VRIJE UNIVERSITEIT

GENETICS OF ALCOHOL USE AND LIVER ENZYMES

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. F.A. van der Duyn Schouten,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Psychologie en Pedagogiek
op woensdag 4 september 2013 om 11.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Jannigje Heiltje Dingena Adriana van Beek

geboren te Waalwijk

promotor:	prof.dr. D.I. Boomsma
copromotoren:	dr. A.H.M. Willemsen
	dr. M.H.M. de Moor

GENETICS OF
ALCOHOL USE AND LIVER ENZYMES

JENNY VAN BEEK

Reading committee:

Prof. dr. C. van Duijn

Prof. dr. C. Kluft

Prof. dr. W. van den Brink

Prof. dr. A.N.M. Schoffelmeer

Dr. H. Mbarek

Dr. N. Smits

Paranimphs:

Lot Geels

Mara van Laaren

Acknowledgments

We thank all participants in the NTR and NESDA studies.

This work has been executed in the Mental Health research program of the EMGO Institute for Health and Care Research and was supported by grants from the Netherlands Organization for Scientific Research (NWO) and MagW/ZonMW (Addiction-31160008, grants 985-10-002, 904-61-193, 480-04-004, 400-05-717, Middelgroot-911-09-032, Spinozapremie 56-464-14192, Geestkracht 10-000-1002), Center for Medical Systems Biology (CSMB, NWO Genomics), NBIC/BioAssist/RK(2008.024), Biobanking and Biomolecular Resources Research Infrastructure (BBMRI –NL, 184.021.007); the European Science Foundation (ESF, EU/QLRT-2001-01254), the European Community's Seventh Framework Program (FP7/2007-2013), ENGAGE (HEALTH-F4-2007-201413); the European Science Council (ERC Advanced, 230374), Rutgers University Cell and DNA Repository (NIMH U24 MH068457-06), the Avera Institute, Sioux Falls, South Dakota (USA) and the National Institutes of Health (NIH, R01D0042157-01A, MH081802, Grand Opportunity grants 1RC2 MH089951). Part of the genotyping and analyses were funded by the Genetic Association Information Network (GAIN) of the Foundation for the National Institutes of Health. This work has been partly executed during a stay at the University of Notre Dame (Indiana, USA). The visit was funded by the EMGO travel Grant and VU-FPP Regeling Talentgeld. Statistical analyses were partly carried out using the facilities of the Center for Research Computing at the University of Notre Dame (Indiana, USA) and SARA Computing and Networking Services (www.sara.nl).

ISBN: 978-94-6191-803-1

Printed by Ipskamp Drukkers B.V., Enschede

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Alcohol use is a serious risk factor for disease. Although beneficial effects of alcohol use have been reported, the total net effect on health is disadvantageous. Light to moderate levels of alcohol use have been related to lower risks for coronary heart disease (CHD) and type 2 diabetes, but for individuals who drink heavily (even occasionally) relative risks are higher. In Western countries, 8-12% of (in)direct aversive health effects are due to alcohol use (calculated in DALYs, disability-adjusted life years) in men, while this is 0.5-3% for women (Rehm et al. 2003). Most alcohol-attributable deaths result from cancer, cardiovascular disease, liver cirrhosis, diabetes and injury (Rehm et al. 2003). The average daily volume of alcohol intake (ADV) associated with increased mortality risk lies around 35-45 grams (~3 glasses). Risk of disease already starts to increase at an ADV of 25 grams (for hypertension, liver cirrhosis, chronic pancreatitis, various cancers), although for some diseases the ADV needed is much higher (>60 grams for type 2 diabetes, stroke; >89 grams for CHD, i.e. >4 and >6 glasses) (Dawson 2011). Other possible negative effects of alcohol use encompass alcohol use disorders (AUD; e.g. addiction), mental health problems, disruption of social relationships, loss of work productivity, as well as aggression, violence, and legal problems (NIAAA 2000).

The vast majority of people consumes alcohol, at least occasionally, which is likely to be explained by the positive effects of alcohol use on stress reduction, mood elevation, relaxation, and increased sociability (NIAAA 2000). Based on data from the World Health Organization, in the Netherlands, 82% of men and 64% of women aged 15 or older consumed alcohol in the past year. The prevalence of current drinking is even higher in the UK (90% and 82%), Germany (96% and 95%), Canada (81% and 74%) and Australia (88% and 80%), but lower in the USA (72% and 59%). The prevalence of heavy drinking however is highest among the Dutch. Twenty percent of Dutch men report to drink heavily (>60 grams of alcohol weekly at least once a week, i.e. >4 glasses) and five percent of the women. Estimates for the other countries are: 10% and 3% (Australia), 13% and 3% (USA), 14% and 2% (Germany) and 16% and 4% (Canada) (no estimates are reported for the UK) (World Health Organization 2011). Data from the Netherlands Twin Register (NTR) indicate that 9-17% of men and 3-11% of women drink heavily (>21 and >14 glasses per week respectively for individuals aged ≥18) (Geels et al. 2013). Given the high prevalence of alcohol use and heavy drinking in combination with the multitude of disease associations, understanding the causes of individual differences in (problematic) alcohol use is important.

1. Drinking guidelines and definitions of (problematic) alcohol use

Drinking guidelines on safe alcohol use are based on its harmful consequences for health and vary across countries (Health Counsel of the Netherlands, 2006; Dawson et al., 2011) as well as within countries across advisory organizations (Health Counsel of the Netherlands, 2006). Differences can partly, but not entirely, be explained by variation in the standard drink size between countries (Dawson et al., 2011) that ranges from 8 grams (UK) to about 14 grams (USA) (Dawson 2011; Health Counsel of the Netherlands 2012). Defining a standard drink size as containing 10 grams of alcohol, the Health Counsel of the Netherlands recommends daily alcohol use not to exceed two glasses of alcohol for men (≤20 grams) and one glass of alcohol for women (≤10 grams). These daily limits do not refer to an *average* daily volume of alcohol intake (e.g. heavy weekend drinking with five days of non-drinking), but emphasize that alcohol use should be kept within daily limits, each day. Countries generally recommend complete abstinence for youth and women that are (trying to get) pregnant, or breastfeeding (Health Counsel of the Netherlands 2012).

Definitions of problematic alcohol use can be separated into those that focus on the amount of alcohol consumption (e.g. excessive drinking) and those that refer to the negative physical and social effects of alcohol use (AUDs) (Fiellin et al. 2000). Excessive alcohol use is defined as drinking more than three alcoholic beverages a day *on average* for men (ADV >30 grams) and more than two alcoholic drinks *on average* for women (ADV >20 grams) (Health Counsel of the Netherlands 2012). Excessive drinking is known to put individuals at risk for alcohol-induced health-related consequences (therefore also known as 'hazardous drinking') (Fiellin et al. 2000). AUD diagnoses are based on manuals as the Diagnostic and Statistical Manual of Mental Disorders (4th edition; DSM-IV) (American Psychiatric Association 2000) and the International Classification of Mental and Behavioral Disorders (10th edition; ICD-10) (World Health Organization 1992). The DSM-IV (American Psychiatric Association 2000) distinguishes two types of AUDs: alcohol dependence and alcohol abuse. Alcohol dependence is defined as a maladaptive pattern of alcohol use, leading to clinically significant impairment or distress, broadly characterized by physical dependence (tolerance, withdrawal symptoms), psychological dependence (craving), loss of control (exceeding intended amount; unable to cut-down use), and physical and social consequences of maladaptive alcohol use. The diagnosis of alcohol abuse can be seen as a lighter version or precursor of alcohol dependence. It can be defined as a maladaptive pattern of alcohol use, leading to clinically significant impairment or distress, that is manifested by interpersonal and social problems due to alcohol use, without having symptoms of dependence or loss of control (Van den Brink 2010).

Although the diagnosis of AUD does not require a specific level of alcohol consumption, excessive alcohol use is a predictor for developing AUD. Both the average volume of consumption and the frequency of heavy drinking are independently associated with the risk of AUD. Drinking five or more drinks at a day for men (≥70 grams) or four or more for women (≥56 grams), was associated with a higher incidence of AUD, and the frequency of drinking these amounts further increased this risk (Dawson 2011). To avoid the development of AUDs, some organizations recommend to avoid daily drinking (Health Counsel of the Netherlands 2012).

Several brief screening instruments have been developed to detect excessive alcohol use and alcohol use disorders, including the CAGE questionnaire (Ewing 1984), and the Alcohol Use Disorders Identification Test (AUDIT) (Saunders et al. 1993). The AUDIT is developed to screen for excessive alcohol use that puts the health of individuals at risk. In addition to screening for excessive alcohol use it performs well in detecting alcohol dependence. Several shortened versions exist that show comparable screening properties (Reinert and Allen 2007). The CAGE, designed to detect alcohol dependence, is also valuable in detecting alcohol abuse, but less so in screening for excessive alcohol use. When combining the CAGE with alcohol consumption items on quantity and frequency, the performance in screening for excessive drinking is much increased (Dhalla and Kopec 2007; Berks and McCormick 2008). An overview of the measures for (problematic) alcohol use and other phenotypes that are used in this thesis is given in Chapter 2.

2. Alcohol metabolism and resulting tissue damage

Health-related consequences of alcohol use may, at least partly, be ascribed to effects of alcohol metabolism that primarily takes place in the liver. In humans, there are three pathways of alcohol oxidation. The bulk of ingested ethanol is oxidized by the alcohol dehydrogenase enzymes (ADH) which are present in the cytosol. At high alcohol concentrations and during chronic alcohol abuse, the cytochrome P450 isozymes, including CYP2E1, that are present in the endoplasmic reticulum, contribute to alcohol metabolism. This system is also called the microsomal ethanol oxidizing system (MEOS). A third, but minor, oxidative pathway is formed by the enzyme catalase that is located in the peroxisomes. Alcohol that is oxidized through these pathways results in the formation of acetaldehyde, a highly reactive and toxic byproduct that, with the enzyme aldehyde dehydrogenase (ALDH), is oxidized to acetate in the mitochondria (Zakhari 2006). The toxicity of acetaldehyde diminishes the capacity of the mitochondria to metabolize acetaldehyde, leading to higher levels of acetaldehyde in the mitochondria which contributes to alcohol-related tissue damage (Caballeria 2003). Other conditions that can result from alcohol oxidation and that can

induce tissue damage are fatty liver, oxidative stress, hypoxia (through increased oxygen consumption) and inflammation. See Figure 1 for an overview of the metabolic changes in the liver associated with alcohol metabolism (taken over from Callallería (2003)).

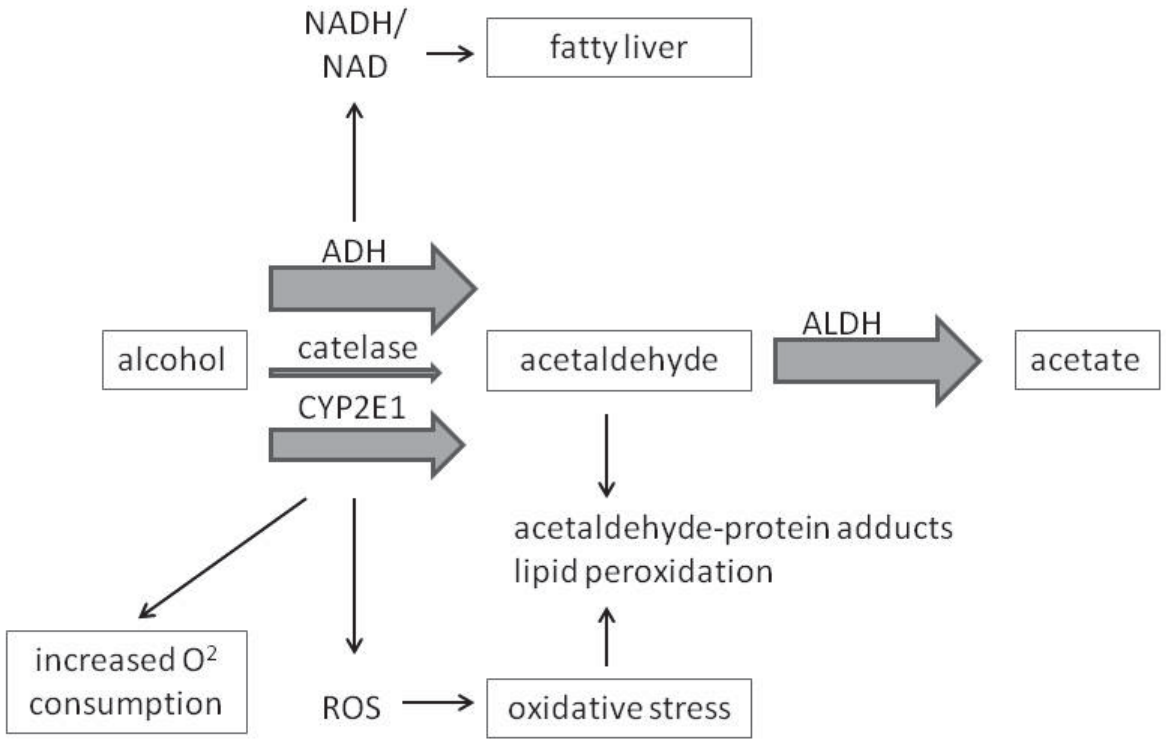


Figure 1 Metabolic changes in the liver associated with alcohol metabolism (taken over from Caballería, 2003)

The development of fatty liver, an excess of triglycerides in the liver, results from a change in the redox (*red*uction-*ox*idation) state in the cytosol and mitochondria. The conversion of ethanol to acetaldehyde by ADH and from acetaldehyde to acetate by ALDH, involves an intermediate carrier of electrons, nicotinamide adenine dinucleotide (NAD+), which is reduced to NADH. This results in an increased NADH/NAD+ ratio in the cytosol and mitochondria (Zakhari 2006). This redox imbalance leads to an excess of triglycerides in the liver by complex mechanisms that increase fatty acid synthesis, decrease hepatic lipoprotein secretion, increase mobilization of fatty acids from adipose tissue favoring their hepatic reuptake and decrease fatty acid oxidation (Caballeria 2003).

The oxidation of ethanol and acetaldehyde results in the generation of oxygen-containing free radicals, called reactive oxygen species (ROS). ROS are toxic because they react with

macromolecules including proteins, lipids and DNA, what can cause their degradation. A variety of enzymes as well as (non-enzymatic) anti-oxidants protect cells from ROS. A disturbance in the balance between ROS generating and protecting factors, resulting in an excess of ROS is called oxidative stress. The presence of certain metals in the cell, especially iron, promotes the interaction between some free radicals and thus catalyzes ROS. Chronic alcohol consumption increases iron levels in the body through increased iron absorption from food (a characteristic not limited to iron-rich red wine) and therefore ameliorates the toxic effects of alcohol on the body (Wu and Cederbaum 2003).

Ethanol tends to increase the oxygen intake by hepatocytes which can induce other liver cells in a state called hypoxia. Oxygen is needed for NADH (the reduced form of NAD+ that results from ethanol oxidation) to pass through the mitochondrial electron transport system in the mitochondria. During this process, H+ is removed from NADH, to obtain NAD+ that can be used again for ethanol oxidation. Oxygen is used to bind to the H+ molecules to form H_2O (Zakhari 2006). If liver cells that are close to the fresh blood supply (near the hepatic artery) take up increased amounts of oxygen, not enough oxygen may be left for liver cells that take up their oxygen supply later in the blood track (near the hepatic venule), leaving those cells in a state called hypoxia (Caballeria 2003). After chronic alcohol consumption, the liver cells near the hepatic venule are the first to show cell death (Cunningham and Van Horn 2003).

The immune system can be modulated by the effects of alcohol in several ways. One is the activation of the body's inflammatory response to the various biochemical metabolic effects that result from alcohol metabolism. Fatty acid accumulation in the liver induces synthesis of pro-inflammatory cytokines. Acetaldehyde can bind to proteins of various cellular membranes forming acetaldehyde-protein complexes that induce antibodies to bind to these complexes and destroy the hepatocytes that contain them. Death cells, for instance those that result from hypoxia or mitochondrial damage, also activate the inflammatory response (Wang et al. 2010). Second, long term alcohol exposure causes a change in the cytokine milieu resulting in a systematic inflammatory condition that can damage various tissues (Wang et al. 2010). Chronic alcohol consumption can cause leakiness of the digestive tract, which can result in the release of endotoxins in the blood that activate Kupffer cells that reside in the liver (Kovacs and Messingham 2002). These Kupffer cells stimulate the release of several inflammation-promoting cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-8. Kuppfer cells are a major source of ROS, further increasing possible tissue damage (Vidali et al. 2008). In addition, alcohol use is known to have immunosuppressive effects on both the innate and the adaptive immune system,

increasing the risk of infections (Molina et al. 2010; Kovacs and Messingham 2002). Finally, liver injury that results from inflammation can diminish the liver's ability to regulate inflammatory responses, which further ameliorates injury through the inflammatory response (Wang et al. 2010).

It should be noted that ethanol is also metabolized through non-oxidative pathways and can take place outside the liver as well. Non-oxidative pathways of ethanol metabolism include formation of molecules called fatty acid ethyl esters (from the reaction of alcohol with fatty acids) and formation of phospholipids that are known as phosphatidyl ethanol (Zakhari 2006). ADH activity is present in the stomach and colon mucosa. Marked gender differences have been observed for ethanol metabolism in the stomach. Gastric ADH activity is lower in women, which may be one reason why women are more susceptible to the toxic effects of alcohol. It is suggested that bacteria, that make up most of the flora in the human colon, can oxidize ethanol to acetaldehyde by bacterial ADH and subsequently to acetate by bacterial ALDH (Caballeria 2003).

3. Epidemiological research on the effect of alcohol use on fatty liver and oxidative stress

Epidemiological research on alcohol use supports the effects on liver injury described above, at least for heavy drinking. A longitudinal study showed that an increase in the quantity of alcohol consumed was associated with a higher risk of developing a fatty liver and a decrease in alcohol consumption with reduction of fat tissue within the liver (Bedogni et al. 2007). In most studies, a higher prevalence of fatty liver was observed among heavy drinkers (>40 grams ADV or alcohol abuse diagnosis) compared to individuals who drink less than 40 grams alcohol per day or abstainers (Hiramine et al. 2011; Bellantani et al. 1997; Pares et al. 2000; Chen et al. 2004; Fan et al. 2005). Studies among Japanese individuals consistently show that moderate drinking is associated with a lower prevalence of fatty liver when compared to abstaining (Yamada et al. 2010; Hiramine et al. 2011; Omagari et al. 2009; Gunji et al. 2009). This higher prevalence of fatty liver among abstainers could not be attributed to individuals who had stopped drinking because they received a diagnosis of fatty liver. Participants who had received the diagnosis of fatty liver during a previous examination were comparable in their level of alcohol use to participants who received the diagnosis of fatty liver for the first time (Hamaguchi et al. 2012). Furthermore, whereas for Japanese women, the prevalence of fatty liver among heavy drinkers was comparable to that among abstainers (Omagari et al. 2009; Hamaguchi et al. 2012; Yamada et al. 2010), for Japanese men, in some studies the prevalence of fatty liver among heavy drinkers was lower than that of abstainers (Hamaguchi et al. 2012; Yamada et al. 2010), although not in all (Omagari et al. 2009).

Effects of oxidative stress are detectable at both moderate and heavy drinking levels. Although the detection of alcohol-induced ROS generation in vivo is difficult (Wu et al. 2006), alcohol exposure has an effect on anti-oxidant levels and on surrogate markers of oxidative stress. Anti-oxidant carotenoid concentrations were lower in excessive drinkers (>25 grams ADV) than in abstainers (Sugiura et al. 2005). After drinking either beer, wine or spirits for 30 days (40 grams alcohol/day), levels of lipid peroxidation were increased and anti-oxidants levels decreased (Addolorato et al. 2008). Increased levels of oxidative stress, as measured by urid acid concentrations, were detected among normal weight excessive drinkers (>30 grams alcohol/day (Oliveira et al. 2010) as well as moderate drinkers (1-21 glasses alcohol/week) (Alatalo et al. 2009a) relative to abstainers (BMI <25). Note however, that in the study of Alatalo et al. (2009a), urid acid levels for women were comparable to those for abstainers (taking effects of BMI into account). Thus, the effect of oxidative stress by alcohol may be dependent on sex.

4. Liver enzyme levels and its relation to alcohol use

This thesis focuses on alcohol use and blood levels of three enzymes that give an indication of injury to the liver: gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Blood levels of these liver enzymes were originally proposed as biomarkers for detection of heavy drinking (Whitfield 2001), but, overall, their clinical value has proven to be limited.

GGT is a glycoprotein enzyme (a large molecule made up of both proteins and carbohydrates) that is situated on the membrane of cells in several tissues, including cells that are involved in bile production (Peterson 2004). It is involved in maintaining adequate levels of intracellular glutathione, the most abundant intracellular antioxidant, that are needed to protect the cell from oxidants that result from the metabolism of ethanol and other products (Whitfield 2001). Increases in GGT activity are thus expected during ethanol metabolism, to protect the cell from oxidative stress. Studies consistently show that GGT levels and the incidence of elevated levels is increased for heavy drinkers (>40 grams ADV) relative to abstainers as well as to moderate drinkers (Alatalo et al. 2009b; Chen et al. 2003; Ioannou et al. 2006; Nakanishi et al. 2000; Conigrave et al. 2002). The odds ratios associated with elevated GGT levels due to heavy alcohol use range from 3.85 to 18.4 (Scouller et al. 2000; Lee et al. 2001; Nakanishi et al. 2000; Higashikawa et al. 2005; Sillanaukee et al. 2000b; Steffensen et al. 1997; Arndt et al. 1998). Most studies report increased GGT levels for moderate drinkers in comparison to those for abstainers (Lee et al. 2001; Nakanishi et al. 2000; Higashikawa et al. 2005; Arndt et al. 1998; Liangpunsakul et al. 2010),

although in some studies GGT levels are similar to that of abstainers (Sillanaukee et al. 2000b; Steffensen et al. 1997). Whereas increasing levels of alcohol consumption are associated with increasing levels of GGT, its value as a marker of chronic alcohol use/abuse is limited. This is because an increase in GGT levels is not specific to heavy drinking (and conversely, not every heavy drinker has elevated GGT levels). Sensitivity estimates for GGT in detecting heavy drinking (>40 grams ADV) or a diagnosis of alcohol abuse or dependence, ranged from 7% to 65% for males; and from 7% to 61% for females, at a specificity of 90-95%. For studies that estimated the specificity to be 80-85%, the sensitivity was estimated to be around 39%-71% (Salaspuro 1999; Allen et al. 2000; Sillanaukee and Olsson 2001; Alte et al. 2004; Aertgeerts et al. 2001; Conigrave et al. 2002). The large differences in the sensitivity/specificity estimates can be explained by differences in the definition of problematic alcohol use that were used (>40/ >60/ >80 grams ADV/current/lifetime disorder of alcohol abuse/dependence), as well as by differences between studies in the prevalence of heavy drinking. Lower sensitivities are expected, and were detected, among the general population, in which the prevalence of heavy drinking is lower, than in medical wards and primary health care settings (Salaspuro 1999).

The association of alcohol use with aminotransferase levels (AST and ALT) is weaker than that with GGT. Blood levels of AST and ALT are markers of hepatocellular injury, that increase when damage to the liver cell membrane leads to increased leakage of AST and ALT into the circulation (Pratt and Kaplan 2000). ALT is present in several organs and muscle, but mostly in the liver and is therefore more specific to liver injury than AST which is found in the liver and skeletal muscle (Hannuksela et al. 2007), but also in the heart, and kidneys (Pratt and Kaplan 2000). An AST/ALT ratio of ≥2:1 is reported to be suggestive of alcoholic liver disease (Hannuksela et al. 2007; Sorbi et al. 1999; Nyblom et al. 2004). Although there is evidence that AST levels are increased by heavy drinking, they are not or only to a small degree increased by moderate levels of alcohol use. One study detected higher AST levels among moderate drinkers than among abstainers (Liangpunsakul et al. 2010), but another did not (Alatalo et al. 2009b). In three other studies the incidence of elevated AST levels among moderate drinkers was comparable to that of abstainers (Lee et al. 2001; Steffensen et al. 1997; Arndt et al. 1998). Heavy drinking was associated with increased (incidence of elevated) AST levels, compared to abstainers, in most studies (Arndt et al. 1998; Alatalo et al. 2009b; Chen et al. 2003; Conigrave et al. 2002), but not in all (Lee et al. 2001) and in one study AST levels were elevated only among women (Steffensen et al. 1997). The sensitivity of AST to detect problematic alcohol use has been estimated around 11-45% at a specificity of 90-95% and around 50% at a specificity of 80% (Salaspuro 1999; Conigrave et al. 2002).

There is some suggestion that ALT levels are increased by heavy alcohol use, but clear evidence is absent. Moderate drinking was associated with elevated ALT levels in two studies (Alatalo et al. 2009b; Ioannou et al. 2006) (in Alatalo et al. (2009b) for men only), but not in three others (Lee et al. 2001; Steffensen et al. 1997; Arndt et al. 1998). Of four studies that looked at the effect of heavy drinking (Lee et al. 2001; Steffensen et al. 1997; Alatalo et al. 2009b; Arndt et al. 1998), two studies detected higher ALT levels or an increase in elevated levels among heavy drinkers compared to abstainers (Arndt et al. 1998; Alatalo et al. 2009b). One study did not detect such an effect (Lee et al. 2001), and another only among women (Steffensen et al. 1997). The sensitivity of ALT to detect problematic alcohol use is estimated between 35% and 47% at a specificity of 85% (Salaspuro 1999).

Liver enzyme levels are associated with disease and mortality. GGT, ALT and AST all strongly predict liver disease and liver-related mortality (Ruhl and Everhart 2009; Kazemi-Shirazi et al. 2007; Hyeon et al. 2004; Lee et al. 2008), with GGT and ALT showing relations with other diseases also. Strong associations have been found for GGT and ALT with type 2 diabetes (Fraser et al. 2009), and for GGT with cardiovascular disease (Targher 2009; Fraser et al. 2007) as well as cancer and chronic kidney disease (Targher 2009). These disease associations cannot be solely explained by the negative effects of alcohol use alone, since most disease associations are still present when taking alcohol use into account (Targher 2009; Ruhl and Everhart 2009; Fraser et al. 2009).

In clinical practice, GGT, ALT and AST are used as markers of liver injury. To explain their role in other disease, GGT and ALT have been proposed as surrogate markers of fatty liver (Targher 2009; Schindhelm et al. 2006) and GGT as marker of oxidative stress as well (Lee et al. 2004). The role of GGT and ALT as marker of fatty liver comes from the positive correlations of GGT and ALT with liver fat content. ALT is suggested to be most closely related to the amount of liver fat (Targher 2009), which is further underlined by the association of the I148 allele of the PNPLA3 gene (chromosome 22) with ALT. The I148 variant of the PNPLA3 gene is associated with hepatic fat content and fatty liver, and predisposes individuals to an 28% increase in ALT activity (Vernon et al. 2011). ALT, GGT, and (to a lesser degree) AST levels increase with increasing levels of BMI (Alatalo et al. 2008). Partial correlations taking alcohol consumption or BMI into account, show that ALT levels depend more on BMI, whereas GGT levels are most dependent on differences in ethanol intake (partial correlations for AST were not reported) (Alatalo et al. 2008).

The role of GGT as marker of oxidative stress is complex. Although GGT is involved in protecting the cell from oxidative stress by maintaining adequate levels of the intracellular

antioxidant glutathione, the presence of iron is suggested to trigger pro-oxidant effects of GGT (Lee et al. 2004). Thus on the one hand, increases in GGT can mark increases in glutathione that actually present anti-oxidant effects to protect the cell from oxidative stress during normal metabolism, e.g. that of alcohol. On the other hand, increases can indicate the presence of ROS. Indeed, higher levels of GGT were associated with higher levels of markers for oxidative stress (Lee et al. 2004; Yamada et al. 2006; Bo et al. 2005) and lower levels of serum anti-oxidants (Sugiura et al. 2005; Lee et al. 2004; Lim et al. 2004). In addition, GGT and ALT correlate positively with CRP, a marker of inflammation (Yamada et al. 2006; Kerner et al. 2005).

5. Aim and research question

Alcohol use, especially heavy alcohol use, is associated with several changes in the body, including liver injury. Liver enzyme levels are used as markers of liver injury, and likely reflect, at least partly, effects of alcohol-related injury. The exact nature of the association between alcohol use and liver enzyme levels is not yet entirely clear however. Not every drinker has elevated liver enzyme levels, which may reflect genetic differences among individuals causing individual differences in liver enzyme levels. If alcohol use is associated with increased liver enzyme levels, and both alcohol use and liver enzyme levels are genetically mediated, then the question arises whether, and if so, to what extent, the genetic epidemiology of alcohol use and liver enzyme levels is shared, which is the focus of this thesis.

This thesis takes a genetic perspective on alcohol use and liver enzyme levels to elucidate the association between them. It first describes what amount of the variance in alcohol use and liver enzyme levels, and the overlap among them, can be ascribed to genetic and environmental effects. These analyses are based on data from twins and their family members registered with the Netherlands Twin Register (NTR) (Boomsma et al. 2002c; Boomsma et al. 2006). Twin-family designs can determine the importance of genetic risk factors for alcohol use and liver enzyme levels, and the amount of genetic overlap in risk factors among these traits, by the fact that mono- and dizygotic twin pairs and their family members differ in genetic relatedness to different degrees. Next, genetic marker data are analyzed to estimate what part of the (twin-family based) heritability estimates for alcohol use and liver enzyme levels are attributable to assessed genetic marker data. It is then examined if the association between alcohol use and liver enzyme levels can be traced back to genetic marker data. These analyses are based on data from NTR individuals as well as from participants of the Netherlands Study on Depression and Anxiety (NESDA) (Penninx et al. 2008).

