



JAANA LATVALA

Effect of Alcohol Consumption  
and Acetaldehyde  
on Blood Cells and Molecules

Pathogenic and Diagnostic Implications



ACADEMIC DISSERTATION

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**To my family**

# Abstract

Alcohol abuse is known to have a wide array of adverse effects on serum proteins, blood cells and their progenitors in bone marrow. The mechanisms by which it does so have not yet been established, however. Recent studies have suggested that acetaldehyde, the first metabolite of ethanol, is responsible for many of its harmful effects, being a highly reactive molecule and capable of forming stable condensates with proteins and cellular constituents. Acetaldehyde-derived protein condensates have also been suggested as possible new markers of alcohol abuse.

The present work is focused on the effects of ethanol and acetaldehyde on blood cells and proteins. The formation of acetaldehyde-derived protein modifications *in vivo* was studied using antibodies recognizing acetaldehyde-modified epitopes in proteins independently of the nature of the carrier protein. The subjects were patients representing a wide range of ethanol consumption and abnormalities in haematological parameters, alcoholics admitted for detoxification and patients with alcoholic liver disease. The controls were individuals without any history of excessive alcohol consumption.

Routine haematological examinations of patients presenting with cytopenia and a history of hazardous drinking showed the incidence of anaemia to be lower than that in the corresponding non-alcoholic controls. Abnormal platelet and leukocyte levels were common, especially in the anaemic alcoholics. Both an elevated mean cell volume of erythrocytes (macrocytosis) (67% vs. 18%) and elevated mean cell haemoglobin (63% vs. 22%) were more frequent in the alcoholics. Reticulocytosis, thrombocytopenia and combined cytopenias were all common findings in the alcoholic patients. Blood smears from the alcoholics typically showed round macrocytes, stomatocytes and knizocytes. Bone marrow aspirates revealed vacuolization of pronormoblasts and megakaryocytes, especially in individuals with recent intoxication.

Immunocytochemical analyses of peripheral blood erythrocytes indicated that acetaldehyde-derived epitopes occurred both on the cell membrane and inside the erythrocytes. Bone marrow aspirates also showed positive staining for acetaldehyde adducts in erythropoietic cells. Separation of erythrocyte proteins by high-pressure liquid chromatography revealed the formation of fast-eluting haemoglobin fractions which reacted with antibodies against acetaldehyde adducts.

Antibodies raised in rabbits against condensates with acetaldehyde and lipoproteins (LDL, VLDL) or bovine serum albumin reacted with protein adducts generated at physiologically relevant concentrations of acetaldehyde *in vitro*. The antibody prepared against the acetaldehyde-VLDL condensate was found to provide the most effective *in vivo* detection of acetaldehyde adducts in erythrocyte and serum proteins from alcoholic patients.

The generation of autoimmune responses to the acetaldehyde-derived epitopes was studied in heavy drinkers admitted for detoxification and in patients with alcoholic liver disease. The mean anti-acetaldehyde adduct IgA levels in the alcohol abusers, who had mild or no clinical and biochemical signs of liver injury, were significantly higher than those in the moderate drinkers or abstainers. The highest titres occurred in patients with alcoholic liver disease. IgM antibodies were also significantly higher in the alcohol consumers without liver disease, whereas IgG levels were high only in the patients with alcoholic liver disease. The cytokine profiles of the alcoholic patients also showed significant differences, with the highest levels of both pro-inflammatory cytokines (IL-2, IL-6, IL-8, TNF- $\alpha$ ) and the anti-inflammatory cytokine (IL-10) occurring in the patients with alcoholic liver disease.

These results suggest that alcohol abuse is an important risk factor for abnormalities in a number of haematopoietic cell lines and in circulating proteins. Acetaldehyde-erythrocyte adducts appear to be formed *in vivo* in the blood and bone marrow of patients with excessive alcohol consumption, which may contribute to the generation of erythrocyte abnormalities. Acetaldehyde adducts are also immunogenic. Antibodies raised against acetaldehyde-VLDL adducts could provide the basis for a new diagnostic assay to assess excessive alcohol consumption. The data also show autoimmune responses to acetaldehyde-modified proteins in alcoholics, which may also prove to be of value in the differential diagnosis of alcohol-related diseases.



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Jaana Latvala



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# Abbreviations

ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
ALDH	aldehyde dehydrogenase
ALT	alanine aminotransferase
apoB	apolipoprotein B
AST	aspartate aminotransferase
AUDIT	alcohol use disorders identification test
BSA	bovine serum albumin
CAGE	alcohol use identification questionnaire
CDT	carbohydrate-deficient transferrin
CMI	combined morphological index
CYP2E1	cytochrome P450 IIE1
CYP3A	cytochrome P450 3A
DTT	dithiothreitol
GGT	$\gamma$ -glutamyl transferase
HbA1c	glycosylated haemoglobin
HD	heavy drinkers
HDL	high-density lipoprotein
HER	hydroxyethyl radical
HNE	4-hydroxynonenal
HPLC	high-pressure liquid chromatography
IgA, IgG, IgM	immunoglobulin (A, G, M)
IL	interleukin
LDL	low-density lipoprotein
MAA	hybrid adduct with malondialdehyde and acetaldehyde
MAST	Michigan alcoholism screening test
MCH	mean corpuscular haemoglobin
MCV	mean corpuscular volume
MDA	malondialdehyde
MEOS	microsomal ethanol oxidizing system
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NASH	non-alcoholic steatohepatitis
NCA	National Council on Alcoholism

PBS	phosphate-buffered saline
SA	sialic acid
TGF- $\beta$ 1	transforming growth factor- $\beta$ 1
TNF- $\alpha$	tumor necrosis factor- $\alpha$
VLDL	very low-density lipoprotein

# List of original publications

- I Latvala J, Parkkila S and Niemelä O (2004): Excess alcohol consumption is common in patients with cytopenia: studies in blood and bone marrow cells. *Alcohol Clin Exp Res* 28: 619-624.
- II Latvala J, Parkkila S, Melkko J and Niemelä O (2001): Acetaldehyde adducts in blood and bone marrow of patients with ethanol-induced erythrocyte abnormalities. *Mol Med* 7: 401-405.
- III Latvala J, Melkko J, Parkkila S, Järvi K, Makkonen K and Niemelä O (2001): Assays for acetaldehyde-derived adducts in blood proteins based on antibodies against acetaldehyde/lipoprotein condensates. *Alcohol Clin Exp Res* 25: 1648-1653.
- IV Latvala J, Hietala J, Koivisto H, Järvi K, Anttila P and Niemelä O (2004): Immune responses to ethanol metabolites and cytokine profiles differentiate alcoholics with or without liver disease. Submitted for publication.

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

# 1 Introduction

Excessive alcohol consumption is a common cause of hospital admission throughout the Western world (Lieber 1995, Charalambous 2002). Health problems are known to arise when acute alcohol consumption exceeds a level of 5-6 standard drinks per day (men) or 3-4 drinks per day (women), or when chronic consumption exceeds 300 g of ethanol per week (men) or 200 g per week (women). Surveys have indicated that 15-30% of all admissions to general hospitals may be related to excessive drinking. Diagnostic methods for detecting problem drinking in hospital settings are currently inefficient, however (Allen et al. 1997, Niemelä 2002, Reinert and Allen 2002). Questionnaires designed to assess alcohol consumption, such as CAGE, MAST or AUDIT, are unreliable because they depend on self-reports, although they may be useful for obtaining information on the drinking patterns of individual patients. This is important, since some adverse consequences are related to occasional heavy drinking bouts whereas others are found in individuals with continuous drinking. A wide variety of laboratory methods have also been evaluated for the detection of alcohol abuse, but all those proposed so far have suffered from a lack of sensitivity or specificity.

Alcohol abuse is known to have a wide array of adverse effects on blood cells and circulating proteins (Lindenbaum 1987, Rosman and Lieber 1994). Heavy ethanol intake may affect both blood cells and their bone marrow progenitors, although the mechanisms underlying such effects have remained obscure. Previous research has suggested that acetaldehyde, the first metabolite of ethanol, may play a crucial role in the haematological derangements observed in alcoholic patients (Eriksson and Fukunaga 1993, Eriksson 2001). Acetaldehyde-derived condensates in proteins have been found in the liver of patients with excessive alcohol consumption (Niemelä et al. 1991b, Holstege et al. 1994, Paradis et al. 1997), and it has been suggested that acetaldehyde-modified epitopes in proteins could serve as diagnostic markers of alcohol abuse (Lin et al. 1990, Niemelä and Israel 1992, Hazelett et al. 1998).

The present work was designed to investigate ethanol-induced changes in blood cells and molecules and the generation of acetaldehyde condensates with proteins and cellular constituents in alcohol consumers. The data indicate that acetaldehyde may play a pivotal role in ethanol-induced changes in circulating proteins and in haematopoiesis.

## 2 Review of the literature

### 2.1 Ethanol and health problems

Alcohol misuse is a major cause of health problems throughout the world (Lieber 1995, Schenker 1997, Simpura et al. 1999). Since virtually all tissues in the body may be affected by heavy ethanol intake, the increased ethanol consumption per capita recorded in countries such as Finland can be expected to lead to an increased incidence of health problems. Recent Finnish health surveys have indicated an increasing prevalence of alcohol abuse, especially among women and young adults (Aalto et al. 1999, Lunetta et al. 2001). While accidents and intoxication account for a high number of alcohol-related deaths, various ethanol-related diseases, including liver cirrhosis, pancreatitis and various forms of cancer, are also significant causes of death in Finland. Therefore, there is an urgent need for research into the primary mechanisms of alcohol-related diseases and for the development of new diagnostic tools to detect alcohol-induced health problems at an early stage.

### 2.2 Main features of ethanol metabolism

Although a small amount of ingested ethanol is excreted unchanged in the breath and urine, over 95% is oxidatively metabolized to acetaldehyde and ultimately to acetate. Ethanol is absorbed from the gastrointestinal tract by rapid diffusion, mostly in the duodenum and upper jejunum, although some is metabolized in the stomach (Roine et al. 1991, Oneta et al. 1998).

There are three metabolic pathways for the oxidation of ethanol to acetaldehyde: the alcohol dehydrogenase (ADH) pathway, the microsomal ethanol oxidizing system (MEOS) and the catalase pathway. The further oxidation of acetaldehyde to acetate is catalyzed by aldehyde dehydrogenase (ALDH).

### *2.2.1 Alcohol dehydrogenase (ADH)*

The main enzyme that catalyzes the oxidation of ethanol to acetaldehyde is ADH (Bosron and Li 1986), a cytosolic enzyme that is expressed in tissues as several isoenzymes with different kinetic properties and substrate preferences. The incidence of these isoforms varies between racial groups (Jörnvall and Höög 1995, Eriksson et al. 2001). Some studies of its tissue distribution have indicated that ADH is enriched in zone 3 hepatocytes (Buehler et al. 1982, Yamauchi et al. 1988), although it has been reported elsewhere to be distributed homogeneously throughout the lobule (Watabiki et al. 1999). Consequently, since the liver is the primary site for ethanol metabolism, it is also a major target for ethanol toxicity (French 1989, Nanji et al. 2002). Some positive associations between ADH genotype and alcohol-induced liver injury, or even alcoholism, have been reported (Sherman et al. 1993, Nakamura et al. 1996).

### *2.2.2 Cytochrome P450 in ethanol metabolism (MEOS)*

A cytochrome P450-mediated pathway also contributes to ethanol metabolism (Lieber 1988, Asai et al. 1996, Caro and Cederbaum 2004). An important characteristic of this pathway is that it is induced by chronic ethanol consumption, so that its capacity to metabolize ethanol is increased (Lieber and DeCarli 1968), as commonly observed after long-term alcohol drinking. It is generally agreed that this effect is due to an increase in hepatic ethanol-inducible cytochrome P450 IIE1 (CYP2E1), which is the major catalytic component of MEOS (Ingelman-Sundberg et al. 1988, Lieber 1999). CYP2E1 catalyzes the oxidation of ethanol to acetaldehyde, a reaction in which the oxidation of ethanol is coupled to the reduction of oxygen, using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a hydrogen donor.

