
<tr>
<td>HIAA</td>
<td>Hydroxyindole-acetic acid</td>
</tr>
<tr>
<td>HTOL</td>
<td>Hydroxytryptophol</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgA, IgG, IgM</td>
<td>Immunoglobulin A, G, M</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LOA</td>
<td>Late onset alcoholics</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAST</td>
<td>Michigan Alcoholism Screening Test</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MEOS</td>
<td>Microsomal ethanol oxidizing system</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PEth</td>
<td>Phosphatidylethanol</td>
</tr>
</tbody>
</table>
PINP     Aminoterminal propeptide of type I collagen
PIIINP   Aminoterminal propeptide of type III collagen
PPV      Positive predictive value
ROC      Receiver-operating characteristic
SA       Sialic acid
SD       Standard deviation
SIJ      Sialic acid index of apolipoprotein J
TGF-β1   Transforming growth factor-β1
TLFB     Timeline follow-back -method
TNF-α    Tumor necrosis factor-α
List of original publications


The original articles are referred to in the text with the above Roman numerals.
1. Introduction

Excessive alcohol consumption and consequent medical disorders are considerable problems in many Western countries (Österberg 2006, Blocker, Jr. 2006). In addition to medical and social problems at the individual level, excessive drinking causes significant problems for the national economy. Resources should therefore be focused on reducing the prevalence of alcoholism through more effective diagnosis and early intervention (Anderson 1993, Sharpe 2001, Fleming et al. 2002, Latt and Saunders 2002, Niemelä 2002, Rehm et al. 2006). It has been estimated that only 20–50% of patients with alcoholism are actually identified in health care and thus more reliable and accurate methods of recognizing excessive alcohol consumption in clinical work are urgently needed (Reid et al. 1986, Moore et al. 1989, Sharpe 2001).

The diagnosis of excessive alcohol consumption is often based on patients' own reports and answers to questionnaires. This approach suffers from a lack of reliability, because patients are usually unwilling to admit excessive drinking (Rosman and Lieber 1994, Sharpe 2001, Niemelä 2002). Laboratory markers have been integrated into the diagnostics and may lead to a considerable improvement in detecting excessive drinking (Lieber 1995, Niemelä 2002). The main advantage of laboratory markers is their objective nature. The results of laboratory tests revealing excessive alcohol consumption and possible tissue injury could motivate patients more than a verbal report by a clinician (Allen et al. 1992, Rosman and Lieber 1994). It has also been suggested that a better understanding of the physiological effects of ethanol and the mechanisms by which alcohol exerts its effects on different tissues would lead to improved diagnostics and treatment of alcohol use disorders.

This work was aimed at investigating the usefulness of various laboratory markers and their combinations for detecting excessive drinking. The effect of abstaining and moderate drinking on levels of these markers was studied. The aims were also to clarify the effects of alcohol on specific antibody and cytokine responses and to investigate the possibility of using such responses as markers of alcohol consumption and tissue injury.
2. Review of the literature

2.1. Ethanol: past and present

Alcohol has been used in human societies from the beginning of recorded history. The earliest alcoholic beverages were fermented drinks, and distilled alcohol products emerged about 3000 years ago. Alcohol has been used over the centuries for both medicinal and refreshment purposes (Milkikan 1999), and the production of alcoholic beverages has gradually become industrialized and has turned into a worldwide business (Walsh 1997, Room 1997). After the temperance movements of late 19th and early 20th centuries, alcohol consumption has become more accepted and is no longer viewed as a threat to all individuals (Blocker, Jr. 2006). Consequently, consumption volumes have grown continuously in many industrialized countries since World War II, with the exception of certain Mediterranean countries, which have shown a slow downward trend during recent decades (Pyörälä 1990, Medical Research Council 1998, Stakes 2005, Blocker, Jr. 2006, IAS 2006). Alcohol consumption by women and young people in particular has increased considerably since the war, and attention has also been focused more recently on the increasing use of alcohol among the elderly.

It has been known for centuries that alcohol consumption is somehow associated with increased illness and death, and the scientific study of alcohol-related mortality that started in the 1920s has continued with the establishment of a connection between alcohol and liver disease (Mann et al. 2003). Over the past few decades, alcohol and alcoholism have been studied intensively in order to achieve more effective diagnosis, treatment and prevention.

Alcoholism and alcohol-related disorders place a considerable burden on society nowadays, and alcohol strategies should therefore be focused on the prevention and early detection of alcoholism (Wilson 1995, Room et al. 2005, Rehm et al. 2006). The simultaneous use of alcohol and drugs has also become more common, posing a new challenge to health care. The price and availability of alcoholic beverages are among the major factors that have contributed to alcohol consumption, and these should be controlled in order to reduce consumption at the population level (Chaloupka et al. 1998, Room et al. 2005).

2.2. Main features of ethanol metabolism

Ethanol is a simple molecule, which can affect cell membranes and signaling pathways throughout the body. Ethanol in itself is not very toxic, but its first metabolite, acetaldehyde, is highly toxic and may cause various adverse effects in cells and tissues (Niemelä 2001, Eriksson 2001). Acetaldehyde is carcinogenic, mutagenic and can interfere with DNA synthesis and repair. It can also react with
proteins and cellular constituents, forming stable or unstable acetaldehyde adducts. The process of ethanol metabolism also generates free oxygen radicals, which are extremely reactive and may thereby cause major damage to cells (Molina et al. 2003, Caro and Cederbaum 2004).

After ingestion, ethanol is absorbed in the gastrointestinal tract, mainly in the small intestine. Approximately 2–10% of the ethanol absorbed will be removed unaltered in the respiration and urine (Lieber 2005), while the rest will be metabolized. The oxidation of ethanol to acetaldehyde begins in gastric wall through the action of the alcohol dehydrogenase enzyme (ADH), in a process referred to as gastric first-pass metabolism (Julkunen et al. 1985, Frezza et al. 1990, Seitz and Pöschl 1997). The microbes in the alimentary tract are also capable of metabolizing ethanol. The resulting acetaldehyde may accumulate in the colon because of insufficient aldehyde dehydrogenase (ALDH) activity and may cause adverse effects there (Salaspuro 1996, Koivisto and Salaspuro 1996).

Ethanol is rapidly distributed among the various tissues of the body. Its metabolism takes place primarily in the liver, where it is oxidized to acetaldehyde, mainly via the ADH pathway. The minor metabolic pathways are the microsomal ethanol oxidizing system (MEOS) and the catalase pathway. The MEOS pathway, in which hepatic P450 cytochromes (mainly CYP2E1) are responsible for ethanol oxidation, is induced by high blood ethanol concentrations and chronic ethanol consumption (Lieber and DeCarli 1968, Salaspuro and Lieber 1978, Asai et al. 1996, Lieber 2004). The catalase pathway requires hydrogen peroxide, which is produced only in small amounts under normal circumstances (Thurman and McKenna 1975, Thurman and Handler 1989, Molina et al. 2003). More recently, however, Bradford and co-workers (1999) have reported that the catalase pathway appears to be ethanol- and methanol-inducible and that the Kupffer cells in the liver participate in the regulation of the hydrogen peroxide supply. It has also been suggested that the catalase pathway may account for most of the ethanol oxidation occurring in brain (Aragon et al. 1991, McBride et al. 2002). This is of importance because acetaldehyde, unlike intact ethanol, cannot readily pass from the blood to the brain because of the abundance of ALDH in the blood-brain barrier (Zimatkin and Deitrich 1997).

Acetaldehyde is further oxidized to acetate in liver by hepatic ALDH. The resulting acetate is transported to the muscles and heart and finally converted to carbon dioxide and water and eliminated from body.

2.3. Effects of ethanol on health

The health hazards associated with excessive alcohol consumption are numerous and well known. Almost all tissues in the body are affected by ethanol and it is related to more than 60 medical conditions (Rehm et al. 2003). The adverse health effects of excessive or even moderate ethanol consumption include both physiological and mental problems (Lieber 1995, Damström Thakker 1998, Adrian and Barry 2003, Corrao et al. 2004). Some beneficial effects of moderate drinking have also been suggested recently. The adverse and beneficial effects of alcohol consumption habits on health are summarized in Table 1.
When evaluating the health effects of ethanol, attention should be paid both to the actual amount consumed and to drinking patterns, as some adverse effects are related to occasional heavy drinking and intoxication and some to chronic consumption (Niemelä 2002, Rehm et al. 2003). Social harm and the possible health benefits seem to be more closely linked to drinking patterns than are the adverse health effects (Damström Thakker 1998). There are also several factors that affect individual susceptibility to the effects of alcohol, such as age, gender, race, body mass and genetic factors (Lieber 1995, Damström Thakker 1998).

Table 1. Possible effects of various levels of ethanol intake on health.

<table>
<thead>
<tr>
<th>Alcohol-related disorder</th>
<th>Abstaining</th>
<th>Moderate drinking</th>
<th>Excessive drinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver disease</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nervous system</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fetal effects</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Immune system</td>
<td>0</td>
<td>0 or +</td>
<td>–</td>
</tr>
<tr>
<td>Psychiatric disorders</td>
<td>0</td>
<td>0 or +</td>
<td>–</td>
</tr>
<tr>
<td>Accidents and injuries</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

+ protective effects – harmful effects 0 no effects


2.3.1. Ethanol and the liver

Since the liver accounts almost exclusively of ethanol metabolism, it is also the main target for ethanol toxicity (Lieber 1977, Jaeschke et al. 2002, Niemelä 2002). Many studies have reported a dose-response relationship between alcohol consumption and various types of liver damage (Coates et al. 1986, Norton et al. 1987, Corrao et al. 1999, White et al. 2002). Some of the adverse effects of ethanol on the liver are likely to be caused by acetaldehyde and free radical production rather than the ethanol itself (Eriksson 2001, Molina et al. 2003, Chase et al. 2005, Dey and Cederbaum 2006), and it has also become evident that the immune system plays a key role in the pathology of liver diseases (Leevy and Elbeshbeshy 2005).

The earliest abnormality to appear in the liver due to excessive alcohol consumption is the accumulation of lipids, which may lead to steatosis (fatty liver). This condition is transient and usually reversible if the alcohol intake ceases. Upon continuing ethanol intake, steatosis may develop into an inflammation process, hepatitis, which involves cell death and massive migration of immune cells into the liver. This is a potentially lethal condition and may easily recur in patients who re-
cover and resume drinking. Alcoholic cirrhosis is a condition in which the normal liver structure has gradually become fibrotic as a consequence of persistent inflammation and abnormal connective tissue metabolism. The normal functions of liver become impaired as fibrogenesis proceeds and eventually this process may lead to liver failure and death. It has been estimated that 10–15 % of all chronic alcoholics will eventually develop cirrhosis (Niemelä 2002, Mann et al. 2003).

Advanced liver disease may also contribute to brain function. When the venous blood flow to the liver is obstructed due to extensive cirrhosis, toxic substances and metabolic by-products may leak into the systemic circulation and pass to the brain, interfering in the actions of neurotransmitters (Oscar-Berman et al. 1997).

2.3.2. Ethanol and extrahepatic tissues

Even a moderate level of ethanol intake has been found to be associated with several types of cancer, including cancers of the upper aerodigestive tract, colorectal area and liver, and breast cancer in women. There seems to be a dose-response relationship between ethanol intake and the related cancer risk (Corrao et al. 1999, Boffetta and Hashibe 2006). Genetic factors have also been found to affect individual susceptibility to development of alcohol-related cancer, mainly via polymorphisms of enzymes associated with ethanol metabolism, folate metabolism and DNA repair (Boffetta and Hashibe 2006). The exact mechanisms by which ethanol induces cancer have not been fully defined but several possibilities have been discussed (Wight and Ogden 1998, Molina et al. 2003, Dumitrescu and Shields 2005, Boffetta and Hashibe 2006). Alcohol has been estimated to cause 3 % of all cancers worldwide (Medical Research Council 1998).

Continuous ethanol consumption is known to have adverse effects on the nervous system, particularly in the brain, including brain shrinkage, emotional and personality changes and learning and memory defects. It is also known to affect sleep patterns, muscular coordination and the regulation of body temperature (Oscar-Berman et al. 1997). The mechanisms by which ethanol affects the nervous system are complex and not yet fully understood, and there appear to be additional factors such as age, gender, family history, coexisting health problems and nutritional deficiencies that play a role in the development of neurological defects (Oscar-Berman et al. 1997). It is well established that ethanol can activate the dopamine system in the brain, which contributes to its rewarding effects (Di Chiara 1997, Tupala and Tiitinen 2004) and it also interferes with the actions of various other neurotransmitters (Oscar-Berman et al. 1997, Eckardt et al. 1998). Unlike other addictive drugs, however, it does not have specific receptors on cell surfaces.

Ethanol is known to penetrate the placental barrier and cause adverse effects on the growing fetus but the exact mechanisms are still unclear. Fetal alcohol syndrome (FAS), or its milder form, fetal alcohol effects (FAE), may result from alcohol consumption during pregnancy and is associated with growth and mental retardation and certain facial features and also with abnormalities in various organs (Jones and Smith 1973, Chaudhuri 2000, Jacobson and Jacobson 2002). FAS seems to be related to relatively high levels of ethanol consumption but milder symptoms such as reduced birth weight and learning problems have already been observed at moderate levels of ethanol intake (Ogston and Parry 1992, Allebeck and Olsen 1998, Eckardt et al. 1998, Sood et al. 2001). Fetal damage has been shown to be dose-dependent (Allebeck and Olsen 1998, Sood et al. 2001), al-
though high occasional blood alcohol levels have been reported to be a greater risk for the fetus than lower continuous consumption (Maier and West 2001). However, not all heavy-drinking mothers give birth to a FAS/FAE child, so that additional risk factors have been suggested to exist (Jacobson et al. 1996, Maier and West 2001). The number of children born with FAS/FAE has been increasing with the growing percentage of alcohol-consuming pregnant women and this problem needs special attention in current health care (Chaudhuri 2000, Warren and Foudin 2001, Eustace et al. 2003).

Excessive drinking may also lead to disorders in the gastrointestinal, cardiovascular, respiratory, reproductive and endocrine systems (Medical Research Council 1998, Damström Thakker 1998, Adrian and Barry 2003). High alcohol intake has been reported to cause damage to heart muscle and to increase the risk of arrhythmias (Piano and Schwertz 1994), and also to elevate blood pressure, which is a risk factor for brain infarction, for instance (Hillbom and Kaste 1990, Puddey and Beilin 2006). Excessive alcohol consumption is a major cause of acute and chronic pancreatitis, its relationship to the risk and severity of pancreatitis being dose-dependent (Durbec and Sarles 1978, Jaakkola et al. 1994, Corrao et al. 1999, 2004). Alcohol is also known to cause various adverse effects in the gastrointestinal tract, such as disruptions in the gastric mucosal barrier, which may lead to deficient absorption of many nutrients and vitamins (Bujanda 2000, Bode and Bode 2003).

2.3.3. Ethanol and the immune system

It is well known that the immune system is greatly influenced by excessive drinking (Cook 1998). It was found in the early 20th century that alcoholics are more susceptible to pneumonia than non-alcoholics, and it was later observed that alcoholics are also more susceptible to other types of infection, such as septicaemia and tuberculosis. More recent studies on immunity and alcohol have focused mostly on immune mechanisms in liver pathology although these mechanisms may also play a part in alcohol-related cardiac, endocrine and neurological damage. Ethanol is known to interfere with the functioning of cells in the immune system such as B and T cells, macrophages, monocytes and dendritic cells and may thus contribute to an increased susceptibility to infection (Cook 1998, Saeed et al. 2004, Szabo et al. 2004, Chase et al. 2005). An imbalance in the T cell subsets TH1 and TH2 has been suggested as a factor contributing to immunological disturbances in alcoholics (Cook 1998). Decreased B-cell numbers have been reported in alcoholics with liver disease, but they often show elevated immunoglobulin levels, particularly IgA levels, and increased IgA deposition in the kidneys, skin and liver (Swedlow et al. 1983, van de Wiel et al. 1988, Amore et al. 1994, Cook 1998). Excessive alcohol consumption may lead to the specific production of antibodies against ethanol-induced antigens (Israel et al. 1986, Koskinas et al. 1992, Tuma and Klassen 1992), and also to alterations in the cytokine balance, which both may contribute to the pathogenesis of tissue damage (McClain et al. 1993, Crews et al. 2006).

2.3.4. Ethanol, psychiatric disorders and accidents

The association between excessive drinking and psychiatric disorders has been studied since the 1950s. It has been reported that mental problems tend to emerge before the onset of excessive drinking but may also follow excessive drinking or the two may coincide in onset (Kessler et al.
1997, Berglund and Öjehagen 1998). Alcohol abuse or dependence can worsen the prognosis for psychiatric disorders (Berglund and Öjehagen 1998), and alcohol dependence is also a significant risk factor for suicide, particularly if associated with mental disorders (Berglund and Öjehagen 1998, Pirkola et al. 2004, Preuss et al. 2006). Alcohol has been found in the blood of 20–55 % of patients who have committed suicide (Öhberg et al. 1996, Crombie et al. 1998, Bedford et al. 2006). At the population level, suicide rates are usually high in countries with a high alcohol consumption and a rise in suicide rates is observed when total alcohol consumption increases (Berglund and Öjehagen 1998).

In addition to diseases, excessive alcohol consumption accounts for numerous cases of trauma, violence and motor vehicle accidents (Brismar and Bergman 1998, Cunningham et al. 2002, Savola et al. 2005). Alcohol-related accidents are common: it has been estimated that over 50 % of injured emergency unit patients have alcohol in their blood (Savola et al. 2005). In connection with alcohol-related violent behaviour, it has been speculated that alcohol may either weaken the normal inhibitory impulses or stimulate aggressive behaviour, but the theories are not conclusive (Källmén and Gustafson 1998, Badawy 2003). Either way, the amount of alcohol consumed is clearly associated with the number of reported cases of violence. Alcohol is also involved in significant proportions of traffic accidents and the risk is known to increase with increasing blood alcohol levels (Hingson and Winter 2003, Bedford et al. 2006, Heng et al. 2006). A recent report has also stated that even moderate drinking (blood alcohol concentration within the legal limits) may significantly increase the risk of motor vehicle accidents (Heng et al. 2006).

