



PÄIVIKKI ALATALO

Markers of Liver Function and Oxidative Stress
in Alcohol Consumers
with or without Overweight



ACADEMIC DISSERTATION

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To my family

Abstract

Alcohol consumption and excess body weight create a major burden for modern health care. Both heavy drinking and obesity lead to derangements in liver function and increased oxidative stress; however, little is still known about the early-phase effects of ethanol intake and adiposity.

The relationships between alcohol consumption, body mass index (BMI) and various laboratory markers (e.g., alanine and aspartate aminotransferases (ALT, AST) and albumin as markers of liver status, uric acid reflecting the status of oxidative stress, and γ -glutamyl-transferase (GGT) and ferritin being associated with both liver function and oxidative stress) were studied here in a large number of volunteers originally recruited by the NORIP survey for establishing common reference intervals in Nordic countries. The population consisted of apparently healthy abstainers and moderate drinkers (1–21 measures of alcohol/week, 75% of men, 62% of women), and also included subjects who were overweight (BMI 25–30 kg/m², 41% of men, 22% of women) or obese (BMI >30 kg/m², 4% of both men and women). In addition, the present study included a group of heavy drinkers who were devoid of liver disease. The dose-response effects of ethanol and adiposity and their interactions on marker levels were assessed in the categories of drinking habits and BMI. The impacts were also evaluated by calculating the reference limits from different subpopulations and by comparing them with the widely used NORIP recommendations, which have been determined from the total survey population.

The levels of serum ALT and GGT were significantly different between male moderate drinkers and abstainers, whereas AST, albumin and ferritin in men and the markers in women only differed between heavy drinkers and abstainers. When the BMI was included in the analyses on liver enzymes (ALT, AST, GGT), activities increased as a function of body weight throughout the BMI scale. The activities were further higher in moderate drinkers than in abstainers of the corresponding BMI. Some observations also suggested an interaction between the effects of the subject's drinking habits and BMI on these enzymes, but the results of the statistical tests were not significant. For uric acid, the concentrations were similarly higher across the categories of BMI, and higher in male moderate drinkers than abstainers. The calculations of reference limits revealed that especially in men, a substantial increase has been introduced into the currently recommended limits by moderate drinking and excess body weight. The calculated upper reference limits from normal weight male abstainers for ALT were 50 U/l (while the current recommendation is 70 U/l), and for GGT 44 U/l in those <40 years (80 U/l) and 69 U/l in those \geq 40 years (115 U/l).

It may be concluded that moderate drinking and an increased BMI have notable effects on markers of liver function and oxidative stress at the population level. These should be recognized in order to improve the clinical value of such measurements for diagnostic purposes and for preventive medicine.

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Abbreviations

ALD	Alcoholic liver disease
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
AUDIT	Alcohol Use Disorders Identification Test
BMI	Body mass index
CDT	Carbohydrate-deficient transferrin
CYP2E1	Cytochrome P450 2E1 enzyme
EtG	Ethyl glucuronide
EtS	Ethyl sulfate
FAEE	Fatty acid ethyl esters
FASD	Fetal alcohol spectrum disorders
GGT	γ -Glutamyltransferase
HDL	High-density lipoprotein cholesterol
HPLC	High performance liquid chromatography
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
LC-MS	Liquid chromatography-mass spectrometry
MCV	Mean corpuscular volume
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NORIP	Nordic Reference Interval Project
PEth	Phosphatidylethanol
SD	Standard deviation
TLFB	Timeline follow-back method
TNF- α	Tumor necrosis factor- α

List of original publications

- I Alatalo P, Koivisto H, Puukka K, Hietala J, Anttila P, Bloigu R and Niemelä O (2009): Biomarkers of liver status in heavy drinkers, moderate drinkers and abstainers. *Alcohol Alcohol* 44:199–203.
- II Alatalo P, Koivisto H, Hietala J, Puukka K, Bloigu R and Niemelä O (2008): Effect of moderate alcohol consumption on liver enzymes increases with increasing body mass index. *Am J Clin Nutr* 88:1097–1103.
- III Alatalo P, Koivisto H, Hietala J, Bloigu R and Niemelä O (2009): Gender-dependent impacts of body mass index and moderate alcohol consumption on serum uric acid – an index of oxidant stress status? *Free Radic Biol Med* 46:1233–1238.
- IV Alatalo P, Koivisto H, Kultti J, Bloigu R and Niemelä O (2010): Evaluation of reference intervals for biomarkers sensitive to alcohol consumption, excess body weight and oxidative stress. *Scand J Clin Lab Invest* 70:104–111.

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

1. Introduction

Alcohol consumption, excess body weight, and related health problems have increased rapidly in our society (Färkkilä 2009). More than half of the Finnish population is currently overweight or obese and the per capita ethanol consumption has increased fourfold since the 1960s (Peltonen et al. 2008, Figure 1). Most recently, alcohol consumption has decreased for three consecutive years in conjunction with increases in alcohol taxation in 2008 and 2009 (after a relaxation of taxation in 2004 and a sharp increase in consumption). Similarly, the increase of adiposity also seems to have subsided if assessed in terms of the mean conscript weight (Figure 1). Nevertheless, the degree of ethanol intake and extra weight remains very high, and these need attention.

The health of the liver is affected by both ethanol consumption and excess body weight. With the increasing prevalence of heavy drinking and obesity, cirrhosis is among the leading causes of death, especially in the middle-aged (Heron 2010). Hepatic status is often mirrored by measuring the activities of liver enzymes from serum. It appears, however, that little is known about the effects of ethanol and adiposity in their earliest phase. Further understanding would also be important, since liver enzymes have been suggested to associate as well with cardiovascular diseases and type 2 diabetes (Fraser et al. 2009, Targher 2010).

The aim of this study was to investigate the effects of rather low levels of alcohol drinking and excess body weight on the liver enzymes and other laboratory markers. These effects were studied both separately for each factor and in combination. The aim was also to clarify the mechanisms of pathology and to evaluate whether increased alcohol consumption and adiposity have influenced the concept of what is considered to be normal for various laboratory analytes.

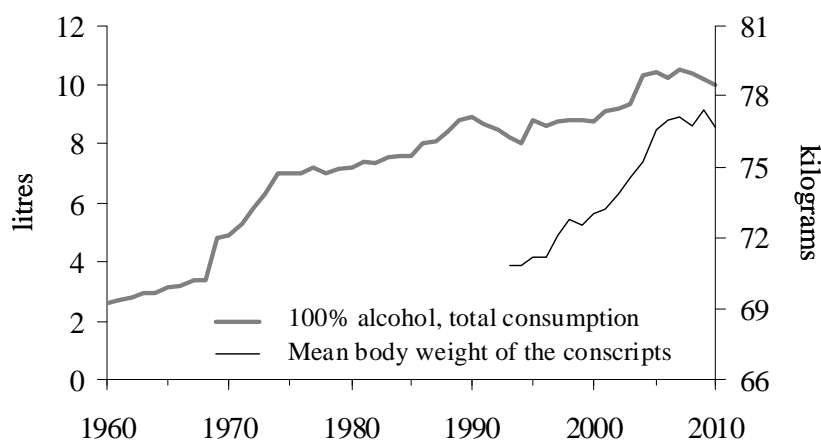


Figure 1. Per capita alcohol consumption and mean conscript weight over time (Finnish Defence Forces 2010, THL 2010). (Mean conscript height remained unchanged.)

2. Review of the literature

2.1 Alcohol through history

Alcohol is a product that has provided a variety of functions for people throughout history (Hanson 1995). While no one knows when beverage alcohol was first used, it was presumably the result of a fortuitous accident occurring at least tens of thousands of years ago. The discovery of late Stone Age beer jugs has established the fact that purposely fermented beverages existed at least as early as about 12,000 years ago, and it has been suggested that beer may have been used as a staple before humans learned to make bread. The levels of amino acids and vitamins in beverages increase during fermentation, which may explain the frequent lack of nutritional deficiencies in populations whose diets were generally poor. Alcoholic beverages have also long served as thirst quenchers, since water supplies in the past have often been either unhealthy or questionable at best. Experience had shown that alcoholic drinks were safer than plain water, which was usually taken from sources used to dispose of sewage and garbage. Other antiseptic and medicinal uses of alcohol were widespread as well and related was the interest of contemporary health professionals in the discovery of distillation. In medieval Europe, distilled alcohol was hoped for as a cure to ailments. While this obviously was an illusion, alcohol was, however, important for example during the horrors of the Black Death by providing relief from sickness through relaxation, by improving mood and by easing of pain. From the earliest times to the present, alcohol has also played an important role in religion and worship, as a social lubricant and as an enhancer of eating pleasure.

Today, many of the historical functions of alcohol have become obsolete and its harmful effects often exceed the benefits. It has been known for centuries that excess alcohol consumption is somehow associated with increased illness and death. In the 1920s, studies of the death rates among the various types of drinkers found that heavy drinkers had higher rates of overall mortality and of mortality from cirrhosis than did lighter drinkers or abstainers (Pearl 1926). Since then, the diseases linked to alcohol consumption have continued to grow and become an enormous burden to modern society.

2.2 Effects of ethanol on health

2.2.1 Alcohol-related general health problems

Almost all tissues in the body are affected by ethanol and it is closely related to more than 60 medical conditions (Rehm et al. 2003). The adverse health effects of excessive or even moderate ethanol consumption include both physiological and mental problems. There are also distinct differences in the effects resulting from the different patterns of ethanol intake, with the chronic pattern producing a different array of health hazards than acute (binge) drinking. Acute ethanol intake is typically presented in injuries and poisonings, and in increased suicide and violence rates. For example, the studies of trauma patients have shown that alcohol is involved in about two-thirds of their head injuries, and that the relative risk starts to increase sharply above a blood alcohol level of 1.5‰ (Savola et al. 2005).

Alcohol metabolism occurs mainly in the liver, which is also therefore a major target for chronic ethanol toxicity. As a consequence, only a few days' consumption of excess alcohol may cause fatty changes in the liver (Lieber 1995), although these are usually reversible (Diehl 1998, Younossi 1998, Mann et al. 2003). Nonetheless, with continued drinking, the accumulation of fat is a common finding (Younossi 1998, Bellentani et al. 2000) and an early sign of alcoholic liver disease (ALD), which can further progress to alcoholic hepatitis and fibrosis. An often life-threatening condition of cirrhosis develops in about 10–15% of all alcoholics (Mann et al. 2003). In addition to the liver problems, adverse health effects related to chronic alcohol intake also include pancreatitis, hypertension, arrhythmias, cardiomyopathy, gastrointestinal problems, increased susceptibility to infections, skin problems, hormonal disturbances, gout, several types of cancers, neurologic symptoms and psychiatric disorders (MacGregor and Louri 1997, Rehm et al. 2003, Choi et al. 2004, Bhole et al. 2010, Qureshi et al. 2010).

One of the most devastating manifestations of ethanol-related harm is fetal alcohol spectrum disorders (FASD), which are developmental defects caused by prenatal alcohol exposure (Autti-Rämö et al. 2008). FASD vary in severity, ranging from isolated dysmorphism to profound abnormalities in craniofacial features, growth and intellectual functioning. It is not currently known what amount of ethanol, if any, can safely be consumed during pregnancy. Studies suggest that even very low levels of alcohol can lead to adverse consequences at a group level (Sood et al. 2001, Sayal et al. 2007, van Faassen and Niemelä 2011). The consensus is that the risk of damage to the fetus increases significantly after about 1–2 drinks per day or with recurrent consumption of more than 5 drinks on a single occasion, the high occasional blood alcohol levels further possessing a greater risk than lower continuous consumption (Autti-Rämö et al. 2008). In Finland approximately 3,000 pregnancies yearly are carried by substance-dependent mothers, and at least 550 newborns per year suffer from FASD (Autti-Rämö et al. 2008).

2.2.2 Suggested positive effects of alcohol

During the past few decades, the possibility that small amounts of alcoholic beverages could promote longevity has been discussed repeatedly. A recent meta-analysis comprising 34 follow-up studies and more than a million subjects showed the lowest total mortality to be at approximately half a drink daily (corresponding to 6 g of alcohol), but up to 2–4 drinks daily in men and 1–2 drinks daily in women still conferred benefit (Di Castelnuovo et al. 2006). The typical dose-response curve between alcohol and total mortality is J-shaped: compared to abstainers, the risk declines with a low level of alcohol consumption, while excess consumption is associated with increased risks.

The benefits of alcohol drinking are thought to be mainly due to the prevention of cardiovascular diseases. Protection can result from increased or modified high-density lipoprotein (HDL) cholesterol, improved insulin signalling, from favorable changes in hemostatic profiles, and from reduced inflammation (Liisanantti et al. 2004, Bau et al. 2007, Mäkelä et al. 2008, Liisanantti and Savolainen 2009). Positive effects have been shown to derive from both ethanol itself, as well as from other components in alcoholic beverages. Especially red wine has often been mentioned. Interestingly, some studies have suggested acutely improved coronary blood flow from red wine but not from de-alcoholized red wine or from cognac (Kiviniemi et al. 2007, Kiviniemi et al. 2008). Thus far no uniform evidence, however, exists as to the type of beverage which produces the most benefit.

It remains controversial as to whether patients with a high risk of cardiovascular disease should be advised to drink small amounts of alcohol, as the benefits appear to be a matter of context. For instance, HDL-cholesterol concentration continues to rise beyond moderate drinking and can, in fact, sometimes be used as a marker of recent excessive alcohol intake (Szegedi et al. 2000). The benefits are also disproportionate across ages: a direct dose-response relation between alcohol consumption and death has been reported in men aged 16–34 and in women aged 16–44, and a U-shaped relation starting at age 35–44 in men and at age 55–65 in women (White et al. 2002). Another important issue with respect to the health effects of ethanol has been raised by the extraordinary fluctuations in life expectancy in parts of the former Soviet Union since the mid-1980s (Leon et al. 1997, Shkolnikov et al. 2001). There the large changes in alcohol consumption and deaths from acute alcohol poisonings and coronary heart disease occur in parallel. It has been argued that binge drinking, a pattern of consumption common in Russia, may be leading to sudden cardiac deaths that are not classic deaths from coronary heart disease (McKee and Britton 1998, McKee et al. 2001). In Finland, acute drinking of intoxicating amounts has been shown to associate with an increased risk of cardiogenic brain embolism in subjects who have an existing source of thrombus (Hillbom et al. 1999).

Currently, the data are not yet sufficient to encourage those who do not drink alcohol to start, especially as some individuals always will find it difficult to drink moderately, and because the risks among teenagers and young adults outweigh any benefits that may accrue later in life (National Institute on Alcohol Abuse and Alcoholism 2000, Bau et al. 2007). Moreover, the same beneficial effects could in most cases probably be achieved with regular exercise and proper diet, without the risks related to alcohol consumption.

2.2.3 Gender-dependent consequences of alcohol intake

The maximum amount of ethanol recommended for women is about half of that for men and likewise with heavier consumption, the health hazards appear at lower doses in women than in men (White et al. 2002). Several reasons have been postulated for that, which are also likely to exist in combinations: 1) Women are usually smaller in size and therefore experience higher blood ethanol concentrations than men after consuming the same amount of ethanol; 2) Even with the same amount ingested per kg of body weight, the concentrations are higher in women because of their having less body water to dilute the ethanol; 3) In addition, the rate of alcohol metabolism in the stomach (first-pass metabolism) is lower and more ethanol enters the circulation (Schenker 1997); 4) In premenopausal women, the potential beneficial effects of drinking are weakened by their inherently low risk of cardiovascular diseases; 5) Women are also more susceptible to liver damage, possibly relating to the actions of sex hormones (Sato et al. 2001); 6) And finally, the risk of breast cancer is increased by about 10% for each additional daily drink (Longnecker 1994, Ellison et al. 2001).

2.2.4 Other aspects determining the individual susceptibility to alcohol-related health problems

In addition to the previously discussed effects of gender, and drinking pattern (2.2.2), various other factors also influence an individual's susceptibility to alcohol-related adverse health effects. Of these, perhaps the most commonly known difference in the effects of alcohol is caused by the genes encoding alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzymes. Certain gene variants encode enzyme forms that metabolize ethanol faster than the others or are unable to process acetaldehyde, the first metabolite of ethanol. The concomitant build-up of acetaldehyde results in the Oriental flushing syndrome, which is characterized by facial flushing, palpitations, nausea, headache, and other symptoms and which also associates with cancers of the head and neck (Yokoyama and Omori 2003, Brennan et al. 2004). As indicated by its name, the related gene variants are frequently observed in Asians; in Caucasians they are rare. However, some Caucasians also experience alcohol-related flushing, although it is of much shorter duration and intensity, and does not appear to dictate the amount of ethanol consumed. This flush has been suggested to be due to inherited low cytosolic ALDH activity (Ward et al. 1994, Ward et al. 1998), whereas the mutations in Asians affect the enzymes in the mitochondria.

There are also genetic differences in one's susceptibility to liver damage, although in our population, environmental factors appear to predominate in varying the risk. According to a 2006 review, the only independently replicated observation of an association between genes and ALD in Caucasians has been with the tumor necrosis factor- α (TNF- α) -238 polymorphism (Day 2006). Among environmental factors, the universal evidence on the role of obesity and smoking is solid (Klatsky et al. 2006). In addition, coexisting hepatitis B or C infections and the use of drugs may also influence the individual course of alcohol-related liver damage. On the other hand, coffee consumption has been shown to protect against liver dysfunction (Klatsky et al. 2006). Protective mechanisms have been suggested both at the cellular level through both anti-

inflammatory and anti-fibrotic effects, and at the epigenetic level through the reversal of ethanol-related histone acetylations in pro-inflammatory genes and the consequential changes in the chromatin conformation that have facilitated transcription (Shukla and Aroor 2006, Kendrick and Day 2007, Kendrick et al. 2010).

2.3 Assessment of ethanol consumption

2.3.1 Definition of drinking patterns

The risks associated with ethanol consumption are influenced both by the amount and the way of drinking. Based on these two, the different drinking behaviors can be classified into five basic patterns as follows (Saunders and Lee 2000, Saunders 2006):

- 1) Alcohol abstainers
- 2) Moderate drinkers, whose drinking does not generally cause problems either for the drinker or for society, and who generally avoid becoming intoxicated
- 3) Hazardous drinkers, who consume large amounts alcohol on certain occasions (binging), or relatively large amounts frequently, but who show no obvious immediate disorders. Alcohol-related problems, nevertheless, are likely to emerge in the future, if the same drinking behavior is continued
- 4) Harmful drinkers, who have physical or mental problems due to their drinking, although they do not fulfil the criteria for alcohol dependence
- 5) Alcoholics, who meet the criteria for alcohol dependence including alcohol craving and the presence of tolerance and withdrawal symptoms.

In practice, the differentiation between moderate and excessive alcohol consumption is difficult and, consequently, the thresholds for defining the different patterns also often exhibit variation, depending on the source and country. In Finland, drinking is considered to be excessive in males consuming 24 standard drinks or more per week on average, or 7 standard drinks or more per occasion weekly (Sillanaukee et al. 1992). In females, the corresponding limits are 16 drinks or more and 5 drinks or more, respectively. A Finnish standard drink is 12 g of pure alcohol, which is the amount contained in each of 33 cl regular beer, 12 cl wine and 4 cl spirits. Compared to many other countries, our national guidelines can be considered to tolerate quite heavy consumption. In the United Kingdom, for instance, the limits are set at 168 g (about 14 Finnish drinks) for men and 112 g (about 9 Finnish drinks) for women weekly, including 2 or 3 days without any alcohol (Royal College of Physicians 1987). In the United States, the recommended criteria for drinking that associates with an increased risk of alcohol-related problems is more than 4 drinks a day, or more than 14 per week for men, and more than 3 drinks a day, or more than 7 per week for women with the assumption of 14 g of ethanol in one drink (National Institute on Alcohol Abuse and Alcoholism 2005). Probably the most referenced guideline is that of the American Heart Association, which stipulates that alcohol consumption should be limited to no more than 2 drinks per day for men and 1 drink per day for women, and that ideally, consumption occurs with meals (Lichtenstein et al. 2006). In addition to describing

the current drinking pattern with the help of pre-defined criteria, the introduction of a category of former drinkers is often important in research settings to avoid bias due to individuals who stopped drinking because of health problems (Di Castelnuovo et al. 2006).

2.3.2 Self-reporting of drinking habits

Self-reporting is used in health care settings to detect adverse drinking habits, and is used in research to quantify the amount of ethanol consumed. The method itself is considered to be powerful, but its reliability decreases if the patient has memory problems, difficulties in understanding questions, problems in performing calculations to quantify drinking or has a tendency for intentional dissimulation. In addition, unintentional misestimating may also bias the results. One study on college students revealed a clear overestimation of the amount of fluid that should be poured to create a standard drink (White et al. 2003). The volume of one serving has also increased in recent years in both restaurants and grocery stores, now more often deviating from a standard drink and thus possibly increasing underestimation.

The Alcohol Use Disorders Identification Test (AUDIT) is a specific questionnaire for screening alcohol abuse (Saunders et al. 1993). It consists of a total of 10 questions, which focus mainly on the level and frequency of consumption and the adverse consequences of drinking. AUDIT is considered as the most sensitive among the current questionnaires to detect hazardous and harmful drinking (Seppä et al. 1995, MacKenzie et al. 1996, Reid et al. 1999) and its shorter versions have also been found to be effective and can be chosen for use in busy medical offices and emergency rooms (Tuunanen et al. 2007, Aalto et al. 2009). In case of a positive screen test, brief intervention of at least 5–10 minutes should be delivered to let the patient know the risks that follow from such drinking, and to motivate and give tools to reduce consumption (Kaner et al. 2007, McQueen et al. 2009). In addition to AUDIT, the other well-known questionnaires are the four-question CAGE (acronym of the key words in its questions: cut down, annoyed, guilty, eye-opener) (Ewing 1984) and the Michigan Alcoholism Screening Test (MAST) (Selzer 1971), which is the most extensive of these with 25 questions directed at the recognition of drinking problems, help-seeking behavior and alcohol-related disabilities.

