CARBOHYDRATE-DEFICIENT TRANSFERRIN (CDT) AND SERUM ANTIBODIES AGAINST ACETALDEHYDE ADDUCTS AS MARKERS OF ALCOHOL ABUSE

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ABSTRACT

In the search for more reliable blood markers for excessive alcohol consumption, considerable effort has been devoted to measurements of carbohydrate-deficient transferrin (CDT), which increases in body fluids as a result of prolonged alcohol intake. In the present work, three CDT methods, CDTect (Pharmacia & Upjohn), %CDT radioimmunoassay (%CDT RIA) by Axis (Oslo, Norway), and Axis %CDT turbidimetric immunoassay (%CDT TIA) were examined for their diagnostic performance in cases of alcohol abuse with or without liver disease.

The diagnostic performance of CDT as a marker of alcohol abuse correlates positively with alcohol consumption. As compared with g-glutamyltransferase (GGT) and mean corpuscular volume of erythrocytes (MCV), which are conventionally used as laboratory markers of excessive ethanol consumption, CDT (CDTect) has the highest sensitivity (64%) at the specificity level of 100% in heavy drinkers consuming >100 g ethanol/day, but its sensitivity decreases to 34% in cases with an alcohol intake of <100 g/day, which hampers the use of CDT as a community screening method.

Patients with alcoholic liver disease (ALD) have significantly higher CDT values than alcoholics with non-liver pathology. However, CDT is primarily increased in cases with an early stage of ALD, so that there is a weak negative correlation between CDT and disease severity, which may prove to be of diagnostic value.

Especially in men, CDTect seems to achieve greater sensitivity than %CDT RIA or %CDT TIA for detecting recent alcohol abuse among heavy drinkers, but it does have a significant correlation with serum transferrin, especially in individuals reporting social drinking or no alcohol intake. This should be considered when interpreting the assay results in patients with increased serum transferrin. %CDT methods achieve greater specificity than CDTect when analyzing samples from patients with high serum transferrin concentrations.

Acetaldehyde-protein adducts are formed in the body after excessive ethanol intake, and their formation triggers antibody production, which may contribute to some forms of tissue damage seen in alcohol abusers. To obtain more information on the association between serum antibodies against acetaldehyde adducts, ALD and alcohol consumption, assays for antibodies against albumin and haemoglobin adducts were performed.

Antibodies of the immunoglobulin (Ig) isotypes A, G, and M against acetaldehyde-adducts are formed in patients with prolonged heavy alcohol consumption. IgA titres in ALD patients are significantly higher than those found in patients with non-alcoholic liver disease, non-drinking controls, or heavy drinkers with no signs of liver disease. Anti-adduct IgG titres, in turn, are increased both in ALD and in heavy drinkers with no signs of liver disease as compared with non-alcoholic liver disease patients or non-drinking controls. It appears that anti-adduct IgA, IgG and IgM titres in ALD patients correlate with the severity of the liver disease. Although this association is a limitation for the usefulness of these antibodies as markers of alcohol abuse, it may serve as a basis for the differential diagnosis of alcohol-induced liver disease.

Keywords: Liver disease, transferrin, ethanol metabolism, immunoglobulins.

I dedicate this thesis to my family

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Katja Viitala

Abbreviations

Ach	Acetaldehyde
ADH	Alcohol dehydrogenase
ALB	Albumin
ALD	Alcoholic liver disease
ALDH	Aldehyde dehydrogenase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ASGP	Asialoglycoproteins
AST	Aspartate aminotransferase
BIL	Bilirubin
CCLI	Combined clinical and laboratory index
CDT	Carbohydrate-deficient transferrin
%CDT	Amount of CDT expressed as a percentage of total transferrin
CMI	Combined morphological index
*CYP*2E1	Cytochrome P450 2E1
CV	Coefficient of variation
d	Day
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
GGT	γ-Glutamyltransferase
Hb	Haemoglobin
Hex	β-Hexosaminidase
HNE	4-Hydroxynonenal
HPLC	High performance liquid chromatography
IB	Immunoblotting
IDL	Intermediate density lipoprotein
IEF	Isoelectric focusing
Ig	Immunoglobulin
IL	Interleukin
kD	Kilodalton
LD	Laser densitometry
LDL	Low density lipoprotein
MAEC	Minicolumn anion-exchange chromatography
mAST	Mitochondrial AST
MCV	Mean corpuscular volume (of erythrocytes)
MDA	Malondialdehyde
MEOS	Microsomal ethanol oxidizing system

NALD	Non-alcoholic liver disease
NMR	Nuclear magnetic resonance spectroscopy
O.D.	Optical density
PBC	Primary biliary cirrhosis
PBS	Phosphate-buffered saline
pI	Isoelectric point
PICP	Carboxyterminal propeptide of type I collagen
PIIINP	Aminoterminal propeptide of type III collagen
RIA	Radioimmunoassay
r_S	Correlation coefficient for Spearman's rank-correlation test
ROC	Receiver-operating characteristic
SA	Semi-automatic
SD	Standard deviation
SE	Standard error of the mean
sMAST	Short Michigan Alcoholism Screening test
TfB	Transferrin phenotype B
TfD	Transferrin phenotype D
TIA	Turbidimetric immunoassay
TIV	Type IV collagen
TNF	Tumour necrosis factor
U	Unit
WB	Western blotting
VLDL	Very low density lipoprotein

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Niemelä O, Sorvajärvi K, Blake JE & Israel Y (1995) CDT as a marker of alcohol abuse: relationship to alcohol consumption, severity of liver disease and fibrogenesis. Alcohol Clin Exp Res 19: 1203–1208.

II Sorvajärvi K, Blake JE, Israel Y & Niemelä O (1996) Sensitivity and specificity of CDT as a marker of alcohol abuse is significantly influenced by alterations in serum transferrin: comparison of two methods. Alcohol Clin Exp Res 20: 449–454.

III Viitala K, Lähdesmäki K & Niemelä O (1998) Comparison of the Axis %CDT TIA and the CDTect method as laboratory tests of alcohol abuse. Clin Chem 44: 1209–1215.

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

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1. Introduction

Ethanol abuse is a world-wide problem causing social, physical and mental injuries to the abusers themselves and their families. The monetary contributions needed to cover the expenses arising from these defects are immeasurable and not all the injuries can be cured. Thus, early intervention or prevention of alcohol abuse is extremely important to avoid profound hazards. Traditionally, prolonged excessive alcohol abuse is diagnosed on the basis of a clinical history, questionnaires concerned with alcohol consumption, and various laboratory tests, e.g. γ-glutamyltransferase (GGT), total or mitochondrial aspartate aminotransferase (AST or mAST, respectively), alanine aminotransferase (ALT) or mean corpuscular volume of erythrocytes (MCV) (for reviews, see Stibler 1991, Rosman & Lieber 1994, Sillanaukee 1996).

Since potentially harmful alcohol consumption is often concealed and questionnaires give very subjective and unreliable responses (Poikolainen 1985), laboratory markers have an important role in the diagnosis of alcohol abuse. However, all the conventional markers available are either indicators of disease in a particular organ, with poor specificity for the various aetiological possibilities, or else they have poor sensitivity for detecting alcohol consumption before the stage of organic complications (for reviews, see Stibler 1991, Rosman & Lieber 1994). Blood ethanol concentration is a specific marker of alcohol consumption, but the short half-life of ethanol limits its use (for review, see Salaspuro 1986). Thus, new sensitive diagnostic tools are needed (Walsh *et al.* 1991, Irwin *et al.* 1988, Crabb 1990, Watson *et al.* 1986, Conigrave *et al.* 1993). Ideally, markers of alcohol abuse should be specifically related to the presence or metabolism of ethanol and the amount of ethanol consumed, they should be sensitive to excessive ethanol consumption, they should not be affected by short periods of abstinence, and their kinetics during abstinence should be defined (Stibler 1991, Niemelä 1993).

The main interest in the present work is focused on the characteristics of carbohydrate-deficient transferrin (CDT), one of the most promising markers of alcohol abuse available today, and the acetaldehyde-protein adducts, which may offer a basis for several useful future applications to the diagnosis of alcohol abuse and alcohol-related organ damage.

2. Review of the literature

2.1. Definition of alcohol abuse and alcoholism

The risk of medical problems due to alcohol abuse is related to the amount of alcohol consumed. Most people are able to limit their intake to amounts that produce no serious health or social consequences. Some people remain teetotalers, who drink no alcohol, and other are moderate drinkers, who are able to control their drinking and whose alcohol consumption is so low that no health problems are to be expected. People who either drink large amounts on rare occasions or consume moderate amounts frequently are termed heavy drinkers. Health problems are known to arise at levels corresponding to 50–60 g of daily alcohol consumption. (Sanchez-Graig & Israel 1985, Niemelä 1998). Thus it has been recommended that men should consume no more than 4 drinks/day and 16 drinks/week, and women no more than 3 drinks/day and 12 drinks/week at maximum (Sanchez-Graig *et al.* 1995). It is, however, very difficult to define any exact dividing line between moderate and heavy drinking. This is apparently an underlying reason for the diversity of the cut-off values above which drinking has been considered harmful. (National Council on Alcoholism 1972, Nilssen *et al.* 1992, for a review, see Werner 1996). Alcohol abuse refers to heavy drinking that results in health consequences, social problems or both, and patients of this kind suffer from mental or physical complications brought on by alcohol even though the criteria for alcoholism may not have been fulfilled. Alcoholism is the most severe problem related to alcohol consumption, a disease in which severe dependence and increased tolerance has been developed and withdrawal symptoms appear after drinking has stopped. Blood or breath alcohol exceeding 1.5‰ (35 mmol/l) without obvious evidence of intoxication or 3‰ (70 mmol/l) at any time are the first-level criteria for alcoholism. (National Council on Alcoholism 1972, Niemelä 1998).

2.2. Alcoholic liver disease and laboratory markers

The liver is the primary site of alcohol metabolism, and therefore it is also vulnerable to the harmful effects of excessive alcohol intake. The spectrum of alcoholic liver disease (ALD) includes fatty liver, alcoholic hepatitis, fibrosis and cirrhosis. These lesions usually

develop sequentially, although they may coexist in any combination. Alcoholic fatty liver can be detected after only two days of excess alcohol intake and is usually a fully reversible lesion, whereas alcoholic hepatitis is an acute necrotizing lesion. Fibrosis may be an early feature of ALD, showing a pericellular distribution. With continuing hepatic inflammation, progressive fibrosis and scarring can occur. (For a review, see Rubin & Farber 1994). Perivenular fibrosis at the fatty liver stage is likely to progress to more severe stages of alcoholic liver disease if the patient continues to consume alcohol (Worner & Lieber 1985). In about 15% of alcoholics, hepatocellular necrosis, fibrosis and regeneration eventually lead to the formation of fibrous septa surrounding the hepatocellular nodules, which are characteristic of cirrhosis. Cirrhosis is a condition involving the entire liver, in which the parenchyma is changed into a large number of nodules separated from one another by sheets of fibrous tissue. Consequently, there are also systemic effects of altered metabolism, changes in hormone levels, protein abnormalities and defective coagulation. (For reviews, see Rubin & Farber 1994, Niemelä 1998). Cirrhosis of the liver (usually as a complication of alcoholism), is the fourth most frequent cause of death in urban populations between 25 to 64 years of age (for a review, see Lieber *et al.* 1993).

The laboratory tests most frequently used to confirm a suspicion of alcoholic liver disease include serum AST and ALT, bilirubin (BIL), alkaline phosphatase (ALP) and GGT, but these are of limited diagnostic utility for predicting the histological stage of the disease. A chronic increase in serum GGT or AST activity may suggest cirrhosis, but it may be difficult to rule out other possible causes such as recent heavy drinking or coexistent viral hepatitis. BIL, albumin (ALB) and prothrombin time are of prognostic value in cases of severe liver damage. An important target of the laboratory markers is to exclude non-alcoholic causes in cases where signs of liver disease are present. However, it may be difficult to distinguish between alcoholic liver disease and non-alcoholic conditions such as drug-induced liver disease, viral liver disease, haemochromatosis, Wilson's disease, autoimmune hepatitis, primary biliary cirrhosis, or liver disease associated with α_1-antitrypsin deficiency (for a review, see Rosman & Lieber 1994).

The progress of fibrogenesis can be studied by means of serum markers associated with connective tissue metabolism (Orrego *et al.* 1987, Annoni *et al.* 1989, Niemelä *et al.* 1992, Nouchi *et al.* 1987; for reviews, see Risteli *et al.* 1995, Niemelä 1998). The majority of the collagen in the liver is of type I or type III, although types IV, V and VI can also be found (Seyer *et al.* 1977, Rojkind *et al.* 1979; for reviews, see Risteli & Risteli 1995, Niemelä 1996). Several connective tissue markers, e.g. the aminoterminal propeptide of type III collagen (PIIINP), type IV collagen (TIV), and markers of basement membrane formation such as laminin appear to correlate significantly with the severity of liver disease (Niemelä *et al.* 1990a, González-Reimers *et al.* 1990), with the histological severity of alcoholic hepatitis (Niemelä *et al.* 1990a, González-Reimers *et al.* 1990, Annoni *et al.* 1989, Bell *et al.* 1989, Ramadori *et al.* 1991), and with alcohol consumption (Niemelä *et al.* 1990a).

As noted, laboratory tests are also useful as prognostic indicators. Monitoring of the effectiveness of treatment for alcoholic liver disease involves the use of variables that are of prognostic significance and are unaffected in unspecific ways by the treatment. Histological variables, although important for defining the characteristics of the sample, entail several practical problems. (For reviews see Blake and Orrego 1991, Niemelä 1998).

Of the histological variables, necrosis, Mallory's hyalin and inflammation are significantly related to the mortality risk. (Orrego *et al.* 1987, Niemelä 1998). Clinical and laboratory variables, like prothrombin time, BIL, and ALB are most effective when used in combinations, e.g. global indices such as the Combined Clinical and Laboratory Index, the Child-Turcotte-Pugh Index, or the Cox proportional hazards model. Prognostic indicators can be used in individual cases not only to assess recovery or deterioration but also to assign treatment modalities. (For reviews, see Rosman & Lieber 1994, Blake & Orrego 1991, Niemelä 1998).

2.3. Carbohydrate-deficient transferrin

CDT is at present one of the most promising and most intensively investigated markers of alcohol abuse (for reviews, see Stibler 1991, Rosman & Lieber 1994, Anton & Moak 1994, Allen *et al.* 1994). The transferrins are monomeric, iron binding glycoproteins which are synthesized in the liver. They are found in the biological fluids of both invertebrates and vertebrates. Transferrin normally shows microheterogeneity both in its amino acid composition and in its iron and carbohydrate content. Variation in the primary structure of the transferrin polypeptide is seen in the rare phenotypes designated TfB and TfD. TfB has a lower isoelectric point (pI) than TfC, the most common phenotype in all human populations, and TfD a higher one. There are also subtypes of these phenotypes. Genetic polymorphism may lead to different iron binding capacities for transferrin and may possibly influence other functions as well. (For a review, see de Jong & van Eijk 1988). The normal main isoform of transferrin has a pI of 5.4 and four terminal sialic acid residues, two in each of the bifurcated chains consisting of varying amounts of four carbohydrates: N-acetylglucosamine, mannose, galactose and sialic acid. The usual minor isoforms with higher pI values are tri- and disialotransferrins and those with lower pI values penta- and hexasialoproteins. (For a review, see Stibler 1991). It was observed by Stibler & Kjellin as early as 1976 that transferrin has abnormal microheterogeneity in alcoholics. Later it turned out that the difference was associated with defects in the carbohydrate content of the protein. Transferrin fractions with disialylated (pI 5.7), monosialylated (pI 5.8) and asialylated (pI 5.9) carbohydrate chains were found to be present in the serum of alcohol abusing patients, and their transferrin was also observed to lack neutral carbohydrates. (Stibler *et al.* 1979, Stibler & Borg 1981, Stibler & Borg 1986, Stibler *et al.* 1986, Jeppsson *et al.* 1993, Landberg *et al.* 1995). Recently the proportion of the trisialylated fraction of transferrin (pI 5.6) has been reported to be increased in patients with excessive ethanol intake (Heggli *et al.* 1996), although this is in contrast with the results of Mårtensson *et al.* (1997), who could not find any alcohol-induced increase in the trisialylated or more sialylated transferrin subfractions. It has been estimated that a minimum consumption of 50–80 g of alcohol/day for at least one week is needed to increase the blood carbohydrate-deficient transferrin (CDT) concentration and that levels are normalized during abstinence, with a half-life of about two weeks (Lesch *et al.* 1996a, Werle *et al.* 1997; for a review, see Stibler 1991).

