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Biomarkers of Ethanol Consumption

Studies on Carbohydrate-deficient Transferrin, Sialic Acid and Traditional Markers

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ACADEMIC DISSERTATION

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Abstract

Although excessive ethanol consumption is a major cause of health problems throughout the Western world, the detection of excessive alcohol consumption by laboratory methods continues to lack sensitivity and specificity. Increasing evidence is currently available to indicate that carbohydrate-deficient transferrin (CDT) is a useful marker for assessing potentially harmful alcohol consumption, but due to heterogeneity in the methods available for its measurement, conflicting views have been available on the validity of CDT assays for detecting alcohol abuse. It has also been suggested recently that diagnostic improvements may be achieved by combining CDT and γ-GT measurements into a marker defined as γ-CDT. In addition, serum sialic acid (SA) has been proposed as a new marker of alcohol consumption. Little research has yet been carried out into the clinical value of such measurements, however.

In this work various CDT assays, SA and the conventional laboratory markers of ethanol consumption (γ-GT, AST and MCV) are compared in the context of the assessment and follow-up of alcoholics with or without liver disease determined by combined clinical, laboratory and morphological indices. The controls were healthy volunteers who were either social drinkers or abstainers, without any history of hazardous drinking. A new approach for defining a combined γ-CDT marker of ethanol abuse was also developed using data obtained from Axis %CDT turbidimetric assays. The data obtained from the CDT assays were compared with a wide variety of conventional laboratory markers and serum sialic acid determinations.

The %CDT method, which excludes the trisialotransferrin isoform from the measurement, showed equal or better sensitivity and higher specificity in detecting hazardous drinking than CDTect, which has been the most widely used assay for measuring CDT during last decade. The differences were especially clear when the patients diagnosed were women. Both of these methods showed higher sensitivities than the old %CDT-TIA method that reacts to the trisialotransferrin fraction of serum transferrin. ROC analyses showed the highest diagnostic accuracies to be achieved with γ-GT, CDT and SA measurements, while self-reported alcohol consumption over a period of one month prior to admission had closest correlations with the %CDT and SA results. The presence of liver pathology was reflected in the results of CDTect, γ-GT, AST and PIIINP, a marker of fibrogenesis, whereas the %CDT method and SA were less sensitive in this respect. The combined γ-%CDT method exceeded the diagnostic accuracy

of all the markers, was less dependent on liver status, and showed the highest correlation with self-reported alcohol consumption. During follow-up with supervised abstinence the mean %CDT values were found to show a slower rate of normalization than CDT values measured with the CDTect method.

The new %CDT method appears to have advantages over previous versions of CDT methods, and its improved characteristics may be most useful in assays for excessive alcohol consumption in female alcoholics, patients with liver disease and patients with abnormal serum transferrin concentrations. While CDT and γ-GT appear to achieve the highest overall accuracy in the detection of problem drinking, serum sialic acid and PIIINP measurements may possess additional value, particularly when there is a need to differentiate between the effects of alcoholic liver disease and ethanol drinking per se. The data also indicate that the new γ-%CDT method yields improved diagnostic accuracy for the detection of excessive ethanol consumption. The present findings may prove to be of value when assessing alcohol markers for use in monitoring abstinence.

Contents

Abbreviations

AA	Acetaldehyde adducts
ALB	Albumin
ALD	Alcoholic liver disease
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
Apo J	Apolipoprotein J
AST	Aspartate aminotransferase
AUC	Area under curve
AUDIT	Alcohol Use Disorders Identification Test
BIL	Bilirubin
BMI	Body mass index
CAGE	Cut down, Annoyed, Guilty, Eye-opener (acronym)
CCLI	Combined clinical and laboratory index
CDT	Carbohydrate-deficient transferrin
%CDT	Amount of CDT as a percentage of total transferrin
CE	Capillary electroforesis
CHD	Coronary heart disease
CMI	Combined morphological index
CV	Coefficient of variation
EOA	Early onset alcoholics
EtG	Ethyl glucuronide
EtOH	Ethanol
FAE	Fetal alcohol effects
FAS	Fetal alcohol syndrome
GT, γ-GT	γ-Glutamyl transferase
HD	Heavy drinkers
HDL	High density lipoprotein -cholesterol
HIAA	Hydroxyindole-acetic acid
HPLC	High-performance liquid chromatography
HTOL	Hydroxytryptophol
IEF	Isoelectric focusing
LOA	Late onset alcoholics
MAST	Michigan Alcoholism Screening Test
MCV	Mean corpuscular volume
NALD	Non-alcoholic liver disease
NPV	Negative predictive value
PE	Phosphatidylethanol

PIIINP	Aminoterminal propeptide of type III collagen
PPV	Positive predictive value
ROC	Receiver-operating characteristic
SA	Sialic acid
SD	Standard deviation
SIJ	Sialic acid index of apolipoprotein J
TLFB	Timeline follow back -method

List of original publications

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

II Anttila P, Järvi K, Latvala J and Niemelä O (2004): Method-dependent characteristics of carbohydrate-deficient transferrin measurements in the follow-up of alcoholics. Alcohol Alcohol 39:59–63.

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

IV Romppanen J, Punnonen K, Anttila P, Jakobsson T, Blake J and Niemelä O (2002): Serum sialic acid as a marker of alcohol consumption: effect of liver disease and heavy drinking. Alcohol Clin Exp Res 26:1234–1238. (Published earlier in Romppanen J (2003): Serum Sialic Acid in Clinical Diagnostics, Kuopion yliopiston julkaisuja D. lääketiede 309.)

V Anttila P, Järvi K, Latvala J, Romppanen J, Punnonen K and Niemelä O (2004): Biomarkers of alcohol consumption in patients classified according to the degree of liver disease severity. Submitted for publication.

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

1. Introduction

Excessive alcohol consumption is a continuously growing problem in modern health care. Recent Finnish statistics point to about 3100 alcohol-related deaths in 2002, including 1465 cases of alcohol-related disease or poisoning, 700 secondary alcohol-related deaths, 913 cases of ethanol-induced accidents or violence, and 53 cases where persons had died as victims of the actions of drunken persons (Stakes 2003). The rapid rise in alcohol consumption among women and young people in recent years has been a matter of special concern, and the trend seems to be similar in many other Western countries: In the UK, for instance, young people under the age of 16 years are drinking twice as much as their counterparts did 10 years ago. Alcohol misuse is costing up to £6.4 billion per year in the UK, where each general practitioner sees an average of 364 heavy drinkers a year (Eaton 2004). The estimated costs to society (direct and indirect) of alcohol abuse in Finland were between EUR 2.9 billion and 5.3 billion in 2001 (Stakes 2003), in relation to a total annual budget of EUR 36.1 billion.

The prevention of alcohol-related mortality and morbidity has been shown to be more successful if hazardous drinking practices are identified in an early phase (Rosman and Lieber 1992, Fleming et al. 2002, John and Hanke 2002, Latt and Saunders 2002, Niemelä 2002). However, since patients' verbal reports about their drinking habits are often of questionable validity, there is a need for sensitive and specific laboratory tests to assess ethanol consumption. Furthermore, the motivation for a patient to reduce his/her drinking might be greater if a laboratory test suggests problematic consumption rather than if the evidence is based primarily on a verbal report (Allen et al. 1992). A similar effect has been shown to exist among diabetic patients, for instance, for whom a knowledge of the markers reflecting long-term glucose balance has been shown to improve compliance (Larsen et al. 1990). It is known that drinking habits are not easily changed (Pageaux et al. 2003), which is why it is highly important to intervene in cases of potentially hazardous drinking habits as early as possible.

Laboratory methods for detecting alcohol abuse should be highly sensitive in order to be able to identify problem drinking at an early phase, but they should also be highly specific in order to achieve positive predictive value, especially since a positive test result can also have far-reaching social consequences. Based on various discussions with clinicians, their other basic needs with regard to an alcohol marker seem to be a correlation with the amount of ethanol consumed, an optimal normalization time (not too short, so that real abstinence is required, but

not too long, either, in order to maintain the patient's motivation for abstinence), and small personal fluctuation in the results when there is no consumption of ethanol.

This work was undertaken in order to explore the clinical value of various biomarkers for use in the detection of alcohol abuse. The diagnostic characteristics of the laboratory markers were examined in populations representing a wide variety of ethanol consumption and various degrees of liver disease.

2. Review of the literature

2.1. Alcohol consumption and health

2.1.1. General aspects of alcohol-related health problems

Ethanol is a commonly used addictive substance. Virtually all tissues in the body are affected by its excessive consumption and the adverse health effects are numerous: liver problems, neurological symptoms, cardiovascular diseases, cancers of the alimentary tract, mental disorders, infectious conditions, hormonal and spermatogenic disorders, injuries and poisoning, and increased violence and suicide rates (Gruenewald et al. 1995, Lieber 1995, Rossow and Amundsen 1995, Pajarinen et al. 1996, National Institute on Alcohol Abuse and Alcoholism 2000a, Adrian and Barry 2003). The dose-effect relationships between ethanol abuse and the induction of various ethanol-related diseases are not known, however. It appears that the risk for developing liver cirrhosis increases linearly with the ethanol intake, and it has even been suggested that the estimated relative risk of developing liver disease may start to increase at a consumption rate as low as 6 drinks per week (Becker et al. 1996). It is generally accepted, however, that the weekly consumption limits for harmful ethanol intake are 280 g for men or 160 g for women. There are also distinct differences in the health effects resulting from different patterns of ethanol intake, in that chronic intake produces a different array of health problems from acute (binge) drinking (Lieber 1995). While chronic drinking may typically lead to liver disease or various neurological problems, acute ethanol intake is clearly over-represented in cases of trauma, for instance (Cunningham et al. 2002, Savola et al. 2004), and in cases of embolic stroke (Hillbom et al. 1999). Studies of trauma patients have shown that the risk of injury is greater in persons under the influence of alcohol (Cunningham et al. 2002), and that the relative risk of head injury starts to increase sharply above a blood alcohol level of 1.5 ‰ (Savola et al. 2004).

The consumption of alcohol by women, even during pregnancy, is also a continuously growing problem in many Western countries (Eustace et al. 2003). Fetal alcohol syndrome (FAS) is a developmental disorder involving the clinical features of craniofacial abnormalities, growth deficiency and deficits in intellectual functioning (National Institute on Alcohol Abuse and Alcoholism 2000b). It is not known at present what amount of alcohol, if any, can safely be consumed during pregnancy, although the risk of damage to the fetus may start to

increase significantly after about 1–2 drinks daily, or a binge of 5 or more drinks on any single occasion during the pregnancy (Streissguth et al. 1990, Forrest et al. 1991, Olsen 1994, Larroque et al. 1995, Larroque and Kaminski 1998, Stoler et al. 1998, Eustace et al. 2003). Many of the problems linked to FAS also seem to exist in children whose mothers drink moderate amounts of alcohol during pregnancy. This milder combination of developmental problems is referred to as fetal alcohol effects (FAE), the incidence of which may be several times higher than that of FAS (National Institute on Alcohol Abuse and Alcoholism 2000b).