A commonly used means of quantifying the amount of ethanol consumed is to use the timeline follow-back (TLFB) method (Sobell and Sobell 1992), or alternatively to simply ask about the average, e.g., weekly consumption in the preceding, e.g., 12 months. In the TLFB approach, the patient is asked his or her specific recollections of drinks and volumes during a given time period, which contrary to asking an average, also gives an opportunity to assess the individual's patterns of drinking.

2.3.3 Markers of ethanol consumption

Self-reporting or clinical observation are often limited in means of detecting excessive drinking and therefore various laboratory tests are used for more objective assessments (Hannuksela et al. 2007). Some of the tests assess alcohol consumption directly by measuring ethanol or other

specific compounds in the body fluids, and these are discussed below. An assessment can also be made indirectly through the evaluation of liver function, but since hepatic health is affected by various other factors than ethanol as well, such markers will be covered later in section 2.7.5 (2.7.5.1 γ -Glutamyltransferase (GGT), 2.7.5.2 Aminotransferases (ALT, AST)).

2.3.3.1 Ethanol concentration in body fluids

The ethanol concentration of blood, breath, or urine can be used to assess recent alcohol consumption, and when combined with clinical observations, they may also provide information on long-term drinking habits (Niemelä 2007). According to the National Council on Alcoholism (1972), concentration of blood alcohol exceeding 1.5‰ (150 mg/dl, 33 mmol/l) without obvious evidence of intoxication, 3‰ (300 mg/dl, 65 mmol/l) on any given occasion, or 1‰ (100 mg/dl, 22 mmol/l) when occurring in routine examination, is suggestive of alcoholism. Already concentration less than 1‰ in routine examination may be interpreted as being risky alcohol behavior (Käypä hoito 2010). In trauma patients, the blood alcohol concentration at the time of admission has been reported to be the best indicator of hazardous drinking practises and alcohol dependence (Ryb et al. 1999, Savola et al. 2004).

2.3.3.2 Carbohydrate-deficient transferrin (CDT)

Carbohydrate-deficient transferrin (CDT) is defined as an aberrant glycoform of transferrin, characterized by a reduced number of carbohydrate (sialic acid) moieties (Stibler and Kjellin 1976, Stibler 1991). Whereas the predominant isoform of transferrin in healthy individuals is tetrasialotransferrin, with four sialic acid moieties, excessive drinking has been shown to increase asialo- and disialotransferrin, with none and two sialic acids, respectively (Mårtensson et al. 1997, Helander et al. 2001). To account for the variation in total transferrin, CDT is further presented as a percentage of all isoforms (Sorvajärvi et al. 1996).

CDT is currently the most widely used marker for chronic excessive alcohol consumption. There is thus far no uniform opinion, however, on the amount and pattern of drinking needed to increase CDT. It is considered that rather large amounts, at least 50–80 g of ethanol daily for at least a week, may have to be consumed (Stibler 1991) and the consensus has become that CDT is not suitable for the screening of alcohol abuse in the general population, but rather should be measured when excess consumption is suspected. The sensitivities of CDT to detect alcohol abuse have mostly varied from 30% to 90% in different studies, depending on the population (Bortolotti et al. 2006). Lower sensitivities have been detected in women than in men, in overweight than in normal weight individuals, and in non-smokers than in smokers (Whitfield et al. 2008). Especially high sensitivities have been shown in relapsed alcohol abusers in whom CDT may increase at relatively lower levels of consumption, possibly due to sensitization (Walter et al. 2001). Subsequently, one special application in the field is to monitor CDT in the drunk drivers applying for licence regranting (normalization in about two weeks (Stibler 1991)). In addition to population differences, the comparison between different studies concerning the sensitivity of CDT has also been hampered by the lack of standardization, which nevertheless is

now in progress. While different methods at present may measure different isoforms, the recommendations of the working group have defined disialotransferrin as the primary target molecule for CDT measurement and as the single analyte on which standardization is to be based (Jeppsson et al. 2007). Recently, the successful establishment of a network of reference laboratories and candidate reference material was also reported (Oberrauch et al. 2008, Helander et al. 2010). In addition, a calibration trial among laboratories covering the current routine assays was presented, showing a marked reduction in the total coefficient of variation and practical functionality and accompanied by a conclusion that an appropriate reference system for CDT may soon become a reality (Helander et al. 2010).

The advantage of CDT as an analyte is its high specificity, in that it is not influenced by the presence of liver disease unless very severe (Stibler 1991, DiMartini et al. 2001, Berlakovich et al. 2004). A limited number of false results may follow from conditions such as congenital disorders of glycosylation or from genetic transferrin variants (Stibler 1991). These problems are especially related to immunological methods, except the latest launch N Latex CDT from Dade Behring (A Siemens Company), which should be insensitive to genetic transferrin variants (Delanghe et al. 2007). The other available methods, high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), are generally more laborious and time-consuming procedures, but they are beneficial in visibly documenting and the concomitant possibility for detecting analytically divergent transferrin patterns.

2.3.3.3 Mean corpuscular volume (MCV)

Increased mean corpuscular volume (MCV, red blood cell size), or macrocytosis, is typical of people with chronic excessive ethanol intake (Niemelä 2007, Käypä hoito 2010). In non-anaemic men, increased MCV is nearly always due to a problem drinking. In women, about one-third of non-anaemic cases are related to alcohol. MCV may also increase due to deficiencies of vitamin B₁₂ and folic acid, hypothyroidism, reticulocytosis, liver diseases, blood diseases and smoking. In another third of macrocytic women, the etiology nevertheless remains unsolved. The sensitivity of MCV in detecting harmful drinking and alcohol dependence is generally rather high, but less than that of CDT or GGT in detecting hazardous drinking. Some studies have, however, suggested MCV to be the best of the traditional markers for assessing female patients (Sillanaukee et al. 1998, Mundle et al. 2000).

The normalization time of MCV is longer than that of other commonly employed markers, lasting 2–4 months. Such a slow response to abstinence places limitations on its use for the short-term monitoring of drinking cessation, but offers a possibility for supervising longer periods (Morgan et al. 1981, Mundle et al. 1999).

2.3.3.4 Research markers not yet established in clinical routine

Ethyl glucuronide (EtG) and ethyl sulphate (EtS)

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are promising new markers for ethanol consumption (Schmitt et al. 1995, Helander and Beck 2004, Høiseth et al. 2008). They are formed by the enzymatic conjugations of ethanol with glucuronic acid and sulfate, respectively, and remain detectable much longer than the parent compound. After ethanol has been eliminated from the body, EtG and EtS are measurable in the urine up to about 5 days, depending on the initial alcohol dosage but also showing great inter-individual variation (Helander et al. 2009a, Høiseth et al. 2009). In addition, small amounts may be detected after unintentional ethanol exposure (ethanol in mouth wash or hand sanitizer, endogenous ethanol from digestive tract bacteria) or due to the ingestion of the marker itself (in non-alcoholic wine) (Politi et al. 2005, Costantino et al. 2006, Rosano and Lin 2008, Høiseth et al. 2010). Recently, the first commercial EtG assay was launched (DRI Ethyl Glucuronide Enzyme Immunoassay (DRI-EtG EIA), Microgenics Corp.) (Böttcher et al. 2008, Arndt et al. 2009), which now also makes this marker available to facilities other than research laboratories. The drawback of EtG is, however, that both false-positive and -negative results may follow from the microbial contamination of specimens (Helander and Dahl 2005, Helander et al. 2007, Baranowski et al. 2008, Thierauf et al. 2008, Helander et al. 2009b). EtS has thus far proved to be reliable in all but one study (Halter et al. 2009), in which degradation was observed after days of storage under conditions resembling post-mortem putrefaction, and it has been suggested that EtS should possibly replace EtG in the future (Helander et al. 2007).

Phosphatidylethanol (PEth)

Phosphatidylethanol (PEth) is an ethanol-phospholipid adduct that is formed only in the presence of ethanol via the action of phospholipase D, at the expense of the natural product phosphatidic acid (Yang et al. 1967). PEth has been shown to be a sensitive (94.5–100%) and specific (100%) marker of the excess ethanol consumption in alcoholics with a detection window of up to 3–4 weeks after the person's last drinking (Wurst et al. 2004, Hartmann et al. 2007, Wurst et al. 2010). The threshold of total ethanol intake that yields detectable PEth has been considered to around 1,000 g, with a mean daily intake of about 50 g, whereas a 50 g dose on a single day has not been detected (Varga et al. 1998). However, recent advances in methodology suggest that with liquid chromatography-mass spectrometry analysis (LC-MS), instead of previous HPLC, it could be possible to detect low frequency or even single heavy drinking as well (Gnann et al. 2009). LC-MS can also identify individual PEth species, which could allow for its standardization and use as a legally defensible confirmatory analysis (Helander and Zheng 2009). In addition, the successful production of specific monoclonal antibodies also show promise for having an assay for total PEth available later for common expertise and for inexpensive laboratory equipment (Nissinen et al. 2008). The special characteristic of PEth that needs consideration, both in research and in routine, is its unsuitability for storage at room temperature and at –20 °C due to the risk of a false-positive result from the post-collection synthesis in the presence of ethanol; the samples may be stored at +4 °C up to 5 days and further at –80 °C (Aradóttir et al. 2004, Helander and Zheng 2009).

Fatty acid ethyl esters (FAEE)

Fatty acid ethyl esters (FAEE) are non-oxidative metabolites of esterification of ethanol with fatty acids. In serum, the levels of FAEE remain elevated for up to 12–24 hours after the cessation of drinking (Doyle et al. 1996, Bisaga et al. 2005). In addition, a specific FAEE species – ethyl oleate – has been suggested as being higher in chronic alcoholics than in episodic drinkers (Soderberg et al. 2003). FAEE may also serve as long-term markers of ethanol intake when assessed, for example, from hair, in which concentrations have been shown to be different between alcoholics, moderate drinkers and abstainers (Auwärter et al. 2001, Yegles et al. 2004). Some FAEE may be found in the hair of strict abstainers as well, but a concentration above 1 ng/ml can be taken as strong evidence of excessive drinking behavior. The level of elevation with heavy consumption, however, does not seem to quantitatively correlate with self-reported consumption. Rare false positive cases may result from contamination with ethanol-containing hair care products so that in doubtful cases, pubic hair should be analyzed for comparison (Hartwig et al. 2003). A promising application of FAEE is the assessment of FASD from neonatal hair, which begins to grow around six to seven months of fetal life and is collectable until shedding around three months after birth (Caprara et al. 2006). In general, the sample processing and methods for FAEE analysis are labor- and time-intensive, including gas chromatography-mass spectrometry, and may prevent its routine use in clinical laboratories.

Marker combinations: GGT-CDT

The new combinations of old markers have also showed interesting possibilities in the recent literature, especially the mathematical formulation of GGT and CDT. The rationale for combining these two is that they frequently increase in different individuals and may represent different types of ethanol-induced pathophysiological processes (Löf et al. 1994, Helander et al. 1996). The attempt is thus to gain the benefits of two diagnostic entities. Moreover, since both markers belong to the set of routine assays, their combination marker would be cost-effective and easy to manage in hospital laboratories. Conventionally, GGT and CDT have been combined by seeing whether either is elevated, but with this approach, the improved sensitivity is often accompanied by a loss of specificity (Salaspuro 1999, Anton et al. 2001, Schwan et al. 2004). The equation $0.8 \times \ln(\text{GGT}) + 1.3 \times \ln(\text{CDT})$, using absolute CDT concentrations, was first introduced by Sillanaukee and Olsson (2001) and Sillanaukee et al. (2000), and later improved by Anttila and co-workers (2003), who replaced the absolute CDT concentrations with a CDT percentage of total transferrin. With these combinations, decreased specificity appears no longer to be an issue, while the sensitivity in most studies has exceeded that of the traditional markers (Sillanaukee et al. 2000a, Chen et al. 2003, Berner et al. 2006, Hietala et al. 2006, Bianchi et al. 2010). In clinical work, it could make sense to interpret the results of both the combination marker and the single CDT to reach objective conclusions on a patient's alcohol consumption. Until the implementation of CDT standardization, the values of these measures may nevertheless differ between laboratories (Helander et al. 2010).

2.4 Obesity: past and present

Reviewing the history of obesity reveals that it is an age-old condition. The first sculptural representations of the human body about 20,000–35,000 years ago depict obese females (Conard 2009). The sculptures are collectively described as "Venus" figurines in reference to the Roman goddess of beauty, since the prehistorians of the early 20th century assumed they represented an ancient symbol of wealth and fertility.

In the course of time, the perception of obesity has varied. Once symbolizing fertility, ancient Egyptians considered obesity to be a disease and depicted their enemies as obese individuals. The Aztecs believed that obesity was supernatural, but rather than a blessing, it was an affliction of the gods. In cultures struggling with food scarcity, however, obesity has been viewed if not as ideal beauty, at least as a sign of security and welfare. For example, obesity was common among high officials in Europe in the Middle Ages and the Renaissance. In fact, even today, female fatness is viewed as a sign of social status and is a cultural symbol of beauty, prosperity and fertility in some areas of Africa (Mokhtar et al. 2001).

The unique feature of the last century is the deviation of the concepts of ideal beauty and healthy body weight in Western society. The weight that is viewed as an ideal has become lower since the 1920s. This is illustrated by the fact that the average height of the Miss America pageant winners increased by 2% from 1922 to 1999, while their average weight decreased by 12% (Rubinstein and Caballero 2000). On the other hand, people's views concerning healthy weight have moved in the opposite direction. In Britain, the weight at which people considered themselves to be overweight was significantly higher in 2007 than in 1999 (Johnson et al. 2008). This change is believed to be due to the increasing rates of adiposity, leading to an increased acceptance of extra body fat as being normal. With all the gathering of information on the dangers of obesity, this adjustment is considered alarming by many.

2.5 Obesity as a health problem

Obesity is a medical condition in which excess body fat has accumulated to the extent that health may be impaired (World Health Organization 2000). It is considered a chronic disease in its own (Burton and Foster 1985), in addition to which it further increases the risk of various other disorders, either directly or indirectly, through a shared cause of, for instance, poor diet or sedentary lifestyle. The most common co-morbidities include type 2 diabetes, hypertension, the metabolic syndrome, coronary heart disease, cerebral infarction and hemorrhage, sleep apnea, gout, gallbladder disease, non-alcoholic fatty liver disease (NAFLD), osteoarthritis, asthma, and at least cancers of the breast, cervix, large intestine and kidney (Käypä hoito 2006). Moreover, excess body weight may also have adverse effects on mental health and it may decrease fertility. In general, the higher the degree of obesity and the earlier its onset, the higher the risks of health problems. In a follow-up study conducted between 1948 and 1990, obesity at age 40 was estimated to reduce life expectancy by on average six to seven years (Peeters et al. 2003). Similar figures were also suggested in a recently published analysis of 57 follow-up studies,

which estimated that a body mass index (BMI) of 30–35 kg/m² was associated with a two- to a four-year reduction in life expectancy, while a BMI of 40–45 kg/m² had shortened life by eight to ten years (Prospective Studies Collaboration 2009).

2.6 Assessment of excess body weight

2.6.1 Body mass index (BMI)

The most commonly used method for assessing obesity is to calculate the body mass index (BMI), which is defined as weight in kilograms divided by the square of height (kg/m²) (Eknoyan 2008). According to the recommendations by the World Health Organization (2000), BMI <18.50 kg/m² denotes underweight, BMI 18.50–24.99 kg/m² normal weight, BMI 25.00–29.99 kg/m² overweight, and BMI ≥30.00 kg/m² obesity, so that the risk of co-morbidities usually is low with underweight (but the risk of other clinical problems is increased), average with normal weight, increased with overweight, and moderate to very severe in obesity, depending on its degree. These guidelines fit well with the white population and are considered to be the international classification; however, for some ethnicities, the risks are already substantial at a lower BMI than 25 kg/m². In Asian and Pacific populations, a specific BMI reflects a higher percentage of body fat than the same BMI in, for example, Europeans, and therefore additional trigger points for public health actions for these have been recommended at 23 kg/m² (BMI 23.00–27.49 kg/m², increased risk) and at 27.5 kg/m² (BMI 27.50 kg/m² or higher, high risk) (World Health Organization Expert Consultation 2004).

2.6.2 Waist circumference

Another tool for detecting obesity, and especially the related risks, is waist circumference. It is widely accepted that central obesity is more detrimental than peripheral obesity, and thus waist circumference is often a better predictor of diseases than BMI. Accordingly, waist circumference belongs to the definition of the metabolic syndrome, which is known as a cluster of risk factors for cardiovascular disease and type 2 diabetes (Eckel et al. 2005). The other risk factors include raised blood pressure, triglycerides and fasting glucose, and lowered HDL-cholesterol. There have, however, been discrepancies between the different guidelines (Adult Treatment Panel III 2002, Alberti et al. 2005, Grundy et al. 2005) as to whether or not an increased waist circumference should always be present for diagnosis and how to set its thresholds. In an effort to unify the criteria, several major organizations lately created a joint interim statement (Alberti et al. 2009), suggesting that waist measurement would continue to be a useful preliminary screening tool, but should not be an obligatory component of the metabolic syndrome. Instead, a total of any three risk factors must be met. Furthermore, the cut-off points for elevated waist circumference should be population- and country-specific, but at least at this time, it would be up to local decision-making groups to choose between thresholds at which risk starts to increase (≥94/80 cm for white men/women of European origin), or at which it already has substantially

increased ($\geq 102/88$ cm similarly). In general, the metabolic syndrome is estimated to double the risk of developing cardiovascular disease over the next five to ten years, while the risk over the lifetime undoubtedly is even more increased (Alberti et al. 2009). The risk of type 2 diabetes is approximated to become fivefold.

2.7 Alcohol, obesity, and the liver

2.7.1 Main features of alcoholic liver disease (ALD)

Alcoholic liver disease (ALD) covers a wide spectrum of morphological abnormalities, ranging from fatty liver to alcoholic hepatitis, fibrosis and cirrhosis. These injuries usually develop sequentially, but may then exist in combinations (Diehl 1998). The most important factor for both the short-term and long-term survival of patients with ALD is abstinence from alcohol, although the disease may progress despite complete sobriety as well (Parés et al. 1986, Diehl 1998).

Alcoholic fatty liver (steatosis) is the earliest and most common histological consequence of alcohol abuse. It may appear even after a few days of excessive ethanol consumption (Lieber 1995), and is usually fully reversible (Diehl 1998, Younossi 1998, Mann et al. 2003). This condition may be due to several mechanisms (Donohue 2007). Traditionally, it was considered to result from an altered redox state generated by ethanol metabolism, providing more substrate availability for lipogenesis and inhibiting mitochondrial fatty acid oxidation. Later discoveries have also characterized ethanol- and acetaldehyde-related changes in cell signalling processes and transcription factor activities that eventually alter the expression of genes involved in lipid biosynthesis, and in fatty acid transport and oxidation. Fatty liver is often accompanied by hepatomegaly, but typically occurs without any clinical symptoms (Diehl 1998, Mann et al. 2003).

Alcoholic hepatitis, mostly presenting in the form of steatohepatitis, is a clinically severe condition characterized by fat accumulation, inflammation and liver cell death (necrosis) (Diehl 1998). Patients with alcoholic hepatitis may be asymptomatic, only have an enlarged liver and perhaps some abdominal pain, or have a full-blown picture with tender hepatomegaly, jaundice, fever, malaise, anorexia, nausea and vomiting, liver failure and bleeding (Diehl 1998, Mann et al. 2003).

Fibrosis is considered to be an early feature of ALD, elicited by chronic hepatic necroinflammation and eventually leading in some subjects to cirrhosis (Diehl 1998). Fibrosis is defined as a gradual deposition of excessive connective tissue (scarring), whereas cirrhosis is a condition involving the whole liver, in which replacement of normal parenchyma with broadening bands of collagen causes a loss of liver function. Cirrhosis is irreversible and the most serious form of ALD associating with high mortality and many serious symptoms (Younossi 1998, Heron 2010).

2.7.2 ALD and obesity

Obesity potentiates the severity of alcohol-induced liver damage. Results of several population-based studies raise the concern that both the prevalence and severity of alcohol-related liver disease may be rising in conjunction with the obesity epidemic, since obesity seems to increase all stages of ALD from fatty liver to cirrhosis (Naveau et al. 1997, Bellentani et al. 2000, Raynard et al. 2002). Similarly, obesity is also a risk factor in the liver diseases of other etiologies, such as chronic hepatitis C (Nieminen et al. 2009). It is currently unclear whether the hepatotoxic consequences of obesity and ethanol ingestion are additive or synergistic (Diehl 2004), although the most recent studies are supportive of synergism (interaction), i.e., the combined effects of obesity and alcohol would be greater than the simple additive effects of each factor separately (Hart et al. 2010, Liu et al. 2010).

Several mechanisms have been postulated by which obesity could promote ALD. It has become apparent that adipose tissue is not an inert storage of excess fat, but in fact is a very active organ, which may contribute to the development and the severity of alcoholic liver disease by at least increasing insulin resistance and the production of cytokine TNF- α , as well by producing angiotensin II, norepinephrine, neuropeptide Y, and leptin (summarized by Diehl 2004). TNF- α , for example, belongs to the proinflammatory cytokines and plays an important role in initiating the inflammatory cascade in alcoholic hepatitis. Moreover, TNF- α is produced in significantly greater amounts by visceral fat than by subcutaneous fat, and ethanol may further increase the production by influencing fat distribution, i.e., by decreasing the subcutaneous fat and by increasing the visceral fat. Insulin resistance is also related especially to visceral fat and frequently causes steatosis. Given that the converse similarly is true, that the accumulation of fat in the liver causes insulin resistance, the combined effect of obesity and alcohol on steatosis could be of greater magnitude than that of a single component. Angiotensin II, norepinephrine, neuropeptide Y, and leptin in turn have been characterized as fibrogenic agents. For more detailed descriptions and references on all of these, see the above-mentioned article. Yet one other possible mechanism, oxidative stress, will also be discussed in 2.7.4.