2.3.1. Mechanisms contributing to CDT formation

The exact mechanism by which chronic alcohol consumption induces CDT formation has remained unclear (for a review, see de Jong *et al.* 1990). Studies of human alcoholics have indicated that transferrin synthesis is accelerated in patients with fatty liver but diminished in the presence of cirrhosis (Potter *et al.* 1985). Other mechanisms postulated for increased CDT levels in alcoholics include disturbed glycoprotein synthesis in the hepatocytes. Investigations into hereditary carbohydrate-deficient glycoprotein syndromes have suggested defects in N-linked oligosaccharide processing or attachment of the sugar chains to the protein (Stibler & Jaeken 1990, Yamashita *et al.* 1993), although defective synthesis and transfer of nascent dolichol-linked oligosaccharide precursors has been documented more recently (Powell *et al.* 1994). Increased sialidase activation in the particulate fractions of the rat and human liver and a decrease in transferrin glycosyltransferases in the hepatic Golgi apparatus have also been reported in the presence of heavy alcohol consumption (Ghosh *et al.* 1993, Marinari *et al.* 1993, Xin *et al.* 1995, Ghosh & Lakshman 1997). The activities of several other serum glycosyltransferases have likewise proved to be reduced in alcoholic patients (Stibler & Borg 1991), and microheterogeneity has also been found in some other glycoproteins as well as transferrin (Tsutsumi *et al.* 1994). Defects in glycosylation may also lead to important functional alterations in proteins, such as under-glycosylation of gonadotrophic hormones, resulting in hypogonadism (Powell *et al.* 1994, Sairam *et al.* 1990; for a review, see McDowell & Gahl 1997), which on the other hand, readily occurs as a complication of chronic alcohol abuse in any case (Villalta *et al.* 1997).

The newly formed transferrin present in alcoholics during abstinence seems to have a higher sialic acid content than most of the transferrin already present in the blood, suggesting impaired uptake of sialic acid-deficient transferrin by the hepatocytes in alcoholics, due to membrane dysfunction, rather than a defect in the sialylation process (Petrén & Vesterberg 1988). Indeed, it has also been demonstrated that asialoglycoprotein receptors in the liver cells of rats fed on alcohol are inactivated and their synthesis is impaired, leading to decreased binding of asialoglycoproteins (ASGP) to hepatocytes (Casey *et al.* 1989, Casey *et al.* 1990, Casey *et al.* 1991, Tworek *et al.* 1996, Heggli *et al.* 1996). ASGP receptors are structurally related to the receptors for the carbohydrate-rich glycoprotein laminin, and to sex steroid binding protein receptors (Fortunati *et al.* 1993). Potter *et al.* (1992) also conclude that long-term alcohol intake by rats may result in a defect in the membrane receptor recycling mechanism in the hepatocytes. Furthermore they maintain that, as a consequence of this, hepatic iron uptake from transferrin is diminished. On the other hand, iron mobilization from the liver in particular has been found to be responsible for the increase in serum CDT in hereditary haemochromatosis patients (Jensen *et al.* 1994). It has been suggested that acetaldehyde, the main ethanol metabolite, which is known to form conjugates with proteins (Niemelä *et al.* 1990b, Niemelä *et al.* 1990c, Lin & Lumeng 1990, see Chapter 2.4.), is involved in the impairment of enzyme function (Stibler & Borg 1991, Marinari *et al.* 1993) and is associated with disturbances in the functioning of various liver cell receptors (Miller *et al.* 1996, Thiele *et al.* 1996).

It is in any case evident that there are several factors which lead to increased serum CDT concentrations as a consequence of alcohol abuse, and further investigations are needed in order to elucidate the primary mechanism.

2.3.2. Methods for determining CDT

The first qualitative assessments of transferrin variants were made by isoelectric focusing, which was later combined with zone immunoelectrophoresis (Vesterberg *et al.* 1984) or immunofixation (Kapur *et al.* 1989) to achieve quantitative determinations. Anion-exchange chromatography in minicolumns (Stibler *et al.* 1986, Stibler *et al.* 1991, Kwoh-Gain *et al.* 1990, Schellenberg *et al.* 1996) or chromatofocusing (Storey *et al.* 1985, Storey *et al.* 1987) together with radioimmunoassay or nephelometric assay have also been used for this purpose, or alternatively, isoelectric focusing has been combined with Western blotting (Xin *et al.* 1991; for a review, see Lieber *et al.* 1993), or with immunoblotting followed by laser densitometry (IEF/IB/LD, Bean & Peter 1993). A semi-automatic isoelectric focusing assay for CDT (SA-IEF-CDT) employing a Phast System has also been introduced (Löf *et al.* 1993). By virtue of its visible banding patterns, IEF/IB/LD has shown good diagnostic ability in identifying the genetic D variants of transferrin, which may give false positive results when monitoring alcohol abuse in terms of CDT (Bean & Peter 1994). On the other hand, even dry blood spots may be used as a sample for IEF/IB/LD (Bean *et al.* 1996). Ion-exchange chromatography for the quantification of transferrin isoforms has also been used in determinations performed by HPLC (Jeppsson *et al.* 1993, Heggli *et al.* 1996, Bean *et al.* 1997, Renner *et al.* 1997, Werle *et al.* 1997). In any case, charged-based separation appears to be the basis of all the procedures used for measuring transferrin variants in biological fluids.

Kit-type tests for easy, time-saving CDT detection have been developed recently (Stibler *et al.* 1986, Stibler *et al.* 1991, Bean *et al.* 1997). The assay protocols include minicolumn separation of desialylated serum transferrin isoforms and subsequent radioimmunoassay (CDTect by Pharmacia & Upjohn, Uppsala, Sweden, or %CDT RIA by Axis Biochemicals AS, Oslo, Norway), or turbidimetric immunoassay (%CDT TIA by Axis Biochemicals AS, Oslo, Norway). In CDTect, serum transferrin isoforms with pI values higher than 5.7 and minor amounts of those with pI values of 5.7 are included to the CDT fraction (Stibler *et al.* 1991), while according to the manufacturer, %CDT RIA detects transferrin variants carrying 0-2 terminal sialic acid residues. Similarly, the isotransferrins quantified in %CDT TIA are those with 0-2 sialic acid residues, but additionally 50% of the trisialotransferrins are included (Bean *et al.* 1997). The cut-off limits of the %CDT methods given by the manufacturer are accordingly different (2.5% for %CDT RIA versus 6% for %CDT TIA), while the main difference between CDTect and %CDT methods is that the former measures the absolute amount of serum CDT and the latter measure it as a proportion of serum total transferrin. Contrasting opinions are expressed in the literature on the advantages of relative vs. absolute determinations of CDT. Although some groups have admittedly demonstrated a positive correlation between serum transferrin and CDT concentrations, so that relative CDT values may give more useful information for diagnosing alcohol abuse than absolute ones (Huseby *et al.* 1997a,

Schellenberg *et al.* 1989, Bean & Peter 1993, Anton & Bean 1994), there are many reports in which absolute CDT concentrations have been shown to be more accurate than relative values (Xin *et al.* 1991, Xin *et al.* 1992, Mårtensson *et al.* 1997, Sillanaukee *et al.* 1994, Behrens *et al.* 1988a, Bell *et al.* 1993).

2.3.3. Diagnostic performance of CDT as a marker of alcohol abuse

2.3.3.1. Factors influencing the diagnostic performance of CDT

Many authors have reported excellent sensitivities (>80%) for CDT as a marker of alcohol abuse (Stibler *et al.* 1986, Behrens *et al.* 1988a, Kapur *et al.* 1989, Kwoh-Gain *et al.* 1990, Stowell *et al.* 1997; for a review, see Stibler 1991). The duration and amount of alcohol ingestion and the duration of abstinence seem to be, however, crucial factors as the sensitivity of CDT is concerned. It has been assumed that consumption of 50–80 g of ethanol for at least one week is required to reach sensitivities of 81–94% (for a review, see Stibler 1991). Spies *et al.* (1995) found that sampling before the administration of large volumes of fluid increases the sensitivity of CDT by about 10%. On the other hand, sensitivities of less than 30% have been observed for CDT in series including heavy drinkers who are not alcoholics (Nyström *et al.* 1992, Sillanaukee *et al.* 1993, Löf *et al.* 1994). Observed sensitivities may also be low in alcohol-dependent subjects, if their daily ethanol consumption does not exceed that mentioned above, if the time since last period of heavy drinking is long enough, or if the last bout of drinking was of short duration (Jeppsson *et al.* 1993, Helander *et al.* 1997, Löf *et al.* 1994, Lesch *et al.* 1996b). On the other hand, in a population study performed by Nilssen *et al.* (1992), CDT showed its best discriminatory power at a lower alcohol intake (30 g/day for males and 13 g/day for females). It is significant, however, that the sensitivities obtained for CDT at these levels of alcohol intake were only 38.5% for males and 37.1% for females at specificity levels of 80.8% and 75.6%, respectively.

Serum CDT concentrations are also sex-specific (Nyström *et al.* 1992, Sillanaukee *et al.* 1993, Sillanaukee *et al.* 1994, Anton & Bean 1994, Anton & Moak 1994, Löf *et al.* 1994, Konig *et al.* 1995), since the association of CDT with alcohol consumption may be less evident in females, and the diagnostic performance of CDT as a marker of alcohol abuse has often been reported to be lower for women than for men. On the other hand, actual serum CDT concentrations are higher in women (La Grange *et al.* 1994, Stibler *et al.* 1991, Löf *et al.* 1994, Anton & Bean 1994, Anton & Moak 1994, Grønbæk *et al.* 1995). The reasons for these observations remain obscure, but it seems that the sex difference in CDT amounts is focused on the serum concentrations of asialylated and monosialylated transferrin, which are higher in women than in men (Mårtensson *et al.* 1997). Serum CDT values seem not to vary as a function of the menstrual cycle or with serum oestrogen or progesterone concentrations (La Grange *et al.* 1995, Stauber *et al.* 1996a, Stauber *et al.* 1996b). However, it has been reported, that premenopausal women have higher CDT levels than postmenopausal ones, and that CDT levels are increased in women receiving postmenopausal oestrogen replacement therapy (Grønbæk *et al.* 1995, Stauber *et al.*

(1996a). The effects of oral contraceptives on serum CDT concentrations are apparently fairly weak, although the information is partly controversial (Nyström *et al.* 1992, Anton & Moak 1994, La Grange *et al.* 1995, Stauber *et al.* 1996a).

In addition to factors related to alcohol intake or sex, other factors not related to alcohol may influence the sensitivity of CDT. Many authors have reported relatively low sensitivities (about 65%) even in alcoholics with severe ethanol dependence and recent excessive alcohol intake (Meregalli *et al.* 1995, Bell *et al.* 1993, Löf *et al.* 1994), and it has been suggested that insulin sensitivity or hyperinsulinaemia, for instance, may influence the diagnostic accuracy of CDT (Fagerberg *et al.* 1994a, Fagerberg *et al.* 1994b, Arndt *et al.* 1997). One possible reason for the lack of CDT sensitivity (or specificity), seen especially in females, but also occasionally in males, may be an association between serum transferrin and CDT variation. Although it has been reported that no such correlation exists (Stibler *et al.* 1986, Behrens *et al.* 1988a, Werle *et al.* 1997), the opposite opinion has also been put forward (Bell *et al.* 1993, Simonsson *et al.* 1996). According to Stauber *et al.* (1996b), it may be the serum transferrin concentration which is the influential factor in CDT variation rather than any iron deficiency. On the other hand, Anton & Moak (1994) found a weak correlation also between serum iron and CDT in females with an alcohol consumption of less than 15 g/day. Abnormally high non-alcohol related serum transferrin and CDT concentrations, and a significant correlation between these, have also been found in pregnant women (Härlin *et al.* 1994, Stauber *et al.* 1996b). This contradicts suggestions that increasingly more complex carbohydrate chain structures form during pregnancy (van Eijk *et al.* 1987, de Jong & van Eijk 1988). The week of pregnancy and human placental lactogen have been observed to correlate with maternal CDT (Härlin *et al.* 1994, Stauber *et al.* 1996b), and likewise serum transferrin concentrations seem to be associated with gestational age or either oestradiol or progesterone (de Jong & van Eijk 1988, Härlin *et al.* 1994, Stauber *et al.* 1996b). Thus the low CDT values reported in pregnant women by Löf *et al.* (1994) may be due to the relatively early gestational age (16th week). Interestingly, Whitty *et al.* (1997) discovered that cord blood CDT concentrations are even higher than maternal. Taken together, total serum transferrin values may offer important information for interpreting CDT results.

Although there are reports indicating that CDT is not significantly affected by liver disease (Stibler *et al.* 1986, Stibler & Borg 1986, Jeppsson *et al.* 1993, Rubio *et al.* 1997), there are also suggestions that it primarily marks alcoholic liver disease rather than the amount of alcohol consumed (Yamauchi *et al.* 1993, Tsutsumi *et al.* 1994). Also, considerable amounts of data exist to suggest that non-alcoholic liver disease is not usually associated with high concentrations of CDT, indicating a high specificity of the marker with this respect (Stibler *et al.* 1986, Stibler & Borg 1986, Kwoh-Gain *et al.* 1990, Fletcher *et al.* 1991, Kapur *et al.* 1989, Bell *et al.* 1993, Stibler & Hultcrantz 1987, Storey *et al.* 1987, Xin *et al.* 1991; for reviews, see Stibler 1991, Allen *et al.* 1994). False positive CDT values have nevertheless occasionally been detected in cases of hepatic insufficiency due to primary biliary cirrhosis (PBC), chronic active hepatitis, or drug hepatopathy, and in patients with carbohydrate-deficient glycoprotein (CDG) syndrome along with 25% of healthy carriers (Bell *et al.* 1993; for a review, see Stibler 1991). In the absence of chronic alcohol abuse, increased CDT concentrations may occur in patients with liver cirrhosis, hepatocellular carcinoma, or chronic viral hepatitis (Takase *et al.* 1985, Murawaki *et al.* 1997, Perret *et al.* 1997). In any case, the presence of liver disease seems to have less

influence on serum CDT concentrations than on MCV or GGT results (Meregalli *et al.* 1995). Also, methodological aspects may affect the diagnostic accuracy of CDT determinations in patients with liver diseases (Bean *et al.* 1995, Lesch *et al.* 1996c).

There are some reports revealing age-related differences in CDT results, but the issue remains somewhat unclear. Stauber *et al.* (1996a) found a significant negative correlation between age and CDT in females but not in males, but this may be associated with differences in hormone status rather with age as such. Huseby *et al.* (1997a) in turn observed higher CDT values in middle-aged alcohol-dependent patients (36–50 years) than in younger or older patients, but they speculate that this may be explained by the drinking histories of the participants. There are nevertheless many studies in which no consistent relation between age and CDT levels has been observed (La Grange *et al.* 1995, Stibler *et al.* 1986, Schellenberg *et al.* 1989, Xin *et al.* 1992, Konig *et al.* 1995).

2.3.3.2. Comparisons between CDT methods

The results of comparisons between CDT methods serve to illustrate the effects of methodological aspects on the diagnostic value of this marker for detecting alcohol abuse (Table 1). As described above, the various methods available, including minicolumn anion-exchange chromatography (MAEC), discriminate and detect transferrin isoforms differently, which is apparently an important reason for the differences in diagnostic performance between them. The MAEC methods fail to detect genetic transferrin variants and may therefore result in false positive (transferrin-D) or false negative (transferrin-B) CDT findings. This may be one cause of the discrepancies between the results of these methods and those obtained using isoelectric focusing or HPLC, i.e. methods which are readily capable of discriminating between the genetic variants. (Jeppsson *et al.* 1993, Bean & Peter 1994, Simonsson *et al.* 1996). Such genetic variants are rare, however, and therefore more probable reasons for the differences may be the greater precision of an automated procedure relative to a manual one and the stronger effect of serum transferrin variations on absolute CDT results than on relative ones, as speculated by Werle *et al.* (1997).

Table 1. Analytical characteristics of the different CDT methods.

Reference		Subjects, EtOH intake, (n)		Method		Cut-off limit (Females/Males*)		Sensitivity-% (Females/Males*)		Specificity -% (Females/Males*)		Overall accuracy-% in ROC-analysis (Females/Males*)
Xin *et al.* 1992	A	Male alcoholic patients admitted for detoxification, >80 g EtOH/d (n = 53)	I	MAEC/RIA	I	20.6 mg/l[1]	I	60%A 67%[B] 58%[C]	I	63%[D] 100%[E]	I	86%A+B+C+F 68%[B+C+D+E]
	B	Male alcoholic patients with steatosis or perivenular fibrosis in liver biopsy, >80 g EtOH/d (n = 12)	II	IEF/WB	II	100 mg/l[1]	II	76%[A] 75%[B] 75%[C]	II	100%[D] 100%[E]	II	92%[A+B+C+F] 97%[B+C+D+E]
	C	Male alcoholic patients with extensive fibrosis or alcoholic hepatitis in liver biopsy, >80 g EtOH/d (n = 12)										
	D	Abstinent (≥30 d) male alcoholics with liver disease (n = 8)										
	E	Non-drinking male patients with liver disease (n = 7)										
	F	Healthy male controls, <40g EtOH/d (n = 16)										
Jeppsson *et al.* 1993	A	Heavily intoxicated patients, 70-500g EtOH/d (n = 60)	I	HPLC	I	<0.8%[2]	I	100%A 55%[B]	I	91%	I	–
	B	Patients reporting daily ethanol consumption of 40-70 g (n = 45 ?)										
	C	Teetotalers and occasional drinkers (n = 56)										
Yamauchi *et al.* 1993	A	Patients with alcoholic liver disease (n = 55)	I	CDTect	I	32.9 U/l[1]	I	35.6%[A] 8.0%[B]	I	97.3%[C] 84.0%[D]	I	–
	B	Alcoholics without liver disease (n = 25)	II	%CDT RIA	II	2.5%[1]	II	43.7%[A] 12.0%[B]	II	92.0%[C] 76.0%[D]	II	–
	C	Healthy adults (n = 37)										
	D	Patients with non-alcoholic liver disease (n = 25)										

Table 1. Continued.