Upon chronic ethanol consumption, both the content and catalytic activity of CYP2E1 will increase, and consequently acetaldehyde production by MEOS will be enhanced. Immunoblot analysis of human liver microsomes has shown that their CYP2E1 content is several times higher in alcoholics who have been drinking recently than in non-drinkers. In both rats and man, CYP2E1 is normally localized in the perivenular region, or zone 3, of the liver lobule, and its induction by prolonged alcohol consumption occurs primarily within this acinar region (Tsutsumi et al. 1989, Cohen et al. 1997, Fang et al. 1998). CYP2E1 is also present in hepatic Kupffer cells, suggesting that, under conditions where it is induced, metabolites generated in the Kupffer cells may increase toxicity and lipid peroxidation in these cells and induce hepatic injury (Cederbaum 1989, Koop et al. 1991, Nakamura et al. 1999).

### *2.2.3 Catalase*

Minor amounts of ethanol may be oxidized by peroxisomal catalase. As its activity depends on hydrogen peroxide production, which is very low in the liver under normal circumstances, the role of catalase in ethanol oxidation is probably very small, although it could be implicated in other tissues such as the brain (Cohen et al. 1980, Aragon et al. 1991, Rintala et al. 2000). Nevertheless, it has been suggested that some of the ethanol metabolism in the liver may be catalase-dependent (Thurman and Handler 1989).

### *2.2.4 First-pass metabolism of ethanol*

The gastric wall is a major site for the metabolic pathway defined as first-pass metabolism of ethanol (Gentry et al. 1994). The stomach also contains ADH, and both it and the liver have the same class I and III ADH isoenzymes. In addition, the class IV ADH isoform (Hernández-Muñoz et al. 1990) accounts for a substantial proportion of the total ADH activity in the stomach, which may also be regarded as a barrier against the penetration of excess alcohol into the body. A significant correlation exists between overall gastric ADH activity and first-pass metabolism (Frezza et al. 1990). Women have lower total gastric ADH activity and higher blood ethanol levels after ethanol consumption, and it has been speculated that this may explain their higher susceptibility to ethanol-induced toxicity (Frezza et al. 1990, Schenker 1997).

### *2.2.5 Intestinal metabolism*

It has been shown recently that the gastrointestinal microflora can also metabolize ethanol, as the microbes of the alimentary tract participate in the metabolism of exogenous ethanol (Salaspuro 1996). High concentrations of acetaldehyde may be generated through this pathway in humans, since the intestinal capacity for acetaldehyde removal is low, causing acetaldehyde to accumulate in the gastrointestinal tract.

### *2.2.6 Acetaldehyde oxidation*

The acetaldehyde formed from ethanol is effectively oxidized to acetate by hepatic ALDH (Lieber 1988, Eriksson 2001). This oxidation is coupled to the reduction of  $\text{NAD}^+$  to NADH, adding to the redox change induced by ethanol oxidation in the hepatocyte. ALDH activity is found both in human hepatocyte mitochondria and in the cytosol (Hempel et al. 1985, Yoshida et al. 1985).

## 2.3 Formation of acetaldehyde-protein adducts

Acetaldehyde, the first metabolite of ethanol, and the aldehydic products of lipid peroxidation that are generated during ethanol-induced oxidative stress can bind to proteins and cellular constituents to form stable adducts (Israel et al. 1986, Tuma and Sorrell 1987, Trudell et al. 1991a). Acetaldehyde can bind to reactive amino acid residues in several target proteins (Stevens et al. 1981, Israel et al. 1986, Behrens et al. 1990, Wehr et al. 1993, Tuma and Sorrell 1995, Zhu et al. 1996). *In vitro* studies have shown the formation of acetaldehyde adducts with haemoglobin and plasma proteins, including albumin (Donohue et al. 1983, Israel et al. 1986), and there are several other proteins that can also efficiently bind acetaldehyde, including erythrocyte membrane proteins (Gaines et al. 1977), tubulin (Smith et al. 1989), lipoproteins (Wehr et al. 1993, Lin et al. 1995a, Paradis et al. 1996a), collagens (Jukkola and Niemelä 1989, Behrens et al. 1990), ethanol-inducible CYP2E1 (Behrens et al. 1988) and ketosteroid reductase (Lin et al. 1988, 1998).

The acetaldehyde adducts can be either unstable or stable in nature (Tuma and Sorrell 1995). Unstable Schiff base adducts are formed by the binding of acetaldehyde to lysine residues, which are then further stabilized by reduction. Free  $\epsilon$ -aminolysine groups are important targets for adduct formation (Tuma et al. 1987). Lysine residues may become modified even at low acetaldehyde concentrations under appropriate reducing conditions, especially when there is a lysine residue in a critical location, as reported for tubulin (Smith et al. 1989) and for lysine-dependent enzymes (Mauch et al. 1986). The binding reactions may also involve other amino acids, including cysteine, valine and tyrosine (Stevens et al. 1981, San George and Hoberman 1986). Spontaneous stabilization of linkages has been demonstrated to occur between acetaldehyde and the  $\alpha$ -amino groups of haemoglobin in reactions yielding stable imidazolidinone derivatives (San George and Hoberman 1986, Fowles et al. 1996).

## 2.4 Acetaldehyde adducts with haemoglobin

### 2.4.1 Adduct structure

Acetaldehyde-haemoglobin condensates may be generated both *in vitro* and *in vivo* following ethanol ingestion (Sorrell and Tuma 1987, Nicholls et al. 1992). Cyclic imidazolidinone derivatives are formed between acetaldehyde and the free  $\alpha$ -amino groups of the amino-terminal valine of haemoglobin (San George and Hoberman 1986). Lysine residues have been implicated as major targets for acetaldehyde adducts. It has been suggested that the site of the haemoglobin that is modified by acetaldehyde *in vivo* being primarily located in a surface-

accessible domain near the centre of the  $\beta$ -chain, where several lysine residues are clustered (Braun et al. 1995). The peptides Val-His-Leu-Thr-Pro and Val-His-Leu-Thr-Pro-Val-Glu-Lys in the amino terminus of the  $\beta$ -globin chain of haemoglobin have at least one potential site for adduct formation (Fowles et al. 1996, Braun et al. 1997). In the octapeptide, the N-terminal amino group of valine and the  $\epsilon$ -amino group of lysine can be modified by acetaldehyde.

Haemoglobin adducts appear to be stable at 37°C for up to 14 days, which means that these stable Schiff base products can serve as markers of ethanol consumption and explain some clinical consequences of ethanol abuse (Stevens et al. 1981, Niemelä and Israel 1992, Braun et al. 1997).

#### 2.4.2 Adduct measurements on erythrocyte proteins

Methods have been reported previously for measuring adducts in haemoglobin and erythrocyte proteins of alcoholic patients and in proteins labelled with acetaldehyde *in vitro*, and these findings have stimulated research aimed at developing methods for the routine laboratory detection of acetaldehyde-modified haemoglobin in the blood of alcoholics. Such measurements would be analogous to the use of glycosylated haemoglobin (HbA1c) as an index of long-term glycaemic control in cases of diabetes (Nguyen and Peterson 1984a, b).

The immunological methods used to date for adduct measurements have been based on the use of affinity-purified rabbit polyclonal antibodies that react with acetaldehyde-protein condensates independently of the nature of the carrier protein. Such approaches have shown elevated levels of acetaldehyde-haemoglobin condensates in chronic alcoholics and healthy volunteers after occasional heavy ethanol intake (Niemelä et al. 1990a, Niemelä and Israel 1992, Lin et al. 1993a). Acetaldehyde-haemoglobin condensates remain elevated longer than the time for which ethanol is measurable in the blood (Niemelä et al. 1990a). Haemoglobin-acetaldehyde adducts in alcoholic patients have also been measured using a sandwich ELISA with antibodies raised against acetaldehyde-modified keyhole limpet haemocyanin. This method has shown high sensitivity in comparisons between alcoholics and teetotallers (Lin et al. 1993a).

Chromatographic methods and isoelectric focusing have also been used for adduct detection, pointing to the generation of fast-eluting haemoglobin fractions as a result of acetaldehyde exposure *in vitro* (Stevens et al. 1981, Hoberman and Chiodo 1982, Huisman et al. 1983, Nguyen and Peterson 1984a, b, Gordis and Herschkopf 1986, Sillanaukee and Koivula 1990, Sillanaukee et al. 1991). Alcohol abusers have been found to have an elevated concentration of minor haemoglobins, but normal or subnormal amounts of glycosylated haemoglobin (HbA1c). The reaction of acetaldehyde with haemoglobin A produced chromatographic variants, some of which migrated in the haemoglobin A1a-c

region. The analysis of stable acetaldehyde-haemoglobin adducts demonstrated that the reaction sites were the valine, lysine and tyrosine residues of globin. The acetaldehyde-modified amino acid residues appear to exist in interconvertible conformations, only one of which is reducible by an experimental reducing agent, sodium cyanoborohydride. The amount of these adducts was found to be significantly elevated in haemoglobin from alcoholics as compared with normal volunteers (Stevens et al. 1981).

Sillanaukee and Koivula (1990) described a HPLC method for the separation of haemoglobin-acetaldehyde which was able to differentiate between alcoholic and non-alcoholic populations, although there was a significant amount of interference from glycosylated haemoglobin. Acetaldehyde-modified haemoglobins were nevertheless found to have at least the same sensitivity (50%) in the detection of heavy drinking as the most widely accepted conventional biochemical markers of alcohol abuse,  $\gamma$ -glutamyl transferase (GGT) (39%) and mean corpuscular volume (MCV) (17%) (Sillanaukee et al. 1992). Hazelett et al. (1993) used a polyaspartic acid anion exchange column method (Poly CAT A) for such measurements and found no interference from glycosylated haemoglobin. In later studies, Hazelett and coworkers (1998) measured haemoglobin-acetaldehyde (HbA1-AcH) using cation exchange HPLC and noted a weak but statistically significant correlation between its levels and reported drinking. HbA1-AcH appears to be devoid of biases due to gender, illegal drug use or degree of liver damage, and capable of detecting heavy drinking before liver damage occurs (Niemelä and Israel 1992, Hazelett et al. 1998). The work of Hazelett and coworkers further demonstrated that HbA1-AcH levels reaches a plateau when self-reported drinking exceeds 20 drinks per day. The best combination of sensitivity (67%) and specificity (77%) came with a cut-off score of 0.030 area-percentage of total haemoglobin when heavy drinking was defined as more than 6 drinks per day. HbA1-AcH levels had no significant correlation with any of the liver enzymes or MCV.

Peterson and coworkers (1988, 1989) showed by chromatographic methods that the level of haemoglobin-associated acetaldehyde increases rapidly and remains elevated for 28 days, suggesting that this measurement might be a useful marker of relatively acute and chronic ethanol ingestion. The adduct value remained high following an acute dose of ethanol, even after the ethanol had been eliminated from the body. These investigators also found a correlation between haemoglobin-acetaldehyde levels and reported alcohol consumption.

In the case of pregnant women who consumed alcohol, the highest concentration of haemoglobin adducts was found in those who subsequently delivered children with fetal alcohol syndrome (Niemelä et al. 1991a), although the association between adduct levels and the extent of alcohol consumption was not consistent in such patients. Adduct measurement also failed to serve as a

clinical marker of individuals delivering infants with fetal alcohol effects (Sarkola et al. 2000).

## 2.5 Lipoprotein modifications by acetaldehyde

A number of alterations in lipoprotein metabolism are often associated with alcohol consumption. It increases the hepatic output of large, chylomicron-like very low-density lipoprotein (VLDL), the primary cause of the alcoholic hypertriglyceridaemia that is frequently observed at the early stages of alcoholic liver injury. The removal of VLDL is also increased, but production exceeds removal, so that the circulating levels increase (Savolainen et al. 1987). Alcohol consumption reduces the resulting low-density lipoprotein (LDL) levels and apolipoprotein B (apoB) levels.