2.7.3 Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is characterized by a hepatic fat accumulation in the absence of significant ethanol consumption, viral infection, or other specific causes of liver pathology. Its prevalence has been increasing parallel with the increasing prevalence of obesity, diabetes and the other components of the metabolic syndrome, and subsequently NAFLD has become accepted as the hepatic manifestation of the metabolic syndrome (Marchesini et al. 2001) and recognized as the most common chronic liver disease in the Western societies.

Similarly to alcoholic liver disease, NAFLD includes a spectrum of clinicopathological entities ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), with the possibility of progression to cirrhosis (Matteoni et al. 1999). However, contrary to the alcoholic etiology, NASH is usually a silent disease with few or no symptoms (Ali and Cusi 2009). The occurrence of NAFLD has been estimated to be 20–30% in the general population and much

higher in the obese, being an almost universal finding in obese patients with type 2 diabetes (Neuschwander-Tetri and Caldwell 2003, Bellentani and Marino 2009). About at least 10% of the NAFLD-subjects have NASH, with the progression rate of approximately up to one-third to cirrhosis (Neuschwander-Tetri and Caldwell 2003). However, the most prevalent cause of death in those with NAFLD is due to cardiovascular diseases (Ong et al. 2008). To date no single intervention has been reliably effective for NAFLD and NASH, and the cornerstones of the current recommendations are the treatment of comorbid conditions associated with the metabolic syndrome and the reversal of predisposing factors (Lam and Younossi 2009). In relation to weight loss, it appears that physical activity may be of benefit even without a reduction in body weight (Caldwell and Lazo 2009). This can have great motivational importance for patients attempting lifestyle changes.

Insulin resistance is considered to be important in the development of NAFLD (Almeda-Valdés et al. 2009). Peripheral insulin resistance causes triglyceride lipolysis within the adipose depots and inhibits adipocyte uptake of fatty acids, both these increasing the delivery of fatty acids to the liver. The compensatory hyperinsulinemia in turn promotes hepatic *de-novo* lipogenesis and esterification of fatty acids into triglycerides, and decreases fatty acid oxidation. Hence obesity, which often leads to insulin resistance and particularly in such cases, presents a fatty challenge to the liver.

It was long uncertain as to whether or not ALD and NAFLD are similar in their pathologies. However, that controversy has been considered as having been solved by the work of Xu and co-workers in 2003, showing the fat-derived hormone adiponectin to be effective in alleviating both the alcohol-induced and obesity-induced liver abnormality in mice. The improvement was suggested in part due to a suppression of TNF- α production and also an antagonism of its function, in that adiponectin and TNF- α elicit many opposite functions, for example, TNF- α being a causative factor of insulin resistance, whereas adiponectin increases insulin sensitivity. Similarly, TNF- α is a proinflammatory cytokine, whereas adiponectin has direct anti-inflammatory effects. Although the primary etiology of NAFLD and ALD is different, these liver diseases apparently share similarities in their progression. Interestingly, Wang and co-workers (2010) recently demonstrated that experimental NASH is aggravated already by moderate ethanol administration.

2.7.4 Postulated mechanism: oxidative stress

One of the most advocated mechanisms that could explain why alcohol and obesity, for example, potentiate each other in progression from fatty liver to steatohepatitis and fibrosis, is oxidative stress. This mechanism is called "the theory of two hits", and the editorial by Day and James (1998) introducing it has since become extensively cited in related research. Briefly, the first hit is the deposition of fat in the liver triggered by different factors, which renders the hepatocytes susceptible towards the second hit, the oxidative stress.

The origins of the theory lie in the difficulties to develop animal models of severe alcoholic liver disease by administering simple ethanol. In other words, further manipulations are needed to produce necroinflammation and fibrosis (Lieber 1988). Similarly, many patients with non-

alcoholic steatosis never progress in their disease. Although some degree of oxidative stress can be seen in the steatosis of most etiologies (Lettéron et al. 1996), it is believed that an alternative or extra source of oxidative stress is needed to escape the cellular defence systems and to progress from a fatty liver. For example, most reactive oxygen species generated in the livers of ethanol-fed rats are produced in microsomes through the cytochrome P450 enzyme system (Lieber 1997), and further induction of this system by polyunsaturated fatty acids aggravates liver injury (Nanji et al. 1994). In addition, iron can be used to increase the oxidative toxicity in experimental animals (Tsukamoto et al. 1995) (ferrous iron exacerbates oxidative stress by a Fenton chemistry reaction with hydrogen peroxide and superoxide to yield more potent oxidants (Halliwell and Gutteridge 1984, Aust et al. 1985)) and, indeed, hepatic iron overload is associated with the liver damage severity in alcoholics and possibly also in NAFLD (Ganne-Carrié et al. 2000, Bonkovsky et al. 2003, Machado et al. 2009, Wallace and Subramaniam 2009). Insulin resistance on the other hand has been suggested as being capable of playing the roles of both the first and the second hit, since it often leads to fatty liver, which further decreases insulin sensitivity (Day 2002). The connections between insulin resistance, steatohepatitis and oxidative stress have been supported by the observations that individuals with NASH are significantly more insulin resistant than those with a fatty liver alone and that oxidative damage is more pronounced in NASH than in simple steatosis (Dixon et al. 2001, Marchesini et al. 2001, Sanyal et al. 2001).

In conclusion, the theory of two hits postulates that in the presence of one etiology strong enough to induce fatty liver in a considered individual, the additional oxidative stress by the second etiology markedly increases the risk for progressive liver damage (Day and James 1998). However, to add to the complexity, it must be noted that liver damage may be aggravated without an indication of increased oxidative damage as well (Wang et al. 2010). It was also recently emphasized by Choi and Diehl (2008) that fat accumulation itself may not necessarily be harmful and the first hit that sensitizes the liver for more serious damage, as is often interpreted; rather, the authors showed that the inhibition of fat accumulation worsened the hepatic injury in an experimental model of progressive NAFLD, and concluded that the deposition of triglycerides was actually protective in these animals and simply a reflection of the ongoing burden and impending lipotoxicity by the free fatty acids.

2.7.5 Related laboratory tests

Advanced liver disease induces abnormalities in several laboratory markers (Rosman and Lieber 1994). For example, the levels of serum albumin usually decrease due to a decreased synthetic capacity of the hepatic tissue as there are elongations in the prothrombin time (causing defective coagulation) as well. Impaired bilirubin removal and subsequent build-up may even be observed as a yellowish colour of the patient. Both in the advanced and earlier stage of pathology, the measurements of serum liver enzymes also serve as important diagnostic information. Analogously to the scope of the present design, these are discussed more below. Serum uric acid is not a liver marker *per se*, but is included owing to its role as a possible indicator of oxidative stress.

2.7.5.1 γ -Glutamyltransferase (GGT)

Serum γ -glutamyltransferase (GGT) is an enzyme derived from the liver, the changes in its activity having been used to monitor excessive alcohol consumption for several decades. In fact, GGT has been the most commonly used laboratory marker of heavy drinking. Several studies have reported a positive correlation between the amount of ethanol ingested and serum GGT levels, but the sensitivity of GGT has varied greatly between populations, from 10 to 90%, being lower in samples from the general practice and in the young (Salaspuro 1999). In addition, GGT tends to have a relatively low specificity for alcohol consumption, because it may also elevate, e.g., due to diabetes, obesity, pancreatitis, hyperlipidemia, cardiac insufficiency, severe trauma, nephrotic syndrome, renal rejection, and medication, and in patients with other than alcoholic forms of liver diseases (Niemelä 2007). Although the factors affecting GGT are numerous and individual variability in the reactivity to the changes in the body may be great, it has been suggested that even moderate alcohol consumption for a few consecutive nights might increase the activity of GGT in the blood for some days, as compared to the individual baseline value (Freer and Statland 1977a, Freer and Statland 1977b, Nemesánszky et al. 1988). With heavier consumption, normalization may take up to about 4–5 weeks (Anton et al. 2002), and as patients with non-ethanol related liver abnormality usually do not show profound changes in their GGT in the short-term, e.g., one week (Pol et al. 1990), documentation of decreased activity can be helpful in the differential diagnosis in pointing toward alcohol etiology.

Markedly increased GGT activities are usually considered to reflect tissue damage, especially if associated with abnormalities in other liver enzymes, but an increase may also be due to other reasons. Mild elevations of GGT can relate to its biological function to maintain the intracellular levels of glutathione and to metabolize glutathione conjugates (Zhang and Forman 2009). Glutathione is an important antioxidant, and its removal causes loss of viability, for example, in the cytochrome P450 2E1 (CYP2E1) enzyme expressing cells (Wu and Cederbaum 2001). The key features of CYP2E1 are its leakage of free radicals and subsequent oxidative stress, and the fact that ethanol is among the substrates of CYP2E1, especially when the alcohol dehydrogenase pathway is saturated by heavy drinking (Lieber 1997). In general and by various ways, oxidative stress is associated with diseases of the modern life, including cancer, neurodegenerative diseases, rheumatoid arthritis, atherosclerosis, NAFLD, obesity, diabetes, the metabolic syndrome, and others (Sihvo et al. 2002, Willcox et al. 2004, Roberts and Sindhu 2009). Interestingly, GGT levels have been shown similarly to associate with many of these diseases. In the recent literature, GGT has also been shown to have an independent predictive value (that cannot be explained by its association with any of the other risk factors) at least for cardiovascular diseases and type 2 diabetes (reviewed recently in Targher 2010), and the metabolic syndrome (Nakanishi et al. 2004, Nannipieri et al. 2005, Jo et al. 2009, Ryu et al. 2010). The mechanisms for such relationships have not yet been fully resolved.

2.7.5.2 Aminotransferases (ALT, AST)

Aminotransferases are measured primarily to assess the condition of the liver and are not as good indicators of excess drinking as GGT (Sillanauke 1996). Serum alanine aminotransferase (ALT) originates rather specifically from the hepatocytes, whereas aspartate aminotransferase (AST)

can also arise in clinically relevant activities from heart and skeletal muscle tissue (Pratt and Kaplan 2000). In an asymptomatic patient, the activities of serum aminotransferases may be increased at least due to alcohol abuse, medication, chronic hepatitis B and C, steatosis and NASH, autoimmune hepatitis, hemochromatosis, Wilson's disease (<40 years), α_1 -antitrypsin deficiency, celiac sprue, genetic errors in muscle metabolism, acquired muscle diseases, and strenuous exercise, with some of these being easily ruled out by specific tests (Pratt and Kaplan 2000). In addition, the interpretation of aminotransferases together may give specific information, as the activity of AST clearly over that of ALT is often supportive if alcoholic etiology is suspected (Nalpas et al. 1984, Salaspuro 1987, Sheth et al. 1998, Pratt and Kaplan 2000). In association with heavy alcohol consumption and most other etiologies, increased aminotransferases are considered a hallmark of hepatonecrosis and liver damage.

As with GGT, aminotransferases also seem to associate with general health so that ALT appears to have a much stronger association with BMI than with alcohol consumption (Adams et al. 2008). ALT associates especially with the hepatic fat content, and thus with NAFLD (Schindhelm et al. 2006) which, as a component of the metabolic syndrome, is highly related especially to the development of type 2 diabetes (Ghouri et al. 2010). In line with this view, several studies have shown that ALT has a predictive value on the aforementioned diseases, and apparently so even after an adjustment has been made for the classical risk factors (Vojarova et al. 2002, Hanley et al. 2004, Sattar et al. 2004, Hanley et al. 2005, Wannamethee et al. 2005, Goessling et al. 2008, Yun et al. 2009), although a few studies have also not found such independent associations (Nakanishi et al. 2004, Schindhelm et al. 2005). When considering possible pathologies, elevated serum ALT in NAFLD may be a consequence of leakage from the damaged hepatocytes or due to increased gluconeogenesis (alanine is an effective substrate) in the absence of insulin (Vojarova et al. 2002, Jadhao et al. 2004, Schindhelm et al. 2006).

2.7.5.3 Uric acid

Traditionally, uric acid is predominantly a marker of gout. It is an end product of purine metabolism in humans and in increased concentrations crystallizes in the joints, causing the disease. Both increased uric acid concentrations and gout are typical in alcoholics, which is due to increased adenine nucleotide turnover, related to metabolism of acetate to acetyl coenzyme A (energy for this reaction provided by adenosine triphosphate, ATP), and due to decreased renal excretion of uric acid, secondary to increased blood lactate levels (competition in tubular transport) (Puig and Fox 1984). Other reasons for hyperuricemia may be a high dietary intake of purine-rich foods (also including alcoholic beverages, especially beer), a high dietary fructose intake, conditions with elevated rates of cellular turnover, mutations in proteins involved in uric acid metabolism, reduced excretion by diseased kidneys, local ischemia, and circumstances associated with enhanced sodium reabsorption, such as a diuretic therapy, a low-sodium diet, obesity, insulin resistance or hyperinsulinemia, and hypertension (Strazzullo and Puig 2007, Lippi et al. 2008).

Uric acid has also recently gained attention as a marker for oxidative stress (Becker 1993, Glantzounis et al. 2005), although its role in associated diseases still remains controversial. Uric acid is a major antioxidant accounting for up to 60% of serum free radical scavenging capacity

(Maxwell et al. 1997) and since its administration increases plasma antioxidant potential (Waring et al. 2001) and reduces exercise-associated oxidative damage in healthy volunteers (Waring et al. 2003), for instance, it has been hypothesized to be protective. On the other hand, uric acid has been suggested to become a pro-oxidant in certain situations (Patterson et al. 2003, Sautin and Johnson 2008), particularly when exceeding normal concentrations so that in these cases, the antioxidative properties are probably overwhelmed by the detrimental effects and the relationship becomes causal (Lippi et al. 2008). So far, the largest body of research has been gathered on the association between uric acid and cardiovascular diseases, but according to recent studies, there may also be an independent link between elevated serum uric acid and the development of the metabolic syndrome and its components (Strazzullo and Puig 2007, Lippi et al. 2008).

2.8 Reference intervals of laboratory tests

2.8.1 Concept, definition, determining

Reference intervals provide a range of acceptable values for healthy individuals. These intervals serve as a basis for laboratory testing and are useful in determining whether the patient is well or not. If the result is not within the reference interval, the value is flagged in the laboratory information system (LIS) in order to indicate to the physician that the patient may need further examinations.

The concept of reference values was originally introduced by Gräsbeck and Saris (1969) (Gräsbeck 2004). According to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), the current concept of reference values can be considered at various levels (Solberg 1987): A *reference individual* is a healthy person fulfilling certain defined criteria. All those who would meet these criteria belong to a *reference population*, from which an adequate number of reference individuals is selected to form the *reference sample group*. A *reference value* is then measured from the reference individual of the reference sample group, and the statistical distribution of the reference values, the *reference distribution*, is used to produce the *reference limits*. Finally, the area between and including two reference limits is called the *reference interval*.

A reference interval is usually defined as the interval that 95% of the reference values fall into, leaving 2.5% of the reference values below the reference limit, and 2.5% of the reference values above. The calculation of limits is mostly based on two methods, i.e., parametric and nonparametric analyses, although other methods have also been developed (Horn and Pesce 2003). Parametric calculations estimate the reference intervals using a mean and two (exactly 1.96) standard deviations (SD) of the data for a Gaussian distribution. If the distribution is not Gaussian, the data can be adjusted by, for instance, logarithmic or square root transformations. The mean \pm 2SD is then calculated from the transformed data and the reference limits are obtained by transforming these back to the original units. This approach can be used only if the distribution of the data or transformed data is Gaussian. The nonparametric method, in turn,

makes no assumption about the distribution of the reference values. The values are simply ranked by ordering them from the lowest to the highest and the 2.5 and 97.5 percentiles are obtained as the $0.025 \times (n + 1)$ and $0.975 \times (n + 1)$ ordered observations. If the obtained values are not integers, then linear interpolation is carried out.

In addition to properly selecting the calculation method, the size of the reference sample group is important to achieve a reasonable degree of precision. For the nonparametric method, at least 120 values are needed to produce the 90% confidence interval of the obtained limit, and at least this amount of reference values is also recommended by the Clinical and Laboratory Standards Institute (CLSI) (2008). At least 39 values are needed to produce the limit itself (with 39 values, the limits become to the 1st and 39th values). For the parametric method, the reference limits and confidence intervals are calculable with even smaller sample sizes and in this regard, the quality management becomes more the responsibility of the investigator.

2.8.2 The Nordic Reference Interval Project (NORIP)

The modern lifestyle has created a need for a harmonization in reference intervals. Historically, the laboratories have been advised to establish their own reference intervals, which is, however, a highly demanding and costly process for a single laboratory to be performed according to the recommendations (Clinical and Laboratory Standards Institute 2008). In practise, laboratories have often used reference intervals obtained from the literature or have adjusted old intervals when introducing new methods in the laboratory. As a consequence, considerable variation has occurred in the reference intervals between laboratories. Recently, alongside advances in methodological consensus, the production of common reference intervals has become a potential approach to overcome these problems.

The Nordic Reference Interval Project (NORIP) set out to establish common reference intervals for 25 of the most commonly measured properties in clinical chemistry (Rustad et al. 2004a). The project was launched in March, 1998 in Oslo and supported by the Nordic Society of Clinical Chemistry (NFKK). The external quality assurance programs had shown that especially in Norway, the reference intervals for the same quantity in the same age and gender groups varied more than the corresponding analytical deviation could account for (Gadeholt 2004). It was also believed that the population was too homogenous to allow for a biological variation. After the Norwegians were the driving force, and based on a decentralized design from Denmark, the NORIP project was a joint action of all five countries (Denmark, Finland, Iceland, Norway, and Sweden), the key project members being elected by the national societies of clinical chemistry. At least 25 samples, evenly distributed on age and gender, were collected from a total of 102 Nordic laboratories mainly in 2000. In 2004, the project group published their recommendations for the common reference intervals after having concluded that partitioning by country was not necessary (Lahti 2004, Rustad et al. 2004a). Since then, these common reference intervals have been implemented in many, if not most, routine clinical chemistry laboratories in Finland.

3. Aims of the present research

Health problems due to alcohol consumption and excess body weight are rapidly growing in our society. Although both heavy drinking and obesity are known to cause derangements in liver function and increased oxidative stress, the early-phase effects of ethanol intake and adiposity on the corresponding laboratory markers have remained less understood.

The aims of the present work were as follows:

1. To explore the effects of various levels of alcohol consumption on liver enzymes and proteins, and the effects of reference population selection on diagnosing heavy drinking
2. To study the relationships between moderate drinking, excess body weight, and liver enzymes
3. To study the relationships between moderate drinking, excess body weight, and uric acid
4. To examine the impacts of moderate drinking and excess body weight on reference limits.

4. Materials and methods

4.1 Subjects

The subjects in these studies mainly consisted of those recruited for the NORIP survey, which was organized to establish common reference intervals for use in the Nordic countries (Rustad et al. 2004a). A total of more than 3,000 subjectively healthy individuals participated in this trial, and in addition to the blood samples, they provided data on a number of demographic characteristics, e.g., gender, age, ethnic origin, height and weight (self-report), alcohol consumption and smoking habits, menstrual status, use of estrogen preparations, and use of drugs or food supplements (Felding et al. 2004). The survey excluded individuals who had any evidence of current or recent illnesses, had consumed more than two standard drinks during the preceding 24 hours (one drink equivalent to 12 g of pure ethanol), had donated blood in previous five months, had diabetes (known or by survey results), were pregnant or breastfeeding, as well as most of those on prescribed drugs. A fasting (≥ 12 hours) state was recommended, and monitored by recording the time since the last meal. Smoking was not permitted one hour prior to sampling.

The distributions of the study population across the most relevant characteristics are shown in Table 1. The data from Sweden were excluded from these calculations, as well as from all the analyses in the present study, due to the inapplicable questioning of ethanol consumption (Felding et al. 2004). In addition, only subjects reporting 0 measures of alcohol per week (later referred to as abstainers) or 1–21 measures/week (moderate drinkers) were qualified from the other countries. The assessment was conducted by asking, "Do you normally drink 0, 1–21, or more than 21 measures of alcohol per week?" (0.5% more than 21 measures/week, 1.0% unanswered). In the final study sample, 96% of the subjects were of Nordic origin, 80% were non-smokers (3% unanswered), and 76% had not used drugs or food supplements (5% unanswered).

In addition to the NORIP participants, study I also included 133 heavy drinkers. This sample consisted of 105 men (age 45 ± 10 years) and 28 women (age 43 ± 11 years) who had been admitted for detoxification. They all showed a history of continuous or binge drinking, the mean alcohol consumption having been 64 (range 23–315) measures/week from the period of 4 weeks prior to the sampling, as assessed by questionnaires asking the number of standard drinks consumed during the last 24 hours, 1 week and 4 weeks. None of these heavy drinkers had any known liver disease or apparent symptoms of such a condition.