Furthermore, a gene finding study is performed to detect genetic risk loci that explain differences alcohol use.

6. Outline thesis

First, a method section explains the methodology that has been applied to investigate the genetic architecture of alcohol use and liver enzyme levels (**Chapter 2**). Chapters 3 to 8 can be subdivided into two parts. In the first part, studies are presented that rely on twin-family data to obtain estimates of the heritable component of variation in alcohol use and liver enzyme levels. In these studies the genetic relatedness between individuals is inferred and the influence of genetic effects is estimated based on the correlations between the phenotypic values of family members. In the second part of this thesis, studies are presented that make use of genetic marker data (single nucleotide polymorphisms, SNPs) with the aims of gene finding and studying the genetic architecture of alcohol use and liver enzyme levels explained by SNPs.

Chapter 3 studies the importance of genetic and environmental influences in explaining individual differences in alcohol intake. For this study, the resemblance in alcohol intake levels among twins pairs, their siblings and parents is analyzed to obtain an estimate of the heritability. Besides effects of genetic transmission, effects of cultural transmission from parents to offspring are tested. Does the level of alcohol intake by the parents affect that of their children, e.g. through social modeling, even when the transmission of the genetic risk affecting alcohol intake levels is taken into account?

Chapter 4 describes a study on the development of symptoms of alcohol abuse and dependence (AAD) from adolescence into young adulthood, an important timeframe for the development of alcohol use, and how this development can be explained. For this study, longitudinal data on the CAGE are analyzed (Ewing 1984), collected in twins aged 15-32 years old. Specifically, it is examined what the relative contribution is of genetic and environmental effects on the development of symptoms of AAD in adolescence and young adulthood. Can this development be explained by one set of genes, or do different genes get expressed along adolescence and young adulthood, for instance when individuals tend to leave the parental home?

Chapter 5 describes what part of individual differences in blood levels of the liver enzymes GGT, ALT and AST can be ascribed to genetic effects. By analyzing the resemblance in liver enzyme levels among twin- and sibling pairs and parents and offspring, an estimate on the heritability is obtained. By analyzing data from twins, siblings and parents, possible effects of spousal

resemblance on the heritability can be taken into account, and non-additive genetic effects and effects of shared environment can be estimated simultaneously.

Chapter 6 presents a study on the association of alcohol intake with GGT. The relation between alcohol intake levels and GGT among twins and their family members can inform the underlying mechanism of association. Do individuals have elevated GGT levels because they drink higher amounts of alcohol ? (i.e. alcohol intake causes increased GGT levels) Or is there a common cause, e.g. shared genetic factors that influence both alcohol consumption as well as variation in GGT levels which explains the association between alcohol use and GGT at the population level?

In **Chapter 7**, a candidate gene study is performed on the association of loci in the ADH gene cluster with measures of alcohol use. This gene cluster harbors functional variants in the ADH1B and ADH1C genes which have been associated with risk for alcoholism previously (Edenberg 2007). Relatively little is known about genetic variants in other ADH genes and how these are related to measures of alcohol use other than alcoholism however, which is the focus of this study.

Chapter 8 examines what part of the variance in liver enzyme levels and their covariance with alcohol use can be explained by genetic marker data. Two relatively novel methods are applied to estimate the heritability of alcohol use and liver enzyme levels based on SNPs (genetic marker data that differ from the DNA reference variant by a single base pair, A, C, T, G). A first method is based on the genetic relatedness among individuals implemented in the software package GCTA (Yang et al. 2011a) and a second method on density estimation (proposed by So et al. (2011)). In addition, it is explored if part of the variation in liver enzyme levels that can be explained by SNPs, is shared with that for alcohol use.

This thesis is concluded with a summary and general discussion (**Chapter 9**) and Dutch summary (**Chapter 10**).

CHAPTER 2

DATA (COLLECTION) AND METHODS

This chapter consists of two parts. The first part briefly introduces the two cohorts that provided the data for the studies in this thesis and gives an overview of the variables analyzed. The second part presents an in detail description of the methodology that was used to study the genetic architecture of alcohol use and liver enzyme levels.

1. Data and data collection

The data on alcohol use and liver enzyme levels analyzed in this thesis come from twin-families registered with the Netherlands Twin Register (NTR) and from participants of the Netherlands Study on Depression and Anxiety (NESDA). First a short description of the NTR is presented and its longitudinal survey study on health, lifestyle and personality in which alcohol data were collected, followed by an overview of the actual measures on alcohol use that were studied for this thesis. Next, the NTR biobank study is introduced that provided the information on the liver enzyme levels. To give information on the comparability of the NTR biobank participants to the general NTR sample with regards to alcohol use, data are presented on alcohol use for those participating and not participating in the NTR biobank. After this, the NESDA study is introduced. Finally, the phenotypic relation between alcohol use and liver enzyme levels is shortly described.

1.1 The Netherlands Twin Register (NTR) survey studies on alcohol use

The NTR was established in 1987 to study genetic and environmental influences on traits related to health, lifestyle, personality, psychopathology, cognition, and development (Boomsma et al. 2002c). To this purpose, a longitudinal survey study on health, lifestyle and personality was started in 1991 in adolescent and adult twins and their family members (Adult NTR; ANTR), see Willemsen et al. 2013 for the most recent description). Surveys have been sent out every 2-4 years and at present, nine surveys have been conducted. In the first years, only twins and their parents were invited to participate, but later the invitation was extended to siblings, spouses and most recently children of twins. A separate NTR cohort was followed from birth (the Young NTR; YNTR, see Beijsterveldt et al. (2012) for a detailed description). Since 2009, these twins, once they reach the age of 18, and their family members, are also invited into the ANTR surveys. The studies presented in this thesis make use of the data on alcohol use that were collected between 1991 and 2012 as part of the first eight surveys. Nearly all individuals who participated in ANTR research at

least once, filled out questions on alcohol use (98.5% of individuals aged ≥15). At present, data on alcohol use are available for >31,000 participants of the ANTR (Table 1). Only a small percentage of these individuals was life-time abstainer (2.7% of men; 4.5% of women).

Table 1 Number of NTR and NESDA participants with data on alcohol use, liver enzyme levels and SNPs

			Alcohol data	**Liver enzyme data**	**SNP data** [a]
			N	N	N
NTR	twins	males	5,277	1,302	1,419
		females	9,232	2,497	2,930
	siblings	males	1,630	531	294
		females	2,582	1,334	620
	parents	males	4,439	1,170	840
		females	5,340	1,462	1,032
	spouses	males	1,302	224	171
		females	719	142	100
	other	males	157	9	4
		females	345	87	20
	total		31,023	8,758	7,430
NESDA [b]		males	736	744	744
		females	1,473	1,491	1,491
	total		2,209	2,235	2,235

[a] Numbers refer to individuals with genotyping data who also had data on alcohol use *or* liver enzyme levels. Genotyping of individuals is still ongoing. Note that these numbers differ from the number of participants in Chapter 8, as here in contrast to Chapter 8 related individuals are included.

[b] These numbers reflect a subgroup of all NESDA participants for whom quality controlled genotyping data and data on liver enzyme levels and alcohol use were available at the time Chapter 8 was written.

1.2 Measures of alcohol use

Data on alcohol use comprised questions on alcohol consumption (drinking quantity and drinking frequency), symptoms of alcohol abuse and dependence (AAD) as measured by the four CAGE items, age at onset of alcohol use (experimental alcohol use/regular alcohol use/getting drunk), physical reactions after alcohol use, and beverage preference. Table 2 provides an overview of the measures available for alcohol use, and the years in which they were collected.

Table 2 Overview of alcohol use measures [a] available through ANTR surveys

	Survey number (year of start data collection)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	1991	1993	1995	1997	2000	2002	2004-2008	2009-2012
Initiation	X	X	X	X	X	X	X	X
Frequency		X	X	X	X	X	X	X
Quantity		X	X	X	X	X	X	X
CAGE			X	X	X	X	X	X
AUDIT								X
Frequency of getting drunk		X	X		X			X
Frequency binge drinking [b]			X					X
Max. no. drinks in 24 hours in past year								X
Age at onset experimental use	X	X	X	X	X			X
Age at onset regular use		X	X	X	X			X
Age at onset getting drunk		X	X	X				X
Physical reactions after use		X	X					X
Beverage preference			X		X			X
Situation-specific urges to drink								X
Reasons not to drink at a regular basis								X

[a] Measures shown in italic are analyzed for this thesis.

[b] Defined as drinking ≥5 alcoholic beverages on one occasion.

Drinking frequency In surveys 2-7, drinking frequency was assessed by the question 'How often do you drink alcohol? Also count the times that you only drank a small amount (e.g. half a pint of beer or a few sips of wine)'. The response categories were 'I don't drink alcohol', 'once a year or less', 'a few times a year', 'about once a month', 'a few times a month', 'once a week', 'a few times a week' and 'every day'. In survey 8, individuals were asked to take the past year in mind with the question being phrased as 'How often did you drinking alcohol in this period?' Response categories were 'I don't drink alcohol', 'monthly or less', 'two to four times a month', 'two to three times a week', 'four to five times a week', 'six times a week or daily'. Response categories from surveys 2-7 and survey 8 were combined into: 'I don't drink alcohol or less than once a year' 'monthly or less', 'two to four times a month', 'two to five times a week', 'six times a week or daily'.

Drinking quantity Data were available from survey 2 (1993) onwards. In surveys 2-7, drinking quantity was surveyed by the question 'How many glasses a week do you drink on average (including the weekend)?'. There were seven response categories: 'less than 1 glass', '1-2 glasses a week', '3-5 glasses a week', '6-10 glasses a week', '11-20 glasses a week', '21-40 glasses a week' and 'more than 40 glasses a week'. In survey 8, individuals were asked to report for each day of the

week the number of glasses of beer, wine and spirits they drank in an average week. These numbers were summed and then categorized as in the earlier surveys.

Symptoms of AAD The CAGE, which indicates symptoms of alcohol abuse and dependence/ risk for problematic alcohol use (problem drinking liability), was included in all surveys from survey 3 (1995) onwards. The CAGE derives its name from the phrasing of its four questions (Ewing 1984): 'Have you ever felt you should **C**ut down on your drinking?', 'Have people **A**nnoyed you by criticizing your drinking?', 'Have you ever felt bad or **G**uilty about your drinking?', 'Have you **E**ver had a drink first thing in the morning to steady your nerves or to get rid of a hangover?'. In survey 8, CAGE items could be answered with 'yes, not in the past year', 'yes, in the past year', and 'no'. In other years, each item could be answered with 'yes' or 'no'. The frequency of the two 'yes categories' in survey 8 combined, was comparable to the one 'yes category' of earlier years, and therefore combined into one 'yes' category.

Age at onset experimental alcohol use In surveys 2-5, age at onset of experimental alcohol use was measured with the question 'If you have ever used any of the substances listed below, please indicate the age at which you used them for the first time' followed by the item 'Tried an alcoholic drink'. In surveys 2-4 there were eight response categories: '11 or younger', '12', '13', '14', '15', '16', '17', and '18 or older'. In survey 5 response categories were brought back to five: '11 or younger', '12-13', '14-15', '16-17' and '18 or older'. In surveys 1 and 8, the question was phrased as an open-ended question, of which the answers were brought back to the categories described above if information needed to be combined over different surveys. In survey 1, the question was phrased as 'At what age did you start using alcohol?'. In survey 8 the question was 'At what age did you try alcohol for the first time?'.

Age at onset regular alcohol use Age at onset of regular alcohol use was determined in surveys 2-5 (1993-2000) in a similar way as described for experimental alcohol use with the question being followed by 'regularly drank alcohol'. Response categories were the same as described for experimental alcohol use. In survey 8, age at onset of regular alcohol use was determined by an open ended question 'At what age did you start using alcohol regularly?' for which answers could be brought back to the categories in surveys 2-5, if age at onset was to be combined over surveys.

Age at onset getting drunk In survey 2-4, information on age at onset of getting drunk was obtained in the same manner as described for experimental alcohol use with the item now being followed by 'gotten drunk or tipsy'. Again, the same age categories apply. In survey 8, age at onset of getting drunk was measured by the open-ended question 'At what age did you get drunk or very

tipsy for the first time?' For survey 8, age reported by the participants could be categorized as in surveys 2-4, if necessary.

Physical reactions to alcohol Whether persons experienced physical reactions after alcohol use was determined in surveys 2, 3 and 8 with the question 'Do you experience unpleasant physical reactions, like flushing of face or body, itching, sleepiness or heart beating after drinking one or two glasses of alcohol?' with response categories being 'always', 'sometimes' and 'never'.

Beverage preference Beverage preference was assessed with the question 'What do you drink preferably?' In surveys 3 and 5, response categories were 'wine', 'beer' and 'spirits (gin, whisky, liquor etc.)' with the question followed by 'you can indicate one preference only'. In survey 8, the question was not followed by the addition that only one preference could be given. Response categories were 'wine', 'beer', 'spirits (e.g. gin, mix drinks, cocktail)', and a fourth option 'no preference'.

1.3 Blood collection and liver enzyme levels

In the period 2004-2009, 9,530 adults registered with the NTR participated in a large blood collection study. As part of this study, adult NTR participants were visited at home, or when preferred at work, to provide blood and urine samples. Sampling took place in the morning (between 7.00 and 10.00 a.m.). Participants had been asked to fast from 10 p.m. the night before, to refrain from smoking one hour before the home visit, and, at the day of the home visit, to abstain from physical exertion and medication if possible. Compliance was checked in a brief interview, that also obtained information on health and smoking status. In addition, height, weight and hip and waist circumference were determined (for a detailed description of the NTR biobank and the blood collection procedures, see Willemsen et al. 2010).

The liver enzyme levels were determined in heparin plasma. At the time of blood sampling, blood was collected in two heparin plasma tubes that were turned 8-10 times immediately after collection to prevent clotting and stored in melting ice during transport. Processing of the tubes took place within six hours (average transport time 196 minutes) at one central laboratory in Leiden. There was no association between transportation time and hemolysis. In the central laboratory, plasma was collected and six samples of 500 µL were snap-frozen and stored at -30 °C. These aliquots were used to determine the level of gamma-glutamyl transferase (GGT), alanine (ALT) and aspartate aminotransferase (AST) in units per liter (U/L) by Vitros assays (Vitros 250, Ortho-Clinical diagnostics) (Johnson & Johnson, Rochester, USA). The reference intervals were as

follows: <50 U/L (males) and <35 U/L (females) for GGT; <45 U/L (males) and <40 U/L (females) for ALT; <40 U/L (both sexes) for AST.

For this thesis, SNP data were analyzed that were genotyped on five platforms: Affymetrix 6.0, Affymetrix 5.0-Perlegen (Affymetrix, Santa Clara California), Illumina 660, Illumina Omni Express 1M and Illumina 370 (Illumina, San Diego, California). In total, liver enzyme levels were determined for 8,758 participants and genotypic information was available for 5,834 persons (see Table 1).

1.4 Alcohol use and liver enzyme levels within the Netherlands Study on Depression and Anxiety

Chapter 8 of this thesis also presented data on alcohol use, liver enzyme levels and SNP data from individuals who participated in the NESDA study (N=2,027). The NESDA study is a longitudinal cohort study on the course and consequences of anxiety and depressive disorders and includes 2,981 individuals, who currently or in the past, suffered from depression or anxiety disorders, or participated as healthy control. At the baseline assessment, information was obtained on mental health outcomes and psychosocial, clinical, biological and genetic determinants through questionnaires, psychiatric interviews and blood samples. During the baseline assessment, data on alcohol use were obtained by questionnaires (AUDIT questionnaire to measure alcohol consumption and severity of alcohol problems) as well as by psychiatric interviews (DSM-IV diagnosis of alcohol use disorders). For the study presented in Chapter 8, questionnaire data on drinking quantity were analyzed and data on liver enzyme levels (GGT, ALT and AST).

To obtain a measure on drinking quantity that was comparable to that in the NTR, the first two questions of the AUDIT questionnaire were combined. AUDIT item 1 assessed drinking frequency: 'How often do you have a drink containing alcohol?' with response categories 'never', 'monthly or less', 'two to four times a month', 'two to three times a week', 'four or more times a week'. AUDIT item 2 assessed drinking quantity at a drinking day: 'How many drinks containing alcohol do you have on a typical day when you are drinking? with response categories '1 or 2', '3 or 4', '5 or 6', '7, 8 or 9', '10 or more'. To calculate alcohol intake levels, (each category of) drinking frequency was expressed as the number of drinking days per year and (each category of) drinking quantity as the midpoint number of glasses consumed at a drinking day. The last category of drinking quantity was given the midpoint value of 11 based on the median number of drinks per day, reported by NTR participants who drank more than 10 glasses per day. If individuals reported to 'never' drink alcohol on the first AUDIT item, they were assigned an alcohol intake level of 0

glasses alcohol per year. Drinking quantity per week could then be calculated by dividing the number of drinks per year by 52.

Blood samples collected at the baseline assessment were used to determine liver enzyme levels and to obtain genotypic information (Penninx et al. 2008). Before the start of the blood sample collection, NTR and NESDA protocols for processing and storage of blood samples were largely harmonized (Boomsma et al. 2008). For NESDA, blood sampling took place at seven field sites that were within walking distance of a laboratory between 8.30 and 9.30 a.m. after an overnight fast. Participants did not receive further instructions regarding smoking or strenuous exercise. At the laboratory, blood samples were processed within an hour (Boomsma et al. 2008; Penninx et al. 2008), including determination of GGT, ALT and AST levels in U/L with Roche Diagnostic assays (Chambers et al. 2011).

The total number of NESDA participants with data on alcohol use, liver enzyme levels and SNPs is given in Table 1.

1.5 Alcohol use NTR biobank sample versus general NTR sample

Tables 3 and 4 give for each drinking category, the number of NTR biobank participants with liver enzyme data and the number of NTR participants who did not participate in the biobank study and therefore did not provide data on liver enzyme levels. To test whether individuals who participated in the NTR biobank sample were a selected sample, drinking patterns were compared for a random set of unrelated individuals who differed in whether or not they participated in the NTR biobank study. Not-current drinkers were included in these comparisons and were assigned a drinking quantity of <1 glas per week. For males, drinking patterns were similar for those who participated in the biobank study and those who did not (drinking frequency $\chi^2(4)=12.14$, $p=.02$; drinking quantity $\chi^2(4)=6.75$, $p=.15$). Females who participated in the biobank study drank somewhat more frequent ($\chi^2(4)=37.31$, $p<.001$), but consumed less at a time ($\chi^2(4)=47.84$, $p<.001$) than females who did not participate in the biobank study, though differences were minor. Among female participants of the biobank study, 16.2% drank ≥4 times per week and 69.0% less ≤5 glasses per week. For females who did not participate in the biobank study, this was 12.9% and 62.9% respectively.

1.6 Alcohol use NTR biobank sample versus NESDA sample

Tables 3-6 show mean liver enzyme levels stratified by levels of drinking frequency, quantity and risk for problematic alcohol use (as indicated by the CAGE and AUDIT), for NTR and

NESDA. For NESDA, 39.3% of male participants drank ≥4 times a week versus 26.5% for NTR (27.4% vs. 26.5% for females respectively). Among NESDA participants, 13.3% of males drank ≥21 glasses per week and 3.4% of females. Among NTR, this was 9.0% and 2.1% respectively. Risk for problematic alcohol use as measured with the AUDIT was estimated to be present for 34.9% and 17.4% of male and female NESDA participants respectively, and for 10.6% and 4.4% of male and female NTR participants. In Tables 3-6, mean levels of BMI did not differ over drinking categories. Thus the level of alcohol use is suggested not to be dependent on BMI.

1.7 Relation alcohol use and liver enzyme levels

With regression analyses it was estimated what amount of variance in liver enzyme levels could be attributed to different measures of alcohol use. Regression analyses were performed including age as fixed effect, separately over source (NTR vs. NESDA) and sex. For the NTR sample, only unrelated individuals were included in the analyses. Measures of alcohol use explained a significant amount of variance in GGT, but not in ALT. For AST, results were mixed. The CAGE and AUDIT, but not drinking quantity, explained a significant amount of variance among male NTR participants, whereas drinking quantity, but not the AUDIT, explained variance among female NTR and male NESDA participants. For drinking quantity and the AUDIT, the amount of explained variance was highest. Around 2-4% of the variance in GGT could be explained by these measures of alcohol use (see Table 7).

Table 3 Number of male NTR participants with and without liver enzyme data and distribution of liver enzyme levels [a] over alcohol use categories

			data liver enzymes							
			yes						**no**	
data	alcohol use [bc]		N (%) [d]	GGT	ALT	AST	BMI	age	N [d]	age
yes	abstainers		18 (0.7%)	31.8 (13.9)	11.6 (4.5)	23.3 (5.7)	24.5 (2.2)	47.5 (13.5)	108 (1.6%)	35.5 (15.1)
	not-current drinkers		80 (3.1%)	34.1 (18.8)	11.2 (4.2)	23.2 (7.0)	26.4 (5.2)	51.8 (17.1)	224 (3.2%)	43.2 (16.6)
	current drinkers		2,472 (96.1%)	40.2 (41.7)	12.0 (7.0)	23.5 (9.3)	25.6 (3.6)	46.6 (15.2)	6,568 (94.9%)	39.3 (15.5)
		frequency <=1x p/month	250 (10.1%)	36.8 (30.8)	12.8 (8.5)	23.4 (8.3)	25.9 (4.3)	45.1 (14.4)	750 (11.4%)	36.7 (15.1)
		2-4x p/month	558 (22.6%)	34.3 (25.6)	12.6 (7.8)	23.1 (7.9)	25.5 (3.8)	42.8 (14.9)	1,733 (26.4%)	34.9 (14.4)
		2-3x p/wk	990 (40.0%)	37.9 (37.7)	11.9 (6.4)	23.4 (10.3)	25.4 (3.4)	44.0 (14.8)	2,522 (38.4%)	37.8 (14.9)
		>=4 p/wk	654 (26.5%)	50.0 (57.7)	11.5 (6.4)	24.1 (9.1)	25.7 (3.3)	54.6 (13.3)	1,509 (23.0%)	48.3 (14.2)
		missing	20 (0.8%)						54 (0.8%)	
		quantity <1 gl p/wk	299 (12.1%)	35.7 (29.9)	12.5 (8.3)	22.9 (6.6)	26.2 (4.5)	45.2 (14.2)	667 (10.2%)	36.7 (14.7)
		1-5 gl p/wk	773 (31.3%)	34.0 (21.0)	12.2 (6.9)	23.5 (11.4)	25.4 (3.6)	46.1 (15.4)	1,931 (29.4%)	39.5 (15.0)
		6-10 gl p/wk	552 (22.3%)	37.9 (43.1)	11.8 (6.8)	23.2 (8.3)	25.4 (3.3)	46.3 (15.4)	1,448 (22.0%)	40.8 (15.1)
		11-20 gl p/wk	570 (23.1%)	46.5 (54.2)	11.6 (6.5)	23.4 (7.2)	25.6 (3.3)	48.0 (15.2)	1,469 (22.4%)	40.0 (15.9)
		>=21 gl p/wk	222 (9.0%)	57.9 (61.3)	12.2 (6.3)	25.0 (10.0)	25.8 (3.3)	48.3 (15.1)	628 (9.6%)	39.5 (16.5)
		missing	56 (2.3%)						425 (6.5%)	
		CAGE 0	1678 (67.9%)	37.8 (39.0)	12.0 (7.0)	23.2 (7.9)	25.7 (3.6)	47.2 (15.1)	4,311 (65.6%)	39.6 (15.5)
		1	427 (17.3%)	41.2 (40.1)	12.0 (6.9)	23.2 (7.2)	25.2 (3.4)	44.6 (15.0)	1,239 (18.9%)	39.0 (15.5)
		2	221 (8.9%)	48.6 (50.1)	12.5 (7.4)	25.7 (17.1)	25.2 (3.6)	44.4 (15.8)	596 (9.1%)	39.5 (15.5)
		>=3	139 (5.6%)	53.1 (57.3)	11.7 (6.5)	25.5 (12.2)	25.5 (4.0)	47.8 (15.0)	380 (5.8%)	36.7 (14.6)
		missing	7 (0.3%)						42 (0.6%)	
		AUDIT <8	1295 (52.4%)	38.6 (36.3)	11.7 (6.7)	23.1 (8.3)	25.6 (3.5)	47.7 (14.8)	3,159 (48.1%)	40.0 (15.6)
		≥8	263 (10.6%)	48.6 (49.1)	12.2 (6.5)	25.5 (15.1)	25.5 (3.3)	45.0 (14.5)	793 (12.1%)	34.5 (15.5)
		missing	914 (37.0%)						2,616 (39.8%)	
	missing [e]		3 (0.1%)						18 (0.3%)	
no			659	39.1 (32.3)	12.2 (6.3)	24.1 (7.3)	25.7 (3.7)	46.1 (16.6)	0	
total			3,232						6,918	

Table 4 Number of female NTR participants with and without liver enzyme data and distribution of liver enzyme levels [a] over alcohol use categories

			data liver enzymes yes						**no**	
data	alcohol use [bc]		N (%) [d]	GGT	ALT	AST	BMI	age	N (%) [d]	age
yes	abstainers		103 (2.2%)	25.4 (16.1)	8.8 (4.1)	19.8 (5.7)	27.0 (5.7)	53.0 (16.2)	317 (3.0%)	39.7 (18.1)
	not-current drinkers		338 (7.3%)	29.1 (54.1)	9.5 (6.2)	20.3 (9.3)	25.4 (4.9)	46.1 (13.8)	692 (6.6%)	38.1 (15.1)
	current drinkers		4,179 (90.3%)	26.6 (26.9)	9.2 (5.3)	19.9 (6.7)	24.9 (4.4)	44.1 (13.6)	9,425 (90.1%)	36.3 (14.8)
		frequency <=1x p/month	1,191 (28.5%)	25.2 (22.1)	9.2 (5.2)	19.3 (5.4)	25.7 (4.9)	41.6 (13.0)	2,610 (27.7%)	34.0 (13.9)
		2-4x p/month	1,164 (27.9%)	23.6 (13.8)	9.0 (4.8)	19.4 (5.9)	24.6 (4.4)	40.5 (13.2)	3,174 (33.7%)	32.4 (13.8)
		2-3x p/wk	1,089 (26.1%)	25.5 (18.5)	9.0 (5.3)	20.0 (6.3)	24.5 (3.9)	45.2 (13.3)	2,315 (24.6%)	37.5 (14.6)
		>=4 p/wk	675 (16.2%)	35.7 (51.2)	10.0 (6.1)	21.6 (9.5)	24.8 (3.7)	52.8 (11.5)	1,218 (12.9%)	49.0 (11.6)
		missing	60 (1.4%)						108 (1.1%)	
		quantity <1 gl p/wk	1,334 (31.9%)	24.8 (21.0)	9.1 (5.1)	19.3 (5.6)	25.4 (4.9)	41.9 (13.2)	2,230 (23.7%)	35.3 (13.4)
		1-5 gl p/wk	1,549 (37.1%)	24.7 (16.8)	9.2 (5.5)	19.8 (6.1)	24.7 (4.3)	43.0 (13.5)	3,694 (39.2%)	35.8 (14.7)
		6-10 gl p/wk	665 (15.9%)	25.8 (17.3)	9.3 (4.9)	20.2 (6.8)	24.5 (3.8)	46.1 (13.5)	1,614 (17.1%)	37.5 (15.2)
		11-20 gl p/wk	471 (11.3%)	32.9 (38.5)	9.4 (4.9)	20.8 (7.9)	24.6 (3.9)	49.3 (13.3)	1,002 (10.6%)	39.9 (15.8)
		>=21 gl p/wk	86 (2.1%)	63.4 (105.8)	11.0 (8.3)	25.1 (14.8)	25.3 (3.4)	50.5 (10.6)	209 (2.2%)	39.3 (16.4)
		missing	74 (1.8%)						676 (7.2%)	
		CAGE 0	3,330 (79.7%)	25.7 (24.7)	9.2 (5.2)	19.8 (6.5)	25.0 (4.4)	43.8 (13.7)	7,524 (79.8%)	36.1 (14.7)
		1	472 (11.3%)	27.5 (27.2)	9.5 (5.4)	20.3 (6.6)	24.6 (3.9)	44.4 (13.4)	1,084 (11.5%)	36.0 (14.9)
		2	243 (5.8%)	31.1 (37.7)	9.4 (5.9)	20.6 (8.8)	24.5 (4.1)	46.2 (12.2)	534 (5.7%)	38.6 (14.9)
		>=3	94 (2.2%)	40.3 (55.6)	9.9 (6.4)	21.4 (7.4)	25.0 (5.1)	42.9 (13.5)	208 (2.2%)	38.0 (14.4)
		missing	40 (1.0%)						75 (0.8%)	
		AUDIT <8	2,634 (63.0%)	26.0 (26.7)	9.1 (5.0)	19.8 (6.3)	24.9 (4.3)	43.9 (13.3)	5,640 (59.8%)	35.6 (14.8)
		≥8	185 (4.4%)	32.7 (33.1)	9.8 (5.9)	20.9 (7.5)	24.1 (3.9)	42.9 (12.8)	593 (6.3%)	31.4 (14.6)
		missing	1,360 (32.5%)						3,192 (33.9%)	
	Missing [e]		6 (0.1%)						26 (0.2%)	
no			889	28.8 (27.3)	9.5 (5.8)	20.2 (6.7)	25.8 (5.0)	47.9 (15.8)	0	
total			5,515						10,460	

Footnotes table 3 and 4

[a] Liver enzyme levels are untransformed

[b] Individuals are included in the overview if they were aged ≥ 18 at the time of the data collection (in contrast to Table 2 that includes individuals aged ≥ 15)

[c] data on alcohol use defined as data collected in surveys 6-8 (2002-2012)

[d] Percentage of individuals who are abstainer, not-current drinker, current drinker or drinker with missing information on alcohol use calculated as proportion of all individuals with data on alcohol use (N=2,573 and N=6,918 for table 3; N=4,626 and N=10,460 for table 4). Percentage of individuals in drinking categories calculated as proportion of number of current drinkers.