Lipoproteins are readily modified by alcohol-derived acetaldehyde *in vivo*, with acetaldehyde adducts occurring especially in apoB-containing lipoproteins. *In vivo* modification of apoB apparently takes place in the liver (prior to its secretion) rather than in the circulation (Kervinen et al. 1991, Wehr et al. 1993). The LDL of alcoholic patients contains less vitamin E, is chemically modified and exhibits altered biological function, while LDL samples from alcoholics without serious liver disease have been shown to be more negatively charged than those from non-drinking controls. Both acetaldehyde-modified and oxidative-modified epitopes exist in the LDL fraction of many alcoholic patients. LDL samples from most alcoholic patients induce apolipoprotein E secretion in mouse peritoneal macrophages. Acetaldehyde-modified LDL activates apolipoprotein E synthesis in macrophages and may thereby promote atherogenesis in alcoholics (Lin et al. 1995a, Hill et al. 1998).

## 2.6 Other protein adducts in alcoholics

Aldehydic lipid peroxidation products also form Schiff base adducts with proteins in patients with excessive alcohol consumption (Niemelä 2001). A number of species have been identified, including adducts with malondialdehyde (MDA) (French et al. 1993, Niemelä et al. 1994, Tsukamoto et al. 1995), 4-hydroxynonenal (HNE) (Kamimura et al. 1992, French et al. 1993, Niemelä et al. 1994, 1995, Tsukamoto et al. 1995, Chen et al. 1998, Ohhira et al. 1998, Chen et al. 1999, Niemelä 1999), MDA-acetaldehyde adducts (MAA) (Tuma et al. 1996, Xu et al. 1998) and hydroxyethyl radicals (HER) (Moncada et al. 1994, Clot et al. 1995, Albano et al. 1996, Clot et al. 1996, 1997). MDA is a reactive dialdehyde originating from the non-enzymatic lipid peroxidation of a variety of unsaturated fatty acids, from lipid peroxidation that occurs during phagocytosis

by monocytes and from arachidonic acid catabolism in thrombocytes (Palinski et al. 1990, Esterbauer et al. 1991). HNE, produced by the free-radical-mediated oxidation of long-chain polyunsaturated fatty acids, can react with the sulfhydryl groups of proteins through a mechanism of the Michael addition type (Palinski et al. 1990, Esterbauer et al. 1991, Stadtman 1992). MDA adducts have been found in the liver of alcohol consumers (Niemelä et al. 1994) and hybrid MAA in the liver of ethanol-fed rats (Tuma et al. 1996, Xu et al. 1998). HER can also form adducts with hepatic proteins (Moncada et al. 1994, Albano et al. 1996).

In addition to alcohol related diseases, oxidative modification of proteins with MDA or HNE has been found to occur in other conditions including atherosclerotic lesions of arterial walls (Palinski et al. 1990).

## 2.7 Adducts in tissues

### 2.7.1 Liver

Early studies by Medina and coworkers (1985) demonstrated that acetaldehyde can form both stable and unstable adducts with hepatic proteins, the unstable adducts being liable to undergo conversion to form stable adducts during incubation. Immunohistochemical studies of the liver have subsequently shown different types of adducts to be enriched in zone 3 hepatocytes in alcoholics (Niemelä et al. 1991b, 1994, Li et al. 1997). These are formed in the early phases of ethanol-induced liver damage, indicating that this mechanism may be one of the key events in the pathogenesis of alcoholic liver disease (Niemelä et al. 1991b, Holstege et al. 1994, Paradis et al. 1996b, Li et al. 1997). While patients with early stages of alcoholic liver disease (ALD) show acetaldehyde-modified epitopes around the central vein, the distribution of these is more widespread at the advanced stages (Niemelä et al. 1991b)

Both acetaldehyde adducts and MDA adducts are co-localized with areas of fatty change in alcohol-induced liver injury (Niemelä et al. 2002a) and aldehyde adducts also co-localize with centrilobular fibrosis (Niemelä et al. 1994). These results have enabled increased lipid peroxidation and aldehyde-protein adduct formation to be linked with central-central bridging fibrosis. A comparable liver pathology is also associated with the induction of CYP2E1. Adduct formation in hepatocytes appears to occur at the expected sites of acetaldehyde production. In addition, the detection of adducts in human and rat stellate cells further suggests that acetaldehyde can diffuse into these cells and bind to cytoplasmic proteins to form local adducts. As hepatic stellate cells are the main effector cells of liver fibrosis, the detection of adducts in these points to their possible involvement in liver fibrogenesis (Paradis et al. 1996b).

The mechanisms modulating adduct formation *in vivo* have remained unclear. The formation of protein adducts with acetaldehyde and MDA appears to be accompanied by induction not only of CYP2E1 but also of other cytochromes such as CYP3A, which is a major component of the hepatic cytochrome mass (Jeong et al. 2000, Niemelä et al. 2000). The occurrence of acetaldehyde-derived and lipid peroxidation-derived protein adducts together with enhanced expression of CYP2E1 and CYP3A characterizes the early phase of ALD (Nanji et al. 1994, Niemelä et al. 1998).

Kupffer cells, which are the main phagocytic cells in the liver and are located on the endothelial surface of the sinusoidal space, may also be involved in the generation of protein adducts with both acetaldehyde and MDA (Niemelä et al. 2002a). Their inhibition influences ethanol metabolism and they may contribute to the hepatotoxic effects of ethanol. Kupffer cells contain ALDH2, which can participate in the oxidation of acetaldehyde. A superoxide anion is formed in these cells during ethanol oxidation (Nakamura et al. 1999), and this together with lipopolysaccharide-enhanced free radical generation by the liver may also be of pathogenic significance with regard to resistance to infection (Bautista and Spitzer 1992). Under conditions where CYP2E1 is induced, metabolites generated by Kupffer cells may further contribute to Kupffer cell toxicity (Koop et al. 1991).

Other factors in addition to ethanol, such as a high-fat diet (Tsukamoto et al. 1995), an iron excess (Niemelä et al. 1999a) or the castration of experimental animals (Niemelä et al. 1999b), have been shown to be associated with enhanced adduct levels in the liver.

### 2.7.2 *Extrahepatic tissues*

There is only limited information available on the occurrence of protein adducts in extrahepatic tissues. The alimentary tract (Biewald et al. 1998) and pancreas (Iimuro et al. 1996, Horne et al. 1997, Casini et al. 2000) may evidently also be potential sites of adduct formation, and intestinal bacteria are able to metabolize ethanol and thereby increase acetaldehyde concentrations and local toxicity in the gut (Salaspuro 1996). Acetaldehyde administered in drinking water has been shown to result in hepatotoxicity and adduct formation (Jokelainen et al. 2000). In the pancreas, ethanol intake leads to the adduct formation with HER (Iimuro et al. 1996) and HNE (Casini et al. 2000), which suggests a role for free radical generation in ethanol-induced pancreatitis. Lipid peroxidation-derived adducts also occur in response to pancreatic iron overload (Horne et al. 1997).

Evidence of acetaldehyde-protein adducts in the rat brain has been provided by Nakamura and coworkers (2000) and Rintala and coworkers (2000). After

life-long ethanol consumption, acetaldehyde adducts were found in the large neurons of the deep layers of the frontal cortex and in the molecular layer of the cerebellum, particularly in a rat strain that has high concentrations of acetaldehyde during ethanol oxidation (ANA).

Ethanol metabolites and enhanced lipid peroxidation may also be involved in the generation of muscle pathology in alcoholics, as evidenced by the recent demonstration of adducts in experimental ethanol-induced myopathy (Worrall et al. 2001, Niemelä et al. 2002b, 2003) and by the association between high acetaldehyde levels and myocardial apoptosis in experimental animals (Jänkälä et al. 2002).

## 2.8 Possible functional effects of adduct generation in tissues

Adduct formation may have many adverse functional consequences in physiological processes, such as interference with protein function, stimulation of fibrogenesis and induction of immune responses (Tuma and Sorrell 1995, Niemelä 1999). Consequently, tissue function and structure may be altered in a variety of ways. Acetaldehyde favours protein retention, with associated swelling of hepatocytes (Lieber 1988). Various mitochondrial functions are altered, particularly after chronic ethanol consumption, which sensitizes the mitochondria to the toxic effects of acetaldehyde (Lieber 1988). Acetaldehyde stimulates collagen production in cultured myofibroblasts (Brenner and Chojkier 1987, Casini et al. 1991), and acetaldehyde-protein adducts also stimulate the production of antibodies directed against the acetaldehyde-derived epitopes (Niemelä et al. 1987, Hoerner et al. 1988, Izumi et al. 1989, Worrall et al. 1991, Koskinas et al. 1992, Viitala et al. 1997).

Adduct formation interferes with protein function particularly when there is a lysine residue in a functionally critical location, such as in tubulin and in lysine-dependent enzymes (Mauch et al. 1986, 1987, McKinnon et al. 1987, Sorrell and Tuma 1987, Jennett et al. 1989, Smith et al. 1989, Tuma et al. 1991). Altered microtubule function may impair protein secretion and plasma membrane assembly, and the generation of reactive aldehydes may also contribute to ethanol-induced impairment of receptor-mediated endocytosis (Casey et al. 1991). Acetaldehyde binding with cellular constituents can also stimulate fibrogenesis (Moshage et al. 1990, Pares et al. 1994) and activate carcinogenesis (Fraenkel-Conrat and Singer 1988). Alcoholics who have higher acetaldehyde levels due to polymorphisms and/or mutations in the genes coding for the enzymes responsible for acetaldehyde generation or detoxification have an increased risk of cancer (Yokoyama et al. 1996).

### *2.8.1 Stimulation of fibrogenesis*

Not all alcohol abusers develop liver fibrosis even with high alcohol consumption. On the other hand, the appearance of early fibrosis in zone 3, adjacent to the hepatocytes that are the site of protein-adduct deposition in the early phase of ALD, predicts the subsequent development of irreversible cirrhosis (Nakano et al. 1982, Worner and Lieber 1985, Niemelä et al. 1995).

Previous cell culture studies have shown that aldehydic products derived from ethanol metabolism and lipid peroxidation increase collagen mRNA levels and the expression of connective tissue proteins (Brenner and Chojkier 1987, Chojkier et al. 1989, Casini et al. 1991, 1994, Friedman 1993, Halsted et al. 1993, Parola et al. 1993, Lee et al. 1995). Acetaldehyde is thus able to increase the production of extracellular matrix components (Casini et al. 1994, Houghlum 1996). The reduction of adduct formation by scavengers of reducing equivalents abolishes such increases (Chojkier et al. 1989). It has also been demonstrated that hepatic stellate cells (Ito cells) become readily activated under conditions involving acetaldehyde generation, enhanced oxidative stress and lipid peroxidation (Houghlum et al. 1991, Bedossa et al. 1994, Lee et al. 1995, Houghlum 1996).