In study IV, which focused on the assessment of reference intervals for different groups, several further exclusion criteria to the above mentioned *a priori* rules were used. These were both analytical and individual aspects, which followed those applied to the calculation of the

NORIP recommendations (Rustad et al. 2004a). With respect to the analytes in the present study, the most common reason for rejection was the incompatibility of the methods used for the analysis of liver enzymes with the IFCC primary reference procedures (Schumann et al. 2002a, Schumann et al. 2002b, Schumann et al. 2002c, Strømme et al. 2004). It is likely that the data not fulfilling this criterion (13–46%) have biases that are both negative and positive so that the rest-effect would be an increase in variance, widening the reference intervals. These results can, however, be expected to distribute across the population, and therefore in studies I–III, such procedures were not used in order to maintain as large a data as possible. Furthermore, for GGT in paper IV, results were rejected only for person exclusion criteria, as the number of IFCC-compatible measurements for this analyte was too few to enable the study purposes. The distributions of GGT for the IFCC- and non-IFCC-compatible method types were ascertained as being essentially identical for high values, which should indicate that combined data could be used for reference interval calculations.

Table 1. Population characteristics. The data are percentages (%), except for age (mean \pm SD).

Characteristic	Male	Female
Gender	47	53
Age	47 \pm 18	46 \pm 17
Body mass index (BMI, kg/m ²)		
<19	1	5
19–25	54	69
25–30	41	22
>30	4	4
Alcohol consumption (measures/week)		
0	25	38
1–21	75	62

4.2 Ethical aspects

The NORIP project was approved by the ethics committees in each of the five Nordic countries (Felding et al. 2004) and the studies on heavy drinkers were approved by the Ethics Committee of the Seinäjoki Central Hospital, Finland. All research was carried out according to the provisions of the Declaration of Helsinki.

4.3 Measurements of laboratory markers

The measurements for the individuals representing the NORIP material were carried out between 1999 and 2001 in several Nordic routine clinical biochemistry laboratories, including the laboratory at the Seinäjoki Central Hospital (accredited according to SFS-EN ISO/IEC 17025).

The measurements were performed on thawed samples (stored at $-20\text{ }^{\circ}\text{C}$ for up to one month) in one series. Agreement between laboratories was ascertained by an analysis of the calibration material together with the samples and adjustment of the results to meet a common level in case of non-enzyme analytes; for enzymes, only the results obtained by assay conditions at $37\text{ }^{\circ}\text{C}$ which were compatible with and traceable to IFCC reference methods were included (Rustad et al. 2004a). The measurements from heavy drinkers were performed locally at Seinäjoki and applying the same procedures for harmonization.

4.4 Statistical methods

The SPSS 13.0 and 15.0 packages were used for the statistical analyses (SPSS Inc., Chicago, IL, USA), and a two-sided p-value <0.05 was taken as the level for statistical significance. The correlations were calculated as Pearson correlations for continuous Gaussian distributed parameters and as Spearman correlations if one or both parameters were categorical or skewed. The differences between correlations were analyzed with the z-test for correlation coefficients. The comparisons of frequency data were made using the χ^2 -test. The comparisons between two groups regarding parameters with Gaussian distributions were made by the t-test, and the comparisons between groups with skewed distributions with the Mann-Whitney test. Three or more groups were compared using one-way analysis of variance (ANOVA) and the Bonferroni *post hoc* test in case of Gaussian distributions and equal variances. Otherwise, the analyses were made with the Kruskal-Wallis test and the Tamhane's T2 *post hoc* test. The interactions among gender, drinking habits and BMI on different biomarkers were analyzed using factorial ANOVA. Linear regression was used to assess the proportion of variability and to determine the predictors of laboratory values. For factorial ANOVA and linear regression, a mathematical transformation was performed for skewed analytes to meet the prerequisites of these tests.

In paper IV, the Analyse-it 2.11 for Microsoft Excel (Analyse-it Software Ltd., Leeds, UK) was also used to calculate the reference limits. The limits were always calculated as 2.5 and 97.5 percentiles, similarly to the NORIP protocol (Rustad et al. 2004a). On the contrary, enzyme outliers were not excluded by identification of results lying outside the mean $\pm 4\text{SD}$ of the gender specific logarithmic data, but by performing the Dixon's outlier test for each reference data set separately (Dixon 1953, Clinical and Laboratory Standards Institute 2008). The Dixon's test identifies an extreme value as an outlier if the difference between the extreme and the next extreme value exceeds one third of the range of values in the sample. In study I, the upper reference limits for albumin and transformed ferritin were also calculated parametrically as the mean + 2SD.

5. Results

5.1 Effects of various levels of drinking and gender on liver enzymes, albumin, and ferritin (I)

In paper I, the levels of liver enzymes (GGT, ALT, AST) and proteins (ferritin, albumin) in serum were compared in a large population of abstainers, moderate drinkers and heavy drinkers. A significant correlation ($r > 0.3$) was found between the self-reported ethanol consumption and four of the five analytes (GGT, $r = 0.50$; ALT, $r = 0.39$; AST, $r = 0.45$; ferritin, $r = 0.49$; $p < 0.001$ for all). In the comparisons between groups, male heavy drinkers showed significantly higher ($p < 0.001$) values than both abstainers and moderate drinkers in all the variables, and also between abstainers and moderate drinkers in their GGT ($p < 0.001$) and ALT ($p < 0.05$). In women, heavy drinkers similarly showed the highest values, although somewhat less pronounced, but no significant differences between abstainers and moderate drinkers were noted.

This study also explored the influence of reference population selection on the reference limits and diagnosis of heavy drinking. Again, marked differences were found for the liver enzymes and ferritin, especially for men, in whom the increase from the upper reference limits based on abstainers to the limits based on moderate drinkers varied between 15% for AST and 68% for GGT, as calculated from Table 2. Among women, an increase was noted for ferritin and GGT, these being 35% and 25%, respectively.

Table 2. The upper reference limits for liver enzymes and proteins based on either abstainers or moderate drinkers as the reference population.

Analyte	Upper reference limit			
	Men		Women	
	Abstainers	Moderate drinkers	Abstainers	Moderate drinkers
γ -Glutamyltransferase (GGT, U/l)	63	106	63	79
Alanine aminotransferase (ALT, U/l)	58	73	49	48
Aspartate aminotransferase (AST, U/l)	39	45	41	37
Ferritin ($\mu\text{g/l}$)	274	342	141	191
Albumin (g/l)	47	48	46	46

Table 3 compares the marker-specific sensitivities resulting from the different reference limits. As expected, the abstainer-based limits identified more heavy drinkers with increased

values than the limits based on moderate drinkers. In addition, up to 9% of moderate drinkers also showed values above the limit. If the data using these lower limits were reported for genders separately, the proportions were slightly higher for men and on average about one-third lower for women, compared to those presented in the table. When using the limits from moderate drinkers, the decrease in diagnostic power was more obvious in men than in women.

Table 3. The percentage of heavy drinkers exceeding the limits from the different reference populations.

Analyte	Reference population →	% of heavy drinkers with value above ref. limit	
		Abstainers	Moderate drinkers
γ-Glutamyltransferase (GGT)		62	40
Alanine aminotransferase (ALT)		39	30
Aspartate aminotransferase (AST)		53	50
Ferritin		34	27
Albumin		20	13

5.2 Effects of moderate drinking and BMI on liver enzymes (II) and uric acid (III)

Paper II studied the effects of both drinking habits and BMI on the liver enzymes in abstainers and moderate drinkers, and found the effects of excess body weight and alcohol consumption parallel. When the participants were classified into subpopulations, each subgroup with a higher BMI had higher mean enzyme activities, and the activities in moderate drinkers were higher than those in the group of abstainers with the same BMI. The highest mean levels were thus found in obese moderate drinkers. The effects of these two lifestyle factors were especially strong for GGT and ALT in men (Figure 2).

In statistical analyses, the interactions between the BMI and drinking status did not reveal significance for any of the enzymes, i.e., the effects were additive. Interestingly though, for example, for GGT in men, the activities in obese moderate drinkers were higher than expected from the separate effects of an increased BMI and moderate drinking: compared to normal weight abstainers, the mean GGT activities were increased by 24% in normal weight moderate drinkers, by 36% in obese abstainers, and by 116% in obese moderate drinkers (theoretically $1.24 \times 1.36 = 1.69$, which is less than 2.16, i.e., less than an increase of 116% (calculated from Figure 2)). Also the correlation coefficient between the BMI and the enzyme level was 0.32 ($p < 0.001$) in moderate drinking men, whereas only 0.22 in abstainers ($p < 0.01$).

The observations on mean enzyme activities and the differences in partial correlations further indicated that GGT may be dependent more on ethanol intake and that ALT may be more strongly associated with BMI. For GGT, the increase in enzyme activity from abstainers to

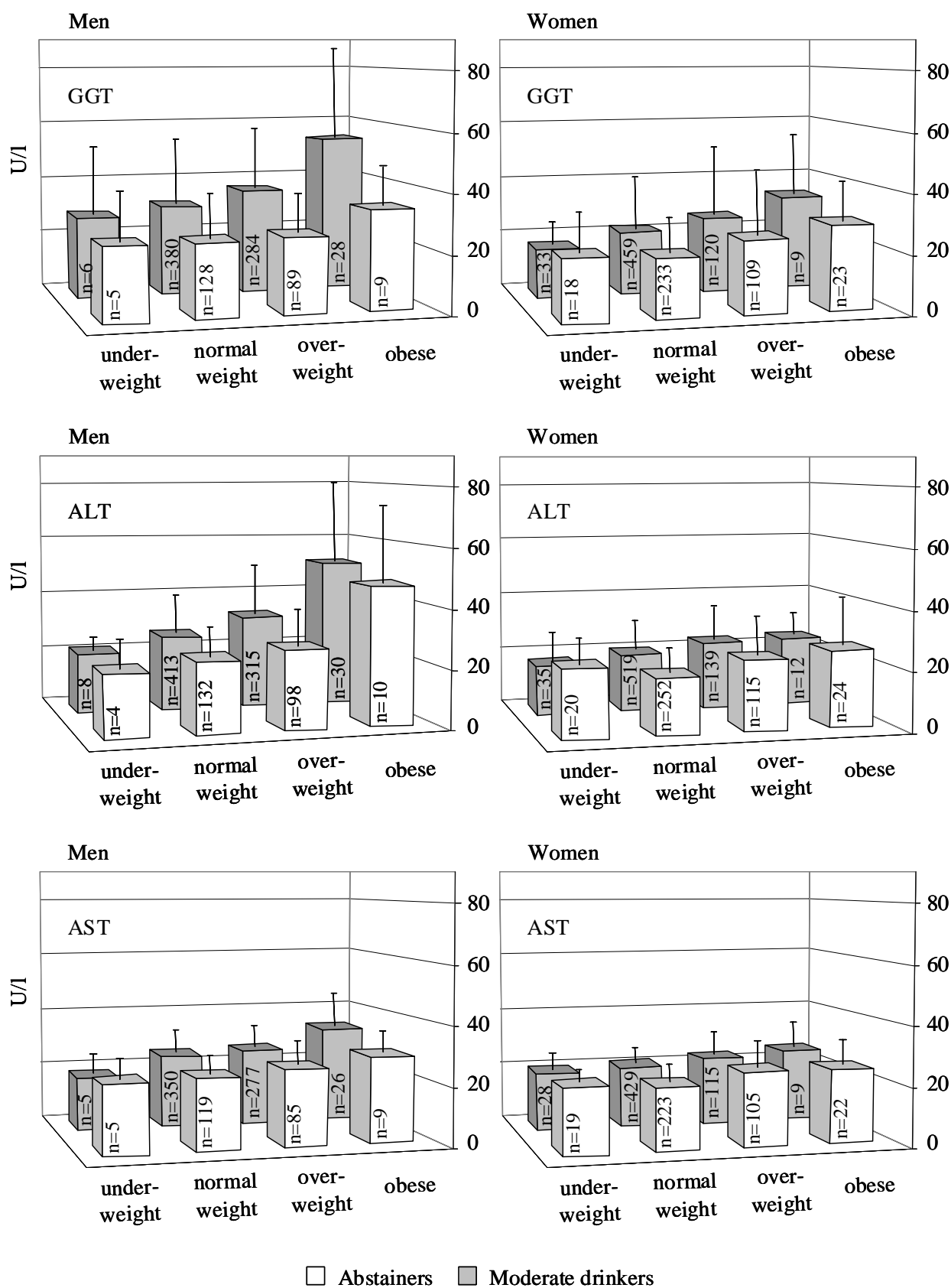


Figure 2. The activities (mean + SD) of γ -glutamyltransferase (GGT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) according to the BMI and drinking habits.

moderate drinkers among overweight men was 38%, whereas only 19% for ALT. In those with normal weight, they were the previously mentioned 24% for GGT and 8% for ALT. In the partial correlations controlling for drinking status, the correlation coefficient for an association between the BMI and the enzyme activity was higher for ALT ($r = 0.31$, $p < 0.01$) than for GGT ($r = 0.23$, $p < 0.01$).

In addition, the ratio of AST to ALT varied greatly in this study population as a function of the two lifestyle factors. The ratio >1 was significantly less common in moderate drinkers than in abstainers ($p < 0.002$), and in overweight individuals than in normal weight individuals ($p < 0.001$). Among the BMI subgroups, the distributions showed significant differences according to drinking status in participants who were underweight ($p < 0.05$ for the higher portion of ratios >1 among moderate drinkers) and overweight ($p < 0.001$ for the lower portion of ratios >1 among moderate drinkers).

Paper III studied the effects of moderate drinking and BMI on uric acid and predominately found very similar patterns to those with the liver enzymes. At maximum, the mean concentrations were increased by about one-third (Figure 3). The concentrations of uric acid also correlated with the liver enzymes, the strongest correlation being with GGT ($r = 0.41$; ALT, $r = 0.32$; AST, $r = 0.32$; $p < 0.001$ all).

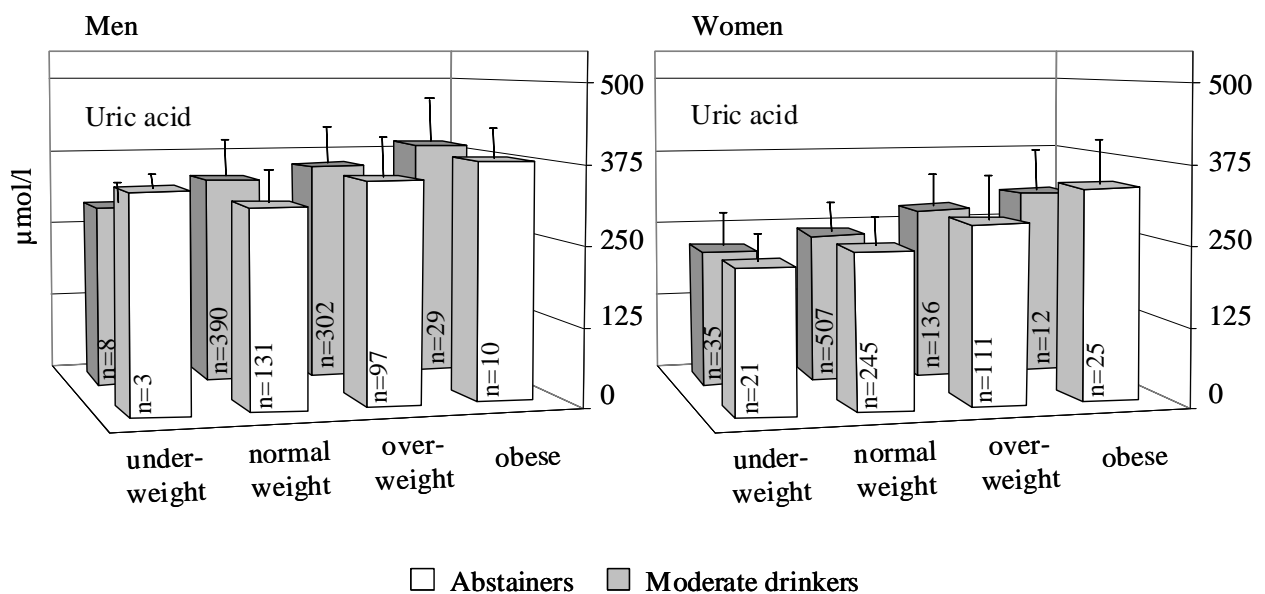


Figure 3. The concentrations (mean + SD) of uric acid according to the BMI and drinking habits.

5.3 Gender-dependent effects of moderate drinking and BMI on liver enzymes (II) and uric acid (III)

Gender issues were specifically addressed in papers II and III. In paper II, the effects of excess body weight and alcohol consumption were noticed not to be equal in men and women. The mean enzyme activities (Figure 2) and the correlations in different groups suggested the male subjects to have stronger positive associations especially between moderate drinking and enzyme levels. Accordingly, the 2-factor interactions of drinking status \times gender were statistically significant for each enzyme (GGT, $p < 0.001$; ALT, $p < 0.05$; AST, $p < 0.05$).

Paper III examined the effects of BMI and moderate drinking on uric acid, and found both effects to be different with respect to gender. The mean uric acid concentration increased as a function of an increasing BMI regardless of sex (Figure 3), but in women, this association was stronger. The correlation coefficients were 0.38 and 0.27 for women and men respectively, their difference being significant ($p < 0.02$). In the BMI-specific comparisons between drinking habits, men showed significantly higher concentrations in moderate drinkers than in abstainers among those with normal weight ($p < 0.001$), whereas in the other groups the concentrations did not differ significantly. In women, the concentrations were unchanged or even decreased slightly. Moreover, the factorial analyses showed interactions between gender and the BMI ($p < 0.02$), and between gender and drinking status ($p < 0.001$). In addition, the interaction between the BMI and drinking status was nearly significant in men ($p = 0.056$).

Among women age also influenced their variation in uric acid. In women, nearly 9% of the variation was due to age, whereas in men, the effect was virtually absent (0.1%). Interestingly, the menopausal status appeared to determine not only the uric acid concentrations, but also the drinking-related changes. First, the concentrations of uric acid were significantly ($p < 0.001$) higher in the subgroup of postmenopausal women (>60 years and without any estrogen medication) as compared to the subgroup of premenopausal women (<40 years and without any estrogen medication). Second, in the BMI-specific comparisons of these subjects, the decrease in concentration by alcohol drinking was noted significant in normal weight premenopausal women ($p = 0.024$), whereas this was not noted in normal weight postmenopausal women. However, in the overweight groups, neither of the comparisons was significant.

5.4 Effects of body weight and alcohol consumption on the population-derived reference intervals (IV)

Study IV included a large panel of analytes which had been shown to differ according to the BMI in the NORIP population (Rustad et al. 2004b), and examined the effects of body weight and drinking habits on their upper reference limits. The co-existence of excess body weight and moderate drinking in a reference population influenced the upper limits of several biomarkers, and moderate drinking alone influenced the upper limits of GGT and ALT. When the upper limits calculated from moderate drinkers with a BMI of $>27 \text{ kg/m}^2$ were compared against the limits from normal weight abstainers, marked changes were noted for GGT (+11% – +186%),

ALT (+51% – +146%) and AST (0% – +30%), but also for total cholesterol, creatine kinase, fasting glucose, HDL-cholesterol, fasting triglycerides, and uric acid. When normal weight moderate drinkers and the corresponding abstainers were compared, the differences were +13% – +74% for GGT and +14% – +27% for ALT. Statistically, alcohol drinking was a significant predictor variable of GGT, total cholesterol (≥ 50 years) and uric acid in men, and of HDL-cholesterol in both men and women, but not of ALT in either sex.

The impacts of a high BMI and alcohol drinking were also studied by calculating the relative risks of increased values for different subgroups. When the reference limits from normal weight abstainers were used as a baseline, the portion of liver enzyme activities above the limit gradually increased from this baseline to normal weight moderate drinkers, to overweight abstainers, and to overweight moderate drinkers, respectively. The trend was clearer in men than in women. At its greatest, the portion (relative risk) in men was 3–7-fold as compared to the theoretical 2.5% in normal weight abstainers, being the lowest for AST and the highest for GGT. A similar pattern and a more than 3-fold higher risk were also found for uric acid. In addition, changes were seen in serum lipid profiles (total cholesterol; HDL-cholesterol, for which risks were assessed by values below the lower reference limit; and triglycerides), but these were not as prominent as for the liver enzymes.

Table 4 summarizes the upper reference limits from normal weight abstainers and contrasts them to the NORIP recommendations. It appears that an increase of up to 82% and 67% in GGT (men <40 years, men ≥ 40 years) and 40% in ALT (men) occurs for the currently recommended limits by excess body weight and alcohol consumption. Respectively 9.2%, 7.6% and 7.2% of the corresponding NORIP participants had a value greater than the limits calculated here.

Table 4. The upper reference limits of liver enzymes according to the NORIP recommendations and as calculated from normal weight abstainers.

Analyte	Upper reference limits		
	Recommendation (NORIP)	Normal weight abstainers	
γ -Glutamyltransferase (GGT, U/l)			
Men <40 years	80	44	(n = 59)
Men \geq 40 years	115	69	(n = 64)
Women <40 years	45	38	(n = 88)
Women \geq 40 years	75	75	(n = 145)
Alanine aminotransferase (ALT, U/l)			
Men	70	50	(n = 109)
Women	45	37	(n = 217)
Aspartate aminotransferase (AST, U/l)			
Men	45	40	(n = 100)
Women	35	40	(n = 192)

6. Discussion

6.1 Different levels of drinking and markers of liver status (I)

Study I was conducted on a population with a wide range of alcohol consumption and the results show that excessive alcohol drinking, even in individuals without apparent liver disease, can induce the activities of liver-derived enzymes (GGT, ALT, AST) and elevate the concentrations of hepatic proteins (ferritin, albumin). Moreover, the marker levels may differ already by moderate consumption. When the reference limits from abstainers were further used as a baseline, not only were more heavy drinkers diagnosed as having values above the limits, but an increased portion of moderate drinkers also exceeded these limits. This knowledge on the early-phase alterations is important in an effort to improve the effectiveness of diagnosing heavy drinking. Subsequently, action can be taken to reduce consumption (Kaner et al. 2007, McQueen et al. 2009) and thereby to prevent the onset of health hazards or their progression, in that with abstinence, the survival rate in ALD is usually fairly good (Diehl 1998, Mann et al. 2003).