2.3.5. Suggested positive effects of ethanol

Although the positive effects of ethanol consumption are usually thought to lie in psychological benefits such as mood elevation, stress relief and more social behaviour, reports concerning the beneficial effects of moderate drinking on somatic health have also been published recently. Studies on coronary heart disease (CHD) have suggested that people drinking approximately 20 g of ethanol per day have the lowest relative risk of CHD, although there is a rapidly increasing risk at higher consumption levels (Doll 1997, Corrao et al. 2000, Agarwal 2002). It has been suggested that red wine may confer a greater protective effect than other types of alcoholic beverage (Grønbæk 2006). Ethanol is known to elevate high-density lipoprotein (HDL) levels and this, along with other possible factors, has been suggested to contribute to a reduction in the CHD risk (De Oliveira e Silva et al. 2000, Agarwal 2002). Moderate drinking may also be associated with a decreased risk of diabetes (Howard et al. 2004). The thresholds for "healthy" drinking are not definitive, however, as there is wide variation in individual susceptibility to the harmful effects of alcohol (Damström Thakker 1998, Enoch 2003, Fromme et al. 2004). Whether clinicians should advise patients with a high risk of cardiovascular disease, for example, to have one drink per day remains a controversial issue (Ellison 2002, Fisher Wilson 2003), especially as the same beneficial effects could in most cases be achieved with regular exercise and a low-fat diet, without the risks associated with alcohol (Damström Thakker 1998, Goldberg 2003). The benefits of moderate drinking with respect to cardiovascular disease, for instance, may quickly be outweighed by increases in liver disease and cancer, and the net effect is likely to be negative (Friedman and Klatsky 1993, Damström Thakker 1998, Goldberg 2003).
2.4. Assessing alcohol consumption

2.4.1. Amount and pattern of drinking

The limits for moderate ethanol intake and the definition of a standard drink show variation depending on the source and country. The upper limits quoted here for moderate drinking, 280 g of ethanol per week for men and 190 g for women, are those defined for Finland (Sillanaukee et al. 1992a), while in the United Kingdom, for instance, the weekly limits are set at 168 g for men and 112 g for women (Sharpe 2001). One unit or drink is defined as containing ethanol in amounts ranging from 8 g to as much as 20 g, so that ethanol consumption should preferably be assessed in grams in order to avoid confusion (Turner 1990, Dufour 1999, Brick 2006).

The methods for identifying excessive drinking include verbal reports, questionnaires and laboratory tests. In clinical practice, interviews and questionnaires form the basis for detecting excessive drinking in its early phase, whereas laboratory tests are most useful for monitoring the treatment of alcoholism, although they can also be integrated into the detection of excessive drinking (Treatment of alcohol abuse. Current Care Guideline 2005).

On the basis of their drinking habits, individuals can be divided into five groups based on limits that are currently used in Finland.

1. Abstainers, who do not drink any alcohol,
2. Moderate drinkers, who have a weekly ethanol consumption below 280 g (men) and 190 g (women) and do not drink over 80 g (men) and 60 g (women) on any single occasion,
3. Hazardous drinkers, who consume alcohol in amounts exceeding the limits of moderate drinking, but do not show any obvious immediate disorders,
4. Alcohol abusers, who consume ethanol in large amounts and have various medical, social and financial problems due to their drinking, although they do not fulfill the criteria for alcoholism, and
5. Alcoholics, alcohol abusers who meet the criteria for alcohol dependence including pathological alcohol consumption, social impairment, and the presence of tolerance and withdrawal symptoms.

Alcoholic individuals may further be classified into two subtypes according to the onset of their drinking (Buydens-Branchey et al. 1989, Johnson et al. 2000, Enoch 2003). If excessive drinking has started before the age of 20, the drinker is classified as an early onset alcoholic (EOA), also called type II alcoholism. It is often associated with antisocial behaviour and a high familial or genetic loading towards alcoholism. Late onset alcoholics (LOA) or type I alcoholics usually start drinking later in life, often as a consequence of psychosocial stress.

2.4.2. Self-reported alcohol consumption

Since there are no exact symptoms of early-phase alcohol problems, clinicians have limited means of assessing patients' drinking based on external evaluations (MacKenzie et al. 1996, Sharpe 2001).
Patients with alcohol problems often tend to deny having any, and it may be difficult to make a clinical diagnosis when based solely on an interview.

Structured questionnaires can be of assistance in identifying alcohol abusers and are well suited for screening purposes (Seppä et al. 1995, Ryb et al. 1999, Aertgeerts et al. 2001). The best-known questionnaires are AUDIT, CAGE and MAST. AUDIT (Alcohol Use Disorders Identification Test) is a quite recently developed questionnaire, which consists of 10 questions and focuses mainly on the level and frequency of consumption and the adverse consequences of drinking (Saunders et al. 1993). It has been reported that AUDIT is the most sensitive among the current questionnaires for detecting hazardous and harmful drinking (Seppä et al. 1995, MacKenzie et al. 1996, Reid et al. 1999). Shorter forms of this questionnaire (three to five questions) have also been developed and tested in clinical populations more recently, and the results have so far been quite promising (Piccinelli et al. 1997, Aertgeerts et al. 2001, Gual et al. 2002, Hodgson et al. 2002). The CAGE questionnaire includes four questions concerning drinking habits and alcohol-related problems (Ewing 1984), its name being an acronym for the key words in the questions (Cut down, Annoyed, Guilty, Eye-opener). The most extensive questionnaire is the Michigan Alcoholism Screening Test (MAST), which consists of 25 questions directed at the recognition of drinking problems, help-seeking behaviour and alcohol-related disabilities (Selzer 1971). Both CAGE and MAST have been found to perform well in detecting alcohol dependence but to be less effective in identifying hazardous drinkers (MacKenzie et al. 1996, Reid et al. 1999). Combinations of these questionnaires and novel variations have also been introduced, with efficacies comparable or superior to the older ones (Seppä et al. 1998, Merikallio-Pajunen et al. 2004, Patton et al. 2004).

A commonly used means of assessing the amounts of ethanol consumed is the timeline follow-back (TLFB) method, in which the patient is not asked to estimate the mean consumption of alcohol but to fill in a calendar and report how many drinks he or she has consumed during a specified period of time (Allen et al. 1992). This method is used mostly for research purposes and to a lesser extent in clinical interviews.

2.4.3. Laboratory markers of alcohol consumption

The introduction of laboratory tests for detecting excessive alcohol intake has improved the assessment of ethanol consumption. Laboratory tests and structured questionnaires may be used in combination in order to achieve the most efficient detection of excessive alcohol use (Sharpe 2001, Dolman and Hawkes 2005, Berner et al. 2006).

An ideal biomarker for detecting excessive drinking should be
1. Specific to ethanol, in order to avoid false positives
2. Sensitive enough to provide a useful screening tool for identifying excessive drinking also in its early phases
3. Related to the amount of alcohol consumed.
4. Suitable for monitoring abstinence (e.g. decreasing consistently after cessation of drinking) and
5. Easy and cost-effective in routine use.
None of the existing biomarkers fulfills all these criteria. The most frequently used markers today are carbohydrate-deficient transferrin, gamma-glutamyltransferase and the mean corpuscular volume of erythrocytes.

The development of a new marker usually begins by contrasting alcoholics with abstainers, which shows whether the method gives any differences between these two groups in the first place. The process continues with studies in clinical populations, since the ultimate purpose of a marker is to identify excessive drinkers in a general population, where various sources of unspecificity may occur. If the marker appears to be of value in these studies, its use may be considered in routine clinical contexts.

Basically, biomarkers can be divided into markers of acute and chronic alcohol consumption (Rosman and Lieber 1992). The former, which remain elevated for a few days, may be used for detecting relapses in alcoholic patients undergoing treatment, for example. The latter, which are usually elevated after a longer period of drinking or several shorter periods of binge drinking, are useful for identifying alcohol abuse and to some extent for screening purposes. Some of these long-term markers can also be useful for detecting relapses during treatment.

Among the measures used to describe the diagnostic performance of a marker (Boyd 1997), sensitivity is the percentage of positive test results of all the positive patients and specificity is the percentage of negative test results from all the negative patients. Thus, if an alcohol marker has a sensitivity of 60 % and a specificity of 90 %, it means that 60 % of the alcoholics will have abnormal test results and 90 % of the control population will have normal test results. An ideal assay should provide both a specificity and a sensitivity close to 100 %. The other commonly used measures are predictive values. The positive predictive value (PPV) is the percentage of true positive results of all positive results (including true and false positives), and the negative predictive value (NPV) is the percentage of true negative results of all negative results (including true and false negatives). The closer these values are to 100 %, the better the predictive value of the marker.

The cut-off values, sensitivities and specificities of markers can be assessed with the receiver-operating characteristic (ROC) curves (Robertson and Zweig 1981, Zweig and Campbell 1993, Boyd 1997). A cut-off value is what separates a normal from an abnormal (usually elevated) result. ROC analysis gives sensitivity and specificity values for all possible cut-offs, and a measure called the area under the curve (AUC), which describes the accuracy of the test. With ROC analysis, it is easy to compare the performance of different markers and cut-off values.

One important aspect of the use of laboratory markers is the definition of "normal" values and reference limits. Reference intervals are defined based on values for apparently healthy individuals. This reference population should be carefully selected and the effects of age, gender, race, nutritional status and preferably alcohol drinking habits must be considered in order to avoid misleading reference limits and future misinterpretation of laboratory results. The most common methods for determining reference intervals are the mean ± 2SD for populations following a Gaussian distribution (or a Gaussian distribution is obtained by transformation of the data) and the non-parametric method, which does not require Gaussian distributions but uses 2.5 and 97.5 percentiles as the limits (for a review, see Horn and Pesce (2003)).
2.4.4. Laboratory markers of ethanol-induced tissue injury

Alcohol-induced tissue injury may be examined with blood tests, although a tissue biopsy is the only confirmative test (Niemelä 2002, Phillips et al. 2003). The liver enzymes commonly used for measuring alcohol consumption (GGT, AST, ALT, see next section) reflect the status of the liver and may also be used to assess liver damage. Serum albumin, bilirubin and alkaline phosphatase may also be used for this purpose. Markers of the progression of liver disease are usually associated with collagen and connective tissue metabolism, the most commonly used being the aminoterminal propeptide of type III procollagen (PIIINP). Other postulated fibrosis markers include the aminoterminal propeptide of type I procollagen (PINP) and hyaluronic acid (HA).

2.4.5. Gender issues

Gender has been reported to influence both the effects of alcohol consumption on health and the detection of alcohol abuse. Women are known to be more susceptible to the effects of alcohol than men, particularly to liver disease, for several physiological and metabolic reasons (Lieber 1995, Schenker 1997, Damström Thakker 1998). Immune responses have also been observed to vary greatly between the genders, probably due to hormonal factors (Kovacs and Messingham 2002). When assessing individuals' alcohol consumption, questionnaires appear to be more effective for men than for women, with AUDIT being the most accurate (Seppä et al. 1995, Aertgeerts et al. 2001). Laboratory tests seem in general to be more accurate for detecting alcohol abuse in men than in women, and significant gender-dependent differences in the diagnostic usefulness of markers have been observed (Chalmers et al. 1980, Anton and Moak 1994, Löff et al. 1994, Allen et al. 2000, Sillanaukee et al. 2001, Neumann and Spies 2003).

2.5. Routinely used biomarkers of ethanol consumption

2.5.1. Ethanol concentration in blood, breath or urine

Measurement of the ethanol concentration in the blood, breath or urine is suitable for acute health care purposes and provides a specific marker for alcohol intake. It can also give information on the patient's drinking habits, as blood ethanol levels exceeding 150 mg/l (1.5 % or 33 mM) without obvious signs of intoxication or 300 mg/l (3.0 % or 65 mM) on any occasion indicate significant tolerance, which is typically associated with alcohol dependence (Niemelä 2002). The blood alcohol concentration at the time of admission has been reported to be the best indicator of hazardous drinking practices and alcohol dependence in trauma patients (Ryb et al. 1999, Savola et al. 2004).

2.5.2. Gamma-glutamyl transferase

The liver enzyme gamma-glutamyl transferase (GGT) is one of the most commonly used laboratory tests for detecting excessive alcohol intake, although it may also be elevated by other causes of liver damage, e.g. drugs or non-alcoholic liver diseases. Coffee, smoking, obesity and age have also been
found to influence GGT values (Cushman 1992, Aubin et al. 1998, Daeppen et al. 1998, Puukka et al. 2006a, 2006b). There have been several reports of a positive correlation between the amount of ethanol ingested and serum GGT levels, but the sensitivity of GGT has varied greatly between populations, from 15 to 85 %, being lower in samples from the general practice (Bagrel et al. 1979, Chick et al. 1981, Papoz et al. 1981, Anton and Moak 1994, Yersin et al. 1995, Sillanaukee et al. 1998, Hock et al. 2005, Berner et al. 2006). GGT tends to have a relatively low specificity as compared with carbohydrate-deficient transferrin (CDT), for example, because of many sources of unspecificity (Reynaud et al. 2000, Niemelä 2002, Miller and Anton 2004, Hock et al. 2005).

The ethanol consumption needed for an elevation in GGT has been estimated to be over 40 g per day (Sharpe 2001, Miller and Anton 2004) although no clear threshold has been observed (Schellenberg et al. 2005). GGT values have been estimated to normalize in 2–5 weeks in alcoholics, depending on the original level and the presence or absence of liver pathology (Orrego et al. 1985, Cushman 1992, Conigrave et al. 2003).

2.5.3. Carbohydrate-deficient transferrin

The first reports on the existence of carbohydrate-deficient transferrin (CDT) in alcoholics were published almost 30 years ago (Stibler and Kjellin 1976, Stibler et al. 1978, 1979), since when CDT has emerged as a highly useful marker of excessive ethanol consumption (Stibler 1991, Allen et al. 1994, Golka and Wiese 2004, Bortolotti et al. 2006, Niemelä 2007). Its sensitivity has varied from 20 to 90 %, depending on the population, being higher for men than for women in most studies, and lower in detecting hazardous drinking than in identifying alcoholism (Bell et al. 1994, Anton and Moak 1994, Anton and Bean 1994, Litten et al. 1995, Lesch et al. 1996b, Sillanaukee et al. 1998, Allen et al. 2000, Anton et al. 2001, Berner et al. 2006). The advantage of CDT over other markers is its high specificity (Stibler 1991). False positives have been reported to occur in rare cases of genetic transferrin variants (Stibler et al. 1988, Helander et al. 2001) and in certain other conditions (Sillanaukee et al. 2001, Fleming et al. 2004). Some variation in the results was also observed with earlier methods that measure the absolute amounts of CDT (U/l) due to variations in total transferrin levels (Sorvajärvi et al. 1996, Keating et al. 1998, Viitala et al. 1998, Anton et al. 2001). More recently developed methods, which express the CDT results as a percentage of total transferrin, have improved the performance of this marker (Keating et al. 1998, Helander 1999, Anton et al. 2001, Helander et al. 2005).

CDT includes isoforms that have fewer carbohydrate (sialic acid) moieties than normal transferrin (Stibler 1991). The predominant isoform in a healthy individual is tetrasialotransferrin, with four sialic acid moieties, and excessive alcohol consumption has been shown to increase the proportion of the asialotransferrin and disialotransferrin isoforms, which are generally considered to constitute CDT. The trisialo fraction is not usually included in CDT measurements and has actually been found to reduce the diagnostic accuracy of CDT (Mårtensson et al. 1997, Helander et al. 2001, Arndt et al. 2002, Legros et al. 2002). It is not yet clear how ethanol induces the elevation of CDT, but it has been suggested that acetaldehyde might either inhibit the enzymes that participate in transferrin sialylation or activate the deglycosylating enzymes, or both (Stibler and Borg 1991, Xin et al. 1995, Arndt 2001, Sillanaukee et al. 2001). CDT has been reported to become elevated at daily ethanol consumptions ranging from 40 to 80 g, with duration of 2 to 3 weeks (Stibler 1991,
Schellenberg et al. 2005) although it has been suggested that even higher consumption may be needed to increase CDT in the general population (Lesch et al. 1996a). The half-life of CDT has been estimated to be approximately two weeks (Behrens et al. 1988, Stibler 1991, Allen et al. 1994).

2.5.4. Mean corpuscular volume

An increase in the mean corpuscular volume of erythrocytes (E-MCV), or macrocytosis, is typical of people with chronic excessive ethanol intake, having sensitivities ranging from 20 to 90% (Cushman 1992, Bell et al. 1994, Yersin et al. 1995, Reynaud et al. 2000, Mundle et al. 2000). MCV has proved to be diagnostically more sensitive among women, in whom it may even be superior to other markers (Chalmers et al. 1980, Morgan et al. 1981, Seppä and Sillanaukee 1994, Sillanaukee et al. 1998, Mundle et al. 2000). Several haematological diseases, hypothyroidism, reticulocytosis and vitamin B₁₂ or folic acid deficiency (often related to excessive drinking) and smoking have been shown to elevate MCV and thus reduce its specificity as an alcohol marker (Sharpe 2001, Niemelä 2002). Alcohol abuse is frequently the underlying cause of elevated MCV in hospital patients with non-anaemic macrocytosis (Seppä et al. 1991).

The normalization time for MCV is longer than that for any other commonly employed marker, 2 to 4 months, which places limitations on its use for the short-term monitoring of abstinence but offers a possibility for supervising abstinence over longer periods (Morgan et al. 1981, Mundle et al. 1999, Niemelä 2002).

2.5.5. Aminotransferases

Other liver enzymes commonly used for detecting excessive drinking are the aminotransferases (aspartate aminotransferase, AST and alanine aminotransferase, ALT). Although these are frequently elevated in patients with excessive alcohol consumption, they are more directly related to liver status, so that increased AST and ALT values can be found in other conditions where the liver is damaged, e.g. due to viral hepatitis or medications (Pratt and Kaplan 2000, Niemelä 2002, Conigrave et al. 2003). It has been suggested that the ratio of AST to ALT may indicate the aetiology of liver disease: a ratio over 2 often being seen in alcohol-related liver disease, particularly in advanced states (Rosman and Lieber 1994, Nyblom et al. 2004). Aminotransferase levels have been reported to normalize in 2 to 3 weeks after cessation of drinking, depending on the original level (Niemelä 2002, Chrostek et al. 2006).