### *2.8.2 Immune mechanisms*

The triggering of immune mechanisms may represent one of the main pathways by which adduct formation contributes to the progression of ALD (Marshall et al. 1983, Israel et al. 1988, Lieber 1988, Zetterman 1990). Aldehyde-protein adducts and hydroxyl radicals stimulate immunological responses directed against the specific modifications of proteins (Israel et al. 1986, Niemelä et al. 1987, Hoerner et al. 1988, Izumi et al. 1989, Trudell et al. 1991b, Worrall et al. 1991, Koskinas et al. 1992, Wehr et al. 1993, Clot et al. 1995, 1996). Such responses can also be measured in serum specimens by immunological assays (Israel et al. 1986, Niemelä et al. 1987, Hoerner et al. 1988, Israel et al. 1988, Izumi et al. 1989, Worrall et al. 1991, Israel et al. 1992, Koskinas et al. 1992, Tuma and Klassen 1992, Wehr et al. 1993, Worrall et al. 1994, Clot et al. 1995, Albano et al. 1996, Clot et al. 1996, Viitala et al. 1997, Xu et al. 1998, Viitala et al. 2000). Chronic ethanol administration to experimental animals has been shown to lead to the generation of circulating immunoglobulins with anti-acetaldehyde adduct, anti-MDA-adduct or anti-MAA adduct specificity. Similar immunoglobulins and autoantibodies recognizing CYP2E1-HER adducts have also been found in the blood of alcoholics (Israel et al. 1986, Clot et al. 1995, 1996). Both IgA and IgG responses have been reported (Worrall et al. 1991, Clot et al. 1995, Viitala et al. 1997). The highest titres of such autoantibodies have been observed previously in patients with severe ALD (Niemelä et al. 1987, Hoerner et al. 1988, Izumi et al. 1989, Worrall et al. 1991, Koskinas et al. 1992,

Wehr et al. 1993, Clot et al. 1995, Rolla et al. 2000). Viitala and coworkers (1997) showed that IgA titres are elevated in 69% of patients with ALD, these titres being significantly higher than those for patients with non-ALD, non-drinking controls, or heavy drinkers without any clinical and biochemical signs of liver disease. Anti-adduct IgGs have been found both in some ALD patients and in heavy drinkers without liver disease. It has further been shown that experimental liver disease can be induced by ethanol challenge in animals immunized with acetaldehyde adducts (Yokoyama et al. 1995). Alcoholics also develop increased amounts of IgG autoantibodies against apoB-containing lipoproteins (Wehr et al. 1993). Plasma IgG reactivity increased when VLDL and LDL were isolated from alcoholics who had recently been drinking (Wehr et al. 1993), indicating that alcohol consumption increases both the formation of the antigen and the humoral immune response.

Enhanced production of cytokines by leukocytes is also associated with ethanol abuse, suggesting that excessive ethanol intake may also affect the regulation of immune responses (Deviere et al. 1992). Studies of ALD patients (Khoruts et al. 1991, Peters 1996) and experimental animals (Fang et al. 1998) have also shown that there are increases in circulating levels of pro-inflammatory (TNF- $\alpha$ , IL-6, IL-1 $\beta$ ) and anti-inflammatory (IL-4) cytokines. Cytokine-mediated cell-cell interactions may be important factors in the onset of ethanol-induced liver damage, including the stimulation of fibrogenesis (Border and Noble 1994) and inflammatory changes (Yin et al. 1999, Li et al. 2003), although the mechanisms are poorly characterized. It has recently been suggested that the production of cytokines in the liver of alcoholic patients may be induced by increased levels of endotoxin (Neuman et al. 2001, Hoek and Pastorino 2002).

## 2.9 Effects of ethanol in blood cells and bone marrow

Heavy ethanol intake may affect both blood cells and their bone marrow progenitors (Malik and Wickramasinghe 1986, Lindenbaum 1987, Niemelä and Parkkila 2004). Elevated MCV (macrocytosis) is the most typical abnormality occurring as a result of heavy drinking (Morgan et al. 1981, Wymer and Becker 1990). Macrocytosis in alcohol abusers is usually relatively mild in degree (MCV of 96-100), but other abnormalities in the erythropoietic cell lineage, including erythroblast vacuolization in bone marrow, have also been reported (Lindenbaum 1987).

Anaemia is common in alcoholics with liver disease, as also are folate deficiency and megaloblastic alterations in bone marrow (Lindenbaum 1987, Niemelä and Parkkila 2004). Ethanol consumption with an inadequate diet has been associated with the appearance of ring sideroblasts in bone marrow, and ethanol has also been reported to inhibit a number of enzymatic steps in heme

synthesis (Lindenbaum 1987). There may also be changes in cell structure in the erythropoietic cells, including membrane convolutions adjacent to vacuoles which lack organelles or any organized structure, suggesting an effect of alcohol on the cell membranes (Lindenbaum 1987).

Megakaryocytes, the precursors of platelets, are typically normal or increased in number in the bone marrow of alcoholics (Lindenbaum 1987), and a low platelet count is a common finding after heavy ethanol intake. Rebound thrombocytosis (an elevated platelet count) usually occurs within a week of alcohol withdrawal. It has been postulated that alcohol intoxication itself is the cause of thrombocytopenia, but it is likely that the mechanism is a combination of ineffective thrombocytopoiesis (normal in the presence of increased numbers of marrow megakaryocytes with decreased production of platelets) and a shortened platelet life span (Lindenbaum 1987).

## 2.10 Current biochemical markers of ethanol consumption

There are several biochemical markers currently available for detecting alcohol-related health problems. It should be emphasized that clinical experience with the use of such markers indicates that many of them may reflect changes in liver status rather than alcohol consumption per se.

Measurement of the ethanol concentration in the blood, breath or urine is a specific marker of alcohol ingestion and can be used to diagnose ethanol intoxication (for reviews, see Salaspuro 1986, Sillanaukee 1996, Niemelä 2002). High blood ethanol levels may reveal tolerance, so that levels exceeding 150 mg/L (33 mM) without obvious evidence of intoxication or 300 mg/L (65 mM) at any time can be taken to indicate significant tolerance and alcoholism. Ethanol has a short half-life, however, which hampers its use as a tool for diagnosing chronic drinking problems.

Elevations in the activity of GGT, a membrane-bound glycoprotein enzyme that catalyses transfer of the  $\gamma$ -glutamyl moiety of glutathione to various peptide acceptors (Salaspuro 1986, Goldberg and Kapur 1994, Rosman and Lieber 1994, Cook 2003), are not specific to alcohol-induced damage, as they can be induced by both ethanol consumption and liver disease. Abnormal values in patients who have not consumed ethanol may suggest the presence of the latter.

MCV is known to increase as a result of ethanol consumption, particularly in women (Chalmers et al. 1980, Morgan et al. 1981, Seppä and Sillanaukee 1994, Seppä et al. 1996, Niemelä 2002, Cook 2003). It responds slowly to abstinence, requiring 2-4 months to normalize. MCV in combination with GGT are preferred

laboratory markers for detecting and monitoring alcohol consumption in several clinical settings. For instance, they have been found to be associated with the amount of alcohol consumed by heavily drinking mothers who give birth to children with fetal alcohol effects (Sarkola et al. 2000).

The carbohydrate-deficient transferrin (CDT) assay measures isoforms of transferrin that have a reduced number of carbohydrate moieties. Stibler and coworkers (1976, 1986) were the first to report that the amount of desialylated isoforms of transferrin in biological fluids increases as a result of ethanol consumption. CDT measurements have been widely used to detect alcohol abuse in recent years and have improved the specificity with which alcohol-related health problems can be detected. They may lack sensitivity, however, since hazardous drinkers in the early phase of their drinking problems often remain undetected, and they have also shown lower diagnostic sensitivity and specificity in the detection of alcohol abuse in women than in men, possibly due to the fact that the assay results obtained with some CDT methods are significantly influenced by changes in serum levels of total transferrin (Anttila et al. 2003). The reasons for the high concentrations of sialic acid-deficient transferrin in alcoholics are not known, but a role for acetaldehyde in interfering with the enzymes mediating addition of the carbohydrate moieties has been suggested (Arndt 2001, Sillanaukee et al. 2001).

Serum concentrations of the liver-derived enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are frequently elevated in patients with chronic alcohol consumption (Salaspuro 1989), but this is usually associated primarily with hepatocyte injury. It can also be found in abstinent alcoholics with chronic liver disease. The ratio of AST to ALT is often over 2 in ALD, while it is usually below 1 in patients with non-ALD (Rosman and Lieber 1994).

Changes in some other laboratory parameters may also be associated with alcohol abuse. High-density lipoprotein (HDL) cholesterol increases as a consequence of prolonged alcohol consumption, although this effect occurs with fairly moderate consumption (3-5 drinks per day). High HDL cholesterol levels should therefore alert clinicians to investigate a patient's recent pattern of alcohol consumption. HDL levels have also been shown to correlate with GGT, ALT, AST and MCV in alcohol-consuming patients (Szegedi et al. 2000). Blood triglyceride concentrations are elevated even after rather short periods of heavy drinking.

Since the elevation in blood acetate concentrations in alcoholics is induced by hepatic ethanol oxidation (Korri et al. 1985, Nuutinen et al. 1985, Salaspuro 1986), acetate concentrations should correlate with the rate of ethanol metabolism. This marker can be used when there is ethanol in the blood. Other markers of alcohol intake that have been postulated include circulating levels of

the hepatic lysosomal glycosidase,  $\beta$ -hexosaminidase. These are quite sensitive, but fall rapidly during abstinence, and they can also be elevated in conditions other than alcohol intake (Kärkkäinen et al. 1990, Hultberg et al. 1991, 1995, Sharpe 2001). Urinary dolichols are long-chain 2,3-dihydroxy-polyprenols which serve as lipid carriers in the biosynthesis of glycoproteins, but their clinical use is limited by their short half-life and lack of specificity (Pullarkat and Raguthu 1985, Roine et al. 1987).

The ratio of the serotonin metabolite 5-hydroxytryptophol to 5-hydroxyindole-3-acetic acid (5-HTOL/5-HIAA) in urine has a high diagnostic accuracy for detecting recent alcohol consumption and relapse (Helander and Eriksson 2002). 5-HTOL remains elevated for 6-20 h after the disappearance of ethanol (Carlsson et al. 1993, Helander et al. 1996, Helander and Tabakoff 1997).

Serum levels of sialic acids, acetylated derivatives of neuraminic acid which attach to non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids (Crook et al. 1996, Pönniö et al. 1999, Sillanaukee et al. 1999, Romppanen et al. 2002), increase significantly after a short period of drinking (Pönniö et al. 2002). Other potential markers of alcohol intake include urinary ethyl glucuronide (Sarkola et al. 2003), fatty acid ethyl esters (Doyle et al. 1996), phosphatidyl ethanol (Lundqvist et al. 1994), erythrocyte ALDH (Agarwal et al. 1983) and urinary salsolinol (Haber et al. 1995), data on their use in clinical settings and on comparisons between them are limited.

### 3 Purpose of the research

Alcohol abuse is known to have a wide array of adverse effects on blood cell formation, but the mechanisms underlying such effects remain unclear. Many of the toxic effects of ethanol have been attributed to its toxic metabolite, acetaldehyde. The aims of the present research were as follows:

1. to examine the role of ethanol and acetaldehyde in creating abnormalities in blood cell indices and bone marrow morphology,
2. to explore the relationship between ethanol consumption and laboratory and morphological findings in patients undergoing bone marrow aspiration,
3. to examine the formation of acetaldehyde-derived protein modifications in erythrocytes and their bone marrow precursors using antibodies recognizing acetaldehyde-modified epitopes in proteins independently of the nature of the carrier protein,
4. to develop new approaches for the detection of acetaldehyde adducts using antibodies against protein condensates with acetaldehyde, and
5. to examine the generation of autoimmune responses to acetaldehyde-derived protein condensates in alcoholics.

## 4 Materials and methods

### 4.1 Patients and control subjects

The cases for the blood and bone marrow studies (papers I, II) were consecutive patients undergoing bone marrow examinations. Blood smears and bone marrow aspirates from all patients were prepared for routine diagnostic purposes. Erythrocyte haemolysates prepared from EDTA-anticoagulated blood (I-IV) and serum samples (III-IV) from alcoholic patients were kept frozen at  $-70^{\circ}\text{C}$  until used. Informed consent was obtained from all the subjects. The work was approved by the institutional ethical committee and it was carried out according to the provisions of the Declaration of Helsinki.

The series reported on in paper I included 144 consecutive general hospital patients undergoing bone marrow examinations due to abnormalities in peripheral blood counts without any previously established diagnoses of specific haematological diseases, malignancies, or infections. It included 57 patients who were classified as alcohol abusers and showed a history of ethanol consumption exceeding a mean of 60 g per day either continuously or during repeated inebriations. Of these, 14 patients met the DSM-IV criteria for ethanol dependence on the grounds of their case history and clinical examinations. They had been actively drinking in amounts exceeding a mean of 120 g per day for 1-2 weeks prior to sampling. Thus the series included both alcohol abusers whose primary pattern was binge drinking and patients with more continuous dependent drinking. Clinical and laboratory tests nevertheless showed the patients to be free of any severe liver dysfunction. Eighty-seven patients (referred to as non-alcoholics) were non-drinkers or social drinkers with a mean ethanol consumption of less than 20 g per day or 40 g on any single occasion. A follow-up of supervised abstinence for a period of 7-8 days was initiated for 14 patients with ethanol dependence.