Interestingly, many of the marker elevations observed here may be linked to the processes of oxidative stress. For instance, GGT is critical for maintaining glutathione, an antioxidant of the cells, and as such, GGT-deficiency results in oxidative stress and a susceptibility to oxidative injury (Zhang and Forman 2009). By breaking down extracellular glutathione into its constitutive amino acids, GGT helps to provide cysteine, the rate-limiting amino acid, for cellular glutathione synthesis. Similarly, elevated serum ferritin (iron-storage protein) has been suggested to reflect increased body defence against the oxidative stress caused by excess free iron, but as well by other stressors (Lee and Jacobs 2004). Given that alcohol consumption is a well known inducer of oxidative stress (Lieber 1997) and that even mild to moderate alcohol consumption has been shown to elevate the indices of iron stores (Ioannou et al. 2004), both mechanisms may play a role in the present study population. Finally, elevated values were also noted for albumin, which is quantitatively the most important circulating antioxidant (Roche et al. 2008). Due to this antioxidative nature and to the fact that oxidative stress can significantly modify albumin, affecting its biological function (Arroyo 2009), it could be hypothesized that albumin is increased to keep up with the increased need. Usually, however, increased oxidative stress has been found to associate with decreased total albumin (Bito et al. 2005), whereas a relationship between increased albumin (albumin is a negative acute-phase reactant) and the anti-inflammatory effects of alcohol has been suggested (Imhof et al. 2001, Imhof et al. 2004). Such effects may relate to the more direct consequences of ethanol or its metabolites on gene expression, e.g., through the modulation of transcription factor activity (Mandrekar et al. 1996, Mandrekar et al. 2006). It also cannot be excluded as to whether the other above effects were at least in part due to similar ways, since various epigenetic modifications by alcohol drinking are possible as well, and the understanding of them is only beginning to emerge (Shukla and Aroor 2006).

The relative sensitivities of the liver enzymes in detecting heavy drinking were as expected. In accordance with several lines of literature, GGT was most often elevated, followed by AST and ALT (Sillanauke 1996, Salaspuro 1999). Also in line with recent work by others, ALT had the strongest association with the BMI (study II) (Schindhelm et al. 2006, Adams et al. 2008).

6.2 Moderate alcohol consumption and excess body weight (II, III)

According to study II, the liver enzyme activities were also markedly influenced by excess body weight so that i) the mean enzyme activities increased with an increasing BMI and already by overweight, and that ii) the effects of an increased BMI and moderate drinking were additional to each other. Significant BMI \times drinking status interactions were not found by the corresponding statistical tests, although several other observations were supportive. Briefly, the enzyme levels in obese moderate drinkers were higher than estimated from the isolated additive effects of obesity and drinking, and the correlation coefficients between the enzyme levels and the BMI were stronger in moderate drinkers than in abstainers. In addition, the ratios of AST \div ALT were significantly lower in moderate drinkers than in abstainers among overweight but not normal weight subjects, which further supports the view that the effects of alcohol are not equal with normal and excess body weight. Previously, only a few of the studies that have addressed the potential modification by adiposity on the effects of alcohol have been population-based, but those published have noted statistically significant positive interactions between alcohol and the BMI on the liver enzyme levels (Poikolainen and Vartiainen 1997, Ruhl and Everhart 2005, Loomba et al. 2009), except for Adams et al. (2008), yet with the difference that in these studies, as opposed to the current data, heavy drinkers were not excluded from the analyses.

The ratios of the transaminase enzymes (AST \div ALT) are generally helpful in the differential diagnosis of alcoholic liver damage as compared to those with non-alcoholic damage, or in predicting fibrosis in non-alcoholic steatohepatitis (NASH), but in the present study, differences were also noted among apparently healthy individuals with only mild changes in their ALT and AST activities. Typically, the ratio of AST to ALT over 1.5–2.0 is suggestive of alcoholic etiology (Cohen and Kaplan 1979, Salaspuro 1987), whereas in non-alcoholic etiology, the ratio is usually less than one, although it increases as fibrosis advances (Angulo et al. 1999, Sorbi et al. 1999). In the present series, the ratios were lower, i.e., ALT exceeded AST more often in overweight individuals than in those with normal weight, and also (paradoxically) in moderate drinkers than in abstainers. In adiposity, a high ALT is considered to mark fat accumulation in the liver (Schindhelm et al. 2006), but the reason for this increase in moderate drinkers remains to be elucidated. In more advanced liver disease, the predominance of AST over ALT may be explained by a depressed hepatic ALT activity due to a drinking-related deficiency of pyridoxal-5'-phosphate (vitamin B6, an enzyme cofactor) (Diehl et al. 1984), which often, however, is supplemented in an assay mixture (supplemented in here), or by hepatic mitochondrial damage (Nalpas et al. 1984) and skeletal or cardiac muscle injury (alcoholic myopathy), which release AST into the circulation. Irrespective of the etiology, there may also be disturbances in the hepatic clearance of AST through the sinusoidal liver cells (Sheth et al. 1998).

The observations on uric acid in study III support the interpretation of an increased oxidative stress level in alcohol consumers discussed under 6.1. Uric acid has recently been proposed as a marker of oxidative stress (Becker 1993, Glantzounis et al. 2005), and in the present study, moderate drinking as well as excess body weight were found to produce similar patterns of increases on its concentrations than on the liver enzyme activities. On the other hand, it could be argued that various other factors also may have affected the uric acid concentrations. For example, these factors include a high fructose intake, purines in alcoholic beverages and decreased excretion due to obesity and related conditions (Strazzullo and Puig 2007, Lippi et al. 2008). Nevertheless, several studies have shown increased oxidative stress in obesity (Keaney et al. 2003) and also associations of GGT and ALT with the direct markers of oxidative stress, independent of the metabolic syndrome (Yamada et al. 2006).

6.3 Gender issues (II, III)

The ethanol-related changes in the liver enzymes appeared to occur in a gender-dependent manner (study II). The interaction analyses indicated the positive associations between alcohol consumption and the liver enzyme activities to be stronger in men than in women. In general this observation is in line with the fact that susceptibility to alcohol-related adverse health effects vary according to sex. However, this would then suggest inverse kinetics between the enzyme levels and tissue pathology when considering that the risks are known to be higher in women rather than men (Becker et al. 1996, Schenker 1997). In other words, it is as if men were protected by their greater increases in the enzyme activities. Although it may well be that GGT, due to its role in maintaining the antioxidative glutathione (Zhang and Forman 2009), is induced in an effort to confer protection against the harms of alcohol, it should still be considered to reflect the degree of tissue damage or burden, and not the efficacy of defence. It is also possible that the present observations could be explained by differences in alcohol consumption levels. The NORIP protocol assessed drinking habits categorically, and thus within a same category, the true amounts consumed may differ for men and women.

It is tempting to speculate that the gender difference in the effects of alcohol on uric acid (decreased concentrations in women and increased concentrations in men) that was observed in study III, could predispose women to greater risks of alcohol-related health hazards. The hypothesis is supported by the following rationalization: 1) The concentration of serum uric acid is influenced, among others, by nitric oxide, which is an inhibitor of the uric acid synthesizing pathway (Pitocco et al. 2008); 2) Nitric oxide in turn is important in maintaining normal vascular function, and is inducible by estrogen (Qiao et al. 2008). Accordingly to points 1 and 2, uric acid seems to increase during menopause when the relative cardioprotection of premenopausal women as compared to men of the corresponding age is lost (Maxwell 1998); The next point, 3), given that alcohol intake can increase both pre- and postmenopausal estrogen (Ginsburg 1999, Onland-Moret et al. 2005), it may be suggested that alcohol consumption could, through increasing nitric oxide, eventually lead to lowered uric acid in women, as was noticed here particularly among premenopausal normal weight women. Unchanged uric acid in normal weight postmenopausal women could be due to the unresponsiveness of estrogen receptors, which has recently been proposed to account for the lack of vascular benefits of hormone therapy

for aging women (Meyer et al. 2008, Qiao et al. 2008). In overweight or obese women, excess body weight probably overpowers the impact of moderate alcohol intake, as seen also with men, in whom the increase in concentration from abstainers to moderate drinkers was noted to be significant among those having normal weight but not among overweight individuals; And finally point 4), to conclude, it remains to be established whether nitric oxide increases due to moderate alcohol intake and associates with beneficial cardiovascular effects specifically in normal weight premenopausal women and whether the related decrease in uric acid renders these women at the same time more susceptible to damage in other tissues, such as the liver. It is important to note, however, that the latter conclusion is based on the assumption of the antioxidative effects of uric acid.

Study III also demonstrated a gender difference in the effects of the BMI on uric acid, i.e., the association was stronger in women than in men. Thus far, the mechanisms behind the difference seem to be unknown, and studies addressing this issue more specifically are needed to elucidate the phenomenon. Interestingly though, the link between uric acid and diabetic findings or coronary heart disease has also been suggested to be stronger in women than in men (Fang and Alderman 2000, Chou et al. 2001, Lin et al. 2004, Kodama et al. 2009), yet conflicting data also exist (Wheeler et al. 2005).

6.4 Upper reference limits (IV)

Paper IV summarized the findings in studies I–III and showed that moderate drinking and excess body weight markedly influence the current liver enzyme reference limits. Based on the data obtained from normal weight abstainers, the upper reference limit for GGT in men <40 years would be around 44 U/l, while the current recommendation is 80 U/l. For men ≥ 40 years, the limit would be 69 U/l instead of the current 115 U/l. And also for ALT, the limit from normal weight abstaining men was found to be 50 U/l, as against the recommended 70 U/l. It is interesting to note that, before the establishment of the NORIP reference limits in 2004, the common limits in men were 80 U/l for GGT (all ages), and 50 U/l for ALT. Moreover, in the early 1990s, the limit for GGT in men was only 50 U/l.

Establishing reference intervals that take into account the individual's BMI and drinking habits would undoubtedly have both benefits and drawbacks. If reference limits were set separately, e.g., for BMI-subgroups, it could easily cause confusion, especially in case the limits were not very different. The NORIP project also reported differences in the upper limits for GGT and ALT between the subjects with a BMI above and below 28 kg/m^2 , but considered these modest and did not suggest separate limits (Strømme et al. 2004). On the other hand, producing higher limits for the adipose subjects would seem inappropriate in considering the increased liver enzyme activities to nonspecific biochemical interference (Prati et al. 2002). Alternatively, the problems related to heterogeneity of a reference population could be handled by producing the reference limits from a single population by strengthened inclusion criteria, as has also been suggested by Prati and colleagues (2002) (Kaplan 2002, De Rosa et al. 2003, Senior 2003).

Several reasons support the view that increasing criticism in selecting reference populations would yield benefits that are greater than the possible expenses caused by the increased frequency of values above the limits. First, the detection of the early changes in the liver enzyme activities may be important for the early detection of heavy alcohol consumption. At least before the full onset of an epidemic of obesity, experts considered roughly 70% of the cases of increased GGT activity to be explained by excessive drinking (Sillanaukee 1996, Käypä hoito 2010). Nevertheless, it should be noted that there are a number of more sensitive and specific markers (e.g., EtG/EtS, CDT, PEth, FAEE), which ideally would often be more preferable when the investigation of alcohol consumption or lack thereof is the main reason for testing, whereas the liver enzymes rather perform at best to prompt the suspicion of excess use.

Second, mild to moderate increases in aminotransferases are often a sign of NAFLD, which is currently recognized as the hepatic manifestation of obesity and the metabolic syndrome, and which in itself also has possible detrimental consequences (Matteoni et al. 1999). Clark et al. (2004) found nearly 70% of the aminotransferase elevations unexplained by traditional causes (alcohol, hepatitis B or C, or iron overload), and suspected most of these being caused by NAFLD. In addition, increased reference limits may affect the referral for a liver biopsy when considering that the level of aminotransferases repeatedly more than twice the upper limit has been recommended as the decision-making point, for example, in cases of an unknown cause to rule out serious disorders (Pratt and Kaplan 2000) or in NAFLD-suspects to distinguish those needing the closest follow-up (Day 2002, Kaplan 2002, McNair 2002). Similarly, the established reference limits may affect the management of various other conditions (Pratt and Kaplan 2000).

Third, the liver enzymes have also recently emerged as markers of general well-being. But rather than simply increasing as a consequence of, for instance, alcohol consumption or obesity, changes in the liver enzymes (and in uric acid) seem also to have an independent predictive value for cardiovascular diseases, type 2 diabetes, and for the metabolic syndrome (Vozarova et al. 2002, Hanley et al. 2004, Nakanishi et al. 2004, Hanley et al. 2005, Nannipieri et al. 2005, Strazzullo and Puig 2007, Lippi et al. 2008, Jo et al. 2009, Targher 2010). The reasons for these independent associations are currently not known. They can be a reflection of so early changes that cannot be observed by any current outcome measures. Or, they can be a reflection of thus far undiscovered disease mechanisms (Ioannou 2008). These biomarkers can as well be the real culprits in disease development, as suggested in the case of increased uric acid concentrations (Lippi et al. 2008), but also in fact for GGT in atherosclerotic plaques (Paolicchi et al. 1999). On the other hand, independent-looking associations may also emerge owing to the imperfectness of controlling measures such as the BMI or waist circumference (Ioannou 2008) or because of not knowing what to control for. Recently, Lee and Jacobs (2009) suggested that the association of GGT with many diseases may reflect the harmful effects of background exposure to various environmental pollutants. Nevertheless, whether causal, protective, or inert, the higher the levels of the liver enzymes and uric acid become, the higher the risks apparently are, and therefore the aim should be to modify the factors that are known to elevate these markers and that one can have an influence on (Ioannou 2008). Moreover, prediction has also been shown with those values clearly below the currently recommended reference limits (Sattar et al. 2004, Wannamethee et al. 2005, Goessling et al. 2008, Yun et al. 2009, Ryu et al. 2010, Targher 2010). And finally, another important argument is that an objective proof of unhealthy lifestyle may

motivate change. Altogether these reasons clearly suggest that an effort should be made to prevent the increase in the liver enzyme upper reference limits.

The data here also showed changes in the serum lipid profiles as a result of moderate drinking. The most interesting was the increased HDL-cholesterol concentration, which in several studies has been suggested to play a role in the positive impacts of moderate alcohol intake on cardiovascular mortality (Rimm et al. 1999, Mukamal et al. 2005). In addition, drinking can also affect the composition and function of HDL-cholesterol (Brinton 2010). However, given that the sensitivity of the liver to alcohol consumption in general appears to be greater than perhaps previously anticipated, the changes in liver status by moderate drinking may exceed the benefits observed for other organs. Apparently, further studies are warranted on this matter.

6.5 Possible limitations of this study

In practice, all scientific studies entail some limitations. In the present study, the possibility of some erroneous classification of alcohol consumption cannot be excluded. The permitted alcohol intake were similar for women and men in the NORIP protocol, and may have led to the misclassification of some female individuals who were in fact on the edge of excessive consumption rather than true moderate drinkers. Moreover, since the alcohol consumption data were based on the subjects' own assessments, some underreporting may have taken place throughout the population as well. Whether some misclassification happened or not, e.g., some moderate drinkers were true excessive drinkers, it however does not affect the key observation on the reference limits but, if anything, it underscores the need for better control in qualifying reference individuals.

6.6 Future considerations

The findings of this study provide evidence that the diagnosis and prevention of liver problems could be improved by better taking into account and understanding the effects of different factors on related biomarkers. In addition to BMI (or perhaps preferably waist circumference) and alcohol consumption, other factors can also influence the liver enzyme activities, such as smoking, coffee consumption and ethnic origin. According to recent studies, there might be synergism between the effects of alcohol consumption and smoking on the liver enzymes, and thus possible aggravation of the related health hazards (Whitehead et al. 1996, Breitling et al. 2009, Wannamethee and Shaper 2010). On the other hand, the protective effect of coffee drinking is an interesting field of study in Finland (Poikolainen and Vartiainen 1997, Bidel et al. 2008, Hu et al. 2008), as Finns are some of the highest consumers of this beverage in the world. Furthermore, both the baseline liver enzyme activities and the susceptibility to induction by alcohol or obesity may differ between races (Horn and Pesce 2002, Stewart 2002, Horn and Pesce 2003, Stranges et al. 2004, Samadi et al. 2007). With increasing international mobility, recognizing these differences is becoming more and more important.

In respect of establishing reference limits based on more carefully defined populations, it should be noted that for some analytes in the present study, the limits for normal weight abstainers were calculated from a relatively small number of individuals. In these cases, the corresponding upper limits provided here should be considered as estimates warranting more studies in larger populations to set the exact boundaries. In ideal future scenarios, the results of those studies and the gathering of information on the links with the diseases of affluence would further translate into a diagnosis of increased oxidative or metabolic burden when exceeding a certain threshold value of liver enzyme activity.

7. Conclusions

The summary of and conclusions to be drawn from the main findings in the present series are as follows:

1. The biomarkers of liver status may respond to even moderate drinking. If the reference limits were based on moderate drinkers, the diagnosis of heavy drinking is also compromised. Consequently, only abstainers should be included in the reference populations in order to achieve the best sensitivity for these tests used to detect alcohol abuse.
2. The liver enzyme activities increased as a function of body weight throughout the BMI scale, and the activities were yet higher in moderate drinkers than in abstainers with a corresponding BMI. The statistical results were not significant for the interactions between the effects of moderate drinking and the BMI, although the mean activities in moderate drinkers with an increased BMI were slightly higher than expected from the separate additive effects. Furthermore, the impacts of moderate drinking were stronger in men than in women, but this may be also due to differences in true drinking levels.
3. The concentrations of uric acid were higher across the categories of BMI, and higher in male moderate drinkers than in abstainers. The findings support the hypothesis that oxidative stress plays a pivotal role in many pathologies related to heavy alcohol use and obesity, and suggest that oxidative stress is generated early in the course of possible health hazards and by rather low degrees of stimuli. In premenopausal women, moderate drinking may decrease uric acid, but future studies are needed to confirm and understand this phenomenon.
4. Moderate drinking and excess body weight have markedly influenced the current upper reference limits of especially GGT and ALT in men. The effects of BMI and drinking should be better taken into consideration when selecting reference individuals for the liver enzymes, which associate with heavy drinking and liver problems in general, and also with cardiovascular diseases, type 2 diabetes, and the metabolic syndrome.

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Biomarkers of liver status in heavy drinkers, moderate drinkers, and abstainers

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ABSTRACT

Aims: Although a wide variety of biomarkers reflecting liver status are known to be influenced by excessive ethanol consumption, the dose-response relationships between ethanol intake and marker changes have remained less understood.

Methods: Serum gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, and ferritin and albumin protein concentrations were compared in a large population of heavy drinkers (105 men, 28 women), moderate drinkers (781 men, 723 women), and abstainers (252 men, 433 women), who were devoid of apparent liver disease.

Results: In heavy drinkers, serum GGT, AST, ALT, ferritin and albumin were all significantly higher than in moderate drinkers or abstainers ($P < 0.001$ for all comparisons). The highest incidences of elevated values were found for GGT (62 %) followed by AST (53 %), ALT (39 %), ferritin (34 %) and albumin (20 %). Serum GGT ($P < 0.001$), ALT ($P < 0.01$) and ferritin ($P < 0.05$) in moderate drinkers were also higher than the levels observed in abstainers. When the study population was further divided into subgroups according to gender, significant differences between moderate drinkers and abstainers in GGT and ALT were noted in men whereas not in women.

Conclusions: The data demonstrate that biomarkers of alcohol abuse and liver function may respond to even rather low levels of ethanol intake in a gender-dependent manner, which should be implicated in studies on the early-phase interactions of ethanol and the liver and in the definition of normal ranges for such biomarkers.

INTRODUCTION

Ethanol consumption and associated medical disorders continue to grow in most Western countries (Lieber, 1995; Room *et al.*, 2005; Leon and McCambridge, 2006). Therefore, the need for more effective diagnostic procedures for detecting problem drinking in its early phase has been widely acknowledged (Conigrave *et al.*, 2002). Because nearly all ethanol consumed is metabolized by the liver, it is also a primary target of ethanol-induced adverse health effects. Previous studies in alcoholic patients have demonstrated several liver-derived biomarkers, which are associated with excessive ethanol intake and alcoholic liver disease (Rosman and Lieber, 1994; Sharpe, 2001; Niemelä, 2007). Heavy drinkers typically show increased activities of serum GGT and transaminases (ALT, AST), whereas upon progression of alcoholic liver disease there may be elevations in liver enzymes together with abnormally low serum concentrations of hepatic proteins.

Recent studies have indicated a gradual effect of alcohol on GGT enzyme induction, which may be initiated at rather low levels of ethanol intake (Hietala *et al.*, 2005). The status of oxidative stress has also been closely linked with serum GGT activities (Whitfield, 2001; Lee and Jacobs Jr., 2004; Puukka *et al.*, 2006). However, the dose-response relationships between various liver enzymes, proteins, and ethanol intake have, however, continued to be poorly known. Comparisons of the different variables between abstainers and moderate drinkers have also been limited.

In order to gain further insight on the interpretation of the interactions between ethanol intake and biomarkers of liver status, we compared here the serum levels of liver-derived enzymes and proteins in individuals with a wide range of ethanol consumption including i: abstainers, ii: moderate drinkers and iii: heavy drinkers, who were all devoid of apparent liver disease.