[e] Information on ever alcohol use (yes) is present, but not on current drinking measures (drinking frequency, quantity, CAGE, AUDIT)

Table 5 Distribution of liver enzyme levels [a] over alcohol use categories for male NESDA participants

			data liver enzymes yes					
data alcohol use [b]			N (%) [c]	GGT	ALT	AST	BMI	age
yes	not-current drinkers		103 (11.3%)	31.6 (25.7)	32.2 (18.5)	28.9 (12.5)	27.3 (4.7)	44.9 (11.2)
	current drinkers		807 (88.7%)	35.6 (35.6)	30.6 (18.3)	29.4 (11.4)	26.0 (4.5)	43.7 (13.0)
		frequency <=1x p/month	96 (11.9%)	35.3 (34.4)	33.5 (25.2)	29.1 (12.3)	26.5 (5.6)	43.0 (12.6)
		2-4x p/month	155 (19.2%)	31.2 (32.6)	32.3 (20.4)	29.2 (10.8)	26.2 (5.1)	39.3 (12.6)
		2-3x p/wk	239 (29.6%)	31.3 (31.4)	27.6 (14.3)	27.6 (10.0)	25.6 (4.1)	41.2 (13.6)
		>=4 p/wk	317 (39.3%)	41.2 (39.6)	31.2 (17.3)	30.9 (12.2)	26.1 (4.0)	47.8 (11.6)
		quantity <1 gl p/wk	82 (10.2%)	35.7 (35.9)	30.9 (15.7)	27.9 (8.1)	26.5 (5.9)	43.0 (12.6)
		1-5 gl p/wk	243 (30.1%)	30.5 (30.7)	31.7 (21.9)	29.1 (12.4)	26.1 (4.4)	41.6 (12.9)
		6-10 gl p/wk	207 (25.7%)	29.3 (23.7)	27.3 (13.9)	28.0 (8.8)	25.6 (4.2)	43.6 (12.9)
		11-20 gl p/wk	166 (20.6%)	39.5 (42.9)	29.4 (16.5)	29.5 (10.9)	25.7 (3.9)	45.8 (13.1)
		>=21 gl p/wk	107 (13.3%)	53.9 (45.7)	35.4 (19.2)	33.2 (14.8)	26.8 (4.6)	45.4 (13.3)
		missing	2 (0.2%)					
		AUDIT <8	525 (65.1%)	31.8 (31.6)	30.4 (18.8)	29.0 (10.8)	26.1 (4.5)	44.3 (12.9)
		≥8	282 (34.9%)	42.9 (41.4)	31.0 (17.4)	30.2 (12.3)	26.0 (4.4)	42.5 (13.2)
no			9					
total			919					

[a] Liver enzyme levels are untransformed.

[b] Individuals are included in the overview if they were aged ≥ 18 at the time of the data collection. Data on alcohol use defined as having data on AUDIT item 1.

[c] Percentage of individuals who are not-current or current drinker calculated as proportion of all individuals with data on alcohol use (N=910). Percentage of individuals in drinking categories calculated as proportion of number of current drinkers.

Table 6 Distribution of liver enzyme levels [a] over alcohol use categories for female NESDA participants

			data liver enzymes yes					
data alcohol use [b]			N (%) [c]	GGT	ALT	AST	BMI	age
yes	non-current drinkers		322 (18.1%)	19.5 (14.1)	19.5 (10.5)	24.0 (8.6)	26.6 (6.2)	42.4 (13.3)
	current drinkers		1,461 (81.9%)	20.6 (25.5)	19.7 (12.6)	23.4 (8.4)	25.0 (4.9)	40.7 (13.1)
		frequency <=1x p/month	325 (22.2%)	19.0 (17.1)	20.7 (18.4)	23.7 (8.0)	26.3 (6.0)	38.0 (12.9)
		2-4x p/month	372 (25.5%)	18.0 (15.8)	18.6 (10.0)	22.9 (8.1)	24.7 (4.7)	36.1 (12.9)
		2-3x p/wk	363 (24.8%)	18.4 (15.2)	18.7 (9.1)	22.6 (7.3)	24.6 (4.6)	39.3 (12.5)
		>=4 p/wk	401 (27.4%)	26.4 (40.4)	20.6 (11.4)	24.9 (9.8)	24.5 (3.9)	48.5 (10.4)
		quantity <1 gl p/wk	295 (20.2%)	19.0 (17.6)	20.9 (19.2)	23.8 (8.2)	26.2 (6.0)	38.2 (13.1)
		1-5 gl p/wk	573 (39.2%)	17.6 (14.9)	18.8 (9.5)	22.9 (7.6)	24.9 (4.9)	38.1 (12.8)
		6-10 gl p/wk	340 (23.3%)	20.0 (19.6)	19.2 (10.0)	22.9 (7.8)	24.2 (3.7)	44.2 (12.5)
		11-20 gl p/wk	198 (13.6%)	26.9 (45.0)	20.1 (9.3)	24.2 (8.3)	24.7 (4.1)	44.7 (12.7)
		>=21 gl p/wk	50 (3.4%)	42.9 (57.0)	24.1 (18.5)	28.9 (16.9)	25.0 (5.4)	45.8 (12.3)
		missing	5 (0.3%)					
		AUDIT <8	1,204 (82.4%)	19.1 (17.6)	19.6 (12.7)	23.3 (8.0)	25.1 (5.0)	41.0 (13.2)
		≥8	254 (17.4%)	27.9 (46.9)	19.8 (11.7)	24.0 (10.0)	24.3 (4.1)	39.4 (12.8)
		missing	3 (0.2%)					
no			16					
total			1,799					

[a] Liver enzyme levels are untransformed.

[b] Individuals are included in the overview if they were aged ≥ 18 at the time of the data collection. Data on alcohol use defined as having data on AUDIT item 1.

[c] Percentage of individuals who are not-current or current drinkers calculated as proportion of all individuals with data on alcohol use (N=1,783). Percentage of individuals in drinking categories calculated as proportion of number of current drinkers.

Table 7 Variance of liver enzyme levels [a] explained by measures of alcohol use

		NTR		NESDA	
		males	**females**	**males**	**females**
GGT	frequency	1.6%	n.s.	n.s.	n.s.
	quantity [b]	3.5%	1.0%	3.1%	1.6%
	CAGE	1.9%	0.8%	--	--
	AUDIT	2.8%	1.2%	4.0%	1.7%
ALT	frequency	n.s.	n.s.	n.s.	n.s.
	quantity	n.s.	n.s.	n.s.	n.s.
	CAGE	n.s.	n.s.	--	--
	AUDIT	n.s.	n.s.	n.s.	n.s.
AST	frequency	n.s.	n.s.	n.s.	n.s.
	quantity	n.s.	0.4%	1.1%	n.s.
	CAGE	0.8%	n.s.	--	--
	AUDIT	1.6%	n.s.	n.s.	n.s.

n.s.= not significant (at α=.01)

[a] Liver enzyme levels are *log*-transformed.

[b] Analyses in Chapter 3, 6 and 8 are based on drinking quantity expressed in the amount of alcohol consumed in grams per day. Explained variance for GGT by alcohol intake in grams per day was 4.0%, 1.7%, 5.0% and 2.4% for male NTR participants, female NTR participants, male NESDA participants and female NESDA participants respectively.

2. Methods to study genetic effects

To study the genetics of alcohol use and liver enzyme levels two sets of methods were used. The first set of methods estimated the heritability of traits based on the known genetic relatedness between monozygotic (MZ) and dizygotic (DZ) twin pairs and their family members (registered with the NTR). The second set of methods used genetic marker data (single nucleotide polymorphisms, SNPs) that were available for a subset of NTR participants as well as for NESDA participants. SNP data were used to estimate the SNP-based heritability as well as to perform gene finding studies. The basis of all these methods is given by the biometric model, the cornerstone of quantitative genetics. The discussion of the different methods is therefore preceded by a short introduction to the biometric model.

2.1 Biometric model

Quantitative genetics is concerned with the polygenic inheritance of quantitative differences between individuals (Falconer 1996), in contrast to Mendelian genetics that describes the inheritance of a dichotomous trait by a single gene (Kwan et al. 2007). Quantitative genetics (and its biometric model) lies at the heart of methods to estimate heritability as well as gene finding.

The biometric model describes how genetic variation is related to phenotypic variation. Genetic variation affects the mean phenotypic level in a population. In the simplified case of a

trait that is influenced by one gene with two alleles (A_1, A_2 with allele frequencies p and q respectively, where $p+q=1$), there are three possible genotypes, A_1A_1, A_1A_2, A_2A_2, that occur with the following frequencies: p^2, $2pq$, and q^2 (under the assumption of Hardy Weinberg equilibrium, HWE). Having assigned these genotypes genotypic values of $+a$, d and $-a$ respectively, the mean phenotypic level in the population can be expressed as the sum of the product of genotype frequencies and genotypic values (Falconer 1996):

$$\mu = a(p-q) + 2dpq \tag{1}$$

The phenotypic variance in a population can be expressed as the sum of variation due to genetic effects (G) and variation due to environmental effects (E) (Fisher 1918). If the genetic variance ($var(G)$) and environmental variance ($var(E)$) are assumed to be independent, the covariance between G and E, $2cov(G,E)$, is zero. The phenotypic variance can then be formulized as follows (Falconer 1996):

$$\mathrm{var}(P) = \mathrm{var}(G) + \mathrm{var}(E) \tag{2}$$

The genetic variance can be obtained by taking the sum of the difference between the genotypic values and the population mean multiplied by their respective genotypic frequencies. Starting of with a one gene (two-allele) model, the genetic variance can be separated into variance due to additive genetic effects (average allelic effects or breeding values; $var(A)$) and variance due to dominance genetic effects (interaction between two alleles at one locus; $var(D)$). Then the genetic variance can be given as (Falconer 1996):

$$\begin{aligned} \mathrm{var}(G) &= p^2(a-\mu)^2 + 2pq(d-\mu)^2 + q^2(-a-\mu)^2 \\ &= 2pq(a+d(q-p))^2 + (2pqd)^2 \\ &= \mathrm{var}(A) + \mathrm{var}(D) \end{aligned} \tag{3}$$

The ratio of $var(G)$ to the total phenotypic variance ($var(P)$) expresses the broad-sense heritability of a trait (total h^2). The ratio of $var(A)$ to the total phenotypic variance, is called the narrow-sense heritability (h^2) (Falconer 1996). To obtain estimates on the amount of variance that can be ascribed to genetic effects, data from genetically informative individuals can be modeled (e.g. data from twins), or genetic variants can be analyzed (e.g. SNP data). These methods will be described below.

2.2 Methods to estimate the heritability based on the inferred genetic relationship among family members

Phenotypic values can be correlated among relatives to obtain estimates on the relative influence of genetic and environmental influences on these traits. The classic approach to study the relative influence of genetic and environmental variation underlying a trait is to study the phenotypic similarity of MZ twins reared apart (twin-adoption design) or to compare phenotypic similarity among MZ twins with that of DZ twins (classical twin design; CTD) (Boomsma et al. 2002a). In this thesis, different variations/ extensions of the CTD are applied which are described below. In Chapter 3, two variants of the CTD were applied, a multivariate CTD and a longitudinal simplex model. In Chapter 4, 5 and 6, data were modeled with the extended twin-family design (an extension of the CTD), which allows for the inclusion of data from family members other than twins. Variances due to genetic and environmental effects according to these models were estimated with classic Mx (Neale et al. 2006) and OpenMx (Boker et al. 2011), two statistical programs for structural equation modeling (SEM).

2.2.1 The classical twin design

The relative contribution of genetic and environmental effects can be estimated by comparing the phenotypic similarity between the members of MZ twin pairs versus that of DZ twin pairs, under a number of assumptions (Jinks and Fulker 1970).

The cornerstone of the CTD is the equal environment assumption (EEA), that postulates that the difference between the similarity between DZ twins as compared to MZ twins can be ascribed to effects of genes alone, not to differences in environmental effects. It is thus assumed that the amount of environmental variance that is shared between twins is equal for MZ and DZ twins (Jinks and Fulker 1970). Research on the validity of the EEA shows that for some traits, MZ twins are more similar in their environmental experiences than DZ twins (Felson 2009). With regard to alcohol use, research on whether contact and/or closeness between twins predicts similarities in alcohol use is mixed. Whereas cohabitation, social contact, and overlap in friends were related to similarity in alcohol consumption (Hettema et al. 1999; Horwitz et al. 2003; Rose et al. 1990), similarity in childhood treatment, co-socialization, and perceived similarity did not predict alcohol dependence concordance (Kendler and Gardner 1998). Importantly, increased environmental similarity in itself does not necessarily invalidate the EEA. From the concept of gene-environment correlation it is expected to find that MZ twins who share all their genes will be more similar in environmental exposures elicited by their genotype and thus in closeness and contact, than DZ twins who share 50% of the genetic material on average. The right question to ask is then whether twins experience more similar environments because they are treated as identical, instead of whether they elicit similar environments based

on their genotype (Eaves et al. 1978). From this perspective it is interesting to examine whether perceived zygosity has an additional effect on trait similarity when genetic similarity is taken into account (as captured by the 'true' zygosity) (Scarr 1968), given there is a substantial proportion of twins who is misinformed about their zygosity. DZ twins who are perceived by themselves and their direct environment as being MZ (e.g. by their parents) may behave more similarly and may be expected to be more alike, which may increase their trait similarity. In contrast, MZ pairs incorrectly labeled as DZ pairs may be less similar for the trait of interest than MZ pairs who were labeled correctly. Perceived zygosity had little impact on twin similarity for alcohol dependence (Xian et al. 2000; Kendler et al. 1993). Neither was there an effect of physical similarity on concordance for alcoholism (Hettema et al. 1995), supporting the notion that MZ twins are not treated more similarly because they are identical.

Predictions following from the invalidation of the EEA hypothesis (Lykken et al. 1990), are supported to some degree however, which suggests that the EEA does not entirely hold true. First, it is predicted that if greater frequency of contact leads to more similarity, individuals, including spouses, become more similar to each other the longer they are together (in this thesis also called 'cohabition effects'). Whereas spousal resemblance for alcohol use is partly due to partner choice guided by one's own phenotype (and hence by one's own genotype, because $var(P)=var(G)+var(E)$, a process called phenotypic assortment), additional cohabitation effects have been observed (that is, after taking effects of phenotypic assortment into account) (Grant et al. 2007) especially during the first years of the relationship (Leonard and Eiden 2007; Ask et al. 2012). Second, it is predicted that MZ twins reared apart, who did not have contact with each other, would be less similar than MZ twins reared together. Pedersen et al. (1992) found that for MZ twins who differed in the degree to which they were reared apart, greater similarity in rearing environment, predicted greater similarity in total alcohol consumption, although effects accounted for only 2% of the variation. Thus whereas there is some evidence for additional environmental similarity among MZ twins pairs compared to DZ pairs, on the whole, the validity of the EEA is supported and overall the influence of any additional environmental similarity among MZ twins on heritability estimates is likely to be small.

According to the CTD, MZ twins are assumed to share all their genetic material with their co-twin (all additive and dominance genetic variation). DZ twin pairs are assumed to share about half of their segregating genetic material, that is, they are assumed to share 50% of the additive genetic variation (having 25% chance to inherit none of the parents alleles in common, 50% to inherit one allele in common and 25% to inherit two alleles in common at a specific locus). Given that sharing variation due to genetic dominance requires that both alleles at a locus are shared, resultantly, DZ twins are assumed to share 25% of the dominance variation.

Both types of twins are assumed to share some part of their environmental effects, and this part is called shared environmental effects (*C*). The environmental effects that twins do not share with their co-twin are called non-shared, unique or individual-specific environmental effects (*E*). This also includes measurement error of the trait. Incorporating variation due to these effects, equation (2) is then extended to (Falconer 1996):

$$\text{var}(P) = \text{var}(A) + \text{var}(D) + \text{var}(C) + \text{var}(E) \quad (4)$$

Given that the *A*, *D*, *C*, and *E* factor means are fixed at zero, for univariate phenotypes, the phenotypic value p_j for individual *j* can be described as a linear function of the sum of the regression weight (β_a, β_d, β_c, β_e) times the respective factor score (a_j, d_j, c_j, e_j) denoting the additive genetic, genetic dominance values, shared environmental and non-shared environmental values for that individual respectively (Martin and Eaves 1977):

$$p_j = \beta_o o_j + \ldots + \beta_a a_j + \beta_d d_j + \beta_c c_j + \beta_e e_j \quad (5)$$

The first term $\beta_o * o_j$ +.. indicates that for subject *j* any fixed effects o_j with regression weight β_o can be included in the equation (e.g. age). For here, it is assumed that the factor variances are fixed at 1 for identification purposes. An example of this model is given in Figure 1a.

The genetic influence on a trait (*var(G)*), can be estimated from the degree to which MZ twins are more alike than DZ twins, whereas non-shared environmental effects (*var(E)*) are estimated from the degree to which MZ twins are dissimilar to each other. An estimate of the variance due to shared environmental effects (*var(C)*) is obtained from the notion that twins share 100% of the variation due to shared environmental influences with their co-twin (by definition), but not with other twins. Covariances for MZ (*cov(MZ)*) and DZ twin pairs (*cov(DZ)*) can then be formulized as follows:

$$\begin{aligned} \text{cov}(MZ) &= \text{var}(P) - \text{var}(E) \\ &= \text{var}(A) + \text{var}(D) + \text{var}(C) \end{aligned} \quad (6)$$

$$\text{cov}(DZ) = 0.5\,\text{var}(A) + 0.25\,\text{var}(D) + \text{var}(C) \quad (7)$$

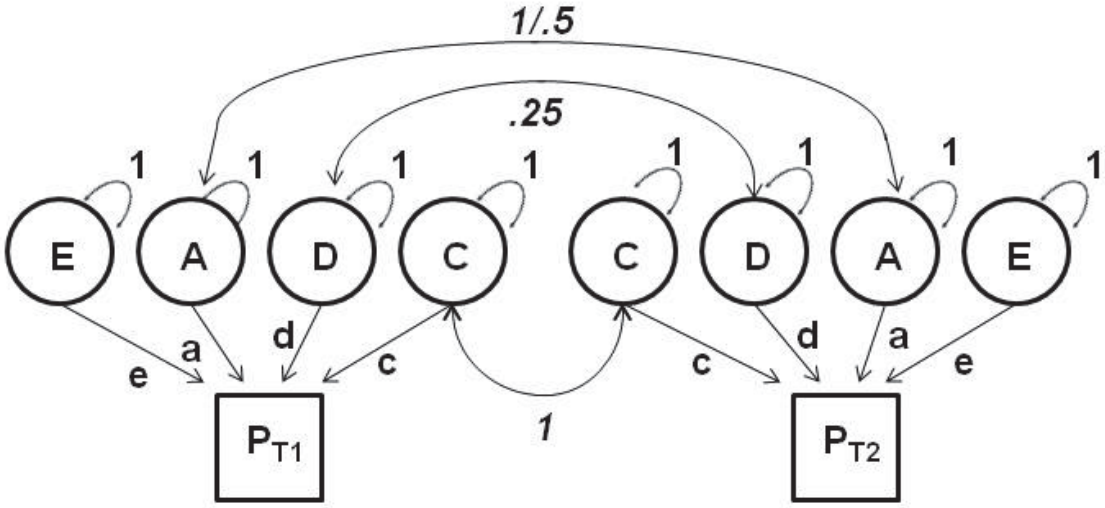


Figure 1a Path diagram for variance decomposition with classical twin design (CTD)

P_{T1}-P_{T2}= phenotype of twin 1, phenotype of twin 2

A=additive genetic factor (correlates 1 within MZ twin pairs, .5 within DZ twin pairs)

D= genetic dominance factor (correlates 1 within MZ twin pairs, .25 within DZ twin pairs)

C=shared environmental factor (correlates 1 within twin pairs)

E=individual-specific environmental factor (uncorrelated within or across twin pairs)

a,c,d,e = path loadings (c-paths estimated in ACE model with d-paths fixed to zero; d-paths estimated in ADE model with c-paths fixed to zero)

In the equations above, four parameters need to be estimated: *var(A)*, *var(D)*, *var(C)* and *var(E)*. Given that there are three observed values (*var(P)*, *cov(MZ)* and *cov(DZ)*), this poses a problem of underidentification. The common solution is to determine whether shared environmental influences or effects of dominance contribute most to the phenotypic variation and fix the other variance component to zero. If twice the DZ correlation is equal to or larger than the MZ correlation, *var(D)* is fixed to zero and the so-called ACE model is fitted to the data. If the MZ correlation is larger than twice the DZ correlation, *var(C)* is fixed to zero and the ADE model is fitted. In the ADE model, if *var(C)* is nonzero, *var(A)* is overestimated by a factor *3var(C)*, *var(D)* underestimated by *2var(C)* and *var(C)* by its true value. Conversely, in the ACE model, if *var(D)* is nonzero, *var(A)* is overestimated by a factor *1.5var(D)*, *var(C)* underestimated by *0.5var(D)*, and *var(D)* by its true value (Keller and Coventry 2005). The CTD provides useful estimates on the genetic architecture of a trait with this knowledge in mind and as long as the estimates are broadly interpreted. That is, *var(C)* also reflects other types of effects, including phenotypic assortment and *var(D)* is reflective of effects due to dominance as well as epistasis (Keller et al. 2010) that results from the interaction between two alleles at different loci. The interacting effects of alleles resulting from genetic dominance and epistasis are also referred to as non-

additive genetic effects, in contrast to additive genetic effects that assume a linear relation between the genetic variants and the phenotypic values.

In the CTD, variation component due to genetic dominance is completely confounded with effects of (additive-by-additive) epistasis, which are correlated 1 for MZ twins and .25 for DZ twins (Heath et al. 1984). Additive-by-additive epistasis reflects that additive effects of two alleles interact (Falconer 1996). If higher-order epistatic interactions among alleles underlie the phenotypic variance, then the overall correlation between non-additive variation among DZ pairs (consisting of all possible interactions among alleles) will be lower than .25. The reason for this is that higher-order interactions between alleles are less frequently shared within DZ pairs. Since estimating additive genetic effects, non-additive genetic effects and the true correlation between the non-additive genetic variance components for DZ twins in the CTD will result in model under-determination given that there are only three observed statistics (phenotypic variance, MZ covariance, DZ covariance), the correlation between variance components due to non-additive genetic effects is usually fixed at .25 assuming the absence of higher-order interactions between alleles (Keller and Coventry 2005).

If partner choice is random with regard to the phenotype in question (random mating), the correlation between additive genetic values of DZ twins and sibs is estimated at 0.5. If instead partners choose their spouses based on the phenotype of interest (that is, phenotypic assortment), the additive genetic variation among DZ twins increases (Jinks and Fulker 1970) which in the CTD mimics shared environmental variance. The reason for this is that because most, if not all, traits are partly genetically determined, phenotypic assortment induces the genetic influences on the spousal phenotypes to be correlated. When partners are more alike genetically, they will transmit genes that are more alike and therefore, under phenotypic assortment, the additive genetic covariance among DZ twins will increase (that is, the correlation among the additive genetic values for DZ twins >.5). The effect of non-random mating is dependent on the correlation between the additive genetic effects on the phenotypes of the parents. Since the correlated phenotypes of the spouses cannot increase the genetic covariance among (genetically identical) MZ twins, phenotypic assortment increases the similarity among DZ twins relative to MZ twins, coming up as effects of the shared environment (Lynch and Walsh 1998). There are other mechanisms underlying spousal resemblance that do not induce the genetic effects on the two parental phenotypes to be correlated. If the causes of spousal resemblance are wholly environmental in origin, there will be no consequences for the genetic variation, for instance in the case of social homogamy which describes assortment between individuals from the same social background (Heath and Eaves 1985). The same is true when spousal resemblance results from cohabitation effects. By this mechanism spousal resemblance is a function of relation length, having the consequence that spouses become more

alike phenotypically the longer they are together (Van Grootheest et al. 2008). The pattern of correlations among twins and spouses and the spouses of their cotwin can inform the mechanism of assortment (Heath and Eaves 1985).

Effects of cultural transmission, that is effects of parental trait values on that of their children which cannot be accounted for by genes they have in common (Eaves 1976), are suggested by social learning theory (Bandura and McClelland 1977) that views parents as being role models for their children. With regard to alcohol use, parental alcohol use may set the reference for their children, with the effect that when children see their parents drink, they may imitate that behavior (Quigley and Collins 1999). If cultural transmission is present, the genes that parents transmit will covary with the environmental influences they present for their children, leading to passive gene-environment (*A*-*C*) correlation. Effects of cultural transmission as well as effects of *A*-*C* correlation will increase the shared environmental variance component (Eaves 1976).

Gene-environment correlation and gene-environment interaction affect the CTD estimates (Jinks and Fulker 1970). Active gene-environment (*A*-*E*) correlation arises when an individual creates or seeks out his own environment based on his or her genotype. A person with a genetic predisposition to use alcohol may seek out friends who use alcohol as well. Passive gene-environment (*A*-*C*) correlation occurs when parents provide an environment for their offspring that is correlated with the genes they transmit (Plomin et al. 1977). An alcoholic may for instance not only transmit genes that predispose his or her offspring to alcoholism, but also show an allowing attitude towards drinking and/or provide a home environment in which drinks are abundant and thus more accessible. Positive *A*-*E* correlations will be incorporated in the additive genetic variance component, and positive *A*-*C* correlations will come up as shared environmental effects. Negative *A*-*E* and *A*-*C* correlations will decrease the respective variance components (Purcell 2002). Gene-environment interaction refers to a different reaction to different environments for individuals with the same genotype or to different reactions to the same environment depending on the genotype. Positive *AxE* interactions will be estimated as variance due to non-shared environmental effects (*E*), whereas positive *AxC* interactions will be estimated as variance due to additive genetic effects (*A*). Gene-environment interaction is not estimated as a separate variance component in the CTD, but can be incorporated (e.g. in the so-called moderator model) (Purcell 2002) or can be studied in other ways. When the genetic correlation is less than 1 for a trait that is studied in two different environment, gene-environment interaction is suggested (Boomsma et al. 2002b). Otherwise gene-environment interaction can also be studied by correlating the MZ pair differences with the MZ pair sums. A correlation between MZ pair differences and pair sums shows that the distribution of

environmental influences within families is not independent of the total variance, and is thus indicative of *GxE* interaction (Jinks and Fulker 1970).

Another assumption of the CTD postulates that data of twins are representative for non-twins and conclusions generalizable to the general population. Criticism focuses on the unusual prenatal and postnatal circumstances in which twins grow up compared to non-twins (Eaves et al. 1978). Comparing data of twins to their non-twin siblings can answer whether twins and non-twins are comparable for the trait of interest. Poelen et al. (2005) already showed that within the NTR, data of twins on alcohol use collected in surveys 2-5 are largely comparable to those of singletons. In Chapters 3 and 5, this will be examined for the data that were used in this thesis.

In summary, estimates based on the CTD give valuable information on the underlying genetic architecture of a trait, as long as the variance components are interpreted broadly. That is, *var(A)* is reflective of additive genetic effects, as well as *A*-*E* correlation and *AxC* interaction. *Var(D)* indicates variance due to effects of dominance as well as additive-by-additive epistasis. *Var(C)* reflects shared environmental effects, but may also indicate effects of phenotypic assortment, cultural transmission and *A*-*C* correlation. *Var(E)* reflects non-shared individual-specific environmental effects (including measurement error) as well as *AxE* interaction.

2.2.2 Multivariate CTD

In the multivariate case, the variances of the separate phenotypes as well as the covariance between them, can be decomposed into sources of variation due to additive genetic effects, dominance, shared environmental effects and non-shared individual-specific environmental effects (Eaves and Gale 1974). An additional advantage of multivariate modeling is the increase in power to detect genetic and environmental effects (given that the measurements are correlated) (Schmitz et al. 1998). The identification of the (co)variance components is similar to that described above and is given by equations (6) and (7). One representation of the variance decomposition of multiple traits is the Cholesky decomposition which is represented for the bivariate case in Figure 1b. This decomposition constrains the matrices (that is, variance components) to be positive definite by modeling the variance components as the product of a triangular matrix with its transpose. In the multivariate case, the Cholesky decomposition specifies a first genetic factor to influence all the phenotypes, a second genetic factor to influence all phenotypes but the first, a third to influence all the phenotypes but the first and second (Neale and Cardon 1992), etcetera. The same holds true for the C, D and E factors.