The series considered in paper II comprised 138 patients (85 males, 53 females) with elevated MCV, of whom 68 (49%; 45 males and 23 females, mean age 51 years) had a history of excessive alcohol consumption (exceeding a mean of 60 g ethanol per day either continuously or during repeated bouts). 28 of these patients (23 males, 5 females, mean age 54 years) met the DSM IV criteria for alcohol dependence. They had been drinking amounts exceeding a mean of 120 g per day for 1-2 weeks, with an abstinence time of between 0-3 days prior to

sampling. The series was compiled separately and contained different patients from those in paper I, except for 12 alcoholics.

For paper III, EDTA blood samples and serum samples were collected from 32 alcoholic patients who had been hospitalized for detoxification (30 males, 2 females, mean age 46 years, range 32-59 years). They were treated and followed up at the Department of Psychiatry, Seinäjoki Central Hospital. All the patients had a history of heavy drinking (a mean of more than 60 g of ethanol per day). The time of abstinence prior to sampling was between 0-7 days, blood samples being taken from 22 patients on the day of admission, from 5 patients 1-3 days after admission and from 5 patients 4-7 days after admission. The controls were 22 healthy individuals (11 males, 11 females, mean age 45 years, range 25-59 years) who were either teetotallers or social drinkers consuming less than 20 g of ethanol per day with a maximum weekly consumption of 60 g. None of them had consumed alcoholic beverages during the week prior to sampling.

Paper IV is based on a series of 86 male alcoholics. 54 of them had biopsy-confirmed liver disease and had consumed ethanol in amounts exceeding 80 g per day either continuously or in repeated episodes of binge drinking. Liver histology ranged from mild fibrosis and fatty changes to cirrhosis with a wide distribution of morphological abnormalities related to alcoholic hepatitis, as assessed according to the combined morphological index (CMI) of liver disease severity (Niemelä et al. 1990b, Blake and Orrego 1991). In addition, there were 32 patients, who had been admitted for detoxification, showing a well-documented history of continuous ethanol consumption or binge drinking, amounting to a mean of 130 g of ethanol per day during the 4 weeks prior to sampling, as assessed by detailed personal interviews using a time-line follow-back technique. The mean duration of abstinence prior to sampling was  $2 \pm 2$  days. A follow-up with supervised abstinence for  $8 \pm 2$  days was completed for 17 of these patients. The male reference subjects (mean age  $48 \pm 17$  years) were either abstainers ( $n=6$ ) or moderate drinkers ( $n=14$ ) whose recent mean daily ethanol consumption was 20 g (range 1-60 g).

## 4.2 Preparation of erythrocyte proteins and acetaldehyde labelling *in vitro* (II-IV)

Human erythrocyte protein (haemoglobin) was prepared from the EDTA blood of a teetotaller. The cells were separated out by centrifugation and washed three times with an equal volume of phosphate-buffered saline (PBS: 7.9 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , 137 mM NaCl, 2.7 mM KCl, pH 7.4). The erythrocytes were haemolyzed with twenty volumes of 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

haemolysate was brought to a haemoglobin protein concentration of 12 mg/mL and was dialyzed twice against PBS. Thereafter, 1 mL of the haemolysate was incubated for 3 h at room temperature with various concentrations of  $^{14}\text{C}$ -acetaldehyde-solution in PBS including 10  $\mu\text{L}$  of 1 M  $\text{NaCNBH}_3$  as a reducing agent. The reaction mixture was subsequently dialyzed against PBS and the labelled proteins stored at  $-70^\circ\text{C}$  until used.

### 4.3 HPLC separation of erythrocyte protein adducts (II)

The erythrocyte proteins were separated into subfractions using a HPLC procedure. The column was a cation exchanger, Mono S HR5/5 (Pharmacia Fine Chemicals, Uppsala, Sweden), with lithium as the elution cation. The flow rate was 2.0 mL/min, and 0.5 mL fractions were collected. Haemoglobin fractions were monitored at 405 nm. All fractions were subsequently tested for immunological reactivity by ELISA using previously characterized rabbit polyclonal affinity-purified antibodies against acetaldehyde adducts (Israel et al. 1986, Niemelä et al. 1991b) raised against bovine serum albumin (BSA) conjugated with 1 mM acetaldehyde under reducing conditions. The antibodies reacted with acetaldehyde-protein condensates prepared at 5  $\mu\text{M}$  to 10 mM concentrations of acetaldehyde independently of the nature of the carrier protein.

### 4.4 Preparation of lipoprotein modifications for immunizations (III)

Blood samples for the preparation of the various derivatized lipoproteins were collected from a healthy non-drinking control subject. LDL and VLDL fractions were prepared by ultracentrifugation. Previously established methods were subsequently followed to prepare acetaldehyde condensates with lipoproteins (Niemelä et al. 1991b, 1994), erythrocyte proteins and BSA (Israel et al. 1986, Niemelä et al. 1991b, 1994). Essentially, the proteins were incubated in PBS containing various concentrations of acetaldehyde (range 0-10 mM) and the condensates were stabilized with sodium cyanoborohydride (10 mM) (Sigma Chemical Co., St Louis, MO.). The solutions of acetaldehyde in buffer were prepared by appropriate dilutions of stock solutions of 1,2- $^{14}\text{C}$ -acetaldehyde (185 MBq/mmol, NEC-374, Lot 2212-171, New England Nuclear, Boston, MA) with unlabelled acetaldehyde.  $^{14}\text{C}$ -acetaldehyde was used to check the amount of acetaldehyde actually bound to proteins.

Lipoproteins were modified with MDA by incubating them for 3 h at  $37^\circ\text{C}$  with 0.5 M MDA (Haberland et al. 1982, Palinski et al. 1990, Niemelä et al.

1994). The MDA was freshly generated from malondialdehyde bis-dimethylacetal by acid hydrolysis.

Human LDL was acetylated according to the procedure of Basu et al. (1976). In a typical preparation, 2 mL of 0.15 M NaCl containing 2 mg of LDL protein was added to 2 mL of a saturated solution of sodium acetate with continuous stirring in an ice-water bath. Acetic anhydride was then added in multiple small aliquots over a period of 1 h with continuous stirring. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of the protein used, the mixture was stirred for an additional 30 min without further additions. The reaction solution was then dialyzed for 24 h at 4°C against PBS containing 10 µM EDTA, pH 7.4.

Human LDL was oxidized by exposing 200 µg/mL apolipoprotein to copper sulphate (5 µM free copper concentration) in PBS at 37°C for 20 h.

Antisera against acetaldehyde-modified lipoprotein fractions, acetaldehyde-modified BSA, MDA-modified lipoproteins, acetylated LDL and oxidized LDL were generated by immunizing rabbits with homologous protein modifications which had been freshly prepared and stored at 4°C until used for the immunizations. The primary immunization consisted of intradermal injections of 500 µg (BSA-acetaldehyde, MDA-modified lipoprotein, acetylated and oxidized lipoproteins) or 250 µg (LDL-acetaldehyde) or 125 µg (VLDL-acetaldehyde) suspended in PBS and Freund's complete adjuvant (Difco Laboratories, Detroit, MI) (1:1 ratio). Booster injections of 250 µg of the antigen in Freund's incomplete adjuvant were given at 3-4 week intervals and antisera were collected one week after the last booster injection.

#### 4.5 Preparation of acetaldehyde-modified antigens *in vitro* for ELISA assays (II-IV)

Acetaldehyde diluted in PBS was added to aliquots of the freshly prepared haemoglobin 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 adding sodium cyanoborohydride (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 use at -70°C. Samples representing unmodified haemoglobin were prepared and treated similarly to that of the modified haemoglobin except for the addition of acetaldehyde.

## 4.6 Immunological assays (II-IV)

For the antibody titre determinations, microtitre plate wells were coated with different freshly prepared antigen preparations with which the various antisera were allowed to react. Antigen concentrations of 1-5  $\mu\text{g}/\text{well}$  representing different degrees of modification were used. After treatment with gelatin, the rabbit anti-acetaldehyde-adduct antiserum (diluted 1:200 in PBS-Tween) was added. The solutions containing antigen were allowed to react with various dilutions of antisera for 1 h at 37°C.

After extensive washes, goat anti-rabbit immunoglobulin labelled with alkaline phosphatase was incubated on plates overnight at 4°C. P-nitrophenylphosphate solution was used as a colour reaction substrate (Alkaline Phosphatase Substrate Kit, Bio-Rad Laboratories, Hercules, CA). The colour reaction was stopped by adding 100  $\mu\text{L}$  of 0.4 M NaOH. The intensities of the reactions were measured using an Anthos HTII microplate reader (Anthos Labtec Instruments, Salzburg, Austria).

For the assays of erythrocyte proteins, microtitre plates were first coated with ammonium sulphate-precipitated, affinity-purified antibodies (1:50 dilution) and the reaction allowed to proceed overnight at 4°C. The haemolysate samples (120  $\mu\text{g}$  protein/well) were added after blocking with 0.2% gelatin in PBS. The plates were incubated at 37°C for 1.5 h and washed with PBS containing 0.04% Tween-20. Sheep anti-human haemoglobin antiserum (Biogenesis) was then added (dilution 1:1500) and the reaction was allowed to proceed for 30 min at 37°C. After extensive washes, alkaline phosphatase-linked rabbit anti-sheep IgG (H+L) (Zymed) was added and allowed to react for 1 h at 37°C to form antigen-antibody complexes. An Alkaline Phosphatase Substrate Kit from Bio-Rad was used for the colour reaction.

Similarly, for the assays of serum proteins, microtitre plates were first coated with the purified antibodies, and after addition of the patient serum samples (50  $\mu\text{g}$  protein/well), incubated at 37°C for 1.5 h and washed with PBS-Tween. Goat anti-human serum antiserum (Jackson ImmunoResearch Laboratories, Inc) was then added (dilution 1:10000) and allowed to react for 30 min at 37°C. After extensive washes, alkaline phosphatase-conjugated affinity-purified rabbit anti-goat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc) was added at a dilution 1:5000 and allowed to react for 1 h at 37°C to form antigen-antibody complexes.

In the studies of autoimmune responses in alcoholics (IV), the microtitre plates (Nunc-Immuno Plate, Maxisorb<sup>TM</sup>, InterMed, Denmark) were coated with acetaldehyde-modified haemoglobin, or the corresponding unmodified haemoglobin (background) in PBS (3  $\mu\text{g}$  protein in 100  $\mu\text{L}/\text{well}$ ) and incubated

for 1.5 h at 37°C. Non-specific 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 containing 0.04% Tween-20 (PBS-Tween). The final volume of 50 µL of each serum dilution was allowed to react with the coated proteins for 1 h at 37°C, followed by extensive washing with PBS-Tween. Alkaline phosphatase-linked goat anti-human immunoglobulins IgA, IgG and IgM (Jackson ImmunoResearch Laboratories, Inc.) diluted in PBS-Tween containing 8 mM MgCl<sub>2</sub> and a small amount of dithiothreitol (DTT) were used to label the antibody-antigen complexes (50 µL/well). The plates were allowed to incubate at 4°C overnight. After washing, 100 µL of p-nitrophenylphosphate solution was added as a colour reaction substrate (Alkaline Phosphatase Substrate Kit, Bio-Rad Laboratories, Hercules, CA). The colour reactions were stopped by adding 100 µL of 0.4 M NaOH and the optical densities read at 405 nm using an Anthos HTII microplate reader.