2.1.2. Suggested positive effects of alcohol

Alcohol has been used for medicinal purposes in more or less questionable ways in the course of history. During recent decades some research articles have suggested that alcohol might also have some beneficial health effects when consumed in small-to-moderate amounts, e.g. it may reduce the risk of coronary heart disease (CHD) and some other health problems (Doll 1997, Ellison 2002, Fisher Wilson 2003). This is thought to be at least partly due to the effects of alcohol on lipid metabolism, particularly increases in serum HDL cholesterol (Gaziano et al. 1993, Sillanaukee et al. 1993). It should be noted, however, that such effects are limited to the consumption of no more than 1–2 drinks per day.

Another area in which alcohol is regarded as having some positive effects is the social life of individuals. The most commonly reported positive effects are stress relief, mood elevation, increased sociability and relaxation. These benefits are greatly influenced by culture, however, and by preconceived expectations, and there is extensive evidence to show that people who rely on alcohol to relieve stress are more likely to develop alcohol abuse and dependence (National Institute on Alcohol Abuse and Alcoholism 2000a, Kuntsche et al. 2004, Okoro et al. 2004, Prescott et al. 2004).

It should also be noted that the definitions of "moderate/excessive alcohol consumption" and "standard drink" vary between reports (Doll 1997, Burke et al. 1998, Niemelä 2002, 2003). Such variation might not appear significant, but considering the net effect of lowering the limit of harmful alcohol consumption from 200 g ethanol/week to 160 g/week (for women) and setting the ethanol concentration of a standard drink at 15 g instead of 8 g, one can see that fluctuations in these limits can be clinically significant.

2.1.3. Balancing the risks and benefits

Combining the death rates from ischaemic heart disease, external injury and poisoning, as in the developed countries in 1990, it seems that alcohol is unlikely to produce any reduction in total mortality in the population under the age of 45

years. Especially among teenagers and young adults, the risks of alcohol use outweigh any benefits that may accrue later in life (Doll 1997, National Institute on Alcohol Abuse and Alcoholism 2000a).

It should also be noted that, since vulnerability to alcohol dependence varies greatly among individuals, it is difficult to determine any definite ethanol consumption limits for the risk of developing dependence. Two persons exposed to alcohol at the same intensity and frequency may not have the same outcome, for many reasons, including genetic differences, personality, behavioural features and environmental factors (Heath et al. 1994, Prescott et al. 1997, National Institute on Alcohol Abuse and Alcoholism 2000a, Enoch 2003, Fromme et al. 2004, McBride et al. 2004).

Although Ellison (2002) recently supported the opinion that patients should be informed of the potential decrease in the risk of coronary heart disease upon moderate alcohol consumption, other authors have expressed considerable doubts regarding the overall benefit of even moderate alcohol consumption (Friedman and Klatsky 1993, Goldberg 2003). Goldberg (2003) states that even when considering the potential decrease in the risk of CHD, one must remember that substitution of one disease for another is not a medical advance, and a change in life-style in general would yield better results: "If alcohol were a newly discovered drug, we can be sure that no pharmaceutical company would develop it to prevent cardiovascular disease. Nor would many physicians use a therapy that might reduce the rate of myocardial infarction by 25 to 50 percent, but that would result in thousands of additional deaths per year due to cancer, accidents, and liver disease."

2.1.4. Gender-dependent consequences of ethanol intake

The optimum amount of alcohol consumed is lower for women because 1) they are smaller in size, 2) their risk of heart disease is lower, 3) their susceptibility to liver damage is higher, and 4) the risk of breast cancer is increased by about 10% for each additional daily drink (Longnecker 1994, Becker et al. 1996, Doll 1997). Additionally, 1) because of the decreased volume of distribution, blood ethanol levels are higher in women (with the same amount ingested per kg of body weight), 2) the acute effects of alcohol last longer as alcohol metabolism in the stomach is slower, and 3) the rate of ethanol oxidation, creating acetaldehyde and other toxic products, in the liver is higher (Baraona et al. 2001). Taken together, these effects enhance the vulnerability of women to alcohol-related diseases. Furthermore, it has been suggested that the reduction of life expectancy attributable to alcohol is more drastic in women than in men (John and Hanke 2002). Thus the guidelines on moderate vs. harmful drinking should be gender-specific.

2.2. Definition of alcohol consumption patterns

The thresholds for defining "excessive alcohol consumption" are often arbitrary, but they can be considered to reflect the level of drinking that can increase the risk of organ damage (Rosman and Lieber 1992). The commonly accepted limits for a harmful level of alcohol consumption are 4–6 standard drinks on one occasion or over 280 g of ethanol (16 drinks) per week for men and 3–4 drinks on one occasion or over 160 g of ethanol (12 drinks) per week for women (Sanchez-Craig et al. 1995, Niemelä 2002). Sanchez-Craig and Israel (1985) proposed that the drinking pattern that best separates subjects who are problem free from those whose drinking may lead to increasing problems is an average consumption of 4 drinks on three days per week. The authorities in the USA have defined moderate drinking as one drink per day or less for women and two or less for men (National Institute on Alcohol Abuse and Alcoholism 2000a).

In theory, the alcohol consumption patterns can be classified in five basic groups:
1. teetotallers: do not drink alcohol,
2. moderate drinkers: are able to control their alcohol consumption and usually do not exceed 30 g on any given occasion,
3. heavy drinkers: consume large amounts of alcohol on certain occasions or moderate amounts frequently,
4. alcohol abusers: drink such amounts of alcohol that health or social problems, or both, are unavoidable and suffer from mental or physical complications caused by alcohol even though the criteria for alcoholism may not be fulfilled,
5. alcoholics: a stage at which the consumption of alcohol is so high that it causes severe dependence and increased tolerance.

Alcoholics can be further classified into two "subtypes" based on the age of onset of drinking (Buydens-Branchey et al. 1989, Johnson et al. 2000, Enoch 2003, Wetterling et al. 2003). For early-onset alcoholics (EOA) problem drinking usually starts before the age of 20 years, with increased susceptibility to antisocial behaviour and high predisposition to biological disease. There is increased familial loading among first-degree relatives. These are sometimes referred to as type II alcoholics. Typical characteristics of late-onset alcoholics (LOA, type I alcoholics) are feelings of anxiety, guilt and high harm avoidance. LOA patients also tend to show higher compliance with treatment. Although this categorization does not define entirely homogeneous groups, it could be of clinical importance if a specific age range at onset could be linked with different reactions to pharmacological and/or psychological treatment (Johnson et al. 2003, Kranzler et al. 2003).

2.3. Assessing ethanol consumption

2.3.1. Self-reporting of drinking habits

Self-reports of ethanol consumption are often used as a way of estimating patients' drinking habits. The data on the validity of such self-reports are controversial, however. Some studies support the hypothesis that self-reports are valid for estimating the outcome of abstinence treatment (Mundle et al. 1999a), while other clinical trials have led to the conclusion that alcoholic patients tend to underestimate their consumption when monitored. Some relapsed patients have been described as totally denying any drinking during the monitoring period (Orrego et al. 1979, Peachey and Kapur 1986, Fuller et al. 1988). The main problems with the validity of self-reports seem to be difficulties with memory, with understanding the questions and with performing the mental calculations needed to quantify drinking, and finally the prevalence of intentional dissimulation (Allen et al. 1992, Laatikainen et al. 2002). A study of whether college students were able to estimate their alcohol consumption correctly in terms of standard drinks, indicated that they over-poured all the drinks, thus underestimating their own alcohol consumption (White et al. 2003).

The timeline follow back (TLFB) procedure is widely employed to assess the amount of ethanol consumed during a given period. In this approach the patient fills in a blank calendar for the given period together with an experienced interviewer, identifying his or her most common daily drinking pattern, weekend or holiday drinking style, and drinking on special occasions (Allen et al. 1992). This method differs from traditional self-reports in that it relies on specific recollection rather than asking the patient to estimate his or her average drinking within a certain time frame.

Structured questionnaires such as the Alcohol Use Disorders Identification Test (AUDIT) are often used in combination with laboratory tests to assess ethanol consumption. AUDIT has been reported as being the most accurate of the questionnaires aimed at detecting potentially hazardous drinking habits (Seppä et al. 1995, MacKenzie et al. 1996, Reid et al. 1999), and even as being equal to laboratory tests in predicting alcohol-related medical problems (Conigrave et al. 1995). The existing screening questionnaires are, however, mainly capable of detecting alcohol dependence (Cherpitel 1999, Reid et al. 1999), and it has been argued in a recent study that a more accurate measure for assessing binge drinking would be achieved by asking directly for the largest number of drinks consumed in a single drinking session (Matano et al. 2003). Other commonly used questionnaires are CAGE (an acronym referring to the four questions in the test) and the Michigan Alcoholism Screening Test (MAST) (Buchsbaum et al. 1991).

The most commonly used questionnaires are less accurate when used with women than with men (Seppä et al. 1995, Cherpitel 1999). This may partly be due to the increased stigma experienced by women who drink, tempting them to underreport their alcohol consumption and related problems (National Institute on Alcohol Abuse and Alcoholism 2000b).

2.3.2. Biomarkers for ethanol consumption

The above considerations regarding the assessment of excessive ethanol intake by means of self-reports imply a clear need for sensitive and specific biomarkers that reflect the extent of drinking. Laboratory markers of ethanol consumption and alcoholic liver disease can help clinicians to raise the issue of excessive drinking as a possible cause of health problems. These markers should become positive when the limits of hazardous drinking are exceeded (see chapter 2.2.), but they should be devoid of any reactivity towards other health variables.

Many of the currently used biochemical measures of alcohol consumption give information only on abstinence vs. drinking or moderate vs. excessive drinking (Allen et al. 1992). This may be enough for some purposes, but there are situations where a test with "more grey tones" (a continuous variable) would be preferable. For common health care purposes, it is sufficient to find those who are at risk of developing alcohol dependence or some level of organ damage, but a far more sensitive marker is needed for testing the aeroplane pilots, for example. Different markers are also used for monitoring abstinence during detoxification and for monitoring sobriety by law enforcement agencies.

Markers reflecting the amount of alcohol consumed can be divided into markers of chronic consumption and markers of acute consumption (Rosman and Lieber 1992). The former can be used to screen for patients whose drinking habit could result in long-term behavioural or medical problems, e.g. for the identification of drivers with evidence of a chronic problem who should be referred to a treatment programme. These markers are also of value in epidemiological studies and in the screening of workers involved in areas of public safety. Markers of acute consumption may be used in detecting relapses in alcoholic patients undergoing treatment. Such a marker should be sensitive to low levels of drinking and remain elevated for at least a few days after the last dose of alcohol consumed. Some of the markers currently proposed for assessing chronic and acute alcohol consumption are summarised in Table 1, although the borderline between the two groups is not always easy to determine. Some markers of chronic ethanol consumption increase rapidly after relapse and could thus also be used as markers of acute consumption.