Reference	Subjects, EtOH intake, (n)	Method	Cut-off limit (Females/Males*)	Sensitivity-% (Females/Males*)	Specificity -% (Females/Males*)	Overall accuracy-% in ROC-analysis (Females/Males*)
Anton & Bean 1994	A Alcohol-dependent patients, >60g EtOH/d, (n = 59)	I IEF/IB/LD	I 7 DU/ 5 DU[1]	I 33%/85%	I 98%/93%	I 88%
	B Controls, <15g EtOH/d (n = 61)	II CDTect	II 22 U/l/ 16 U/l[1]	II 44%/66%	II 100%/98%	II 73%
Bell *et al.* 1994	A Consecutive patients including heavy drinkers, >50 g EtOH/d (n = 26)	I CDTect	I 27 U/l/ 20 U/l[3]	I 69%	I 92%	I –
	B Patients consuming <50 g EtOH/d (n = 421)	II %CDT RIA (version 1)	II 2.5%[4]	II 69%	II 76%	II –
		III %CDT RIA (version 2)	III 2.5%[4]	III 50%	III 90%	III –
Sillanaukee *et al.* 1994	A Male alcoholics, >1000g EtOH/wk (n = 28)	I CDTect	I -/20 U/l[4]	I 43%[A] 89%[B]	I 85%	I 63%[A vs. C] 87%[B vs. C]
	B Male heavy drinkers, 50-600g EtOH/wk (n = 28)	II FPLC	II (see ref.)[1]	II 29-32%[A] 71-75%[B]	II 92%	II 59-61%[A vs. C] 81-83%[B vs. C]
	C Healthy male controls, <105g EtOH/wk (n = 26)	III IEF with immunofixation	III -/4.4%[5] or [1] (see ref.)	III 18-59%[A] 36-89%[B]	III 88-100%	III 57-74%[A vs. C] 67-89%[B vs. C]
Schellenberg *et al.* 1996	A Male alcoholics, 80-250 g EtOH/d (n = 74)	I Anion-exchange separation and nephelometric assay	I - /70 mg/l[6]	I 73%	I 90%	I 89%
	B Healthy male controls, 0-150g EtOH/wk (n = 90)					
Bean *et al.* 1997	A Alcohol abusers, 100-400g EtOH/d (n = 32)	I IEF/IB/LD	I 7 DU[1]	I 83%	I 94%[B+C]	I 89%[A vs. (B+C)]
	B Social drinkers, <40g EtOH/d (n = 33)	II %CDT HPLC	II 6%[1]	II 87%	II 100%[B+C]	II 93% A [vs. (B+C)]
	C Total abstainers (n = 8); Abstinent pregnant women (n = 7)	III %CDT TIA	III 5-6%[1]	III 87%	III 98%[B+C]	III 96%[A vs. (B+C)]

Table 1. Continued.

Reference	Subjects, EtOH intake, (n)	Method	Cut-off limit (Females/Males*)	Sensitivity-% (Females/Males*)	Specificity -% (Females/Males*)	Overall accuracy-% in ROC-analysis (Females/Males*)
Huseby *et al.* 1997a	A Alcohol-dependent patients group I, 0-920 g EtOH/d, (n = 137)	I CDTect	I 26 U/l/ 20 U/l[4]	I 76^{A} 51^{B}	I 86%	I –
	B Alcohol dependent patients group II, low EtOH consumption/d (n = 57)	II %CDT RIA	II 2.5%[4]	II 77^{A} 44^{B}	II 92%	II –
	C Teetotalers and subjects with normal alcohol consumption (n = 145)					
Renner & Kanitz 1997	A Currently drinking alcohol-dependent inpatients (n = 40)	I HPLC	I 80 mg/l[7]	I $82.5\%^{A}$	I $100\%^{B+C}$	I –
	B Alcohol-dependent inpatients with abstinence of >2 weeks (n = 34)					
	C Teetotalers (n = 39)					
Stowell *et al.* 1997	A Older male alcoholics and heavy drinkers, >60g EtOH/d (n = 19 ?)	I %CDT RIA	I >26 U/l / >20 U/l[4]	I $83\%^{A}$ $20\%/40\%^{C}$ $22\%/43\%^{D}$	I $88\%^{B}$ $97\%/88\%^{E}$	I –
	B Older male moderate drinkers, <60g EtOH/d, and non-drinkers (n = 34)	II CDTect	II 2.5%[4]	II $78\%^{A}$ $40\%/44\%^{C}$ $26\%/35\%^{D}$	II $94\%^{B}$ $92\%/83\%^{E}$	II –
	C Young heavy drinkers, ≥ 16 drinks/wk (n = 30)					
	D Young moderate drinkers, ≥6 and <16 drinks/wk (n = 81)					
	E Young light drinkers, >0 and <6 drinks/wk, and non-drinkers (n = 101)					

Table 1. Continued.

Reference	Subjects, EtOH intake, (n)	Method	Cut-off limit (Females/Males*)	Sensitivity-% (Females/Males*)	Specificity-% (Females/Males*)	Overall accuracy-% in ROC-analysis (Females/Males*)
Werle *et al.* 1997	A Alcoholic inpatients, 162 ± 96 g EtOH/d, (n = 51), including subgroups: a: Patients with S-ASAT >30 U/l and b: patients with S-ASAT ≤ 30 U/l	I CDTect	I 26 U/l/ 20 U/l[4] or 31.9 U/l/ 23.6 U/l[1]	I 62.5%/ 71.4%[Aa, 4] 54.5%/ 61.1%[Ab, 4] 62.5%/ 57.1%[Aa, 1] 45.4%/ 44.4%[Ab, 1]	I 83%/83%[B+C, 4] 100%/95.7%[B+C, 2]	I 73%/79%[A vs. C]
	B Patients with non-alcoholic liver disease, <30 g EtOH/d, (n = 20) C Healthy persons, <30 g EtOH/d, (n = 30)	II HPLC	II 1%[1]	II 75%/ 92.9%[Aa] 64%/ 83.3%[Ab]	II 96.6%[B+C]	II 92%/97%[A vs. C]

*included if the results are given in the original reference,
Cut-off limit: [1]mean of control values + 2SD, [2]determined by Jeppsson *et al.* 1993, [3]determined by Bell *et al.* 1993, [4]given by the manufacturer, [5]determined by Löf *et al.* 1993, [6]determined to give a specificity of 90%, [7]determined by Godsell *et al.* 1995.

2.3.3.3. CDT vs. other markers of alcohol abuse

Several previous reports indicate that CDT is one of the most valuable among the various available markers of chronic alcohol abuse, e.g. GGT, MCV, AST, ALT, or mAST (Kwoh-Gain *et al.* 1990, Nyström *et al.* 1992, Bisson & Milford-Ward 1994, Konig *et al.* 1995, Spies *et al.* 1995, Grønbæk *et al.* 1995, Stauber *et al.* 1995, Meregalli *et al.* 1995, Schellenberg *et al.* 1989, Rubio *et al.* 1997, Helander *et al.* 1997; for reviews, see Stibler 1991, Sillanaukee 1996), although AST, mAST and GGT have been reported to distinguish heavy drinking better from lower levels of alcohol consumption than CDT does (Sharpe *et al.* 1996). Furthermore, the AST/ALT ratio and mAST have been found to achieve a better diagnostic performance than other markers, including CDT, when distinguishing alcoholics from non-alcoholic liver disease patients (Sharpe *et al.* 1996). In fact, even in alcoholics with liver disease, the sensitivity of CDT at the cut-off levels recommended by the manufacturers has been reported to be lower than that of GGT or MCV, although its specificity seems to be higher (Meregalli *et al.* 1995). A combination of CDT and AST has proved to be a better marker of both harmful alcohol intake (>35 drinks/day) and alcohol intake above the recommended level of 21 drinks/week than either CDT or AST alone or the short Michigan Alcoholism Screening test (sMAST) in men, whereas neither CDT, AST, CDT/AST nor sMAST seems to be useful as a marker of alcohol intake in women (Grønbæk *et al.* 1995).

A considerable amount of research has been done into the usefulness of CDT and GGT as markers of heavy alcohol consumption (for a review, see Litten *et al.* 1995). CDT and GGT are statistically independent of each other and could therefore be used in combination (Behrens *et al.* 1988b, Nilssen *et al.* 1992, La Grange *et al.* 1994, Löf *et al.* 1994, Anton & Moak 1994, Huseby *et al.* 1997a). GGT is elevated in all forms of liver disease, but can identify only 30–50% of patients consuming excessive amounts of alcohol before organic damage becomes manifest (for a review, see Goldberg & Kapur 1994). Thus treatment and follow-up studies have shown the change in CDT from pre-treatment levels to be more sensitive to drinking status than GGT (Anton *et al.* 1996, Huseby *et al.* 1997b). There are nevertheless some patients for whom GGT may be a more effective marker of relapse than CDT (Mitchell *et al.* 1997). It has been reported that in order to increase the possibility of identifying excessive alcohol consumption and to improve the detection of relapse into heavy drinking during the long-term monitoring of outpatients, it would be advisable to measure both CDT and GGT, so that the more sensitive individual marker could then be determined by following the changes in these two measures during a period of alcohol withdrawal (Helander *et al.* 1996).

The receiver operator characteristic analysis (ROC) curves simultaneously show the proportion of both true positive results (sensitivity) and false negative results (1 - specificity) obtained with various cut-off points in the tests. The area under an ROC curve describes the diagnostic performance of the test, i.e. its ability to classify the subjects correctly into clinically relevant subgroups. (Hanley & McNeil 1982, Hanley & McNeil 1983). The ROC analysis performed for GGT and CDT (CDTect) by Anton & Moak (1994) showed GGT to have a higher sensitivity in alcohol-dependent females at high specificity levels (>50%) than CDT, although the differences between the total areas under the curves of the markers were not significant (0.76 and 0.75, respectively). No clear

difference between the ROC areas for GGT and CDT could be noted in the case of males (0.85 and 0.95, respectively), but the performances of both tests seem to be higher than for females.

Jaakkola *et al.* (1994) found that the sensitivity of CDT for detecting an alcoholic cause of acute pancreatitis was 75%, while the lipase/amylase ratio index, MCV and GGT could not distinguish these cases from ones of non-alcoholic origin. CDT was also significantly higher in the patients with alcoholic acute pancreatitis or a suspicion of this than in ones with non-alcoholic disease. Fletcher *et al.* (1991) have in turn reported that the ratio of desialylated transferrin to total transferrin has greater specificity (98%) and sensitivity (81%) in detecting alcohol abuse in patients with steatohepatitis than total AST, mAST/ total AST, GGT, MCV or ALT, for which the specificities were 66%, 50%, 55%, 79%, and 50%, respectively, and the sensitivities 69%, 92%, 69%, 73% and 58%.

β-Hexosaminidase is a lysosomal enzyme that exists in many human tissues and has both N-acetylglucosaminidase and N-acetylgalactosaminidase activity. Human lysosomal β-hexosaminidase (Hex) consists of several glycoprotein isozymes: Hex B, I_1, I_2, P, A, and S, in decreasing order of isoelectric points. (Price & Dance 1972, Stirling 1972, Nakagawa *et al.* 1977, Pamplos *et al.* 1980). Hex P has been noted to increase markedly in alcoholism, in different forms of liver disease and in pregnancy (Hultberg *et al.* 1981, Hultberg & Isaksson 1983, Stirling 1972, Hultberg *et al.* 1991, Hultberg *et al.* 1995). The only difference found between Hex B and Hex P is that the latter contains more sialic acid (Isaksson *et al.* 1992). Interestingly, alcohol abuse appears to have the opposite effect on the Hex pattern (hypersialylation) to that on transferrin (desialylation). Hultberg *et al.* (1995), comparing certain biochemical and diagnostic properties in the total amounts of serum Hex B and Hex P ("Hex B") determined by enzyme-linked immunosorbent assay (ELISA) with CDT, found the sensitivity of "Hex B" in detecting alcohol abuse at a cut-off level obtained using a control group to be higher (90%) than that of CDT (83%). Additionally, "Hex B" and CDT were reported to have similar time-course variations and half-lives, and to correlate highly significantly with each other, whereas neither serum GGT nor AST was found to correlate with either.

2.3.4. Usefulness of CDT as a marker of alcohol abuse in various clinical conditions

Tests for the identification of alcohol abuse are required as screening procedures in the general population as well as for specific diagnosis in cases of hospital inpatients or outpatients presenting with signs of liver disease or a suspicion of such. CDT has also been reported to be useful as a marker of relapse in alcoholic patients and as a corrective tool for assessing patients' reports of their own alcohol consumption in connection with outpatient treatment, even in cases of severe liver disease. (Rosman *et al.* 1995, Borg *et al.* 1994, Borg *et al.* 1995, Mitchell *et al.* 1997, Huseby *et al.* 1997b, Caldwell *et al.* 1995, Henriksen *et al.* 1997). The possibilities of detecting relapses by CDT during long-term monitoring of alcohol-dependent outpatients have been found to be further improved by introducing individualized cut-off points between normal and increased CDT levels (Borg *et al.* 1994, Borg *et al.* 1995). It is also known that disulfiram treatment

(Antabuse, ALDH inhibitor) does not influence the serum concentration of CDT, at least when its intake has not continued during relapse (Helander & Carlsson 1996). On the other hand, CDT seems not to be sensitive in detecting short-term heavy drinking by healthy subjects (Salmela *et al.* 1994, Lesch *et al.* 1996b), and its sensitivity also drops quickly after a relatively short period of abstinence in cases of chronic alcoholism (Konig *et al.* 1995, Henriksen *et al.* 1997). However, provided that its biological turnover is taken into account, CDT determination may be a useful test for the diagnosis of alcohol-related neurological disorders, for example, or for screening for excessive drinking (Yersin *et al.* 1995, Stibler 1993), although it is only of limited value when screening unselected, non-hospitalized subjects (for a review, see Goldberg & Kapur 1994). As CDT is related to alcohol intake, it may also be used as an indicator of the severity of biological and psychosocial dysfunction induced by drinking that may require further intervention (Saini *et al.* 1997). It has also shown considerable promise as a post mortem marker of chronic alcoholism (Sadler *et al.* 1996).

2.4. Acetaldehyde adducts

Acetaldehyde is the main metabolite of ethanol, and its formation in the hepatocyte is mediated by three alcohol metabolizing systems: the alcohol dehydrogenase (ADH) pathway of the cytosol, or soluble fraction of the cell, the microsomal ethanol oxidizing system (MEOS), involving the ethanol-inducible cytochrome P450 2E1 (*CYP*2E1), and a catalase located in the peroxisomes. Apart from the stomach, extrahepatic metabolism of alcohol is minimal. (For reviews, see Lieber 1988, Lieber 1994), although it has been suggested that catalase-mediated acetaldehyde formation in foetal brain tissue may be an important factor in the neurotoxic effects of in utero exposure (Hamby-Mason *et al.* 1997). Acetaldehyde is normally converted rapidly to acetate by aldehyde dehydrogenase (ALDH), but prolonged alcohol consumption can induce pathophysiological abnormalities which have been attributed to the accumulation of acetaldehyde in the liver and blood (for reviews, see Lieber 1988, Israel *et al.* 1988, Niemelä 1993, Lieber 1997). The toxicity of acetaldehyde is associated with its impairment of the capacity of the liver to utilize oxygen. It also promotes depletion of reduced glutathione, free radical mediated toxicity and lipid peroxidation. (For a review, see Lieber 1997). The sex differences observed in the adverse effects of alcohol appear to be related in part to lower gastric ADH activity in young women (with consequent reduction of first pass ethanol metabolism), less hepatic fatty acid binding protein, higher free fatty acid levels and less pronounced omega-hydroxylation, all of which result in increased vulnerability to alcohol (for review, see Lieber 1994). It has also been suggested recently that an increase in oestrogen-related acetaldehyde could be the key factor explaining sex differences in alcohol drinking and its effects (Eriksson *et al.* 1996). A significant proportion of the toxic effects of acetaldehyde *in vivo* arises from the formation of acetaldehyde-protein adducts, which may lead to tissue damage via alterations in protein function or via the triggering of immunological responses (for reviews, see Israel *et al.* 1988, Lieber 1997).