2.6. New biomarkers of ethanol consumption

2.6.1. Acetaldehyde adducts and anti-adduct antibodies

Acetaldehyde adducts are formed when acetaldehyde, the first metabolite of ethanol, reacts with proteins and cellular constituents (Gaines et al. 1977, Stevens et al. 1981, Niemelä 2001) and may
persist in the blood for 1 to 3 weeks after the last dose of ethanol (Niemelä and Israel 1992). The measuring of these adducts in either erythrocytes or plasma proteins could be of use for determining recent alcohol consumption (Niemelä and Israel 1992, Sillanaukee et al. 1992b, Lin et al. 1993, Niemelä 2002). Elevated levels have been reported in moderate drinkers as compared with abstainers (Niemelä and Israel 1992). The specificity of this approach may nevertheless be reduced to some extent by endogenous acetaldehyde production (Rosman and Lieber 1992).

Acetaldehyde adducts have been shown to induce an immune response, and circulating IgA, IgG and IgM antibodies, which recognize sequential and conformational epitopes in adducts, have been found in alcohol-consuming populations (Israel et al. 1986, Koskinas et al. 1992, Tuma and Klassen 1992). Of these, anti-adduct IgAs have emerged as a possible indicator of excessive drinking (Worrall et al. 1994, 1996, 1998, Niemelä 2007).

2.6.2. 5-Hydroxytryptophol

The ratio of two serotonin metabolites, 5-hydroxytryptophol (5HTOL) and 5-hydroxyindole-3-acetic acid (5HIAA), has been reported to increase in the urine after heavy drinking (Helander et al. 1992, Voltaire et al. 1992) and to remain elevated for 6–20 h after the disappearance of ethanol (Helander et al. 1993, Carlsson et al. 1993, Helander et al. 1996a). This marker has been suggested as being of use for assessing recent ethanol ingestion e.g. for monitoring treatment or in forensic investigations, and also for verifying subjects' self-reports in alcohol studies (Helander et al. 1996a, Helander and Eriksson 2002, Beck and Helander 2003).

2.6.3. Ethyl glucuronide

Ethyl glucuronide (EtG) is a direct non-oxidative metabolite of ethanol that has been reported to increase in amount in the urine after ethanol ingestion (Dahl et al. 2002, Sarkola et al. 2003). Levels of EtG may remain detectable for up to 5 days (Wurst et al. 2003) and as an intermediary marker it could fill up the gap between the known short-term and long-term markers of alcohol consumption (Wurst et al. 1999, Wurst and Metzger 2002, Wurst et al. 2003). Use of the EtG to creatinine ratio in the urine has also been suggested, because drinking large volumes of water can lower the EtG concentration but does not affect the EtG/creatinine ratio (Dahl et al. 2002, Bergström et al. 2003). EtG has been found in serum, urine, tissues and even hair and may have significant applications in both clinical and forensic medicine (Wurst et al. 1999, 2003, Bergström et al. 2003).

2.6.4. Fatty acid ethyl esters

Fatty acid ethyl esters (FAEES), which are formed when ethanol reacts with fatty acids, were first found in organs damaged by ethanol and also suggested as mediators of organ damage (Laposata and Lange 1986, Laposata 1997). Later, their presence in serum and hair after ethanol intake was also confirmed (Doyle et al. 1994, Pragst et al. 2001). FAEES have been proposed as markers of alcohol consumption, both in short term (measurements in serum) and long term (hair analysis) (Doyle et al. 1996, Laposata 1997, Auwärter et al. 2001). They have been reported to remain de-
detectable in serum for up to 24 h after ethanol ingestion (Doyle et al. 1994), and for even longer periods in heavy drinkers (Borucki et al. 2004).

2.6.5. Phosphatidylethanol

Phosphatidylethanol (PEth) is formed from phospholipides when ethanol is present, in a reaction catalyzed by phospholipase D (Gustavsson 1995). Blood PEth has been reported to correlate closely with ethanol consumption, but detectable PEth levels may not occur until ethanol consumption is relatively high (Hansson et al. 1997, Varga et al. 1998, Aradottir et al. 2006). PEth may be detected for more than two weeks after last dose of ethanol (Hansson et al. 1997, Gunnarsson et al. 1998) so that it has been suggested as a possible marker of excessive drinking (Varga et al. 1998, Aradottir et al. 2006).

2.6.6. Sialic acid

Studies have suggested that concentrations of sialic acid (SA), a carbohydrate predominantly found in the oligosaccharide chains on the surface of cell membranes and macromolecules (Traving and Schauer 1998), are often elevated in serum and saliva of alcoholics and might serve as an alcohol marker (Pönniö et al. 1999a, Sillanaukee et al. 1999b, Romppanen et al. 2002). However, SA concentrations seem to increase in a number of other conditions as well, e.g. in inflammatory and cardiovascular diseases, diabetes and cancer, which limits its use as a marker of excessive drinking (Stefenelli et al. 1985, Sillanaukee et al. 1999a). In addition, body mass index (BMI), blood pressure, ageing, hormonal factors and smoking may cause variations in SA levels (Pönniö et al. 1999b). The presence of SA in the saliva of alcoholics could be of use when considering non-invasive methods of assessing alcohol intake (Pönniö et al. 1999a, Sillanaukee et al. 1999b, Anttila et al. 2005).

Another application involving sialic acid is the plasma sialic acid index (SIJ) of apolipoprotein J (ApoJ), referring to the number of sialic acid moieties of ApoJ. This index has been found to decrease as a consequence of chronic ethanol intake and could possibly serve as a marker of excessive drinking (Ghosh et al. 2001).

2.6.7. Other postulated markers

In addition to other direct ethanol metabolites (EtG, FAEEs, PEth), ethyl sulphate (EtS) in the urine has been introduced as a potential marker of alcohol consumption. It appears to have quite similar characteristics to EtG (Wurst et al. 2006).

Other suggested markers of alcohol intake include hepatic lysosomal enzyme β-hexosaminidase, which has been shown to increase in alcoholics (Isaksson et al. 1985, Kärkkäinen et al. 1990, Hultberg et al. 1991), but also in non-alcoholic liver disease and in some other conditions, which limits its usefulness (Rosman and Lieber 1992, Javors and Johnson 2003).
Urinary dolichols are polyprenol compounds, which have been reported to increase in chronic alcoholics (Pullarkat and Raguthu 1985, Roine et al. 1987). Their use as a marker, however, suffers from a lack of both sensitivity (Stetter et al. 1991) and specificity (Roine et al. 1989, 1991).

In addition, 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 indicators of excessive drinking. More research into these postulated markers is needed in order to establish their clinical value and usefulness.

2.7. Marker combinations

Despite the abundance of potential markers, none of them has so far achieved perfect diagnostic accuracy. Some researchers during the past 20 years have suggested that a diagnostic improvement could be achieved by combining two or more alcohol markers (Monteiro and Masur 1985, Salaspuro 1987, Niemelä and Israel 1992, Yersin et al. 1995, Anton et al. 2001, Sillanaukee and Olsson 2001, Hock et al. 2005). An ideal combination would consist of markers which are affected in different ways by alcohol intake. GGT and CDT, for example, do not correlate in the alcoholic population and seem to be elevated in partially different populations, possibly reflecting independent mechanisms by which ethanol induces their elevation (Anton and Moak 1994, Helander et al. 1996b, Huseby et al. 1997, Anton et al. 2001, Conigrave et al. 2002).

The first researchers who studied the use of test results in combination integrated a large panel of laboratory tests into a single score by mathematical methods (Beresford et al. 1982, Hillers et al. 1986, Hartz et al. 1997, Harasy miw et al. 2000, Harasy miw and Bean 2001). The use of large test panels with dozens of assays would not, however, be either rational or cost-effective, and thus more interest has been shown in combining two or three markers of alcohol consumption. The conventional manner of combining markers is to see whether either is elevated. This approach has been found to give improved assay sensitivity but is frequently associated with a decrease in specificity (Rosman and Lieber 1992, Anton and Moak 1994, Helander et al. 1996b, Huseby et al. 1997, Anton et al. 2001, Schwan et al. 2004). Researchers have also introduced the idea of combining markers by means of mathematical equations (Sillanaukee 1992, Sillanaukee and Olsson 2001), whereupon the sensitivities appear to increase without significant loss of specificity (Sillanaukee and Olsson 2001, Anttila et al. 2003, Chen et al. 2003). The most frequently used components of such combinations are CDT and GGT, and some researchers have also included MCV as a third component (Sillanaukee et al. 1998, Sillanaukee and Olsson 2001, Anttila et al. 2003, Hock et al. 2005). A promising approach was introduced by Sillanaukee and Olsson (2001), who combined GGT and CDT by means of the equation $0.8 \times \ln(GGT) + 1.3 \times \ln(CDT)$ and reported sensitivities of 79 % (men) and 72 % (women) for this marker. A further improvement in this combination was achieved by replacing absolute CDT values with %CDT (Anttila et al. 2003). The marker has been reported to be more sensitive for detecting excessive drinking in men than in women (Sillanaukee and Olsson 2001, Anttila et al. 2003, Chen et al. 2003). Chen and co-workers (2003) also found that integrating clinical information with the combined marker would be useful for the accuracy of detection.
More research into combinations of markers is needed in order to establish their usefulness in screening for excessive drinking. Their cost-effectiveness would also need to be evaluated in order to assess whether the improvement in detection is worth the increase in assay costs. The long-term effects of any improvement in early detection and treatment should also be taken into consideration in cost-effectiveness analyses.

2.8. Laboratory markers for the follow-up of alcoholics

Laboratory markers play an important role in the monitoring of abstinence in treatment units and in health care, and they are also used for legal purposes, e.g. for the supervision of drunken drivers. A suitable marker for follow-up purposes should normalize at a steady rate with as little interindividual variation as possible and should also react to relapses of drinking during follow-up.

For the moment, CDT is the most favoured marker for follow-up purposes, as it has been observed to decrease steadily during abstinence and to be suitable for detecting relapses (Schmidt et al. 1997, Allen et al. 2001, Sharpe 2001, Anton et al. 2002). Determination of the patient’s CDT baseline could be of use in the monitoring process, as a 30 % elevation from the baseline may indicate a relapse (Borg et al. 1995, Anton et al. 2002). CDT appears to be especially suitable for follow-up in the case of men and of patients with liver disease (Bell et al. 1993, Anton et al. 1996, Allen et al. 1999, Anton et al. 2002), since the use of GGT and other liver enzymes in follow-up may be complicated by the fact that they are affected by liver status (Rosman and Lieber 1994). Thus they have usually not performed as well as CDT in follow-up studies (Bell et al. 1993, Salaspuro 1999, Anttila et al. 2004).

Markers of recent alcohol consumption (particularly the direct metabolites of ethanol) may prove to be of significant value for follow-up purposes (Hansson et al. 1997, Wurst et al. 1999, Helander and Eriksson 2002, Bisaga et al. 2005, Wurst et al. 2005), and as these markers have different time frames with respect to detection (from hours to weeks) it might be possible to select suitable markers for different situations.

Combinations of markers have also been proposed for the follow-up of treatment. CDT together with the 5HTOL/5-HIAA ratio in urine could serve as an accurate marker for detecting a recent relapse (Carlsson et al. 1993), and another useful combination for this purpose could be GGT and CDT (Allen et al. 1999, Anton et al. 2002).

2.9. Immune responses related to alcohol consumption

2.9.1. Immune responses to ethanol metabolites

Acetaldehyde is a highly reactive molecule and can form stable condensates with various proteins and cellular constituents (Gaines et al. 1977, Stevens et al. 1981, Wehr et al. 1993, Svegliati-Baroni et al. 1994, Braun et al. 1997, Niemelä 2001), and other ethanol metabolites such as malondialde-
hyde and 4-hydroxynonenal are also capable of generating protein adducts (Niemelä 2001). Acetaldehyde adducts may result in altered protein function and play a part in the stimulation of fibrogenesis and the induction of immune responses (Tuma and Sorrell 1987, Niemelä 1999). They have been found in both the circulation and tissues of alcoholics in increased amounts as compared with control populations (Stevens et al. 1981, Peterson and Polizzi 1987, Niemelä and Israel 1992, Sillanaukee et al. 1992b, Niemelä 2001). Haemoglobin-acetaldehyde adduct levels seem to correlate with self-reported ethanol consumption (Sillanaukee et al. 1991a, 1991b, Hazelett et al. 1998) and have thus been suggested as markers of alcohol intake (Niemelä et al. 1990, Sillanaukee et al. 1991b, Gross et al. 1992, Niemelä and Israel 1992, Sillanaukee et al. 1992b, Lin et al. 1993, Hazelett et al. 1998). They have been found to persist in the blood for 1 to 3 weeks (Niemelä and Israel 1992). The earliest methods for measuring haemoglobin-acetaldehyde adducts were based on chromatography and isoelectric focusing (Stevens et al. 1981, Huisman et al. 1983, Nguyen and Peterson 1984, Gordis and Herschkopf 1986, Sillanaukee and Koivula 1990), and antibody-based assays have been developed more recently (Lin et al. 1990, Niemelä et al. 1990, Niemelä and Israel 1992).

Of the adducts localized in tissues, liver adducts have been the most intensively studied, and their association with alcohol-induced liver damage has been well established (Niemelä et al. 1991, Holstege et al. 1994, Paradis et al. 1996, Li et al. 1997, Niemelä 2001). There is only limited information on adducts in other tissues, but other potential sites of adduct formation are the alimentary tract and pancreas (Iimuro et al. 1996, Salmela et al. 1997, Biewald et al. 1998, Niemelä 2001). Adducts have also been localized in the muscles and brain (Rintala et al. 2000, Worrall et al. 2000, Niemelä 2001, Worrall et al. 2001) and in erythrocyte cell membranes (Niemelä and Parkkila 2004).


It is still unclear whether the antibody responses to acetaldehyde adducts reflect protective or harmful effects. It has been suggested that the generation of these responses may contribute to the pathogenesis of alcoholic liver disease (Niemelä et al. 1987, Israel et al. 1988, Izumi et al. 1989), but it is also possible that the antibodies may serve as neutralizing factors by binding and removing acetaldehyde adducts from the circulation (Israel et al. 1988). It has been speculated that immunological damage to tissues may develop in the presence of both high antibody levels and continuous adduct formation i.e. continuous alcohol intake (Israel et al. 1988).
2.9.2. Alcohol-induced cytokine responses

The involvement of cytokine responses in alcohol-related liver injury has been widely studied in recent years (Khoruts et al. 1991, Deviere et al. 1992, McClain et al. 1993, Border and Noble 1994, Peters 1996, Yin et al. 1999, Neuman 2003, Bode and Bode 2005). Cytokines are a group of proteins that modulate immune responses and participate in cell growth and differentiation and they can be classified as either pro-inflammatory or anti-inflammatory, although they may have overlapping or multiple functions depending on the cell type. An important function of cytokines is to recruit the cells of the immune system to a site of inflammation (McClain et al. 1997). Cytokines include several classes of molecules: interleukins (IL), tumor necrosis factor (TNF), transforming growth factor (TGF) and interferons (IFN). The currently known family of cytokines has over 100 members altogether, working in concert to produce either anti-inflammatory or pro-inflammatory effects depending on the target cells and organs.

Under normal circumstances the liver tissue produces only minimal levels of cytokines (Neuman 2003), but when the liver suffers damage, e.g. due to excessive alcohol consumption, their production increases and the resulting cytokines mediate the regeneration of liver tissue (Peters 1996). This process includes an inflammatory response, which is required for normal tissue healing, and the cytokine levels return to baseline levels when the inflammation is under control (Neuman 2003). It appears that acute moderate alcohol intake leads to inhibition of this inflammatory process (through increases in anti-inflammatory and decreases in pro-inflammatory cytokine production), while chronic alcohol consumption shifts the cytokine balance towards persistent inflammation (Crews et al. 2006). Patients with alcoholic liver disease appear to have elevated pro-inflammatory (e.g. IL-1, IL-6, TNF-α, and IL-8) and lowered anti-inflammatory (IL-10, IL-4) cytokine levels (McClain et al. 1997, Cook 1998, Crews et al. 2006). The continuous presence of pro-inflammatory cytokines and inflammation in the liver (alcoholic hepatitis) may ultimately lead to scar tissue formation (fibrosis) and cirrhosis (Neuman 2003). Apoptosis has also been shown to contribute to the tissue damage that occurs in alcoholic liver disease and this process may be induced by ethanol (Casey et al. 2001, Neuman 2003).

The increased intestinal permeability observed in alcoholics has been shown to lead to elevated bacterial lipopolysaccharide (LPS) concentrations in the circulation, whereupon the resulting endotoxaemia may lead to the activation of liver macrophages and induces them to produce pro-inflammatory cytokines. This cascade leads to inflammation in the liver and may initiate or promote the pathogenesis of alcoholic liver disease (Wheeler 2003, Bode and Bode 2005). The LPS pathway may in fact represent a significant contributor to the onset of alcoholic liver disease (Bode and Bode 2005).
3. Aims of the present research

Despite advances in methods of detecting excessive alcohol consumption, the incidence of alcoholism and its associated medical, social and economic problems continue to increase in many Western societies. Accurate screening tools and a better understanding of the primary mechanisms involved in the adverse health effects of alcohol are clearly needed.

The aims of the present work were as follows:

1. To study the influence of the reference population on the reference intervals of gamma-glutamyltransferase and other markers of excessive alcohol consumption.
2. To study the clinical behaviour of the marker combination GGT-CDT in assessing alcohol consumption.
3. To examine the association between immune responses to acetaldehyde adducts, alcohol-induced liver disease and alcohol consumption.
4. To develop a new marker for excessive drinking based on a specific immune response to acetaldehyde adducts.
4. Materials and methods

4.1. Subjects

The excessive drinkers included in these studies were alcoholics who were recruited from a detoxification and alcoholism treatment unit. They had all been classified for alcohol dependence by reference to the DSM-IV criteria and had a well-documented history of excessive drinking. Detailed interviews on their alcohol consumption were carried out using a timeline follow-back method, according to which the patients were asked how many drinks of alcohol (standard drink = 12 g of ethanol) they had consumed during the past 24 h, the past week and the past 4 weeks before admission. The mean duration of abstinence prior to sampling was 2 ± 2 days. The patients with alcoholic liver disease had all had a history of continuous ethanol consumption for at least five years (> 80 g/day) and the liver disease had been assessed by a previously established combined morphological index (CMI) or a combined clinical and laboratory index (CCLI) (Orrego et al. 1983, Blake and Orrego 1991). All the excessive drinkers were negative for hepatitis B antigen or hepatitis C serology. The reference population consisted of apparently healthy abstainers and moderate drinkers who had mostly been recruited from hospital personnel and who had no previous social or medical history of excessive drinking. The mean daily alcohol consumption of the moderate drinkers, as assessed with questionnaires, was 1–40 g. Blood sampling was performed by trained laboratory personnel. The analyses of liver enzymes and blood cell counts were performed immediately and the serum samples separated by centrifugation were then stored at –70 °C until used for additional analyses. The participants gave their informed consent and the research was carried out in accordance with the provisions of the Declaration of Helsinki.