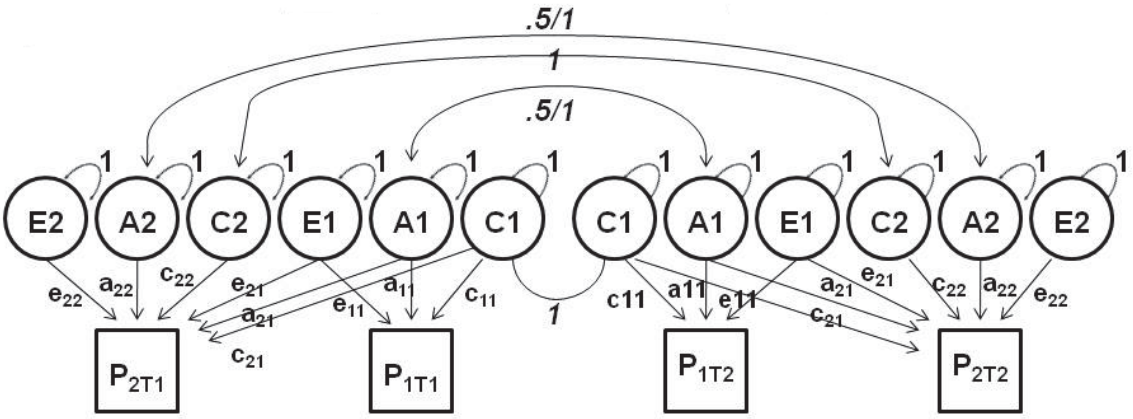


Figure 1b Path diagram for variance decomposition with bivariate ACE model

$P_1T_1, P_2T_1, P_1T_2, P_2T_2$ = phenotype 1 of twin 1, phenotype 2 of twin 1, phenotype 1 of twin 2, phenotype 2 of twin 2

A_1, A_2 = additive genetic factors for phenotype 1 and 2 (correlates 1 within MZ twin pairs, .5 within DZ twin pairs)

C_1, C_2 = shared environmental factors for phenotype 1 and 2 (correlates 1 within twin pairs)

E_1, E_2 = individual-specific environmental factors for phenotype 1 and 2 (uncorrelated within or across twin pairs)

a_{11},a_{21}, a_{22} = additive genetic path loading from first factor to phenotype 1, additive genetic path loading from first factor to phenotype 2 (indicating additive genetic covariance path), additive genetic path loading from second factor to phenotype 2 (indicating unique variance to phenotype 2)

c_{11},c_{21}, c_{22} = shared environmental path loading from first factor to phenotype 1, shared environmental path loading from first factor to phenotype 2 (indicating additive genetic covariance path), shared environmental path loading from second factor to phenotype 2 (indicating unique variance to phenotype 2)

e_{11},e_{21}, e_{22} = non-shared individual-specific environmental path loading from first factor to phenotype 1, individual-specific environmental path loading from first factor to phenotype 2 (indicating individual-specific environmental covariance path), individual-specific environmental path loading from second factor to phenotype 2 (indicating unique variance to phenotype 2)

The covariance decomposition in variation due to genetic effects (additive/non-additive) and environmental effects (shared/non-shared) provides information on the nature of the association, as explained by De Moor et al. (2008). Do the phenotypes correlate phenotypically, because they are (partly) influenced by the same genes (i.e. through genetic pleiotropy)? Or because they are (partly) influenced by the same environmental factors? Or is one phenotype causing the other, which predicts that both the genetic factors and the environmental factors are correlated?

The causes of covariation are typically expressed in terms of the genetic and environmental correlations. These reflect the correlations between the genetic or environmental effects on two (or more) traits. The additive genetic correlation (r_a) reflects the correlation between the additive genetic effects on two traits, for example, alcohol use (ALC) and liver enzyme levels (LIV), and is a standardized measure of the additive genetic covariance ($cov(A_{ALC,LIV})$) divided by the square root of the product of the genetic variances of the separate

phenotypes (*var(A_{ALC}), var(A_{LIV})*). The correlation between non-additive genetic effects on two traits (r_d), between shared environmental effects on two traits (r_c) and between non-shared individual-specific environmental effects on two traits (r_e) can be similarly derived. For example, the correlation between non-shared individual-specific effects on alcohol use and liver enzyme levels ($r_{e,\,ALC,LIV}$) is calculated by dividing the environmental covariance among the traits (*cov($E_{ALC,LIV}$)*) by the square root of the product of the separate environmental variances of the traits (*var(E_{ALC}), var(E_{LIV})*) (Martin and Eaves 1977). The additive genetic and non-shared individual-specific environmental correlations $r_{a,ALC,LIV}$ and $r_{e,ALC,LIV}$ for the association of alcohol use (ALC) and liver enzyme levels (LIV) can thus be given as:

$$r_{a,\,ALC,\,LIV} = \frac{\mathrm{cov}(A_{ALC,\,LIV})}{\sqrt{\mathrm{var}(A_{ALC}) + \mathrm{var}(A_{LIV})}} \tag{8}$$

$$r_{e,\,ALC,\,LIV} = \frac{\mathrm{cov}(E_{ALC,\,LIV})}{\sqrt{\mathrm{var}(E_{ALC}) + \mathrm{var}(E_{LIV})}} \tag{9}$$

The genetic contribution to the observed correlation among phenotypes is calculated by multiplying the genetic correlation with the heritabilities of the separate phenotypes. This shows that a high genetic correlation will make a large contribution to the observed phenotypic correlation only if both traits are highly heritable. The same is true for the environmental contribution to the phenotypic correlation. Large proportions of variance that can be attributed to environmental effects in combination with a large environmental correlation will result in a large contribution to the observed correlation that is environmental in origin (Falconer 1996).

2.2.3 Longitudinal simplex modeling

A specific example of the analysis of multiple phenotypes is given when the same variable is measured repeatedly for the same individuals. When the correlations among the measurements are highest for adjoining observations and decrease when the time interval between the observations increases, the correlation structure forms a 'simplex'. When longitudinal data conform a 'simplex' structure, these cannot be analyzed in the same way as multiple phenotypes, especially when the number of time points increases (Boomsma and Molenaar 1987). The autoregressive process among observations that are ordered in time can be modelled in a 'simplex' model. In a first-order nonstationary autoregressive model, each latent variable at time point *t* is predicted by the latent variable at time point *t-1* (Boomsma and Molenaar 1987).

The aim of longitudinal twin data analysis is to estimate the genetic and environmental processes as they unfold over time. Questions that can be asked are whether additive genetic variance increases over time, and if so, whether this is due to amplification of existing genetic influences or whether other genes are expressed and new genetic variance comes into play (Molenaar et al. 1991). To answer these questions, the (single) latent factors (genetic and environmental) that figure in the CTD (see equation (5)) are replaced by simplex structures that causally relate the latent factor at time point *t* to that at time point *t-1*. A representation of this model is given by Figure 1c.

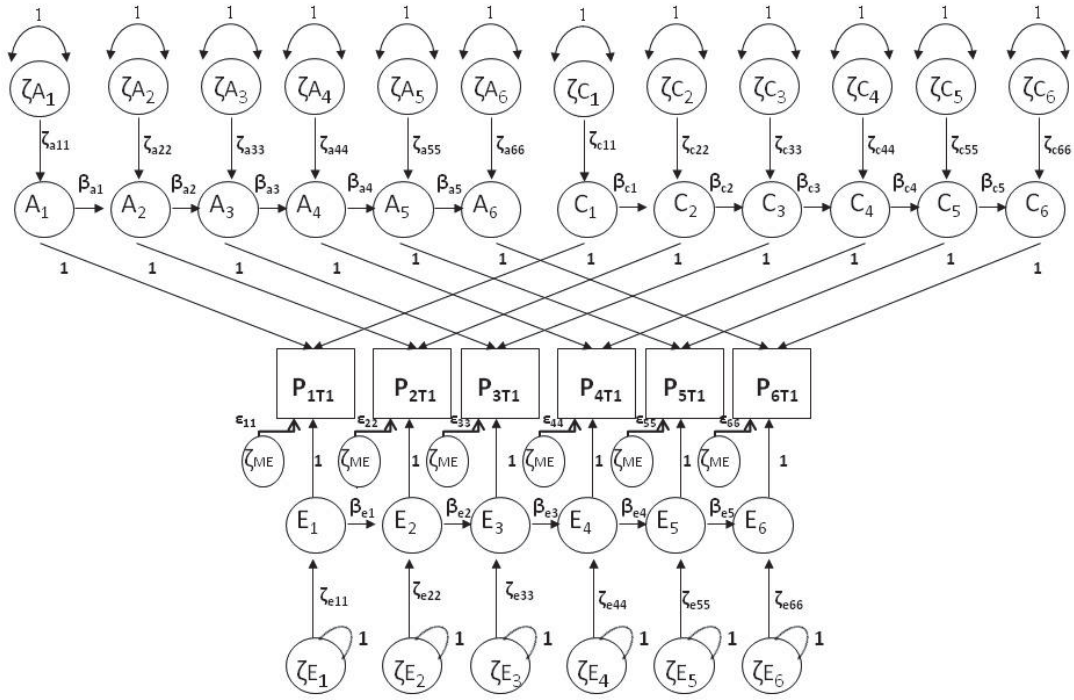


Figure 1c Path diagram for variance decomposition with longitudinal simplex model (6 time points) (shown for 1 twin only)

P_1T_1-P_6T_1= phenotype of twin 1 at time point 1-6 (phenotypes for twin 2 left out of the model for clarity of presentation)

A_1-A_6 = additive genetic factors for time points 1-6

C_1-C_6 = shared environmental factors for time points 1-6

E_1-E_6 = non-shared individual-specific environmental factors for time points 1-6

β_{a1}-β_{a6} = regression coefficients representing transmission of additive genetic variance for time points 1-6

β_{c1}-β_{c6} = regression coefficients representing transmission of additive genetic variance for time points 1-6

β_{e1}-β_{e6} = regression coefficients representing transmission of additive genetic variance for time points 1-6

ζ_A1- ζ_A6 = additive genetic innovation terms for time points 1-6

ζ_C1- ζ_C6 = shared environmental innovation terms for time points 1-6

ζ_E1- ζ_E6 = non-shared environmental individual-specific innovation terms for time points 1-6

ζ_{ME} = factor representing measurement error (equal over time points 1-6)

For the ACE model, with $a_{j,t}$, $c_{j,t}$, and $e_{j,t}$ representing the additive genetic, shared environmental and non-shared environmental values for subject *j* at time point *t* respectively, the structural model can be given by:

$$a_{j,t} = \beta_{a,t} * a_{j,t-1} + \zeta_{a,j,t}$$
$$c_{j,t} = \beta_{c,t} * c_{j,t-1} + \zeta_{c,j,t}$$
$$e_{j,t} = \beta_{e,t} * e_{j,t-1} + \zeta_{e,j,t} \quad (10)$$

Thus, the additive genetic value for subject *j* at time point *t* is a function of the additive genetic value at time point *t-1* that is transmitted, $\beta_{a,t} * a_{j,t-1}$, plus the additive genetic innovation term $\zeta_{aj,t}$. Shared environmental and non-shared environmental values at time point *t* for subject *j* are similarly related to earlier time points. The regression coefficients ($\beta_{a,t}$, $\beta_{c,t}$, $\beta_{e,t}$) in the transmission paths indicate the magnitude of the amplification or attenuation of the genetic or environmental influences that were present at the earlier time point. Transmission paths represented by regression coefficients >1 indicate that the genetic influences that were present at an earlier time point are augmented, whereas regression coefficients <1 indicate that the genetic influences that were present at the earlier time point are attenuated. The innovation terms ($\zeta_{a,t}$, $\zeta_{c,t}$, $\zeta_{e,t}$) indicate whether new genetic influences have emerged that were not present at the earlier time points. The innovation terms at time point *t* are thus uncorrelated with the effects of the latent factor at time point *t-1*.

In the simplex model, non-shared environmental influences can be separated from effects due to measurement error. Measurement error is modeled to be time specific and not to influence subsequent time points, whereas the innovation terms can influence the following observations. Measurement error variances for the first and last time point cannot be distinguished from the innovation terms and thus need to be constrained to other measurement error variances for reasons of model identification (Boomsma et al. 1989). In the notation above, the factor loadings that relate the observed values to the latent factors are fixed to 1 and the innovation and transmission paths are estimated, but the simplex model can be specified in other ways as well (Neale and Cardon 1992).

2.2.4 The extended twin-family design

The CTD can be extended to include parents and siblings into a so-called extended twin-family design (ETFD) in which effects due to the shared environment, phenotypic assortment, cultural transmission, and passive (*A-C*) gene-environment correlation can be separately estimated. By including data of siblings, it can be examined whether variances and covariances of data on twins are comparable to that of siblings, to test for a special twin environment. By

including data of parents, two extra pieces of information are added: the covariance among parents (*cov(FM)*) and that between parents and offspring (*cov(PO)*). Including parents in the design allows for two extra parameters to be estimated above those that can be estimated in the CTD. Two models that are commonly employed that make use of this extra information are the ACDE model and the ACE model with cultural transmission from parents to offspring (shown in Figure 1d).

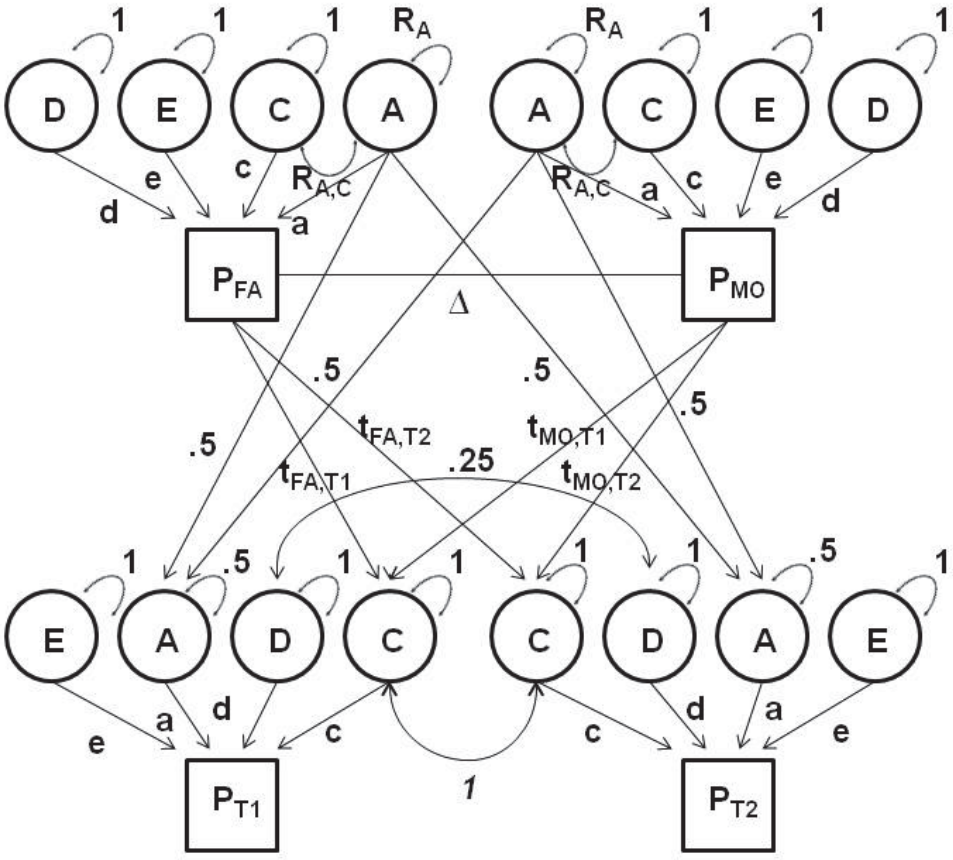


Figure 1d Path diagram for variance decomposition with extended twin-family design (ETFD) (shown for DZ twin pair with parents)

P_{FA}, P_{MO}, $P_{T}1$, $P_{T}2$ = phenotype of father, mother, twin 1, twin 2

A= additive genetic factor with variance R_A (differs from 1 in the case of phenotypic assortment)

D= non-additive genetic factor

C=shared environmental factor with variance R_C (differs from 1 in the case of shared environmental transmission)

E=individual-specific environmental factor

a,c,d,e= path loadings (d-paths estimated in ACDE model; set to zero in ACE model with shared environmental transmission)

$t_{FA,T}1$, $t_{FA,T}2$, $t_{MO,T}1$, $t_{MO,T}2$ = shared environmental transmission paths from father to twin 1, father to twin 2, mother to twin 1, mother to twin 2 (estimated in model with shared environmental transmission; set to zero in ACDE model)

$R_{A,C}$=covariation between A and C (differs from 1 in the case of shared environmental transmission)

Δ =path representing correlations between the latent factors induced by phenotypic assortment

In both models the effect of phenotypic assortment can be modeled by introducing two correlations: *s*, the correlation between the phenotypic values of the parents (*s*var(P)*) and *m*, the correlation among the additive genetic effects (breeding values) resulting from phenotypic assortment (*m*var(A)*). If phenotypic assortment is the sole reason for covariation among the parental phenotypes, then the relation between *s* and *m* can be expressed as $m=sh^2$, with h^2 being the narrow-sense heritability of the phenotype in question (Falconer 1996).

In the ACDE model *var(A)*, *var(D)*, *var(C)* and *var(E)* are estimated as well as the covariance among parents (*cov(FM)*). The covariances for MZ and DZ twins, parents and parents and offspring are given by (Falconer 1996):

$$\begin{aligned}\mathrm{cov}(MZ) &= \mathrm{var}(P)-\mathrm{var}(E)\\ &= \mathrm{var}(A)+\mathrm{var}(D)+\mathrm{var}(C)\end{aligned} \quad (6)$$

$$\mathrm{cov}(DZ) = 0.5\,\mathrm{var}(A)*(1+m)+0.25\,\mathrm{var}(D)+\mathrm{var}(C) \quad (7b)$$

$$\mathrm{cov}(PO) = 0.5\,\mathrm{var}(A)*(1+s) \quad (11)$$

$$\mathrm{cov}(FM) = s*\mathrm{var}(P) \quad (12)$$

If spousal resemblance, *s*, is zero, the equations above reduce to:

$$\begin{aligned}\mathrm{cov}(MZ) &= \mathrm{var}(P)-\mathrm{var}(E)\\ &= \mathrm{var}(A)+\mathrm{var}(D)+\mathrm{var}(C)\end{aligned} \quad (6)$$

$$\mathrm{cov}(DZ) = 0.5\mathrm{var}(A)+0.25\mathrm{var}(D)+\mathrm{var}(C) \quad (7)$$

$$\mathrm{cov}(PO) = 0.5\mathrm{var}(A) \quad (11b)$$

$$\mathrm{cov}(FM) = 0 \quad (12b)$$

As a result of phenotypic assortment, the between-family variation in the offspring generation due to additive genetic effects is increased. The within-family variation in the offspring generation due to additive genetic effects, also called segregation variance, is unaffected by phenotypic assortment (provided that the number of genes at play is large) (Falconer 1996). Segregation variance results from the fact that parents do not transmit average genetic effects to their offspring (which would eliminate within-family additive genetic variation), but transmit one of every two alleles for each locus (thus giving rise to additive genetic within-family variation). In the presence of phenotypic assortment that results in correlated additive genetic effects for spouses, the additive genetic variation in the offspring generation (between-family variation + within-family variation) will therefore be greater than 1 (Keller et al. 2009).

In the ACE model with cultural transmission, cultural transmission can be modeled as well as the resulting passive-gene environment (*A*-*C*) correlation that is induced when parents transmit both their genes and environment to their children (Eaves 1976). Under the ACE model with cultural transmission the covariances among the twins, parents and parents and offspring can be given as follows (based on Neale et al. (1994)):

$$\text{var}(P) = \text{var}(A) + \text{var}(C) + \text{var}(E) + 2\,\text{cov}(A,C) \quad (5b)$$

$$\begin{aligned}\text{cov}(MZ) &= \text{var}(P) - \text{var}(E) \\ &= \text{var}(A) + \text{var}(C) + 2\,\text{cov}(A,C)\end{aligned} \quad (6b)$$

$$\text{cov}(DZ) = 0.5\,\text{var}(A)*(1+0.5\,\text{cov}(A,P)*s) + \text{var}(C) + 2\,\text{cov}(A,C) \quad (7c)$$

$$\text{cov}(PO) = \text{cov}(C,P)*(1+s) + 0.5\,\text{var}(A)*(1+s)*\text{cov}(A,P) \quad (11c)$$

$$\text{cov}(FM) = s*\text{var}(P) \quad (12)$$

Note that when estimating the cultural transmission path *t*, another parameter needs to be set to zero to keep the model identified, usually *var(D)*. As described above, the ETFD can model effects of phenotypic assortment and passive gene-environment (*A*-*C*) correlation. By including data of family members other than twins (parents, siblings), the number of individuals is increased and the power to detect effects of dominance as well as effects of the shared environment is augmented (Posthuma and Boomsma 2000). Furthermore, by including siblings in the design, a specific twin environment can be tested for. The absence of a specific twin environment suggests that twins resemble each other to the same extent as non-twins (after e.g. correction for age differences), thus that twin-specific prenatal or postnatal effects (e.g. low birth weight) are not affecting the trait of interest.

It should be noted that the ETFD also depends on assumptions that are not readily tested. As described above, the correlation between the non-additive genetic variance components among DZ twins needs to be fixed to avoid under-determination, assuming absence of higher-order interaction between alleles. In addition, in the ETFD, additive-by-additive epistasis tends to overestimate *var(A)* (Keller et al. 2010). This follows from the parent-offspring covariance in equations (11), (11b) and (11c) that do not explicitly model effects of epistasis, whereas epistatic effects are shared among offspring and parents and offspring. Overall however, *Var(D)* appears to only slightly underestimate the effects of all non-additive genetic variation (effects due to dominance and epistatis) and in practice ETFD estimates agree well with the true values broad-sense heritability estimates (Keller et al. 2010).

2.3 Methods to estimate genetic effects based on SNP data

The second set of methods used in this thesis to study genetic effects relied on genetic marker data, called SNPs. As estimated in the 1000 Genomes project (Altshuler et al. 2010b), ~0.6% of the human sequence variation consists of SNPs. SNPs are variations in the DNA sequence that differ among individuals by a single nucleotide (*A, T, C, G*) and form the most prevalent type of genetic markers among humans (Frazer et al. 2009). Approximately, 15 million SNPs have been catalogued by the 1000 Genomes Project (minor allele frequency (MAF) >.01 for non-functional variants and >.001 for functional variants) (Altshuler et al. 2010b). As a reference, the entire human genome sequence consists of nearly three billion base pairs. Around ~80% has been assigned biochemical functions with a higher fraction of bases thought to be involved in gene regulation than in the coding of proteins (ENCODE Project Consortium 2012). The correlation structure in the genome, called linkage disequilibrium (LD), makes it possible to capture most of the genetic SNP variation with only a subset of all SNPs, called tagging SNPs (Frazer et al. 2009). This LD among SNPs results from the fact that blocks of DNA are generally inherited together. For example, based on the Hapmap II data reference set, around 550,000 SNPs were needed to tag most of the 2.6 million common SNPs in Caucasians (MAF > .05; $r^2 \geq .8$). With around 1 million SNPs all common SNP variation was captured (MAF > .05; $r^2 = 1$) (Frazer et al. 2007).

Four methods are discussed that were applied in this thesis and made use of SNP data. The first method, association analysis, focuses on individual SNP associations with the aim of gene finding. The other methods, polygenic risk score prediction, methods based on genetic relatedness (Yang et al. 2010) and density estimation (So et al. 2011), examine what proportion of the phenotypic variation can be ascribed to the total effect of all SNPs.

2.3.1 Genetic association analysis

Association analysis is concerned with the question whether phenotypic variation can be explained by genotypic differences at a locus (Long et al. 1997). It can be performed for a subset of loci (in a candidate gene study) or genome-wide (in a genome-wide association, GWA, study) (Balding 2006). GWA studies have the advantage that no prior information on susceptibility loci is needed. It has the disadvantage of running many tests which renders the need to correct for multiple testing (Hirschhorn and Daly 2005). A SNP with a p-value $<5 \times 10^{-8}$ is considered genome-wide significant in a GWA study (Stranger et al. 2011).

In association analysis, the phenotypic value for a quantitative trait p_j for subject j is modeled as a linear function of the additive genetic effect of SNP i and a residual ε_j:

$$p_j = \nu + \beta_o o_j + \ldots + \beta_i x_{ij} + \varepsilon_j \quad (13)$$

Here, ν denotes the intercept and β_o the regression coefficient for any fixed effect o_j for subject j. x_{ij} refers to the number of A_1 alleles for SNP i for subject j and the regression weight β_i represents the additive genetic effect for SNP i. According to the biometric model, the regression weight β_i corresponds to the average effect of allelic substitution (α), that is, the effect of having an A_1 allele instead of having an A_2 allele (α_1- α_2) (Kwan et al. 2007). For categorical data, logistic regression models can be applied instead of linear regression models. By taking the logit of disease risk (π), *log* ($\pi/(1-\pi)$), risk of disease is transformed to the 0-1 scale. The regression models can be extended to include effects due to dominance (the genotypic values minus the additive effect for that genotype or breeding value).

Given that most association studies are restricted to the usage of tagging SNPs, a SNP that is significantly associated with the trait of interest is unlikely to be the causal variant. The power to detect the causal variant is dependent on the LD between the tagging SNP and the causal variant, r^2, that itself is dependent on sample size and allele frequencies. A low r^2 between the tagging SNP and the causal variant implies that a large sample size is needed to detect the LD between the variants (relative to the sample size that is needed to detect an association for directly genotyping the causal SNP), whereas in the case of a high r^2, no additional increase in sample size is needed to detect the disease association via the tagging SNP (Balding 2006). The dependence of r^2 on differences in MAF between SNPs has the consequence that tagging SNPs (that often have a MAF >.01) are in low LD with rare causal variants (often MAF<.01) even if they are in the same genomic region (Visscher et al. 2012).

2.3.2 Methods to estimate the heritability based on all SNPs

Although GWA studies have successfully detected SNPs that contribute to trait variation, the aggregated effect of genome-wide significant SNPs only explains a small part of the heritability for these traits (with the exception of diseases as age-related macular degeneration and type 1 diabetes) (Stranger et al. 2011). Explanations for the so-called 'missing heritability' (Maher 2008) include (a) small SNP effects that do not cross the threshold for multiple testing, (b) underestimated effect sizes of associated SNPs because of incomplete LD between the tagging SNPs and causal variants, (c) the specific contributions of rare alleles (MAF <.01), as well as structural variation, that are poorly tagged by the current available chips due to incomplete LD between tagging SNPs and causal variants, and (d) overestimated heritability estimates (Stranger et al. 2011). Several methods have been proposed to test whether the first

explanation holds true, that is to what extent SNP effects are captured by commonly available chips.

2.3.2.1 Polygenic risk score prediction

In their classic paper, Purcell et al. (2009) tested the hypothesis that the genetic risk is due to the additive effects of 'potentially thousands' of individual SNP effects that are too small to pass the stringent significance thresholds. The polygenic risk score approach consists of selecting (partially overlapping) sets of SNPs that are associated with the trait of interest in a discovery sample for varying thresholds of association (Purcell et al. 2009). The selected subsets of SNP associations will contain false positives, but if the signal of truly associated SNPs is strong enough, polygenic risk scores based on these SNP sets may still predict trait variation in an independent target sample (Demirkan et al. 2010). For an independent target sample, a polygenic risk score for subject *j* can be calculated as follows (based on additive SNP effects for SNPs *i* to *n* that resulted from a GWA study in a discovery sample):

$$y_j = b_i x_{ij} + \cdots + b_n x_{nj} \quad (14)$$

Thus, for every individual *j* in the target sample, for each SNP *i* (selected in that particular SNP set) the number of risk alleles x_{ij} is counted (0,1,2) and multiplied by the regression weight for that SNP (b_i) that resulted from the association study in the discovery sample, and then summed into a polygenic risk score y_j. The calculation of genetic risk scores can be performed in e.g. Plink (Purcell et al. 2007). The genetic risk score can then be used as a predictor of the phenotypic values in the target sample in a regression model. Additionally, it can be examined whether the genetic risk score contributes to other traits to test for genetic overlap among traits (Purcell et al. 2009).

The utility of polygenic risk scores in predicting trait variation depends on the (narrow-sense) heritability of the trait. If the heritability is low, the prediction of trait variation with the polygenic risk score will be poor. The opposite does not necessarily hold true. Whether the polygenic risk score predicts phenotypic trait variation does not only depend on the heritability, but also on (not mutually exclusive factors as) LD between SNPs selected for polygenic prediction and the causal variants, the accuracy of the estimated effect sizes of the selected SNPs, and sample size (Wray et al. 2008). A larger sample size implies better estimates of effect sizes and will therefore give a better representation of which alleles are truly associated, whereas a larger target set will increase the significance of the prediction of polygenic risk score (for a similar amount of variance explained). For quantitative traits, the amount of variance that can be explained by the polygenic risk score can be directly compared to the heritability estimate resulting from twin and family studies. For categorical traits, the prediction of the trait

depends on the prevalence. To compare the polygenic risk score prediction for categorical traits, the proportion of variance that can be explained by SNPs can be best expressed on the underlying liability scale (that is not dependent on the prevalence of disease) (Lee et al. 2012a).

2.3.2.2 GRM method

To estimate the heritability explained by SNPs, Yang et al. (2010) developed a method that relied on estimating the genetic relatedness for each pair of individuals (expressed in a Genetic Relatedness Matrix, GRM) and including this pairwise genetic relatedness as a random effect in a linear mixed model to predict the phenotypic similarity among these individuals. If most trait variation is due to SNPs that are poorly tagged by SNPs on commercially available chips, the GRM-based estimate will not be much higher than the total variance that can be explained by all genome-wide significant SNPs that already have been detected. If there are many SNPs with effects that are too small to pass the significance threshold however, then the GRM-based estimate will be higher (Visscher 2010).