## 4.7 Immunocytochemical methods (II)

Isolated peripheral blood cells permeabilized with saponin (0.05%) were spread onto microscope slides and fixed in 4% formaldehyde for 20 min. For confocal laser scanning microscopy, the samples were excited with a laser beam at a wavelength of 568 nm using an air-cooled argon-krypton laser (75 mW output; Leitz CLSM, Leica Laser Technics, Germany). The emission light was focused through a pinhole aperture and the full field was scanned in square image formats of 512 x 512 pixels. Built-in software was used to reconstruct the images obtained from the confocal sections. Digital image analysis was performed with a Nikon Diaphot-TMD microscope (Nikon, Tokyo, Japan) combined with a Dage model 72E CCD camera with remote control (Dage-MTI, Michigan City, IN) and a MCID-M2 image analyzer (Imaging Research, St. Catharines, Ontario, Canada) with a spatial resolution of 1280 x 1024 pixels.

## 4.8 Other methods

Blood cell counts were determined using a Coulter STKS Hematology Analyzer (I-II). Measurements of AST, ALT, GGT, erythrocyte folate, serum vitamin B<sub>12</sub> levels, serum albumin and serum bilirubin were carried out using standard clinical chemical methods in the accredited laboratory of Seinäjoki Central Hospital, Finland (SFS-EN 45001, ISO/IEC Guide 25).

The concentrations of cytokines (IL-2, IL-6, IL-8, IL-10, TNF- $\alpha$  and TGF- $\beta$ 1) in serum were determined using Quantikine high sensitivity ELISA kits (R&D Systems, Abingdon Science Park, U.K.). The concentration of CDT in

serum was measured by a competitive radioimmunoassay after microcolumn separation (CDTect).

## 4.9 Statistical methods

Values are expressed as mean  $\pm$  SD. Statistical comparisons were evaluated with Student's t-test or the Mann-Whitney U-test. Logarithmic transformation was used for variables with an asymmetrical distribution (measurements performed on haemolysates). ANOVA or Kruskal-Wallis test was used to analyse the differences between groups, which were considered statistically significant at  $p < 0.05$ . Square root transformation was used to yield non-skewed distributions before the ANOVA analyses. Either the Pearson or the Spearman rank correlation test was used to calculate correlations between the variables, as required. Incidences were compared with Fisher's exact probability test or the Chi Square test for trends, as appropriate.

## 5 Results

The main observations are summarized in Table 1. A more detailed account of the data is also provided in the following chapters and in the original papers I-IV.

**Table 1. Summary of the main observations**

Study	Subjects	Main findings
I	144 patients with cytopenias (57 alcoholics, 87 non-alcoholics)	High MCV: 67% of alcoholics 18% of non-alcoholics
		High MCH: 63% of alcoholics 22% of non-alcoholics
		Reticulocytosis: 37% of alcoholics 25% of non-alcoholics
		Thrombocytopenia: 41% of alcoholics 22% of non-alcoholics
II	138 patients with macrocytosis (68 alcoholics, 70 non-alcoholics)	Acetaldehyde adducts on the cell membrane and inside the erythrocytes of alcoholics
III	32 alcoholics, 22 healthy non-alcoholic controls	Acetaldehyde adducts in serum, as analysed with the anti-acetaldehyde VLDL adduct antibody
IV	86 male alcoholics, 20 healthy male controls	IgA, IgG and IgM antibodies against acetaldehyde adducts in alcoholics Elevated levels of IgAs, IL-2, IL-8 and TNF- $\alpha$ in patients with alcoholic liver disease

MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin

### 5.1 Haematological abnormalities in the blood of alcoholics (I)

40% (57/144) of the patients referred for bone marrow examination due to cytopenia were found to be hazardous drinkers of alcohol, their mean blood haemoglobin levels ( $124 \pm 24$  g/L) being higher than in the non-alcoholics ( $112 \pm 26$  g/L) ( $p < 0.01$ ) and the incidence of anaemia lower (51% vs. 69%;  $p < 0.05$ ) (Table 2). This alcoholic group had significantly higher MCV ( $p < 0.0001$ ) and

mean cell haemoglobin (MCH) ( $p < 0.0001$ ) than the non-alcoholic group. Elevated MCV (macrocytosis) was found in 67% (38/57) of this group as opposed to 18% (16/87) of the non-alcoholic group, whereas MCH was elevated in 63% vs. 22%. The levels of peripheral blood platelets and leukocytes did not differ significantly between the groups. Erythrocyte folate ( $1261 \pm 750$  nmol/L and  $940 \pm 679$  nmol/L, normal value  $>300$  nmol/L) and serum B<sub>12</sub> levels ( $486 \pm 340$  pmol/L and  $350 \pm 336$  pmol/L, normal range 139-543 pmol/L) similarly did not differ significantly. Haematological parameters by gender are summarized in Table 2.

**Table 2. Characteristics of the series studied in paper I, by gender**

	n	Age (years)	Haemoglobin (g/L)	MCV (fL)	MCH (pg)	Platelets ( $\times 10^9$ /L)	Leukocytes ( $\times 10^9$ /L)	Reticulocytes (%)	Incidence of anaemia	
<b>All</b>	Alcohol abusers	57	55 $\pm$ 11	124 $\pm$ 24	98 $\pm$ 8	33 $\pm$ 3	214 $\pm$ 149	7.2 $\pm$ 5.0	1.5 $\pm$ 1.0	51% (29/57)
	Non-alcoholics	87	59 $\pm$ 15	112 $\pm$ 26	88 $\pm$ 12	30 $\pm$ 5	259 $\pm$ 144	8.0 $\pm$ 6.8	1.2 $\pm$ 1.0	69% (60/87)
	p Value		0.016	0.0065	< 0.0001	< 0.0001	0.074	0.51	0.045	0.036
<b>Males</b>	Alcohol abusers	51	55 $\pm$ 11	124 $\pm$ 25	98 $\pm$ 8	33 $\pm$ 3	210 $\pm$ 147	7.2 $\pm$ 5.2	1.4 $\pm$ 1.0	49% (25/51)
	Non-alcoholics	45	63 $\pm$ 15	110 $\pm$ 31	90 $\pm$ 13	31 $\pm$ 6	255 $\pm$ 177	7.7 $\pm$ 5.7	1.3 $\pm$ 1.2	71% (32/45)
	p Value		0.0004	0.017	0.0001	0.0034	0.18	0.61	0.17	0.038
<b>Females</b>	Alcohol abusers	6	55 $\pm$ 10	122 $\pm$ 14	100 $\pm$ 6	33 $\pm$ 2	244 $\pm$ 177	7.3 $\pm$ 2.9	2.0 $\pm$ 0.9	67% (4/6)
	Non-alcoholics	42	54 $\pm$ 14	114 $\pm$ 19	86 $\pm$ 10	29 $\pm$ 4	98 $\pm$ 15	8.3 $\pm$ 7.9	1.1 $\pm$ 1.0	67% (28/42)
	p Value		0.84	0.33	0.0025	0.022	0.43	0.85	0.049	1.0

Normal values:

Haemoglobin: 130-180 g/L (men) and 120-160 g/L (women); MCV: 76-96 fL; MCH: 27-32 pg; platelets: 150-400  $\times 10^9$  /L; leukocytes: 4.0-10.0  $\times 10^9$  /L; reticulocytes: 0.5-1.5%. Anaemia refers to a reduction in haemoglobin concentration below the normal

In addition to macrocytosis, high incidences of reticulocytosis (37%) and thrombocytopenia (41%) were seen in the alcoholic group (Table 3). When both groups were divided according to the presence or absence of anaemia, the incidences of macrocytosis and an elevated MCH were found to be significantly higher among the alcoholics regardless of the presence of anaemia, while platelet and leukocyte levels were found to differ between the alcohol abusers and non-alcoholics among the anaemic patients, but not among the non-anaemic ones. Erythrocyte folate content and serum vitamin B<sub>12</sub> levels did not show any significant differences between the groups.

**Table 3. Haematological findings in all the alcoholic patients (the alcoholic patients discussed in paper I), total and by gender**

Parameter	Range of values <sup>a</sup>	Incidence of observation		
		All	Males	Females
<b>Haemoglobin</b>	65–161 g/L	51%	49%	67%
Anaemia				
<b>MCV</b>	76–114 fL	67%	65%	83%
Macrocytosis				
Normocytosis				
Microcytosis				
<b>Reticulocytes</b>	0.3–4.6%	37%	35%	0%
Reticulocytosis				
Normal				
Reticulocytopenia				
<b>Platelets</b>	26–677 x 10 <sup>9</sup> /L	14%	12%	33%
Thrombocytosis				
Normal				
Thrombocytopenia				
<b>Leukocytes</b>	2.2–33.9 x 10 <sup>9</sup> /L	21%	22%	17%
Leukocytosis				
Normal				
Leukopenia				

<sup>a</sup> For normal values, see Table 2

The haemoglobin levels of 14 alcohol-dependent patients decreased significantly during a follow-up of 7-8 days of abstinence, while their red blood cell indices remained fairly constant. Blood platelets showed an increase during this period, although the change did not reach statistical significance.

## 5.2 Morphological and bone marrow abnormalities in alcoholics (I)

Bone marrow aspirates from the patients with hazardous drinking practices showed excessive vacuolization of pronormoblasts in 24% of cases and ring sideroblasts in 4%, while vacuolization of pronormoblasts was observed in only two (2%) of the non-alcoholic patients. The peripheral red blood cell

morphology of the alcoholic patients showed round (instead of oval) macrocytes, stomatocytes (41%) and knizocytes (33%), whereas the incidences of stomatocytosis and knizocytosis in the non-alcoholic group were only 12% and 4%, respectively. Excessive vacuolization of the cell periphery of megakaryocytes was observed in 20% of the alcoholic patients, all of whom had a history of recent intoxication, whereas this morphological feature was found in only one patient in the non-alcoholic group.

### 5.3 Acetaldehyde adducts in patients with macrocytosis (II)

The sample of 138 patients with macrocytosis undergoing bone marrow aspiration or biopsy included 68 patients (49%) with a history of excessive alcohol consumption, 28 (20%) of whom were suffering from severe dependence. Separation of the erythrocyte proteins by HPLC chromatography in the case of the alcoholics revealed the formation of fast-eluting haemoglobin fractions which reacted with antibodies against acetaldehyde adducts.

Bone marrow aspirates from the alcoholic patients showed vacuolization of pronormoblasts in 12 cases (18%) and abnormal sideroblasts in 8 (12%). Bone marrow samples from 11 consecutive alcoholic patients and 9 non-alcoholic controls were processed for immunocytochemical staining of acetaldehyde-modified epitopes, and 8/11 (73%) of the former showed positive staining was found in the late (non-nucleated) erythropoietic cells, whereas no specific staining was seen in the bone marrow cells of the control patients. The erythrocyte plasma membrane was found to be the primary site of adduct deposition. The early erythroblasts and other marrow cell lineages were usually devoid of specific staining. Blood smears prepared from the alcoholic patients with macrocytosis also contained stomatocytes and knizocytes, and immunocytochemical analyses of peripheral blood erythrocytes pointed to the occurrence of acetaldehyde-derived epitopes both on the cell membrane and inside the erythrocytes. Membrane adducts were present in all of the immunopositive alcoholic patients and intracellular adducts in 60%. The patients exhibiting positive staining in their bone marrow also had immunopositive erythrocytes in their circulation. The degree of macrocytosis in the alcohol-consuming patients showed a slight correlation with self-reported ethanol intake ( $r=0.26$ ,  $p<0.05$ ), but that in the alcoholics with elevated MCV was not found to correlate significantly with quantity of erythrocyte adducts ( $r=0.15$ ) as assessed using confocal laser scanning microscopy and digital image analysis of the staining data.

## 5.4 Immunogenic adducts of acetaldehyde and lipoproteins (III)

The antibodies raised against LDL, VLDL and BSA-acetaldehyde all reacted with protein adducts generated at physiologically relevant concentrations of acetaldehyde *in vitro*, whereas those raised against MDA-LDL, oxidized LDL and acetylated LDL were not found to cross-react with the acetaldehyde-derived adducts. In assays for acetaldehyde adducts in the erythrocyte proteins and serum proteins of alcoholic patients (n=32) and healthy controls (n=22), the antibody prepared against the acetaldehyde-VLDL condensate was found to provide the most effective detection of acetaldehyde adducts *in vivo*.