2.4.1. Formation and structure of acetaldehyde adducts

Acetaldehyde has been shown to be capable of conjugating covalently with various proteins, particularly under reducing conditions, but also under non-reducing ones (Donohue *et al.* 1983, Mauch *et al.* 1986, Tuma *et al.* 1987, Behrens *et al.* 1988c, Jennett *et al.* 1989, Jukkola & Niemelä 1989, Niemelä *et al.* 1991a, Niemelä *et al.* 1994, Niemelä *et al.* 1995, Holstege *et al.* 1994, Worrall *et al.* 1994, Paradis *et al.* 1996a, Lin *et al.* 1995a, Lin *et al.* 1995b, Sillanaukee *et al.* 1996). The prevailing acetaldehyde concentration appears to have an effect on the structure of the resulting adducts (Lin *et al.* 1993b). These protein adducts may be stable or unstable, of which the latter may be rendered detectable with reducing agents, e.g. cyanoborohydride or ascorbic acid (Donohue *et al.* 1983, Tuma *et al.* 1987). Even in the absence of reducing agents, stable cyclic imidazolidinone structures are formed in a reaction between acetaldehyde and the free alpha-amino group of the aminoterminal valine of haemoglobin (San George & Hoberman 1986, Fowles *et al.* 1996). Primary amino groups of lysine residues (–NH2) react rapidly with acetaldehyde to form Schiff bases [–N=CH(CH3)] (Tuma *et al.* 1987, Braun *et al.* 1997), so that proteins with large amounts of reactive lysine residues appear to become modified even at low concentrations of acetaldehyde under appropriate reducing conditions (Stevens *et al.* 1981, Donohue *et al.* 1983, Tuma *et al.* 1987, Jennett *et al.* 1989). However, even lysine residues located in close vicinity one to another in a peptide are not equally reactive in forming stable acetaldehyde adducts (Lin *et al.* 1995a). Tryptophan analogues and tyrosine, for instance, have also been implicated as target structures for the acetaldehyde adducts resulting from alcohol consumption (Stevens *et al.* 1981, Austin & Fraenkel-Conrat 1992). The structures of protein adducts have been studied by isotopic methods (Stevens *et al.* 1981, Donohue *et al.* 1983, San George & Hoberman 1986, Tuma *et al.* 1987, Gross *et al.* 1992), mass spectrometry (Austin & Fraenkel-Conrat 1992, Gross *et al.* 1992, Lin *et al.* 1995a, Sillanaukee *et al.* 1996, Braun *et al.* 1997), nuclear magnetic resonance spectroscopy (NMR) (Austin & Fraenkel-Conrat 1992, Fowles *et al.* 1996, Braun *et al.* 1997), Raman spectroscopy (Braun *et al.* 1997) and neutron diffraction (Wess *et al.* 1996).

2.4.2. Acetaldehyde-protein adducts detected in blood

2.4.2.1. Haemoglobin adducts

Many authors have reported increased concentrations of acetaldehyde adducts in both chronic alcoholics and heavy drinkers who are not alcoholics (Niemelä & Israel 1992, Sillanaukee *et al.* 1992, Gross *et al.* 1992, Lin *et al.* 1993a). Moreover, they appear to increase in the erythrocyte proteins of non-alcoholic volunteers even after a single heavy drinking bout, whereas GGT and MCV concentrations are not influenced, and the adduct concentration remains elevated after the ethanol has been eliminated from the body, returning to normal levels in 1–3 weeks (Niemelä & Israel 1992, Sillanaukee *et al.* 1992).

Acetaldehyde adducts have also been found in women who continued to drink during pregnancy and subsequently gave birth to children with foetal alcohol effects (Niemelä *et al.* 1991b).

The site in the haemoglobin that is modified by acetaldehyde *in vivo* is primarily located in a surface-accessible domain near the centre of the beta chain of Haemoglobin-A, where a number of lysine residues are clustered (Lin *et al.* 1993a). Measurements of acetaldehyde-modified blood proteins have been performed by HPLC or immunological techniques (Sillanaukee *et al.* 1992, Sillanaukee *et al.* 1992, Gross *et al.* 1992, Wickramasinghe *et al.* 1994, Wickramasinghe *et al.* 1996, Hurme *et al.* 1998, Israel *et al.* 1986, Niemelä *et al.* 1990b, Niemelä *et al.* 1991b, Lin *et al* 1993a, Niemelä & Israel 1992, Israel *et al.* 1992, Lin *et al.* 1993a, Klassen *et al.* 1994), while Peterson *et al.* have detected increased concentrations of haemoglobin-acetaldehyde adducts in chronic alcoholics using fluorigenic labelling with 1,3-cyclohexanedione for aldehyde quantification (Peterson & Polizzi 1987; Peterson & Scott 1989). Immunization of animals with acetaldehyde adduct antigen results in the production of antibodies which recognize acetaldehyde-modified structures irrespective of the nature of the carrier protein (Israel *et al.* 1986, Israel *et al.* 1992, Niemelä *et al.* 1991a, Lin *et al.* 1993b, Klassen *et al.* 1994, Lin *et al.* 1995b). Such antibodies are able to recognize adducts prepared at 20–100 µM concentrations of acetaldehyde, which have been reported to occur in the blood of individuals consuming alcohol (Nuutinen *et al.* 1983, for reviews, see Eriksson 1983, Eriksson & Fukunaga 1993, Niemelä 1998). On the other hand, if protein adducts are produced under different conditions, antibodies raised against them recognize different epitopes (Lin *et al.* 1993b). Even so, antibodies produced against adducts prepared in high, non-physiological concentrations of acetaldehyde may be useful for protein conjugate measurements (Yokoyama *et al.* 1995a).

2.4.2.2. Haemoglobin adducts as markers of alcohol abuse

Adduct measurements from erythrocytes based on immunological assays have shown sensitivities of about 50–70% with specificities of >95% (Lin *et al.* 1993a, Niemelä & Israel 1992, Sillanaukee *et al.* 1992; for a review, see Goldberg & Kapur 1994). A comparison of methods for detecting acetaldehyde-haemoglobin adducts showed the overall sensitivities and specificities of an immunological assay and a chromatographic method to be rather similar, although the HPLC method achieved a slightly higher sensitivity in alcoholics (55%) than in heavy drinkers (50%) and the immunological method had a lower sensitivity among alcoholics (40%) than among heavy drinkers (50%) (Sillanaukee *et al.* 1992). Sensitivities as high as 75–90% have recently been reported for adduct detection in alcoholic women using HPLC separation of blood specimens (Hurme *et al.* 1998). The sensitivities obtained for the adduct measurements in preliminary experiments were also found to be comparable to those of GGT and CDT and higher than that of MCV (Niemelä & Israel 1992, Sillanaukee *et al.* 1992, Niemelä 1993, Hurme *et al.* 1998). The comparison by Wickramasinghe *et al.* (1994) nevertheless showed poorer

diagnostic performance for haemoglobin-adduct detection in chronic alcoholics by HPLC than for the conventional GGT or AST methods, CDT, or a combination of CDT and GGT.

2.4.2.3. Other acetaldehyde-modified proteins in blood

In addition to erythrocytes, acetaldehyde adducts occur in detectable amounts in serum proteins, particularly those synthesized in the liver and thereby exposed to acetaldehyde. In this respect albumin is a quantitatively important protein, having a half-life of 17–20 days. (Rothschild *et al.* 1988). Acetaldehyde conjugates with albumin more efficiently *in vitro* than with erythrocyte proteins (Israel *et al.* 1986). Overall, serum seems to have a high acetaldehyde carrying capacity, since it has been shown to bind >447 mM acetaldehyde without alteration in its fluorescence (Brecher *et al.* 1997). Some authors have reported acetaldehyde-protein condensates in plasma protein (Wickramasinghe *et al.* 1986; Peterson & Polizzi 1987, Nicholls *et al.* 1994), where adducts have a half-life of 4.8 weeks (Nicholls *et al.* 1994). Acetaldehyde-modified lipoproteins have also been reported to occur in the blood of alcoholics (Wehr *et al.* 1993, Lin *et al.* 1995b, Melkko *et al.* 1996). Interestingly, lipoprotein modification *in vivo* may cause the activation of apolipoprotein E synthesis in macrophages, which has been suggested as a mechanism promoting atherogenesis in alcohol abusers (Lin *et al.* 1995b). Acetaldehyde also reacts with apoprotein B prior to its secretion from the liver, the altered very low density lipoproteins (VLDL) being thought to be partially removed prior to their conversion to low density lipoprotein (LDL). It has also been speculated that alteration of VLDL-B by acetaldehyde *in vivo* may be associated with the low intermediate density lipoprotein (IDL) and LDL levels observed in alcoholics. (Wehr *et al.* 1993, Kervinen *et al.* 1995).

2.4.3. Acetaldehyde-protein adducts in tissue specimens

2.4.3.1. Methods for detecting adducts in tissue samples

Protein adducts show altered electrophoretic mobility relative to native proteins, and therefore Western blot systems and other electrophoretic techniques have been widely used to study them (Lin *et al.* 1988, Lin & Lumeng 1989, Lin & Lumeng 1990, Jennett *et al.* 1989, Behrens *et al.* 1988c, Koskinas *et al.* 1992, Lin *et al.* 1995b, Zhu *et al.* 1996, Paradis *et al.* 1996a, Li *et al.* 1997, Ma *et al.* 1997). Acetaldehyde-modified structures have also been detected on the surfaces of hepatocytes and splenocytes by flow cytometry (Trudell *et al.* 1990, Trudell *et al.* 1991, Lin *et al.* 1992, Braun *et al.* 1995) and in the mitochondrial fraction, membranes and cytosolic compartments of hepatic tissue by an enzyme-linked immunosorbent assay (ELISA) method (Nicholls *et al.* 1994, Tuma *et al.* 1996). Antibody-based methods have furthermore been used for the

microscopic detection of protein-aldehyde adducts in tissue specimens (Niemelä *et al.* 1991a, Halsted *et al.* 1993, Lin *et al.* 1993c, Yokoyama *et al.* 1993a, Niemelä *et al.* 1994, Holstege *et al.* 1994, Niemelä *et al.* 1995, Paradis *et al.* 1996b).

2.4.3.2. Adduct findings in tissue samples

Aldehyde adduct formation in tissues is thought to be an important factor in various disturbances of biological functions following extensive ethanol consumption, and reactive aldehydic products resulting from ethanol metabolism and ethanol-induced oxidative stress have been reported to play an important role in the pathogenesis of alcoholic liver injury (Cederbaum 1989, French 1989, Tsukamoto *et al.* 1990, Nordmann *et al.* 1992, Tuma & Sorrell 1995, Lin *et al.* 1998; for reviews, see Lieber 1988, Niemelä 1998). Such products could serve as a basis for the development of specific markers of ALD, which is difficult to differentiate from NALD either histologically or on the basis of conventional laboratory markers. It has been observed that modified cytosolic liver proteins decline with a half-life of 2.3 weeks (Nicholls *et al.* 1994). Covalent binding of acetaldehyde to protein is known to interfere with the functioning of tubulin and lysine-dependent enzymes (Sorrell & Tuma 1987, Jennett *et al.* 1989, McKinnon *et al.* 1987, Smith *et al.* 1989, Tuma *et al.* 1991, Mauch *et al.* 1986, Mauch *et al.* 1987). Reactive aldehydes may also play a role in alcohol-related changes in protein-protein interactions (Paradis *et al.* 1996a), in the impairment of receptor-mediated endocytosis (Casey *et al.* 1991, Kervinen *et al.* 1991, Miller *et al.* 1996, Thiele *et al.* 1996), and in ethanol-induced stimulation of fibrogenesis (Brenner & Chojkier 1987, Chojkier *et al.* 1989, Moshage *et al.* 1990, Parola *et al.* 1993, Casini *et al.* 1991, Casini *et al.* 1993, Casini *et al.* 1994, Friedman 1993, Lee *et al.* 1995, Yokoyama *et al.* 1995b, Niemelä *et al.* 1994, Niemelä *et al.* 1995, Halsted *et al.* 1993, Holstege *et al.* 1994, Hartley & Petersen 1997). Koskinas *et al.* (1992) demonstrated the existence of a 200 kD protein in the cytosolic liver fraction which appeared to be a preferential target for acetaldehyde modification, and Behrens *et al.* (1990) reported a similar cytosolic adduct which was thought to be a condensate with procollagen type I. There is evidently an association between the existence of this adduct and parameters of liver disease activity in human patients (Svegliati-Baroni *et al.* 1994). The influence of acetaldehyde on fibrogenesis may be associated with transcriptional activation of collagen synthesis (Holt *et al.* 1984, Brenner & Chojkier 1987, Niemelä *et al.* 1990c, Casini *et al.* 1993, Parés *et al.* 1994). Ma *et al.* (1997) have suggested that accelerated collagen production by liver stellate cells is stimulated in part by acetaldehyde adduct formation on the carboxyl-terminal propeptide of procollagen, which uncouples the normal feedback regulation of collagen synthesis by the propeptide and causes collagen accumulation. Similar interference by acetaldehyde-modified proteins with the feedback inhibition system has been suggested in the interleukin (IL)-2 secretion system of alcohol consumers, resulting in immunobiological changes (Braun *et al.* 1995). Acetaldehyde-protein conjugates may also influence blood clotting (Koterba *et al.* 1995) and lead to ethanol-associated gastric injury (Salmela *et al.* 1997). In addition, Lin *et al.* have demonstrated the existence of a stable cytotoxic adduct of Δ2-3-ketosteroid 5β-reductase (37 kD, a key enzyme in bile acid synthesis) in cytosolic fractions prepared

from the livers of ethanol-fed rats or from isolated hepatocytes cultured in the presence of ethanol (Lin *et al.* 1988, Zhu *et al.* 1996, Lin *et al.* 1998). The ethanol-inducible microsomal enzyme *CYP*2E1 has correspondingly been found to form adducts with metabolic derivatives of ethanol *in vivo* (Behrens *et al.* 1988c, Clot *et al.* 1996).

Acetaldehyde-protein adducts have been observed microscopically in the centrilobular region of the liver in the early phase of ALD in both human alcohol abusers and experimental animals (Niemelä *et al.* 1991a, Halsted *et al.* 1993, Niemelä *et al.* 1994, Holstege *et al.* 1994, Niemelä *et al.* 1995, Paradis *et al.* 1996b). Adducts are present even when excessive alcohol consumption has led to no obvious clinical, biochemical or histological signs of alcoholic liver disease, although the staining is more widespread at the advanced stages of ALD (Niemelä *et al.* 1991a, Niemelä *et al.* 1994, Niemelä *et al.* 1995, Halsted *et al.* 1993). Acetaldehyde adducts can form around both the portal and perivenular areas of the liver, but the latter seems to be exposed to a higher concentration after ethanol intake, since acetaldehyde-protein adducts appear predominantly in the perivenous zone after short-term ethanol exposure (Yokoyama *et al.* 1993a, Lin *et al.* 1993c). Holstege *et al.* (1994) have shown that the prognosis for alcoholic patients is related to the presence of sinusoidal acetaldehyde adducts, and acetaldehyde adducts were similarly found to be most abundant in those experimental animals which showed withdrawal symptoms, indicating that individual high blood alcohol levels may account for adduct positivity (Niemelä *et al.* 1994). Acetaldehyde-protein adducts have also been detected in Ito cells by immunohistochemical staining, and as these cells are the main effectors of liver fibrosis, the finding supports the possible involvement of such adducts in liver fibrogenesis (Paradis *et al.* 1996b).

2.4.4. Other alcohol-associated adducts

It has been proposed that adduct formation may also be of pathogenic importance for alcoholic and other liver diseases with respect to aldehydic products of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Houglum *et al.* 1990, Niemelä *et al.* 1994, Niemelä *et al.* 1995). MDA is a highly reactive dialdehyde generated during non-enzymatic peroxidation of unsaturated lipids, from lipid peroxidation that occurs during phagocytosis by monocytes and as a by-product of arachidonic acid metabolism (for reviews, see Esterbauer *et al.* 1991, Niemelä 1998). The free radical-mediated oxidation of long-chain polyunsaturated fatty acids leads to the production of HNE, which can react with the sulfhydryl groups of proteins through a Michael addition type of mechanism (Palinski *et al.* 1990; for reviews, see Esterbauer *et al.* 1991, Stadtman 1992, Niemelä 1998). Oxidative protein modification with MDA and HNE have been demonstrated in the arterial vessel walls of atherosclerotic lesions (Palinski *et al.* 1989, Haberland *et al.* 1988; for a review, see Steinberg *et al.* 1989), in the liver specimens from patients with alcoholic liver disease, in liver biopsies from ethanol-fed micropigs and in animals with an experimental iron overload (Niemelä *et al.* 1994, Houglum *et al.* 1990, Parkkila *et al.* 1996). When a high-fat diet containing ethanol is supplemented with iron a marked potentiation of adduct formation is seen, coinciding with increased concentrations of liver-derived enzymes in the serum and progressive

histopathology (Tsukamoto *et al.* 1995). It has also been suggested that as a consequence of enhanced lipid peroxidation resulting from prolonged alcohol consumption, increased HNE levels may compromise the cellular elimination of ethanol-derived acetaldehyde and thus participate in the potentiation of alcoholic liver fibrosis (Hartley & Petersen 1997). However, according to Li *et al.* (1997), the degree of liver protein modification with HNE shows no correlation with the severity of liver disease, although such a correlation does emerge between the stage of liver injury and modification by acetaldehyde.