Table 1. Markers of chronic and acute alcohol consumption.

			Reference range	Previously reported normalization time
Markers of chronic alcohol consumption	Carbohydrate-deficient transferrin	%CDT CDTect	< 2.6 – 3.0 % men <20 U/l women <26 U/l	2 to 3 weeks
	γ-Glutamyl transferase	GT	men <80 U/l women <50 U/l	3 to 5 weeks
	Aspartate aminotransferase	AST	men <50 U/l women <35 U/l	2 to 3 weeks
	Alanine aminotransferase	ALT	men <50 U/l women <35 U/l	2 to 3 weeks
	Mean corpuscular volume	MCV	76 – 96 fl	2 to 4 months
	Sialic acid	SA	not determined	3 to 5 weeks
	Acetaldehyde adducts	AA	not determined	up to 2 weeks
Markers of acute alcohol consumption	Ethanol	EtOH	0 mmol/l	1g /1 hour /10kg
	5-Hydroxytryptophol (to 5-Hydroxyindole-3-acetic acid ratio)	5HTOL /5HIAA	<15 nmol/µmol	15 hours
	Ethyl glucuronide	EtG	0 mg/l	up to 3 days
	Phosphatidylethanol	PE	0 pmol	up to 23 hours

(Rosman and Lieber 1992, Lundqvist et al. 1994, Pönniö et al. 1999a, Wurst et al. 1999, 2000, Helander and Eriksson 2002, Niemelä 2002, Sarkola et al. 2003)

An ideal marker for alcohol abuse would correlate significantly with the amount of ethanol consumed (state markers). It should also be specific for ethanol-induced organ damage and be capable of predicting the risk of alcohol dependence (trait markers or genetic markers) (Rosman and Lieber 1992, Niemelä 2002).

To achieve an objective comparison of alternative methods for measuring alcohol consumption, the patients need to be carefully described, the base drinking rates in the population noted, the refusal rates in taking the test considered, and sensitivity and specificity reported consistently (Allen et al. 1992).

2.3.3. The clinical chemistry of disease markers

Common measures of test accuracy include sensitivity and specificity, which describe the test's diagnostic capability for differentiating between positive and negative patients. The sensitivity of a test refers to its ability to make a correct positive diagnosis in patients with the disease (50% sensitivity means that half of the positive samples give negative values), whereas specificity describes the test's ability to identify true negative patients correctly (50% specificity means that half of the negative samples give positive values) (Boyd 1997). The diagnostic accuracy of a test can also be determined by means of predictive values. The positive predictive value (PPV) describes the test's capability for identifying true positive samples, and the negative predictive value (NPV) its capability for identifying true negative samples. If a test's PPV is 0.50, for example, only half of all the positive results are true positives (Bean et al. 1997, Boyd 1997, Alte et al. 2004). Precision is the ability of a test to produce the same result when a sample is measured on several occasions (Allen et al. 1992).

A more detailed statistical evaluation of the performance of a diagnostic test can be achieved by the receiver-operating characteristic curve analysis (ROC), which is a statistical tool that shows sensitivities and specificities for all possible cut-offs (decision levels) of the test analysed (Robertson and Zweig 1981, Bean et al. 1997, Boyd 1997). The greater the area under the ROC curve (AUC), the more efficient the test is in discriminating between true positive and true negative samples. This is an efficient means of comparing different methods and optimising the cut-off limits.

When comparing disease markers, one must also consider the effect of pre-analytical and analytical variables. These include the measuring method, reagents, assay and storage conditions, use of technicians and the measurement instrument (Allen et al. 1992). The less vulnerable to pre-analytical and analytical variables a test is, the more generalizable it is (i.e. the easier it is to use the same test in different laboratories).

Finally, to ensure adequate statistical power in the measures of a test's accuracy, an appropriately large sample of patients must be studied, including patients representing all stages of the disease, from mild to severe, otherwise the diagnostic value of the test will be overestimated (e.g. if the sample includes only patients with severe stages of the disease) (Boyd 1997).

2.4. Traditional biomarkers of ethanol consumption

2.4.1. Blood ethanol concentration

The ethanol concentration in the blood, breath or urine can be used to indicate recent alcohol consumption (Table 1), but combined with clinical observations it can also give information about long-term alcohol consumption. A concentration of alcohol in the blood or breath exceeding 150 mg/l (1.5‰) without obvious evidence of intoxication or 300 mg/l (3‰) on any given occasion is an indication of alcoholism (National Council on Alcoholism 1972). The rapid rate at which ethanol is metabolised, about 1 g/hour /10 kg (also influenced by drinking practices), limits its usefulness as a marker to measuring only very recent intake (Rosman and Lieber 1992).

2.4.2. Gamma-glutamyl transferase (GT)

Gamma-glutamyl transferase (or transpeptidase) is traditionally known as a liver enzyme, although it has been quite successfully used as a marker of alcohol abuse. It is actually one of the most commonly employed laboratory tests for detecting possible chronic heavy ethanol consumption. In addition to chronic alcohol consumption and liver disease, serum GT concentration may be elevated by diabetes, obesity and certain drugs and diseases, including biliary tract disease, severe heart and kidney diseases, trauma and hyperthyroidism (Cushman 1992, Allen et al. 2000).

The usefulness of this marker is basically derived from the pharmacological effects of ethanol on the liver, so that its usefulness as an alcohol marker in patients without liver disease may be different from that in patients with liver disease (Nalpas et al. 1997). Also, false positive results may be obtained in patients with non-alcoholic liver disease. It has nevertheless been suggested that since hospitalised patients with non-ethanol-associated liver disease usually do not show profound changes in serum GT in the short term, documentation of decreases in serum GT in hospitalised liver disease patients can be helpful in distinguishing alcoholics from those with non-alcohol-related liver disease (Pol et al. 1990). It has been suggested that serum GT in alcoholics is normalised in about 4 weeks during supervised abstinence (Anton et al. 2002).

Although the variables affecting GT concentration are numerous and individual variability in the reactivity of GT levels to the changes in the body is great, it has been suggested that even moderate short-term ethanol consumption (0.75 g/ kg of body weight on two or three consecutive nights) might increase the GT concentration in blood (Freer and Statland 1977a, 1977b). The suggested increase, however, was determined in relation to the individual's baseline and it

seemed to be temporary, as GT levels returned to baseline in all patients within 100 hours.

2.4.3. Serum aminotransferases

Serum aminotransferases (aspartate aminotransferase, AST and alanine aminotransferase, ALT) are commonly used as laboratory markers for chronic excessive alcohol consumption, although they are more directly related to liver status. AST and ALT values may give more specific information on the alcoholic aetiology of liver disease when interpreted together (Niemelä 2002). A ratio of AST to ALT of over two is suggestive of alcoholic aetiology, while the ratio for patients with non-alcoholic liver disease is normally below one (Rosman and Lieber 1992, 1994).

2.4.4. Mean corpuscular volume (MCV)

Mean corpuscular volume (MCV, red blood cell size), which can be used to measure injury to the red blood cells or the red blood cell precursors, has also been used to diagnose excessive ethanol consumption. It is not very efficient in men, but it may be more sensitive as a means of assessing women (Morgan et al. 1981). Some studies have even suggested that it is superior to all the other markers when analysing female patients (Mundle et al. 2000).

The long normalization time (2 to 4 months) limits the usefulness of MCV for the follow-up of alcohol abstinence, and its specificity as an alcohol marker is limited in the case of patients with vitamin B_{12} or folic acid deficiency, liver diseases, several haematological diseases, hypothyroidism, reticulocytosis and smoking (Aubin et al. 1998, Niemelä 2002, Nordin et al. 2004).

2.4.5. Serum HDL levels

Ethanol consumption also increases the serum HDL cholesterol concentration, but due to its complex effects on HDL metabolism and the high variability of serum HDL in the normal population, HDL´s use as an alcohol marker is limited (Rosman and Lieber 1992). Other indices affecting the serum HDL concentration are age, sex, smoking, exercise, estrogens, severe liver disease and certain drugs (Cushman 1992). Very high HDL cholesterol levels should nevertheless alert clinicians to investigate the patient's recent alcohol consumption (Szegedi et al. 2000).

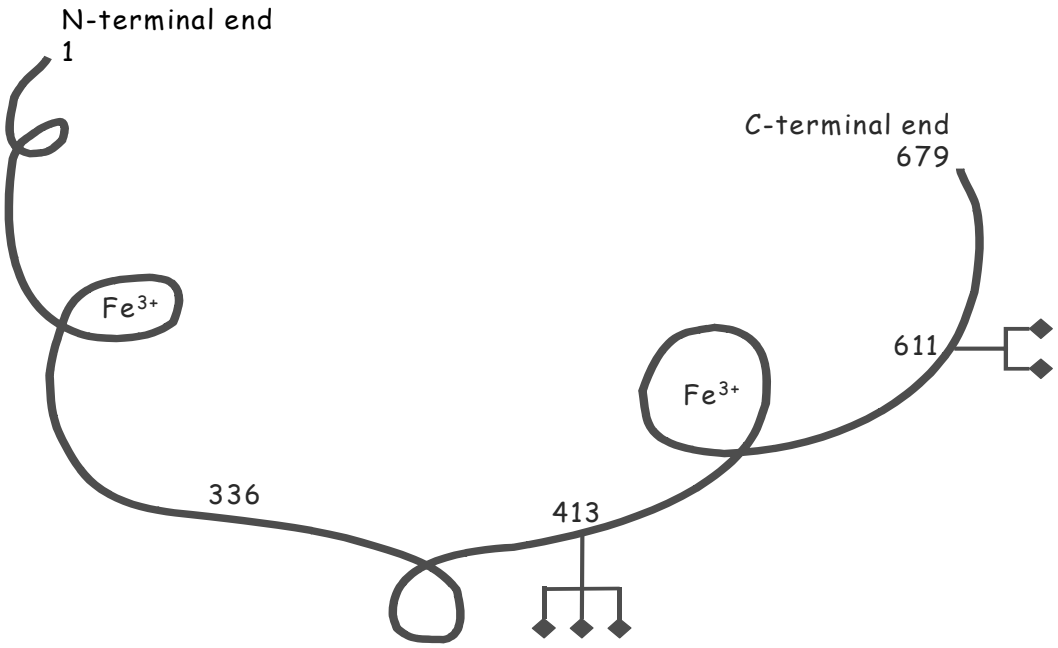
2.5. Carbohydrate-deficient transferrin (CDT)

Carbohydrate-deficient transferrin (CDT) is a relatively new marker for alcohol abuse in routine use (Stibler 1991, Nyström et al. 1992, Allen et al. 1994, Bean et al. 1997, Salaspuro 1999, Scouller et al. 2000, Arndt 2001, Helander et al. 2001, Sillanaukee et al. 2001, Conigrave et al. 2002). The first reports were published over 25 years ago (Stibler and Kjellin 1976, Stibler et al. 1978), and since then significant amounts of data have been published on its validity and reliability.