When the antibodies were compared in immunoassays for acetaldehyde adducts from erythrocyte proteins of 32 problem drinkers and 22 control individuals, the acetaldehyde adduct levels, as analyzed using the anti-VLDL-acetaldehyde adduct antibody, were significantly higher in the former. Anti-LDL adduct antibodies and anti-BSA adduct antibodies also revealed a trend towards higher adduct levels in the alcohol abusers, but the difference did not reach statistical significance.

Measurements with the anti-VLDL adduct antibody in serum samples similarly revealed significantly higher concentrations of immunoreactive adducts in the alcoholic group, and a parallel trend was observed using the anti-LDL adduct antibodies and anti-BSA adduct antibodies, but the differences were less striking.

## 5.5 Immune and cytokine responses in alcoholics (IV)

Titres of IgA, IgG and IgM antibodies against acetaldehyde-modified epitopes were studied in alcoholics with or without liver disease, in moderate drinkers and in abstainers. The series in this case comprised 86 male alcohol consumers and 20 male abstainers. The ALD patients showed the highest titres of IgA and IgG antibodies, and IgA titres were also significantly higher in the alcoholics without liver disease than in the moderate drinkers ( $p < 0.01$ ) or the abstainers ( $p < 0.001$ ), whereas IgG titres did not differ between these groups. IgM titres were most notably elevated in the alcoholics without liver disease. Interestingly, the values for the moderate drinkers were also higher than those for the abstainers ( $p < 0.05$ ).

The patients with ALD also showed markedly elevated values of pro-inflammatory cytokines (IL-2, IL-8 and TNF- $\alpha$ ), whereas in the patients without liver disease these cytokines (except for IL-8) remained at the levels of healthy controls. IL-6 was elevated in the alcoholics both with and without liver disease

as compared with the moderate drinkers or abstainers ( $p < 0.001$  for both comparisons), and the levels of the anti-inflammatory cytokine IL-10 also varied significantly between the groups, with all the alcohol consuming groups, including the moderate drinkers, showing higher values than the abstainers ( $p < 0.001$ ). By contrast, the levels of TGF- $\beta$ 1 showed less variation. Interestingly, relatively low values of this cytokine were observed in patients with ALD.

Seventeen ethanol-dependent patients underwent a supervised period of abstinence for  $8 \pm 2$  days, after which their anti-adduct IgA levels were significantly lower ( $p < 0.05$ ), whereas IgG and IgM levels did not change significantly. Significant changes in the levels of IL-6 (-47%), IL-10 (-82%) and TNF- $\alpha$  (-41%) were noted after this period of abstinence, whereas IL-2 ( $\pm 0\%$ ), IL-8 (-26%) and TGF- $\beta$ 1 (+3%) remained relatively stable. IL-6 levels nevertheless remained higher after abstinence than they were in the reference groups ( $p < 0.05$  vs. moderate drinkers;  $p < 0.001$  vs. abstainers).

## 6 Discussion

### 6.1 Haematological findings in patients with excessive ethanol intake

Excessive ethanol consumption was found to be common (40%) in these general hospital patients referred for bone marrow examinations on account of occult abnormalities in peripheral blood cell counts or red cell indices. The data suggest diverse patterns of haematological effects in alcohol abusers, with macrocytosis, reticulocytosis, thrombocytopenia, leukopenia and combined cytopenias standing out as frequent findings. The high prevalence of problem drinking in such patients suggests the importance of the systematic inclusion of a history of ethanol consumption in their clinical assessment. These findings also suggest that chronic alcohol use is an underdiagnosed cause of cytopenia.

#### *6.1.1 Anaemia in alcoholics*

Anaemia was found in approximately 50% of the alcohol abusers and was characteristically normocytic or macrocytic, whereas there were no patients with detectable numbers of hypochromic microcytes (even in cases with iron deficiency). Macrocytic anaemia has been reported to be common in patients with ALD, who may also suffer from folate deficiency and megaloblastic alterations in bone marrow, or acute blood losses (Savage and Lindenbaum 1986, Maruyama et al. 2001). In the present series, however, from which patients with severe liver injury had been excluded, erythrocyte folate and serum vitamin B<sub>12</sub> levels did not differ between the anaemic and non-anaemic alcoholics. It should also be noted that the present findings apply to a restricted group of patients, so that the findings regarding the incidence of anaemia cannot be extended to general populations of alcohol abusers or non-alcoholics.

#### *6.1.2 Alcoholic macrocytosis (I, II)*

Ethanol consumption seems to be an increasingly common cause of macrocytosis in general hospitals (Wu et al. 1974, Morgan et al. 1981, Savage et al. 2000), but most patients of this kind tend to escape detection. Recent reviews of adult outpatients focusing on macrocytosis have shown that 65% of such

patients may have an underlying alcohol problem (Seppä et al. 1991, 1996, Savage et al. 2000). Elevated MCV and MCH appear to be characteristic of alcohol abusers both in the presence and absence of anaemia. Elevated MCV has frequently been used as part of the screening procedure for detecting alcohol abuse, despite the fact that it has a relatively low sensitivity for this purpose (Wu et al. 1974, Chalmers et al. 1980, Thiele and Thiele 1987). It responds slowly to abstinence and it may require 2 to 4 month to normalize (Niemelä 2002). It has also been shown that red blood cell size may correlate with the amount of reported drinking more closely than other conventional biochemical markers of ethanol consumption such as GGT (Anttila et al. 2004). In accordance with previous observations (Morgan et al. 1981), macrocytosis appears to be especially common in women. Thus men and women may show different susceptibilities in developing haematological aberrations in response to ethanol intake. It should be noted, however, that the number of female patients in the present series was small and future research will be necessary to address this issue.

### *6.1.3 Changes in blood platelets*

When anaemia was present, alcohol abusers had concurrent abnormalities in leukocyte or platelet levels more commonly than non-alcoholic patients. A low platelet count (thrombocytopenia) is a common abnormality after heavy ethanol intake (Levine et al. 1986, Lindenbaum 1987, Michot and Gut 1987, Niemelä 2002), implying that ethanol is able to suppress platelet production, since its ingestion may lead to thrombocytopenia both in chronic alcoholics (Nakao et al. 1991) and after experimental ethanol administration (Cooper et al. 1984, Levine et al. 1986). Thrombocytopenia was seen in approximately 40% of the present alcohol-abusing patients, whereas thrombocytosis occurred only in the anaemic alcoholics, with a prevalence of 28%. Interestingly, the anaemic patients also showed granulocytopenia significantly more often, which may suggest generalized marrow suppression in heavy drinkers. It is possible that close interactions may exist between the different bone marrow cell lines in their response to ethanol toxicity. Thrombopoietin and erythropoietin serum profiles have been shown previously to be closely associated in alcoholics (Schmitt et al. 1999). Thrombocytopenia may also be a result of cytotoxic ethanol effects, as supported by the present findings on the occurrence of excessive megakaryocyte vacuolization in patients with recent heavy drinking, being found typically in the cell periphery, in close proximity to the plasma membrane. Such vacuoles can be expected to disappear with abstinence (Lindenbaum 1987). When ethanol is withdrawn, platelet counts return from low levels to either normal or supernormal levels (rebound thrombocytosis) in 1-3 weeks. Thrombocytosis may also occur following withdrawal of alcohol in patients who are not thrombocytopenic at the time of admission.

#### 6.1.4 Ethanol-induced effects on blood cell morphology and bone marrow

Morphological examinations of blood films typically revealed round rather than oval macrocytes in the case of alcohol abusers, the oval form being in turn more characteristic of megaloblastic alterations. The erythrocyte morphology also revealed excessive stomatocytosis and knizocytosis. Alcohol abuse is known to alter the structural order of lipids in human erythrocyte membranes (Beauge et al. 1988), and there may also be a direct cytotoxic effect of ethanol on erythropoiesis. This concept is supported by current observation of erythroblast vacuolization in the bone marrow of a significant percentage of the alcohol-abusing patients, and by previous evidence showing that abstinence after heavy alcohol intake is usually followed by reticulocytosis (Schmitt et al. 1999). Such vacuoles usually disappear with abstinence (Lindenbaum 1987, Michot and Gut 1987).

Where pathological ring sideroblasts in bone marrow have been previously reported in 25-30% of anaemic alcoholic patients, we found a markedly lower incidence (<10%), suggesting that other factors such as malnutrition or liver disease may also contribute to the generation of these sideroblastic changes in alcoholics (Savage and Lindenbaum 1986, Lindenbaum 1987, Maruyama et al. 2001).

## 6.2 Role of acetaldehyde in haematotoxicity

The current data suggest a direct role for acetaldehyde in creating haematotoxicity, as evidenced by the presence of acetaldehyde-derived structural modifications in erythrocyte constituents in the heavily drinking subjects. Although the measurement of acetaldehyde in erythrocytes is known to be problematic (Fukunaga et al. 1993), high acetaldehyde concentrations have been found previously in chronic alcoholics (Hernández-Muñoz et al. 1992). Acetaldehyde has also been shown to form stable adducts with erythroid proteins and cellular constituents *in vitro* (Gaines and Sorrell 1979, Stevens et al. 1981, Magnani et al. 1989) and *in vivo* (Stevens et al. 1981, Israel et al. 1986, Peterson et al. 1988, Lin et al. 1995b). The occurrence of acetaldehyde-derived epitopes in peripheral blood erythrocytes and in their bone marrow precursors supports the notion of a pivotal role for acetaldehyde in producing haematological derangements (Meagher et al. 1982, Levine et al. 1986, Lindenbaum 1987, Michot and Gut 1987). Suppression of haematopoietic progenitor cell proliferation by ethanol and acetaldehyde has also been reported previously (Meagher et al. 1982, Levine et al. 1986).

Wickramasinghe and Malik (1986) reported that even relatively low concentrations of acetaldehyde, but not of ethanol, can cause an impairment of cell proliferation and an abnormality in cell growth in cultures of human cell lines (Raji, MOLT-4, WI-L2 and K562), supporting a specific role for acetaldehyde in increasing the mean cell volume of erythrocytes. Such erythrocytes may be more vulnerable to damage and haemolysis, and may have a shortened biological half-life *in vivo*. This view is also supported by the results of the present follow-up study, showing a decrease in blood haemoglobin levels during one week of supervised abstinence after heavy drinking. The other haematological parameters were not found to change substantially during this period.

Acetaldehyde-derived erythrocyte modifications in the bone marrow appear to be most abundant at the late stages in erythrocyte development. No apparent correlations exist, however, between marrow positivity and either self-reported ethanol intake or the degree of macrocytosis. Although the reasons why the marrow specimens from some alcoholic patients stained positively and others did not are unclear, it may be speculated that factors such as the duration of abstinence prior to sampling, the amount of alcohol ingested and different individual susceptibilities may all play a role. Previous *in vitro* studies have shown that ethanol and acetaldehyde inhibit colony formation by committed stem cells, with the erythroid cells being particularly vulnerable (Meagher et al. 1982). Interestingly, we observed that the early blastic cells were usually devoid of specific staining for acetaldehyde adducts, suggesting that adducts are not required for erythroblast vacuolization.

### *6.2.1 Possible implications of acetaldehyde adducts in blood cells*

Based on the present findings, future work is warranted to examine the possibility that immunological and chromatographic assays for acetaldehyde-derived protein modifications in peripheral blood erythrocytes and bone marrow could be used for the differential diagnosis of alcohol abuse as an underlying cause of haematological symptoms. Such assays should perhaps be targeted at erythrocyte membrane proteins or haemoglobin fractions enriched by chromatographic techniques rather than total haemoglobin (Stevens et al. 1981, Niemelä and Israel 1992, Sillanaukee et al. 1992, Hazelett et al. 1998, Sarkola et al. 2000, Takeshita and Morimoto 2000). Since thrombopoiesis is also clearly affected by ethanol, future studies should investigate the possibility that acetaldehyde adducts may also be present in platelet protein lysates and that acetaldehyde could play a role in the effects of ethanol on platelet metabolism.