A significant colocalization of acetaldehyde and malondialdehyde adducts and histological tissue damage has been observed (Niemelä *et al.* 1994, Niemelä *et al.* 1995). Tuma *et al.* (1996) have further demonstrated the formation of hybrid adducts with acetaldehyde and malondialdehyde (MAA adducts). A cyclic fluorescent adduct of defined structure has been identified as the epitope recognized by a MAA adduct antibody, in addition to which the MAA adducts include other non-fluorescent products (Xu *et al.* 1997). The appearance of hydroxyethyl adducts formed in the conjugation of ethanol-derived hydroxyethyl radicals with proteins in the presence of iron has also been described recently in the liver microsomes of ethanol-fed animals (Moncada *et al.* 1994, Clot *et al.* 1995). According to Albano *et al.* (1996), there seems to be a link between the induction of *CYP*2E1 by ethanol, the formation of hydroxyethyl radicals, the stimulation of lipid peroxidation and the onset of alcohol-related liver injury.

Alcohol drinking may also result in the formation of DNA adducts of acetaldehyde, lipid peroxidation products and reactive oxygen species. This may be associated with the carcinogenic effect of ethanol. (For a review, see Brooks 1997). Fang & Vaca (1997), for instance, report the presence of acetaldehyde adducts in granulocytic and lymphocytic DNA from alcoholic patients and were able to measure them by 32P-postlabelling using reversed-phase HPLC with on-line detection of radioactivity. The same investigators had detected these adducts earlier in liver DNA from alcohol-fed mice (Fang & Vaca 1995).

2.4.5. Immunogenicity of alcohol-altered proteins

Aldehyde-protein adducts and hydroxyethyl-protein condensates, have been shown to stimulate immunological responses which are detectable in blood (Fleisher *et al.* 1988, Izumi *et al.* 1989, Israel *et al.* 1986, Lung *et al.* 1990, Israel *et al.* 1992, Wehr *et al.* 1993, Teare *et al.* 1993, Niemelä *et al.* 1987, Niemelä *et al.* 1994, Niemelä *et al.* 1995, Koskinas *et al.* 1992, Worrall *et al.* 1991, Worrall *et al.* 1994, Hoerner *et al.* 1988, Yokoyama *et al.* 1993b, Yokoyama *et al.* 1995b, Lin *et al.* 1995b, Moncada *et al.* 1994, Clot *et al.* 1995, Clot *et al.* 1996, Albano *et al.* 1996). Chronic administration of ethanol to animals has been shown to lead to the generation of circulating immunoglobulins with anti-adduct specificity, and such antibodies have been found in sera from patients with alcoholic hepatitis or cirrhosis, but also in sera from patients with non-alcoholic liver disease (Israel *et al.* 1986, Niemelä *et al.* 1987, Hoerner *et al.* 1988, Izumi *et al.* 1989, Worrall *et al.* 1991, Worrall *et al.* 1994, Worrall *et al.* 1996, Koskinas *et al.* 1992). Furthermore, a proportion of patients with alcoholic heart muscle disease have been shown to develop cardiac protein-acetaldehyde adducts and antibodies against them

(Harcombe *et al.* 1995). Lung *et al.* (1990) reported that rabbits immunized with MDA-albumin conjugate produce high titres of IgG antibodies against the adduct structures, while human alcoholics appear also to have serum autoantibodies recognizing *CYP*2E1 hydroxyethyl radical adducts, the highest titres of which have been found in samples from patients with severe liver disease (Clot *et al.* 1995, Clot *et al.* 1996). In addition to humoral immune responses, acetaldehyde-modified structures on the cell surface have been shown to induce the generation of cytotoxic T lymphocytes specific to acetaldehyde-altered cells (Terabayashi & Kolber 1990). On the other hand, patients with severe alcoholic hepatitis apparently fail to improve after discontinuation of alcohol intake on account of a persistent cell-mediated immune dysfunction (Marshall *et al.* 1983, Mutchnick *et al.* 1990). The clinical significance of the immune responses to ethanol and acetaldehyde and the corresponding protein modifications is uncertain. According to Izumi *et al.* (1989), patients with serum antibodies to alcohol-altered liver cell membranes show severe advanced liver disease characterized by a tendency to progress with continued alcohol ingestion. Although there are many findings to support the theory that alcohol-altered proteins have a role in mediating alcoholic liver injury, it is not known for certain whether the immune responses to such proteins represent a cause or a consequence of alcoholic liver disease (for reviews, see Tuma & Klassen 1992, Klassen *et al.* 1995).

Koskinas *et al.* (1992) reported the existence of a serum IgA antibody recognizing a 200-kD cytosolic acetaldehyde adduct in patients with alcoholic hepatitis. Increased IgA responses to acetaldehyde-modified albumin epitopes also emerge in alcohol abusers, as measured from plasma samples, but the corresponding IgG or IgM responses appear to be similar to those obtained from social drinkers (Worrall *et al.* 1991, Worrall *et al.* 1996). On the other hand, hydroxyethyl adducts have been reported to trigger both IgA and IgG responses (Clot *et al.* 1995). It should be noted that the serum total IgA concentration is also known to be frequently increased in alcoholic patients (van de Wiel *et al.* 1988a, Mili *et al.* 1992, McMillan *et al.* 1997; for reviews, see Johnson & Williams 1986, Brown & Kloppel 1989, Kerr 1990). Nevertheless, Worrall *et al.* (1991) observed a lack of correlation between total serum IgA and serum anti-adduct IgA titres in a population with a wide range of total IgA concentrations, which supports the possibility that the serological IgA response in alcoholics may be antigen-driven.

According to Worrall *et al.* (1991, 1996), increased IgA reactivity with acetaldehyde-modified epitopes correlates moderately well with patients' own reports of their alcohol intake, but not with plasma transaminases, GGT activity or MCV, nor with plasma ALB, ALP or BIL. Furthermore, anti-adduct IgA reactivity shows higher sensitivities in detecting alcohol abuse among heavy drinkers, both men and women (63.3% and 53.3%, respectively), than the conventional markers (≤ 48.1% and ≤ 43.3, respectively), even though their sensitivities appear to be similar among alcoholics, about 50–70% (Worrall *et al.* 1996). Thus it seems that the anti-adduct IgA assay could provide a suitable means of detecting of heavy drinking. However, as noted above, antibodies against protein adducts produced under different conditions may bind with different epitopes (Lin *et al.* 1993b), and therefore the results of anti-adduct immunoglobulin assays must be highly dependent on the nature of the acetaldehyde adduct antigens used for coating the microtitre plates.

Sinusoidal IgA deposits and circulating IgA immune complexes have been reported in patients with alcoholic liver disease (van de Wiel *et al.* 1987a, van de Wiel *et al.* 1988a, van de Wiel *et al.* 1988b, Amano *et al.* 1988; for a review, see van de Wiel *et al.* 1987b), while mesangial IgA deposits have been found in alcoholic patients with nephropathy related to hepatocellular injury (see Amore *et al.* 1994). Once deposited, these immune complexes may lead to recruitment of inflammatory cells and macrophages to the site, cells which upon activation will release tissue-damaging mediators such as proteases and oxygen radicals (Johnson *et al.* 1994). According to van de Wiel *et al.* (1987a), IgA deposits along the liver sinusoids are seen more often in alcoholic patients (76%) than in non-alcoholic ones (12%) and seem not to be related to the serum IgA concentration or composition but may represent a distinct effect of alcohol on the liver related to the role of this organ in IgA metabolism. On the other hand, IgA deposits are not observed in any other conditions associated with high levels of serum IgA, such as IgA myeloma (for a review, see Brown & Kloppel 1989). There is evidence that circulating IgA and IgA deposits in patients with advanced stages of ALD stimulate IL-6 production and thus initiate an autoamplification process (Deviere *et al.* 1992). Simultaneously, an acute-phase response may be activated, initiating synthesis of C-reactive peptide (Castell *et al.* 1990). Attached IgA may also trigger superoxide secretion and activate monocytes to secrete fibrogenic cytotoxic factors (for a review, see Border & Noble 1994). Deviere *et al.* (1991) have shown that secretion of the inflammatory and immunoregulatory cytokine tumour necrosis factor alpha (TNFα) by peripheral blood mononuclear cells is enhanced synergistically in the presence of solid phase monomeric IgA, and it has been suggested that TNF may have a role in the reduced immune response to infections in alcoholics (Nelson *et al.* 1990, Nair *et al.* 1994).

Low or moderate alcohol consumption alone seems not to affect serum total IgG or IgM concentrations (McMillan *et al.* 1997), but it has been claimed that IgM concentrations increase along with alcohol consumption, whereas IgG levels decrease (Mili *et al.* 1992). By contrast, Drew *et al.* (1984) found that plasma IgG concentrations are similar in controls and alcoholic patients, whereas ALD patients appear to have higher concentrations than do alcoholics without any evidence of liver damage. The relative increase in IgG synthesis nevertheless appears to be lower than that of IgA in cultured peripheral blood mononuclear cells from alcoholics (Drew *et al.* 1984). IgG antibodies are known as the primary mediators of a variety of harmful immunological consequences, including activation of the complement system, and both IgG and IgM antibodies are capable of inducing cytotoxic reactions affecting cell surfaces or connective tissues (for reviews, see Johnson & Williams 1986, Israel *et al.* 1988, Brown & Kloppel 1989, Zettermann 1990).

3. Purpose of the research

The purpose of the present research was to examine the diagnostic properties of different CDT methods as markers of alcohol abuse and the existence of serum antibodies against acetaldehyde-derived adducts in heavy drinkers with or without liver disease. More specifically the aims were as follows:

1. to study the clinical usefulness of serum CDT measurements in a large population of alcohol abusing patients with or without liver disease,
2. to compare the sensitivities and specificities of CDT determined by the different methods as a marker to study the effect of transferrin variation on the specificity and sensitivity of CDT as measured by the different methods,
3. to apply an enzyme-linked immunosorbent assay (ELISA) technique for detecting serum antibodies against acetaldehyde-derived epitopes, and
4. to clarify the association between antibodies against acetaldehyde-derived epitopes, alcoholic liver disease and alcohol consumption.

4. Materials and methods

4.1. Subjects

The characteristics of the subjects examined in Papers I–IV are presented in Table 2. Many of them were included in more than one of the papers. All the serum and biopsy samples were taken for routine diagnostic purposes and the research was conducted according to the provisions of the Declaration of Helsinki. Serum samples were stored at –70 °C until analysed.

Table 2. Subjects evaluated in Papers I–IV.

Paper	Subjects	n	Sex M/F	Alcohol consumption /week
I	ALD-patients	173	128/45	560–1500 g
	Heavy drinkers without liver disease	200	166/34	250–3400 g
	Controls (healthy volunteers)	42	27/15	0–210 g
II	ALD-patients	20	10/10	560–1500 g
	Heavy drinkers without liver disease	63	48/15	250–1000 g
	Controls:	89	30/59	0–210 g
	Healthy volunteers	36	23/13	
	Hospitalized patients with non-alcoholic liver disease	5	0/5	
	Hospitalized patients with iron deficiency	19	7/12	
	Pregnant women	29	–/29	
III	ALD-patients with cirrhosis	22	13/9	250–1000 g
	Heavy drinkers with (n = 47) or without liver disease (n = 21)	68	51/17	250–1000 g
	Controls:	114	16/56	0–210 g
	Healthy volunteers	42	20/22	
	Hospitalized patients with non-alcoholic liver disease	15	3/12	
	Hospitalized patients with iron deficiency	20	8/12	
	Hospitalized patients with low serum transferrin	12	5/7	
	Pregnant women	25	–/25	
IV	ALD-patients	86	62/24	560–1500 g
	Heavy drinkers without liver disease	54	37/17	250–2300 g
	Controls:	64	39/25	0–95 g
	Healthy volunteers	35	26/9	
	Hospitalized patients with non-alcoholic liver disease	19	10/9	
	Myeloma patients (IgA-type, n = 5; IgG-type, n = 5)	10	3/7	

ALD, alcoholic liver disease; Ig, immunoglobulin

Alcohol abusers both with and without liver disease were examined, the majority of the patients with alcoholic liver disease being enrolled for monitoring purposes at a specialized liver clinic at the Addiction Research Foundation, Toronto. These subjects had a history of either regular ethanol consumption in amounts exceeding 80 g/day, or repeated prolonged inebriations over a period of at least 5 years. The Combined Clinical and Laboratory Index (CCLI) and/or the Combined Morphological Index (CMI) were used to assess the severity of liver disease. The biopsy series included patients with minimal fibrosis or fat and some with cirrhosis, and covered the full range of morphological abnormalities related to alcoholic hepatitis. A small number of alcohol abusers with liver disease were patients hospitalized at the Central Hospital of Southern Ostrobothnia who had clinical and laboratory evidence of an early stage of alcoholic liver disease, or biopsy data indicating liver disease (III).

The alcohol abusers with no clinical or laboratory evidence of liver dysfunction were cases admitted for detoxification with a history of severe alcohol dependence, hospitalized patients, outpatients, or participants in volunteer health screening programmes with a history of heavy drinking (>250 g/week). All of these were alcohol abusers in terms of both their case history and the clinical examinations.

The control subjects used to determine the specificity of the various markers were either abstainers or social drinkers, including healthy volunteers, and also non-drinking patients with abnormalities in iron balance (II, III) or non-alcoholic liver disease (II–IV), pregnant women (II, III), or myeloma patients (IV). All of these had an alcohol consumption of less than 30 g per day, as confirmed by questionnaires and interviews on important collaterals, and the drinking of alcohol had not induced any disabilities in social or occupational functioning, nor did they have any medical or social records of alcohol-related hospital admissions or disorderly behaviour.

4.2. Methods

4.2.1. CDT analyses

Three methods were used here for performing the CDT analyses. Firstly, CDT was measured by anion exchange chromatography followed by radioimmunoassay using a commercially available assay kit (CDTect, Pharmacia & Upjohn, Uppsala, Sweden) according to the manufacturer's instructions (I–IV). This procedure separates out serum transferrin isoforms with pI values higher than 5.7 in a microcolumn, with minor amounts of isotransferrin with pI values of 5.7 also included (Stibler *et al.* 1991). The eluted transferrin fraction, which is deficient in its carbohydrate moieties, is subsequently quantified by a radioimmunoassay in which the CDT in the eluate competes with ^{125}I-labelled transferrin for antibody binding sites. Bound and free transferrin are separated by the addition of a second antibody immunoadsorbent, followed by centrifugation and decanting. The radioactivity measured in the pellet is inversely proportional to the quantity of CDT in the sample. The reference range in this assay is 0–20 U/litre for men and 0–26 U/litre for women.

The second method used for CDT measurements (II, III) was the Axis %CDT radioimmunoassay (%CDT RIA, Axis Biochemicals AS, Oslo, Norway), in which serum transferrin is radiolabelled with antibody fragments before separating out the transferrin variants with 0-2 sialic acid residues on an ion exchange chromatography minicolumn. Since the transferrin from the serum is present in excess of the transferrin-binding ^{125}I-labelled antibody fragments, the labelled antibodies should be distributed between the transferrin variants according to the proportions of the latter. The quantity of labelled antibody-transferrin complexes eluted is expected to be independent of the total transferrin concentration in the serum, and the proportion of CDT (%CDT) is obtained by measuring the radioactivity of the eluted fraction with a Gamma counter and interpolating it on the standard curve for %CDT. In this procedure amounts exceeding 2.5% are considered elevated.

In Paper III, CDT was analyzed by the Axis %CDT turbidimetric immunoassay (%CDT TIA, Axis Biochemicals AS, Oslo, Norway), in which serum transferrin is first saturated with Fe^{3+} before separating out the low sialic acid transferrin (CDT) on an ion exchange chromatography minicolumn (Bean *et al.* 1997). The %CDT TIA method measures asialylated, monosialylated and disialylated serum transferrin isoforms and 50% of the trisialylated ones (Bean *et al.* 1997). The CDT content of the eluate and the total transferrin content of the Fe^{3+}-saturated serum sample are measured separately by a turbidimetric method using the same anti-transferrin antibodies. The measurements are evaluated using a calibration curve, and the %CDT is calculated. According to the manufacturer, amounts exceeding 6% should be considered elevated. A Kone Optima Analyzer (Kone Instruments, Espoo, Finland) was used for the measurements.

4.2.2. *Transferrin analyses*

Serum total transferrin concentrations were assayed with the Array® Protein System (Beckman Instruments, Inc., USA) which measures nephelometrically the rate of light-scatter formation resulting from an immunoprecipitin reaction with the protein (II, III). The reference range for transferrin is 1.7–3.4 g/l. The method is not affected by the degree of transferrin desialylation.

4.2.3. *Collagen markers*

The concentrations of the carboxyterminal propeptide of type I procollagen (PICP), PIIINP and the basement membrane-related components, TIV and laminin were measured radioimmunologically for the work reported in Paper I, and also in Paper IV in the case of PIIINP. The PICP and PIIINP assays were equilibrium radioimmunoassays based on the use of human standard antigens (Melkko *et al.* 1990, Niemelä 1985, Risteli *et al.* 1988), and the TIV and laminin assays were sequential radioimmunoassays of the saturation type based on the use of polyclonal antibodies against the 7-domain of TIV

and the laminin fragment P1 (Niemelä *et al.* 1990b). The upper normal limits for healthy subjects in these analyses (mean + 2SD) were: PICP, 170 μg/l for females and 202 μg/l for males; PIIINP, 4.5 μg/l; TIV collagen, 8 μg/l; and laminin, 90 μg/l.