METHODS

Study protocol

The sample of heavy drinkers consisted of 133 patients, 105 men (mean age 45 ± 10 years) and 28 women (mean age 43 ± 11 years), who had been admitted for detoxification in a consecutive manner. All patients underwent detailed clinical examinations and personal interviews on the amounts and patterns of ethanol consumption using a time-line follow-back technique, which indicated a history of continuous ethanol consumption or binge drinking, the mean consumption being 110 grams (range 40–540 grams) of ethanol per day from the period of 4 weeks prior to sampling. The percentage of patients reporting a mean consumption less than 60 g/day or 80 g/day was 5 % and 11 %, respectively. In addition to the above, the patients were also advised to sum up their intake from the last one week and 24 hours, which indicated considerably higher levels of daily ethanol intake from the past one week prior to admission. All patients were devoid of any clinical signs of liver dysfunction (collateral circulation, oedema, ascites, encephalopathy, spider nevi, anorexia, or weakness).

In addition, samples were collected from 1504 moderate drinkers, 781 men (mean age 46 ± 17 years), 723 women (mean age 45 ± 16 years) and from 685 abstainers, 252 men (mean age 49 ± 20 years), 433 women (mean age 49 ± 19 years). All of these subjects were apparently healthy volunteers who participated in a Nordic survey for establishing reference intervals for common laboratory tests (Puukka *et al.*, 2006). The mean recent alcohol consumption in the population of moderate drinkers had been less than 40 grams per day, the maximum amount during the past twenty-four hours prior to sampling being two standard drinks (each containing 12 grams of alcohol). The survey excluded individuals who had clinical or laboratory evidence of current or recent illnesses or infections, who were pregnant, had donated blood during the past five months or had used any prescription drugs

during the preceding one week. Smoking had not been allowed for one hour prior to sampling.

Measurements of serum gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities and the concentrations of ferritin and albumin were carried out using standard clinical chemical methods. The procedure was approved by the institutional review board. Informed consent was obtained from the participants and the study was carried out according to the provisions of the Declaration of Helsinki.

Statistical methods

Values are expressed as mean \pm SD and as 95th-97th percentiles, as appropriate. Comparisons between groups were made with Kruskal-Wallis test and Tamhane's T2 post hoc test. Upper normal limits were calculated using mean + 2SD if the data came from Gaussian distribution or by using nonparametric method if the data came from skewed distribution after Dixon's test to remove outliers, as recommended by Horn and Pesce (2003). Correlations were calculated using Spearman's rank correlation. The statistical analyses were carried out using SPSS for Windows 15.0 (Chicago, Illinois, USA) software. A P -value < 0.05 was considered statistically significant.

RESULTS

Table 1 summarizes the values of the various biomarkers of liver status in the groups of heavy drinkers, moderate drinkers and abstainers. Among heavy drinkers, serum GGT, AST, ALT activities and ferritin and albumin concentrations were all significantly higher than those observed in either abstainers or moderate drinkers ($P < 0.001$ for all comparisons).

Interestingly, in moderate drinkers the activities of GGT ($P < 0.001$) and ALT ($P < 0.01$) as well as ferritin ($P < 0.05$) levels were also significantly higher than those in abstainers (Table 1). When the study population was further divided into subgroups according to gender, the alcoholic men showed significantly higher values than both moderate drinkers and abstainers in all of the study variables (Table 1). Male moderate drinkers and abstainers also differed in their serum GGT ($P < 0.001$) and ALT ($P < 0.05$) activities. In women, the heavy drinkers also showed the highest values, whereas in comparisons between moderate drinkers and abstainers no significant differences were noted (Table 1).

The amount of self-reported ethanol intake correlated significantly with serum GGT ($r = 0.50$, $P < 0.001$), AST ($r = 0.45$, $P < 0.001$), ALT ($r = 0.39$, $P < 0.001$), and ferritin ($r = 0.46$, $P < 0.001$). Among the different biomarkers, GGT was found to correlate strongly with AST ($r = 0.42$), ALT ($r = 0.53$), and ferritin ($r = 0.49$), AST with ALT ($r = 0.62$), and ferritin ($r = 0.61$), and ALT with ferritin ($r = 0.46$) ($p < 0.001$ for all) (Table 2).

In order to explore the interpretation of these variables as possible biomarkers of alcohol abuse, we also determined their upper normal limits based on the present population of abstainers or moderate drinkers (Table 3). Notable differences in the cut-offs based either on the use of the database obtained from moderate drinkers or abstainers were found for serum GGT, AST, ALT and ferritin. The incidences of observations, which exceeded the abstainer-based upper normal limits among heavy drinkers and moderate drinkers, are summarized in Figure 1. The highest incidences of elevated values in heavy drinkers were found for GGT (62 %) followed by AST (53 %), ALT (39 %), ferritin (34 %) and albumin (20 %).

DISCUSSION

The present study in a large population of subjects with a wide range of alcohol consumption indicates that excessive drinking even in individuals without apparent liver disease induces the activities of several liver-derived enzymes and elevates the concentrations of hepatic proteins, which have recently been linked with defence mechanisms towards oxidative stress (Whitfield, 2001; Lee and Jacobs Jr., 2004; Faure *et al.*, 2008). Moderate drinkers also show higher enzyme activities than abstainers underscoring an early occurrence of the biochemical responses in response to ethanol intake. While GGT and transaminase enzymes are also known to specifically increase as a result of obesity (Lawlor *et al.*, 2005; Puukka *et al.*, 2006; Alatalo *et al.*, 2008), the presence or absence of overweight should not account for such differences here, since the groups of abstainers and moderate drinkers showed essentially similar BMIs (24.3 ± 3.5 and 24.0 ± 2.9 , respectively). Obviously, overweight when occurring together with alcohol drinking could, however, aggravate the metabolic burden and hepatic enzyme responses, as recently observed for both GGT (Puukka *et al.*, 2006) and ALT (Ruhl and Everhart, 2005a; Alatalo *et al.*, 2008). Current BMIs (24.5 ± 3.8) of the heavy drinkers also exhibited similar mean levels. Although the relatively small number of observations in the heavy drinker group here does not provide enough statistical power for assessing independent effects of ethanol drinking and overweight on hepatic enzymes and proteins within this subgroup, it should be noted that obesity has been previously found to be a risk factor for cirrhosis in the alcoholics (Naveau *et al.*, 1997).

When compared to abstainers, the group of moderate drinkers also showed elevated levels of serum ferritin, a marker of stored body iron. Thus, ethanol-related biochemical consequences in iron homeostasis may also be expected to occur at rather low levels of ethanol consumption. Previously, heavy drinking has been shown to increase ferritin levels, and secondary hepatic iron overload is a typical characteristic of alcoholic patients (Fletcher,

1996; Whitfield *et al.*, 2001). Recent studies have indicated that even consumption of > 2 alcoholic drinks/day may be associated with a significant elevation in the risk of iron overload (Ioannou *et al.*, 2004). Deposition of excess iron in hepatic tissue is in turn an important secondary risk factor for the development of alcoholic liver disease. In experimental animals, iron and alcohol have been shown to act in a synergistic manner to enhance lipid peroxidation and liver injury (Bacon and Britton, 1990; Tsukamoto *et al.*, 1995; Cederbaum, 2003; Harrison-Findik, 2007). Alcohol consumption also increases the risk of liver injury in human patients with iron overload (Fletcher *et al.*, 2002). It has recently been hypothesized that serum ferritin may be produced in order to sequester catalytically active free iron and increases in serum ferritin could actually reflect a defence mechanism, which occurs in response to ethanol-induced oxidative stress (Lee and Jacobs Jr., 2004). Increased ferritin levels could thereby protect from oxidative stress and consequent pathology due to free iron. In a similar manner, the responses in serum GGT, which is responsible for extracellular metabolism of glutathione, the main antioxidant in mammalian cells, could be linked to protection from reactive oxygen species (Whitfield, 2001; Puukka *et al.*, 2006). On the other hand, acetaldehyde, the main oxidative metabolite of ethanol, has been previously shown to alter the gene expression of several proteins, such as collagen (Parés *et al.*, 1994; Moirand *et al.*, 1995; Niemelä, 2001; Thiele *et al.*, 2005; Purohit and Brenner, 2006). Since ferritin concentration is also under tight genetic control it is also possible that its concentration may be affected in a similar way rather than through the cellular iron concentration.

The present data also show increased levels of serum albumin among heavy drinkers suggesting increased rates of albumin protein synthesis in response to regular ethanol intake prior to the development of liver dysfunction, whereas in patients with advanced liver disease the rates of hepatic protein synthesis are obviously decreased. Although the mechanisms underlying this observation remain unclear it should be noted that previous studies in cell cultures have shown elevated hepatic protein synthesis rates as a result of

chronic ethanol administration (Potter *et al.*, 1985; Ohtake *et al.*, 1986; Rothschild *et al.*, 1988). Recently, Tyulina and co-workers (2006) found elevated plasma albumin levels in alcoholics, which correlated with elevated protein carbonyls, suggesting that covalent modifications of proteins by acetaldehyde could also be associated with albumin protein expression among heavy drinkers. Serum albumin is also an important antioxidant agent, whereas any structural modification of albumin induced by ethanol metabolites, glucose or free radicals has been suggested to impair its antioxidant properties (Faure *et al.*, 2008).

The ethanol-induced biochemical changes in hepatic tissue appear to occur in a gender-dependent manner. It remains to be established whether such findings would also correlate with the differences in the individual susceptibility to tissue damage, which is known to be not equal between men and women (Schenker, 1997). It should also be noted that previous studies have indicated effects of smoking (Whitehead *et al.*, 1996; Steffensen *et al.*, 1997) and coffee consumption (Nakanishi *et al.*, 2000; Ruhl and Everhart, 2005b) on the activities of hepatic enzymes. Unfortunately, in this study the possible confounding effects of smoking or coffee consumption could not be addressed. Since people who drink heavily usually also smoke a lot we cannot rule out the possibility of additional increasing effects of smoking on hepatic enzymes among the heavy drinkers. However, in comparisons between the present population of moderate drinkers and abstainers, the enzyme activities or protein levels were not found to be significantly different between smokers and non-smokers (data not shown). In light of recent evidence indicating that coffee drinking could protect against liver injury and lead to reduced activities of liver enzymes (Nakanishi *et al.*, 2000; Ruhl and Everhart, 2005b; Hu *et al.*, 2008), we feel, that the interactions between ethanol intake, smoking and coffee consumption clearly warrant further studies in large populations.

Our data also emphasize the view that due to the possible effects of even rather low ethanol doses on hepatic enzymes and proteins the clinical interpretations of any ethanol-sensitive biomarkers as diagnostic tests in health care need further attention. Current surveys indicate

a trend towards permanent increases in GGT activities at population level (Hietala *et al.*, 2005; Lee *et al.*, 2006; Niemelä, 2007). On the other hand, serum activities of hepatic enzymes have recently been suggested to be useful as general indicators of health and disease and long term survival (Kazemi-Shirazi *et al.*, 2007; Kim *et al.*, 2008) Therefore, in order to improve the discriminative power of any biomarker reflecting liver status, the normal ranges should perhaps be re-defined based on databases of healthy individuals who abstain from ethanol. Moreover, the possible roles of a wide variety of biochemical markers as players in defense mechanisms towards oxidative stress warrant further studies.

FIGURE LEGENDS

Figure 1.

The incidences of elevated values (%) of serum gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and ferritin and albumin concentrations in moderate drinkers and heavy drinkers when compared to upper normal limits obtained from the population of abstainers.

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Table 1. Liver enzyme and protein levels in heavy drinkers, moderate drinkers and abstainers.

	Heavy drinkers	Moderate drinkers	Abstainers
GGT (U/l)			
All	177 ± 317 (578–1612) ¹	29 ± 23 (70–97) ***	24 ± 15 (50–64) ***, ††
Men	193 ± 347 (949–1664)	34 ± 24 (79–106) ***	26 ± 14 (53–63) ***, ††
Women	119 ± 158 (561–607)	23 ± 20 (48–79) **	22 ± 16 (45–68) **
AST (U/l)			
All	63 ± 51 (171–208)	24 ± 8 (38–43) ***	24 ± 7 (36–41) ***
Men	65 ± 49 (160–204)	26 ± 8 (40–45) ***	25 ± 7 (35–43) ***
Women	55 ± 57 (235–263)	21 ± 7 (33–37) *	23 ± 7 (36–41) *
ALT (U/l)			
All	67 ± 65 (211–273)	25 ± 15 (51–64) ***	23 ± 12 (46–52) ***, †
Men	71 ± 68 (220–282)	29 ± 17 (59–73) ***	26 ± 12 (49–58) ***, †
Women	51 ± 48 (195–214)	20 ± 11 (40–48) *	21 ± 11 (40–49) *
Ferritin (µg/l)			
All	212 ± 207 (687–777)	100 ± 83 (246–333) ***	70 ± 63 (245–257) ***, †
Men	258 ± 222 (750–853)	136 ± 90 (332–378) ***	101 ± 72 (257–257) ***
Women	105 ± 112 (396–465)	65 ± 55 (188–231)	48 ± 45 (184–225) *
Albumin (g/l)			
All	44 ± 4 (50–51)	41 ± 3 (46–47) ***	41 ± 3 (46–47) ***
Men	44 ± 4 (50–50)	42 ± 3 (47–48) ***	42 ± 3 (47–47) ***
Women	44 ± 4 (51–53)	41 ± 3 (45–46) **	40 ± 3 (45–46) ***

¹ mean ± SD (95th–97,5th percentile) (all such values).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared to heavy drinkers.

† $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ when compared to moderate drinkers.

GGT, gamma-glutamyltransferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 2. Correlations between study variables.

	GGT	AST	ALT	Ferritin
AST	0.42 ***			
ALT	0.53 ***	0.62 ***		
Ferritin	0.49 ***	0.61 ***	0.46 ***	
Albumin	0.10 ***	0.11 ***	0.15 ***	0.12 *

* $P < 0.05$, *** $P < 0.001$

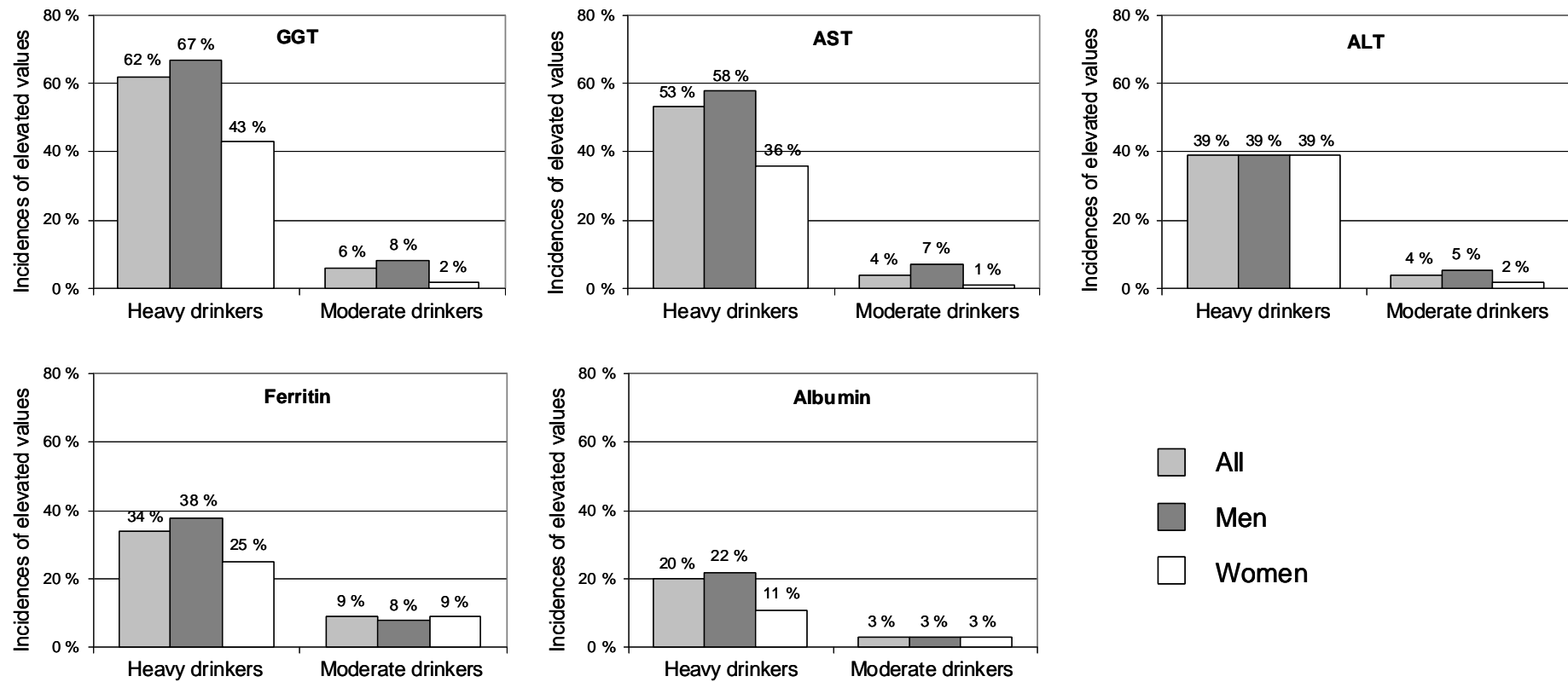
Table 3. Upper normal limits for the study parameters, as based either on the data from abstainers or moderate drinkers.

	Upper normal limit			
	Men		Women	
	Abstainers	Moderate drinkers	Abstainers	Moderate drinkers
GGT (U/l) ¹	63	106 (+68 %)	63	79 (+25 %)
AST (U/l) ¹	39	45 (+15 %)	41	37 (–9 %)
ALT (U/l) ¹	58	73 (+26 %)	49	48 (–2 %)
Ferritin (µg/l) ²	274	342 (+25 %)	141	191 (+35 %)
Albumin (g/l) ²	47	48 (+2 %)	46	46 (0 %)

For abbreviations, see Table 1.

The percentages in brackets indicate the relative change in the upper normal limits in moderate drinkers as compared to abstainers, calculated upper normal limits calculated as 97,5th percentile¹ or as mean + 2SD², as required.

Figure 1





Original Contribution

Gender-dependent impacts of body mass index and moderate alcohol consumption on serum uric acid—an index of oxidant stress status?

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ABSTRACT

Uric acid seems to be causally involved in a variety of medical disorders involving oxidative stress. Although alcohol abuse and obesity are known to increase serum uric acid, the interactions between moderate drinking, adiposity, and uric acid metabolism have remained poorly understood. We examined serum uric acid concentrations from 2062 apparently healthy volunteers (970 men, 1092 women) reporting either no alcohol (abstainers) or <40 g of ethanol consumption per day (moderate drinkers). The study population was further classified according to BMI as follows: <19 (underweight), 19–25 (normal weight), 25–30 (overweight), and >30 (obese). Serum uric acid concentrations in male moderate drinkers were significantly higher, and in females they were lower, than in the corresponding groups of abstainers. In the BMI-based subgroups, the highest concentrations were found in those who were overweight or obese. Significant two-factor interactions occurred between gender and drinking status ($p < 0.001$) and between gender and BMI ($p < 0.02$). Serum uric acid also correlated with indices of hepatocellular health (GGT, ALT, AST). The data indicate distinct gender-dependent impacts of alcohol consumption and BMI on serum uric acid. These findings should be applicable to the assessment of oxidative stress status and associated morbidity in alcohol consumers and individuals with excess body weight.

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Recent studies have indicated that the development of metabolic syndrome, a cluster of cardiovascular disease risk factors, correlates with alterations in serum uric acid levels [1,2]. Although the primary mechanisms of such interactions have not been defined, mounting evidence has suggested a role for uric acid as an antioxidant owing to its ability to chelate transition metal ions and to react with biological oxidants such as hydroxyl radicals [1,3–5]. However, high concentrations of uric acid in circulation may also have pro-oxidant properties [6,7].

Current surveys have indicated that more than half of the population in the developed world suffers from overweight or obesity [8,9]. Obesity is largely responsible for the high prevalence of metabolic syndrome as well as other adverse health effects, such as nonalcoholic fatty liver disease, which has increased in an epidemic manner during the past few years [10,11]. Simultaneously, there has also been a trend toward increasing ethanol consumption and associated medical disorders [12,13]. Both excess body weight and heavy alcohol consumption induce oxidative stress, which can lead to alterations in hepatic lipid, carbohydrate, protein, lactate, and uric acid metabolism [14]. As yet, little information is, however, available on the

joint effects of alcohol consumption and adiposity on uric acid turnover. It is also unclear whether the changes in the concentrations of uric acid in response to oxidative stress represent a protective response or a primary cause.

To explore further the biological significance of alterations in serum uric acid levels in circulation, we investigated here the relationships between serum uric acid, ethanol consumption, and body mass index (BMI) in a cohort of apparently healthy abstainers and moderate drinkers.

Materials and methods

Study protocol

Subjects in this study were participants in a survey collected for establishing reference intervals for common laboratory parameters in Nordic countries and, therefore, they partially overlap with our previous study on γ -glutamyl transferase (GGT) [15]. Subjects were recruited following the rules of the Nordic Reference Interval project (NORIP protocol) [16]. The study population consisted primarily of hospital personnel and their relatives or acquaintances, of whom 97% were Scandinavians and 3% of other Caucasian origin. This population included 2062 apparently healthy volunteers: 970 men, mean (\pm SD) age 47 ± 18 years, and 1092 women, mean (\pm SD) age 47 ± 17 years, classified as either abstainers ($n = 643$: 241 men, age 49 ± 20 years;

Abbreviations: BMI, body mass index (kg/m^2); GGT, γ -glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

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Table 1
Distribution of study participants into BMI subgroups

	BMI			
	<19	19–25	25–30	>30
Men				
Abstainers	3	131	97	10
Moderate drinkers	8	390	302	29
Women				
Abstainers	21	245	111	25
Moderate drinkers	35	507	136	12

402 women, age 49 ± 19 years) or moderate drinkers ($n = 1419$: 729 men, age 46 ± 17 years; 690 women, age 45 ± 16 years) and further according to BMI (kg/m^2): <19 (underweight), ≥ 19 and <25 (normal weight), ≥ 25 and <30 (overweight), and ≥ 30 (obese) (Table 1).