Many of the studies on CDT have examined the difference in serum levels between heavily dependent alcoholics and teetotallers or social drinkers, and the results in such cases are of course more optimistic than in studies with more heterogeneous populations. The sensitivity of detection has varied from 20% to 90% depending on the method used and the population examined (for a review, see Litten et al. 1995).

2.5.1. Structure and formation of CDT in alcoholics

Transferrin is a glycoprotein synthesized and secreted by the liver. It is the most important iron-transport protein, and has a comparatively simple basic structure (Figure 1). It consists of a single polypeptide chain of 679 amino acids, two independent metal ion-binding sites, and two N-linked glycan chains at positions 413 and 611 in the C-terminal region (amino acids 337–679), to which the sialic acid residues are linked (MacGillivray et al. 1983, Sillanaukee et al. 2001). The microheterogeneity of human serum transferrin is very complex, however, due to the varying iron load at the metal ion-binding sites, the complexity of the glycan chains, the numbers of terminal sialic acid residues bound to the glycan chains, and the existence of rare genetic variants of the polypeptide chain (Arndt 2001).



■ = *sialic acid residue;* Fe^{3+} = *iron*

Figure 1. Basic structure of the transferrin molecule (adapted from Sillanaukee et al. 2001).

Chronic ethanol consumption is known to impair the synthesis (incorporation of sialic acid moieties), secretion and membrane assembly of secretory and membrane-bound glycoproteins (Tuma et al. 1981, Sorrell et al. 1983, Mailliard et al. 1984). The precise mechanism underlying the formation of desialylated forms of transferrin (CDT) as a result of excessive ethanol consumption is currently unclear, however (Allen et al. 2000). It has been suggested that alcohol or its metabolites may reduce the enzymatic activity of certain glycosyltransferases which are found predominantly in the hepatic Golgi complexes and responsible for the addition of carbohydrate groups to the transferrin molecule, namely sialyltransferase, galactosyltransferase and N-acetylglucosaminetransferase. Alternatively, ethanol or acetaldehyde (the first metabolite of ethanol oxidation) may enhance the activity of the sialidase enzyme that removes the carbohydrate groups from transferrin. These actions may also be operative simultaneously (Stibler and Borg 1991, Xin et al. 1995, Allen et al. 2000).

The predominant isoform in both healthy and alcohol abuse populations is tetrasialotransferrin, which accounts for approximately 80% of the total transferrin concentration (Legros et al. 2002). The diagnostically most interesting isoforms of transferrin for examining excessive ethanol consumption are nevertheless asialotransferrin and disialotransferrin, which increase in alcohol abusers, the asialo form being totally absent in samples from teetotallers or patients consuming

moderate amounts of alcohol (Mårtensson et al. 1997, Legros et al. 2002, 2003). There also seems to be a slight gender dependence in the CDT isoform distribution (Mårtensson et al. 1997).

There has been some debate recently about whether or not to include trisialotransferrin in the definition of CDT, and the growing consensus is that this should not be done. This view is based on findings that the mean trisialotransferrin concentration is not affected by alcohol abuse and that the inclusion of trisialo-Fe_2-transferrin will actually reduce the diagnostic accuracy of CDT in determining hazardous drinking (Mårtensson et al. 1997, Viitala et al. 1998, Helander et al. 2001, Arndt et al. 2002, Legros et al. 2002).

There is currently no uniform opinion about the amount and pattern of drinking which is needed to elevate serum CDT values. It is believed that levels of CDT increase once alcohol consumption exceeds 50–80 g/day for 2 to 3 weeks, at least in patients who are alcohol-dependent (Stibler 1991), but it has been suggested elsewhere that the amount of 60 g, or even 80 g, of alcohol per day is not sufficient to increase CDT values over the reference limit in the general population (Salmela et al. 1994, Lesch et al. 1996b, Oslin et al. 1998). A few studies of relapsed alcoholics indicate, however, that CDT may be a sensitive relapse marker giving increased values with even lower levels of consumption in patients with a previous history of alcohol abuse (Anton et al. 1996, Schmidt et al. 1997). Mikkelsen et al. (1998) suggested that CDT might be a better marker of alcohol dependence than of actual ethanol consumption. Their results, however, were reached by using an old version of the CDT method, so their findings may not be comparable to the data obtained by more recent methods. Nevertheless, it appears that even if CDT may not be sensitive for screening alcohol consumption at the population level, it can be used as a continuous variable to detect changes in alcohol intake in chronic alcohol abusers (Burke et al. 1998, Whitfield et al. 1998). The type of alcoholic beverage consumed (grain-based vs. grape-based) does not seem to have a significant effect on CDT levels (Whitfield et al. 1998).

One of the postulated advantages of CDT over the traditional markers of alcohol consumption is that it may not be influenced by the presence of liver disease (Stibler 1991, Nalpas et al. 1997). Thus it can also be used to study patients with non-alcoholic liver disease.

2.5.2. Methods for measuring CDT

The sensitivities and specificities of most alcohol markers in unselected clinical series have remained unclear (Allen et al. 1997). In the case of CDT this may in part be due to a lack of methodological standardization (Arndt 2003). This is one of the main problems in comparing CDT with other markers of alcohol consumption. There has also been a lack of uniformity in the populations tested

(sex, age, alcohol consumption and duration of abstinence before sampling), and the ability of the marker to distinguish between alcohol consumption and the secondary effects of liver disease has received little attention so far (Mundle et al. 1999b, Niemelä 2002).

Two main kinds of CDT test have been in common routine use. The most widely used method has been CDTect, which expresses the total serum concentration of CDT (U/l), while %CDT expresses the CDT concentration as a percentage of total transferrin. These methods also differ with respect to the number of desialylated transferrin isoforms included in the measurement, as CDTect measures a-, mono- and minor amounts of disialotransferrin, while the new version of %CDT measures a-, mono- and all of the disialotransferrin (Helander et al. 2001). These two methods have been shown to have a positive correlation and to be highly comparable for the detection of alcohol abuse disorders (Anton et al. 2001). It is possible, however, that %CDT may show improved performance when diagnosing cases among women, since it takes account of the natural variability in serum transferrin concentration (Huseby et al. 1997, Keating et al. 1998, Helander 1999, Anton et al. 2001). Their ease of use makes both of these methods practicable for most clinical laboratories.

It could be argued that determining the amount of CDT as a percentage of total transferrin may give rise to both false-positive and false-negative results, because of the independent variations in total transferrin concentration, since it has been suggested that total transferrin is unrelated to CDT (Renner and Kanitz 1997). This does not seem to be the case, however, for it has been found that there is a correlation between total transferrin concentration and CDT in the non-drinking population, but that it becomes obscured in the drinking population, partly due both to the highly differing amounts of alcohol consumed and to the highly variable individual CDT response to a given alcohol dose (Sorvajärvi et al. 1996, Stauber et al. 1996a, Helander et al. 1998, Helander 1999).

CDTect and many of the %CDT methods employ microcolumn anion-exchange chromatography and the separation is based on the different charge characteristics of the transferrin isoforms. Another method for detecting CDT based on the charge difference is isoelectric focusing (IEF), the results of which seem to correlate well with the CDTect method (Anton and Bean 1994).

For more detailed determination of the transferrin isoforms, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) methods are available as well as IEF. These give additional information on the isoform distribution of the CDT and can be more accurate especially in the rare cases of genetic transferrin variants (Helander et al. 2001, Arndt and Kropf 2002a). These methods have the advantage of visualizing the total transferrin isoform pattern, and should be employed for confirmation purposes, especially in cases where a positive CDT result may have significant social consequences, where samples

have been stored for extended periods at room temperature (Renner and Kanitz 1997, Arndt and Kropf 2002b) and in cases where the CDT result is not congruent with the clinical information.

2.5.3. Gender-dependent characteristics in CDT measurements

The clinical characteristics of CDT have been shown to vary depending not only on the method used but also on gender. CDT measurements seem to be more accurate in men than in women (Bell et al. 1993, Anton and Bean 1994, Anton et al. 2001), and questions have been raised as to whether CDT can be used as a marker of alcohol abuse with women. There seem to be several aspects which need to be considered when using CDT for assessing female patients, although, the reasons underlying the gender-dependent characteristics are still unclear.

Mean levels of CDT have been shown to be different in male and female patients when the assays are carried out with the CDTect method (Sorvajärvi et al. 1996). This problem may be solved by using %CDT methods. CDT is more sensitive in men than in women – a trait typical of almost all the markers except for MCV (Huseby et al. 1997, Mundle et al. 2000). It has been suggested that women as a group express higher CDT levels under natural conditions, but may produce less CDT in response to heavy drinking (Anton and Moak 1994).

Sillanaukee and co-workers (2000) previously studied the effects of hormone balance on CDT in female social drinkers, testing for the effects of fertility stage (fertile, perimenopausal or postmenopausal), pregnancy, the use of oral contraceptives, the use of an intrauterine contraception device, hormone replacement therapy and hormone treatment for infertility. Pregnancy seemed to increase CDT levels, which needs to be considered when using CDT to detect alcohol abuse in pregnant women (Sarkola et al. 2000). Stauber et al. (1996a) have previously reported a correlation between CDT and the week of pregnancy. Conversely, low CDT values occur in postmenopausal women, and CDT levels in women who are close to the late menopause seem to be close to the values for men. Whitfield et al. (1998) also reported a difference in mean CDT values between women under and over the age of 50.

Results regarding the possible correlation between CDT and the use of oral contraceptives have been inconsistent, however. La Grange and co-workers (1995) found high CDT values among women using oral contraceptives, whereas Sillanaukee and co-workers (2000) reported precisely the opposite result. Still others have failed to find any differences in CDT levels among women in relation to their use of oral contraceptives (Anton and Moak 1994, Stauber et al. 1996b).

The differences in the results may be partially explained by sociological differences. Even though the differences between the study populations may not

be significant, females using oral contraceptives tend as a group to consume more alcohol than those not taking oral contraceptives, and this effect might be especially striking when comparing the results of La Grange and co-workers (1995) with all the others, since their subjects were all university students.

Sillanaukee and co-workers (2000) did not find menopausal hormone replacement therapy to have an effect on CDT levels. This finding, however, is inconsistent with other reports, where postmenopausal estrogen replacement therapy has appeared to increase serum levels of CDT (Stauber et al. 1996b). CDT values do not appear to vary as a function of the menstrual cycle (La Grange et al. 1995), and there does not appear to be any direct correlation with the estrogen level (Stauber et al. 1996a, 1996b).