In a linear mixed model, the phenotypic value p_j for subject j is expressed as a function of fixed effects o_j, the total genetic effects w_j and residual effects e_j (with the W and E factor means fixed at zero) (Yang et al. 2011b):

$$p_j = \beta_o o_j + \ldots + \beta_w w_j + \beta_e e_j \quad (15)$$

$$W \sim N(0, R * \mathrm{var}(W)) \quad (16)$$

$$E \sim N(0, I * \mathrm{var}(E)) \quad (17)$$

β_o represents the regression coefficient for any fixed effect o_j for subject j. W is normally distributed as $W \sim N(0, R*var(W))$. R is the N by N genetic relatedness matrix with realized additive genetic relationship coefficients for each pair of N individuals, who, although unrelated in the conventional way, share part of their DNA based on the correlation structure in the DNA. *Var(W)*, is the polygenic additive genetic variance explained by the SNPs that is estimated via residual maximum likelihood (REML) analysis. Residual effects (*E)* are normally distributed as $E \sim N(0, I*var(E))$ with I being the N by N identity matrix and *var(E)*, the variance component for residual effects estimated via REML analysis. The phenotypic variance *var(P)* can be decomposed into variance resulting from polygenic additive genetic SNP effects *var(W*) and residual effects *var(E)* (Yang et al. 2011a):

$$\mathrm{var}(P) = \mathrm{var}(W) + \mathrm{var}(E) \quad (18)$$

The proportion of variance that can be explained by all SNPs is calculated as the ratio of *var(W)* to *var(P)*. Note that the covariance between the phenotypic values of two individuals is proportional to their genetic relationship, and not influenced by residual effects (only 'unrelated' individuals are included and shared environmental effects are thus assumed to be zero; that is the off-diagonal elements of the matrix *I* are zero) (Yang et al. 2011b). An additional reason to exclude related individuals is that they may share causal variants above those that are captured by SNPs, thus confounding SNP effects with genetic effects not captured by SNPs (Yang et al. 2011b). Yang et al. (2010) have excluded one of each pair of individuals with a genetic relatedness >.025 (corresponding to third or fourth cousins).

For two individuals *j* and *k*, the pairwise additive genetic relatedness (R_{jk}) based on SNPs *i* to *n* can be calculated as follows (Yang et al. 2010):

$$R_{jk} = \frac{1}{n}\sum i \frac{(xij - 2p_i)(x_{ik} - 2p_i)}{2p_i(1-p_i)},\ j \neq k$$

$$R_{jk} = 1 + \frac{1}{n}\sum_i \frac{x_{ij}^2 - (1+2p_i)x_{ij} + 2p_i^2}{2p_i(1-p_i)},\ j = k \qquad (19)$$

If $j \neq k$, the correlation between the additive genetic values between two individuals *j* and *k* based on a given SNP *i* is calculated as the product of the number of risk alleles x_i for SNP *i* minus the mean number of risk alleles for SNP *i* (*np* with *n*=2 representing the mean for a binary SNP variable with frequency *p*) for subject *j* and subject *k*, standardized by the heterozygosity of SNP *i* (*np(1-p)* with *n*=2 representing the variance of a binary variable with frequency *p*). The genome-wide additive genetic relationship for individuals *j* and *k* is then obtained by taking the mean over the additive genetic correlations for each SNP *i* indexed 1 to *n*. If *j=k*, the formula is slightly different and describes the correlation of additive genetic values of an individual with him/herself. This correlation is estimated and is not exactly 1 to minimize sampling variation (Yang et al. 2011a).

The significance of the variance components *var(W)* and *var(E)* (and that of fixed effects) can be tested by comparing the model fit of the full model to that of the reduced model with the likelihood ratio test. The significance is dependent on the standard error of the GRM-based estimates that decreases as sample size increases (Yang et al. 2010). If the causal SNPs have a distribution of MAF that is similar to that of tagging SNPs, GRM-based estimates are unbiased, in contrast to the polygenic risk score approach that is based on effect sizes that are estimated in a discovery set that do not provide unbiased estimates of the effect sizes in the target set (Visscher 2010). When causal SNPs have a distribution of MAF that, on average, is lower than that of the tagging SNPs, the GRM method will underestimate the heritability due to

all SNP effects (Yang et al. 2010). Denser SNPs will provide higher LD with the causal SNPs, and can thereby increase the GRM-based estimate of total variance that can be explained by SNPs, but if there are systematic differences between the MAF of tagging SNPs and causal SNPs, then the GRM-based estimates will still be biased downwards (Visscher 2010).

Note that for categorical data stringent SNP data quality control (QC) is important since artificial allele differences between cases and controls will appear as genetic variance. See Lee et al. (2011) for SNP QC step when working with categorical data. Since GRM-based estimates are dependent on SNP density and differences in the distribution of MAF of tagging SNPs and causal SNPs however, too stringent QC may avertedly cause too many SNPs to be excluded, resulting in GRM-based estimates that are biased downwards.

2.3.2.3 Density estimation (DE) method

So et al. (2011) developed a density estimation (DE) method that estimates the proportion of phenotypic variance explained by SNPs by comparing the distribution of observed test statistics from a GWA study to that expected under the null hypothesis of no effect. They reasoned that if sample size would be infinite, all susceptibility variants would be detected, regardless how small their effect. Thus if the 'true' effect sizes of SNPs would be known (that is, effect sizes that would result if there was no random noise) then the total variance explained by all SNPs (*var(W)*) could be obtained by summing these 'true' effect sizes for all independent SNP signals. Their method thus relies on recovering the true effect sizes of SNPs from the observed effect sizes. Expressed mathematically: the expected value of the true effect size, δ_i for SNP i given an observed effect size z_i, that is $E\{\delta_i|z_i\}$.The observed effect sizes can be expressed as z-scores and result from a GWA study or meta-analysis. To estimate the expected effect size δ_i given an observed effect size z_i, they refer to Efron (2009) who proved that the expected value of the effect size δ given an observed effect size z is equal to taking the derivative of ψ with respect to z (with ψ being the *log* of the observed z-statistics divided by the z-statistics under the null):

$$E(\delta \mid z) = \psi'(z), \; with \; \psi(z) = \log \frac{f(z)}{\phi(z)} \qquad (20)$$

The DE method assesses how much the observed distribution of effect sizes ($f(z_i)$) differs from what is expected when all SNP effects are zero ($f(z_i|\delta=0)$). Whereas the majority of the observed effect sizes will be close to zero, some SNPs will have non-zero effects which will be reflected in a distribution $f(z_i)$ with somewhat heavier tails than what is expected under the null distribution. The expression of the distribution of expected z scores $f(z_i)$ takes the assumption

that z given δ is normally distributed with mean δ and a standard deviation of 1. With $\gamma(\delta)$ denoting the prior density function of δ and $\varphi(z)$ the standard normal density function, the marginal distribution of $f(z_i)$ can then be expressed as follows (following Bayes' theorem that $P(B)=P(B|A)*P(A)$):

$$f(z)=\int_{-\infty}^{\infty} f(z|\delta)\gamma(\delta)\mathrm{d}\delta=\int_{-\infty}^{\infty}\varphi(z-\delta)\gamma(\delta)\mathrm{d}\delta \tag{21}$$

For continuous traits, the variance explained by SNP i ($var(W_i)$) can be obtained by the formula for ANOVA. That is, sums of squares (SS) due to regression divided by the sums of squares about the mean is equal to SS due to regression divided by SS due to residuals + SS due to regression:

$$\mathrm{var}(W_i)=\frac{[E(\delta|z)]^2}{n-2+[E(\delta|z)]^2} \tag{22}$$

Denoting the function to convert the observed to the expected effect sizes with ξ, the total variance that can be explained by SNPs ($var(W)$) is obtained by summing the expected effect sizes for all independent SNPs.

$$\mathrm{var}(W)=\sum_i \xi\{\psi'(\mathbf{Z}_i)\} \tag{23}$$

To perform the DE method, So et al. (2011) provide a R script available at the developer's webpage https://sites.google.com/site/honcheongso/software/total-vg
The script provides two Kernel estimation procedures. So et al. (2011) note that the so-called unconditional Kernel estimator is more accurate and stable than the so-called conditional Kernel estimator. To avoid total $var(W)$ to be inflated by redundant SNP effects, SNPs that are in high LD with other SNPs need to be removed by pruning. Pruning is dependent on the LD pattern and thus independent of the SNP associations with the trait (So et al. 2011). When raw SNP data are available, these can be used. Otherwise pruning can be performed based on the LD pattern in a reference set (for example, the HapMap CEU reference set). Based on SNP data sets consisting of .1-2.6 million SNPs, So et al. (2011) recommend pruning based on a r^2 threshold of .25, a 100-SNP sliding window and proceeding by 25 SNPs in each step (with the Plink command "--indep-pairwise 100 25 0.25"). In addition, So et al. (2011) recommend to randomly permute the sign of the z-statistics (positive or negative) a 100 times, then run the DE method for each permutation and calculate a mean estimate over the 100 permutations, to ensure more

stable estimates. A loop function to run this permutation is present in the script for binary variables.

Since the DE method proposed by So et al. (2011) does not rely on any distributional assumptions, standard errors cannot be derived analytically. When multiple pruned sets are extracted (that show <50% overlap in SNPs), the density method can be performed multiple times and an estimate of variability can be calculated. One way to obtain multiple pruned sets that overlap <50%, is to prune the SNP data set with the command above, then select *n* random samples that contain 50% of the SNPs that are included in the pruned set. Then the *full* SNP data set is pruned *n* times, each time excluding another random sample of the SNPs from this full SNP data set when starting to prune (with the Plink command "--exclude myrandomset.dat --indep-pairwise 100 25 0.25"). To obtain a single number that represents the total amount of variance that can be explained by all SNPs, the mean of the *n* estimates that resulted for the *n* sets can be calculated. The estimate of variability is then obtained by taking the standard deviation of the *n* mean estimates for the *n* pruned sets.

2.4 Summary

The methods that are applied in this thesis to study genetic effects on alcohol use and liver enzyme levels can be distinguished into methods that use the known genetic relatedness among family members to estimate genetic effects (twin-family based heritability studies, e.g. the CTD and the ETFD) and methods that rely on SNP data (association studies, SNP-based heritability studies).

With the CTD phenotypic variance is usually decomposed in variance due to additive genetic effects (A), non-shared individual-specific effects (E) and either shared environmental effects (C) or effects of genetic dominance (D) that can also reflect epistatic effects. In variations of the CTD, the covariance among traits can be investigated to test whether the association among the traits (e.g. at the population level) may be causal or better explained by shared genetic effects. With data on multiple time points the covariance among the time points can be used to describe the causes of variation that underlie the development of a trait over time (e.g. in a simplex model). When the CTD is extended to include data of siblings and parents (the so-called ETFD), effects of shared environmental effects (C) can be estimated simultaneously with those of genetic dominance (D). In the ETFD further questions can be addressed that include (a) whether twins are comparable to singletons, which if this is the case, supports the generalisability of the conclusions to non-twin populations, (b) whether there are effects of cultural transmission from parents to offspring, beyond the genetic transmission effects from parents to offspring, and (c) whether heritability estimates are affected by effects of phenotypic assortment.

Methods based on SNP data applied in this thesis, were used to further explore the additive genetic variation underlying alcohol use and liver enzyme levels. Association analysis tests whether additive SNP effects significantly influence phenotypic trait variation. SNP-based heritability methods estimate what part of the phenotypic variation is due to the joint effect of all SNPs, assuming an additive model. The DE method proposed by So et al. (2011) is based on comparing the distribution of observed effect sizes (resulting from a GWA study or meta analysis) to that what would be expected if no effects of SNPs on the trait were expected. Given that the (shape of the) distribution of expected effect sizes (for SNPs with effect sizes >0) is correctly assumed, the difference between the observed and expected distribution of effect sizes gives an estimate of the variance that can be explained by all SNPs simultaneously. The GRM method (Yang et al. 2011a) correlates differences in phenotypic values among individuals with differences in genetic values among individuals. The GRM method is thus similar to twin-family based heritability studies in which phenotypic differences among family members are correlated with the inferred genetic relatedness among them. In contrast to the latter however, the GRM method, is not inferring the genetic relatedness among individuals but calculating it from SNP data for 'unrelated' individuals. Since DNA is inherited in blocks, 'unrelated' individuals share part of the correlation structure in the DNA, which can be expressed as the realized genetic relatedness between individuals. By restricting the analysis to 'unrelated' individuals, shared environmental effects on the covariance among individuals can be assumed to be absent and confounding SNP effects with genetic effects that are not captured by SNPs is avoided. Finally, the polygenic risk score approach, uses effect sizes as detected by a GWA (meta-analysis) study in a discovery sample to test how much phenotypic variance can be explained in a target set.

CHAPTER 3

EXPLAINING INDIVIDUAL DIFFERENCES IN ALCOHOL INTAKE IN ADULTS: EVIDENCE FOR GENETIC AND CULTURAL TRANSMISSION? [1]

Abstract

Objective The current study aimed to describe what proportion of individual differences in adult alcohol intake is due to genetic differences among individuals and what proportion to differences in environmental experiences individuals have been exposed to, taking the possible influence of spousal resemblance on heritability estimates into account. It was also tested whether effects of cultural transmission of alcohol intake from parents to offspring were present.

Method In a twin-family design the effects of cultural and genetic transmission, shared and non-shared environmental influences on alcohol intake, and spousal resemblance were tested with genetic structural equation models. Data originated from adult twins, and their siblings, parents (N=12,587) and spouses (N=429) registered with the population-based Netherlands Twin Register (63.5% female, age 18-97).

Results Alcohol intake (grams per day) was higher among men than women and increased with age. Broad-sense heritability estimates were similar over sex and age (53%). Spousal resemblance was observed (r=.39), but did not significantly affect the heritability estimates. No effects of cultural transmission were detected. Overall, 23% of the variation in alcohol intake was explained by additive genetic effects, 30% by non-additive genetic effects and 47% by non-shared individual-specific environmental effects.

Conclusions Individual differences in adult alcohol intake are explained by genetic and individual-specific environmental effects. The same genes are expressed in males and females, as well as over age. A substantial part of the heritability of alcohol intake is due to non-additive gene action. Effects of cultural transmission that have been reported in adolescence are not present in adulthood.

[1] This chapter is under revision for publication in Journal of Studies on Alcohol and Drugs as:
van Beek, JHDA, de Moor, MHM, Geels, LM, Willemsen, G, Boomsma, DI. Explaining individual differences in alcohol intake in adults: evidence for genetic and cultural transmission?

1. Introduction

Alcohol use conveys risk for medical conditions (e.g. cancer) (Rehm et al. 2003; Dawson 2011) as well as psychosocial problems (e.g. alcohol use disorders; AUD) (NIAAA 2000). Despite these well-known risks, the vast majority of people consumes alcohol, at least occasionally. In Western countries, the prevalence of current drinking ranges between 72-96% in men and 59-95% in women. Heavy drinking is also common. For example, 9-17% of Dutch men and 3-11% of women drink heavily (>21 glasses and >14 glasses per week respectively for individuals aged ≥18) (Geels et al. 2013). Given the high prevalence of alcohol use in combination with the aforementioned risks, understanding the causes of individual differences in alcohol intake is important.

Twin studies can provide insight into the role of genetic and environmental factors in the variation in alcohol intake. Genetic and environmental effects on the phenotypic trait variation can be separated by the comparison of resemblance among monozygotic (MZ) twins pairs (assumed to share all their genetic material) and dizygotic (DZ) twins (who share about half of their segregating genes) (Van Dongen et al. 2012). For alcohol intake, heritability estimates range around 50% for adults (Hansell et al. 2008; Dick et al. 2009b; Dick et al. 2011; Kendler et al. 2008c; McGue 1999), with somewhat lower heritabilities for young adults (around age 20-30): between 30% and 40% (Hansell et al. 2008; Dick et al. 2011; Geels et al. 2012). The remainder of variation is mostly due to non-shared individual-specific environmental effects, although some studies have reported evidence for additional effects of the shared environment for young adults (Geels et al. 2012; Kendler et al. 2008c). These findings leave some issues unresolved, that include (a) whether parent-offspring resemblance in alcohol intake levels is fully explained by genetic resemblance, or whether parents' drinking has additional effects on the drinking behavior of their adult offspring that are not accounted for by the transmission of their genes (i.e. effects of cultural transmission), (b) whether non-random mating has an effect on the heritability, (c) to what degree the heritability of alcohol intake reflects effects of additive versus non-additive gene action, and (d) whether genetic and environmental influences on alcohol intake are equally important for men and women and over age.

Parents can affect the drinking level of their offspring by the genes they transmit, and/or the environment they provide for their children. The effect of parental alcohol intake on the alcohol intake level of their children that cannot be accounted for by genes they have in common, is referred to as cultural transmission. Effects of cultural transmission are suggested by social learning theory (Bandura and McClelland 1977) that views parents as being role models for their children. Consequently, when children see their parents drink, they may imitate that behavior (Quigley and Collins 1999). Alternatively, effects of cultural transmission can result from inadequate parenting associated with parental drinking, such as being less

inclined to enforce strict drinking rules, which is known to affect adolescent drinking (Engels and Bot 2006). Although effects of cultural transmission have been reported for alcohol initiation in mid adolescence (Koopmans and Boomsma 1996), results on whether effects of cultural transmission extend into adulthood are mixed. A large extended twin-family study reported evidence for cultural transmission of drinking frequency (Maes et al. 1999) and a study among adults raised by either biological or non-biological parents showed that having a parent with AUD increased the risk of having a diagnosis of alcohol abuse as an adult, even after taking genetic influences into account (Newlin et al. 2006). Not all studies detected effects of cultural transmission however (Kendler et al. 1994; Slutske et al. 2008; Baker et al. 2012). The studies that did were conducted in very large samples (N>14,000) (Maes et al. 1999; Newlin et al. 2006) suggesting that a proper test of cultural transmission of alcohol intake requires large sample sizes, which the current study will provide (N~12,500).

Heritability estimates of alcohol intake may be biased if spouses choose their partner (partly) based on their drinking behavior (i.e. phenotypic assortment), for instance because they enjoy the social activity of drinking together. When spouses choose each other based on his/her level of alcohol intake, individuals indirectly 'choose' their spouse based on the genes that affect alcohol intake levels with the result that spouses become genetically more alike than expected by chance. This will augment the genetic similarity among the offspring. When no phenotypic assortment is at play, the genetic similarity for DZ twins (and full siblings) is expected to be 50% (resulting from the possibility of inheriting 0, 1 or 2 parental alleles in common with the respective probabilities of 25%, 50% and 25%). When the genetic similarity among spouses is increased, the probabilities of inheriting alleles in common is increased for DZ twins (and full siblings), resulting in an increased genetic similarity among these individuals. The increased genetic similarity among spouses cannot increase the genetic similarity among MZ twins any further, as they already share ~100% of their genetic material, so the DZ twin similarity will be increased relative to the MZ twin similarity, resulting in an overestimation of environmental effects that are shared among twins and an underestimation of genetic effects. Alternatively, assortative mating may be explained by mechanisms such as social homogamy and cohabitation effects, which have no effect on heritability estimates. Social homogamy results from choosing a spouse from the same stratum. Cohabitation effects refer to the process whereby spouses become more alike the longer they are together (Van Grootheest et al. 2008). Spousal resemblance for alcohol use has been attributed to all three mechanisms, but mostly to phenotypic assortment and cohabitation effects. Phenotypic assortment has been observed for alcohol use disorders (Grant et al. 2007; Maes et al. 1998; Agrawal et al. 2006; Leonard and Eiden 2007), regular alcohol use (Agrawal et al. 2006) and alcohol intake levels (Reynolds et al. 2006; Ask et al. 2012). After taking effects of phenotypic assortment into account, additional

cohabitation effects have been observed (Grant et al. 2007) especially during the first years of the relationship (Leonard and Eiden 2007; Ask et al. 2012). Effects of social homogamy have also been detected (Maes et al. 1998; Reynolds et al. 2006), but those were smaller than effects of phenotypic assortment (Maes et al. 1998).

Genetic resemblance among relatives may result from additive and non-additive gene action. If the joint genetic effect is equal to the sum of the separate effects, gene action is additive. To the extent that allelic effects interact, either within loci (dominance) and/or between loci (e.g. additive-by-additive epistasis), non-additive gene action is present. Although non-additive effects have been reported for genes involved in alcohol metabolism (Chen et al. 1999; Kuo et al. 2008a) its effects in explaining differences in alcohol intake levels have to our knowledge not been explored.

The large sex differences in alcohol use that have been described as one of the few universal gender differences in human behavior (Holmila and Raitasalo 2005) will likely have their origin in biological factors as well as cultural factors. Biological factors include sex differences in the breakdown and elimination of alcohol. Women have higher amounts of body fat and consequently lower amounts of body water, as well as lower activity of the enzyme alcohol dehydrogenase in the stomach. Since the amount of alcohol that is metabolized in the stomach is lower ('first pass' alcohol metabolism), peak blood alcohol levels among women are higher, which has been hypothesized to reduce their alcohol intake (Wilsnack et al. 2000). Cultural factors influencing drinking behavior include gender roles. The current study will examine whether the sources of variation in alcohol intake differ over sex, including that of additive versus non-additive gene action. The latter were detected for frequency of alcohol use (Maes et al. 1999) but have not been studied yet for alcohol intake levels.

A means to study the magnitude of additive and non-additive genetic effects as well as environmental effects (individual-specific or shared within the offspring generation) is provided by the so-called extended twin-family design that includes data from parents as well as twins and siblings. This design can take effects of phenotypic assortment into account and allows for the estimation of cultural transmission effects (under specific constraints). The current study examines the genetic architecture of alcohol intake by analyzing data from 13,016 twins, siblings, parents and spouses registered with the Netherlands Twin Register (NTR) (Boomsma et al. 2002c). The amount of variation that can be ascribed to additive, non-additive genetic effects and/or shared environmental and non-shared environmental influences will be estimated, while taking effects of spousal resemblance into account. Differences in the importance of genetic and environmental effects over sex and age and effects of cultural transmission will be tested.

2. Methods

2.1 Participants

Data on alcohol intake originated from adult twins and their family members registered with the Netherlands Twin Register (NTR) who participated in the longitudinal survey research on health, personality and lifestyle that is ongoing since 1991 (Boomsma et al. 2002c; Willemsen et al. 2013). For this study we analyzed data on alcohol intake that were collected in the eighth NTR survey, which was sent out in the period 2009-2011. Data on ever alcohol use were available for 16,661 individuals. Data were excluded for 376 individuals who reported to never have drunk any alcohol. For 1,869 individuals data on alcohol intake were missing. For 700 individuals alcohol intake data were considered invalid and therefore excluded (number of drinks per day or week>100, >4 standard deviations above the mean, and/or inconsistent with data on drinking frequency). For 1,129 individuals, data were excluded, since they were not biological relatives of the twins (N=67), zygosity was unknown (N=24), they formed part of a triplet/quadruplet (N=89), were a sibling in a family with more than two same-sex siblings (N=21; a maximum of two same-sex siblings were included per family), or had another relation to the twin than co-twin, sibling or parent (N=499, including 240 spouses for whom data on length of their relationship was not available). Hence, data on alcohol intake were analyzed for 13,016 individuals: 6,619 twins, 1,492 siblings, 4,476 parents, as well as data on alcohol intake and relationship duration from 429 spouses of twins. Overall, 63.5% was female (year of birth 1911-1992). Individuals were categorized into five zygosity by sex groups, based on the zygosity and sex of the twins. See Table 1 for a complete overview of the number of individuals. For same-sex twins, zygosity was determined by DNA comparison (51%) or survey questions on physical similarity when the twins were young. Agreement between DNA zygosity and zygosity based on survey questions was >96% (Willemsen et al. 2013).

2.2 Measures

Respondents were asked to report the number of glasses of beer, wine and spirits they drank for each day of the week keeping the past 12 months in mind. In the analyses described below, alcohol intake was analyzed as the average amount of (grams of) alcohol consumed per day. This was calculated by summing the number of drinks per week, multiplied by 14 grams alcohol per glass, divided by 7 (days in the week).

Table 1 Number of participants per zygosity by sex group

	Number of participants					
Zygosity by sex group [a]	**twins [b]**	**brothers**	**sisters**	**fathers**	**mothers**	**spouses**
MZM	945 (57%)	65	84	172	212	81
DZM	541 (50%)	40	61	143	159	35
MZF	2,413 (65%)	121	180	355	467	157
DZF	1,229 (57%)	75	115	203	288	69
DOS	1,491 (44%)	71	154	314	390	87
Families without twins [c]	0	176	350	663	1,110	0
Total	6,619	548	944	1,850	2,626	429

[a] MZM: monozygotic males, DZM: dizygotic males, MZF: monozygotic females, DZF: dizygotic females, DOS: dizygotic opposite-sex

[b] percentage reflects what proportion of twins is part of a complete twin pair

[c] families in which twins did not participate

2.3 Statistical analyses

The genetic analysis of alcohol intake was performed by fitting saturated and structural equation models to raw data based on maximum likelihood estimation, using the statistical software package Mx (v1.54) (Neale et al. 2006) in five zygosity by sex groups. A saturated model was specified containing 16 sex-specific familial correlations to describe the resemblance in alcohol intake among family members (model 1). These were five twin correlations (for MZ males (MZM), DZ males (DZM), MZ females (MZF), DZ females (DZF), DZ opposite sex (DOS)), three twin-sibling and three sibling-sibling correlations (male-male, male-female, female-female), four parent-offspring correlations (father-son, mother-son, father-daughter, mother-daughter) and one spouse correlation (father-mother). In this model, two means, and two variances (males, females) were estimated. Age was included as a covariate, adding two regression coefficients to be estimated (males, females). This saturated model was used to test for differences in means, variances and associations over sex (models 2a-e). Differences in the familial correlations were tested in four submodels. First, the presence of a special twin environment was examined by testing whether twin correlations could be constrained to be equal to DZ twin-sibling and sibling-sibling correlations (model 3). Second, sex differences in the correlations were tested by equating correlations over sex for DZ twin/sibling pairs (model 4a), parent-offspring pairs (model 4b) and MZ twin pairs (model 4c). Third, age effects on the correlations were tested by equating correlations for parent-offspring pairs, to those for offspring (DZ twin/siblings) pairs. Since parent-offspring pairs differ more in age than offspring pairs, when correlations for both pairs can be constrained to be equal, there are no age effects on the correlations which gives an indication that genetic effects underlying differences in

alcohol intake are similar over age (model 5). Then, spousal resemblance for alcohol intake was tested for significance (model 6). In case of significant spousal resemblance, we explored whether this could be explained by cohabitation effects by estimating the correlation between the absolute difference in alcohol intake for spouses and the duration of their relationship in months (performed on data of 429 twin-spouse pairs and 903 parental pairs with data on relationship duration). If living together influences resemblance between spouses, this correlation will be negative, i.e. smaller differences between spouses who have been together longer. The resulting pattern of familial correlations informed the second step, whether sex differences and/or spousal resemblance needed to be specified in the genetic model.

Structural equation modeling was performed to estimate how much of the phenotypic variance in alcohol intake could be ascribed to genetic differences among individuals and how much to environmental differences. Factor models were specified in which alcohol intake among family members was regressed on latent factors that represented the genetic and environmental contributions to alcohol intake (Neale et al. 1994) (see Figure 1). In these parent-offspring models, alcohol intake of fathers and mothers (depicted as PFA and PMO) and their offspring (depicted as PT1 and PT2 for twin 1 and twin 2, siblings not depicted in the figure for clarity of presentation) was regressed on additive genetic factors (with factor loadings a), shared environmental (factor loadings c) and non-shared environmental factors (factor loadings e). The amount of variance ascribed to genetic and environmental factors can be estimated by the fact that family members share their genetic and environmental background to different degrees. MZ twin pairs are assumed to share all of their genetic material (that is, the additive genetic factors (A) were modeled to correlate 1 within twin pairs), whereas DZ twin pairs/siblings, and parents and offspring are estimated to share half of their segregating genes (A factors were modeled to correlate .5 for DZ twin, sibling and parent-offspring pairs). Shared environmental influences (represented by C factors) were correlated 1 among the offspring generation within each family and 0 with those from other families or the parental generation. Spousal resemblance was modeled to run via a Δ-path that represents the correlations between the latent genetic and environmental factors influencing the phenotypes of the parents, that result from phenotypic assortment. The increase in the additive genetic variance (RA) that results from phenotypic assortment is modeled through the additive genetic variance component that is larger than 1 in the case of phenotypic assortment. The variance of the additive genetic factor in the offspring generation (.5) reflects the segregation variance that emerges due to recombination. This within-family additive genetic variance emerges since parents pass their alleles, not genotypes, giving rise to new genetic variance in the offspring generation (Keller et al. 2009).

Cultural transmission of alcohol intake was tested in the so-called ACE model with cultural (shared environmental) transmission. Cultural transmission would be indicated by significant path loadings from parents to offspring ($t_{FA,T1}$, $t_{MO,T1}$, $t_{FA,T2}$, $t_{MO,T2}$). Since parents also contribute to variation in the offspring by transmission of their genetic values (indicated by the paths from the additive genetic factors in the parental generation to those in the offspring generation), cultural transmission refers to the non-genetic, environmental transmission of alcohol intake from parents to offspring. The presence of both genetic and shared environmental transmission, gives rise to a correlation between the additive genetic and shared environmental factors (A-C correlation, represented by $R_{A,C}$). The remainder of the variance, that is, the amount of variance that was not accounted for genetic, shared environmental, or cultural transmission effects, was estimated as non-shared, individual-specific environmental effects (E).

In the ACDE model, cultural transmission paths were fixed to zero (for model identification reasons) and non-additive genetic influences (D) were estimated (with factor loadings d). These can be estimated by assuming that MZ twin pairs share all their genetic material with each other including non-additive genetic factors (i.e., D factors are correlated 1) whereas DZ twins and sibling pairs share on average a quarter of the non-additive genetic factors (D factors are correlated .25) and parent-offspring pairs share none of their non-additive factors. By setting cultural transmission to zero, A-C correlation was no longer implicated and set to zero as well.