The subjects in paper I included 195 individuals, comprising 103 alcoholics (90 men, 13 women) and 92 reference individuals (54 men, 38 women). The population of alcoholics had no signs of liver damage and their mean consumption of ethanol had been 40–539 g per day during the past 4 weeks. The reference population included 30 abstainers and 62 moderate drinkers with a mean daily ethanol consumption of 1–40 g. For the comparisons between moderate drinkers and abstainers, additional data were included from a survey on 2485 apparently healthy individuals (1174 men, 1311 women) collected for the Nordic Reference Interval Project, as kindly provided by the project coordinator, Professor Pål Rustad, Fürst Medical Laboratory, Oslo, Norway. These subjects were either abstainers (n = 1156: 471 men, 685 women) or moderate drinkers (n = 1329: 703 men, 626 women), the maximum alcohol consumption during the twenty-four hours prior to sampling having been 24 g (two standard drinks). Weekly alcohol consumption was assessed by means of questionnaires employing categories of a) 0 drinks, b) 1–21 drinks and c) over 21 drinks, and category c was excluded from the analyses. The survey also excluded individuals 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 taken any prescription drugs during the preceding week. Smoking had not been allowed for one hour prior to sampling.
Paper II reports on a sample of 165 alcoholics (140 men, 25 women) with a mean daily ethanol consumption of 40–540 g during the past month. They were further classified according to liver status, with 51 patients (38 men, 13 women) designated as having liver disease. In order to assess marker normalization rates, follow-up tests with supervised abstinence periods of 11 ± 4 days (confirmed with repeated breath analyses) were carried out on 44 alcoholics (39 men, 5 women). The reference population comprised 86 healthy volunteers (49 men, 37 women), including 35 abstainers and 51 moderate drinkers.

Paper III consisted of 86 male alcoholic patients (mean age 48 ± 12 years), of whom 54 had biopsy-proven liver disease and 32 had no clinical or laboratory evidence of significant liver disease. A follow-up with supervised abstinence for 8 ± 2 days, controlled by means of hospitalization and repeated breath analyses was carried out on 17 patients. The reference population consisted of 20 apparently healthy male volunteers who were either abstainers (n = 6) or moderate drinkers (n = 14) with a mean daily ethanol consumption of 20 g (a maximum of 60 grams on any one occasion).

The subjects in paper IV consisted of 40 male alcoholics who had no clinical or laboratory signs of liver disease, but had a history of continuous ethanol consumption or binge drinking, the mean recent consumption having been 40–540 g/day for a period of 4 weeks prior to sampling. 19 alcoholics (mean age 42 ± 12 years) volunteered for a follow-up, which was carried out with supervised abstinence over a period of 8 ± 3 days. In addition, there were 41 male reference individuals who were either abstainers (n = 16) or moderate drinkers (n = 25), none of whom had any history or clinical evidence of alcohol abuse, recent illnesses or immunological disorders.

4.2. Measurements of laboratory markers (I-II, IV)

CDT concentrations were measured using a turbidimetric immunoassay (TIA) after ion exchange chromatography (%CDT, Axis-Shield, Oslo, Norway). This assay detects primarily asialo-, monosialo- and disialotransferrin, although there may be some reactivity to the trisialo fraction of CDT, as recently reported by Aldén and co-workers (2005). The %CDT results are expressed as percentages of total transferrin. The measurements were carried out on a Behring Nephelometer II (Dade Behring, Behring Diagnostics GmbH, Marburg, Germany). The within-run precision of the assay was 4.7 %, day-to-day variation 6.0 % and accuracy 12.7 %.

Serum gamma glutamyl transferase (GGT), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), alkaline phosphatase, albumin, bilirubin and mean corpuscular volume (MCV) of erythrocytes were measured by standard clinical chemical methods in an accredited (SFS-EN ISO/IEC 17025) laboratory at Seinäjoki Central Hospital, Finland. The assay characteristics for these were as follows (assay; within-run variation, day-to-day variation, accuracy): GGT; 0.85 %, 0.54 %, 5.0 %, AST; 0.90 %, 1.5 %, 10.6 %, ALT; 0.87 %, 1.1 %, 12.1 %, alkaline phosphatase; 2.0 %, 1.9 %, 10.1 %, albumin; 2.3 %, 2.0 %, 11.2 %, bilirubin; 2.2 %, 2.4 %, 13.4 % and MCV; 0.41 %, 0.37 %, 3.7 %. The cut-offs for these parameters were as follows: GGT < 80 U/l (men), < 50 U/l (women); AST and ALT < 50 U/l (men), < 35 U/l (women); alkaline phosphatase 60–275 U/l; albumin 36–50 g/l; bilirubin 2–20 µmol/l; MCV 76–96 fl.
4.3. Determination of marker combinations (II, IV)

4.3.1. GGT-CDT (II)

The original equation for combining GGT and CDT in the present manner was suggested by Sillanaukee and Olsson (2001), who used absolute CDT values (U/l). These were later replaced with %CDT results (Anttila et al. 2003). In the present work the combined GGT-CDT marker was calculated in the latter manner (Anttila et al. 2003): \( \text{GGT-CDT} = 0.8 \times \ln(\text{GGT}) + 1.3 \times \ln(\%\text{CDT}) \). The assay cut-off values were determined with ROC analyses using Analyse-It for Microsoft Excel software, which yielded a GGT-CDT cut-off of 4.18 for men and 3.81 for women.

4.3.2. IgA-CDT

Several equations for combining %CDT and anti-adduct IgA results were tested for differentiating between alcoholics and the control population. The IgA-CDT marker was eventually calculated according to an equation \( \log_{10}\text{CDT} + \frac{\text{IgA}}{10^3} \), which appeared to be superior to the others tested. The cut-off value was 0.412, as determined by ROC analysis.

4.4. Measurement of antibodies against acetaldehyde adducts (III-IV)

The test antigen was prepared by purifying a human erythrocyte fraction from the EDTA-blood of an abstainer. The cells were separated by centrifugation and washed three times with phosphate-buffered saline (PBS: 7.9 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 137 mM NaCl, 2.7 mM KCl, pH 7.4). They were then haemolyzed with polyoxyethylene ether, 0.1 % V/V in borate buffer (Hemolysis Reagent, DIAMAT\textsuperscript{TM} Analyzer system, Bio-Rad) and incubated for 35 min at 37 °C to remove unstable Schiff bases. The haemolyzate was diluted with PBS to contain 12 mg/ml of protein, dialysed twice against PBS and then mixed with acetaldehyde (in PBS) so that the final concentration of the latter in the reaction mixture was 10 mM. The mixture was allowed to react overnight (18 h) at 4 °C in a tightly sealed container and the adducts generated were reduced by the addition of sodium cyanoborohydride (10 mM) and mixed for 5 h at 4 °C. The reduced protein solutions were then dialysed twice against PBS at 4 °C and stored in aliquots for single use at −70 °C. The unmodified proteins were prepared and treated similarly to the modified proteins except for the addition of acetaldehyde.

For the measurement of antibody titres, microtitre plates (Nunc-Immuno Plate, Maxisorp\textsuperscript{TM}, InterMed, Denmark) were coated with acetaldehyde-modified red cell protein or corresponding unmodified protein in PBS (3 \( \mu \)g protein in 100 \( \mu \)l/well) and incubated for 1½ h at +37 °C. Non-specific binding was blocked by incubation with 0.2 % gelatin in PBS (150 \( \mu \)l/well) for 1 h at +37 °C. The samples were diluted (1:40) in PBS containing 0.04 % Tween 20 (PBS-Tween) and 50 \( \mu \)l of the diluted serum was allowed to react with the coated proteins (1 h, +37 °C). The plates were then washed extensively with PBS-Tween. Alkaline phosphatase -linked goat anti-human immunoglobulins IgA, IgG, or IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove) were
used to detect antibody-antigen complexes. The immunoglobulins (50 µl/well) were diluted in PBS-
Tween containing 8 mM MgCl₂ and a small amount of dithiothreitol (DTT). The plates were incu-
bated overnight at +4 °C and washed with PBS-Tween. After washing, 100 µl of p-
nitrophenylphosphate solution was added as a colour reaction substrate (Alkaline Phosphatase Sub-
strate Kit, Bio-Rad Laboratories, Hercules, CA). This reaction was stopped by adding 100 µl NaOH
(0.4 M) and the optical densities (ODs) were read at 405 nm with an Anthos HTII microplate reader
(Anthos Labtec Instruments, Salzburg, Austria). The anti-adduct IgA results are expressed as
units/litre (U/l), corresponding to OD₄₀₅nm x 10⁻³.

4.5. Serum cytokines

The concentrations of serum cytokines (IL-2, IL-6, IL-8, IL-10, TNF-α and TGF-β1) were deter-
dined using Quantikine high sensitivity ELISA kits (R&D Systems Inc., Minneapolis, USA). The
results are presented in U/l units, which correspond to OD₄₅₀nm–₅₄₀nm x 10⁻³. The analytical charac-
teristics, as given by the assay manufacturer, were as follows (assay; average within-run variation,
average day-to-day variation, detection limit): IL-2; 3.1 %, 4.6 %, 7 pg/ml, IL-6; 2.6 %, 4.5 %, 0.70
pg/ml, IL-8; 5.8 %, 7.7 %, 3.5 pg/ml, IL-10; 3.7 %, 6.9 %, 3.9 pg/ml, TNF-α; 4.7 %, 5.8 %, 1.6
pg/ml, TGF-β1; 5.3 %, 11.0 %, 7 pg/ml. All the assays were linear within their dynamic range.

4.6. Statistical methods

The statistical analyses were carried out using GraphPad Prism (GraphPad Software Inc., San
Diego, CA, USA) and SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and the ROC analyses with Analy-

The t-test was used for comparisons between two groups following a Gaussian distribution, and
the Mann-Whitney test was used for non-Gaussian populations. For comparisons of three or more
groups, a one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test was employed
when the data followed Gaussian distribution and passed the homogeneity of variance test, while
the Kruskal-Wallis test with an appropriate post hoc test was used if a Gaussian distribution or
equal variances in the groups could not be obtained from the original data or from the logarithmi-
cally transformed data.

Correlations were calculated using Pearson product-moment correlation coefficients for con-
tinuous non-skewed parameters or Spearman’s rank correlations for non-continuous or skewed pa-
rameters, as required. A p-value of less than 0.05 was considered statistically significant.

Cut-off values for new markers were either determined with ROC-analyses or calculated as
mean + 2 SD of the control group.
5. Results

5.1. Influence of drinking levels on markers (I, II, IV)

In paper I, GGT was measured in a large population of alcoholics, moderate drinkers and abstainers. The concentration in alcoholics consuming 40–80 g (68 ± 54 U/l) or > 80 g (167 ± 254 U/l) of ethanol per day significantly exceeded those in both the abstainers (p < 0.001) and the moderate drinkers (p < 0.001) consuming ethanol in amounts ranging from 1 to 40 g per day. GGT activities were also found to be significantly higher in the moderate drinkers (28 ± 23 U/l) than in the abstainers (24 ± 17 U/l) (p < 0.001). When experimental reference intervals were calculated from these populations, the upper limits were found to be on average 43 % higher when the moderate drinkers were taken as the source than when calculated for the abstainers. Gender and age were also found to have an effect on the reference intervals; the limits for men were higher than for women and the age group > 40 years had higher levels than the age group 18–39 years in both genders. The fluctuation in reference intervals due to the choice of reference population was found to have a significant effect on the sensitivity of GGT, so that 69 % of the heavy drinkers were identified when contrasted with the abstainers, as opposed to 56 % with the moderate drinkers.

The inclusion of moderate drinkers in the reference population was also found to lead to decreases in the sensitivity of MCV and AST whereas GGT-CDT and CDT were not affected. The anti-adduct IgA responses studied in paper IV were also found to be affected by moderate drinking, as these values were significantly higher in the moderate drinkers than in the abstainers (p < 0.05).

5.2. Clinical characteristics of marker combinations (II, IV)

5.2.1. GGT-CDT (II)

The performance of a mathematical combination of GGT and CDT, assessed in paper II, was found to be superior to that of the conventional laboratory markers in detecting excessive drinking. GGT-CDT reached a sensitivity of 90 % (for a specificity of 98 %), whereas the sensitivity of CDT alone remained at 63 % and that of GGT at 58 %. The sensitivities and specificities of GGT-CDT were high for both men and women. Combining GGT and CDT in a manner which gave a positive result when either of the markers was positive obviously yielded a higher sensitivity (85 %) than either assay alone, but did not reach the sensitivity of the mathematically formulated combination. Liver status was not found to complicate the assessment of alcohol consumption with GGT-CDT, the sensitivities being quite similar for both alcoholics with (93 %) and without (88 %) liver disease, while the performance of GGT, MCV, AST and ALT was clearly dependent on liver status. Elevation of
the GGT-CDT marker was estimated to require a threshold ethanol consumption of 40 g per day, and GGT-CDT was found to correlate more closely with self-reported ethanol consumption \( r = 0.76, p < 0.001 \) than either GGT \( r = 0.71, p < 0.001 \) or CDT \( r = 0.59, p < 0.001 \) alone or any of the other markers.

### 5.2.2. IgA-CDT (IV)

When the possibility of using anti-adduct IgA results in marker combinations was examined in paper IV, the highest sensitivities and specificities were obtained by combining these with CDT in a mathematical equation. This approach yielded a sensitivity of 90 %, a specificity of 98 % and an AUC of 0.966. Preliminary analyses suggest that IgA-CDT may to be comparable or even superior to GGT-CDT (Figure 1).

![Figure 1. Sensitivity of laboratory markers and their combinations in detecting excessive drinking. IgA-CDT, a combination derived from the equation \( \log\text{CDT} + (\text{IgA}/10^3) \); GGT-CDT, a combination derived from the equation \( 0.8 \times \ln(\text{GGT}) + 1.3 \times \ln(\text{CDT}) \); IgAs, immunoglobulin A against acetaldehyde adducts; CDT, carbohydrate-deficient transferrin; GGT, gamma-glutamyl transferase; MCV, mean corpuscular volume of erythrocytes; AST, aspartate aminotransferase.](image-url)
5.3. Antibody and cytokine responses in alcoholics (III–IV)

5.3.1. Alcohol-related changes in cytokine and antibody production (III-IV)

Alterations in anti-adduct immunoglobulin levels were found in alcoholics, particularly in those with liver disease. The anti-adduct titres were measured for immunoglobulin classes IgA, IgG and IgM in paper III, while paper IV focused on the characteristics of anti-adduct IgA as a marker of excessive drinking. The highest anti-adduct IgA and IgG titres were found in the patients with alcoholic liver disease (paper III), the differences relative to the moderate drinkers and the abstainers being significant (p < 0.001), while the alcoholics without any apparent liver disease also showed elevated IgA levels as compared with both control groups (p < 0.01). IgM titres were highest in the alcoholics without liver disease, and the values for the moderate drinkers were also higher than for the abstainers (p < 0.05), as was also the case for class IgA antibodies. There were no significant differences in total serum immunoglobulin levels between the groups.

Cytokine profiles were measured in paper III in order to assess the effects of alcohol intake and liver disease on the regulation of inflammation. The alcoholics with liver disease showed markedly increased levels of pro-inflammatory cytokines IL-2, IL-6, IL-8 and TNF-α, and decreased TGF-β1, while those without liver disease had normal or slightly elevated pro-inflammatory cytokine levels, except for IL-6, which was high in both alcoholic groups as compared with the control groups (p < 0.001). IL-10 levels were higher in all the alcohol-consuming groups than in the abstainers.

Anti-adduct IgA values were found to correlate significantly (p < 0.001) with recent ethanol consumption (r = 0.77), as also were IL-6 levels (r = 0.83, p < 0.001).

5.3.2. Anti-adduct IgAs as a marker of alcohol consumption (IV)

When anti-adduct IgAs and various traditional alcohol markers were measured in a population comprising alcoholics, moderate drinkers and abstainers, the alcoholics showed elevated levels as compared with either the moderate drinkers (p < 0.001) or the abstainers (p < 0.001). The diagnostic characteristics of anti-adduct IgAs for detecting excessive drinking were compared with those of the conventional markers CDT, GGT, MCV and AST by contrasting the data obtained for the alcoholics with those obtained for the abstainers and/or moderate drinkers. The sensitivity of anti-adduct IgAs using abstainers as the reference population was 73 % for a specificity of 94 %, which exceeded the corresponding figures obtained for CDT, GGT, MCV and AST. When moderate drinkers were also included in the reference population, the sensitivity of the anti-adduct IgA assay was 65 % for a specificity of 88 %, showing essentially similar characteristics to CDT while remaining superior to GGT, MCV or AST. The sensitivity of anti-adduct IgAs can be further improved by combining it with CDT (see section 5.2.2.).

The anti-adduct IgA values showed significant correlations with all the conventional markers of alcohol consumption and also with actual recent consumption. Anti-adduct IgAs were found to cor-
relate significantly with serum IL-6 ($r = 0.33, p < 0.05$) and TNF-α ($r = 0.31, p < 0.05$), but not with IL-2, IL-8, IL-10 or TGF-β1.

5.4. Markers in the follow-up of alcoholics (II-IV)

Marker normalization was examined in papers II and IV, and changes in cytokine levels were also addressed in paper III. GGT-CDT was found to decrease in 93% of alcoholics during 11 ± 4 days of supervised abstinence, and the normalization rate was estimated to be 18 ± 9 days, depending on the initial value. GGT-CDT and CDT showed more consistent declines during abstinence than did GGT, MCV, AST or ALT.

Anti-adduct IgG and IgM values in the alcoholics did not show any significant changes upon abstinence, but a significant decrease was observed in anti-adduct IgA levels (papers III and IV), which also remained higher in the alcoholics after abstinence than in the moderate drinkers or the abstainers. Assessment of the normalization rate of anti-adduct IgAs in paper IV showed it to be about 3% per day, with a mean normalization time of about four weeks, depending on the original level.