4.2.4. Preparation of erythrocyte proteins

Human erythrocyte protein (haemoglobin) was prepared using EDTA-blood from a teetotaler as the starting material (IV). The cells were separated from the plasma by centrifugation and washed three times with an equal volume of phosphate-buffered saline (PBS: 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), pH 7.4. The washed cells were lysed with polyoxyethylene ether, 0.1% V/V in borate buffer (Hemolysis Reagent, DIAMAT^{TM} Analyzer System, Bio-Rad), and incubated for 35 minutes at 37°C to remove the unstable Schiff bases. Finally, the haemolysate was brought to a haemoglobin concentration of 12 mg/ml with PBS.

4.2.5. Preparation of acetaldehyde-derived conjugates

Conjugates prepared by incubation of proteins with acetaldehyde in the presence or absence of a reducing agent (IV), are referred to as “acetaldehyde-derived adducts“ (Moncada *et al.* 1994).

4.2.5.1. Reduced epitopes

Acetaldehyde (Ach) in PBS was added to aliquots of freshly prepared haemoglobin (Hb) and bovine serum albumin (Alb) solutions, both containing 12 mg protein/ml, to obtain final acetaldehyde concentrations of 10 mM. The mixture was allowed to react in tightly sealed containers at +4°C overnight (18 h). Samples representing the unmodified proteins were prepared and treated in the same way as those of the modified proteins except for the addition of acetaldehyde. Protein adducts were reduced by adding sodium cyanoborohydride to 10 mM and mixing for 5 hours at +4°C. All the protein solutions were dialyzed twice against PBS at +4°C and stored in small aliquots at –70°C for use on one occasion only.

4.2.5.2 Non-reduced epitopes

Haemoglobin and albumin protein solutions were treated with acetaldehyde as described above (4.2.5.1), except for the final concentration of acetaldehyde in the albumin solution (250 mM). In addition, the reaction time at +4°C was prolonged to 36 hours. Directly after this, the protein solutions were dialyzed twice against PBS at +4°C. Protein samples without any acetaldehyde addition were prepared and treated similarly. All protein solutions were stored in small aliquots at –70°C for use on one occasion only.

4.2.6. ELISA for measurement of antibody titres

The microtitre plates (Nunc-Immuno Plate, Maxisorb™, InterMed, Denmark) were coated with acetaldehyde-modified haemoglobin (Hb-adduct or Ach-Hb), bovine serum albumin (Alb-adduct or Ach-Alb), or corresponding unmodified proteins (background) in PBS (3 µg protein in 100 µl/well) and incubated for 1 hours at +37°C. Non-specific binding was blocked by incubation with 0.2% gelatin in PBS (150 µl/well) for 1 hour at +37°C. The sample sera were serially diluted in PBS containing 0.04% Tween-20 (PBS-Tween). Final volumes of 50 µl of each serum dilution were allowed to react with the coated proteins for 1 h at +37°C, followed by extensive washing with PBS-Tween. Alkaline phosphatase-linked goat anti-human IgA, IgG or IgM (dilutions 1:3000, 1:1500 and 1:3000, respectively) (Jackson Immuno Research Laboratories, Inc., Pennsylvania) was added to label the desired antibody-antigen complexes (50 µl/well). The immunoglobulins were diluted in PBS-Tween containing 8 mM $MgCl_2$ and a small amount of dithiothreitol (DTT). The plates were then incubated at +4°C overnight. After washing the plates, 100 µl of p-nitrophenylphosphate-solution was added as a colour reaction substrate (Alkaline Phosphatase Substrate Kit, Bio-Rad Laboratories, Hercules, CA). The reaction was allowed to proceed for standard periods of time as follows: 8 minutes for Hb-IgM conjugates, 15 minutes for Hb-IgG conjugates, 23 minutes for Hb-IgA conjugates, 5 minutes for Alb-IgG conjugates, 7 minutes for Alb-IgM conjugates and 16 minutes for Alb-IgA conjugates. The colour reactions were stopped by adding 100 µl of 0.4 M NaOH and the optical densities were read at 405 nm with an Anthos HTII microplate reader (Anthos Labtec Instruments, Salzburg, Austria).

4.2.7. Other analyses

The concentrations of TNFα and IL-6 in Paper IV were determined using high sensitivity ELISA kits which employ the quantitative "sandwich" enzyme immunoassay technique (Biotrak, Amersham International plc, Buckinghamshire, England). The total (non-specific) IgA and IgG measurements in the same paper were performed using the Array® Protein System (Beckman Instruments, Inc., USA), which measures nephelometrically the rate of light-scatter formation resulting from an immunoprecipitin reaction with the proteins.

Serum ALB, BIL, ALP, GGT, ALT, AST and MCV were determined using established clinical chemical methods as indicated in the original publications.

4.3. Calculations and statistical methods

Values are expressed as means ± SD unless otherwise indicated. Differences were considered statistically significant at $p < 0.05$. Student's t-test was used to analyze the differences between two groups in Paper I. The data on CDT values in Papers II and III were subjected to logarithmic transformation to yield normal, non-skewed distributions. Square root transformation was used for the same purpose in Paper IV in the case of the difference scores obtained by subtracting the immunoassay values (OD_{405}) for the

reaction with the serum sample and the unconjugated protein from the corresponding values measured for the reaction between the serum sample and the acetaldehyde-protein conjugate. One-way analyses of variance (ANOVA) were performed on the transformed values, followed by Bonferroni's multiple comparisons procedure to test for statistical differences among pairs of groups, as indicated in the original publications. Spearman's rank-correlation test or linear regression analysis was used to calculate correlations between variables, as indicated in the original publications. The abbreviations r and r_S stand for the correlation coefficients for linear regression analysis and Spearman's rank-correlation test, respectively. Additionally, the Bland-Altman plot (Bland & Altman 1986) was used to monitor the agreement between the methods in Paper III.

The 95% confidence intervals indicated in Paper III for observed indices were estimated (P ± 1.96 × (SE), where SE = $\sqrt{P(1-P)/n}$, where n >30 and P is the specificity or sensitivity), or else the exact confidence ranges were calculated according to Armitage and Berry (1994) when appropriate. The receiver-operating characteristic (ROC) plot areas ± SE and the differences between the areas were calculated as recommended by Hanley *et al.* (1982) and Hanley & McNeil (1983).

5. Results

5.1. Relationships of alcohol consumption and the severity of alcoholic liver disease to serum CDT values obtained with CDTect (I)

The concentrations of CDT as measured by the CDTect were found in Paper I to be significantly higher in both the alcohol abusers with liver disease (mean ± 2SE: 29.8 ±3.0 U/l; $p < 1 \times 10^{-15}$) and those without liver disease (20.9 ± 2.3 U/l; $p < 1 \times 10^{-5}$) than in the healthy controls (14.6 ± 1.3 U/l). The sensitivity of CDT for detecting alcohol abuse in the total population of 200 heavy drinkers without any significant liver disease and with an ethanol intake of 710 ± 80 g during the week prior to sampling in comparisons with healthy non-drinking controls was 34% and the specificity 100%. The sensitivity of MCV in a similar comparison was 34 %, whereas GGT was elevated in 47 % of these subjects. Based on the present control material, however, the specificities of GGT and MCV were 97% and 94%, respectively. Further comparisons of assay sensitivities in the case of heavy drinkers pointed to a significant effect of sex, in that MCV was the most sensitive marker in females, reaching 62%, while CDT showed a better sensitivity for detecting alcohol abuse in men (36 %) than in women (27 %). The most sensitive marker of alcohol abuse in men was serum GGT (47%). However, when the patients who had been admitted for detoxification with a recent alcohol intake of 1160 ± 180 g per week (mean ± 2SE) and a history of severe dependence were selected from among the group of heavy drinkers without liver disease for separate analysis, the sensitivity of CDT in this subgroup markedly improved relative to the other markers. The overall sensitivity of CDT in this sample (64%) was higher than that of GGT (55%) or MCV (39%), although the latter continued to be the most sensitive marker among women. The correlation between the amount of alcohol consumed and marker levels was clearly higher for CDT ($r = 0.332$, $p < 0.001$) than for GGT ($r = 0.185$, $p < 0.01$) or MCV ($r = 0.105$, not significant). There was no correlation between CDT and GGT ($r = -0.014$), or between CDT and MCV ($r = 0.001$).

The CDT values of the alcoholics with liver disease were significantly higher than those of the group without such disease ($p < 0.001$), even though CDT was increased relative to the healthy controls only in 60% of the alcoholics with liver pathology whereas the markers of fibrogenesis (PIIINP, type IV collagen and laminin), which were measured for

comparison, were elevated significantly more often (90 %, 80 %, and 80 %, respectively). Increased PICP was found in 50 % of the liver disease patients. There was a significant correlation between the individual values for all the connective tissue markers, whereas no significant correlations emerged between CDT and collagen markers.

For additional comparisons, the alcoholics were then divided according to the laboratory and clinical indices of disease severity. The most consistently elevated CDT values were observed in those with CCLI scores between 1–5, being significantly higher than those in the group with CCLI = 0. On the other hand, the group representing the highest CCLI scores, 6–12, did not differ significantly from those with CCLI = 0. This observation was also in line with a separate analysis of 44 alcoholics with liver disease verified by biopsy, 40 of whom had cirrhosis. When the CDT values were compared with histological indices of disease severity (CMI), the highest values were seen in the patients with mild to moderate liver damage, whereas those with the most severe abnormalities had significantly lower values ($p < 0.02$). There was a significant inverse correlation between CDT and the morphological index of disease severity ($r_S = -0.315$, $p < 0.05$), whereas by contrast, the collagen markers increased with disease severity (I). The ALD patients also showed a significant negative correlation between CCLI indices and total transferrin concentrations ($r_S = -0.599$, $p < 0.01$) and a significant positive correlation between CCLI indices and the calculated CDTect/total transferrin ratios ($r_S = 0.527$, $p < 0.05$) (II).

5.2. Comparisons between the characteristics of the CDT methods

5.2.1. %CDT RIA and CDTect (II)

There was a significant correlation between the results obtained using the CDTect and %CDT RIA ($r = 0.629$, $p < 0.001$), which then improved significantly ($p < 0.05$) when the CDTect values were modified by calculating the ratios of CDTect/total transferrin ($r = 0.770$, $p < 0.001$).

The amounts of CDT (means ± SD) in the total series of alcohol abusers, analyzed either with the CDTect (29.2 ± 18.1 U/l) or with %CDT RIA (2.2 ± 2.2 %), differed significantly from those in the controls (19.0 ± 7.3 U/l, $p < 0.001$ for CDTect and 0.1 ± 0.0 %, $p < 0.001$ for %CDT RIA). Also, when similar comparisons were made separately between the subgroups of alcohol abusers, i.e. the ALD patients and the heavy drinkers without liver disease, these were found to differ significantly in their mean values in the %CDT RIA and CDTect tests, as well as differing from the control subjects. On the other hand, comparison between the heavy drinkers without liver disease and the controls, further distinguished by sex, yielded differences in both the males ($p < 0.001$) and females ($p < 0.01$) only by the %CDT RIA method. In CDTect the difference was significant only for the males ($p < 0.001$), whereas there was no difference between the female heavy drinkers and controls.

The overall sensitivity of CDTect for detecting alcohol abusers was markedly higher than that of %CDT RIA, 59% and 34%, respectively, when the cut-off limits recommended by the manufacturers were used (Tables 3a and 3b). Calculation of the ratio of CDTect to

total transferrin yielded similar distributions to those obtained by the %CDT RIA method, but this ratio was also found to lead to a lower sensitivity for detecting alcohol abusers than with CDTect alone (Tables 3a and 3b).

Table 3a. Sensitivities (% of true positives) of CDTect, %CDT RIA and the ratio of CDTect/ total transferrin as markers of alcohol abusers.

Patient group	CDTect		%CDT RIA		CDTect/ Total Transferrin	
	Sensitivity	n	Sensitivity	n	Sensitivity	n
All alcohol abusers together	59 %	83	34 %	83	45 %	83
Women	64 %	25	32 %	25	56 %	25
Men	57 %	58	34 %	58	40 %	58
ALD-patients	90 %	20	70 %	20	85 %	20
Women	80 %	10	50 %	10	80 %	10
Men	100 %	10	90 %	10	90 %	10
Heavy drinkers	49 %	63	22 %	63	32 %	63
Women	53 %	15	20 %	15	40 %	15
Men	48 %	48	23 %	48	29 %	48

Table 3b. Specificities (100% – % of false positives) of CDTect, %CDT RIA and the ratio of CDTect/ total transferrin as markers of alcohol abusers.

Control group	CDTect		%CDT RIA		CDTect/ Total Transferrin	
	Specificity	n	Specificity	n	Specificity	n
All controls together	81 %	89	100 %	29	99 %	53
Women	78 %	59	100 %	24	99 %	46
Men	87 %	30	100 %	5	100 %	7
Control subjects with normal or low transferrin	88 %	26	100 %	14	96 %	26
Control subjects with high transferrin	48 %	27	100 %	15	100 %	27

5.2.2. %CDT TIA and CDTect (III)

The precision values obtained for CDTect and %CDT TIA in Paper III when analyzing samples of pooled patient sera with low and high CDT concentrations are presented in Table 4. Analyses of the within-run and day-to-day precisions of the CDTect assay in Paper I, as determined for samples representing CDT concentrations between 10 and 60 U/l, yielded coefficients of variation (CV) of 11% (n = 20) and 15% (n = 20), respectively.

The mean %CDT TIA values in the alcohol abusers and the healthy controls were 5.4 ± 2.5% and 2.6 ± 0.8% (mean ± SD), respectively, with corresponding values of 27.5 ± 13.8 U/l and 11.5 ± 3.6 U/l for CDTect. The differences were significant in both of

the above comparisons. There were no sex differences in either the %CDT TIA or CDTect values among the alcohol abusers, but the CDTect values for the women were significantly higher among the healthy controls ($p < 0.01$). The mean %CDT TIA and CDTect values for the non-drinking hospitalized patients were 3.0 ± 0.9% and 19.9 ± 8.9 U/l, respectively, both significantly higher than for the healthy controls.

Table 4. Mean CDT values and precisions determined in serum pools of low and high CDT content, as obtained for CDTect and %CDT TIA (III).

CDT content of serum pool (High/Low)	Method	Within-day variation			Day-to-day variation		
		n	Mean of CDT determinations	Precision (CV)	n	Mean of CDT determinations	Precision (CV)
High	CDTect	10	33.6 U/l	10%	9	32.6 U/l	12%
High	%CDT TIA	10	4.6%	4.8%	9	5.2%	8.6%
Low	CDTect	10	14.7U/l	6.2%	9	13.1 U/l	22%
Low	%CDT TIA	13	3.5%	3.5%	9	3.9%	7.0%

CV = Coefficient of variation, (SD/mean) x 100%

The slope and intercept of linear regression between the CDTect and %CDT TIA results (with 95% confidence limits, n = 192) were 0.13 (0.12–0.15) and 1.16 (0.73–1.59), respectively. The sy|x was 1.51 and the correlation coefficient 0.744. Difference plotting of the %CDT TIA results and the scale transformed CDTect/transferrin ratios pointed to considerable disagreement between the CDTect and %CDT TIA results (see Figure 3 in Paper III). The %CDT TIA method showed a significantly higher correlation with CDTect than did the %CDT RIA method ($r = 0.629$, n = 112, see above, $p < 0.05$). The CDTect and the %CDT TIA results were also compared by ROC analysis, analysing the results for the sexes separately. For the men the area under the curve (mean ± SE) was significantly higher ($p < 0.05$) for CDTect (0.990 ± 0.009) than for %CDT TIA (0.941 ± 0.025, $p < 0.05$), whereas no significant differences were found for the women on the basis of the results obtained from the healthy controls and alcohol abusers (0.923 ± 0.040 and 0.901 ± 0.045, respectively). The area under the ROC curve of the CDTect results for the men was significantly greater than that for the women ($p = 0.05$), whereas no significant sex differences were found in %CDT TIA.

The sensitivities and specificities of the methods for detecting alcohol abuse, when based on the cut-off limits recommended by the manufacturers or on the healthy control group described in Paper III (mean + 2 SD) are presented in Tables 5a and 5b.

Table 5a. Sensitivities (% of true positives) obtained for CDTect and %CDT TIA with cut-off limits recommended by the manufacturers[1] or based on the healthy control group[2] (III).

Subjects	Cut-off limits recommended by the manufacturers[1]				Cut-off limits based on the healthy + control group[2]			
	CDTect Sensitivity	n	%CDT TIA Sensitivity	n	CDTect Sensitivity	n	%CDT TIA Sensitivity	n
Alcohol abusers	59%	90	29%	90	86%	90	61%	90

[1]Cut-off limits for CDTect: 20 U/l (males) and 26 U/l (females); and for %CDT TIA: 6.0%
[2]Cut-off limits for CDTect: 14 U/l (males) and 20 U/l (females); and for %CDT TIA: 4.2%

Table 5b. Specificities (% of true negatives) obtained for CDTect and %CDT TIA with cut-off limits recommended by the manufacturers[1] or based on the healthy control group[2] (III). Specificities are given for healthy controls and hospitalized controls including patients with increased or decreased serum transferrin concentration, pregnant women, and NALD patients.