If the saturated model indicated sex differences in the correlation structure, sex-specific factor loadings were estimated (resulting in a sex-specific variance decomposition). If not, these were constrained to be equal over sex. Qualitative sex differences in environmental factors (shared by the offspring generation) could be examined by testing whether the correlation between the shared environmental factors ($r_{C,OS}$) was lower than 1 in opposite-sex offspring pairs. Along the same line, it could be examined whether qualitative sex differences in the genetic factors were present by testing whether the genetic correlation for opposite-sex parent-offspring pairs was significantly lower than .5. Model comparisons were based on the likelihood ratio test with a significance level of .01 (Bentler and Bonett 1980).

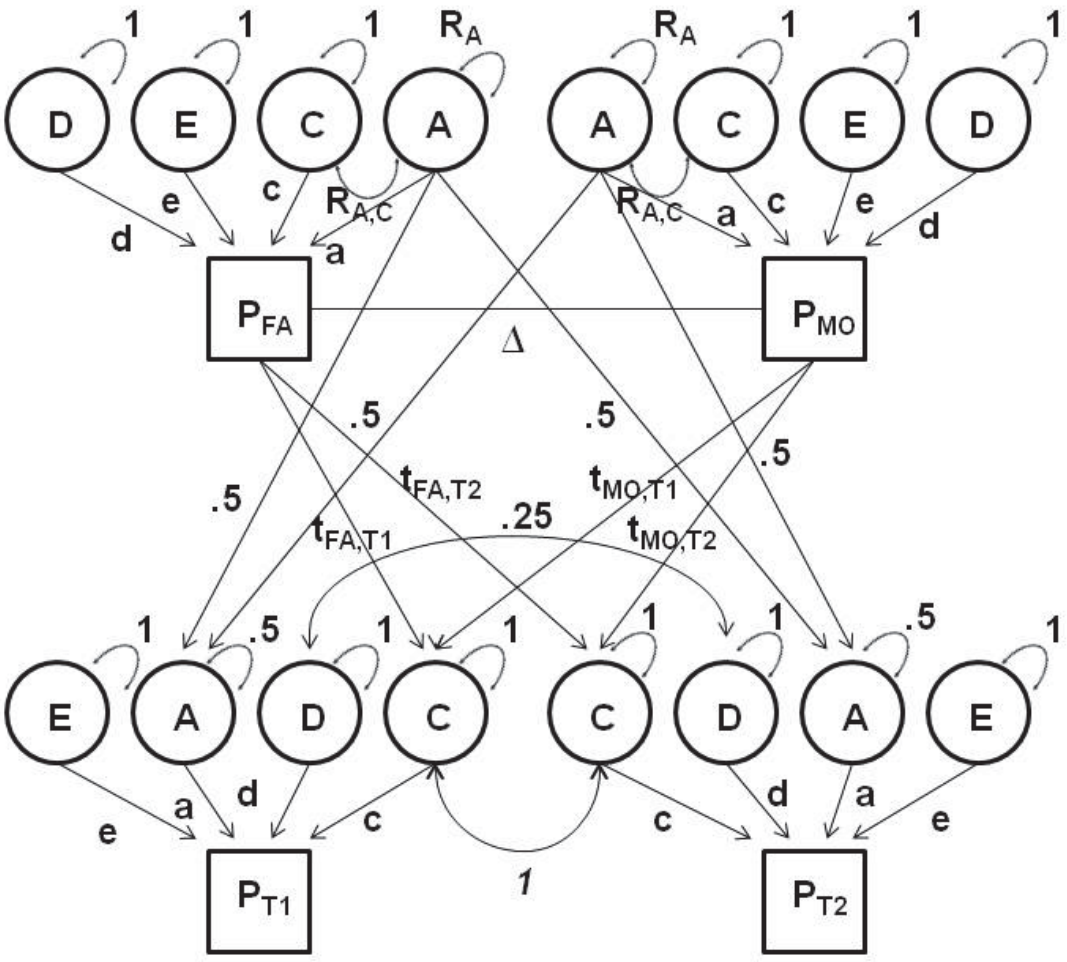


Figure 1 Path diagram for variance decomposition with extended twin-family design (ETFD) (shown for DZ twin/sibling pair with parents)

P_{FA}, P_{MO}, PT1, PT2 = phenotype of father, mother, twin/sibling 1, twin/sibling 2

A= additive genetic factor with variance R_A (differs from 1 in the case of phenotypic assortment)

D= non-additive genetic factor

C=shared environmental factor with variance R_C (differs from 1 in the case of shared environmental transmission)

E=individual-specific environmental factor

a,c,d,e= path loadings (d-paths estimated in ACDE model; set to zero in ACE model with non-genetic shared environmental transmission)

t_{FA},T1, t_{FA},T2, t_{MO},T1, t_{MO},T2 = non-genetic transmission shared environmental paths from father to twin/sibling 1, father to twin/sibling 2, mother to twin/sibling 1, mother to twin/sibling 2 (estimated in ACE model with non-genetic shared environmental transmission; set to zero in ACDE model)

$R_{A,C}$=covariation between A and C (differs from 1 in the case of shared environmental transmission)

Δ =path representing correlations between the latent factors induced by phenotypic assortment

Note: possible sex differences in the a, c, d, e and t path loadings have been omitted from the figure for reasons of clarity in the presentation

3. Results

Table 2 shows the untransformed values of alcohol intake in grams per day, separately for male and female twins, siblings and parents. After taking the natural logarithm of alcohol intake levels, alcohol intake levels became close to normally distributed (skewness -.5, kurtosis -.5 versus skewness 2.2, kurtosis 7.5 for untransformed levels). Subsequent analyses were performed on *log*-transformed values of alcohol intake. Model fit statistics for these models are presented in Table 3.

Table 2 Descriptive statistics for males and females, separately for twins, and their siblings, parents and spouses [a]

		males		females	
		mean (SD)	**N**	**mean (SD)**	**N**
twins	alcohol intake [b]	19.3 (18.7)	2076	10.4 (11.7)	4543
	age	33.0 (14.6)		33.0 (14.0)	
siblings	alcohol intake	20.2 (19.0)	548	10.1 (11.5)	944
	age	38.7 (14.3)		36.9 (13.4)	
parents	alcohol intake	21.5 (18.9)	1850	12.4 (12.4)	2626
	age	56.6 (8.1)		53.4 (8.2)	
spouses	alcohol intake	17.9 (15.9)	273	9.9 (12.0)	156
	age	45.3 (12.0)		40.4 (12.7)	

[a] data of twins, siblings and parents were analyzed to estimate to estimate heritability and, or cultural effects by genetic models; data of parents and spouses of twins were analyzed to study the mechanism of spousal resemblance for alcohol use

[b] untransformed values for alcohol intake in grams/day

Alcohol intake was higher among men than women and increased with age similarly for both sexes (beta=.11; r=.09). Inspection of the scatter plots did not indicate influence of outlier values. Variances were comparable across sex. In subsequent models, two means were specified (males, females) along with one variance and one age regression (equal over sex).

Familial correlations as estimated in the saturated model, are shown in Figure 2. DZ twin correlations were not systematically larger than sibling correlations, indicating that genetic and environmental effects make similar contributions to alcohol intake for DZ twins and siblings, rendering a special twin environment unlikely. There were no significant sex differences in the correlations. MZ correlations (r_{MZM} .53; r_{MZF} .54) could be constrained to be equal over sex (r .53), as could DZ twin/sibling correlations ($r_{DZM/SIB}$ .22; $r_{DZF/SIB}$ .24; $r_{DOS/SIB}$ .16; $r_{OVERALL}$ .20) and parent-offspring correlations ($r_{FATHER\text{-}SON}$ .17; $r_{MOTHER\text{-}DAUGHTER}$ .20; $r_{FATHER\text{-}DAUGHTER}$ .14, $r_{MOTHER\text{-}SON}$ .12; $r_{OVERALL}$ .16). Thus, the same genetic risk factors influence alcohol intake in males and females.

Table 3 Model fit statistics for genetic models of alcohol intake

		NP	-2LL	*df*	vs	$\Delta\chi^2$	Δdf	*p*	
1	saturated model	22	37932.49	12565					
2a	no sex differences mean alcohol intake	21	38504.99	12566	1	572.50	1	<.001	Higher alcohol intake levels among males
2b	no age regression mean alcohol intake	20	38040.05	12567	1	107.55	1	<.001	Alcohol intake levels increase with age
2c	no sex differences age regression mean alcohol intake	21	37934.75	12566	1	2.26	1	.133	Association with age equal over sex
2d	no sex differences phenotypic variance	21	37933.02	12566	1	.53	1	.467	Phenotypic variance equal over sex
2e	saturated model with constraints[a]	20	37935.26	12567	1	2.77	2	.250	
3	DZ twin=sibling correlations	14	37942.50	12573	2e	7.24	6	.299	Equal correlations for twin and sib pairs; no special twin environment
4a	male=female=opp sex DZ twin/sib corr	12	37945.85	12575	2e	10.59	8	.226	No sex diff in twin-/sibling correlations
4b	male=female=opp sex parent-offspring corr	17	37940.37	12570	2e	5.12	3	.164	No sex diff in parent-offspring corr
4c	male=female MZ correlation	19	37935.30	12568	2e	.039	1	.844	No sex diff in MZ correlations
5	DZ twin=sib=parent-offspring corr	8	37953.02	12579	2e	17.76	12	.123	No age diff corr first-degree relatives
6	spouse correlation = 0	19	38093.36	12568	2e	158.11	1	<.001	Significant spousal resemblance
7	ACE model with cultural transmission	11	38002.28	12576	2e	67.02	9	<.001	ACE model with cultural transmission does not fit data
7a	drop cultural transmission	10	38002.28	12577	7	0	1	1	No cultural transmission effects
8	ACDE model	10	37950.59	12577	2e	15.33	10	.121	ACDE model fits data
9	ADE model (drop C to 0)	9	37950.59	12578	8	0	1	1	No shared environmental effects
10	ACE model (drop D to 0)	9	38040.09	12578	8	89.51	1	<.001	Non-additive genetic effects important

NP=number of parameters in statistical model

-2LL=-2 *log*-likelihood, fit function, for model with *df* degrees of freedom

$\Delta\chi^2$=model fit statistic: difference in -2LL of two nested models approximately distributed as χ^2 with $df = \Delta df$, the difference in NP between the models; *p*-value was regarded significant when < .01; diff=differences; corr=correlation; sib=sibling

[a] Constraints included two means (males/females) and one variance and beta for the age regression (equal over sex).

Parent-offspring pairs, who differ more in age than offspring pairs (DZ twin/siblings), showed a similar resemblance in alcohol intake levels as the offspring pairs ($r_{OVERALL}$ .18), indicating that similar genes are expressed over age. Alcohol intake correlated significantly between spouses (*r* .39). Strictly speaking, spousal resemblance did not increase with increasing relationship length (at α=.01), but the correlation was very close to significant ($\chi^2(1)=6.51$, *p*=.011; *r*=-.07, 95% CI -.13, -.02), suggesting that cohabitation effects may explain some of the spousal resemblance for alcohol intake, although large effects are unlikely. In the genetic model, spousal resemblance for alcohol intake was therefore modeled as resulting from phenotypic assortment. Given that the correlations did not differ across sex, path loadings and cultural transmission paths were constrained to be equal over sex.

Cultural transmission of alcohol intake was not significant and hence, the ACDE model, being more parsimonious, gave a better fit compared to the saturated model than the ACE model with cultural transmission. Taking the ACDE model as a reference, non-additive genetic effects were significant, but shared environmental effects were not. In the final model, 23.4% of the variance in alcohol intake was explained by additive genetic effects (95% CI 19.1%, 27.5%), 29.9% by non-additive genetic effects (95% CI 23.9%, 36.0%), and 46.7% by non-shared individual-specific environmental effects (95% CI 43.1%, 50.7%). Quantifying the effect of spousal resemblance on the heritability indicated that .1% of the broad-sense heritability could be ascribed to effects of phenotypic assortment.

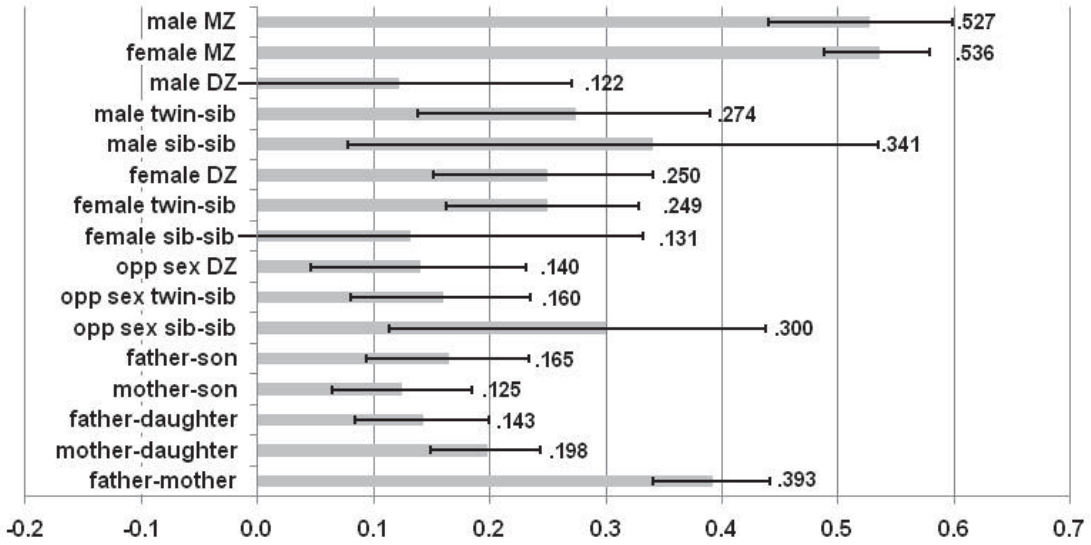


Figure 2 Familial correlations for alcohol intake (with 95% confidence intervals)

4. Discussion

We examined the genetic architecture of alcohol intake in a large sample of adult twins, siblings, and parents. Specifically, we tested (a) whether there were effects of cultural transmission, (b) if heritability estimates were affected by non-random mating, (c) to what degree the heritability was due to additive versus non-additive gene action and (d) if the importance of genetic and environmental risk factors differed over sex and age.

Effects of cultural transmission or the shared environment on adult alcohol intake are not present. Spouses resemble each other in their alcohol intake levels (*r* .39). Large cohabitation effects were not detected however, suggesting that most of the spousal resemblance is due to effects of phenotypic assortment. Taking phenotypic assortment for alcohol intake into account did not significantly influence the heritability estimates. Alcohol intake is higher among men than women, and increased with age. The latter is likely to be explained by increased welfare standards and healthy life years among the Dutch elderly (Geels et al. 2013). Previous research has pointed at a lower heritability of alcohol intake among women (Dick et al. 2009b; McGue 1999), but the current study shows that the genetic architecture underlying differences in alcohol intake is similar over sex (broad-sense h^2 53%) and age. The absence of sex differences in this study and other recent studies (Dick et al. 2009b; Dick et al. 2011; Geels et al. 2012; Sartor et al. 2010) may be explained by the growing convergence in male and female alcohol use due to earlier initiation and augmented alcohol intake by women (Geels et al. 2013; Keyes et al. 2011) which has been associated with changes in gender roles (Keyes et al. 2011; Rahav et al. 2006). Interestingly, a substantial part of the underlying genetics of alcohol intake is due to genes that act in a non-additive manner (30%).

Genes have been hypothesized to impact variation in alcohol use via two broad pathways (Kendler et al. 2012). Genetic effects specific to alcohol intake can reflect those on alcohol metabolism (Hurley and Edenberg 2011; Van Beek et al. 2010) and sensitivity to the response to alcohol (Schuckit 2009; Heath et al. 1999). A second pathway through which genes can have their effect on alcohol intake is by personality characteristics such as impulsivity, disinhibition, sensation seeking (Schuckit 2009) and externalizing psychopathology (Kendler et al. 2011a; Krueger 1999), which are traits that influence risk for substance use in general (Kendler et al. 2012). Interestingly, for both alcohol metabolism (Chen et al. 1999; Kuo et al. 2008a) and personality (e.g. novelty seeking) (Keller et al. 2005), non-additive genetic effects have been detected.

It is tempting to speculate about the underlying mechanism generating these non-additive genetic effects. It can be argued that the genetic non-additivity may reflect a different expression of genes over age. This interpretation is unlikely given that the offspring and parent-offspring correlations were not significantly different from each other. Alternatively, the non-

additive genetic effects can reflect effects of dominant gene action and/or epistasis. Dominant gene action requires that individuals share both alleles at a locus. This is assumed to be the case for all MZ twin pairs, and for one quarter of the DZ twin-sibling pairs, but not for parent-offspring pairs, because parents transmit only one of their alleles to their children. Effects of dominant gene action are thus suggested when DZ twin/sibling correlations are higher than those among parent-offspring pairs. Epistasis represents the effects of interacting risk alleles from different loci, which is assumed to be equal for all first-degree relatives. In the presence of additive-by-additive epistasis, all first-degree relatives are assumed to share 25% of the non-additive genetic variation. Model fitting could be then be performed to disentangle non-additivity due to dominance and that due to additive-by-additive epistasis (Heath et al. 1984). In the case of higher-order epistasis, the correlation between non-additive genetic factors is lower than .25 for first-degree relatives since the more alleles are interacting, the less probable it is that DZ twin or sibling pairs share all these interacting alleles (Posthuma et al. 2003). Higher-order epistasis would thus predict that model fit improves when the correlation among non-additive genetic factors in the offspring correlation is lowered (Keller et al. 2005). When exploring possible effects of dominance and epistasis for the current study, clear effects were not detected. Parent-offspring correlations and offspring correlations were similar, suggesting additive-by-additive epistasis rather than dominance. When assuming that the correlation between non-additive genetic factors for DZ twins and siblings was .20 or .15, an improve in model fit was not observed hence evidence for higher-order epistasis was not detected. However, power may be low to detect these effects in this study. Heath et al. (1984) showed that power was sufficient to disentangle genetic dominance from additive-by-additive epistasis for highly heritable trait. Further research could focus on the power of these tests in less heritable traits, for different levels of genetic dominance and (different types of) epistasis. Nevertheless, a clear effect of non-additive gene action is evident irrespective of the precise mechanism. Future gene finding studies may benefit when taking this non-additivity into account, for instance by using prediction models that involve complex interactions among genetic markers, such as random forests (Molinaro et al. 2011).

There was no evidence for culturally transmitted effects of alcohol intake from parents to offspring. Differences may be partly explained by the use of self-report data. In the current study, all participants reported on their own drinking behavior. Any rater effects (measurement error) were included in the estimates of individual-specific environmental effects. With regard to the two large studies that reported evidence for effects of cultural transmission, Maes et al. (1999) also analyzed self-reported data on alcohol use, whereas Newlin et al. (2006) analyzed data from probands along with their reports on their parents' alcohol use. Similarity between the proband and his/her biological parents was estimated as effects resulting from both cultural

and genetic transmission, but for probands from adoptive or step families, any (reported) similarity in the proband's alcohol use behavior and that of his/her parents, was a direct measure of cultural transmission. Thus to the extent rater effects were present, these were incorporated in the estimates of cultural transmission.

The absence of effects of cultural transmission of alcohol intake does not mean that parents cannot influence the drinking behavior of their children apart from the transmission of their genes. For younger adolescents cultural transmission of alcohol use patterns of parents to children seems to play a role, although peer influences on adolescent alcohol use are generally larger than parental influences (Hopfer et al. 2003; Allen et al. 2003). For adolescents aged 15-16, it might explain up to 10% of the variation in alcohol use (Koopmans and Boomsma 1996). In addition, parents can influence adolescent drinking through certain styles of parenting that may be unrelated to the parents' own drinking behavior (Smit et al. 2008; Petrie et al. 2007; Eijnden et al. 2011).

It might be argued that including the group of not-current drinkers in the study, has introduced bias if these include individuals who have stopped drinking because of their propensity to indulge into heavy drinking (which is likely to be reflective of a high genetic risk for heavy drinking). This seems unlikely however. For 1,426 out of 1,714 not-current drinkers, data were available on their reasons for not drinking and only 0.8% stopped drinking because of problems with alcohol use. Most individuals reported not to drink alcohol because they did not like the taste (52.9%), did not feel the need to drink (16.3%) or because of reasons of health (17.5%). Some only drank at special occasions (2.1%). Other reasons for not drinking were (unpleasant) side effects (3.3%), principles or reasons of belief (2.0%), fear of alcohol problems because of what occurred in their surroundings (2.4%) or other reasons (2.6%).

To conclude, the current study showed that 53% of the variation in adult alcohol intake is explained by genes, taking the significant spousal resemblance into account. Effects of cultural transmission were not significant. The substantive amount of non-additive genetic variation representing effects of dominant gene action and/or epistasis presents an important venue for further study.

CHAPTER 4

STABLE GENETIC EFFECTS ON SYMPTOMS OF ALCOHOL ABUSE AND DEPENDENCE FROM ADOLESCENCE INTO EARLY ADULTHOOD [2]

Abstract

Relatively little is known about how genetic influences on alcohol abuse and dependence (AAD) change with age. We examined the change in influence of genetic and environmental factors which explain symptoms of AAD from adolescence into early adulthood. Symptoms of AAD were assessed using the four AAD screening questions of the CAGE inventory. Data were obtained up to six times by self-report questionnaires for 8,398 twins from the Netherlands Twin Register aged between 15 and 32 years. Longitudinal genetic simplex modeling was performed with Mx. Results showed that shared environmental influences were present for age 15-17 (57%) and age 18-20 (18%). Unique environmental influences gained importance over time, contributing 15% of the variance at age 15-17 and 48% at age 30-32. At younger ages, unique environmental influences were largely age-specific, while at later ages, age-specific influences became less important. Genetic influences on AAD symptoms over age could be accounted for by one factor, with the relative influence of this factor differing across ages. Genetic influences increased from 28% at age 15-17 to 58% at age 21-23 and remained high in magnitude thereafter. These results are in line with a developmentally stable hypothesis that predicts that a single set of genetic risk factors acts on symptoms of AAD from adolescence into young adulthood.

[2] This chapter is published as:
van Beek, JHDA, Kendler, KS, de Moor, MHM, Geels, LM, Bartels, M, Vink, JM, van den Berg, SM, Willemsen, G, Boomsma, DI (2012) Stable genetic effects on symptoms of alcohol abuse and dependence from adolescence into early adulthood. Behav Genet 42: 40-56

1. Introduction

Twin and adoption studies have consistently shown that the risk for a lifetime history of alcohol abuse/dependence (AAD) is strongly influenced by genetic factors with estimates of heritability ranging around 28% in adolescents (Dick et al. 2009b) and from 45 to 64% in adults (Cloninger et al. 1981; Goodwin et al. 1973; Heath et al. 1997; Kendler et al. 1992; Kendler et al. 1997; Knopik et al. 2004; Liu et al. 2004; Pickens et al. 1991; Prescott and Kendler 1999; Reed et al. 1996; Sartor et al. 2010; True et al. 1999). These cross-sectional studies could however not examine whether AAD in adolescence and adulthood is influenced by the same or different genetic factors. For this, longitudinal studies are needed. Within a longitudinal design, the relative contributions of genetic and environmental influences have been explored from adolescence into young adulthood for a wide range of alcohol-related phenotypes. Generally, with increasing age the importance of additive genetic factors increases and that of shared environmental influences decreases for alcohol use measures such as alcohol initiation (Koopmans and Boomsma 1996; Viken et al. 1999), frequency of alcohol use (Geels et al. 2012; Rose et al. 2001; Viken et al. 1999), quantity of alcohol use (Kendler et al. 2008c; Geels et al. 2012) and perceived alcohol availability (Gillespie et al. 2007). In contrast to alcohol use, the change of the genetic underpinnings of symptoms of alcohol misuse during adolescence and early adulthood, has seldomly been explored in a longitudinal design.

In this paper, the primary goal is to investigate how genetic risk factors for symptoms of AAD change across age. In particular, we seek to discriminate between two hypotheses about the developmental pattern of genetic risk factors for AAD symptoms in the key time period of adolescence and early adulthood. In this period drinking habits are commonly formed, levels of alcohol consumption typically peak (Koppes et al. 2000; Midanik and Clark 1994; Moore et al. 2005; Poelen et al. 2005) and symptoms of AAD usually begin (Harford et al. 2006; Schuckit et al. 1998). The *developmentally stable* hypothesis predicts that a single set of genetic risk factors will impact on AAD symptoms from late adolescence through early adulthood. By contrast, the *developmentally dynamic* hypothesis predicts that new genetic influences on AAD symptoms "come on line" at a particular age. These genetic *innovations* give rise to a qualitative change in genetic effects. Regardless of qualitative change, the importance of genetic risk factors may change quantitatively over time. This can result from genetic *amplification*, if the importance of genetic influences increases over age, or from genetic *attenuation* if the importance of genetic factors declines during development.

The current study analyzed longitudinal survey data on symptoms of AAD in a cohort of 8,398 twins from the Netherlands Twin Register aged between 15 and 32 years. Symptoms of AAD were assessed by the CAGE, a four item screening instrument for AAD, and were obtained up to six times. To ensure data from participants of different ages and sex could be meaningfully

compared, measurement invariance of the CAGE was tested over age and sex. In addition to the primary goal of clarifying the nature of genetic effects on AAD symptoms over age, we also examined qualitative and quantitative changes of environmental risk factors. To achieve this aim, simplex models were fitted by which genetic and environmental influences could be separated from variance due to measurement error and the presence of genetic and environmental innovation could be tested.

2. Methods

2.1 Sample

Data came from a sample of twins (aged 15-32 years; N=8,398) who were registered with the Netherlands Twin Register (NTR) and had answered survey questions about symptoms of AAD as part of the ongoing longitudinal study on health, personality and lifestyle that is being conducted in all active participants (twins and their relatives) of the Adult Netherlands Twin Register (ANTR) since 1991 (Boomsma et al. 2002c; Boomsma et al. 2006). Questions about AAD symptoms were included in the surveys in 1995, 1997, 2000, 2002, 2004/2005 and 2009/2010. In 2009/2010, twins of 18 years and older (and their family members) who were followed since their birth by the Young Netherlands Twin Register (YNTR) (Bartels et al. 2007) were also invited to take part in the ANTR study. Extended information about the NTR sample and data collection can be found in Boomsma et al. (2002; 2006).

In our sample, very few participants under the age of 15 reported symptoms of AAD (see also Poelen et al. (2005)). Therefore, to study the genetic architecture of AAD symptoms during adolescence and early adulthood, we included all participants aged 15 or older with data on symptoms of AAD. Because we collected data on symptoms of ADD from 1995 to 2010, spanning a 16 year period, the upper age limit was 32 years. Thus, for this study data on symptoms of AAD were analyzed if obtained between the ages 15 and 32. Zygosity for same-sex twin pairs was based on DNA polymorphisms if available (42%), or otherwise on survey questions about zygosity (58%). Agreement between DNA zygosity and zygosity based on survey questions for same-sex twins was 97% (Willemsen et al. 2005). The 8,398 participants (62% female; year of birth 1964-1991) provided up to six measurements of AAD symptoms. Amongst this group, 1.9% had reported that they had not or rarely drunk alcohol. For 1,588 individuals (18.9%) two measurements on AAD symptoms were analyzed, for 1,175 (14.0%) three, for 778 (9.3%) four, for 344 (4.1%) five and for 24 individuals (0.3%) six measurements. Additional repeated measures at age 14 or younger or age 33 or older were available but not analyzed for 674 of the 8,398 individuals (15,0%). The relative large number of twins with one survey (N=4,489; 53.5%) was mainly due to the entry from participants of the YNTR in

2009/2010 who were invited to participate in the ANTR research for the first time (N=2,151; 47,9%).

2.2 Measure

Symptoms of AAD were assessed by the CAGE, originally developed as a screening instrument for medical settings (Ewing 1984), which gives an indication of the presence of symptoms of AAD (Dhalla and Kopec 2007). The CAGE consists of four items that can be answered with 'yes' or 'no': Have you ever felt that you should **C**ut down your drinking?; Have people **A**nnoyed you by criticizing your drinking?; Have you ever felt bad or **G**uilty about your drinking?; Have you ever had a drink in the morning (as an '**E**ye opener') to steady your nerves or get rid of a hangover? In 2009/2010, there were three response categories: 'yes, during the last year'; 'yes, not during the last year'. These two yes categories were recoded into one, since its frequency was comparable to the frequency of the single yes categories in earlier years. If one of the four CAGE items was missing (N=64; 0.3%), its value was imputed based on the mean of the three other answers. In case of two or more missing answers (N=30; 0.2%), the CAGE was set to missing.

The number of participants aged 15-17 who gave two or more positive responses on the CAGE was low (see Table 1). This leads to numerical problems when analyzing longitudinal twin data. Therefore, the analyses were conducted on the dichotomy of zero versus one or more yes answers. In analyses that are described in more detail below, we investigated whether this dichotomization influenced the results.

2.3 Data file restructuring

The mean age across surveys was 19.7 (SD 2.9) in 1995, 21.6 (SD 3.6) in 1997, 24.2 (SD 3.9) in 2000, 26.4 (SD 3.7) in 2002, and 26.9 (SD 3.6) in 2004/2005. Due to the entry of a large new group of young participants, the mean age was 22.9 (SD 4.9) in 2009/2010. To study age-related changes in genetic influences on AAD symptoms, the data file was restructured from observations by survey to observations by age band. Six age groups were created: 15-17, 18-20, 21-23, 24-26, 27-29, and 30-32, since surveys were sent out every 2-3 years. If two or more observations of one individual fell into the same age band, the most recent measurement was selected (at which the co-twin also answered the CAGE). Table 2 shows the number of observations for each age group, separately for sex and zygosity.