Significant changes were also noted in the levels of cytokines during abstinence, the changes in IL-6 (−47%), IL-10 (−82%) and TNF-α (−41%) being significant, whereas IL-2 (± 0%), IL-8 (−26%) and TGF-β1 (+3%) remained relatively stable. IL-6 levels still remained higher after abstinence than those in the control groups ($p < 0.05$ for moderate drinkers; $p < 0.001$ for abstainers).
6. Discussion

6.1. Influence of moderate drinking on marker levels (I-IV)

The present data indicate that moderate drinking has an influence on several biomarkers of alcohol consumption, which could lead at the population level to changes in reference intervals and subsequently to problems in recognizing excessive alcohol consumption in its early phase. It would therefore be essential to define reference intervals on the basis of abstainers rather than moderate drinkers. In order to obtain universally comparable policies, the concept of moderate drinker should also be defined more accurately in terms of both drinking pattern and alcohol intake.

Serum GGT concentrations were observed to be significantly elevated in alcoholics and also in moderate drinkers as compared with the abstainers (paper I), and the estimated upper normal limits for GGT would be approximately 40% higher if moderate drinkers were used as the reference population instead of abstainers. This situation was also reflected in a recent NORIP survey produced in the Nordic countries, which showed markedly increased GGT reference values as compared with the limits used previously (Stromme et al. 2004). Such a change in GGT limits would obviously lead to decreased sensitivity in the detection of alcoholics, as the present data suggest that 13% of them would escape detection if moderate drinkers were taken as the reference population instead of abstainers. At the same time, it can be argued that with lower GGT limits the specificity of the assay would decrease, leading to false positive values, as approximately 11% of the moderate drinkers studied here would have been labelled as having elevated values with lower limits. This aspect may also be viewed, of course, as a possibility for detecting excessive drinking in an earlier phase, for these reference individuals with slightly elevated GGT may report moderate drinking but actually represent drinkers who are on the edge of excessive alcohol consumption or have other confounding factors such as obesity and diabetes, which can both cause elevated GGT values (Kornhuber et al. 1989, Cushman 1992, Aubin et al. 1998, Daeppen et al. 1998, Puukka et al. 2006a).

The differences between moderate drinkers and abstainers were also investigated in the case of the GGT-CDT, CDT, MCV, AST and ALT (paper II), and the inclusion of moderate drinkers in the reference population was found to affect the diagnostic performance of GGT, AST and MCV but not GGT-CDT. This may be an important consideration when screening for excessive alcohol consumption.

Papers III and IV focused on the immunological responses to alcohol and its metabolites. These responses were also found to show variation as a result of moderate drinking. Anti-adduct IgA values were found to be significantly elevated in moderate drinkers by comparison with abstainers, and the other anti-adduct immunoglobulins, IgG and IgM, were also found to show variation between moderate drinkers and abstainers, the difference in IgM being significant. For cytokines, anti-
inflammatory IL-10 values appeared to be slightly elevated in moderate drinkers. It could be speculated that both the IgM response and IL-10 elevation indicate an early response against the adverse effects of alcohol.

6.2. Marker combinations (II, IV)

Although various markers for detecting excessive alcohol consumption have emerged during past decades, clinicians continue to lack a sensitive tool for the identification of excessive drinking, especially in its early phase. Since the passage of a new marker molecule from its discovery to routine use can take years or even decades, increasing interest has been shown in the idea of combining existing markers. Various marker combinations have been introduced during the past two decades (Salaspuro 1987, Anton et al. 2001, Sillanaukee and Olsson 2001, Anttila et al. 2003, Hock et al. 2005). Traditionally, combinations have included CDT, GGT and/or MCV, usually combined in a manner in which a case is deemed positive when either of the markers is positive. The combining of GGT and CDT in this way, for instance, has been shown to yield a sensitivity of 90 % for a specificity of 81 % in men and a sensitivity of 75 % for a specificity of 87 % in women (Anton et al. 2001). More recently, Schwan and co-workers (2004) have shown that combining GGT and CDT as independent parameters provides a sensitivity of 90 % in alcohol abusers and 99 % in alcohol-dependent subjects, whereas the specificity remains at a level of only 63 %. A further improvement in marker combinations has been achieved by using mathematical equations, the most promising combination of this kind so far being GGT and CDT with an equation of 0.8 x ln(GGT) + 1.3 x ln(%CDT) (Sillanaukee and Olsson 2001, Anttila et al. 2003). In line with this view, Hock and co-workers (2005) recently reported a sensitivity of 83 % with a specificity of 95 % using a simple combination of log GGT and CDT. When MCV was included as a third component in these analyses, the sensitivity was reported to increase to 88 %.

The data presented in papers II and IV clearly support the idea of using marker combinations for detecting hazardous drinking. The mathematically formulated equation based on serum GGT and CDT results, referred to in paper II as GGT-CDT was first introduced by Sillanaukee and Olsson (2001), who used absolute CDT values (U/l) in their equation. The replacement of these with %CDT results has proved to improve the performance of this approach still further (Anttila et al. 2003). The present comparison of the diagnostic characteristics of GGT-CDT with those of various other biochemical markers used in the diagnosis of excessive drinking (II) showed this combination to be superior to all the conventional markers. One remarkable thing is that this improvement in sensitivity was achieved without sacrificing the specificity. The use of MCV or any other parameter as a third component in the combination was not found to lead to any additional improvement over GGT-CDT alone. GGT-CDT also appears to be relatively independent of liver status, despite having GGT as one of its components. Earlier observations suggest that using %CDT in the equation instead of absolute CDT further improves this property (Anttila et al. 2003). On the other hand, the presence of liver pathology was found to affect the performance of GGT, AST, ALT and MCV in the assessment of excessive drinking.

Paper IV introduces briefly a marker combination consisting of anti-adduct IgAs and %CDT, combined by means of a mathematical equation logCDT + (IgA/10^3). The resulting marker, IgA-
CDT, was found to provide improved sensitivity as compared with its parent components or other alcohol markers, without any loss in assay specificity. The diagnostic performance of IgA-CDT also seems to be similar to or even better than that of GGT-CDT.

Ideal markers for combinations should have different mechanisms of induction, so that the combination will gain the diagnostic benefits of both components. GGT and CDT, for instance, frequently increase in different individuals and may represent different types of ethanol-induced pathophysiological processes (Anton et al. 2002, Anttila et al. 2003, Neumann and Spies 2003). This combination of different diagnostic windows could partly explain the distinct improvement that can be obtained with marker combinations. Taken together, this work supports the idea of employing marker combinations to achieve a more sensitive diagnosis of excessive drinking. Anti-adduct IgA measurements are not currently available for clinical use, but since GGT and CDT already belong to the set of routine assays, GGT-CDT would be cost-effective and easy to manage in hospital laboratories.

6.3. Antibody and cytokine responses in alcohol abusers (III-IV)

An understanding of the pathophysiological processes related to alcohol is equally as important as the discovery of a reliable laboratory marker. If the mechanisms of tissue pathology could be clarified, it might provide new tools for effective treatment and enable the use of immune parameters as biomarkers of alcohol consumption and alcoholic liver disease. Alcohol affects almost every tissue in the body, and our knowledge of the mechanisms of these actions is limited. The present study of the antibody and cytokine responses related to alcohol intake (III) and the possibility of using antibodies as markers of alcoholism (IV) was focused on male subjects because the immunological responses in vivo may show significant gender dependence, possibly due to differences in sex hormones (Kovacs and Messingham 2002). Women generally show stronger immune responses, and thus further studies with female populations are clearly warranted.

6.3.1. Changes in antibody and cytokine production attributable to alcohol and liver disease (III-IV)

Some of the adverse consequences of alcohol consumption are clearly mediated by immunological mechanisms. The antibody and cytokine responses studied here seem to be associated with inflammatory processes in tissues, particularly in the liver. The generation of immune responses to acetaldehyde-modified epitopes appears to occur early in the sequence of events leading from excessive alcohol consumption to clinical signs of alcoholic liver injury. Both anti-adduct IgA and IgM already showed an elevation in alcoholics without liver disease, and IgG antibodies also paralleled the evolution of liver injury. These findings, together with the observation that cytokine levels also respond to ethanol intake, suggest that the balance in the immune regulation of tissues may be disturbed by ethanol.

Previous studies focusing on alcoholic liver disease have reported an increase in circulating total IgA concentrations together with increased deposition of IgA in tissues (Swerdlow et al. 1983, van
de Wiel et al. 1987, 1988, Tuma and Klassen 1992, Amore et al. 1994). It has been suggested that this may result from either decreased IgA catabolism or excretion or increased IgA production (van de Wiel et al. 1987, Koskinas et al. 1992, Tuma and Klassen 1992, Viitala et al. 1997). The present findings support the theory that the increase in total IgA is due to antigen-driven antibody production, because the levels of specific antibodies become elevated before any generalized increase in serum total IgA levels.

The specific IgAs against ethanol metabolites may be derived from intestinal B-cells, because IgA is the predominant antibody in the gastrointestinal tract, and gastric immunity may readily respond to environmental and dietary antigens (Kerr 1990, Amore et al. 1994). The epithelial tissues, which are rich in enzymes capable of metabolizing ethanol to acetaldehyde, are continuously exposed to ethanol in excessive drinkers, thus enabling the formation of acetaldehyde adducts and antibodies against them (Seitz et al. 1994, Salaspuro 1996, Salmela et al. 1997, Visapää et al. 1998). This is also supported by the present observations that there is a close correlation between anti-adduct IgA levels and recent ethanol intake, and also by the presence of anti-adduct IgAs in individuals reporting moderate drinking. Excessive ethanol intake has previously been found to increase intestinal permeability and disrupt mucosal barriers (Bode and Bode 2003). This could further enhance the antibody responses to various gut-derived antigens and also increase the absorption of IgA. Alcoholics with liver disease often have high endotoxin levels and they have been reported to produce IgA antibodies to endotoxin (Nolan et al. 1986, Parlesak et al. 2002, Wheeler 2003) and also to human gut luminal aspirates (Douds et al. 1998). Anti-endotoxin antibodies as well as anti-adduct antibodies may contribute to the formation of alcoholic liver disease (Klassen et al. 1995, Parlesak et al. 2002, Leevy and Elbeshbeshy 2005).

The involvement of cytokine signalling in the progression of alcoholic liver disease has become more and more evident in the past decades, and the present studies also show a distinctly different cytokine profiles for alcoholics and for healthy controls. It is not known, however, whether the immune responses in excessive drinkers represent protective or harmful mechanisms for the liver. The positive correlation found between anti-adduct IgAs and the pro-inflammatory cytokines TNF-α and IL-6 may be an indication of early-phase inflammatory response to ethanol-derived antigens, as IL-6 has been reported to participate in the acute phase hepatic response (Khoruts et al. 1991) and also in the control of immunoglobulin production (Deviere et al. 1992). The increased amount of anti-adduct IgM and IL-10 in moderate drinkers and in alcoholics without liver disease may represent an early-phase immune response and the regulation of protective immune mechanisms. IgA antibodies may also contribute to immune protection by excluding and neutralizing the altered protein structures resulting from acetaldehyde modification (Israel et al. 1988). When excessive drinking continues, the anti-adduct IgA increases and the pro-inflammatory cytokines become even more elevated, while anti-inflammatory cytokines show a decline. When antigenic stimulation is excessive, IgA immune complexes may be damaging, since these can cause monocytes to release mediators of tissue damage (Deviere et al. 1991). IgG antibodies are known as mediators of several immunopathogenic consequences, including complement activation and the induction of cytotoxic reactions. These immune responses are likely to play an important role in the progression of liver pathology. Alcoholics with liver disease clearly have a skewed balance in cytokine levels, with distinct elevations in IL-2, IL-6, IL-8 and TNF-α, while TGF-β1 levels are actually decreased, although TGF-β1 has previously been associated with the progression of fibrosis in alcoholics (Chen et al. 2002). An excessive release of pro-inflammatory cytokines can induce the activation of inflammatory cells,
increase the production of reactive oxygen species from hepatocytes and induce apoptosis (Neuman 2003). According to several studies, increased TNF-α production and Kupffer cell activation may play key roles in hepatic inflammation (Iimuro et al. 1997, Yin et al. 1999).

6.3.2. Anti-adduct IgAs as a marker of alcohol consumption

It is shown in paper IV that the specific IgA response to acetaldehyde adducts may serve as a clinically useful tool for diagnosing excessive ethanol consumption. A high sensitivity and specificity was observed for the adduct-specific IgA measurements in differentiating between alcoholics and non-alcoholics, and a close correlation between alcohol intake and anti-adduct IgA levels was also found. The diagnostic usefulness of IgA antibodies has previously been studied with a different test antigen (acetaldehyde-modified bovine serum albumin) and these investigations also gave a significant correlation (r = 0.44) between anti-adduct IgA and alcohol intake (Worrall et al. 1996, 1998). Although the anti-acetaldehyde antibody responses are generated independently of the carrier protein, there may be differences in the characteristics of the test antigen and in the efficiency of the assay in differentiating alcoholics from the control population. The red cell protein (mainly consisting of haemoglobin) appears to suit well for anti-adduct antibody measurements, which may be due to an efficient modification of the carrier protein, perhaps leading to an adduct resembling the naturally occurring ones.

It should be noted that moderate drinkers show elevated IgA values as compared with abstainers. This has to be taken into consideration when defining normal values for such measurements. The diagnostic sensitivity of anti-adduct IgAs exceeds that of the conventional markers if abstainers are used as the reference individuals, but the inclusion of moderate drinkers elevates the reference limit and causes a slight decrease in assay sensitivity. Nevertheless, measurements of specific IgAs against acetaldehyde-modified epitopes in proteins could be used as a sensitive tool for detecting excessive drinking.

6.4. Follow-up studies

Of the markers studied here, GGT-CDT was found to be most suitable for the follow-up of alcoholics during treatment, since it was observed to decrease in 93% of the subjects and the mean normalization rate was estimated to be 2–3 weeks. The time required for the normalization of GGT-CDT was slightly longer than for either of its components separately, suggesting that this mathematical combination follows a slightly different kinetics in its normalization. In line with previous studies, CDT performed well in the follow-up, while the normalization of GGT, AST and ALT was not as consistent as that of either GGT-CDT or CDT alone (Bell et al. 1993, Salaspuro 1999, Anttila et al. 2004). This is perhaps due to the induction of liver pathology, which may be reflected in marker levels even after the cessation of drinking.

Anti-adduct IgA antibodies and IL-6 levels were found to decrease significantly in the follow-up of abstaining alcoholics, and they still remained elevated by comparison with those in healthy controls. Significant changes during abstinence were also observed for IL-10 and TNF-α. The follow-
up studies showed that anti-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 longer appearance of anti-adduct IgAs in serum may be explained by the fact that acetaldehyde adducts in the erythrocytes of alcoholics persist in the circulation for 1 to 3 weeks after the last dose of ethanol (Niemelä and Israel 1992). This consistent decrease in anti-adduct IgAs during abstinence suggests, however, that this marker could also be used for follow-up purposes.

6.5. Possible limitations of this study

It should be noted that this study contrasted apparently healthy controls with alcoholics, while in clinical practice the hospital population may comprise patients with various medical conditions and drinking habits, which may complicate the assessment of alcohol consumption by means of laboratory markers. Data on possible refusers among alcoholics were not collected, but it can be assumed that the significance of this factor is relatively small in large populations. The control individuals used here also entail some limitations. The permitted alcohol intake for women was similar to that for men, which may have led to the inclusion of some female individuals who were in fact on the edge of excessive consumption rather than true moderate drinkers. Also, as the alcohol consumption data are based on individuals' own reports, the possibility of having some excessive drinkers among the male control population as well cannot be excluded.

Most of the methods used in these studies are already in routine use and thus quite well characterized in terms of variation and accuracy as well as clinical usefulness. The anti-adduct IgA assay has so far been used for research purposes only and corresponding figures for its precision have not yet been established. We cannot exclude some additional variation in results due to this unstandardized assay method, and this work should therefore be continued by standardization of the assay.

6.6. Future considerations

The markers and combinations presented here appear to provide some improvements in the diagnosis of excessive alcohol consumption. Since the prevalence of excessive drinking continues to increase and frequently still remains undetected in health care, there is an obvious need for new objective tools. The markers that are already in routine use could also be used in combinations for clinical purposes. In the case of new markers, assay methods need to be standardized both within and between laboratories, and further studies are needed in clinical settings in order to establish their diagnostic value.
7. Conclusions

These findings indicate that the assessment of alcohol consumption in clinical practice could be improved by using laboratory markers and their combinations in a carefully standardized manner.

Moderate drinking at the population level may induce changes in some of the commonly used biomarkers of alcohol consumption, such as GGT. This should be taken into consideration in the clinical use of such markers and when selecting individuals for reference populations. Any ethanol-related deviation in GGT reference intervals will obviously reduce the sensitivity of this marker in detecting excessive drinking.

The diagnosis of excessive drinking can be improved by using combinations of markers. The present data suggest that the diagnostic characteristics of the combined marker GGT-CDT appear to exceed those of the traditional markers and both of its components separately.

Immune responses to alcohol-induced antigens, together with disturbances in cytokine balance, may contribute to the development of tissue injury in alcoholics. Changes in ethanol-induced immune responses may already occur in alcoholics without liver disease, however, and even in moderate drinkers. Anti-acetaldehyde adduct IgAs may be suitable for assessing alcohol consumption, particularly when combined with CDT.
This research was carried out at the Department of Clinical Chemistry and Medical Research Unit, Seinäjoki Central Hospital, during the years 2004–2007.

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SERUM GAMMA-GLUTAMYL TRANSFERASE IN ALCOHOLICS, MODERATE DRINKERS AND ABSTAINERS: EFFECT ON GT REFERENCE INTERVALS AT POPULATION LEVEL

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Abstract — Aims: To clarify in the association between amount of ethanol consumption and serum gamma-glutamyl transferase (GT) levels. Methods: GT values were measured from 195 individuals with a wide variety of well-documented ethanol consumption assessed by detailed personal interviews using a time-line follow-back technique. These included 103 heavy drinkers (90 men, 13 women) and 92 healthy volunteers (54 men, 38 women) who were either abstainers (n = 30) or moderate drinkers (n = 62). For comparisons, data were collected from GT measurements for establishing GT reference intervals from 2485 healthy volunteers including 1156 abstainers and 1329 moderate drinkers. Results: GT values in the individuals whose mean ethanol consumption exceeded 40 g of ethanol per day were significantly higher than those in the moderate drinkers with a mean consumption of 1–40 g/day (P < 0.001) or in abstainers (P < 0.001). The GT values in the group of moderate drinkers also exceeded those of the abstainers (P < 0.001). The upper normal GT limits obtained from the data from abstainers were markedly lower (men 45 U/l, women 35 U/l) than those obtained from the population of moderate drinkers (men 66 U/l, women 40 U/l). Conclusions: Serum GT concentrations may respond to relatively low levels of ethanol consumption, which should be considered when defining GT reference intervals. The continuous increase in alcohol consumption at population level may lead to increased GT cut-off limits and hamper the detection of alcohol problems and liver affection in their early phase.