Subjects	Cut-off limits recommended by the manufacturers[1]				Cut-off limits based on the healthy control group[2]			
	CDTect Specificity	n	%CDT TIA Specificity	n	CDTect Specificity	n	%CDT TIA Specificity	n
Healthy controls	100%	42	100%	42	95%	42	98%	42
Hospitalized controls	71%	72	100%	60	53%	72	88%	60

[1]Cut-off limits for CDTect: 20 U/l (males) and 26 U/l (females); and for %CDT TIA: 6.0%
[2]Cut-off limits for CDTect: 14 U/l (males) and 20 U/l (females); and for %CDT TIA: 4.2%

5.3. CDT results and serum transferrin variation (II, III)

It is observed in Papers II and III that the serum transferrin concentration has a significant influence on CDT values. The CDTect assay in particular appears to be affected by serum transferrin concentrations in both alcohol consumers with or without liver disease and control subjects, whereas %CDT methods do not show such variation as clearly (see Papers II and III). According to Paper II, CDTect correlated with serum transferrin in the alcohol abusers ($r = -0.240$, $p < 0.05$) and still more obviously in the control group ($r = 0.727$, $p < 0.001$). Comparison of serum transferrin and %CDT RIA gave a significant inverse correlation ($r = 0.302$, $p < 0.01$). Likewise the CDTect results in Paper III were found to correlate significantly with serum transferrin, the coefficient in the total series being 0.239 ($n = 192$, $p < 0.001$), while that for the women ($r = 0.425$, $n = 104$, $p < 0.001$) was significantly higher ($p < 0.05$) than for the men ($r = 0.098$, $n = 100$, not significant). As before, there was a particularly close correlation ($p < 0.001$) between the serum transferrin and CDTect results in the subgroups of non-drinking hospitalized patients and healthy controls (III) and serum transferrin was also found to correlate with the %CDT TIA results ($p < 0.05$) in the subgroups of alcohol abusers, non-drinking hospitalized controls and healthy controls, although not in the total series (Table 6). The correlation of %CDT RIA with serum transferrin ($r = -0.302$, $n = 112$, $p < 0.01$) was slightly higher than that of %CDT TIA ($p = 0.07$).

Table 6. Correlation (r) of %CDT TIA and CDTect results with serum transferrin concentrations in alcohol abusers and controls.[1]

Subjects	%CDT TIA vs. Total transferrin			CDTect vs. Total transferrin		
	r	n	p	r	n	p
Total	–0.132	192	<0.1	0.224	204	<0.001
Women	–0.044	94	n.s.	0.425	104	<0.001
Men	–0.123	98	n.s.	0.098	100	n.s.
Alcohol abusers	–0.248	90	<0.05	–0.032	90	n.s.
Hospitalized non-drinking patients	0.274	60	<0.05	0.774	72	<0.001
Healthy controls	–0.297	42	<0.05	0.546	42	<0.001

[1]The results are given for the total series and separately for women and men, and the subgroups.
n.s. not significant

As expected, the variation in transferrin also influences the diagnostic performance of the CDT methods. The specificities of %CDT RIA and CDTect when based on control data that included patients with increased serum transferrin were 100% and 81% (87% in males and 78% in females), respectively. However, the CDTect values were elevated in nine out of the nineteen patients in the control group who had iron deficiency (47%), in five of the eight pregnant women in the third trimester (63%), in two of the twenty-one pregnant women in the first trimester (10%) and in one of the five patients with non-alcoholic liver disease (20%). Serum transferrin was also abnormally high in 14 of these 17 false positive controls (82%). When the assessment was based on this subgroup of control subjects with elevated serum transferrin, the specificity of CDTect was only 48% (II). Considering the total group of hospitalized patients evaluated in Paper III, including cases with decreased and increased transferrin, pregnant women and NALD patients, the specificities of %CDT TIA and CDTect were 100% and 71%, respectively (Tables 5a and 5b). Although both methods improved in sensitivity when cut-off limits based on the present healthy control group (mean + 2 SD) were used (Tables 5a and 5b), their specificities with regard to the hospitalized non-drinkers decreased simultaneously to 88% for %CDT TIA and 53% for CDTect. The more profound ROC analyses of the total series of women (III), including patients with high transferrin, gave an area under the curve (mean ± SE) which was significantly higher ($p < 0.05$) for %CDT TIA (0.861 ± 0.049) than for CDTect (0.740 ± 0.061), whereas no significant differences were found in the total series of men, including hospitalized non-drinkers (0.921 ± 0.027 and 0.899 ± 0.031, respectively). These areas for both methods and both sexes were slightly lower than those obtained when alcohol abusers were contrasted with healthy controls (see Section 5.2.2.).

Significant discrepancies were noted between the individual values measured by the different CDT methods, this being consistently so with patients having abnormal serum transferrin. The group of alcohol abusers considered in Paper II included 24 subjects (6 of whom had alcoholic liver disease) who were correctly classified by the CDTect method but not by %CDT RIA, their mean transferrin concentration (3.15 ± 0.72 g/l, mean ± SD) being close to the upper normal limit (3.4 g/l). On the other hand, there were 3 patients (2 of whom had alcoholic liver disease) for whom the CDTect method yielded normal values while %CDT RIA showed increased concentrations. In these cases, serum

transferrin concentrations were low (1.88 ± 0.17 g/l). Nine alcohol abusers with increased serum transferrin described in Paper III gained false negative results, six in %CDT TIA (66%) and three in CDTect (33%) when the cut-off limits set by the manufacturers were used, whereas 21 out of 35 non-drinking patients with increased serum transferrin (60%) gained elevated (false-positive) values in CDTect but none in %CDT TIA (III).

5.4. Antibodies against acetaldehyde-derived epitopes in the serum of heavy drinkers with or without liver disease

5.4.1. Antibodies against Ach adducts

Significant differences in the titres of anti-Ach adduct antibodies were seen between the non-drinkers and alcohol abusers with or without liver disease in Paper IV, as presented in Tables 7a and 7b, where mean anti-adduct IgA, IgG and IgM titres and incidences of titres exceeding the upper normal limits are given for the various groups of subjects. The cut-off values were the means + 2SD of the values obtained for healthy controls. The anti-adduct IgA titres, as analyzed against either reduced albumin or erythrocyte protein (Hb) condensate, were significantly higher in the alcoholic liver disease (ALD) patients than in the heavy drinkers with no apparent liver disease ($p < 0.001$), the patients with non-alcoholic liver disease (NALD) ($p < 0.001$), or the non-drinking controls ($p < 0.001$). Anti-adduct IgG titres did not differ between the ALD patients and heavy drinkers without apparent liver disease, but were higher in both of these groups than in the patients with non-alcoholic liver disease ($p < 0.001$ for both comparisons), or the non-drinking control subjects ($p < 0.01$ and $p < 0.05$, respectively). It is interesting that the non-drinking control patients with IgA or IgG myeloma did not show any increased titres in the immunoassays for the specific anti-adduct immunoglobulins. Like IgG, the anti-adduct IgM titres of both the ALD patients and the heavy drinkers were higher than those of the NALD patients ($p < 0.001$ for both comparisons). No differences could be found in any of the above comparisons, however, when acetaldehyde-protein conjugates prepared under non-reducing conditions were used as antigens in ELISA (data not shown). Comparison of the immunoassay results obtained with the haemoglobin and albumin adducts pointed to some variation in both the antibody titres and the incidences of elevated values (Tables 7a and 7b). Elevated anti-HB adduct IgG and IgM titres were found in the alcohol abusers, for instance, less frequently than were elevated anti-Alb adducts. The correlations between the titres obtained with the albumin and haemoglobin conjugates were nevertheless significant (see Paper IV).

Table 7a. Mean titres of IgA, IgG and IgM antibodies to haemoglobin-adducts and incidences of titres exceeding the upper normal limits[1] *in patients with alcoholic liver disease, heavy drinkers without apparent liver disease, healthy controls, NALD patients, and myeloma patients.*

Subjects	Anti-Ach-Hb IgA			Anti-Ach-Hb IgG			Anti-Ach-Hb IgM		
	Mean ± SD/10^{-2} x O.D.	n	Incidence	Mean ± SD/10^{-2} x O.D.	n	Incidence	Mean ± SD/10^{-2} x O.D.	n	Incidence
ALD	9.9 ± 10.7	86	57%	16.5 ± 18.4	62	22%	11.4 ± 10.4	86	8%
Heavy drinkers	1.8 ± 2.0	54	13%	14.0 ± 14.7	51	14%	12.4 ± 10.7	54	11%
Healthy controls	1.2 ± 1.6	34	6%	8.3 ± 7.3	34	6%	9.4 ± 8.4	34	9%
NALD	0.4 ± 1.0	17	0%	3.8 ± 6.7	17	6%	3.5 ± 2.7	17	0%
Myeloma patients	0.6 ± 2.1	10	10%	3.5 ± 3.3	10	0%	6.2 ± 3.8	10	0%

Ach-Hb, acetaldehyde-haemoglobin; O.D., optical density (405 nm).
[1]Cut-off value calculated as the mean+2SD of the values for healthy controls.

Table 7b. Mean titres of IgA, IgG and IgM antibodies to albumin-adducts and incidences of titres exceeding the upper normal limits[1] *in patients with alcoholic liver disease, heavy drinkers without apparent liver disease, healthy controls, NALD patients, and myeloma patients.*

Subjects	Anti-Ach-Alb IgA			Anti-Ach-Alb IgA			Anti-Ach-Alb IgA		
	Mean ± SD/10^{-2} x O.D.	n	Incidence	Mean ± SD/10^{-2} x O.D.	n	Incidence	Mean ± SD/10^{-2} x O.D.	n	Incidence
ALD	8.2 ± 8.3	32	69%	13.4 ± 11.0	32	42%	8.5 ± 4.4	32	9%
Heavy drinkers	1.1 ± 1.0	16	6%	9.0 ± 7.0	25	16%	10.8 ± 7.1	26	31%
Healthy controls	0.7 ± 1.1	26	4%	6.6 ± 3.7	28	4%	6.7 ± 3.9	28	4%
NALD	1.8 ± 2.3	19	26%	5.8 ± 6.1	19	11%	6.4 ± 3.9	19	0%
Myeloma patients	0.1 ± 0.2	10	0%	1.5 ± 1.7	10	0%	4.3 ± 3.9	10	0%

Ach-Alb, acetaldehyde-albumin; O.D., optical density (405 nm).
[1]Cut-off value calculated as the mean+2SD of the values for healthy controls.

5.4.2. Correlations between titres of serum antibodies to Ach adducts and other laboratory and clinical data

Anti-Hb adduct IgA correlated with GGT in the ALD patients ($r = 0.420$, $p < 0.01$), but not in the heavy drinkers with no apparent liver disease. In addition, there was a significant correlation between anti-Alb adduct IgA and serum bilirubin in the ALD patients ($r = 0.768$, $p < 0.001$) and a weak negative correlation between anti-Alb adduct IgA and serum albumin ($r = -0.328$, $p < 0.1$). In the heavy drinkers, significant correlations emerged between CDT, a marker of alcohol consumption, and anti-Hb adduct IgG ($r = 0.344$, $p < 0.05$) and between CDT and anti-Hb adduct IgM ($r = 0.393$, $p < 0.01$). Although there was no correlation between total IgA and anti-adduct IgA in the total population ($r = -0.070$) or in the subgroup of controls with a wide range of serum IgA concentrations ($r = -0.075$), a weak correlation existed between total IgA and anti-Alb adduct IgA in the subgroup of alcoholic liver disease patients ($p < 0.05$). Interleukin 6 (IL-6) was found to correlate significantly with anti-Alb adduct IgA ($r = 0.504$, $p < 0.001$), whereas no significant correlation emerged between TNFα and any of the anti-adduct titres, as measured in a sample of 38 ALD patients, heavy drinkers and healthy controls.

5.4.2.1. Serum antibodies against Ach adducts and the severity of liver disease

The anti-adduct IgA titres of the ALD patients correlated significantly with the severity of liver disease as measured with the CCLI index ($r_S = 0.497$, $p < 0.001$ for anti-Hb adduct IgA and $r_S = 0.575$, $p < 0.001$ for anti-Alb adduct IgA), but anti-Hb adduct IgG and IgM also had a slight positive correlation with CCLI ($r_S = 0.361$, $p < 0.01$; $r_S = 0.322$, $p < 0.01$, respectively). The mean anti-adduct IgA and IgG titres were also markedly higher in the group with CMI scores from 3 to 5 than in those with scores from 0 to 2. Of the individual CMI parameters, significant correlations were noted between anti-adduct IgGs and both inflammation ($p < 0.01$) and necrosis ($p < 0.01$). The correlation between antibody titres and the histological grade of fibrosis was insignificant for each immunoglobulin ($r_S = 0.217$ for IgA; $r_S = -0.07$ for IgG, and $r_S = 0.095$ for IgM).

When anti-adduct IgA, IgG and IgM titres were monitored in 11 ALD patients showing a decrease in the CCLI score for disease severity, IgA titres declined in six subjects, remained constant in three and increased in two (data not shown), while the initially elevated IgG titres decreased in six subjects, remained constant (low) in four and increased in one and IgM titres decreased to normal levels in five subjects and remained constant (low) in six. A detailed follow-up was also carried out on a 49-year-old female patient admitted with alcoholic hepatitis who showed clinical deterioration during the first two weeks after admission. IgG-class antibodies were found to be significantly elevated during the first few days of the follow-up and began to decline only after one week. The changes in the titres of this antibody during the follow-up were found to parallel those in serum PIIINP, a marker of fibrogenesis ($r = 0.64$, $p < 0.01$).

6. Discussion

6.1. Characteristics of CDT

6.1.1. CDT as a marker of alcohol abuse in heavy drinkers without liver disease

A number of recent reports have indicated that 15–30% of all admissions to general hospitals are related to alcohol abuse (Stibler *et al.* 1986, Bean *et al.* 1997, Scheig 1991, Bonkovsky 1992, Lieber 1995). In view of their high prevalence and their serious health and social consequences, screening for alcohol problems is most important, but no sensitive methods for doing this yet exist (Rosman & Lieber 1994, Walsh *et al.* 1991, Irwin *et al.* 1988, Crabb 1990, Watson *et al.* 1986, Conigrave *et al.* 1993). It is for this reason that the encouraging results achieved with measurements of carbohydrate-deficient transferrin have stimulated a considerable amount of research to clarify the diagnostic efficiency of this marker. In their review article, Allen *et al.* (1994) conclude that while CDT seems to distinguish alcoholics consuming large amounts of alcohol, many important controversial issues remain concerning its value as a more generalized marker of ethanol abuse. The heterogeneity of alcohol disorders complicates the development of a "gold standard" that can be used to determine the predictive validity of screening tests. It has been reported that alcohol-related health problems arise at levels corresponding to a daily consumption of 50–60 g (Sanchez-Graig & Israel 1985). It is therefore crucial to be able to detect excessive drinking as the underlying cause of morbidity, particularly in patients who are not obvious alcoholics.

We have examined patients with a wide variety of alcohol problems to obtain a representative sample of consecutive admissions of such cases to general hospitals, and the significantly lower sensitivities reported here for CDT (I–III) than in many previous studies in this field (Stibler *et al.* 1986, Behrens *et al.* 1988a, Kapur *et al.* 1989, Kwoh-Gain *et al.* 1990, Stowell *et al.* 1997; for a review, see Stibler 1991) may be due to differences in population selection. There are reports indicating rather low sensitivities for CDT in detecting harmful alcohol consumption in the early phase (Nilssen *et al.* 1992, Nyström *et al.* 1992, Sillanaukee *et al.* 1993, Löf *et al.* 1994), which is in accordance with the finding

that high concentrations of CDT are found in only one third of patients drinking immoderate amounts of alcohol (average 100 g per day) but free of any apparent liver disease (I). On the other hand, our results also suggest that if drinking exceeds 150 g per day the incidence of increased CDT values increases rapidly to 60–70%, together with an improved diagnostic efficiency of this marker as compared with others (I). It should be noted, however, that the individuals identified by CDT determinations with 60–70% sensitivity also represent drinkers with severe alcohol dependence, and that in the comparison of GGT, MCV and CDT it was GGT that showed the highest overall sensitivity among men and MCV among women, although CDT had the closest correlation with the amount of alcohol consumed (I).