Blood samples were collected between December 1999 and January 2002. Before sampling, all participants received detailed instructions to avoid any hard physical activity, avoid any blood donations, and control the time period since the last meal. Samples were taken in fasting state primarily between 7:00 and 10:00 AM. The participants were not paid for their contribution.

The health status and the patterns and amounts of ethanol intake were assessed using specifically designed questionnaires. Those who reported no alcohol intake were classified as abstainers. Moderate drinkers were individuals by whom the amount of alcohol consumed was less than 40 g of ethanol/day and the maximum amount of alcohol during the past 24 h before sampling had been two standard drinks (each providing 12 g of ethanol). None of the participants were former alcoholics or individuals with any social or medical records of heavy drinking and associated medical disorders. The survey excluded individuals who had clinical or laboratory evidence of any current or recent illnesses or infections, who were pregnant, who had donated blood during the past 5 months, or who had used any prescription drugs during the preceding week. Possible use of estrogen-containing preparations was also recorded. Smoking information from this material indicated that 81% of the individuals in the present series were nonsmokers, 6% smoked 1–5 cigarettes per day, and 11% smoked more than 5 cigarettes per day. Smoking information was missing from 2% of the subjects. Smoking had not been allowed for 1 h before sampling.

Serum uric acid concentrations; GGT, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activities; and blood glucose, HDL- and LDL-cholesterol, and triacylglyceride levels were measured using International Federation of Clinical Chemistry-compatible measuring systems [16–18].

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

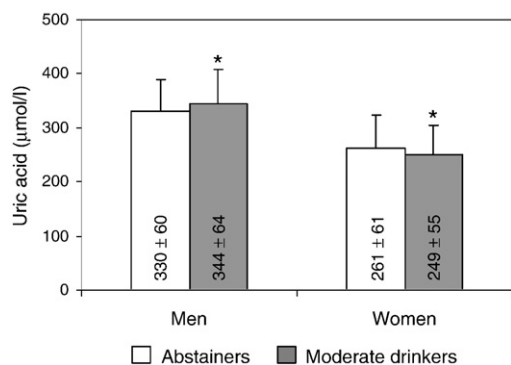


Fig. 1. Serum uric acid concentrations (mean \pm SD) in the study population of 970 men and 1092 women reporting either no alcohol consumption (abstainers) or moderate drinking. * $p < 0.01$ compared to the corresponding group of abstainers.

Statistical methods

Values are expressed as means \pm SD. Differences between groups were determined with Student's t test when comparing two groups or ANOVA with Bonferroni post hoc test for multiple comparisons. Factorial ANOVA was used to investigate the interactions between gender, drinking status, and BMI on serum uric acid. Before the analyses, the data on serum uric acid were subjected to square root transformation to yield nonskewed distributions with homogeneity of variance. Correlations were calculated with Pearson product-moment Correlations coefficients. The differences between correlations were analyzed with the Z test for correlation coefficients. Linear regression analysis was used to measure proportions of variability. The analyses were carried out using SPSS 15.0 for Windows statistical software (Chicago, IL, USA). Tests were considered statistically significant at $p < 0.05$.

Results

Serum uric acid concentrations in male moderate drinkers were significantly higher ($p < 0.01$), and in female moderate drinkers lower ($p < 0.01$), than those in the corresponding groups of abstainers (Fig. 1). When the study population was further classified according to BMI, the highest concentrations were found in overweight and obese individuals (Fig. 2). Interestingly, significantly higher uric acid concentrations in male moderate drinkers (335 ± 62 $\mu\text{mol}/\text{L}$) compared to abstainers (313 ± 52 $\mu\text{mol}/\text{L}$) were found in those with normal weight ($p < 0.001$) and not in those with BMI above $25 \text{ kg}/\text{m}^2$.

Serum uric acid correlated significantly with BMI ($r = 0.42$, $p < 0.001$), and also with the biomarkers of liver status: GGT ($r = 0.41$, $p < 0.001$), AST ($r = 0.32$, $p < 0.001$), and ALT ($r = 0.32$,

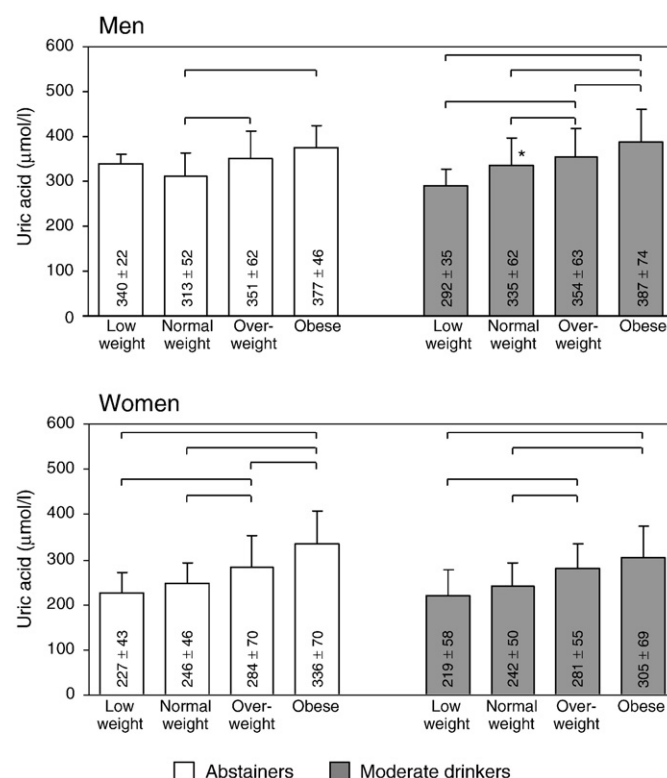


Fig. 2. Serum uric acid concentration (mean \pm SD) in abstainers and moderate drinkers as further classified according to BMI. The highest values were observed in those who were overweight or obese. BMI <19 , underweight; BMI ≥ 19 and <25 , normal weight; BMI ≥ 25 and <30 , overweight; BMI ≥ 30 , obese. Horizontal lines indicate significant ($p < 0.05$) differences between groups within each drinking category, as assessed by ANOVA with Bonferroni post hoc test. * $p < 0.001$ compared to the corresponding group with similar BMI.

Table 2
Correlations between study variables

	BMI	Uric acid	GGT	ALT	AST	Glucose	HDL	LDL
Uric acid	0.42***							
GGT	0.32***	0.41***						
ALT	0.29***	0.32***	0.48***					
AST	0.21***	0.32***	0.34***	0.58***				
Glucose	0.32***	0.30***	0.26***	0.23***	0.26***			
HDL	−0.35***	−0.34***	−0.18***	−0.18***	−0.01	−0.10**		
LDL	0.27***	0.23***	0.32***	0.17***	0.22***	0.33***	−0.07*	
Triacylglycerides	0.37***	0.29***	0.27***	0.23***	0.16***	0.31***	−0.40***	0.34***

HDL, high-density cholesterol; LDL, low-density cholesterol.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

$p < 0.001$), and with blood glucose ($r = 0.30$, $p < 0.001$) and serum lipids (HDL-cholesterol, $r = -0.34$, $p < 0.001$; LDL-cholesterol, $r = 0.23$, $p < 0.001$; triacylglycerides, $r = 0.29$, $p < 0.001$) (Table 2). In separate analyses for men and women, the correlation between uric acid and BMI was significantly ($p < 0.02$) stronger in women ($r = 0.38$) than in men ($r = 0.27$) (Fig. 3) and also when computing partial correlations controlling for drinking status (women, $r = 0.40$; men, $r = 0.26$). In this study, smoking status was not significantly associated with serum uric acid levels.

Fig. 4 illustrates the relative differences in serum uric acid levels between the study groups of men and women using the corresponding population of abstainers with normal weight as baseline. Among abstainers presenting with obesity the mean uric acid levels were 14% (men) and 37% (women) above this baseline, whereas in moderate drinkers the corresponding difference was 24% for both genders.

Factorial ANOVA was subsequently used to assess the relative importance of the various combinations of explanatory variables on serum uric acid. While the three-factor interaction between BMI, drinking status, and gender was not significant, joint effects between gender and drinking status ($p < 0.001$) and between gender and BMI ($p < 0.02$) were observed. In additional gender-specific two-factor analyses the interaction between BMI and drinking status was nearly significant ($p = 0.056$) among men but not among women.

Increasing age has also been suggested to affect the concentrations of uric acid. Multiple regression analyses on the effect of age in this study indicated that age accounts for nearly 9% (multiple $R^2 = 0.089$) of the variation in women but for only 0.1% (multiple $R^2 = 0.001$) among men. Uric acid concentrations were significantly higher in the subgroup of postmenopausal (276 ± 58 $\mu\text{mol/L}$) compared to premenopausal (238 ± 50 $\mu\text{mol/L}$) women ($p < 0.001$). Premenopausal moderate drinkers not using any estrogen medication and having BMI < 25 showed lower serum uric acid levels (226 ± 46 $\mu\text{mol/L}$, $n = 93$) than their abstaining counterparts (242 ± 34 $\mu\text{mol/L}$, $n = 41$; $p = 0.024$), whereas no such differences were seen in corresponding

comparisons among overweight premenopausal or postmenopausal groups, or among estrogen users.

Discussion

This cross-sectional survey among a large population of apparently healthy abstainers and moderate drinkers indicates distinct gender-dependent impacts of increased body weight and alcohol drinking on serum uric acid, which has recently been closely linked with oxidant stress status in humans [1,5]. The concentration of serum uric acid is influenced by nitric oxide (NO) and peroxynitrite production and plays a pivotal role in the human antioxidant defense systems through its ability to scavenge free radicals [4,5,19–21]. Uric acid also prevents the degradation of extracellular superoxide dismutase [22], an important enzyme maintaining normal NO levels and endothelial function.

Both excessive ethanol consumption and adiposity readily induce oxidative stress in vivo. Although the effects of alcohol abuse [23–25] and obesity [1] on serum uric acid levels have been previously acknowledged, as yet the early phase interactions between uric acid, ethanol intake, and adiposity have received less attention. The biological significance of alterations in uric acid levels under such conditions has also remained obscure. It is tempting to speculate that by acting as an antioxidant uric acid could counteract oxidative stress and endothelial dysfunction, which, based on the present data, could occur even in apparently healthy individuals with adiposity and alcohol consumption. Recent studies have suggested a role for uric acid as a mediator of cardiovascular and renal diseases, metabolic syndrome, and type 2 diabetes [26–31]. Although hyperuricemia has in general been regarded as a cardiovascular risk factor [32,33], conflicting data also exist. Experimental administration of uric acid induces a significant increase in serum free radical scavenging capacity in healthy volunteers [34] and improves endothelial function in the forearm vascular bed of smokers and in patients with type 1 diabetes [35]. Elevated uric acid concentrations are also associated with

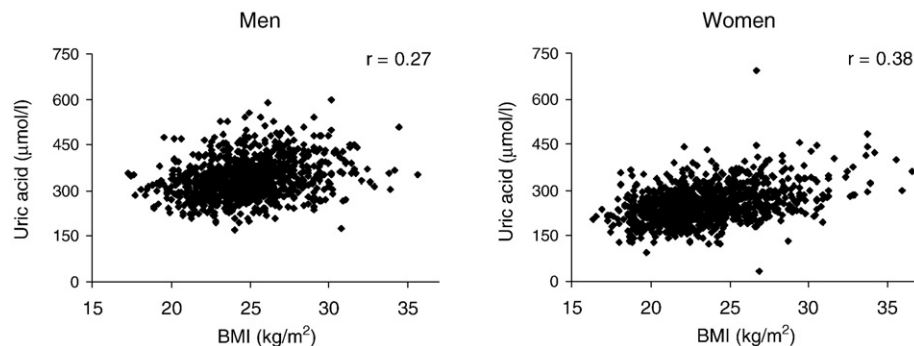


Fig. 3. Scatter plots of the correlations between BMI and uric acid in men and women. A significantly stronger correlation was observed among women ($p < 0.02$).

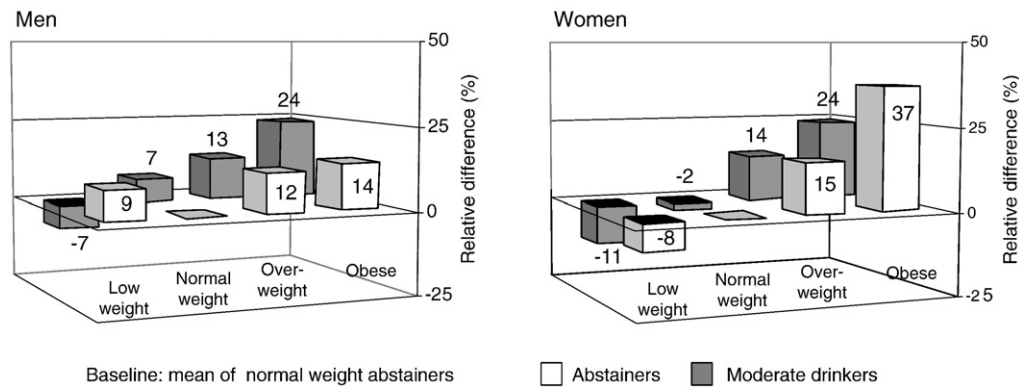


Fig. 4. Relative changes (%) in mean serum uric acid in the study groups of men and women. The mean activity obtained from the corresponding group of abstainers with normal weight is used as baseline. For weight group BMIs, see the Fig. 2 legend.

increased serum antioxidant capacity and reduced oxidative stress during acute physical exercise among healthy subjects [36].

Interestingly, the present data indicate striking gender differences in uric acid responses. Whereas moderate alcohol consumption seems to be associated with low uric acid concentrations among women, men seem to show the opposite. Previous studies have suggested that sex steroids play a key role in the regulation of oxidant stress status in vivo [37–40]. In accordance with the present findings, Gao et al. [41] recently found that a greater intake of added sugars or sugar-sweetened drinks was associated with high plasma uric acid concentrations in men but not in women. Pitocco and coworkers [5] have reported that the initial increase in oxidative stress coincides with a reduction in plasma levels of uric acid in women with type 1 diabetes. Interestingly, uric acid seems to increase in menopause [42], during which the relative cardioprotection of premenopausal women compared to men of corresponding age is also lost [43]. Uric acid can improve NO-mediated vasorelaxation in arteries, and the cardiovascular risk status of women may also be linked with NO owing to the fact that it is inducible by estrogen [44]. Alcohol intake can induce both pre- and postmenopausal estrogen [45,46] and thus possibly potentiate NO and lead to lower uric acid levels, as noticed here particularly among premenopausal normal weight women. Unchanged uric acid in the corresponding group of postmenopausal women could be due to unresponsiveness of estrogen receptors. Indeed, decreased downstream signaling through estrogen receptors has previously been proposed to account for the lack of vascular benefits of hormone therapy in aging women [44,47]. In overweight or obese women excess body weight seems, however, to overpower the impact of moderate alcohol intake. It remains to be established whether estrogen induction by moderate ethanol intake could also be associated with possible beneficial cardiovascular effects specifically in normal weight premenopausal women. It could also be argued that the decrease in uric acid and antioxidant defense could render women more susceptible to oxidative stress in other tissues, such as adverse effects of ethanol in the liver. Indeed, women are known to have a greater propensity than men for ethanol-induced tissue damage at more than moderate drinking levels.

Unfortunately, in this work the preferred type of alcoholic beverage consumed was not recorded systematically enough to allow beverage-specific analyses of the data. Current statistics from corresponding Scandinavian populations have indicated that the share of strong alcohols is approximately 15–20% of the total alcohol consumption among men and about 10% in women, whereas mild beverages, primarily beer, is responsible for more than half of the total consumption in both genders. The rest of the consumption is mostly wine, the proportion of which is slightly higher among women (20–30%) than among men (15–20%). Although we feel that the type of alcoholic beverage consumed by the moderate drinkers in this study is unlikely to

have accounted for the observed changes in serum uric acid, this issue should be a subject of future studies in large samples of individuals with varying levels of drinking different types of alcoholic beverages. Similarly, in light of recent studies indicating a protective effect of coffee consumption on ethanol-induced oxidative stress in hepatic tissue, which may also occur in a gender-dependent manner [48–50], studies on the associations between coffee consumption and uric acid metabolism seem warranted.

The present data further indicate significant correlations between uric acid and indicators of hepatocellular health. Apparently, alcohol drinking occurring together with excess caloric intake is a potent inducer of hepatocellular oxidative stress. Previous studies in experimental liver diseases have indicated that high-fat diets reproduce many of the features found in nonalcoholic steatohepatitis and administration of high-fat diets together with ethanol results in enhanced oxidative stress and more severe liver injury [51]. The ethanol-inducible cytochrome enzyme CYP2E1 may also be induced by obesity, owing to free fatty acids serving as substrates [52,53]. We previously reported that alcohol drinking and obesity increase serum GGT in an additive manner [15]. GGT is responsible for the extracellular metabolism of glutathione, the main antioxidant in mammalian cells, and GGT enzyme induction may be specifically associated with the generation of reactive oxygen species [54–58]. Interestingly, serum ALT, which also seems to readily respond to both ethanol intake and adiposity [59–61], has recently been suggested to be a good indicator of overall health in the context of obesity, metabolic syndrome, and cardiovascular disease [11]. Obviously, the links between serum uric acid, oxidative stress, and biomarkers of liver status, which associate with various biochemical and anthropometric features of the metabolic syndrome [62,63], also warrant future studies.

Our findings are also in accordance with the view that uric acid could serve as a clinical biomarker of oxidative stress provided that the effects of obesity and ethanol intake would be carefully controlled in the definition of normal ranges for such measurements. It may be recommended to use databases of abstainers with normal weight as a reference or, alternatively, BMI-based reference intervals. Combinations of uric acid and liver enzyme levels could possibly be used to develop more accurate models to evaluate biological responses to oxidative stress and associated pathophysiology in the human body.

Conclusions

This study indicates significant interactions between gender, BMI, alcohol consumption, and serum uric acid, which may be associated with the status of oxidative stress in such individuals. Future population studies addressing the prognostic and clinical implications of such responses and whether it might be necessary to formulate BMI-based recommendations for uric acid normal ranges seem warranted.

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ORIGINAL ARTICLE

Evaluation of reference intervals for biomarkers sensitive to alcohol consumption, excess body weight and oxidative stressPÄIVIKKI ALATALO¹, HEIDI KOIVISTO¹, JOHANNA KULTTI¹, RISTO BLOIGU² & ONNI NIEMELÄ¹¹Department of Laboratory Medicine and Medical Research Unit, Seinäjoki Central Hospital, Seinäjoki, Finland and University of Tampere, Tampere, Finland, ²Medical Informatics Group, University of Oulu, Oulu, Finland**Abstract**

Background. Unexplained liver enzyme activities are often found in health screening programs and constitute an increasingly common cause for referral to specialized clinics. Recent studies have indicated that both excess body weight and alcohol consumption may lead to metabolic aberrations which are readily reflected in the activities of liver enzymes in circulation. **Materials and methods.** We compared various laboratory markers and their upper normal limits in relation to information on alcohol consumption and BMI in a large population of apparently healthy individuals collected from Nordic countries. **Results.** Based on the data obtained from normal weight abstainers (BMI 19–25 kg/m²) the upper normal limits in men should be 50 U/L for ALT, and 45 U/L (<40 years) and 70 U/L (≥40 years) for GGT, while the current recommendations are 70 U/L, 80 U/L, and 115 U/L, respectively. Already in comparisons between normal weight abstainers and corresponding moderate drinkers notable impacts (+14% – +74%) on upper limits for these analytes were seen, which further grew when adiposity occurred together with alcohol drinking (+75% – +186%, BMI ≥27 kg/m²). In addition to liver enzymes, similar changes were also found for uric acid. **Conclusions.** Alcohol consumption and excess body weight even in apparently healthy individuals have a significant influence on liver enzyme activities, which may be due to a cumulative oxidative stress burden. The metabolic changes induced by adiposity or ethanol intake should be considered in the definition of normal ranges for all laboratory parameters sensitive to oxidative stress.

Key Words: Alanine aminotransferase, aspartate aminotransferase, body mass index, ethanol, gamma-glutamyltransferase, normal limit, overweight, uric acid

Introduction

During the past few decades several new challenges for health care services have emerged. The rapidly increasing percentage of individuals with excess body weight constitutes a major threat in all Western countries. In the European Union approximately 50% of the population was either overweight or obese in 2003 [1]. Simultaneously, the total per capita ethanol consumption and associated medical disorders have been growing [2–4]. Thus, obesity-related health problems are also more and more likely to co-exist with ethanol consumption.

In laboratory medicine increasing national and international mobility has created a need for harmonization of reference intervals. Historically, because of a wide variety of differences in both methods and

populations, laboratories have been advised to establish their own reference intervals for each analyte. This is, however, a highly demanding and costly process for a single laboratory and, therefore, reference intervals obtained from literature or from small sample sizes are often used. As a consequence, considerable variation between laboratories may also occur. Recently, along with advances in methodological consensus, the production of common reference intervals has become a potential approach to overcome these problems. The Nordic Reference Interval Project (NORIP) [5] showed that partitioning of the reference intervals by country (i.e. Denmark, Finland, Norway and Sweden) was not necessary [6]. This project resulted in recommendations for common reference intervals for 25 biochemical parameters frequently measured

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in serum [7]. In addition to analytical and methodological data a number of demographic characteristics in relation to this reference population were systematically documented [8].

In the present study we explored the effects of alcohol consumption and excess body weight on the reference intervals using the database from the NORIP material.