INTRODUCTION

Gamma-glutamyl transferase (GT) is a commonly used laboratory parameter for detecting excessive alcohol consumption (Zein and Discombe, 1970; Reyes and Miller, 1980). Although several studies have reported a positive correlation between the amount of alcohol consumed and serum GT levels, the reported sensitivities of this marker in previous literature have varied greatly, from 15 to 85% (Bagrel et al., 1979; Chick et al., 1981; Papoz et al., 1981; Persson et al., 1990; Leino et al., 1995; Anttila et al., 2004).

Over the past decades, both the total ethanol consumption per capita and associated medical disorders have continued to increase. Simultaneously, the percentage of individuals fully abstaining from ethanol has decreased. In previous studies and in routine health care, reference intervals for GT determinations have been based on values obtained from mixed populations of apparently healthy moderate drinkers and abstainers, whereas only limited attention has been paid on the exact amounts of ethanol consumption in these individuals.

In this work we explored the relationship between ethanol consumption and GT values in individuals with a wide variety of ethanol consumption. Our data indicate distinct effects of mild to moderate ethanol consumption on serum GT levels, which should be considered in the clinical use of GT measurements as a marker of ethanol abuse and liver status.

METHODS

Study protocol

Serum GT was first measured from a sample of 195 individuals (103 heavy drinkers: 90 men, mean age 42 ± 10 years; 13 women, mean age 40 ± 7 years, and 92 moderate drinkers or abstainers: 54 men, mean age 41 ± 16 years; 38 women, mean age 44 ± 19 years) who underwent detailed personal interviews using a time-line follow-back technique (interview sample). The heavy drinkers had a history of continuous ethanol consumption or binge drinking, the mean consumption being in the range of 40–539 g/day during the period of 4 weeks prior to sampling. In addition, this interview sample included 30 abstainers and 62 moderate drinkers with a mean daily ethanol consumption between 1 and 40 g/day. Measurements of GT levels were carried out using standard clinical chemical methods in an accredited (SFS-EN 45001, ISO/IEC Guide 25) laboratory of Seinäjoki Central Hospital, Finland. For comparisons, data from a survey on 2485 apparently healthy individuals (1174 men, age 47 ± 18 years; 1311 women, age 47 ± 18 years) collected for establishing reference intervals in Nordic countries were also used as kindly provided by the project coordinator, Professor Pål Rustad, Furst Medical Laboratory, Oslo, Norway. These subjects were classified as either abstainers (n = 1156: 471 men, age 49 ± 19 years; 685 women, age 49 ± 19 years) or moderate drinkers (n = 1329: 703 men, age 46 ± 17 years; 626 women, age 45 ± 16 years). In these subjects, the maximum amount of alcohol consumption during the 24 h period prior to sampling had been 24 g (two standard drinks). The survey excluded individuals 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 1 week. Smoking was not allowed for 1 h prior to sampling. All GT measurements were carried out with homogeneous International Federation of Clinical Chemistry (IFCC) compatible measuring systems.

Ethical considerations

The procedure was approved by the institutional review board. Informed consent was obtained from the participants and the
study was carried out according to the provisions of the Declaration of Helsinki.

Statistical methods
Values are expressed as mean ± SD. Comparisons were made with Kruskal-Wallis test and Dunn’s Multiple Comparison Test or Mann–Whitney test when comparing two groups. Correlations were calculated with Pearson product–moment correlation coefficients. Reference intervals were calculated after logarithmic transformation as previously described (Horn and Pesce, 2003). A P-value <0.05 was considered statistically significant.

RESULTS
In the total population, serum GT concentrations (mean ± SD) in the groups of heavy drinkers drinking 40–80 g (68 ± 54 U/l) or >80 g (167 ± 254 U/l) of ethanol per day significantly exceeded the levels of both abstainers (P < 0.001) and moderate drinkers (P < 0.001) (Fig. 1). Male alcoholics had slightly higher GT values (166 ± 267 U/l) than female alcoholics (130 ± 163 U/l), although the difference between genders did not reach significance. Interestingly, GT values in the group of moderate drinkers with a daily consumption of 1–40 g (28 ± 23 U/l) also exceeded the values obtained from the group of abstainers (24 ± 17 U/l) (P < 0.001). The correlation between ethanol consumption and GT values, as calculated from the individuals interviewed with the time-line follow-back method, was significant (r = 0.35, P < 0.001).

Figure 2 demonstrates the previously established changes in national GT reference intervals in Finland in comparison with the yearly changes in ethanol consumption at population level. The data on GT reference intervals, as calculated from the individuals classified according to ethanol consumption data in the detailed personal interviews, are summarized in Table 1. The upper normal limits were found to be on average 43% higher, when the individuals with moderate drinking are contrasted with the population of abstainers. The upper normal limits for men were higher than those for women and the age group ≥40 years had higher levels than the age group 18–39 years in both genders. However, the correlation between GT levels and age per se did not reach significance (r = 0.097).

The effect of the choice of the reference population on the estimated diagnostic sensitivity of GT as a marker of excessive ethanol consumption is demonstrated in Fig. 3. When the heavy drinkers are contrasted with abstainers, 69% of heavy drinkers become correctly classified. If the reference interval and definition of normal values would be based on moderate drinkers, the sensitivity remains at a level of 56%. In separate analyses of men and women, the corresponding percentages were 68 and 54% for men and 77 and 69% for women, showing essentially similar changes.

DISCUSSION
Alcohol abuse and alcoholism rank as one of the most serious health problems in most Western countries (Room et al., 2005). Therefore, a high priority should be given to reduction in the prevalence of alcoholism through more effective diagnosis and early intervention. Objective methods for detecting excessive ethanol consumption in health care are necessary for a majority of heavy drinkers who have not self-identified as having alcohol problems.

Studies in the past have shown that a number of biochemical parameters are altered in alcoholics, of which serum GT has emerged as one of the most efficient tests (Bagrel et al., 1979; Chick et al., 1981; Papoz et al., 1981; Bernadt et al., 1982; Leino et al., 1995; Anttila et al., 2004). The present study indicates that even moderate amounts of ethanol consumption influence serum GT concentrations at population level and this phenomenon may significantly affect the interpretation and the establishment of common reference intervals for GT measurements in health care. The data support the view that in order to improve the diagnostic potential of laboratory markers of excessive ethanol consumption and liver status, the reference intervals of each test should be based on healthy

Table 1. GT reference intervals based on the data from the groups of individuals classified according to ethanol intake in the interview sample

<table>
<thead>
<tr>
<th></th>
<th>All men</th>
<th>Men 18–39</th>
<th>Men ≥40</th>
<th>All women</th>
<th>Women 18–39</th>
<th>Women ≥40</th>
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<tbody>
<tr>
<td>Moderate drinkers</td>
<td>8–66</td>
<td>7–63</td>
<td>11–66</td>
<td>6–40</td>
<td>6–31</td>
<td>6–64</td>
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<tr>
<td>Alcohol consumption (1–40 g/day)</td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Moderate drinkers and abstainers</td>
<td>9–61</td>
<td>8–57</td>
<td>12–62</td>
<td>7–39</td>
<td>7–30</td>
<td>8–44</td>
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<tr>
<td>Alcohol consumption (0–40 g/day)</td>
<td></td>
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<tr>
<td>Abstainers</td>
<td>10–45</td>
<td>9–37</td>
<td>11–52</td>
<td>8–35</td>
<td>7–30</td>
<td>9–37</td>
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<tr>
<td>Alcohol consumption (0 g/day)</td>
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individuals who abstain from ethanol. Since a gold standard for a bona fide social drinker currently does not exist, the concepts of moderate drinking and social drinking should also be defined more accurately and the proportions of abstainers and moderate drinkers considered separately when selecting reference individuals in future studies.

The present data indicate that the estimated upper normal limits for GT measurements would be ∼40% higher if the data based on moderate drinkers would be used as the basis of the reference population instead of abstainers. In accordance with this view, a recent NORIP survey from the Nordic countries showed markedly increased GT reference values (Stromme et al., 2004). The diagnostic sensitivity of GT measurements as a marker of excessive ethanol consumption would obviously improve if reference intervals would be based on the data from abstainers. This work indicates that 13% of alcoholics would escape detection if moderate drinkers are used as the reference population, instead of abstainers. Thus, there may be a need for revising the reference range downwards. It may, however, be argued that setting a lower limit could worsen the specificity of GT assays and lead to a high number of false positive values. According to this work, ∼11% of the moderate drinkers would have shown increased values. However, there may be individuals who are in the upper range of the limits of social drinking. Since the data are based on self-reports, we cannot rule out occult alcohol abuse in these subjects. It should be noted, however, that future studies are clearly warranted to explore the independent effect of various possible sources of unspecificity on GT
values, such as obesity or diabetes in individuals reporting either moderate drinking or no drinking. Our preliminary analyses on moderate drinkers with different degrees of obesity have indicated potentiation of GT activities in individuals with significant obesity (data not shown). The associations between GT, moderate drinking, and obesity have previously been examined by Kornhuber et al. (1989) who also concluded that the definition of GT normal values may need to be readdressed. The correlation (r = 0.35) between alcohol consumption per se and GT values in this study is consistent with previous observations (Bagrel et al., 1979; Chick et al., 1981; Papoz et al., 1981; Leino et al., 1995; Anttila et al., 2004). The correlation was, however, essentially similar in women (r = 0.36) and men (r = 0.32), though some earlier studies have reported higher correlations in populations consisting of men only (Papoz et al., 1981; Anton and Moak, 1994; Sillanaukee et al., 1998). The diagnostic sensitivity of GT has usually been shown to be lower for women than for men (Anton and Moak, 1994; Yersin et al., 1995; Mundle et al., 2000). Based on the present data, these findings may in part be explained by the definition of reference intervals. Furthermore, GT values in women may increase at lower levels of alcohol consumption as a result of women’s increased vulnerability to the toxic effects of alcohol (Anton et al., 1998).

The advent of carbohydrate-deficient transferrin (CDT) testing has recently imposed a new challenge to the use of GT measurements because CDT has shown higher specificities than GT in several trials. Although CDT has become an increasingly important tool for assessing excessive ethanol consumption, it appears that CDT and GT frequently increase in different individuals (Anton et al., 2002; Anttila et al., 2003; Neumann and Spies, 2003). Some studies have concluded that GT is more efficient in identifying female alcoholics than CDT (Anton and Moak, 1994. Anton et al., 2002). Therefore, it seems at this time that CDT alone does not cover all the needs for an alcohol marker in routine clinical practice, and other markers, especially GT are needed as well.

Taken together, the present data indicate that the changes in drinking behaviour at population level may parallel increases in recommended GT cut-offs, which may subsequently lead to problems in recognizing excessive alcohol consumption in its early phase. Therefore, a critical re-evaluation of reference intervals even in the use of the well-established biochemical markers of alcohol consumption may be necessary in order to improve the assessment and treatment of patients with early-stage alcohol problems.

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COMPARISON OF THE COMBINED MARKER GGT–CDT AND THE CONVENTIONAL LABORATORY MARKERS OF ALCOHOL ABUSE IN HEAVY DRINKERS, MODERATE DRINKERS AND ABSTAINERS

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Abstract — Aims: A combined index based on γ-glutamyltransferase (GGT) and carbohydrate-deficient transferrin (CDT) measurements (GGT–CDT) has been recently suggested to improve the detection of excessive ethanol consumption. The aim of this work was to compare GGT–CDT with the conventional markers of alcohol abuse in individuals with a wide variety of alcohol consumption.

Methods: A cross-sectional and follow-up analysis was conducted in a sample of 165 heavy drinkers, consuming 40–540 g of ethanol per day, and 86 reference individuals who were either moderate drinkers (n = 51) or abstainers (n = 35).

Results: GGT–CDT (5.53 ± 1.08) in the heavy drinkers was significantly higher than in the reference individuals (3.30 ± 0.37). The sensitivity of GGT–CDT (90%) in correctly classifying heavy drinkers exceeded that of CDT (63%), GGT (58%), mean corpuscular volume (MCV) (45%), aspartate aminotransferase (AST) (47%), and alanine aminotransferase (ALT) (50%), being also essentially similar for alcoholics with (93%) or without (88%) liver disease. When comparing the data using either moderate drinkers or abstainers as reference population, the sensitivity of GGT–CDT, CDT, and ALT remained unchanged whereas the sensitivity of GGT, MCV, and AST was found to show variation.

Conclusions: GGT–CDT improves the sensitivity of detecting excessive ethanol consumption as compared with the traditional markers of ethanol consumption. These findings should be considered in the assessment of patients with alcohol use disorders.

INTRODUCTION

Although the prevalence of alcoholism and associated medical disorders are continuously growing in most Western countries, patients with hazardous drinking practices continue to escape detection in clinical work (Lieber, 1995; Conigrave et al., 2002; Niemelä, 2002; Room et al., 2005). Therefore, the need for objective laboratory tests, which respond to excessive ethanol intake in a sensitive and specific manner, has been widely recognized. In spite of the fact that a wide variety of biochemical parameters in circulation are altered in alcoholics, none of them has so far provided enough diagnostic accuracy to meet the demands of clinicians in routine use for differentiating between alcoholics and non-alcoholics (Allen et al., 1994; Salaspuro, 1999; Scouller et al., 2000; Arndt, 2001; Conigrave et al., 2002; Niemelä, 2002).

Recent studies have suggested the possibility of using marker combinations, which could improve assay sensitivities without sacrificing specificity (Sillanaukee and Olsson, 2001; Anttila et al., 2003a). A mathematically formulated equation derived from the data of γ-glutamyltransferase (GGT) and carbohydrate-deficient transferrin (CDT) measurements appears to be elevated in a higher percentage of alcoholics than either GGT or CDT alone. This approach, when the latter component of the equation is replaced by the results from %CDT assay, which expresses CDT data as percentage of total transferrin, was recently found to further improve this method as compared with the previous CDTect-based equation (Anttila et al., 2003a). The main advantage achieved by the %CDT method in this context is its ability to avoid the interference of unexpected variation in serum transferrin levels, which complicated the use of the CDTect assay. However, as yet, only limited information has been available on the clinical performance of the new GGT–CDT method and its comparisons with other markers of ethanol intake.

This study was aimed at comparing the diagnostic value of GGT–CDT with several other biomarkers of alcohol abuse in a well-characterized population of heavy drinkers, moderate drinkers, and abstainers.

METHODS

Patients

The sample of alcohol abusers consisted of 165 heavy drinkers (140 men, 25 women), age (mean ± SD) 46 ± 10 years (range 19–73 years). All patients showed a well-documented history of excessive ethanol consumption, as assessed by detailed personal interviews using a time-line follow-back technique and medical and social records. They had consumed ethanol in amounts ranging from 40 to 540 g/day during the past 1 month either continuously or during repeated episodes of binge drinking. The patients also met the DSM-IV criteria of alcohol dependence including pathological alcohol use, social impairment, presence of tolerance, and withdrawal symptoms. There were 51 patients, (38 men, 13 women) age 49 ± 10 years, range 30–67 years, who also showed evidence of liver disease, as assessed by previously established combined morphological index (CMI) or a combined clinical and laboratory index (CCLI) (Orrego et al., 1983; Blake and Orrego, 1991). The mean duration of abstinence prior to sampling was 2 ± 2 days, range 0–6 days. All patients were negative for hepatitis B antigen or hepatitis C serology. Follow-up studies with supervised abstinence for assessing marker normalization rates were carried out in 44 alcoholics, age 42 ± 11 years (range 19–59 years), who were monitored for a period of 11 ± 4 days, range 3–19 days.

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Reference individuals were 86 healthy volunteers (49 men, 37 women), age 48 ± 17 years (range 19–84 years) who were either abstainers (n = 35, age 54 ± 18 years, range 22–84 years) or moderate drinkers (n = 51, age 43 ± 14 years, range 19–76 years) whose mean ethanol consumption per day, as also assessed from the past one month by questionnaires, was between 1 and 40 g. All of them were without any previous social or medical history of alcohol abuse. The weekly ethanol consumption in these individuals did not exceed 280 g of alcohol (men) and 160 g (women) or 6 drinks (men) and 4 drinks (women) on any single occasion.

All 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. The study was approved by the hospital ethical committee.

Laboratory methods

The concentration of CDT was measured by a turbidimetric immunoassay (TIA) after ion exchange chromatography (%CDT, Axis-Shield, Oslo, Norway). The assay detects primarily α-, mono- and disialotransferrin, although there may be some reactivity towards the trisialofraction of CDT, as recently reported by Aldén et al. (2005). The measurements were carried out on Behring Nephelometer II (Dade Behring, Behring Diagnostics GmbH, Marburg, Germany). The within-run precision was 4.7%, day-to-day variation was 6.0%, and accuracy 12.7%. Serum GGT was measured using enzymatic colorimetric assay, as standardized against IFCC (International Federation of Clinical Chemistry and Laboratory Medicine). The imprecision within run was 0.85% and the day-to-day variation was 0.54%. The accuracy of the GGT method was found to be 5.0%. Serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were analysed using pyridoxal phosphate methods according to IFCC. For AST, the within-run precision was 0.90%, the day-to-day precision was 1.5%, and accuracy of the method was 10.6%. For ALT, the corresponding values were: within run 0.87%, day-to-day 1.1%, and accuracy 12.1%. The analyses of GGT, AST, and ALT were carried out with Cobas Integra 800 analyser (Roche Diagnostics, Basel, Switzerland). Mean corpuscular volume (MCV) of erythrocytes was measured using the Sysmex XE-2100 hematology analyser (Sysmex Corporation, Kobe, Japan). The imprecision within run was 0.41% and the day-to-day variation was 0.37%. The accuracy of the method was 3.7%. All assays were carried out in an accredited (SFS-EN ISO/IEC 17025) laboratory of the Seinäjoki Central Hospital, Finland. The cut-offs in the above assays were as follows: GGT, men 80 U/l, women 50 U/l; CDT, 2.6%; MCV, 96 fl; AST, men 50 U/l, women 35 U/l; ALT, men 50 U/l, women 35 U/l.