6.1.2. Usefulness of CDT as a marker of alcoholic liver disease

CDT is significantly higher in alcohol abusers with liver disease than in the earlier phases of drinking problems, but it is increased in only about two thirds of ALD patients (I). Thus the amount of serum desialylated transferrin appears also to be affected by liver status, since the alcoholics with liver disease usually drink similar amounts or often less than abusers admitted for detoxification with no apparent liver disease. Although the heaviest drinkers with no apparent liver pathology and those with documented signs of early-phase liver disease are obviously overlapping groups, the present findings do indicate that CDT could serve as a marker of alcoholic liver disease in its early phase, which may prove to have diagnostic applications. The usefulness of CDT in this context is further supported by several previous studies indicating that non-alcoholic liver disease only exceptionally leads to an increase in concentrations of this marker in the circulation (Stibler *et al.* 1986, Stibler & Borg 1986, Kwoh-Gain *et al.* 1990, Fletcher *et al.* 1991, Kapur *et al.* 1989, Bell *et al.* 1993, Stibler & Hultcrantz 1987, Storey *et al.* 1987, Xin *et al.* 1991). Although low levels of CDT are usually found at the advanced stages of alcoholic liver disease, such conditions rarely pose any diagnostic problems. It should be noted, however, that the present finding of low levels of CDT in severe cases of liver disease (I) should be interpreted as preliminary due to the small number of subjects concerned.

6.1.2.1. CDT and markers of fibrogenesis in ALD patients

Markers of fibrogenesis have been shown previously to correlate with prognostic indicators of alcoholic liver disease (Niemelä *et al.* 1990a, Annoni *et al.* 1989), and our finding that markers reflecting type III collagen and basement membrane metabolism are more frequently elevated than CDT (I) suggests that combined measurements of PIIINP and CDT performed during the follow-up of alcohol abusers with suspected liver disease may yield useful information on the stage which the patient has entered within the continuum of excessive drinking, increased tolerance and progressive liver pathology.

6.1.3. Suggestions on the mechanisms underlying increased serum CDT

Although the amount of desialylated transferrin has long been recognized as a typical characteristic of alcohol abusers, the mechanisms underlying the elevated serum CDT concentrations have remained unknown. Evaluations of human alcoholics have indicated that transferrin synthesis is accelerated in patients with fatty liver but diminished in the presence of cirrhosis (Potter *et al.* 1985), with which our finding of a high incidence of increased CDT values in the early phase of liver disease is consistent (I). Among other suggestions (Stibler & Jaeken 1990, Yamashita *et al.* 1993, Ghosh *et al.* 1993, Marinari *et al.* 1993, Powell *et al.* 1994, Xin *et al.* 1995, Ghosh & Laksham 1997), one postulated mechanism for the increase in CDT in alcoholics is the inability of the ASGP receptors on the hepatocytes to remove sialic acid-deficient transferrin from the circulation (de Jong *et al.* 1990, Potter *et al.* 1992). The receptor for the carbohydrate-rich glycoprotein laminin is structurally related to the ASGP receptor(s) and to the sex steroid binding protein receptor (Fortunati *et al.* 1993), but despite a slightly significant inverse correlation between CDT and laminin, the present data cannot be said to support the notion of a common pathway for eliminating these proteins.

6.1.4. Comparisons between CDTect, %CDT RIA and %CDT TIA

All the CDT methods studied here measure to some extent different carbohydrate-deficient isoforms, which apparently explains the differences in cut-off limits between the %CDT assays (6% versus 2.5%, respectively). Unexpectedly, the present data show that the correlation between CDTect and %CDT improves when the latter measurements are carried out by %CDT TIA instead of %CDT RIA, in spite of the fact that the quantification scheme of %CDT TIA includes 50% of the trisialotransferrins (Bean *et al.* 1997), which should not be measurable in the CDTect procedure (Stibler *et al.* 1991). In any case, the correlation between the %CDT TIA and CDTect results is still low, which supports the view that there are considerable differences in the transferrin isoforms detected by these two assays.

The various CDT methods differ markedly in their analytical characteristics and in their clinical value as blood tests for alcohol abuse (II, III). The finding that the diagnostic performance of CDTect in detecting alcohol abuse is more accurate than that of the %CDT methods is in agreement with a recent report by Bell *et al.* (1994). This is interesting, as it is its lack of sensitivity which has detracted from the more widespread use of CDT as a routine screening tool even though it has been regarded as the most reliable currently available marker of excessive alcohol consumption.

In contrast to our findings, Stowell *et al.* (1997), who used %CDT RIA with the cut-off levels indicated by the manufacturer, achieved sensitivities of 78%–94% for %CDT RIA and 83%–88% for CDTect, ranges which did not differ significantly. This may be related to the fact that they were reporting findings in alcoholics who had been actively drinking amounts ranging from 120 to 342 g of ethanol per day for two weeks before sampling (Stowell *et al.* 1997), whereas our patients represent heavy drinkers with a lower alcohol

consumption per day and longer period of abstinence prior to sampling. When our patients who were the heaviest of the drinkers were analyzed separately, the two assays were found to be of equal sensitivity in the present material as well, 64%–73%, percentages that were markedly higher than in the total population, indicating that the different CDT assays may be equally effective in detecting an advanced stage of heavy drinking (data not shown). As indicated in Paper I, the number of carbohydrate moieties attached to serum transferrin can apparently alter as a function of the amount of alcohol consumed and/or as a function of the severity of liver disease (for a review, see Rosman & Lieber 1994). Thus the assays may differ from each other more, especially, in their detection of binge drinking than in their detection of steady intake of large amounts of alcohol.

6.1.5. Variation in serum transferrin and CDT concentrations

Variations in serum transferrin concentrations markedly affect the sensitivity and specificity of CDTect as a blood test for alcohol abuse, in that its differential diagnostic ability decreases markedly when comparisons are made between alcohol abusers and controls with abnormal serum transferrin concentrations. This may be a particularly serious problem for the detection and follow-up of excessive ethanol consumption in women, who have a high prevalence of iron deficiency, currently the most common form of nutritional deficiency and the most common cause of anaemia in general medical practice (for a review, see Lee 1993). Although Stauber *et al.* (1996b) suggest that the serum transferrin concentration is the influential factor in CDT variation rather than iron deficiency, Anton and Moak (1994) also found a weak correlation between serum iron and CDT in females with an alcohol consumption of less than 15 g/day. Thus it is possible that a depletion of iron reserves may be a reason underlying the higher mean CDTect values in females, leading to a need for higher cut-off limits and an apparent lack of sensitivity in detecting female alcohol abusers, as reported by a number of investigators (Grønbæk *et al.* 1992, Löf *et al.* 1994, Anton & Moak 1994, I–III). It is significant that the diagnostic performance of %CDT TIA in detecting alcohol abuse in men (and women) is at precisely the same level as that of CDTect in women when healthy controls with normal serum transferrin are contrasted with heavy drinkers (II) but markedly higher in women when non-drinkers with high serum transferrin are included among the controls.

Serum transferrin values are known to increase constantly during normal pregnancy. On the other hand, it has been suggested that increasingly more complex carbohydrate chain structures are formed (van Eijk *et al.* 1987, de Jong & van Eijk 1988), so that a reduced carbohydrate deficient isoform content could be expected. The present findings nevertheless indicate that CDT values in about 10% of non-drinking pregnant women in their first trimester and about one half in their last trimester are above the cut-off limit (II). The highly significant correlation between CDTect and serum transferrin in this subgroup indicates that increased CDT values in pregnant women are mostly due to an overall increase in serum transferrin, and therefore this assay should not be recommended for detecting alcohol consumption during pregnancy. Other clinical conditions which could result in abnormal serum transferrin include various (acute phase) inflammatory reactions

and the use of oral contraceptives. Recent work by Bean and Peter (1994) has indicated that increased CDT concentrations may also be caused by a genetic D3 variant of transferrin. Although analyses for such phenotypes were beyond the scope of the present work, it is unlikely that the specificity reported here could have been influenced by such phenotypes, as D3 is an extremely rare variant of transferrin.

As noted above, CDT is most frequently increased in those patients with an early stage of alcoholic liver disease (I). This is definitely associated with the fact that serum transferrin and the severity of liver disease correlate inversely with each other in series of patients covering the full range of disease severity. When CDT is measured by the CDTect method, the low transferrin synthesis capacity entailed in severe liver disease is evidently reflected in low CDT levels, while increased concentrations are recorded at the early stages, when transferrin synthesis is active (Potter *et al.* 1985). Nevertheless, the finding that mean CDTect values were significantly higher in the heavy drinkers with liver disease than in those without, despite rather similar total transferrin concentrations, indicates that alcoholic liver disease also has a quantitative effect on carbohydrate-deficient isoforms over and above that on transferrin concentrations alone (II). These findings together with previous results from several other laboratories (Stibler & Hultcrantz 1987, Storey *et al.* 1987, Fletcher *et al.* 1991, Xin *et al.* 1991, Bell *et al.* 1993; for reviews, see Stibler 1991, Allen *et al.* 1994) support the role of CDT in differentiating the early stages of alcoholic liver disease from other types of liver diseases. As Fletcher *et al.* (1991) indicate, the ratios of CDT to total transferrin could be useful for the differential diagnosis of alcoholic/non-alcoholic steatohepatitis, but calculation of the ratio of CDT to total transferrin in the present patients with ALD resulted in a marked decline in assay sensitivity, so that CDTect showed a markedly higher sensitivity than %CDT RIA or the calculated ratio. This is in agreement with Behrens *et al.* (1988a), who found that the CDT/total transferrin ratio is less sensitive than CDT alone. Xin *et al.* (1991) also reported that CDT alone is more sensitive, but in contrast to the present data, these investigators also reported higher specificities for CDT alone. It should be noted that even the %CDT methods, which measure the ratio of CDT to total transferrin and should thus be independent of serum transferrin concentrations, are affected by alterations in serum transferrin to a slight degree. Contrary to the situation in CDTect, increased serum transferrin may lead to false negative results in %CDT assays.

6.2. Serum antibodies against Ach adducts

One major finding that emerged from Paper IV is the presence of various classes of immunoglobulins with specificity for acetaldehyde-derived protein adducts in alcohol abusers. A correlation is also demonstrated between the antibody titres and indices of liver disease severity, which have previously been established as indices of prognostic importance for the individual patient (Orrego *et al.* 1983, Blake & Orrego 1983, Orrego *et al.* 1987).

6.2.1. Types of serum antibodies against Ach adducts

Our observations are in accordance with previous reports presenting evidence of immune responses directed against acetaldehyde-modified proteins in patients with alcoholic hepatitis and cirrhosis (Niemelä *et al.* 1987, Horner *et al.* 1988, Izumi *et al.* 1989) and demonstrating that the antibody response to acetaldehyde-derived epitopes is primarily an IgA response (Worrall *et al.* 1991, Koskinas *et al.* 1992). The latter report also confirms observations on the high incidence of anti-adduct IgAs in a general population of alcoholics. The present data further indicate, however, that ALD is a major determinant of the production of IgA against acetaldehyde-derived adducts. Increased titres are restricted to such patients, whereas heavy drinkers with no apparent liver disease show insignificant amounts of anti-adduct IgAs. Thus, in contrast to the conclusion reached by Worrall *et al.* (1991), it appears that IgA titres are markers of ALD rather than markers of ethanol consumption. Also opposed to the findings of Worrall *et al.* (1991) is our observation of IgG and IgM responses to acetaldehyde adducts in alcohol consumers. However, as shown here for albumin and haemoglobin adducts, there may be variations in the assays for immune responses when analyzed against different types of *in vitro* modifications with different antigenic characteristics. On the other hand, the discrepancy may also be due to the fact that we used a 10 mM concentration of Ach to prepare the Ach-modified standard protein, which is markedly different from the Ach concentration of 240 mM used in previous studies (Hoerner *et al.* 1988, Worrall *et al.* 1991). Lin *et al.* (1993b) have shown that a 240 mM concentration of acetaldehyde readily crosslinks proteins and generates antigenic determinants which are markedly different from those prepared at lower concentrations. It should be noted, however, that the Ach concentrations occurring in the blood of alcohol consumers are closer to that of 10 mM used by us (IV) than to 240 mM (Nuutinen *et al.* 1983, Eriksson 1983, Eriksson & Fukunaga 1993).

6.2.2. Serum antibodies against Ach adducts and alcoholic liver disease

Both previous observations (Worrall *et al.* 1991) and the present indications of a lack of correlation between total IgA and anti-adduct IgAs in a population with a wide range of total IgA concentrations support the notion that the serological IgA response in alcoholics is antigen-driven. On the other hand, the fairly strong association between the anti-adduct IgA titres and serum bilirubin found in our material may argue in favour of disturbed clearance of IgAs into the bile as a possible mechanism for the increased titres in patients with liver disease.

The present data show a correlation between anti-adduct IgAs and IL-6, which have been shown to mediate acute-phase responses in the liver (Deviere *et al.* 1992, Castell *et al.* 1990). It should be noted in this context that attached IgA may also trigger superoxide secretion and activate monocytes which secrete fibrogenic cytotoxic factors (Border & Noble 1994). The correlation between anti-adduct IgA levels and indices of the severity of

liver disease may be of clinical significance. The finding that anti-adduct IgA titres (particularly anti-Hb-adduct titres) efficiently differentiated patients with ALD from those with NALD may prove to be of diagnostic use.

Although not all heavy drinkers eventually develop liver disease, it is important to note that anti-adduct IgG and IgM antibodies were found to exist in many heavy drinkers who had no significant liver disease and that anti-adduct IgM antibodies seemed to occur in social drinkers as well. IgG and IgM antibodies may be involved in cytotoxic reactions affecting cell surfaces or connective tissues in alcohol abusers. Should IgG, IgM or IgA be generated against a tissue or circulating antigen, immune complexes may also be formed, leading eventually to tissue injuries. Indeed, IgA deposits in tissues and immune complexes have been reported in patients with alcoholic liver disease (Brown & Kloppel 1989, van de Wiel *et al.* 1987a, van de Wiel *et al.* 1987b, Johnson & Williams 1986, van de Wiel *et al.* 1988a, van de Wiel *et al.* 1988b, Israel *et al.* 1988, Zettermann 1990, Amore *et al.* 1994). Since anti-adduct IgGs were shown to be increased during the follow-up of a hospitalized patient with clinical deterioration of hepatitis despite abstinence, it is possible that this type of response could also play a role in the aggravation of liver disease under such conditions. Thus our findings support the view of Marshall *et al.* (1983) that the failure of patients with alcoholic hepatitis to improve after discontinuation of alcohol intake may be mediated by immune mechanisms. A number of recent studies have demonstrated acetaldehyde-derived antigenic epitopes in the centrilobular region of the liver of human alcoholic patients and experimental animals with an early phase of liver disease (Niemelä *et al.* 1991a, Halsted *et al.* 1993, Niemelä *et al.* 1994, Holstege *et al.* 1994, Niemelä *et al.* 1995, Paradis *et al.* 1996b), and it has also been demonstrated previously that hepatic fibrosis can be produced in ethanol-fed animals by immunization with acetaldehyde-protein adducts (Yokoyama *et al.* 1995b). Interestingly, we found that anti-adduct IgG titres correlated with the presence of inflammation and necrosis and that during follow-up they showed changes parallel to those in serum PIIINP, a marker of fibrogenesis.

6.2.3. Serum antibodies against Ach adducts and CDT

Since anti-adduct IgG titres correlated significantly with CDT, a marker of ethanol consumption, in heavy drinkers without signs of liver disease, it may be speculated that ethanol ingestion per se could contribute to the formation of anti-adduct IgG responses, although no correlation between such titres and the patients' own reports of their alcohol intake could be found, and although alcohol drinking per se is actually thought to suppress general IgG synthesis (Drew *et al.* 1984, Mutchnick *et al.* 1990). The fact that no correlation emerged between CDT and IgG titres in patients with established liver disease could be due to the presence of confounding factors which contribute to the concentrations of CDT in liver disease patients (I, II). Similarly, although increased anti-Hb adduct IgM titres were found in both heavy drinkers and ALD patients, the correlation with CDT was seen only in the former.

7. Conclusions

1. Although the CDT concentration correlates with the amount of alcohol consumed, it lacks diagnostic sensitivity in alcohol abusers consuming <100 g of alcohol per day, which hampers its use as a community screening tool.
2. The amount of the serum desialylated transferrin appears to be affected by liver status. CDT could serve as a diagnostic marker of alcohol-related liver disease in its early phase.
3. CDTect seems to be more sensitive in classifying alcohol abusers correctly than either %CDT RIA or %CDT TIA, the assay modifications that express the results as percentages of total transferrin. This is especially the case in males.
4. The diagnostic performance of each method, CDTect, %CDT RIA and %CDT TIA, is hampered by changes in serum transferrin, which should be considered when using CDT measurements as a marker of alcohol abuse in general hospitals. CDTect assay results in particular should be interpreted with caution in all cases of increased serum transferrin, e.g. in the presence of iron deficiency or during pregnancy. On the other hand, low transferrin concentrations associated with acute-phase reactions could result in false negative values, especially when the %CDT methods are used.
5. The %CDT and CDTect methods appear to differ with respect to a number of analytical characteristics, and therefore they are not readily interchangeable in routine laboratory work.
6. Various classes of immunoglobulins with specificity for reduced acetaldehyde-derived protein adducts are present in alcohol abusers.
7. Alcoholic liver disease is a major determinant of the production of IgA against acetaldehyde-derived adducts. There is a correlation between the titres of this antibody and indices of the severity of liver disease.
8. The unique patterns of isotype-specific immunoreactivity to ethanol metabolites may prove to be of value for the treatment and follow-up of alcohol abusers and for the differential diagnosis of alcohol-induced liver disease.

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