2.6. Sialic acid (SA)

2.6.1. Structure and function of sialic acid

The sialic acids, a family of over 40 derivatives of neuraminic acid, represent a group of sugar molecules with an unusual and highly variable chemical structure (Traving and Schauer 1998, Schauer 2000). They are found mostly in the terminal position of oligosaccharide chains on the surface of cell membranes and macromolecules. They are involved in many biological and pathological phenomena, and play a central role in the physiological functioning of humans. Their highly variable structure and exposed position enable the sialic acids to fulfil several important biological functions, such as regulating cellular and molecular interactions by either serving as recognition determinants or masking recognition sites. The most common form of sialic acid in humans is N-acetylneuraminic acid (Neu5Ac or NANA) (Pönniö et al. 1999a).

The mean concentration of sialic acid in serum/plasma is 1.94 mmol/l (varying from 0.64 to 2.18 mmol/l depending on the report), and it does not seem to exhibit any gender difference (Pönniö et al. 1999b, Romppanen et al. 2003). It nevertheless needs to be determined separately for all the methods used, since there are several methodological factors which can influence the results. Furthermore, there is currently no reference method for sialic acid measurements against which other methods could be calibrated.

2.6.2. Sialic acid as an alcohol marker

Previous reports have suggested that sialic acid could also serve as an alcohol marker (Pönniö et al. 1999a, Sillanaukee et al. 1999b), and that it may be especially well suited for use with women.

It has been proposed that excessive ethanol intake may affect post-translational glycosylation processes in the liver and result in the desialylation of transferrin and other glycoproteins (Malagolini et al. 1989, Stibler and Borg 1991, Guasch et al. 1992). Several reports have also indicated that ethanol reduces the activities of the sialyltransferases in the Golgi apparatus and increases the activities of sialidase in the cytosol and plasma membranes (Xin et al. 1995, Hale et al. 1998).

Serum SA concentrations appear to decrease during abstinence from alcohol (Pönniö et al. 1999a, Sillanaukee et al. 1999b), a property which may be useful in the follow-up of alcohol dependence treatment. Pönniö and co-workers (1999a) also found that SA concentrations in both serum and saliva are elevated in alcoholics, which could be of value when considering non-invasive methods for diagnosing alcohol abuse.

The potential clinical usefulness of SA determinations is questionable, however, since increased SA concentrations have been reported in a variety of conditions, including inflammatory diseases, diabetes, chronic renal failure, chronic glomerulonephritis, and cancer (Shamberger 1984, Stefenelli et al. 1985, Plucinsky et al. 1986, Sillanaukee et al. 1999a). A connection between increased SA levels and elevated stroke and cardiovascular mortality risk, and also myocardial cell damage in patients undergoing cardiac surgery, has also been reported (Lindberg et al. 1992, Berkan and Sagban 2002). Several other factors, such as BMI, blood pressure, ageing, pregnancy, use of oral contraceptives and smoking, may also cause changes in SA concentrations (Pönniö et al. 1999b). In addition to this non-specificity, the absolute increases in SA levels are rather small and the reference values for normal healthy controls are greatly dependent on the method used (Sillanaukee et al. 1999a, Romppanen et al. 2003). Mean alcohol consumption over the last week or year does not seem to correlate significantly with SA in the general population (Pönniö et al. 1999b).

2.7. Marker combinations in screening for alcohol abuse

Although a variety of biochemical markers of ethanol consumption have been available for screening programmes in health care, none of them alone has shown adequate diagnostic accuracy in routine clinical work (Allen et al. 1994, Salaspuro 1999, Scouller et al. 2000, Arndt 2001, Conigrave et al. 2002). CDT, the form of

transferrin with 0 to 2 sialic acids, has achieved equal or greater sensitivity than γ-glutamyltransferase (GT) in many studies and generally greater specificity.

The use of combinations of markers may improve assay sensitivities, but it will at the same time detract from their specificities (Rosman and Lieber 1992, Chen et al. 2003). This decrease in specificity may be of especial importance under conditions where the overall occurrence of the condition in the population tested is low, since the number of false-positive results becomes high relative to the actual true positive cases (Allen et al. 1992). This should also be noted when diagnosing conditions for which a positive result may have serious social consequences in the patient's life, as is the case with alcohol abuse.

When CDT is combined with GT (either one being positive), the sensitivity noticeably increases with a no longer excessive, but still a perceptible, decrease in specificity (Anton et al. 2001). Some previous studies using a qualitative method for combining the assay results state that the specificity does not decrease with increasing sensitivity, but these studies may have compared the combined specificity with the original test which was of lower specificity (Anton and Moak 1994). The fact that combining CDT and GT increases the sensitivity of the findings implies that the mechanisms by which alcohol affects these two markers are independent. This hypothesis is also supported by the observation that the CDT and GT do not correlate with each other in either gender (Bell et al. 1993, Löf et al. 1994, Huseby et al. 1997, Anton et al. 2001). Since CDT and GT appear to be independently associated with alcohol consumption, they should be regarded as complementary rather than alternative markers (Helander 1999).

There are also previous studies available that suggest rules or algorithms for combining the quantitative results of multiple tests (Sillanaukee 1992, Hartz et al. 1997, Harasymiw et al. 2000, Harasymiw and Bean 2001). The algorithm developed by Sillanaukee and Olsson (2001) combined the results of CDT (absolute values) and GT. Chen and co-workers (2003) tested this algorithm in a population of volunteers with a wide range of variation in ethanol consumption. They also tested whether adding non-alcohol-related factors (age, BMI, ethnicity or smoking) would increase the accuracy of the resulting diagnosis. They found the algorithm to be efficient, especially with men, and noted that combining clinical information with the test results added to the accuracy of the diagnosis.

One practical aspect that needs to be taken into account when considering the use of combinations of markers for the detection of excessive alcohol consumption is the increased costs involved, but the approach may be rendered cost-effective in the long run by the greater treatment success achieved when choosing diagnostically accurate combinations.

2.8. Comparisons of the effects of biological factors on markers of alcohol abuse

There is a wide selection of existing research on the effects of biological determinants on markers of alcohol abuse, but the results are not consistent. Sensitivities and specificities vary quite considerably from one study to another. There may be differences in the populations studied and even in the definition of excessive alcohol consumption (Scouller et al. 2000, Legros et al. 2002).

Nevertheless, only a limited number of physiological conditions other than excessive ethanol consumption have so far been found that routinely elevate CDT. The known or likely causes of false-positive CDT results include severe hepatic failure (such as primary biliary cirrhosis, chronic viral hepatitis or hepatocellular carcinoma), genetic D-variants of transferrin and carbohydrate-deficient glycoprotein syndrome (Stibler et al. 1988, Stibler 1991, Allen et al. 1994, Stauber et al. 1995, Scouller et al. 2000), while a false negative result may be obtained from a patient with a rare genetic B-variant of transferrin (Stibler et al. 1988).

It has been suggested that CDT may be better than GT or AST for diagnosing high-risk alcohol consumption in men (Allen et al. 1999, Conigrave et al. 2002). Conigrave and co-workers (2002) concluded that all three markers (CDT, GT and AST) were more effective in detecting high-risk than intermediate-risk drinking, although CDT and GT may be influenced by body mass index (BMI), sex and age (Huseby et al. 1997, Whitfield et al. 1998, Mundle et al. 1999b, Conigrave et al. 2002). Whitfield and co-workers (1998) also suggested a decrease in the dose-response curve of CDT in hypertension patients.

Sillanaukee et al. (2003) have recently reported that the only alcohol marker out of those tested (CDT, SA, GT, AST and ALT) that reacted to moderate alcohol consumption over a period of three weeks was CDT, but unexpectedly, this showed a significant decrease. The explanation may lie at least partly in the fact that they used the CDTect method, which can react to the iron status of the body, for measuring CDT (Sorvajärvi et al. 1996, Viitala et al. 1998, De Feo et al. 1999).

Since CDT levels can be affected by pregnancy (chapter 2.5.3.), CDT should be used with caution when diagnosing alcohol abuse at such a time. Under such condition, MCV and GT may be the most efficient markers for detecting excessive alcohol consumption and for predicting the adverse effects of alcohol on the fetus (Sarkola et al. 2000). Neither GT nor MCV has been shown to correlate with gestational age or to change with ongoing pregnancy. Sillanaukee et al. (2000) have reported, however, that GT levels in women are influenced by hormonal status, being increased by hormone treatment for infertility,

postmenopause and the use of oral contraceptives and reduced by pregnancy. GT is also affected by many other factors (chapter 2.4.2.) the best-known of which are various forms of liver disease.

There may also be age-associated effects involved in the sensitivity and specificity of alcohol markers. Huseby et al. (1997) found that even though CDT and GT were quite identical in their overall diagnostic performance, as evaluated by receiver-operating characteristic (ROC) curve analysis, they varied in sensitivity and specificity between patient populations in different ways. The highest sensitivity of CDT among alcohol-dependent patients admitted for acute surgery was found in the middle-aged cases (36 to 50 years), whereas the highest sensitivity of GT was found for those above the age of 51 years. Another group of patients analysed by Huseby et al. (1997) was those hospitalised for detoxification. Here the overall sensitivity of CDT was nearly 75%, whereas among the surgical patients it was below 50%. A striking difference in the sensitivity of CDT between these two groups of patients was noted among the youngest cases (21 to 35 years), where it was less than 20% for the surgical patients but 77% for the detoxification patients. In addition to being related to sex, age and consumption, levels of the two markers (CDT and GT) were considered to depend on the patients' earlier history of alcohol abuse. The reason for the overall similar diagnostic performance of CDT and GT in this study, even when an improved version of %CDT was used, may be connected with the absence of patients with liver pathology from the study population.

Anton et al. (1998) reported that CDT and GT responded differently to certain drinking patterns. In men, CDT levels appear to respond primarily to drinking frequency (number of drinking days), while GT was influenced by drinking intensity (number of drinks per drinking day). In women both CDT and GT responded primarily to drinking intensity. According to Oslin and co-workers (1998), women's CDT levels correlate significantly with drinking intensity and the percentage of days of heavy drinking during the 30-day period before entering treatment only when the amount of alcohol consumed is 6 or more drinks per drinking day.

2.9. Markers of ethanol-induced liver problems

Many of the markers used in the determination of chronic or hazardous ethanol consumption are actually related to liver pathology rather than drinking per se. Parameters that are widely used as markers of liver status include AST, ALT, GT and alkaline phosphatase (ALP), whereas albumin (alb) and bilirubin (bil) have been found to be associated with a poor prognosis in individual patients (Orrego et al. 1983, Blake and Orrego 1991). These tests complement each other and reflect certain morphological tissue alterations, such as cell damage or cholestasis

(Niemelä 2002). Although patients with liver disease can have normal marker values, normal values for all of the markers mentioned above will usually rule out significant liver or biliary tract disease (Balisteri and Rej 1994).