Counting the combined marker

GGT–CDT was counted using an equation based on the data derived from GGT and CDT measurements as follows: 

\[ \text{GGT–CDT} = 0.8 \times \ln(\text{GT}) + 1.3 \times \ln(\%\text{CDT}) \]

(Anttila et al., 2003a). Analyses for assay cut-offs from the present reference population were made with ROC analyses using Analyse-It for Microsoft Excel software, which yielded a cut-off of 4.18 for men and 3.81 for women.

Statistical methods

The values are expressed as means ± SD. The comparisons between groups were carried out using the Kruskal–Wallis test with the Dunn’s test for multiple comparisons. Correlations were calculated using the Pearson product-moment correlation coefficients for continuous non-skewed parameters or the Spearman’s rank correlations for non-continuous variables, as required. Statistical analyses were carried out using GraphPad Prism, version 3.03 (GraphPad Software, San Diego, CA) and Analyse-It for Microsoft Excel software (version 1.68), Leeds, UK. A P-value < 0.05 was considered statistically significant.

RESULTS

The mean values for GGT–CDT, GGT, CDT, MCV, AST, and ALT were all significantly higher in the heavy drinkers than those in the moderate drinkers or abstainers (Fig. 1, P < 0.001 for all comparisons). The sensitivities and specificities of the various markers in differentiating between the heavy drinkers and the reference individuals are summarized in Table 1. GGT–CDT reached a sensitivity of 90% for a specificity of 98%, which clearly exceeded the sensitivities achieved by all the other markers in these comparisons. The sensitivities and specificities of GGT–CDT were high for both men and women (Table 1). Combining GGT and CDT in a manner where either marker is positive obviously yielded a higher sensitivity (85%) than the assays alone, but even this approach did not reach the sensitivity of the mathematically formulated combination (Table 1). When the alcoholic patients were further classified according to the presence or absence of liver disease, GGT–CDT showed essentially similar diagnostic accuracies for both groups, whereas the diagnostic characteristics of GGT, MCV, AST and ALT were found to change as a function of liver status (Table 1). The degree of liver disease severity, as assessed by the combined morphological index (CMI), was not found to correlate significantly with GGT–CDT (r = 0.19).

When the marker data obtained from the heavy drinkers were contrasted alternatively with either moderate drinkers or abstainers, the sensitivity percentages of GGT (+4%), MCV (+17%), and AST (+20%) all increased when abstainers were used as the only control population. In contrast, GGT–CDT and CDT sensitivities were not affected. The threshold ethanol consumption for elevation of GGT–CDT values when plotting daily ethanol consumption and GGT–CDT was found to be ~40 g of ethanol (Figure 2). GGT–CDT also correlated more strongly with self-reported ethanol consumption (r = 0.76, P < 0.001) than either GGT (r = 0.71, P < 0.001) or CDT (r = 0.59, P < 0.001) alone or any of the other markers (Table 2).

In the follow-up of 44 alcoholic patients, GGT–CDT was found to decrease in 93% of these individuals during 11 ± 4 days (range 3–19 days) of supervised abstinence (Table 3). The estimated time for normalization for GGT–CDT, depending on the initial value, was 18 ± 9 days, the mean rate of decay being ~1.5% of the initial value per day.
as compared with 3.4% for GGT and 3.7% for CDT. Nevertheless, the changes towards normalization were found to be more consistent for GGT–CDT and CDT than those for the other markers (Table 3).

**DISCUSSION**

In the diagnostics of alcohol use disorders, it is crucial that the laboratory analyses are accurate. Such analyses are needed...
both for detecting excessive ethanol consumption and for monitoring abstinence. An ideal assay should provide both specificity and sensitivity near 100%. However, to date such assays have not emerged, although marker combinations have been suggested to open new possibilities for improving the situation (Salaspuro, 1987; Anton et al., 2001; Sillanaukee and Olsson, 2001; Anttila et al., 2003a; Hock et al., 2005).

The present work compares a recently developed marker combination, defined here as GGT–CDT, with several other biochemical markers in the diagnosis of alcohol abuse. The data show that the sensitivity of GGT–CDT, which relies on a mathematically formulated equation based on serum GGT and CDT results, exceeds the diagnostic sensitivity of all the conventional markers of alcohol abuse. Interestingly, this advantage is achieved without sacrificing assay specificity. When using standardized methods for the GGT and CDT measurements, the combination can well be standardized for multi-laboratory use, too.

It appears that weighing GGT and CDT in the present manner is important for optimizing assay sensitivity. Previously, combining independent measurements of GGT and CDT (when either of the markers is positive) has been shown to yield a sensitivity of 90% for a specificity of 81% in men and a sensitivity of 75% for a specificity of 87% in women (Anton et al., 2001). Schwan et al. (2004) recently showed that combining GGT and CDT as independent parameters provides a sensitivity of 90% in alcohol abusers and 99% in the alcohol-dependent group, whereas the specificity remains at a level of only 63%. Hock et al. (2005) recently combined log GGT and CDT and obtained a sensitivity of 83% with a specificity of 95%. The sensitivity increased to 88% when MCV was included as a third component in the analyses. The use of MCV or any other parameter as a third component in the present material was, however, not found to lead to any additional improvement as compared with GGT–CDT alone (data not shown).

The first studies employing the combination of GGT and CDT used similar cut-offs for men and women (Sillanaukee and Olsson, 2001; Anttila et al., 2003a). The present work shows, however, that different cut-offs for men and women may be necessary also for this marker. Previously, gender-dependent analytical variation has been observed especially in CDT assays with the CDTect method, which has been later abolished by the use of %CDT assays (Anton and Moak, 1994; Tsutsumi et al., 1994; Niemelä et al., 1995; Viitala et al., 1998; Conigrave et al., 2003). Women are known to be more sensitive to the hepatotoxic effects of alcohol, and it is possible that the activities of serum GGT may also respond to alcohol consumption in a gender-dependent manner, which could explain in part the need for separate cut-offs for men and women. In this material, the presence of liver pathology was found to also affect the interpretation of GGT, AST, ALT, and MCV in the assessment of heavy drinking, whereas GGT–CDT appears to be more resistant towards the variation induced by liver pathology per se. The association between GGT–CDT combination and liver disease may, however, also depend on the method used for analysing the CDT component of the assay (Viitala et al., 1998; Anttila et al., 2003b; Fleming et al., 2004).

The diagnostic potential of GGT–CDT is also supported by its strong correlation with the actual amounts of ethanol consumption from the past 1 month prior to sampling. The values appear to increase after the daily ethanol consumption exceeds 40 g. Previously, CDT has been reported to elevate with daily ethanol consumption ranging from 40 to 80 g, possibly also depending on the method used (Stibler, 1991; Schellenberg et al., 2005). While GGT has also been suggested to increase with a threshold consumption of over 40 g of ethanol per day (Sharpe, 2001), even moderate drinkers may, however, show increased GGT values more often than abstainers (Hietala et al., 2005). Interestingly, recent data have suggested that GGT could actually be considered a marker of oxidative stress (Lim et al., 2004). It is, thus, possible that combining GGT and

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Heavy drinkers</th>
<th>Heavy drinkers with liver disease</th>
<th>Heavy drinkers without liver disease</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>All n = 165</td>
<td>Men n = 140</td>
<td>Women n = 25</td>
</tr>
<tr>
<td>GGT–CDT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>4.18</td>
<td></td>
<td></td>
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<tr>
<td>Women</td>
<td>3.81</td>
<td></td>
<td></td>
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<tr>
<td>GGT U/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men 50</td>
<td>58</td>
<td>66</td>
<td>78</td>
</tr>
<tr>
<td>Women 50</td>
<td>56</td>
<td>68</td>
<td>76</td>
</tr>
<tr>
<td>CDT %</td>
<td></td>
<td></td>
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<tr>
<td>2.60</td>
<td>63</td>
<td>65</td>
<td>67</td>
</tr>
<tr>
<td>GGT or CDT</td>
<td></td>
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</tr>
<tr>
<td>elevated</td>
<td>85</td>
<td>86</td>
<td>89</td>
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<tr>
<td>GGT and CDT</td>
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<td>elevated</td>
<td>96</td>
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<td>96</td>
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<td>MCV fl</td>
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</tr>
<tr>
<td>96</td>
<td>45</td>
<td>46</td>
<td>69</td>
</tr>
<tr>
<td>AST U/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men 50</td>
<td>47</td>
<td>43</td>
<td>59</td>
</tr>
<tr>
<td>Women 35</td>
<td>94</td>
<td>94</td>
<td>94</td>
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<tr>
<td>ALT U/l</td>
<td></td>
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<tr>
<td>Men 50</td>
<td>50</td>
<td>49</td>
<td>92</td>
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<tr>
<td>Women 35</td>
<td>87</td>
<td>88</td>
<td>87</td>
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</table>
CDT could provide new diagnostic windows with synergistic benefits for the assessment of hazardous drinking practices. GGT–CDT seems to recognize ethanol overconsumption in a similar manner whether or not heavy drinkers are contrasted with abstainers or moderate drinkers, which may be a useful characteristic for instance in screening programmes for excessive alcohol consumption. It should be noted that in routine health care reference populations in trials examining diagnostic tests have usually consisted of combined populations of moderate drinkers and abstainers. Here inclusion of moderate drinkers into the reference population was found to affect the diagnostic performance of GGT, AST, and MCV although not GGT–CDT. The combined marker was also found to be suitable for the follow-up of abstinence, showing a rather consistent decline during supervised abstinence, with mean normalization rate of 2–3 weeks. Interestingly, the time required for normalization for GGT–CDT appeared slightly longer than for each marker separately, suggesting that the mathematically formulated combination also follows a slightly different kinetics in its decay.

Taken together, this work supports the idea of using GGT–CDT for achieving a more sensitive diagnosis of alcohol abuse. Since this approach is also cost effective and easy to manage in hospital laboratories, it should be suitable for routine clinical work.

**REFERENCES**


Immune Responses to Ethanol Metabolites and Cytokine Profiles Differentiate Alcoholics with or without Liver Disease

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Department of Laboratory Medicine and Addiction Research Unit, EP Central Hospital, Seinäjoki, and University of Tampere, Finland

OBJECTIVES: Excessive alcohol consumption is associated with the generation of antibodies against neoantigens induced by ethanol metabolism. However, the associations between such immune responses, ethanol consumption, and liver injury remain unclear.

METHODS: Eight-six male alcoholics with (n = 54) or without (n = 32) liver disease, and 20 male volunteers (6 abstainers, 14 moderate drinkers) underwent clinical, morphological, and biochemical assessments of liver status and ethanol consumption.

RESULTS: Antiacetaldehyde adduct IgAs in both groups of alcoholics were significantly higher than those in the controls. Elevated IgGs occurred in patients with liver disease, whereas IgMs were high in the heavy drinkers without apparent liver disease. Liver disease patients had high levels of both proinflammatory (IL-2, IL-6, IL-8, TNF-α) and antiinflammatory (IL-10) cytokines, whereas those without liver disease showed elevated IL-6, IL-8, and IL-10 only. Ethanol consumption correlated significantly with antiadduct IgA and IL-6 levels, which also showed parallel changes upon abstinence.

CONCLUSIONS: Alcoholic liver disease is associated with the generation of IgAs and IgGs against acetaldehyde-derived antigens and enhanced levels of both pro- and antiinflammatory cytokines, whereas elevated IgA, IL-6, and IL-10 characterize alcoholics without liver disease. These data suggest that immunological mechanisms may play a role in the sequence of events leading to liver disease in some patients with excessive drinking.

(Ann Gastroenterol 2005;100:1303–1310)

INTRODUCTION
Alcoholic liver disease in humans has been previously found to be associated with elevated serum IgA and abnormal tissue deposition of IgA (1,2). A growing body of evidence has further indicated that excessive alcohol consumption leads to the production of immune responses, which are specifically directed to sequential and conformational epitopes generated in covalent binding reactions between proteins and ethanol metabolites (3–9). Studies have also suggested a role for such immunological reactions in the adverse effects of ethanol in tissues (4,6,10). IgA antibodies against acetaldehyde-derived neo-antigens have been previously found in patients with alcoholic liver disease, the titers being significantly higher than those in patients with non-alcoholic liver disease, or nondrinking controls (7). However, the sequence of events in the sensitization to ethanol, generation of immune responses, and tissue injury has remained unclear.

The present studies were designed to investigate immunoglobulin isotype-specific responses against acetaldehyde-derived antigens in male alcoholics representing a wide range of alcohol consumption and severity of liver injury. The antibody titers were compared with the levels of proinflammatory (IL-2, IL-6, IL-8, TNF-α) and antiinflammatory (IL-10, TGF-β1) cytokines that are known to modulate immunological functions in vivo. The data indicate distinct differences in the antibody and cytokine responses, which could play a role in the development of liver injury in some alcoholic patients.

METHODS
Patients and Control Subjects
We studied 86 male alcoholic patients (mean age 48 ± 12 yr) all of whom showed a well-documented history of excessive ethanol consumption. The main clinical and laboratory characteristics of the study population are summarized in Table 1. There were 54 patients with biopsy-proven liver disease, who had consumed ethanol in amounts exceeding

1303
### Table 1. Main Clinical and Laboratory Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>Alcoholics (n = 56)</th>
<th>Controls (n = 20)</th>
<th>Reference value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n (yr)</td>
<td>54</td>
<td>32</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>50 ± 12</td>
<td>45 ± 10</td>
<td>43 ± 14</td>
</tr>
<tr>
<td>GT (U/L)</td>
<td>215 ± 298**</td>
<td>168 ± 217**</td>
<td>27 ± 18</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>75 ± 51**</td>
<td>47 ± 35</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>54 ± 32*</td>
<td>44 ± 32</td>
<td>32 ± 11</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>1.4 ± 0.4*</td>
<td>1.1 ± 0.4</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>CDT (U/L)</td>
<td>29 ± 15**</td>
<td>26 ± 15**</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>AP (U/L)</td>
<td>278 ± 161**</td>
<td>179 ± 52</td>
<td>151 ± 32</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>41 ± 6</td>
<td>44 ± 5</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>BIL (μmol/L)</td>
<td>20 ± 13*</td>
<td>17 ± 9</td>
<td>17 ± 4</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01, as compared to abstainers.

The values are mean ± SD.

Abbreviations: GT, gamma glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CDT, carbohydrate-deficient transferrin; AP, alkaline phosphatase; ALB, albumin; BIL, bilirubin.

80 g/day either continuously or during repeated episodes of binge drinking. This sample included patients in whom liver histology ranged from mild fibrosis and fatty change to cirrhosis with a wide distribution of morphological abnormalities related to alcoholic hepatitis, as assessed according to previously established combined morphological index (CMI) of liver disease severity (11,12). In addition, the study population included 32 heavy drinkers, who had been admitted for detoxification, but were devoid of clinical and laboratory evidence of significant liver disease. Because of ethical considerations, these patients were not biopsied. They showed 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. The documentation of ethanol intake was based on detailed personal interviews using a time-line follow-back technique. The patients were asked how many alcohol-containing drinks (standard drink = 12 g of ethyl alcohol corresponding to one beer, one glass of table wine, or three centiliters of 40% proof spirit) they had consumed during (1) 24 h, (2) 1 wk, and (3) 4 wk preceding admission. The mean duration of abstinence prior to sampling was 2 ± 2 days. A follow-up with supervised abstinence and repeated sampling during hospitalization over a period of 8 ± 2 days was carried out from 17 patients.

Blood alcohol concentrations during this time were controlled by repeated ethanol analyses from breath air. All patients were negative for hepatitis B virus antigen or hepatitis C serology.

The reference population consisted of 20 apparently healthy male volunteers (mean age 48 ± 17 yr) who were either abstainers (n = 6) or moderate drinkers (n = 14) whose mean daily ethanol consumption was 20 g (range 1–60 g). In the latter group, the mean duration of abstinence was 3 days and the amount of alcohol had not exceeded two drinks during the 24 h preceding blood sampling.

All serum samples were stored at −70°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.

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### Preparation of Acetaldehyde-Modified Antigens In Vitro

Human erythrocyte protein was prepared from EDTA-blood of a teetotaller. The erythrocytes were separated by centrifugation and washed thrice with an equal volume of phosphate-buffered saline (PBS: 7.9 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), lysed with polyoxyethylene ether, 0.1% V/V in borate buffer (Hemolysis Reagent, DIAMAT™ Analyzer System, Bio-Rad), and incubated for 35 min at 37°C to remove unstable Schiff bases. The hemolysate was brought into a hemoglobin protein concentration of 12 mg/mL with PBS and stored frozen in aliquots at −70°C prior to use. Acetaldehyde diluted in PBS was added to aliquots of the freshly prepared hemoglobin, containing 12 mg protein/mL, to obtain a final acetaldehyde concentration of 10 mM. The mixture was allowed to react in a tightly sealed container at 4°C overnight. Protein adducts were reduced by the addition of sodium cyanoboroxydrichide (10 mM) and mixing for 5 h at 4°C. All protein solutions were dialyzed twice against PBS at 4°C and stored in small aliquots for single use at −70°C. Samples representing unmodified protein were prepared and treated similarly to that of the modified protein except for the addition of acetaldehyde.