Table 1 Prevalence of reporting 0, 1 or 2+ yes answers on the CAGE by sex and age group

	Males				Females				Total
Age	**0**	**1**	**2+**	**No. of obs.**	**0**	**1**	**2+**	**No. of obs.**	**No. of obs.**
15-17	435 (84.1%)	56 (10.8%)	26 (5.0%)	517	629 (91.0%)	45 (6.5%)	17 (2.5%)	691	1208
18-20	956 (68.7%)	268 (19.3%)	167 (12.0%)	1391	1885 (83.8%)	244 (10.8%)	120 (5.3%)	2249	3640
21-23	813 (63.0%)	295 (22.9%)	183 (14.2%)	1291	1832 (83.2%)	242 (11.0%)	129 (5.9%)	2203	3494
24-26	677 (61.8%)	240 (21.9%)	178 (16.3%)	1095	1629 (83.8%)	199 (10.2%)	116 (6.0%)	1944	3039
27-29	585 (67.2%)	171 (19.7%)	114 (13.1%)	870	1393 (82.6%)	183 (10.9%)	110 (6.5%)	1686	2556
30-32	498 (69.1%)	129 (17.9%)	94 (13.0%)	721	1262 (83.7%)	148 (9.8%)	98 (6.5%)	1508	2229

No. of obs.=number of observations

Table 2 Number of observations for twins from (in)complete twin pairs per age and zygosity by sex group

	MZM		DZM		MZF		DZF		DOS		Total
Age	**complete pair**	**incomplete pair**	**complete pair**	**incomplete pair**	**complete pair**	**incomplete pair**	**complete pair**	**incomplete pair**	**complete pair**	**incomplete pair**	**No. of obs.**
15-17	186	18	140	10	318	26	164	19	290	37	1208
18-20	458	104	324	85	896	158	494	140	704	277	3640
21-23	458	93	288	71	844	181	498	150	612	299	3494
24-26	358	116	182	108	742	196	414	145	504	274	3039
27-29	232	124	152	102	618	226	312	172	310	308	2556
30-32	168	129	106	102	514	229	288	166	258	269	2229

No. of obs.=number of observations

2.4 Data analysis

Data analysis consisted of four steps: testing whether the CAGE was measurement invariant over age and sex, estimation of longitudinal and twin correlations, cross-sectional genetic modeling and longitudinal genetic modeling. In all analyses, the CAGE was treated as a threshold character assuming that a standard normal liability distribution underlies the ordered categories.

Measurement invariance We first investigated whether the CAGE was measurement invariant over age and sex. From the dataset as described above one observation per individual was selected. For individuals with repeated measures, observations were selected that gave age groups of roughly the same size. This resulted in the following 12 mutually exclusive age by sex groups: twins aged 15-17 (383 males, 691 females); twins aged 18-20 (698 males, 1142 females); twins aged 21-23 (530 males, 754 females); twins aged 24-26 (353 males, 705 females); twins aged 27-29 (870 males, 1487 females); twins aged 30-32 (343 males, 442 females).

In Mplus 5.1 (Muthen and Muthen 2007) one factor models were specified with the four CAGE items as indicators. First, a configural invariance model was fitted. In this model the factor loadings and thresholds were estimated for each group. Strong factorial invariance and strict factorial invariance models were tested in line with Slof-op 't Landt et al. (2009). In the strong factorial invariance model, the factor loadings and thresholds were constrained to be equal over the 12 groups. The residual variances and means and variances of the latent factor were then estimated. In the strict factorial invariance model, the residual variances were also constrained to be equal over groups. This model tested if for all groups the same proportion of variance is explained by the latent factor. In an additional model, sex differences at the latent factor level were evaluated by constraining the factor means for males and females to be equal. Absence of sex differences at the latent factor level is not a requirement for measurement invariance, but makes the interpretation of results from the cross-sectional and longitudinal analyses easier. Model fit was evaluated with the RMSEA, a measure that indicates the approximate fit of the measurement model in the population, since the χ^2 difference test is directly affected by sample size. An estimate in between .00 and .05 indicates good model fit (Schermelleh-Engel et al. 2003). To correct for the non-independence of twin data, for all models option *complex* was used. This method was shown to perform satisfactory in the context of family data by Rebollo, de Moor, Dolan, and Boomsma (2006).

Estimation of longitudinal and twin correlations To get a first impression of the relative importance of genetic and environmental influences on AAD symptoms, tetrachoric twin correlations for the dichotomous CAGE scores (0 vs. 1+ yes answers) were estimated. These analyses were performed with the package polycor (polychor(var1, var2, ML=T, std.err=T)) in R

(version 2.9.2; http://www.***r***-project.org/) (R Development Core Team 2012) using maximum likelihood estimation. For each of the five zygosity by sex groups, six cross twin-within age correlations (30 in total), 15 cross twin-cross age correlations (75 in total), and 15 within person-cross age correlations were computed.

Sex differences in the correlations and the prevalence of one or more yes answers were tested cross-sectionally by model fitting in Mx (version 3.2) (Neale et al. 2006). In a multigroup analysis of five zygosity by sex groups, qualitative and quantitative sex differences in the correlations were tested for by constraining the correlations to be equal over sex. To examine sex differences in the prevalence, a sex effect on the threshold was tested for significance. The fit of the restricted models was compared to that of the full model using the likelihood ratio test.

To investigate whether the way the CAGE was dichotomized affected the results, correlations based on the dichotomous CAGE were compared to those based on the CAGE with three response categories: 0, 1, and 2+ yes answers. The polychoric correlations for the CAGE with three response categories were estimated for the age groups 18-20, 21-23, 24-26, 27-29 and 30-32 cross-sectionally, using Mx. For the 15-17 year olds the number of 2+ yes answers was too low to estimate polychoric correlations. In the analyses described above on a few occasions empty cells were observed (0.7-1.7%). These were replaced by 1 to be able to run the model estimation.

Genetic analyses The relative importance of genetic and environmental influences on the CAGE was investigated by decomposing the variance-covariance structure of the dichotomous CAGE (0 vs. 1+ yes answers) into latent factors representing additive genetic (A), non-additive genetic (D) or shared environmental influences (C) and unique environmental (E) influences. First, cross-sectional genetic analyses were conducted for each age group separately. Next, longitudinal genetic analyses were performed. All genetic analyses were conducted in Mx (Neale et al. 2006) in two zygosity groups, as sex differences in the twin correlations were absent. For model identification purposes, the variance was fixed at unity for all age groups in both the cross-sectional models as well as in the longitudinal model.

Cross-sectional genetic analyses The decision to fit an ACE or ADE model was based on the correlation structure for a particular age group. If the correlation for MZ twins was smaller than twice the correlation for DZ twins, an ACE model was fitted. If the MZ correlation was more than twice the DZ correlation, the ADE model was fitted to the data. The importance of additive genetic, non-additive genetic and shared environmental factors was tested by dropping the *a*, *d* or *c* parameter from the model and comparing the fit of the restricted model with that of the full model using the likelihood ratio test.

Longitudinal genetic analyses The change in genetic and environmental influences on symptoms of AAD, and specifically whether genetic influences could be best described by the

developmentally dynamic or by the developmentally stable hypothesis, was explored by modeling the data in a longitudinal ACE model. The longitudinal model, which examines the cross-age correlations as well as the within-age correlations, has more power to discriminate between additive genetic and shared environmental factors than the cross-sectional models, which only examine the within-age correlations. In the longitudinal model, one threshold (six in total) and one deviation from the threshold for females (six in total) were estimated in each age group.

In the saturated (full ACE) model the variance-covariance structure was first explored with a Cholesky decomposition (6 A factors, 6 C factors, 6 E factors) (model 1). In model 2, the sex effects on the thresholds were constrained to be equal over the six age groups. Simplex models were specified to test whether the genetic and environmental influences on AAD symptoms showed qualitative or quantitative change. The final model was built by testing a series of restricted submodels. The unique environmental structure was first examined, followed by the development of shared environmental influences and finally by the structure of the genetic influences. All submodels were compared to the preceding model, with the likelihood ratio test.

First, in submodel 3a an unique environmental simplex structure was specified (Gutmann 1955; Boomsma and Molenaar 1987) that allowed for both stable and dynamic influences. This model contained six latent unique environmental variance components, each with one observed measurement as its (only) indicator. The unique environmental variance underlying the observed measurement at a particular age group was determined by the transmission terms (β) that describe the amplification or attenuation of the unique environmental variance that was already present in the previous age group (E_{i-1}), plus the innovation terms (ζ) that allow for new unique environmental variance 'coming on line': $E_i = \beta E_i E_{i-1} + \zeta E_i$ (Neale and Cardon 1992). Since 15-17 was the first age group, no unique environmental variance could be transmitted from an earlier age group. Therefore, the unique environmental variance for this age group reduced to ζE_1^2. For the CAGE at age 18-20, the unique environmental variance equaled $\beta E_1^2 * \zeta E_1^2 + \zeta E_2^2$ and for age 21-23 $\beta E_2^2 * (\beta E_1^2 * \zeta E_1^2 + \zeta E_2^2) + \zeta E_3^2$. The unique environmental covariance over age is modeled to run via the transmission terms only and can therefore be separated from variance due to measurement error that is modeled to influence the variances but not the covariances. For model identification purposes, a constraint has to be applied to the measurement error variance at the outer categories (Neale and Cardon 1992). In this study, variance due to measurement error was constrained to be the same for all age groups (submodel 3a). Whether unique environmental influences were purely age-specific was tested by fixing the transmission terms at zero in submodel 3b.

Secondly, a simplex structure was specified for shared environmental influences allowing for both stable and dynamic influences (submodel 4a). Next, in submodel 4b the shared environmental innovation terms were dropped from the model to test whether shared environmental influences could be described by one factor.

Thirdly, the genetic structure was investigated. In submodel 5a a simplex structure was specified for genetic factors including transmission and innovation terms that allowed for both stable and dynamic genetic influences. Subsequently, in submodel 5b, the innovation terms were dropped from the model allowing for stable genetic influences only. The simplex structure including transmission and innovation terms is shown in Figure 1a and the model without the innovation terms is shown in Figure 1b.

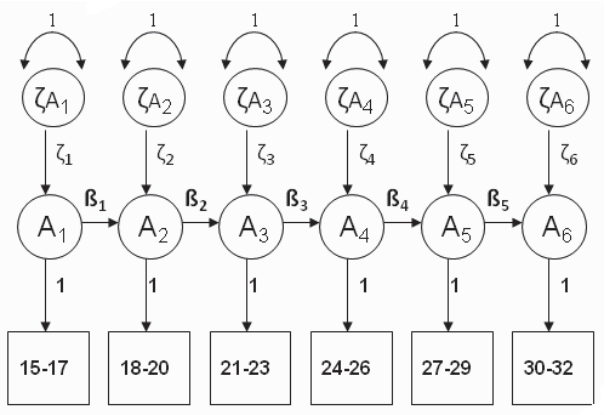


Figure 1a Simplex structure *with innovation* for analyzing 6 age groups (in squares) (shown for 1 twin only), with 6 latent variance components (A1 to A6), 5 transmission terms (β_1 to β_5) and 6 innovation terms (ζ_{A1} to ζ_{A6}).

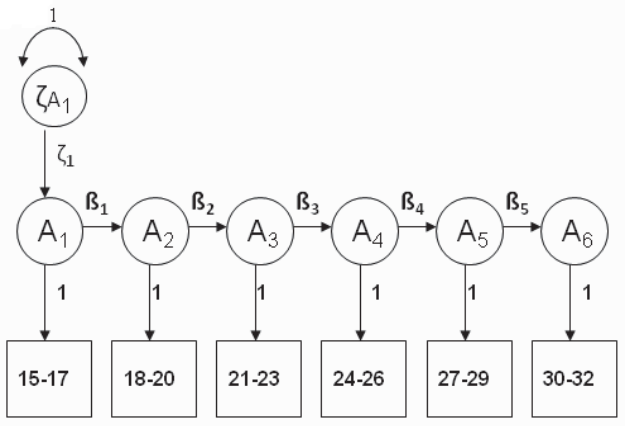


Figure 1b Simplex structure *without innovation* for analyzing 6 age groups (in squares) (shown for 1 twin only), with 6 latent variance components (A1 to A6), 5 transmission terms (β_1 to β_5) and 1 innovation term (ζ_{A1}).

3. Results

3.1 Measurement invariance

The configural invariance model, in which the factor loadings and thresholds were estimated freely for each group, had a good fit (χ^2 (21)=19.84, p=.531; RMSEA=.001). Constraining the factor loadings and thresholds over the groups to be equal was allowed, as indicated by the fit of the strong factorial invariance model (χ^2(36)=43.66, p=.178; RMSEA=.017). Restricting the residual variances to be equal over the groups in the strict factorial invariance model was also permitted, based on the RMSEA (χ^2(71)=119.21, p<.001; RMSEA=.031). This means that for different ages and sexes, the same proportion of the variance can be explained by the latent factor. The CAGE, as it is administered in our surveys, is thus measurement invariant over age and sex. Accordingly, answers on the CAGE can be compared from one age and sex to another. With the CAGE being measurement invariant over age and sex, differences in the prevalence are allowed for when these result from a difference at the latent factor level. In an additional model it was examined whether sex differences existed at the latent factor level by testing whether the factor means for males and females could be constrained to be equal. This changed the model fit based on the RMSEA from good into acceptable (Schermelleh-Engel et al. 2003) (χ^2(73)=246.54, p<.001; RMSEA=.058), suggesting that sex differences exist at the latent factor level.

3.2 Longitudinal and twin correlations

In Table 3 within person-cross age correlations are presented for the dichotomous CAGE scores (0 vs. 1+ yes answers).

Table 3 Estimates of tetrachoric within-person correlations (with standard errors and number of observations) for the CAGE as dichotomy (0 vs. 1+ yes answers)

	15-17	**18-20**	**21-23**	**24-26**	**27-29**	**30-32**
15-17	1					
18-20	.43 (.07)	1				
	N=672					
21-23	.41 (.08)	.59 (.04)	1			
	N=616	*N*=1224				
24-26	.38 (.08)	.45 (.05)	.65 (.03)	1		
	N=540	*N*=1125	*N*=1586			
27-29	.20 (.14)	.47 (.06)	.67 (.03)	.75 (.03)	1	
	N=289	*N*=840	*N*=1270	*N*=1551		
30-32	.29 (.14)	.48 (.08)	.63 (.04)	.69 (.03)	.73 (.03)	1
	N=177	*N*=455	*N*=922	*N*=1147	*N*=1167	

For intervals up to 12 years, the correlations were of similar magnitude. For intervals larger than 12 years, the correlations started to decrease, but the number of individuals for whom data was available on these large time intervals was relatively small.

The tetrachoric cross-twin correlations are shown in Table 4. The cross twin-within age correlations (presented at the diagonal *in italic*) showed a different pattern for MZ and DZ twins. MZ correlations were of similar magnitude for the ages 18-29: .40-.50 for MZF and .50-.60 for MZM. At age 15-17 the correlations were somewhat higher (r_{MZM} = .73; r_{MZF} = .54) and for MZM at age 30-32 somewhat lower (.35). DZ correlations started high at age 15-17 (r_{DZM} = .68; r_{DZF} = .56; r_{DOS} = .48), but were much lower at later ages. This was seen most clearly for DZM, but was also true for DZF and DOS twins. This pattern suggests the influence of shared environmental factors at younger ages and the increase of importance of genetic influences over time.

The cross twin-cross age correlations (shown off-diagonal in Table 4) were lower than the within person-cross age correlations. Overall, cross twin-cross age correlations also decreased over age, although the pattern was less consistent than that seen for the within person correlations.

Table 5 provides the results for testing sex differences in the correlations cross-sectionally. For all age groups, male and female MZ correlations and male DZ, female DZ and DZ opposite sex correlations could be constrained to be equal. Therefore, Table 5 shows the pooled correlations over sex, together with thresholds estimates and deviations from the threshold for females. As described in the section on measurement invariance, there was some indication for true sex differences in CAGE scores (i.e., sex differences that were not due to differences in measurement across sex). To account for these differences, sex effects on the thresholds were tested. Dropping the sex effect on the threshold led to a significantly worse model fit in all age groups (15-17: $\chi^2(1)=15.86$, $p<.001$; 18-20: $\chi^2(1)=109.33$, $p<.001$; 21-23: $\chi^2(1)=153.15$, $p<.001$; 24-26: $\chi^2(1)=157.37$, $p<.001$; 27-29: $\chi^2(1)=67.86$, $p=.001$; and 30-32: $\chi^2(1)=59.65$, $p<.001$). This indicates that the prevalence of giving one or more positive answers differed for men and women. Men more often gave a positive answer on one or more CAGE items than women.

Table 5 also presents the twin correlations and threshold estimates for the trichotomous CAGE (0 vs. 1 vs. 2+ yes answers). The estimates for the dichotomous (0 vs. 1+ yes answers) and trichotomous CAGE (0 vs. 1 vs. 2+ yes answers) were highly similar. Thus, dichotomizing the CAGE using one positive item as cut-off did not bias the observed correlations, nor the thresholds estimates or deviation from the threshold for females as compared to analyzing the CAGE using three response categories. Therefore, further results are presented for the dichotomous CAGE only.

Table 4 Estimates of tetrachoric correlations (with standard errors) for the CAGE as dichotomy (0 vs. 1+ yes answers): cross twin-within age [at diagonal *in italic*] and cross twin-cross age [off-diagonal]

	15-17	**18-20**	**21-23**	**24-26**	**27-29**	**30-32**
MZM						
15-17	*.73*					
	(.14)					
18-20	.29	*.50*				
	(.18)	*(.10)*				
21-23	.19	.43	*.57*			
	(.21)	(.10)	*(.08)*			
24-26	.09	.26	.33	*.59*		
	(.24)	(.11)	(.09)	*(.09)*		
27-29	-.16	.41	.43	.50	*.57*	
	(.36)	(.13)	(.11)	(.09)	*(.12)*	
30-32	.43	-.10	.24	.36	.36	*.35*
	(.33)	(.20)	(.14)	(.12)	(.13)	*(.17)*
MZF						
15-17	*.54*					
	(.15)					
18-20	.43	*.42*				
	(.15)	*(.09)*				
21-23	.53	.43	*.44*			
	(.12)	(.10)	*(.09)*			
24-26	.13	.60	.32	*.43*		
	(.18)	(.08)	(.09)	*(.10)*		
27-29	.09	.43	.43	.39	*.49*	
	(.24)	(.11)	(.08)	(.08)	*(.09)*	
30-32	.21	.51	.36	.40	.50	*.44*
	(.25)	(.14)	(.11)	(.10)	(.08)	*(.11)*

	15-17	**18-20**	**21-23**	**24-26**	**27-29**	**30-32**
DZM						
15-17	*.68*					
	(.17)					
18-20	.19	*.32*				
	(.22)	*(.13)*				
21-23	.11	-.10	*.08*			
	(.26)	(.16)	*(.14)*			
24-26	.08	.26	.34	*.05*		
	(.34)	(.17)	(.15)	*(.17)*		
27-29	.03	.41	.17	.24	*.28*	
	(.44)	(.18)	(.16)	(.14)	*(.19)*	
30-32	.43	.31	.03	.27	-.03	*-.06*
	(.45)	(.22)	(.20)	(.17)	(.18)	*(.23)*
DZF						
15-17	*.56*					
	(.25)					
18-20	.41	*.45*				
	(.33)	*(.11)*				
21-23	.05	.30	*.27*			
	(.30)	(.15)	*(.13)*			
24-26	-.01	.05	.14	*.27*		
	(.31)	(.17)	(.14)	*(.14)*		
27-29	.21	.32	.00	.27	*.27*	
	(.38)	(.16)	(.17)	(.12)	*(.16)*	
30-32	.30	-.16	.33	.23	.22	*.20*
	(.43)	(.28)	(.16)	(.14)	(.14)	*(.18)*

	15-17	**18-20**	**21-23**	**24-26**	**27-29**	**30-32**
DOS						
15-17	*.48*					
	(.17)					
18-20	.10	*.26*				
	(.16)	*(.10)*				
21-23	-.06	-.04	*.06*			
	(.19)	(.12)	*(.11)*			
24-26	.17	-.06	-.16	*.20*		
	(.16)	(.13)	(.10)	*(.12)*		
27-29	-.02	-.06	.04	-.13	*-.11*	
	(.24)	(.15)	(.11)	(.10)	*(.16)*	
30-32	-.28	-.30	.10	.13	-.09	*.48*
	(.28)	(.19)	(.13)	(.11)	(.13)	*(.14)*

Table 5 Test results for equality of tetrachoric correlations over sex for the dichotomous CAGE and comparability with polychoric correlations for the trichotomous CAGE, with thresholds and threshold deviations[d]

	Test results sex differences [a]		**Dichotomous CAGE (0 vs. 1+ yes answers)**				**Trichotomous CAGE (0, 1, 2+ yes answers)**					
Age	**$\chi^2(3)$** [b]	***p***	**MZ corr**	**DZ corr**	**th1**[c]	**Δ th1 females** [d]	**MZ corr**	**DZ corr**	**th1**[c]	**th2**[c]	**Δ th1 females** [d]	**Δ th2 females** [d]
15-17	2.54	0.469	0.62	0.53	0.97	0.39	-- [e]	-- [e]	--	--	--	--
18-20	2.14	0.545	0.47	0.33	0.47	0.52	0.47	0.31	0.47	1.15	0.52	0.46
21-23	2.63	0.453	0.50	0.14	0.33	0.62	0.49	0.14	0.33	1.07	0.62	0.49
24-26	2.17	0.538	0.50	0.19	0.31	0.68	0.45	0.14	0.31	0.99	0.68	0.57
27-29	3.89	0.274	0.51	0.14	0.45	0.50	0.47	0.17	0.44	1.11	0.50	0.40
30-32	4.52	0.210	0.41	0.26	0.50	0.49	0.40	0.31	0.49	1.11	0.49	0.41

[a] results presented for testing sex differences in the correlations for the dichotomous CAGE

[b] testing for the equality of the tetrachoric correlations over sex results in a test with three degrees of freedom. The critical $\chi^2(3)$ value is 7.815 for an alpha level of .05.

[c] threshold estimates

[d] deviation from threshold for females

[e] no polychoric correlations could be computed for the trichotomous CAGE in the 15-17 age group, since the frequency of 2+ yes answers was too low.

3.3 Cross-sectional genetic analyses

Table 6 shows the ACE or ADE variance components estimates and model fitting results for the cross-sectional genetic analyses. Since the MZ correlation was smaller than twice the DZ correlation for the age groups 15-17, 18-20 and 30-32, the ACE model was fitted for these age groups. For the age groups 21-23, 24-26, and 27-29, the MZ correlation was more than twice the DZ correlation and thus the ADE model was fitted. Comparison of the full models showed an increase of unique environmental factors over time, explaining 38% of the variance in AAD symptoms at age 15-17 and 59% at age 30-32. Shared environmental influences decreased over time. While almost half of the variance (45%) was explained by shared environmental influences at age 15-17, and 19% of the variance at age 18-20, no shared environmental effects were present for the ages 21-29. At age 30-32, shared environmental influences accounted for 12% of the variance again. Genetic influences showed an increase in importance over age. At age 15-20, 18% of the variance in AAD symptoms was explained by genetic influences. At age 18-20, this increased to 28% and for the ages 21-29, 50% of the variance could be explained by genetic factors. At age 30-32 genetic influences were less important, explaining 28% of the variance.

For all ages the reduced AE model did not fit significantly worse than the ADE model (model fit statistics shown in Table 6). At the ages 15-17, 18-20 and 30-32, also a reduced CE model fitted the data well, indicating that familial factors were clearly important for this age. Since the AE model described the data well for all age groups, but the CE model was also possible at ages 18-20 and 30-32, the ACE model was selected for the longitudinal analyses.

3.4 Longitudinal genetic analyses

Table 7 presents the model fit results for the longitudinal genetic analyses. Constraining the sex effects (differing thresholds for males and females) to be equal over the six age groups deteriorated the model fit significantly as compared to the saturated model (model 2: $\chi^2(5)=17.69$, $p=.003$). Therefore, in subsequent models separate sex effects for the female threshold deviation from the threshold for males were retained for the different age groups.

The unique environmental simplex model with innovation and transmission terms fitted the data well (model 3a: $\chi^2(9)=7.78$, $p=.557$). Fixing the transmission terms at zero and thereby imposing the matrix with unique environmental factors to be diagonal worsened the model fit (model 3b: $\chi^2(5)=26.66$, $p<.001$). This indicates that unique environmental covariance is present over time and shows that both stable and dynamic unique environmental influences impact the CAGE.

Table 6 ACE and ADE variance component estimates and model fit results for the cross-sectional genetic analyses [a]

Age		A	D	C	E	-2ll	#par	*df*	χ^2	Δ*df*	*p*
15-17	ACE	0.18		0.45	0.38	831.13	5	1204			
	AE	**0.68**			**0.32**	834.00	4	1205	2.87	1	**0.090**
	CE			**0.58**	**0.42**	831.46	4	1205	0.34	1	**0.562**
	E				1.00	869.45	3	1206	38.32	2	0.000
18-20	ACE	0.28		0.19	0.53	3656.37	5	3636			
	AE	**0.51**			**0.50**	3658.00	4	3637	1.63	1	**0.202**
	CE			**0.40**	**0.60**	3658.70	4	3637	2.33	1	**0.127**
	E				1.00	3719.73	3	3638	63.36	2	0.000
21-23	ADE	0.06	0.44		0.50	3644.04	5	3490			
	AE	**0.46**			**0.54**	3645.98	4	3491	1.94	1	**0.163**
	E				1.00	3699.24	3	3492	55.20	2	0.000
24-26	ADE	0.24	0.26		0.52	3130.85	5	3035			
	AE	**0.48**			**0.52**	3131.39	4	3035	0.54	1	**0.462**
	E				1.00	3178.66	3	3036	47.81	2	0.000
27-29	ADE	0.04	0.47		0.49	2620.02	5	2552			
	AE	**0.47**			**0.53**	2621.27	4	2553	1.25	1	**0.264**
	E				1.00	2657.78	3	2554	37.76	2	0.000
30-32	ACE	0.28		0.12	0.59	2211.15	5	2225			
	AE	**0.42**			**0.58**	2211.43	4	2226	0.28	1	**0.597**
	CE			**0.34**	**0.66**	2212.18	4	2226	1.03	1	**0.310**
	E				1.00	2233.51	3	2227	22.36	2	0.000

[a] For models printed in bold, the model fit does not differ significantly from the full (ACE or ADE) model (α =.05)

Subsequent modeling of the shared environmental influences showed that the simplex model including both transmission and innovation terms (model 4a; $\chi^2(10)=3.45$, $p=.969$) fitted the data well. Dropping the shared environmental innovation terms from the model was allowed (model 4b; $\chi^2(5)=.17$, $p=.999$) indicating that shared environmental innovation was not present and that one shared environmental factor influenced the CAGE.

Finally, fitting a genetic simplex model with transmission and innovation terms did not worsen the model fit (model 5a: $\chi^2(10)=11.62$, $p=.311$). The final model without genetic innovation terms did not fit significantly worse than the model with genetic innovation (model 5b: $\chi^2(5)=8.81$, $p=.147$). This final model showed that one stable genetic factor was influencing the CAGE over age.

The parts of the (co)variance that can be explained by additive genetic, shared environmental and unique environmental factors as estimated in the final model are presented in Table 8. Results suggested that 25% of the variance at a particular age could be accounted for by measurement error. In Figure 2 the associated parameter estimates of this model are shown.

Table 7 Model fit results for longitudinal models (ages 15-32)

	AIC/BIC	-2ll	#par	*df*	vs.	Δχ^2	Δ*df*	*p*	Conclusion
M1. Saturated model (full ACE)	-17251.01 -61046.88	14942.99	75	16103					
M2. Saturated model with sex effect on threshold constrained	-17243.32 -61059.31	14960.68	70	16108	M1	17.69	5	.003	Sex by age difference in prevalence
M3a. Unique environmental simplex model with transmission + innovation	-17261.23 -61081.30	14950.77	66	16112	M1	7.78	9	.557	Simplex structure with stable and dynamic unique environmental influences fits data
M3b. Unique environmental simplex model without transmission	-17144.57 -61039.25	15077.43	61	16117	M3a	26.66	5	<.001	Stable unique environmental influences present over time
M4a. Shared environmental simplex model with transmission + innovation	-17277.79 -61122.14	14954.22	56	16122	M3a	3.45	10	.969	Simplex structure with stable and dynamic shared environmental influences fits data
M4b. Shared environmental simplex model without innovation	-17287.61 -61143.33	14954.39	51	16127	M4a	0.17	5	.999	No shared environmental innovation present over time
M5a. Genetic simplex model with transmission + innovation	-17295.99 -61180.09	14966.01	41	16137	M4b	11.62	10	.311	Simplex structure with stable and dynamic genetic influences fits data
M5b. Genetic simplex model without innovation (final model)	-17297.81 -61197.28	14974.19	36	16142	M5a	8.18	5	.147	No genetic innovation present over time

When taking variance due to measurement error into account (letting A+C+E sum up to 100%), it can be seen that shared environmental influences showed rapid attenuation over time. While explaining 57% of the variance at age 15-17, (almost) no environmental effects were present anymore for the ages 21-32. Genetic factors showed quantitative changes in its influences over time, increasing from 28% at age 15-17 to 58% at age 21-23. Genetic influences remained high at later ages, accounting for still 52% of the variance at age 30-32. The influence of unique environmental influences was small at age 15-17 (15%), but amplified during the next years. At age 30-32 unique environmental influences were as important as additive genetic influences in explaining differences in the CAGE. Interestingly, phenotypic stability and change in the CAGE over time could be largely attributed to additive genetic factors, although at later ages unique environmental factors also contributed to the stability and change.