Although the level of cell injury in non-alcoholic liver disease (NALD) is reflected by a marked increase in serum aminotransferase concentrations, the levels of AST and GT do not clearly correlate with the severity of the injury in alcoholic liver disease (ALD). AST and ALT levels may be slightly elevated in alcoholics with mild liver disease (fatty liver), but the bilirubin level is normal except for the combination of fatty liver and cholestasis (this occurs in about 25% of cases of alcoholic fatty liver). The characteristic biochemical abnormalities of acute alcoholic hepatitis are usually more severe: markedly elevated serum GT and an AST/ALT ratio of >1.0 when AST is <300 U/l. Serum AST and ALT are always elevated in alcoholic hepatitis, serum bilirubin is elevated in 90% of cases and ALP in 80%. Serum albumin is usually decreased (Niemelä 2002).

When the patient has a previously established diagnosis of alcohol abuse, the severity of alcoholic liver disease can be assessed on combined clinical and laboratory (CCLI) and morphological (CMI) criteria (Blake and Orrego, 1991). The CCLI index combines laboratory and clinical data which have been shown to be significantly associated with the prognosis for alcoholic patients. The most important prognostic abnormalities in ALD are albumin <25 g/l and bilirubin >136 μmol/l, whereas AST, ALT or GT have no apparent prognostic significance (Orrego et al. 1983). When the prognostic weights of all such variables are added together, the CCLI is obtained, which relates linearly to the risk of mortality over its range 0-25. The CMI index in turn summarizes the morphological findings that are of prognostic significance (necrosis, inflammation and Mallory bodies), which are graded on a scale of 1+ to 3+ (Blake and Orrego 1991).

Several markers of connective tissue metabolism and fibrogenesis, such as the aminoterminal propeptide of type III collagen (PIIINP), also appear to be of independent prognostic value when estimating the severity of ALD. PIIINP, a marker which increases in early fibrosis, is elevated in ALD patients more frequently than the other collagen markers. It correlates with the clinical and histological severity of liver disease and decreases if the patient remains abstinent (Niemelä et al. 1990).

2.10. Use of markers in the follow-up of alcoholics

It is important for treatment purposes to find a marker of alcohol abuse which normalizes with abstinence. Better still, if the same marker were to react comparatively fast to any alterations in the person's alcohol consumption, it could be used as an indicator of both sustained abstinence and possible relapse.

In a recent clinical trial of pharmacological abstinence therapy, Ait-Daoud et al. (2001) found that CDT decreases with increasing duration of abstinence. Although they could not draw any conclusions on a possible direct correlation between CDT and the self-reported amount of alcohol consumed, on account of the small size of their sample, the finding is in accordance with other follow-up and relapse studies, in which CDT appears to correlate with abstinence/relapse and the amount of alcohol consumed, especially in men (Anton et al. 1996, 2002).

CDT has been successfully used as a marker of relapse in follow-up studies, when, instead of using the reference values recommended by the manufacturer, an increase of at least 30% relative to the lowest individual value is considered a significant indication of alcohol consumption (Borg et al. 1995).

Carlsson and co-workers (1993) compared the analytical characteristics of CDT with those of urine 5-hydroxytryptophol (5HTOL) for detecting relapses in alcohol-dependent patients and found that these two markers depicted complementary properties and could be used together for the early detection of relapse and for monitoring treatment.

According to Anton and co-workers (2002), GT can also be used to detect relapse when combined with CDT. Although GT alone also increased following relapse in both genders, the increase did not reach statistical significance, nor did the increase in CDT levels in the women (Anton et al. 2002). The lack of sensitivity in women may, however, have been due to the small number of women suffering a relapse (n=14).

When using GT to detect relapse, it is essential to remember that it can be elevated by chronic liver disease, certain medications and many other non-specific causes even in the absence of recent alcohol consumption. Because of its prolonged half-life, mean erythrocyte cell volume (MCV), which is also a widely used alcohol marker, is not a very suitable means of discriminating between abstinence and relapse. As stated by Bell and co-workers (1993), CDT seems to be a better indicator of abstention than GT, AST or MCV, especially in patients with alcoholic liver disease.

It has also been suggested recently (Pönniö et al. 2002) that SA (sialic acid) measurements could be used as a marker of relapse, since serum SA is significantly increased in abstaining alcoholics even after a short period of heavy drinking. It is not clear from the paper concerned, however, whether there are differences in the sensitivity of serum SA to alcohol drinking depending on previous exposure to alcohol.

2.11. Postulated new markers of ethanol consumption

There are several new markers which have been suggested as possibly being of value for assessing ethanol consumption, although many of them have been studied only to a limited extent (Table 1).

2.11.1. 5-Hydroxytryptophol

The ratio of 5-hydroxytryptophol (5HTOL) to 5-hydroxyindole-3-acetic acid (5HIAA) in urine has been proposed as being at least as sensitive as the measurement of plasma ethanol for indicating recent alcohol consumption (Helander and Eriksson 2002), its main advantage over the latter being its ability to detect recent alcohol consumption several hours after ethanol is no longer measurable. 5HTOL appears to be most useful for measuring acute alcohol ingestion. Since its levels increase in the urine after one day of heavy drinking but decrease 14–15 h after the last dose of ethanol, these measurements may be oflimited use for assessing chronic alcohol abuse (Allen et al. 1992, Sarkola et al. 2003).

2.11.2. Ethyl glucuronide

The concentration of ethyl glucuronide (EtG), a metabolite of ethanol, in urine has been shown to increase after ethanol ingestion (Dahl et al. 2002, Sarkola et al. 2003). It is possible, however, to reduce the concentration by drinking large amounts of water prior to sampling, and this needs to be considered when interpreting EtG measurements. Such manipulation does not influence the EtG/creatinine ratio in urine (Dahl et al. 2002, Bergström et al. 2003), and a further advantage is that the concentration of EtG in the urine is detectable hours after the complete elimination of ethanol from the body (Schmitt et al. 1997, Wurst et al. 1999, Dahl et al. 2002). These findings suggest that the urine concentration of EtG or the EtG/creatinine ratio could be used as a sensitive and specific marker of recent ethanol consumption, with the possibility of filling in the clinically and forensically important time gap between short-term and long-term markers (Wurst et al. 1999, 2000).

2.11.3. Acetaldehyde adducts

Another interesting group of markers, which are currently under investigation in several laboratories, are acetaldehyde adducts (AA). Acetaldehyde, a metabolite of ethanol, binds to a variety of proteins to form stable protein-acetaldehyde adducts (Allen et al. 1992, Niemelä 2001). Even though acetaldehyde itself is

metabolised rapidly after ethanol consumption, these protein-acetaldehyde adducts may persist for up to two weeks after ethanol ingestion (Rosman and Lieber 1992, Niemelä and Israel 1992). A potential problem in these analyses, however, is endogenous acetaldehyde formation.

2.11.4. Phosphatidylethanol

In the presence of ethanol, the phospholipase D enzyme catalyses the formation of phosphatidylethanol (PE) instead of the normal product phosphatidic acid, and significant amounts of this abnormal phospholipid have been detected in human blood cells after ethanol exposure, correlating with the ethanol concentration, thus suggesting that it might serve as a marker of alcohol consumption (Lundqvist et al. 1994, Gustavsson 1995). Furthermore, measurable levels of phosphatidylethanol can be found in alcoholics up to 23 hours after the last intake of ethanol, which enables this marker to be used even after the ethanol has been eliminated from the body.

2.11.5. Sialic acid index of apolipoprotein J

The plasma sialic acid index of apolipoprotein J (SIJ) has recently been suggested as a marker of ethanol consumption (Ghosh et al. 2001). The loss of sialic acid from the apolipoprotein J (Apo J) seems to be positively correlated with both the amount and duration of ethanol consumption. The reason why SIJ can be suspected as being an even more sensitive marker than CDT is that the sialic acid index of Apo J is approximately seven times higher than the index for transferrin (Ghosh et al. 2001). Further studies would be needed to evaluate this hypothesis, however.

3. Aims of the present research

Although a variety of biochemical methods have been used in alcohol screening programmes, data on their accuracy have remained conflicting, with sensitivities ranging from <20% to 100%. There is therefore an urgent need for methodological standardization and comparison of the methods for measuring excessive ethanol intake in well-characterized clinical series.

The aims of the present work were as follows:

1. to study the diagnostic characteristics of different CDT methods for measuring alcohol abuse,
2. to examine CDT methods for the follow-up of alcoholics,
3. to examine CDT as a marker of alcohol consumption when combined with GT,
4. to study the usefulness of sialic acid as a marker of alcohol consumption, and
5. to compare the diagnostic properties of traditional markers of alcohol consumption with those of CDT and sialic acid in patients with different levels of alcohol consumption and degrees of liver disease.

4. Materials and methods

4.1. Patients and control subjects

Paper I reports on a series of 62 alcoholics with a well-documented history of continuous ethanol consumption or binge drinking, comprising 33 biopsy-proven alcoholic liver disease (ALD) patients (26 men, 7 women) and 29 heavy drinkers with no apparent liver disease (25 men, 4 women). The ALD patients all had a history of continuous ethanol consumption for at least five years in amounts exceeding 80 g/day, while the heavy drinkers were patients who had been admitted for detoxification and had a history of heavy drinking consisting primarily of repeated inebriations. Detailed interviews on the amounts and patterns of alcohol consumption were carried out using a time-line follow-back method. The patients were asked how many drinks of alcohol (standard drink = 12 g of ethyl alcohol, corresponding to one beer, one glass of table wine or four centilitres of 40% proof spirit) they had consumed during the last (1) 24 h, (2) 1 week and (3) 4 weeks preceding admission. Total consumption had exceeded 130 g/day (mean) over a period of 4 weeks prior to sampling, and the mean duration of abstinence prior to sampling was about 2 ± 2 days. The healthy controls were 45 volunteers (22 men, 23 women), who did not drink or who were social drinkers (<30 g of ethanol/day on any occasion), as also examined by means of detailed personal interviews. These individuals were primarily hospital personnel.

Paper II compared 36 heavy drinkers admitted for detoxification (31 men, 5 women) with 30 apparently healthy control subjects (22 men, 8 women). A follow-up with supervised abstinence during hospitalization for 8 ± 4 days (range 5–19 days) was carried out with 17 of the drinkers, during which blood alcohol concentrations were checked by means of repeated breath analyses. The controls were either abstainers (n = 20; 16 men, 4 women) or social drinkers (n = 10; 6 men, 4 women) whose ethanol consumption did not exceed a mean of 15 g per day or 40 g on any single occasion.

The patient series described in paper III was essentially the same as that in study I, with the exception of three additional male alcoholics (one with ALD and two heavy drinkers).

The serum samples for paper IV were collected from 51 alcoholics (42 men, 9 women), including 32 with biopsy-proven liver disease (25 men, 7 women) and

19 heavy drinkers (17 men, 2 women). The heavy drinkers had consumed a mean of 81 ± 55 grammes of ethanol per day during the four weeks preceding blood sampling. The time of abstinence prior to sampling was similar in both the heavy drinkers and the ALD patients, varying between 0–7 days. The control population with no history of alcohol abuse consisted of 20 healthy individuals.