Materials and methods

Study protocol

The main characteristics of the NORIP population have been previously described in detail [8]. Originally, blood samples from over 3000 healthy individuals were collected by Nordic routine clinical biochemistry laboratories together with information on sex and age, and various other factors such as drinking and smoking habits, medication, and body mass index (BMI, kg/m²). In this study, alcohol consumption was classified in categories of 0 measures (abstainers), 1–21 measures (moderate drinkers) and >21 measures of alcohol/week, where one measure is equivalent to 12 g of pure ethanol. The data from Sweden were excluded due to non-standardized assessment of alcohol consumption [8]. In the remaining population 31.0% were abstainers and 67.5% moderate drinkers. Individuals who reported more than 21 measures of alcohol per week (0.5%) or had not answered (1.0%) were not included in the analyses. The data gathered on smoking habits indicated that 80% were non-smokers, 6% smoked 1–5 cigarettes/day, and 11% smoked >5 cigarettes/day (3% had not answered). A total of 19% had used medication other than the P-pill or estrogen preparations during the preceding week (76% had not used medication, 5% had not answered). In men 1% were underweight (BMI <19 kg/m²), 54% normal weight (BMI 19–25 kg/m²), 41% overweight (BMI 25–30 kg/m²), and 4% obese (BMI >30 kg/m²). In women the corresponding percentages were 5%, 69%, 22%, and 4%, respectively.

Statistical methods

Data exclusions, calculations of reference limits, and group stratifications were carried out following the NORIP protocol [7]. Outliers were detected by Dixon's test (performed manually to enable successful handling of clusters of two or more outliers) for each reference data set separately, as proposed by IFCC [9]. The influence of the drinking habit (predictor variable) was assessed by linear regression in a model with each analyte as the dependent variable, and by adjusting for body mass index (BMI), age, smoking habit and medication. For skewed analytes logarithmic transformation was used to yield symmetrical distributions. The analyses were made

separately for groups with different reference intervals and further according to sex. A *p*-value <0.05 was considered statistically significant. The analyses were carried out using Analyse-it 2.11 for Microsoft Excel (Analyse-it Software, Ltd., Leeds, UK) and SPSS 15.0 (SPSS Inc., Chigaco, IL, USA) statistical software.

Results

As noted previously, more than half of the analytes included in the NORIP protocol differ significantly between the individuals representing BMI <27 kg/m² and ≥27 kg/m² [10]. For such parameters the data on reference limits in subgroups classified according to both BMI and alcohol drinking are summarized here in Table I. The most striking changes (≥10%) between upper normal limits derived from normal weight abstainers and moderate drinkers with BMI ≥27 kg/m² were observed in alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, creatinekinase (CK), gamma-glutamyltransferase (GGT), glucose, HDL-cholesterol, triglycerides, and uric acid. Drinking habit was found to be a statistically significant independent positive predictor variable of GGT activity, total cholesterol (≥50 years) and uric acid in men, and of HDL-cholesterol concentration in both men and women. When comparing the upper normal limits between normal weight abstainers and the corresponding group of moderate drinkers, clinically relevant alterations were observed for GGT and ALT. Age, smoking habit or use of medication were not found to significantly influence the above associations in this population.

The relative risks for abnormal findings as a function of drinking status were further assessed by comparing the incidences of abnormal values in alcohol drinkers to those found in normal weight abstainers (Figure 1). Particularly in men there was a gradual increase in the relative risks for higher liver enzyme activities (ALT, AST, GGT) from normal weight abstainers to moderate drinkers, and from overweight abstainers to corresponding moderate drinkers. When the reference limits obtained from normal weight abstainers were used as cut-offs the increase in relative incidence of abnormal values from normal weight abstainers to overweight moderate drinking men was up to 3–7-fold, being lowest for AST and highest for GGT. A similar pattern and a more than 3-fold higher relative risk was also found for uric acid. In addition, changes in relative risks were seen in serum lipid profiles, although the changes were less prominent than those for liver enzymes (Figure 1).

The upper normal limits derived from the total NORIP population and from the subpopulation including only normal weight abstainers are compared in Table II. It appears that up to 82%

Table 1. Multiple upper normal limits for subgroups classified by body mass index (BMI, in kg/m²) and drinking habit. The percentages in brackets indicate the relative change from normal weight abstainers.

Upper normal limits (97.5 percentiles)												
Analyte	Unit	Gender	Age	Normal weight abstainers (BMI 19–25)		Normal weight moderate drinkers (BMI 19–25)				BMI ≥27.0 moderate drinkers	90% CI	n
				90% CI	n	90% CI	n	90% CI	n			
Alanine aminotransferase	U/L	F	≥18	37	217	32–49	47 (+27%)	37–56	421	56 (+51%)		60
		M	≥18	50	109		57 (+14%)	49–83	326	123 (+146%)		116
Albumin	g/L	FM	18–39	47.3	168	47.0–54.2	47.7 (+1%)	47.5–48.5	369	50.0 (+6%)		66
			≥40	44.8	225	43.9–46.7	45.5 (+2%)	45.2–45.8	553	46.5 (+4%)	45.0–49.9	153
Alkaline phosphatase	U/L	FM	≥18	108	79		110 (+2%)	97–120	199	112 (+4%)		44
Aspartate	U/L	F	≥18	40	192	32–42	34 (–15%)	32–38	381	40 (±0%)		48
aminotransferase ¹		M	≥18	40	100		43 (+8%)	40–57	290	52 (+30%)		108
Bilirubin	μmol/L	FM	≥18	25	394	21.5–29.4	24 (–4%)	21.8–25.1	929	25 (±0%)	21.2–30.5	219
Cholesterol ^a	mmol/L	FM	18–29	6.00	112		6.21 (+4%)	5.93–6.37	250	6.20 (+3%)		43
			30–49	6.56	108		6.88 (+5%)	6.66–7.38	330	8.18 (+25%)		61
			≥50	7.96	172	7.67–8.61	8.10 (+2%)	7.68–9.07	344	7.83 (–2%)		115
Creatinekinase ²	U/L	F	≥18	211	196	151–270	199 (–6%)	167–218	423	164 (–22%)		56
		M	18–49	300	44		399 (+33%)	321–455	147	485 (+62%)		45
			≥50	255	41		224 (–12%)	208–257	120	522 (+105%)		61
Gamma-glutamyl-transferase ^{b,3}	U/L	F	18–39	38	88		43 (+13%)	41–63	178			17
			≥40	75	145	50–90	99 (+32%)	60–145	273	83 (+11%)		39
		M	18–39	44	59		52 (+18%)	45–71	151	126 (+186%)		41
			≥40	69	64		120 (+74%)	99–151	217	121 (+75%)		89
Glucose	mmol/L	FM	≥18	5.50	125	5.31–5.85	5.85 (+6%)	5.71–6.42	296	6.32 (+15%)		69
		F	≥18	5.65	89		5.84 (+3%)	5.60–6.42	161			19
		M	≥18		36		6.07	5.74–6.83	135	6.61		50
HDL-cholesterol ^c	mmol/L	F	≥18	2.74	249	2.44–3.02	2.65 (–3%)	2.53–2.76	495	2.42 (–12%)		68
		M	≥18	2.13	127	1.89–2.60	2.29 (+8%)	2.16–2.42	391	1.87 (–12%)	1.74–1.99	146
Iron	μmol/L	FM	≥18	34.4	341	31.8–36.9	33.8 (–2%)	31.7–34.9	770	34.0 (–1%)	33.0–38.9	185
LDL-cholesterol	mmol/L	FM	18–29	4.03	41		4.32 (+7%)		104			21
			30–49		38		4.64		117			20
			≥50	5.85	81		5.24 (–10%)	5.03–6.09	148	5.31 (–9%)		51
Phosphate	mmol/L	F	≥18	1.44	244	1.40–1.61	1.50 (+4%)	1.46–1.54	487	1.42 (–1%)		63
		M	18–49	1.70	75		1.66 (–2%)	1.56–1.71	229	1.71 (+1%)		68
			≥50	1.34	56		1.42 (+6%)	1.33–1.54	155	1.33 (–1%)		77
Potassium	mmol/L	FM	≥18	4.63	380	4.56–4.81	4.60 (–1%)	4.58–4.71	871	4.67 (+1%)	4.58–4.75	211

Sodium	mmol/L	FM	≥18	144.8	144.2–145.4	375	144.5 (±0%)	144.3–145.1	883	145.2 (±0%)	144.3–146.3	218
TIBC	μmol/L	FM	≥18	81.5		111	82.7 (+1%)	78.2–89.7	231	86.1 (+6%)		52
Triglycerides	mmol/L	FM	≥18	2.27	1.86–2.80	162	2.28 (±0%)	1.87–2.99	374	3.72 (+64%)		94
Uric acid ^d	μmol/L	F	18–49	325	302–358	135	335 (+3%)	315–363	322			32
			≥50	378		109	353 (–7%)	347–430	181			33
		M	≥18	446	400–472	130	471 (+6%)	457–497	387	505 (+13%)	467–600	143

^aSignificant independent positive prediction by moderate drinking within the total population in men ≥50 years ($p < 0.05$), ^bin men 18–39 years ($p < 0.01$) and ≥40 years ($p < 0.001$), ^cin both men and women ($p < 0.001$), and ^din men ($p < 0.01$). Assessed for analytes in which relative change in upper limit from normal weight abstainers to moderate drinkers with BMI ≥27 kg/m² exceeds ±10%.

¹One outlier in normal weight moderate drinkers in women (62 U/L).

²One outlier (625 U/L) in abstainers and three (>258 U/L) in those with BMI ≥27 kg/m² in women; two outliers (>487 U/L) in abstainers and one (690 U/L) in normal weight moderate drinkers in men 18–49 years of age.

³Two outliers (>108 U/L) in normal weight moderate drinkers in women 18–39 years of age; one outlier in abstainers (88 U/L) and normal weight moderate drinkers (168 U/L) in men 18–39 years of age, and one outlier in abstainers (224 U/L) in men ≥40 of age. For GGT, the person exclusion criteria of those described in NORIP were used for data rejection [5,7,42].

increase in case of GGT and 40% in ALT may be brought about into currently recommended upper normal limits by these factors reflecting life style.

Discussion

The present study supports the view that excess body weight and alcohol drinking create additive effects on several laboratory parameters, especially on liver enzymes. Due to the high incidence of both adiposity and alcohol drinking in our society, these conditions are expected to create a major burden to health care, which should also be reflected in the criteria used for acknowledging normality or abnormality in laboratory values.

Recent Nordic Reference Interval Project (NORIP) introduced a large population of apparently healthy individuals with well-established demographic characteristics [8], which allow the classification of the material according to both BMI and drinking status. The establishment of subgroup-specific reference intervals is, however, a complex task. Continuous variables such as BMI are subject to arbitrary divisions, which can result in the overlooking of clinically important differences. Alcohol consumption in turn is commonly underreported in all alcohol-related questionnaires. The present material is, however, unlikely to include significant amounts of problem drinkers, since individuals reporting over 21 measures/week or over 2 measures in the last 24 hours were excluded. It should also be noted that the levels of safe alcohol consumption may vary between individuals. In Finland the limits of 24 measures per week for men and 16 for women have been previously set for hazardous drinking [11], although adverse health effects are sometimes known to follow from even lower levels of consumption [12,13].

In order to avoid the problems related to heterogeneity of reference populations a single population with strengthened inclusion criteria could be used to produce the normal limits. The present data suggest that the group of normal weight abstainers should be selected for such purposes in case of several variables sensitive to ethanol consumption, excess body weight and oxidative stress. In the total NORIP population the proportions of abnormal values (compared to the theoretical 2.5% in normal weight abstainers) would be 8.2%, 7.2% and 5.3% in men for GGT, ALT and AST, respectively. We feel that in case of liver function assessment lower cut-offs would yield benefits that are greater than the possible expenses caused by increased frequency of abnormal values.

First, the detection of early changes in liver enzyme activities may be important for the early diagnosis of alcohol-related health problems [14]. At least before the full onset of an epidemic

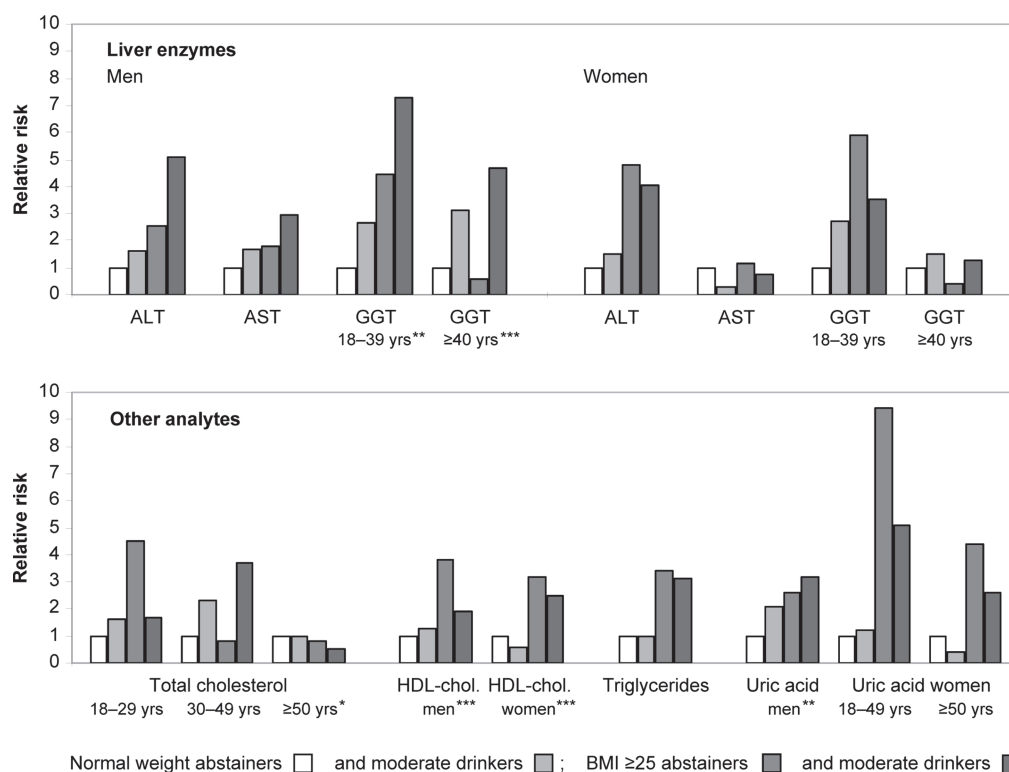


Figure 1. Relative risks for abnormal values in different study groups. The incidence of values over the normal weight abstainer-based upper normal limit (for HDL-cholesterol below the lower normal limit of 1.07 mmol/L (women) and 0.88 mmol/L (men)) was compared to the theoretical reference incidence of 2.5%. The significances indicating positive prediction by drinking habit only are marked as follows: *** $p < 0.001$; ** $p < 0.01$, and * $p < 0.05$. All models were adjusted for BMI, age, smoking habit and medication.

of obesity, experts considered roughly 70% of cases of increased GGT activity to be explained by excess alcohol consumption. In NORIP recommendations the upper normal limits of GGT activity for men were 80 U/L for those <40 years of age and 115 U/L for those ≥ 40 years of age, whereas the present data indicate that for normal weight abstainers the corresponding figures should be set at around 45 U/L and 70 U/L, respectively. On the edge of age 40 especially striking discrepancies between the early phases of liver pathology and interpretation of liver enzyme activities may be expected to occur. Although there currently are rather specific new biomarkers (such as CDT) for revealing alcohol abuse [15], it should be emphasized that they are largely ordered under suspicion and their sensitivity is not high enough for screening.

Second, monitoring early changes in liver enzymes is crucial in the assessment of non-alcoholic fatty liver disease (NAFLD), which has recently been recognized as the hepatic manifestation of obesity and the metabolic syndrome with the prevalence of 20–30% in general population and up to about 85% among obese individuals [16]. The disease constitutes a spectrum of liver injuries ranging from steatosis (fatty liver) to steatohepatitis (NASH), which can further progress to cirrhosis [17,18]. The only clinical evidence of these conditions may be mild to moderate elevations in aminotransferase levels,

although advanced histological damage due to NAFLD can also be present without any alert [19,20]. Recent surveys have shown that nearly 70% of aminotransferase elevations are likely to be explained by factors associated with obesity [21]. The present data suggest that for ALT the upper normal limits based on normal weight abstainers should be 50 U/L instead of 70 U/L, as derived from the total NORIP population. A decrease in ALT cut-offs could possibly yield additional clinical significance through the fact that in routine clinical work the level of aminotransferases twice the normal is frequently used as decision-making point for liver biopsy in order to rule out the most severe liver diseases, as well as to distinguish patients needing the closest follow up [14,22,23].

Third, a number of epidemiologic studies argue against the escaping levels of upper normal limits. Liver enzyme activities have recently been linked not only to hepatocellular health but also to diabetes, the development of metabolic syndrome and cardiovascular diseases (CVD) [24]. Rather than being a sequela of any apparent tissue pathology, liver enzyme changes also seem to have predictive value. Slightly increased GGT activities are associated with increased risk of diabetes and CVD [25,26]. Very recently Goessling et al. [27] reported that ALT, even within its normal range (≤ 40 U/L), could predict diabetes and metabolic syndrome. In addition, AST was

Table II. Comparison of upper normal limits based on the total Nordic Reference Interval Project (NORIP) material [7] and from normal weight abstainers.

Analyte	Unit	Gender	Age	Upper normal limit	
				NORIP suggestions	Normal weight; Abstainers
Alanine aminotransferase	U/L	F	≥18	45	37
		M	≥18	70	50
Aspartate aminotransferase	U/L	F	≥18	35	40
		M	≥18	45	40
Gamma-glutamyltransferase	U/L	F	18–39	45	38
			≥40	75	75
		M	18–39	80	44
			≥40	115	69
Cholesterol	mmol/L	FM	18–29	6.1	6.0
			30–49	6.9	6.6
			≥50	7.8	8.0
HDL-cholesterol	mmol/L	F	≥18	2.7	2.7
		M	≥18	2.1	2.1
Triglycerides	mmol/L	FM	≥18	2.60	2.27
Uric acid	μmol/L	F	18–49	350	325
			≥50	400	378
		M	≥18	480	446

indicative of future diabetes. These associations were independent of the other common measures of adiposity and metabolic derangements, such as interim weight change, and baseline BMI or glucose levels. Accordingly, Ioannou [28] recently concluded that ALT activities should no longer be considered solely as a marker of an underlying liver disease, but also as a biomarker of ectopic fat deposition.

Recent findings have emphasized injuries due to free oxygen radicals and oxidative stress as the underlying cause for liver enzyme elevations [29]. Both alcohol consumption and excess body weight induce oxidative stress, and it appears that especially among obese individuals this could occur in a more striking manner as a result of ethanol intake [30]. Oxidative stress is also thought to play a causal role in the development of cancer and neurodegenerative diseases, and in aging (Figure 2) [31,32]. Interestingly, the cytochrome CYP2E1 enzyme induction, which is one of the central pathways responsible for the ethanol-generated state of oxidative stress in hepatocytes, may also be achieved by obesity alone due to free fatty acids serving as substrates [33]. Since GGT plays a crucial role in maintaining intracellular levels of glutathione, the main antioxidant in mammalian cells, increases in its activity may be a generalized early sign of counteracting mechanisms

to protect hepatocytes against oxidative damage [34,35]. Higher ALT levels in conjunction with alcohol consumption and adiposity may, in turn, be associated with oxidative stress related to increased fatty deposition in the liver [34]. Accordingly, in this study liver enzyme activities showed a gradual increase in upper normal limits and relative risks from normal weight abstainers to overweight moderate drinkers. Intriguingly, similar changes were observed in uric acid, which has also been proposed as an index of oxidant stress status [36,37].

The present data also show changes in serum lipid profiles as a result of moderate drinking. Several studies have emphasized possible positive impacts of moderate ethanol intake especially on HDL-cholesterol, and subsequently, on cardiovascular mortality [38,39]. It should be noted, however, that the changes in liver enzymes and associated relative risks may exceed the benefits observed for serum lipids. Such comparisons apparently warrant future studies in large materials representing different body weights.

In the present material, age, smoking habits and medication were not found to confound the associations reported. It should be noted, however, that in the NORIP material only a few individuals were smokers or used any type of medication and therefore

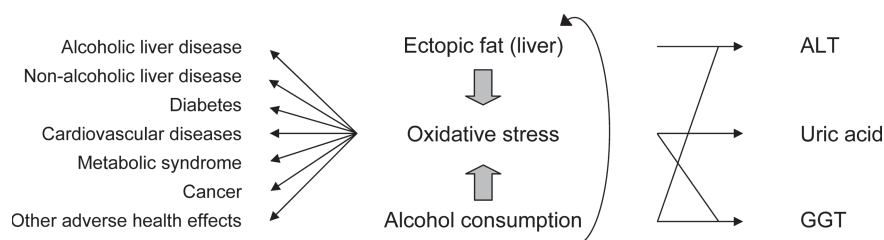


Figure 2. Illustration of the mechanisms and outcomes potentially linking alcohol consumption and excess body fat.

in light of recent findings indicating synergism between alcohol consumption and smoking with respect to liver enzymes [40], further studies appear also warranted to evaluate such effects in individuals with different levels of smoking and ethanol intake.

In conclusion, the material gathered by the NORIP project has provided an excellent tool for testing cut-offs between normality and abnormality in laboratory markers, but also to retrospectively evaluate the reference intervals already in use. It appears that the effects of moderate alcohol consumption and excess body weight on several laboratory parameters need further attention. While it may be argued that narrower intervals would translate into a high percentage of false positives [41], in light of our observations, many of the false positives may actually be true positives, since the presence and consequences of a harmful etiology are identifiable. In the end, abnormal values are those that ultimately prompt the action.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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