## 6.3 Acetaldehyde-lipoprotein condensates

It has previously been established that aldehyde-protein adducts readily induce immunological responses (Israel et al. 1986, Worrall et al. 1991, Viitala et al. 1997). The present data indicate that immunogenic acetaldehyde-lipoprotein adducts are formed *in vivo*. Acetaldehyde-derived adducts in VLDL, possibly involving apoB protein, may be particularly powerful immunogens. Ethylation of apoB-lysine residues in VLDL appears to share the immunological determinants of the adducts generated with proteins *in vivo*. In the liver, where modification of VLDL is expected to take place upon ethanol consumption and the generation of acetaldehyde, the VLDL particles are large and homogeneous, and may contain modified proteins at a higher concentration than the smaller particles found in the circulation.

The ability of acetaldehyde to bind to lipoproteins has also been documented previously (Steinbrecher et al. 1984, Savolainen et al. 1987, Wehr et al. 1993), and previous evidence indicates that such binding also appears to occur in alcohol abusers *in vivo*, creates immunological responses (Wehr et al. 1993), activates apolipoprotein E synthesis in macrophages, and promotes atherogenesis (Lin et al. 1995a).

### 6.3.1 Measurements of lipoprotein adducts

The immunological measurement of adducts in circulating proteins is based on the finding that the antibodies generated against the aldehyde-derived epitopes recognize specific protein adducts independently of the nature of the carrier protein (Steinbrecher et al. 1984, Israel et al. 1986). Antibodies against acetaldehyde-protein condensates have previously been generated using BSA (Niemelä and Israel 1992) or keyhole limpet haemocyanin (Lin et al. 1993b) as the carrier protein, and previous studies have reported high acetaldehyde adduct levels in erythrocytes of chronic alcoholics (Gross et al. 1992, Niemelä and Israel 1992, Sillanaukee et al. 1992, Lin et al. 1993a, Hurme et al. 1998). Some previous studies using relatively cumbersome or indirect techniques have also reported the existence of acetaldehyde adducts in plasma proteins (Wickramasinghe et al. 1986, Peterson and Polizzi 1987, Lin et al. 1990, Chen et al. 1995). Acetaldehyde-modified epitopes have been found on the surface of hepatocytes by flow-cytometry (Trudell et al. 1990, Lin et al. 1992) and in liver tissue in cases of ALD (Niemelä et al. 1991b, Holstege et al. 1994, Niemelä et al. 1994), but examinations using immunological methods have usually failed to detect acetaldehyde-modified proteins in serum. By contrast, the present work demonstrates that VLDL-acetaldehyde antibodies react with adducts in both the erythrocyte and serum compartments in alcoholic patients. It should be noted, however, that the results concern primarily male alcoholics, and it remains to be

established whether the findings are similar in women with excessive ethanol intake.

### *6.3.2 Other types of adducts*

The present data indicate that although MDA, an aldehydic product of lipid peroxidation, also forms Schiff base adducts with proteins, the immunogenic epitopes generated by acetaldehyde and MDA do not significantly cross-react with each other. Acetaldehyde adducts and MDA adducts may coexist in alcoholics, however (French et al. 1993, Niemelä et al. 1995, Xu et al. 1998, Viitala et al. 2000), especially when ethanol consumption is combined with a high-fat diet or iron overload (Tsukamoto et al. 1995, Niemelä et al. 1998). Oxidatively modified proteins have previously been demonstrated in the arterial vessel walls of atherosclerotic lesions (Haberland et al. 1988, Palinski et al. 1989, Steinberg et al. 1989). As yet, no attempt has been made to examine the existence of circulating MDA-modified epitopes in erythrocytes or in serum proteins from alcoholics, although antibodies against oxidative modifications have been detected in the serum of alcoholics with severe liver disease (Viitala et al. 2000, Stewart et al. 2004).

## 6.4 Immune and cytokine responses in alcoholics

The generation of immune responses directed against acetaldehyde-modified epitopes in proteins appears to occur early in the sequence of events leading from excessive alcohol consumption to clinical signs of ALD. IgM-isotype antibodies, the predominant component of early immune responses, and IgA antibodies were both higher in the alcohol consumers without any evidence of liver disease than in the abstainers.

Previous studies of patients with ALD have demonstrated a generalized increase in circulating IgA concentrations together with increased tissue IgA deposition (Drew et al. 1984, Johnson and Williams 1986, van de Wiel et al. 1987, 1988, Brown and Kloppel 1989, Tuma and Klassen 1992). These changes may result from decreased catabolism of IgA by the injured hepatocytes, or from decreased excretion of IgA by the damaged biliary endothelium. The present results indicate that the generation of anti-adduct IgA in alcoholics is antigen-driven, and that the titres become elevated before any generalized increase in serum total IgA levels (data not shown). A weak correlation has previously been shown between anti-adduct IgA levels and serum total IgA levels in patients with ALD, suggesting that the increase in IgA levels with progressing liver disease may reflect multiple mechanisms of IgA turnover (Viitala et al. 1997). Non-

drinking patients with IgA-myeloma and high IgA levels do not show elevated anti-adduct IgA levels, however, suggesting that the ethanol-induced differences in charge and optical and steric conformation between neo-antigens are ethanol-specific (Viitala et al. 1997).

It is possible that IgA antibodies in alcoholic patients may result from intestinally induced B-cell responses, since the epithelial tissues of alcohol abusers are continuously exposed to ethanol. It is significant that the antibody levels also show a close correlation with the actual amounts of ethanol ingested recently. Mucosal immunity is highly adaptable to the antigenic load of its environment, and the formation of IgA antibodies may readily be triggered by intestinal neo-antigens resulting from the presence of ethanol (Kerr 1990, Kunkel et al. 2003). The gastrointestinal tract is also rich in enzymes capable of metabolizing ethanol to acetaldehyde *in vivo* (Seitz et al. 1994, Salaspuro 1996, Visapää 1998). A significant association between intestinal ethanol metabolism and liver damage has also been suggested previously. Endotoxaemia is common in ALD patients, who also have IgA antibodies to components of endotoxin (Nolan et al. 1986) and to human gut luminal aspirates (Douds et al. 1998). Intestinally-induced B lymphocyte responses could lead to the generation of antibodies against several enteric antigens that bypass the liver and reach the antibody-forming organs. However, IgA antibodies against endotoxin-derived components have been reported in patients with ALD but not in alcohol consumers without liver injury (Seitz et al. 1994). A continuous antigen load in patients with ALD and high endotoxin levels may enhance the mucosal response to gut-derived antigens, while IgA absorption may also increase as a result of mucosal injury.

The present observations on the associations between ethanol consumption, antibody profiles, IL-6 levels and IL-10 levels may indicate that the early responses that precede liver disease may 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 the exclusion and neutralization of antigens resulting from acetaldehyde modification of proteins and cellular constituents (Koskinas et al. 1992, Klassen et al. 1995, Niemelä 2001). In line with this view, IgA antibodies and IL-6 levels showed parallel changes during the follow-up of alcoholics undergoing a period of abstinence. IL-6 levels have also been shown previously to respond readily to therapeutic lifestyle modifications and vitamin E interventions in non-alcoholic steatohepatitis (NASH) (Kugelmas et al. 2003). IL-6 release into bile occurs with the same time course as transcellular transport of IgA in the liver (Sonne et al. 1990). A relatively high concentration of IgA after ethanol consumption may also be found in Kupffer cells, and under conditions where Kupffer cell phagocytosis is impaired, there may be increased production of IgG immunoglobulins (Souhami et al. 1975). The generation of IgA immune complexes may be damaging upon

excessive antigenic stimulation, however, 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 (Amore et al. 1994). IgG antibodies, which were observed in the present patients with liver disease, are known to be mediators of several immunopathogenic consequences including complement activation and the induction of cytotoxic reactions. The occurrence of specific T-cell responses to adducted proteins has also been described recently in patients with advanced ALD (Stewart et al. 2004).

The present findings may further indicate that a disturbed balance between ethanol-derived antigen loading and immunological protection mechanisms could play a role in the progression of liver disease in alcoholics. Patients with ALD appear to have a skewed balance in the ratios of pro-inflammatory and anti-inflammatory cytokines. The IL-6 and IL-10 cytokines were found to show early changes, reflecting the current drinking status. IL-6 is a multifunctional cytokine that has previously been implicated in the hepatic acute phase response (Khoruts et al. 1991) and the control of immunoglobulin production (Deviere et al. 1992), but recent evidence has shown that it is also required for normal liver regeneration after injury or partial hepatectomy and that it may provide hepatoprotection by conferring resistance to injury (Taub 2003, Zimmers et al. 2003). IL-6 has been shown previously to inhibit TNF- $\alpha$  production and reduce antigen presentation (Fiorentino et al. 1989, Moore et al. 1990). IL-10 has recently been implicated as having antifibrogenic effects, because IL-10 $^{-/-}$  mice have a tendency for liver fibrogenesis as a result of enhanced inflammation or direct effects on matrix regulation (Louis et al. 1998). Mechanical tissue injury in mice can cause a rapid induction of IL-10 mRNA and promotion of Th2 responses to antigens (Laouini et al. 2003), and IL-6 and IL-10 are both Th2 IgA stimulating cytokines (Wu et al. 1999). Interestingly, although TGF- $\beta$ 1 expression has been previously associated with the development of fibrosis in alcoholics (Chen et al. 2002), the present data show low levels of circulating TGF- $\beta$ 1 in patients with ALD. The pro-inflammatory cytokines IL-8, IL-2 and TNF- $\alpha$  were found to be far 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 survival of cultured human hepatocytes (Joshi-Barve et al. 2003). The levels of IL-2 (influencing the growth and differentiation of Th1-cells) and TNF- $\alpha$  (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 (Yin et al. 1999). Several authors have recently emphasized the importance of increased TNF- $\alpha$  production and Kupffer cell activation as key responses in hepatic inflammation (Iimuro et al. 1997, Yin et al. 1999), and it has been shown in ethanol-exposed rats (Iimuro et al. 1997) and in ob/ob mice, a model for non-alcoholic fatty liver disease (Li et al. 2003), that

treatment with inhibitors of TNF- $\alpha$  may reduce hepatic inflammation and necrosis.

We chose here to study male patients only because immunological responses *in vivo* may show a significant gender dependence (Makkonen et al. 2001). Men and women are known to have markedly different incidences of a variety of autoimmune disorders. Sex steroid hormones are thought to play a role in the regulation of immune responses, although the specific mechanisms have remained obscure. Estradiol, for instance, inhibits the suppressive activity of a subset of T-lymphocytes bearing Fc receptors for immunoglobulin G (Evagelatou and Farrant 1994, Makkonen et al. 2001).

## 7 Conclusions

- I Excessive ethanol consumption was found to affect several cell lines of haematopoiesis. Macrocytosis, reticulocytosis, thrombocytopenia, leukopenia, and combined cytopenias are all common in alcoholics as compared with non-alcoholic controls. Information on ethanol consumption should therefore be systematically included in the clinical assessment of patients with cytopenias.
- II Acetaldehyde-erythrocyte adducts are formed *in vivo* in the blood and bone marrow of patients with excessive alcohol consumption. These adducts may contribute to the generation of the erythrocyte abnormalities that are frequently observed in alcoholic patients.
- III Acetaldehyde generates immunogenic adducts with lipoproteins *in vivo*. Antibodies raised against acetaldehyde-VLDL adducts may provide a basis for a new diagnostic assay to assess excessive alcohol consumption.
- IV The formation of IgA antibodies directed against acetaldehyde-modified epitopes in proteins is an early event in patients with excessive alcohol consumption. These antibodies may result from intestinally induced B-cell responses and show a close association with the actual amount of ethanol ingested recently. Immune responses to the antigens derived from ethanol metabolism, together with the activation of pro-inflammatory and anti-inflammatory cytokines, may be important in creating tissue injury in alcoholic patients.

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