Paper V describes 102 alcoholic patients (87 men, 15 women), of whom 59 (46 men, 13 women) had ALD, the severity of which was assessed by previously established combined clinical, laboratory and morphological indices, and 43 (41 men, 2 women) were heavy drinkers with no clinical evidence of significant liver damage. A follow-up with supervised abstinence during hospitalization was carried out on 15 patients. The controls were 34 healthy volunteers (25 men, 9 women) who were either abstainers or social drinkers.

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

4.2. CDT Analyses

CDT concentrations were measured by two methods: a turbidimetric immunoassay after ion exchange chromatography (%CDT, Axis-Shield ASA, Oslo, Norway), and a competitive radioimmunoassay after microcolumn separation (CDTect, Axis-Shield ASA, Oslo, Norway).

The %CDT assay measures the transferrin variants with 0–2 sialic acid residues and excludes the trisialo fraction containing 3 sialic acids. The concentration of CDT is expressed as a percentage of the total amount of serum transferrin. The measurements were carried out on Behring Nephelometer II (Dade Behring, Behring Diagnostics GmbH, Marburg, Germany).

The CDTect method excludes the trisialo fraction and part of the disialofraction. Thus, the isoforms with 0–1 sialic acid residues (a- and monosialotransferrins) and minor amounts of isotransferrin with 2 sialic acid residues (disialotransferrin) are detected (Helander et al. 2001). The assay results are expressed as absolute CDT concentrations. The commonly accepted reference range in this assay is 0–20 U/L for men and 0–26 U/L for women.

Data obtained by the old %CDT-TIA method, which includes 50% of the trisialylated fraction of serum transferrin in the measurement, were also used for comparison in paper I (Bean et al. 1997, Viitala et al. 1998).

4.3. Determination of the combined marker γ-%CDT (III)

A new approach for a combined marker γ-CDT was developed from a mathematical equation published previously by Sillanaukee and Olsson (2001), which employed measurements of CDT concentrations by the CDTect method and had an optimal cut-off limit of 6.5. Our method was based on %CDT results instead of the CDTect method, the combined γ-%CDT marker being calculated as follows: γ-%CDT = 0.8*ln(GT) + 1.3*ln(%CDT). A cut-off value of 4.0 was established for this assay by ROC analysis.

4.4. Measurement of sialic acid (SA) (IV-V)

The total sialic acid concentration in serum was determined by high-performance liquid chromatography (HPLC) after liberation of sialic acid by acid hydrolysis (Romppanen et al. 2002). Chromatography was performed using an HPLC system with a CarboPac PA-1 anion exchange column of pellicular resin with a PA1 Guard precolumn and pulsed amperometric detection (Dionex Corp., Sunnyvale, CA, USA), with a mobile phase of 100 mM sodium hydroxide and 150 mM sodium acetate solution. The column was then washed with 5 mM acetic acid and equilibrated with 100 mM sodium hydroxide and 150 mM sodium acetate solution.

The calibration curve for sialic acid (range 0.12–7.48 mmol/L) showed a linear relation between the concentration of the compound and the detector response (sialic acid peak height relative to internal standard peak height).

4.5. Other methods

Serum gamma glutamyl transpeptidase (GT), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), albumin, transferrin, bilirubin and mean corpuscular volume (MCV) of erythrocytes and blood ethanol levels were measured by standard clinical chemical methods in an accredited (SFS-EN 45001, ISO/IEC Guide 25) laboratory of Seinäjoki Central Hospital, Seinäjoki, Finland. The reference ranges for these parameters were as follows: GT <80 U/l (men), <50 U/l (women); AST and ALT <50 U/l (men), <35 U/l (women); albumin 36–50 g/l; transferrin 1.7–3.4 g/l; bilirubin 2–20 μmol/l; MCV 76–96 fl. The concentration of the aminoterminal propeptide of type III procollagen (PIIINP), a marker of fibrogenesis, was determined by a radioimmunological procedure (Orion Diagnostics, Oulunsalo, Finland).

4.6. Statistical methods

The data are expressed as mean ± SD. The statistical analyses were carried out using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) and Analyse-it (Analyse-it Software Ltd, Leeds, U.K.) software. The comparisons between the alcoholic and non-alcoholic groups were evaluated using Student's t-test or the Mann-Whitney test for parameters with skewed distributions of values.

One-way analysis of variance (ANOVA), with Bonferroni's method for multiple comparisons, was employed when comparing three or more groups. If a Gaussian distribution or equal variances in the values could not be achieved even after transformations, the comparisons were evaluated using the Kruskal-Wallis test.

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

The comparisons of the analytical characteristics of the markers were based on ROC analyses.

5. Results

5.1. CDT

Pooled sera from control and alcoholic patients with low and high CDT concentrations were first analyzed to determine the analytical characteristics of the different methods. The within-run precision (n=10) for %CDT was 5.2% for the low concentration and 4.4% for the high concentration, while the corresponding values for the CDTect method were 6.2% and 10%, respectively. The day-to-day CVs (n=13) for %CDT were 10% for the low concentration and 5.6% for the high concentration, which were lower than those for the CDTect method.

The mean CDT values in the total population of alcoholics were significantly higher than those in the control group with all the CDT methods tested. Both the new %CDT and CDTect showed approximately two-fold higher sensitivities than the %CDT-TIA method, however, which reacts with trisialotransferrin.

The presence of liver disease was found to influence the results of the CDTect assays, leading to a high incidence of elevated values among the patients with liver pathology as compared with the corresponding group of heavy drinkers. The specificity of CDTect was also significantly affected by the gender of the patient, as it was unable to differentiate efficiently between the heavy drinkers and control patients especially in women. The patients with liver disease had significantly higher values than the controls or the heavy drinkers (paper I). The new %CDT method gave no such differences between these groups of alcoholics, and the alcohol abusing groups with or without liver disease did not differ significantly.

The %CDT and CDTect results correlated significantly with each other in all the series ($r=0.80$, $p<0.0001$), the correlation being markedly closer among the men ($r=0.86$, $p<0.0001$) than among the women ($r=0.57$, $p<0.001$). The same kind of difference in correlations between men ($r=0.81$, $p<0.0001$) and women ($r=0.54$, $p<0.001$) was also seen when comparing the %CDT-TIA and CDTect methods. Self-reported alcohol consumption showed the closest correlation with the results of the new %CDT method ($r=0.6$, $p<0.001$; for CDTect $r=0.4$, $p<0.05$).

5.2. Combining CDT with GT

The mean values for the marker combinations γ-CDT and γ-%CDT in the alcoholic patients were significantly higher than those in the reference population ($p<0.0001$). The previously established combined marker γ-CDT (Sillanaukee and Olsson 2001), yielded a 92% sensitivity and 93% specificity in correctly classifying alcohol abusers in the total series (including both men and women), and when the new index γ-%CDT was determined from the %CDT data with a cut-off of 4.0, as defined by ROC analysis, the assay sensitivity remained at 92% but the specificity improved to 100%. The ROC analyses showed the AUC for γ-%CDT (0.973) to be markedly higher than that for %CDT (0.852) or γ-GT (0.951).

Both of the mathematically formulated markers showed improved performance characteristics relative to their parent components, γ-%CDT showing a higher specificity than γ-CDT particularly in women. The data for the alcoholic patients classified according to the presence or absence of liver disease also showed improved diagnostic accuracies for the combined markers. The highest correlation with self-reported alcohol consumption was observed with the γ-%CDT marker ($r=0.73$).

5.3. Sialic acid

In both papers that made use of sialic acid (SA) measurements (papers IV and V), the mean SA concentration in serum was significantly higher in the alcoholics than in the healthy controls. Furthermore, when the alcoholics were divided into two groups according to the presence or absence of liver disease, the concentrations of serum SA were found to be higher among the alcoholics without liver disease, although the difference was not statistically significant.

The mean concentration of SA differed between the two papers, possibly due to the change of calibrator between the measurements. The optimal cut-off limits with a specificity of 100% yielded a sensitivity of about 50% in both instances, however. The diagnostic accuracy of serum SA was also good according to the ROC analysis.

Serum SA was found to be a sensitive marker of ethanol abuse in the heavy drinkers (HD) without liver disease, but the conventional markers were elevated more often than serum SA in the alcoholic liver disease (ALD) patients.

The correlations observed between serum sialic acid and the conventional alcohol markers (GT, AST or CDT), the clinical and laboratory (CCLI) or

morphological (CMI) indices of liver disease severity, and PIIINP, a marker of fibrogenesis, were all found to be weak and statistically non-significant.

5.4. Other markers of ethanol consumption and liver injury

All the traditional markers of alcohol abuse (GT, AST and MCV) also gave significantly higher values in the alcoholic patients than in the reference population. When the alcoholic population was divided into subgroups according to the presence or absence of liver disease the highest values for GT, AST and MCV were observed in the ALD group.

The highest diagnostic accuracies in the detection of alcohol abuse were found in the ROC analyses in the case of the CDT, GT and SA measurements, the presence or absence of liver disease influencing the sensitivities of the parameters in different manners. More variation was noted, however, when the diagnostic properties of the various methods were further compared in the alcoholics classified by gender.

The amount of alcohol consumed by the alcoholics, as estimated by a time-line follow-back method for different time intervals prior to sampling, correlated significantly with the %CDT, SA and MCV values, whereas the correlations with the CDTect, GT and AST results were markedly lower (papers II and V).

Significant correlations emerged between the CCLI index and serum AST and CDT, whereas the CMI index correlated only with serum AST. PIIINP, a marker of fibrogenesis, correlated with albumin ($r= –0.36$, $p=0.002$) and %CDT ($r= –0.27$, $p=0.03$). Interestingly, PIIINP also correlated positively with %CDT, AST and MCV in the patients without liver disease, and even with monthly and weekly ethanol consumption (paper V).

5.5. Use of markers in the follow-up of alcoholics

The normalization rates of the markers were examined in a follow-up of alcoholics with supervised abstinence. Their mean ethanol consumption over the four weeks prior to admission had been approximately 130 g/day. The patients were followed over a period of 1–2 weeks during hospitalization, and all the markers except for MCV were found to decrease over this period, as reflected both in the incidence of abnormal values and in the marker concentrations. Interestingly, the CDT concentrations obtained by different methods were found to show different rates of normalization (within 14 ± 4 days for %CDT and 10 ± 5

days for CDTect). Among the individual patients, %CDT gave a lower value for all patients after abstinence, whereas occult marker elevations were also seen with CDTect, SA, GT and AST. The estimated times required for normalization of SA, GT, and AST were 13 ± 9, 25 ± 16 and 15 ± 14 days, respectively.