### Measurements of the Antibody Titers

The microtiter plates (Nunc-Immuno Plate, Maxisorb™, Intermed, Denmark) were coated with acetaldehyde-modified hemoglobin, or corresponding unmodified proteins (background) in PBS (3 μg protein in 100 μl/well) and incubated for 1.5 h at +37°C. Nonspecific binding was blocked by incubation with 0.2% gelatin in PBS (150 μl/well) for 1 h at +37°C. The sample sera were diluted (1:40) in PBS, which contained 0.04% Tween-20 (PBS-Tween). The final volume of 50 μl of each serum dilution were allowed to react with the coated proteins for 1 h at +37°C followed by extensive washing with PBS-Tween. Alkaline phosphatase-linked goat antihuman immunoglobulins IgA, IgG, or IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove) were used to label antibody-antigen complexes (50 μl/well). The immunoglobulins were diluted in PBS-Tween containing 8 mM...
Immune Responses in Alcohols

Figure 1. Titers of IgA, IgG, and IgM antibodies against acetaldehyde-modified epitopes in alcoholics, moderate drinkers, and abstainers. The patients with alcoholic liver disease showed the highest titers of IgA and IgG antibodies. The IgA titers were significantly higher in alcoholics with \( (321 \pm 217 \text{ U/L}) \) \((p < 0.001)\) or without \( (143 \pm 118 \text{ U/L}) \) \((p < 0.01)\) liver disease than those in moderate drinkers \( (56 \pm 82 \text{ U/L}) \) or in abstainers \( (22 \pm 24 \text{ U/L}) \). The highest IgG titers occurred in the ALD patients \( (364 \pm 138 \text{ U/L}) \) \((p < 0.05)\) whereas the groups of alcoholics without liver disease \( (293 \pm 168 \text{ U/L}) \), moderate drinkers \( (256 \pm 114 \text{ U/L}) \), and abstainers \( (167 \pm 130 \text{ U/L}) \) were not significantly different. IgM titers were most notably elevated in alcoholics without liver disease \( (656 \pm 143 \text{ U/L}) \) \((p < 0.001)\). The values in moderate drinkers \( (593 \pm 78 \text{ U/L}) \) were also higher than those in abstainers \( (393 \pm 241 \text{ U/L}) \) \((p < 0.05)\) \((\text{U/L} = \text{OD}_{405}\times10^3)\).

MgCl2 and a small amount of dithiothreitol (DTT). The plates were allowed to incubate at \(+4^\circ\text{C}\) overnight. After washing, 100 µL of p-nitrophenolphosphate-solution was added for color reaction substrate (Alkaline Phosphatase Substrate Kit, Bio-Rad Laboratories, Hercules, CA). Color reactions were stopped by adding 100 µl of 0.4 M NaOH and the optical densities were read at 405 nm by Anthos HTII microplate reader (Anthos Labtec Instruments, Salzburg, Austria).

Other Methods
The concentrations of interleukins (IL-2, IL-6, IL-8, IL-10, TNF-α, and TGF-β1) in serum were determined using Quantikine high sensitivity ELISA kits according to the instructions of the manufacturer (R&D Systems, Abington Science Park, UK). The concentration of carbohydrate-deficient transferrin (CDT) was measured by a competitive radioimmunossay after microcolumn separation (CDTect). Serum GT, aspartate aminotransferase (AST), albumin, total immunoglobulins, and bilirubin were measured by standard clinical chemical methods. All analyses were carried out in an accredited laboratory (SFS-EN 45001, ISO/IEC Guide 25) of EP Central Hospital.

Calculations and Statistical Methods
The data are expressed as mean ± SD. In the antibody titer analyses, the immunoassay values \( (\text{OD}_{405}) \) 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 to get a difference score for each sample. The data are expressed as units/liter \( (\text{U/L}) \) referring to the difference score \( (\text{OD}_{405} \times 10^3) \). The comparisons between the alcoholic and the non-alcoholic groups were carried out using Student’s \( t \)-test or the Mann-Whitney test for parameters with skewed distributions of values. The differences between three or more groups were analyzed using ANOVA and the differences were considered statistically significant at \( p < 0.05 \). Square root transformation was used to yield non-skewed distributions before ANOVA analyses when necessary. Linear regression analysis was used to calculate correlations between the different variables. The analyses were carried out using GraphPadPrism statistical software (San Diego, CA).

RESULTS
Titers of IgA, IgG, and IgM antibodies against acetaldehyde-modified epitopes in alcoholics with or without liver disease, in moderate drinkers and in abstainers are shown in Figure 1. The patients with alcoholic liver disease showed elevated mean titers of IgA and IgG antibodies compared to abstainers. The IgA titers were also significantly higher in the alcoholics without liver disease than in moderate drinkers \((p < 0.01)\) or in abstainers \((p < 0.001)\), whereas IgG titers did not differ between these groups. IgM titers were most notably elevated in alcoholics without liver disease. Interestingly, the values in moderate drinkers were also higher than those in abstainers \((p < 0.05)\). The levels of total unspecific IgA, IgG or IgM were not significantly different between any of these groups (data not shown).
Figure 2. The levels of pro- and anti-inflammation cytokines in alcoholics, moderate drinkers, and abstainers. Proinflammatory cytokines IL-2 (A) and TNF-α (C) were elevated only in alcoholic liver disease patients such that the levels in this group were significantly higher than those in any of the other groups ($p < 0.001$). IL-8 (B) was also high in ALD patients ($p < 0.001$) in comparison with all the other groups. The patients without apparent liver disease also showed higher values of IL-8 than those in the moderate drinkers ($p < 0.01$) or abstainers ($p < 0.01$). IL-6 (D) in both groups of alcoholics was significantly higher than those in the control groups ($p < 0.001$ for both comparisons). The levels of the anti-inflammatory IL-10 cytokine (E) were also higher in the alcoholic groups as compared to the abstainers ($p < 0.001$). Some moderate drinkers also showed elevations in IL-10 levels, which, however, did not reach significance when compared to the levels of abstainers ($p = 0.06$). The levels of TGF-β1 were found to be relatively low in ALD patients, although not significantly different from those in the group of abstainers (F).

In order to examine the possible regulation and downregulation of the antibody responses, cytokine measurements were also carried out for each patient. The patients with ALD showed significantly elevated values of proinflammatory cytokines (IL-2, IL-8, and TNF-α), whereas in patients without liver disease these cytokines, except for IL-8, remained at the levels of healthy controls (Figs. 2A–C, respectively). The levels of IL-6 (Fig. 2D) were high both in the groups of alcoholics with and without liver disease, as compared to the groups of moderate drinkers or abstainers ($p < 0.001$ for both comparisons). The mean levels of the antiinflammatory cytokine IL-10 were higher in all the alcohol consuming groups, including moderate drinkers, than that of the abstainers ($p < 0.001$) (Fig. 2E). In contrast, the mean levels of TGF-β1
did not differ significantly in these comparisons (Fig. 2F). Interestingly, relatively low values of this cytokine were observed in patients suffering from liver disease. The CMI of liver disease severity showed a significant correlation with the proinflammatory cytokines, IL-6 ($r_s = 0.59$, $p < 0.001$) and IL-8 ($r_s = 0.63$, $p < 0.001$) whereas not with the other cytokines or with the antibody levels.

Figure 3 shows the mean ($±$SD) titers of IgA, IgG, and IgM antibodies against the acetaldehyde-derived antigens in 17 alcoholics without apparent liver disease at the time of admission and after supervised abstinence, as compared to the levels obtained from moderate drinkers and abstainers. IgA levels were significantly lower in the samples taken after 8 ± 2 days of supervised abstinence ($p < 0.05$), whereas IgG or IgM levels did not change significantly. After this period of abstinence, significant changes were also noted in the levels of IL-6 ($−47$%), IL-10 ($−82$%), and TNF-α ($−41$%), whereas the levels of IL-2 ($±0$%), IL-8 ($−26$%), and TGF-β1 ($±3$%) remained relatively stable. However, the IL-6 levels after abstinence remained higher than those in the reference groups ($p < 0.05$ for moderate drinkers; $p < 0.001$ for abstainers). The mean daily ethanol consumption from the period of 1 month (mean 172 g, range 68−470 g) preceding admission showed a highly significant correlation with the anti-adduct IgA levels ($r = 0.77$, $p < 0.001$) and IL-6 levels ($r = 0.83$, $p < 0.001$).

**DISCUSSION**

The present studies show that the generation of immune responses against acetaldehyde-modified epitopes and enhanced IL-6 and IL-10 cytokine responses occur early in the sequence of events leading from excessive alcohol consumption to clinical signs of alcoholic liver injury. IgM isotype antibodies, the predominant component of early immune responses, and IgA antibodies were both higher in the alcohol consumers without apparent liver disease than in the abstainers. In turn, IgG antibodies and excessive amounts of both pro- and antiinflammatory cytokines parallel the evolution of liver injury suggesting that a disturbed balance in the immune regulation may play a role in the pathogenesis of liver disease (ALD) in some alcoholic patients.

Previous studies in patients with ALD have shown a generalized increase in circulating IgA concentrations together with increased tissue IgA deposition, which have been suggested to result from decreased IgA catabolism or from decreased IgA excretion (2,4,13,14). The present findings on different immunoglobulin isotypes indicate that the generation of excess IgAs in alcoholics is antigen-driven. It is possible that IgA antibodies result from intestinally induced B-cell responses against ethanol-derived neoantigens since the epithelial tissues in alcohol abusers are continuously exposed to ethanol and mucosal immunity has been shown to be highly adaptable to the antigenic load of the environment (15). In line with this view, the IgA responses also show a strong correlation with the actual amounts of recent ethanol ingestion. The gastrointestinal tract is rich in enzymes capable of metabolizing ethanol to acetaldehyde (16−18). Studies in experimental animals have also suggested an association between intestinal ethanol metabolism and the production of liver damage (19,20). Previously, endotoxemia has been shown to be common in ALD patients, and such patients may also have IgA antibodies to components of endotoxin (21) and to human gut luminal aspirates (22). Thus, there may be intestinally induced B lymphocyte responses against several enteric antigens. IgAs against endotoxin-derived components have previously been reported in patients with ALD although not in alcohol consumers without liver injury (17,21). It is possible that in ALD patients a continuous antigen load together with high endotoxin levels could enhance the mucosal response to gut-derived antigens at the same time when IgA absorption is increased as a result of mucosal injury. Endotoxin can also stimulate immune induction by inhibiting T regulatory cells through a T cell-like receptor/interleukin 6 dependent pathway (23).
It is currently not known whether the immune responses occurring in the alcoholic patients represent protective or harmful mechanisms for the liver. The present observations on the associations between ethanol consumption, antibody profiles, IL-6 levels, and IL-10 levels may indicate that the early responses, which precede liver disease, could reflect regulation of tissue damage and immune protection mechanisms. This view is also supported by the differences in the immune parameters between abstainers and apparently healthy moderate drinkers. IgA antibodies may contribute to exclusion and neutralization of antigens resulting from acetaldehyde modification of proteins and cellular constituents (4–6). In line with this view, IgA antibodies and IL-6 levels show parallel changes in the follow-up of alcoholics with abstinence. Previously, IL-6 levels have also been shown to readily respond to therapeutic interventions with lifestyle modifications and vitamin E in non-alcoholic steatohepatitis (NASH) (24). IL-6 release into bile occurs with the same time course as that for transcellular transport of IgA in the liver (25). A relatively high concentration of IgA after ethanol consumption may also be found in Kupffer cells. Under conditions where Kupffer cell phagocytosis is impaired, there may be increased production of IgG immunoglobulins (26). Upon excessive antigenic stimulation, the generation of IgA immune complexes may, however, be damaging since these can cause monocytes to release mediators of tissue damage. Such immune complexes could also play an initiating role in the development of renal lesions in alcoholics (1). IgG antibodies, which were observed in some patients with liver disease, are known as mediators of several immunopathogenic consequences including complement activation and induction of cytotoxic reactions. In patients with advanced ALD, recent studies have also described the occurrence of specific T-cell responses to adducted proteins (10).

The present findings may further indicate that a disturbed balance between ethanol-derived antigen loading and immunological protection mechanisms could also play a role in the progression of liver disease in alcoholics. In ALD patients, there appears to be a skewed balance in the ratios of pro- and antiinflammatory cytokines. The IL-6 and IL-10 cytokines were found to show early changes also reflecting the current status of drinking. IL-6 is a multifunctional cytokine that has been previously implicated in the hepatic acute phase response (27) and control of immunoglobulin production (28). Recent evidence has shown that IL-6 is also required for normal liver regeneration after injury or partial hepalectomy and may thereby provide hepatoprotection by conferring resistance to injury (29,30). IL-6 has been previously shown to inhibit TNF-α production and reduce antigen presentation (31,32). IL-10 has been recently implicated as having antifibrogenic effects since IL-10 deficiency in IL-10−/− mice potentiates liver fibrogenesis as a result of enhanced inflammation or direct effects on matrix regulation (33). In mice, a mechanical tissue injury causes a rapid induction of IL-10 mRNA and promotion of Th2 responses to antigens (34). IL-6 and IL-10 are both Th2 IgA stimulating cytokines (35). Interestingly, although TGβ-β1 expression has been previously associated with the development of fibrosis in alcoholics (36), the present data show no significant differences in circulating TGβ-β1 in patients with ALD as compared to abstainers. The proinflammatory cytokines IL-8, IL-2, and TNF-α were found to be markedly more dominant in the cytokine profiles of the ALD patients than in those without liver disease. Of these, IL-8 is able to attract neutrophils and its enhanced expression also coincides with decreased hepatocyte survival in cultured human hepatocytes (37). The levels of IL-2 (influencing the growth and differentiation of Th1-cells) and TNF-α (inducing activation of inflammatory cells, expression of adhesion molecules, and platelet activation) were especially pronounced in ALD patients. Excessive release of such cytokines can also increase the production of reactive oxygen species from hepatocytes and induce apoptosis (38). Several recent studies have emphasized the importance of increased TNF-α production and Kupffer cell activation as key responses in hepatic inflammation (38,39). Studies in ethanol-exposed rats (39) and in ob/ob mice, a model of non-alcoholic fatty liver disease (40), have shown that treatment with inhibitors of TNF-α may reduce hepatic inflammation and necrosis.

In this work, we chose to study male patients only because immunological responses in vivo may show significant gender dependence (41). Men and women are known to have markedly different incidences of a variety of autoimmune disorders. Sex steroid hormones have been suggested to play a role in the regulation of immune responses, although the specific mechanisms have remained obscure. For instance, estradiol inhibits the suppressive activity of a subset of T-lymphocytes bearing Fc-receptors for immunoglobulin G (41,42). However, a substantial interindividual variation was noted in several study parameters. It is possible that different degrees of liver injury, different amounts and patterns of ethanol intake prior to sampling, and different genetic susceptibilities may all play a role in explaining such variation.

In conclusion, the present data showing characteristic immune responses and cytokine profiles in the expression of ALD indicate that such mechanisms may play a role in the sequence of events leading from excessive ethanol consumption to tissue injury. Future studies appear warranted to examine the possibility whether modulation of the immunopathogenesis could provide new approaches into the treatment of the alcoholic patients and whether the immune parameters could also be used as biological staging markers of ethanol-induced liver disease.

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REFERENCES


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
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°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 Na₂HPO₄, 1.5 mM KH₂PO₄, 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, DIAMATTM Analyzer system, Bio-Rad Laboratories, Hercules, CA), and incubated for 35 minutes at 37°C to remove unstable Schiff bases. The hemolysate containing 12 mg of red cell protein/mL (in PBS) was stored frozen in aliquots at −70°C.

For acetaldehyde labeling, the hemolysate was mixed with acetaldehyde (in PBS) to obtain a final acetaldehyde concentration of 10 mM. After incubation for 18 hours at 4°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°C. Protein solutions were then dialyzed exhaustively against PBS at 4°C and stored in aliquots for single use at −70°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, MaxisorbtM, 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 1h at +37°C. Nonspecific binding was blocked by incubation (1 hour, +37°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°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 MgCl₂ and a small amount of dithiothreitol (DTT). The plates were incubated overnight at +4°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 transpeptidase (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 ± SEM. In the assays for the antiadduct IgAs, the values (OD₄₀₅) 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 OD₄₀₅×10⁵. The combination marker IgA-CDT was calculated with an equation logCDT+(IgA/10⁵). The combination of GGT and CDT (GGT-CDT) was counted using a previously established equation GGT-CDT = 0.8×ln(GGT)+1.3×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
with the major cytokines, antiadduct IgAs were found to correlate significantly with serum IL-6 ($r = 0.33$, $p < 0.05$) and TNF-$\alpha$ ($r = 0.31$, $p < 0.05$) levels, whereas not with IL-2, IL-8, IL-10, or TGF-$\beta$1.

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
aspartate aminotransferase.

transferase; MCV, mean corpuscular volume of erythrocytes; AST, aspartate aminotransferase; CDT, carbohydrate-deficient transferrin; GGT, γ-glutamyl transferase; IgA, immunoglobulin A; AUC, area under curve; SE, standard error of AUC; 1, antiadduct IgA; 2, CDT, carbohydrate-deficient transferrin; 3, GGT, γ-glutamyl transferase; 4, MCV, mean corpuscular volume of erythrocytes; 5, AST, aspartate aminotransferase.

Cut-offs were determined as mean±2SD.

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.

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

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

<table>
<thead>
<tr>
<th>Source of cutoff</th>
<th>Cutoff</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>AUC (SE)</th>
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<tr>
<td><strong>Antiadduct IgA</strong></td>
<td></td>
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<tr>
<td>Abstainers</td>
<td>74</td>
<td>72</td>
<td>94</td>
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<td>65</td>
<td>88</td>
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<tr>
<td>Moderate drinkers</td>
<td>99</td>
<td>65</td>
<td>88</td>
<td>0.806 (0.0545)</td>
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<tr>
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<tr>
<td>Abstainers and moderate drinkers</td>
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<td>96</td>
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<tr>
<td>Abstainers</td>
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<td>63</td>
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<td>43</td>
<td>92</td>
<td>0.690 (0.0656)</td>
</tr>
</tbody>
</table>

Cut-offs were determined as mean±2SD.

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

### Table 2. Correlations Between the Different Markers of Ethanol Intake

<table>
<thead>
<tr>
<th></th>
<th>Antiadduct IgA</th>
<th>CDT</th>
<th>GGT</th>
<th>MCV</th>
<th>AST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiadduct IgA</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDT</td>
<td>0.39**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGT</td>
<td>0.35**</td>
<td>0.43***</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCV</td>
<td>0.51***</td>
<td>0.28*</td>
<td>0.46**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>0.23*</td>
<td>0.46***</td>
<td>0.46***</td>
<td>0.32**</td>
<td>1</td>
</tr>
</tbody>
</table>

*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.
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.

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

Table 3. Sensitivities and Specificities of Marker Combinations

<table>
<thead>
<tr>
<th>Marker Combination</th>
<th>Cutoff</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA-CDT</td>
<td>0.412</td>
<td>90</td>
<td>98</td>
</tr>
<tr>
<td>GGT-CDT</td>
<td>4.20</td>
<td>83</td>
<td>98</td>
</tr>
</tbody>
</table>

CDT, carbohydrate-deficient transferrin.
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