6. Discussion

6.1. CDT as a marker of alcohol consumption

Although the recently introduced %CDT measurements bring about the benefit of expressing CDT concentrations conveniently as percentages of total transferrin, with similar cut-offs for both genders, the previous versions of these measurements have shown poor sensitivities in clinical series (Sorvajärvi et al. 1996, Viitala et al. 1998). The present comparisons between the new %CDT and CDTect methods point to essentially similar sensitivities, which is in accordance with the recent findings of Anton et al. (2001). The new %CDT method even yielded slightly higher sensitivities than the conventional CDTect method or other conventional markers of ethanol consumption when analyzed at the time of admission.

The improved sensitivity appears to stem from the fact that the new assay is devoid of reactivity with the trisialylated fraction of transferrin, which is consistent with the view that the generation of this isoform is not significantly influenced by ethanol consumption *in vivo* (Mårtensson et al. 1997, Arndt et al. 2002, Legros et al. 2002). The data also show a higher specificity of the new %CDT method relative to the conventional CDTect method. This may be due to the higher specificity among women and in patients with liver disease. The findings are in accordance with previous observations that the CDTect method is sensitive to unspecific changes in serum transferrin metabolism (Sorvajärvi et al. 1996, Viitala et al. 1998, De Feo et al. 1999). The analytical precision profiles of the %CDT method are also superior to those of CDTect, suggesting that this method should yield improved reproducibility and thus be more suitable for routine laboratory use (Table 2).

The lack of sensitivity of CDT as a marker of alcohol abuse among women has been recognized previously in several reports (Anton and Moak 1994, Löf et al. 1994, Huseby et al. 1997, Mundle et al. 2000). While the previous data were derived primarily from studies using the CDTect method only, it should be noted that even with the new %CDT method, the diagnostic sensitivity in women continues to be lower than that in men.

Table 2. Characteristics of the CDTect and %CDT methods in clinical laboratory work.

CDTect	%CDT
+ Eluted samples can be refrigerated and stored up to one week	− Eluted samples can not be stored
− Serum transferrin concentration causes significant interference	+ Serum transferrin concentration does not cause interference to the results
− Liver disease affects the interpretation	+ Alcoholic liver disease has no effect on the results
− The reference values are different for men and women	+ Both genders have the same reference values
− Expensive, manual sample processing	+ The clinical accuracy seems to be equal or better than that of the CDTect method
− RIA method is inconvenient	+ Less expensive than the CDTect method, partly automated sample processing
	+ TIA method, which is safe for the laboratory personnel
	− The method is divided into two phases, which increases the possibilities for analytical and pre-analytical errors

The present data also show that the correlation between the marker results and the actual amount of ethanol consumed is closer with the %CDT results than with the CDTect results, indicating that measurements which exclude the trisialo fraction but include the entire disialo fraction in the measurement, reflect ethanol-induced changes in transferrin in a more specific manner. Thus the %CDT method may be recommended for the assessment of alcohol consumption in routine work. No significant differences in CDT values emerged between the abstainers and social drinkers, however, suggesting a lack of correlation between the consumption of low doses of alcohol and CDT values.

6.1.1. CDT as compared with traditional alcohol markers

The majority of previous studies which have compared CDT with other markers of ethanol consumption (GT, MCV, AST) have concluded that the sensitivity of CDT exceeds or equals that of the conventional markers (Scouller et al. 2000). The present data suggest high sensitivities for CDT and GT measurements. It should be noted, however, that GT lacks specificity in assays of hospitalized series where several conditions may lead to elevated values, including non-alcoholic liver diseases, diabetes, obesity, or the use of various drugs (Cushman 1992, Allen et al. 2000). The correlation between the marker results and the

amount of recent ethanol consumption appeared to be closest with the %CDT, SA and MCV values, suggesting that these markers are efficient in reflecting ethanol consumption per se. On the other hand, CDTect, GT and AST appeared to be sensitive to the secondary effects of liver disease.

6.2. γ-CDT combinations as markers of ethanol intake

The present data support the idea of using the combined γ-CDT marker as a means of acquiring a more sensitive and specific diagnosis of alcohol abuse, which is also in line with previous observations by Sillanaukee and Olsson (2001). The improvement in assay performance suggests that a mathematically formulated marker combination can be used even without any loss of assay specificity as is commonly found for combinations of markers.

Instead of using the absolute CDT values (U/L) obtained from CDTect assays (Sillanaukee and Olsson 2001) an equation that is based on the results of the %CDT measurements was used (paper III). This marker was designated γ-%CDT. While the CDTect-based γ-CDT marker also showed a high accuracy, yielding 92% sensitivity and 93% specificity in our material, γ-%CDT was found to achieve a similar sensitivity with a specificity of 100%. Since %CDT assays are also cost-effective and easy to perform, γ-%CDT measurements may similarly be recommended for routine laboratory work. When the combined γ-%CDT marker was further tested in a larger study population similar results were acquired (data not shown).

The biomedical mechanisms underlying the improvement in the detection of alcohol abuse when marker combinations are used have not been established. The changes in CDT and GT obviously reflect different types of ethanol-induced pathophysiological processes, but the %CDT-based combination appears to be less dependent on the presence of liver status than the CDTect-based combination. In line with this view, current data show that the correlation between the marker results and the amount of ethanol consumed during the month prior to blood sampling is closer with the γ-%CDT method than with any of the other methods tested.

6.3. Sialic acid

It is shown in papers IV and V that serum SA concentrations are significantly higher in alcohol abusers than in healthy controls, which is in accordance with earlier studies on serum sialic acid in alcoholics (Pönniö et al. 1999a, Sillanaukee et al. 1999b). The mechanisms underlying the changes and the possible links

between sialic acid and sialic acid deficient transferrin have remained obscure, however. The present data suggest a lack of correlation between these parameters, indicating that they reflect ethanol consumption and ethanol-induced pathophysiological processes in different manners. Interestingly, serum SA was most notably elevated in the heavy drinkers with the lowest degrees of liver damage. At this time we cannot rule out the possibility that the presence of liver pathology could induce biological processes which disturb the reactivity of SA to ethanol consumption. Nevertheless, the data indicate a significant correlation between SA levels and the actual amount of ethanol drinking. This is also notable in the light of the view that sialic acids, which are acetylated derivatives of neuraminic acid, have a variety of biological functions *in vivo* (Pönniö et al. 1999b). Thus there may be several physiological functions that could be disturbed upon an alteration in sialic acid metabolism in vivo.

The present results indicate that serum SA has a higher efficiency than serum CDT (measured by the CDTect method) or ASTfor detecting alcohol abuse in heavy drinkers, but a lower efficiency than serum GT. The presence of liver disease elevates serum AST and GT and reduces the specificity of these markers, especially in hospitalized patients. By contrast, serum SA appeared to be less dependent on liver status in these comparisons. Thus serum sialic acid measurements may prove to be useful for assessing drinking problems, especially under conditions where use of the traditional markers is hampered by the secondary effects of liver disease.

6.4. Follow-up of abstinence

The present follow-up data (papers II and V) show a consistent decrease in %CDT values during abstinence, which is also in line with the view that there is a close association between %CDT marker data and drinking habits. The observed normalization rate for %CDT also seems to be in accordance with an earlier study by Lesch et al. (1996a). The discrepancies between GT, CDTect, AST and abstinence could perhaps be due to the induction of liver pathology, which may also be reflected in the marker values during periods of abstinence. In particular, the CDTect method may be sensitive to such changes even in the early phase of liver disease (Tsutsumi et al. 1994, Niemelä et al. 1995).

The serum SA normalization rate has received very little attention in previous studies. Pönniö et al. (1999a) put forward an estimate of 3 to 5 weeks for the normalization of SA levels, and Sillanaukee et al. (1999b), suggested that serum sialic acid concentrations may remain elevated longer than some of the conventional markers such as GT (Sillanaukee et al. 1999b). It is evident here (V), however, that SA remains elevated for approximately the same time as %CDT but shows a greater degree of individual variation. While %CDT was the only assay

which gave a lower value in all the subjects after abstinence, 27% of the present follow-up samples gave a higher SA value after a short period of abstinence.

6.5. Monitoring fibrogenesis

The fibrosis marker PIIINP has been shown previously to be associated with the severity of liver disease in alcoholics (Niemelä et al. 1990), its values rising markedly at advanced stages, suggesting the activation of hepatic fibrogenesis. According to the present data there is a correlation between PIIINP values and alcohol consumption even in patients without significant liver disease and with normal or only slightly elevated PIIINP levels, which indicates that activation of hepatic stellate cells and initiation of fibrogenesis occurs as an early event in response to hazardous drinking practices. Serum PIIINP could thus be useful as an indicator of the activation of fibrogenesis even prior to the development of severe liver disease, information which could also be of value for assessing the prognosis for alcoholic patients.

6.6. Strengths, weaknesses and future considerations

The results and conclusions presented here should be applied to the general population with caution, since the research was focused on well-defined subgroups of heavy drinkers and controls, in the absence of patients with intermediate levels of ethanol consumption. In clinical situations drinking habits are more varied, and thus the markers may not perform as well as in carefully controlled studies (Alte et al. 2004). For ethical reasons, not all the patients underwent a biopsy, and it is possible that some additional information could have been gathered through additional or repeated biopsies. It should be noted, however, that all the patients who were not biopsied were ones who had no clinical or biochemical evidence of liver disease, and hence there was no clinical justification for this invasive procedure. A wide variety of laboratory tests were used here to examine the alcoholic patients, and bilirubin and albumin measurements, for instance, were included because these parameters are important determinants of liver disease prognosis.

The combined marker γ-%CDT proved to be sensitive and specific, and may also be of value in routine hospital work. If it is to be applied to the general population, however, it must be kept in mind that the same factors that influence %CDT and GT as separate markers may also affect the reliability of the combined marker, and thus further testing of this marker would appear to be warranted in the future.

The present studies also involved a relatively small number of women with alcohol problems, although their number in society seems to be increasing rapidly and ethanol consumption by women is approaching the level reached by men. Furthermore, the consequences of problem drinking for women, including the possible effects on fetus, may be especially severe due to their higher physiological vulnerability to the toxic effects of alcohol (see section 2.1.4.). Future studies for testing the functioning of the markers in more extensive materials appear to be warranted, and research focusing on problem drinking among women deserves special attention in these.

7. Conclusions

The present data indicate distinct differences in the analytical characteristics of the biomarkers for alcohol abuse when ethanol consumption is to be assessed in patients either with or without liver disease.

The new %CDT method, which does not include trisialotransferrin in the measurement but includes all of the disialo fraction, appears to be a sensitive and specific marker of alcohol abuse, which may be recommended for routine use.

A combination of GT and CDT (the %CDT method) values appears to yield a new sensitive and specific tool, γ-%CDT, for diagnosing hazardous ethanol consumption.

Sialic acid (SA) measurements might be valuable for assessing hazardous drinking, especially when it is necessary to differentiate between alcohol consumption and the secondary effects of liver disease.

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