For unique environmental influences a simplex structure with innovation terms was specified, allowing for stable and dynamic influences. At younger ages the largest part of unique environmental influences was due to unique environmental innovation. According to the final model, at age 18-20, 98% of the unique environmental variance was due to innovation ($.58^2/(.34^2 * .27^2 + .58^2) * 100\%$). At age 21-23, this was 83% ($.50^2 /[(.34^2 * .27^2 + .58^2) * .38^2 + .50^2]* 100\%$) and at age 24-26, 65% ($.47^2 /([(.34^2 * .27^2 + .58^2) * .38^2 + .50^2] * .63^2 + .47^2) * 100\%$). At later ages, the largest part of unique environmental influences consisted of those experienced earlier and transmitted to a later age. For example, at age 27-29, only 16% of unique environmental variance was due to innovation ($.23^2 /[([(.34^2 * .27^2 + .58^2) * .38^2 + .50^2] * .63^2 + .47^2)* .91^2 + .23^2] * 100\%$) and 84% to unique environmental factors experienced earlier in life. For age 27-29, 72% of unique environmental influences consisted of those transmitted from earlier ages and 28% was due to innovation ($.32^2 /([([(.34^2 * .27^2 + .58^2) * .38^2 + .50^2] * .63^2 + .47^2)* .91^2 + .23^2] * .89^2 + .32^2) * 100\%$).

Table 8 Percentage of variance [at diagonal *in italic*] and covariance [below diagonal] explained by additive genetic, shared environmental and unique environmental influences for different age groups as estimated in the final model [a]

	Additive genetic factors (A)							**Shared environmental factors (C)**							**Unique environmental factors (E)**					
	15-17	**18-20**	**21-23**	**24-26**	**27-29**	**30-32**		**15-17**	**18-20**	**21-23**	**24-26**	**27-29**	**30-32**		**15-17**	**18-20**	**21-23**	**24-26**	**27-29**	**30-32**
15-17	*.28*						**15-17**	*.57*						**15-17**	*.15*					
18-20	.47	*.36*					**18-20**	.47	*.18*					**18-20**	.06	*.46*				
21-23	.77	.66	*.58*				**21-23**	.20	.08	*.02*				**21-23**	.03	.25	*.40*			
24-26	.91	.78	.69	*.55*			**24-26**	.06	.03	.01	*.00*			**24-26**	.02	.19	.31	*.45*		
27-29	.96	.81	.71	.58	*.56*		**27-29**	.02	.01	.00	.00	*.00*		**27-29**	.02	.18	.28	.42	*.44*	
30-32	.97	.83	.73	.60	.58	*.52*	**30-32**	.01	.00	.00	.00	.00	*.00*	**30-32**	.02	.17	.27	.40	.42	*.48*

[a] ACE simplex model without innovation for A and C with innovation for E (including a term for measurement error)

4. Discussion

Our main goal was to study the temporal pattern of genetic factors for AAD symptoms during the critical developmental period from adolescence into early adulthood. Studying age-related changes was allowed, since the CAGE assessed the same phenotype across age 15-32 and across men and women, as indicated by the analyses on measurement invariance. In particular, we wanted to discriminate between the *developmentally stable* and *developmentally dynamic* hypotheses that predict quite different patterns for cross age genetic effects on symptoms of AAD. Genetic innovation was not detected, which provides evidence in favor of the developmentally stable hypothesis. That is, genetic risk factors for symptoms of AAD appear to be temporally stable across the key developmental period from mid-adolescence into early adulthood. The same genetic risk factors are important at younger and older ages. This is in line with Sartor et al. (2008) who found that one common genetic factor could explain the rate of progression from age at alcohol initiation to age at occurrence of the first symptom of AAD and age at onset of an alcohol dependence diagnosis. Although in our study no qualitative changes in genetic factors were found, quantitative changes were present. Genetic influences amplified from age 15-17 to age 21-23 and then remained high.

Genetic factors can impact on risk for AAD symptoms at a wide range of levels and are likely to share genetic influences on alcohol consumption measures, partly (Whitfield et al. 2004) or (almost) entirely (Grant et al. 2009; Kendler et al. 2010). Genetic influences include those on alcohol metabolism by the ADH and ALDH gene cluster (Edenberg et al. 2006; Luo et al. 2006; Kuo et al. 2008b; Macgregor et al. 2009; Van Beek et al. 2010), the level of response to alcohol (Schuckit et al. 2004) and neurotransmitters related to the reward system that interact directly and indirectly with ethanol (e.g., dopamine, and GABA) (Enoch 2008; Smith et al. 2008; Munafo et al. 2007). Genetic influences on AAD also act via general predispositions to abuse licit and illicit psychoactive drugs (Kendler et al. 2007; Agrawal et al. 2008), predispositions toward externalizing behaviors in general (Cerda et al. 2010) and the tendency to self-select into environments where alcohol is widely available and its misuse socially condoned (Kendler et al. 2007; Gillespie et al. 2007). In addition, genetic factors for AAD can have their influence by acting on co-morbid psychiatric symptomatology, such as depression or anxiety disorders (Cerda et al. 2010; Saraceno et al. 2009).

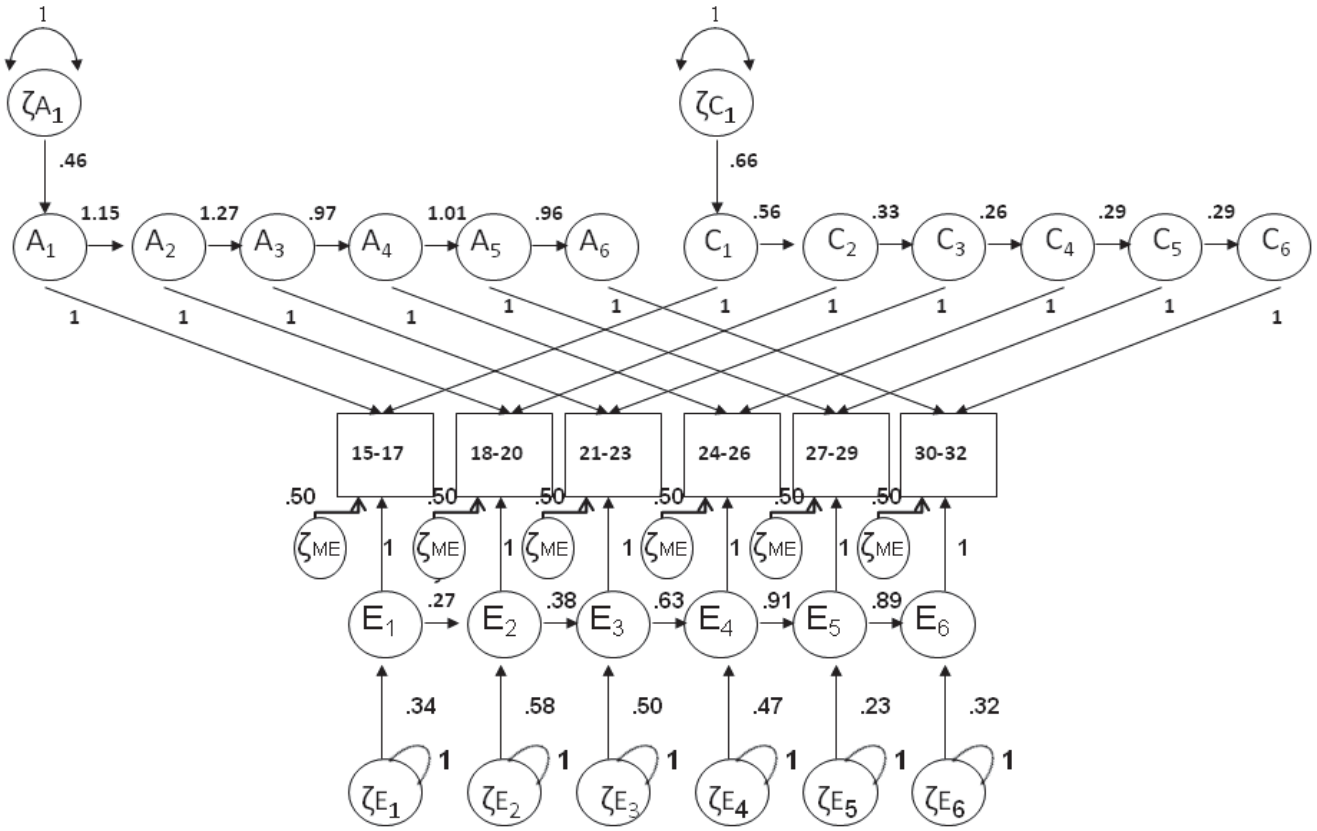


Figure 2 Parameter estimates in final model including measurement error: ACE simplex model without innovation for A and C with innovation for E (shown for 1 twin only)

This project had the additional goal of clarifying the change in environmental influences on symptoms of AAD between the ages 15 and 32. A single shared environmental factor had a large influence on AAD symptoms at age 15-17, but quickly attenuated over time. Consistent with Dick et al. (2009b) shared environmental influences on symptoms of AAD are important in adolescence, but less (during age 18-23) or not (from 24-26 onwards) at later ages. Attenuation of shared environmental influences in this period of life is observed for other phenotypes as well, such as for IQ (Segal and Johnson 2009), social attitudes and nicotine initiation (Bergen et al. 2007) and makes sense from a developmentally point of view in which individuals of this age tend to leave the parental home and sometimes also the neighborhood. The shared environmental effects could be a reflection of parenting practices (Latendresse et al. 2010), drinking family members (Poelen et al. 2007; McGue et al. 1996), or religious upbringing (Kendler and Myers 2009). The home environment that parents provide for their children might also in part reflect the genes the parents transmit to their children. If parents transmit both genes and environment -referred to as genetic and cultural transmission- this induces a passive correlation between G and C (Rijsdijk and Sham 2002). Passive G-C correlation might for instance occur if one of the parents suffers from AAD which also creates an environment in which the availability of alcohol at home is more likely. In addition to familial influences originating at home, the shared environmental effects in twins might also reflect the influence of peers' alcohol use when twins share the same peer group (Fowler et al. 2007b).

The cross-sectional analyses suggested that shared environmental influences were also present at age 30-32 in close correspondence with Kendler et al. (2008c) who found shared environmental effects for alcohol consumption for roughly the same age. For people of this age, who commonly start a family life in this period of life (CBS 2011), a decrease in alcohol consumption is not surprising. In particular in women (62% of our sample), alcohol consumption will change when being pregnant or breastfeeding. Therefore, at least for women, the shared environmental effects at age 30-32 might reflect a decrease in alcohol consumption during pregnancy and breast feeding that is shared within twin pairs, given that age at first birth is partly due to shared environmental influences (Neiss et al. 2002; Rodgers et al. 2008). However, it should be noted that in the longitudinal analyses shared environmental influences were only observed in adolescence and not at age 30-32. It will be interesting to see whether in a few years we can detect evidence for shared environmental factors for people aged 30 and older as we continue our longitudinal data collection and the number of individuals of age 30 and older will increase.

Unique environmental effects increased at age 18-20, the age at which individuals commonly leave the parental home, and remained of similar magnitude at later ages. The variance explained by environmental factors specific to an individual increased from 15% in

adolescence to 40-48% during early adulthood. Whereas at younger ages unique environmental influences were largely age-specific, from age 27-29 onwards the largest part of unique environmental influences resulted from those experienced earlier which were then transmitted to a later age. These unique environmental influences can represent any influences that the twins do not have in common, like a specific group of friends, relationships, life events, or working environment.

Our results show that symptoms of AAD are already reported by adolescents. In 15-17 year olds, 15.8% of men and 9% of women reported one or more symptoms. These percentages are not surprising given the high frequency of alcohol consuming adolescents in the Netherlands. Although according to Dutch legislation people have to be aged 16 or older to buy beer and wine and 18 or older for spirits/hard liquor, the rate of 'success' for obtaining these substances under the age of 16/18 is estimated at 85%. Three in four adolescents report to have consumed alcohol in the last month at least once and a substantial group even more than 10 times (23% of male, 10% of female alcohol-consuming adolescents) (Trimbos Institute 2010).

We did not find evidence for genetic innovation although others have. In Malone et al. (2004) genetic innovations were suggested for symptoms of alcohol dependence at age 17, 20 and 24 years. However, they focused on transmission at the phenotypic level, rather than at the level of latent genetic and non-genetic factors. Under this parameterization, a change in heritability is only possible by including a term for genetic innovation. In our analyses, transmission coefficients were specified at the level of latent genetic, common environmental and unique environmental factors. This is a less constrained model that indicated genetic stability without genetic innovation. When analyzing our data with a phenotypic simplex model, genetic innovations were significant ($\chi^2(5)=41.30$, $p<.001$). This does not reflect new genetic influences however, but the fact that the amounts of genetic, common and unique environmental variance that are transmitted, are not equal to each other. For different phenotypes genetic innovation has been detected, for instance for fears and phobias (Kendler et al. 2008a), anxiety and depression (Kendler et al. 2008b) and externalizing behaviors (Wichers et al. 2013). Our analyses showed that a model without genetic innovation fitted the data as well as a model with genetic innovation. This does not mean that genetic innovation is entirely absent. If we test all genetic innovation parameters separately, parameters at age 21-23 and 24-26 are significant ($p=.038$ and $p=.009$ respectively). Still, our results indicate that the majority of genetic influences are stable over time, and genetic innovation effects are small.

A next step would be searching for susceptibility genes for AAD symptoms. Recently, a number of genome wide association studies have been conducted for alcohol dependence (Bierut et al. 2010; Treutlein et al. 2009; Edenberg et al. 2010; Lind et al. 2010; Kendler et al. 2011b). Two closely linked intergenic SNPs at chromosome 2q35 achieved genome-wide

significance in the combined analysis of the discovery and follow-up sample in Treutlein et al.(2009). In other studies no SNP met criteria for genome-wide significance (Bierut et al. 2010; Edenberg et al. 2010; Kendler et al. 2011b), or genome-wide significant SNPs could not be replicated in a follow-up sample (Lind et al. 2010). Some evidence was found for gene clusters. For instance, in Lind et al. (2010) the top SNPs could be placed in a gene network coding for ion-channels and cell adhesion molecules. Edenberg et al. (2010) found several SNPs on chromosome 11 that were independently marginally associated with alcohol dependence. In regard to the genetic overlap of AAD and alcohol consumption measures (Grant et al. 2009; Kendler et al. 2010), the recent finding of a genome-wide significant association of a SNP at chromosome 7q11.22 with alcohol consumption (Schumann et al. 2011), is also relevant.

The search for susceptibility genes can be aided with a thorough assessment of how genetic influences change across age. When there is no evidence of genetic innovation over time, as for the phenotype under study, a sum score over all available data points might give more power to detect susceptibility loci than analyzing one time point only because sum scores tend to be more reliable than single observations. However, in a scenario in which genetic innovation is present, summing over different observations in time can lead to a considerable drop in power as compared to analyzing one data point only. This was outlined in a simulation study by Minica et al. (2010). With data generated under a one genetic factor model (four time points; heritability of .6; unique environmental effects at .4), the power to detect a genetic risk variant was .96 when a sum score of the four observations was analyzed, and .56 when only one data point was analyzed ($\alpha = .01$; with a power of .99 for the true model). In the scenario with genetic innovation present and a genetic risk variant that entered the model at the fourth time point ($h^2 = .6$; $e^2 = .4$), the power dropped to .03 when a sum score was analyzed, whereas it stayed at .56 when only observations at the fourth time point were analyzed ($\alpha = .01$; power true model > .99).

This study includes some potential limitations. First, there is a question of the degree to which the sample is representative. This was examined in two studies on the effect of non-cooperation in the Netherlands Twin Register (Vink et al. 2004b; Distel et al. 2007). Vink et al. (2004b) found that the proportion of individuals endorsing two or more positive symptoms on the CAGE was greater in families with low versus high cooperation and in incomplete versus complete twin pairs. However, the differences were modest in magnitude and not statistically significant after correction for multiple testing. In a later study no difference was detected between high and low cooperative families in the proportion of individuals that scored two positive items or more (Distel et al. 2007). Therefore, the sample is considered to be relatively unbiased with respect to symptoms of AAD. Secondly, the assessment of symptoms of AAD consisted of a four-item self-report questionnaire. The CAGE has been successfully used as a

screening instrument for AAD (Dhalla and Kopec 2007; Maisto and Saitz 2003; Aertgeerts et al. 2004) and socially undesirable behaviors such as symptoms of AAD may be better assessed using self-administration methods than interviews (Tipping et al. 2010; Bowling 2005). Lastly, the CAGE inquires about 'ever' having symptoms of AAD (Ewing 1984). This would imply that participants cannot answer an item with 'no' when they have answered 'yes' to that item at an earlier assessment. In total, 8,398 subjects took part in the study and for 3,909 there were at least two observations. In this last group, 5.8% of the replies was inconsistent (determined as the number of inconsistent answers as a function of the total number of replies). That is, a no-reply followed an earlier yes-reply. Giving an inconsistent response was relatively more frequent among males ($\chi^2(1)=35.30$, $p<.001$), who on average endorse symptoms of AAD more often than women and therefore have a higher chance of giving an inconsistent response, and individuals who entered the study at a younger age ($\chi^2(5)=133.06$, $p<.001$). Since variance due to measurement error was taken into account in our analyses, the effect of inconsistencies due to measurement error is likely to be minor. It is however likely that certain variability in AAD symptoms has gone undetected by the restriction of the phrasing of the CAGE items to 'ever'. This might have inflated the stability in symptoms of AAD over time which could have had its effect on our findings of genetic stability.

The absence of genetic innovation might be due to a lack of statistical power resulting from the fact that we used a cut-off score of one or more positive answers such that data from the youngest age group (15-17) could be included also, instead of using a cut-off of two or more yes answers as is usually done. In other populations with a low prevalence of AAD a cut-off score of one or more positive answers was also applied (Buchsbaum et al. 1992). More importantly, the cut-off of ≥1 yes answers did not affect the twin correlations or threshold estimates. To fully address the question whether absence of genetic innovation was due to a lack of power since we used the cut-off of ≥1 positive answers, the analyses were repeated using three response categories (0, 1, 2+ yes answers) and four response categories (0, 1, 2, 3+ yes answers) for the age groups spanning from 18 to 32 years. The youngest age group was not included, since in this group the frequency of two or more yes answers was too low. Including more response categories did not change the results. Dropping the terms for genetic innovation did not worsen the model fit significantly for the CAGE as trichotomy ($\chi^2(4)=.530$; $p=.971$), nor when the CAGE was analyzed with four response categories ($\chi^2(4)=.681$, $p=.954$). This renders lack of power as an explanation for the absence of genetic innovation, unlikely.

In conclusion, genetic influences on symptoms of AAD in adolescence and young adulthood are best described by the developmentally stable hypothesis. Symptoms of AAD are influenced by stable genetic risk factors and environmental influences that are largely age-specific.

CHAPTER 5

THE GENETIC ARCHITECTURE OF LIVER ENZYME LEVELS: GGT, ALT AND AST [3]

Abstract

High levels of liver enzymes GGT, ALT and AST are predictive of disease and all-cause mortality and can reflect liver injury, fatty liver and/or oxidative stress. Variation in GGT, ALT and AST levels is heritable. Moderation of the heritability of these liver enzymes by age and sex has not often been explored, and it is not clear to what extent non-additive genetic and shared environmental factors may play a role. To examine the genetic architecture of GGT, ALT and AST, plasma levels were assessed in a large sample of twins, their siblings, parents and spouses (N=8,371; age range 18-90).

For GGT and ALT, but not for AST, genetic structural equation modeling showed evidence for quantitative sex differences in the genetic architecture. There was no evidence for qualitative sex differences, i.e. the same genes were expressed in males and females. Both additive and non-additive genetic factors were important for GGT in females (total heritability h^2 60%) and AST in both sexes (total h^2 43%). The heritability of GGT in males and ALT for both sexes was due to additive effects only (GGT males 30%; ALT males 40%, females 22%). Evidence emerged for shared environmental factors influencing GGT in the male offspring generation (variance explained 28%). Thus, the same genes influence liver enzyme levels across sex and age, but their relative contribution to the variation in GGT and ALT differs in males and females and for GGT across age. Given adequate sample sizes these results suggest that genome-wide association studies may result in the detection of new susceptibility loci for liver enzyme levels when pooling results over sex and age.

[3] This chapter is published as:
van Beek, JHDA, de Moor, MHM, de Geus, EJC, Lubke, GH, Vink, JM, Willemsen, G, Boomsma, DI (2013) The genetic architecture of liver enzyme levels: GGT, ALT and AST. Behav Genet 43:329-339

1. Introduction

High levels of liver enzymes γ-glutamyl transferase (GGT), alanine and aspartate aminotransferase (ALT, AST) increase risk of disease and all-cause mortality. For GGT, strong associations are found with liver disease (2007; Ruhl and Everhart 2009), type 2 diabetes (Fraser et al. 2009) and cardiovascular disease (Targher 2009; Fraser et al. 2009). There is also evidence for a relationship of GGT with cancer and chronic kidney disease (Targher 2009). ALT and AST levels are predictive of liver disease (Kim et al. 2004; Lee et al. 2008) and liver-related mortality (Ruhl and Everhart 2009). In addition, ALT is associated with type 2 diabetes (Fraser et al. 2009).

In clinical practice, blood levels of GGT, ALT and AST are used to index liver injury. GGT is found in liver and biliary epithelial cells, and is a sensitive marker of hepatobiliary disease, although non-specific to its cause. AST and ALT blood levels increase when the liver cell membrane is damaged and thus mark hepatocellular injury (Pratt and Kaplan 2000). To explain their role in other diseases, GGT and ALT have been proposed as surrogate markers of fatty liver (Targher 2009; Schindhelm et al. 2006; Vernon et al. 2011), while GGT is also seen as a marker of oxidative stress (Lee et al. 2004). Variation in liver enzyme levels within the normal reference range predicts disease (Targher 2009) and it is therefore important to investigate the underlying sources of variation that explain individual differences in liver enzyme levels.

Genetic factors influence variation in liver enzyme levels. For adults, heritability estimates for GGT, ALT and AST range between 32%-69%, 22%-44% and 21%-40% respectively (Rahmioglu et al. 2009; Whitfield et al. 2002; Pilia et al. 2006) (studies with N≥3000). For ALT and AST these estimates increase to 64% and 61% if smaller studies are also included (Whitfield and Martin 1985; Makkonen et al. 2009; Sung et al. 2011; Lin et al. 2009; Loomba et al. 2010; Nilsson et al. 2009; Bathum et al. 2001).

The contributions of additive, non-additive genetic factors and shared environmental influences in explaining differences in liver enzyme levels thus vary widely. This may be due to the fact that most studies are based on a comparison of resemblance in monozygotic and dizygotic twins, which gives an overall estimate of heritability (h^2), but cannot distinguish well between additive and non-additive genetic effects (Keller et al. 2010) and in smaller twin studies it is also difficult to estimate the contribution of shared environmental factors (Posthuma and Boomsma 2000). Evidence for non-additive genetic effects that can be attributed to intra-locus (dominance) or inter-locus interaction effects (epistasis), was reported in two studies (Bathum et al. 2001; Makkonen et al. 2009) but not in two others (Whitfield et al. 2002; Rahmioglu et al. 2009). Shared environmental influences on liver enzymes can consist for example of effects of stress or dietary similarity that some families may have been exposed to while others have not (Whitfield and Martin 1985; Rahmioglu et al. 2009). Such factors may be

shared within generations e.g. by the offspring or between spouses, or by all family members. Spousal resemblance is present when the similarity between spouses for a particular trait is higher than expected by chance. If individuals choose a spouse who is similar for a particular trait (phenotypic assortment), the genetic factors influencing the spousal phenotypes become correlated which results in a higher genetic similarity between first-degree relatives. If spousal resemblance is not modeled, estimates of heritability and shared environmental influences may be biased (Van Grootheest et al. 2008; Keller et al. 2010). One form of shared environment is represented by the influence of the parental phenotype on their offspring's phenotype after genetic transmission has been taken into account (sometimes referred to as cultural transmission). Effects of the shared environment on GGT, ALT and AST have been detected in a combined sample of adolescents and adults (estimates 9%-14%) (Herbeth et al. 2010), and by two studies in adults (Rahmioglu et al. 2009; Whitfield and Martin 1985), but not by six other studies (Pilia et al. 2006; Nilsson et al. 2009; Makkonen et al. 2009; Bathum et al. 2001; Whitfield et al. 2002; Sung et al. 2011). The estimation of non-additive genetic and shared environmental effects requires additional data from non-twin family members and large sample sizes (Posthuma and Boomsma 2000; Keller et al. 2010).

Few studies have examined whether the genetic architecture of liver enzymes differed in males and females or across age. Results have been inconclusive. A study among >6,000 Sardinians detected a larger heritability of GGT in females than males, although there was no evidence for qualitative sex differences in the heritability (Pilia et al. 2006). Results of two studies on quantitative sex differences in the heritability of GGT among the elderly were not consistent (Bathum et al. 2001; Nilsson et al. 2009). For ALT, although quantitative sex differences in the heritability were not observed (Bathum et al. 2001; Nilsson et al. 2009; Pilia et al. 2006), a linkage study suggested qualitative sex differences in the association of ALT with quantitative trait loci (QTLs) at the chromosomal region 7p12-15. ALT levels in males, but not in females, were associated with QTLs in this area, suggesting differential gene expression in this region over sex (Brouwers et al. 2006). Results on sex differences in the heritability of AST have not been reported. A recent genome wide association study demonstrated that the effects on GGT by genetic loci at the *GGT1* locus (chromosome 22) interact with age (Middelberg et al. 2012). Several SNPs (including one that affects expression of *GGT1*, *GGT2* and *GGTLA4* in the human liver) decreased GGT levels in adults, whereas it increased GGT levels in adolescents. Other SNPs (located between *GGT1* and *PIWIL3*) increased GGT levels in adolescents, but not in adults. Additional age heterogeneity was observed for SNPs in the *CELF2* gene at chromosome 10 that may affect GGT expression through the coding for specific transcription factors (Middelberg et al. 2012). Evidence for age by genotype interaction effects on GGT and AST also comes from the rather low genetic resemblance between parents and their offspring in a study

by Herbeth et al. (2010), although Pilia et al. (2006) did not detect differences in the (standardized estimates of) heritability of either GGT, ALT or AST.

Our study examines to what extent variation in liver enzyme levels can be attributed to genetic factors (additive and non-additive) and environmental factors (shared and non-shared among family members) and if there is evidence for sex and age by genotype interaction. Data on plasma GGT, ALT and AST levels were collected in a large sample of twins, their siblings, parents and spouses (N=8,371) as part of the Netherlands Twin Register biobank project (Willemsen et al. 2010; Willemsen et al. 2013) .

2. Methods

2.1 Participants

Data originate from twins and their family members who participated in the NTR biobank study that was conducted from 2004 to 2008. Details can be found in Willemsen et al. (2010). The study protocol was approved by the Central Ethics Committee on Research Involving Human Subjects of the VU University Medical Center, Amsterdam.

Data on GGT, ALT and AST plasma levels were assessed in 8,758 participants. We excluded data for 387 family members, who were (a) not biological parents, or full siblings of twins (N=37), (b) twins from pairs for whom information on zygosity was not yet known (one twin was randomly selected to be included, N=4), (c) triplets if data from their two brothers or sisters were already included (N=6), (c) siblings from families with more than two same-sex siblings (a maximum of two siblings were included per family; N=135), or (d) relatives other than twins, siblings or parents, such as children of twins or siblings, grandparents, aunts, uncles, cousins (N=205).

Families were categorized into five groups, based on sex and zygosity (monozygotic, MZ; dizygotic, DZ) of the twin pair. Data of 257 spouses of twins were included in analyses of spouses resemblance. Analyses were performed on 8,371 individuals (8,114 individuals for the genetic analyses and 257 spouses of twins; 62.8% female; age range 18-90) (see Table 1).

2.2 Assessment of liver enzymes

Liver enzymes were determined in plasma that was collected in heparin plasma tubes. Vitros assays (Vitros 250, Ortho-Clinical Diagnostics; Johnson & Johnson, Rochester, USA) were used to determine GGT, ALT and AST levels in units per liter (U/L) (Willemsen et al. 2010). Reference values provided by the laboratory were as follows: for GGT <50 U/L (males) and <35 U/L (females); for ALT <45 U/L (males) and <40 U/L (females); for AST <40 U/L (both sexes). These reference values were based on the guidelines of the Dutch Association of Clinical

Chemistry and Laboratory Medicine (NVKC). Enzyme levels were compared to the reference values.

Table 1 Number of participants as a function of zygosity by sex of twins within the family

Zygosity by sex group[a]	twins[b]	siblings	parents	spouses
MZM	603 (78.8%)	131	298	53
DZM	329 (68.1%)	81	213	24
MZF	1,391 (81.5%)	268	536	102
DZF	697 (78.3%)	188	352	40
DOS	767 (66.8%)	210	455	38
Families without twins		835	760	
Total	3,787	1,713	2,614	257

[a] MZM: monozygotic males, DZM: dizygotic males, MZF: monozygotic females, DZF: dizygotic females, DOS: dizygotic opposite-sex

[b] percentage reflects what proportion of twins is part of a complete twin pair

2.3 Statistical analyses

Liver enzyme values were *ln*-transformed to approximate normality. Age, transformed to *z*-scores, was used as a covariate (*z*-score=0 corresponding to mean age 45.5, SD=14.7; range *z*-scores -1.86–3.07). Seasonal effects on liver enzyme levels were first explored with ANOVA in SPSS. The decrease in liver enzyme levels for individuals whose blood was sampled in spring, was tested for significance by including it as an (additional) fixed effect (dummy coded as 0/1) in the models fit with Mx v3.2 (Neale et al. 2006). Model comparisons were based on the likelihood ratio test (Bentler and Bonett 1980) with a significance level of .01.

A saturated model served to obtain familial correlations, which were computed while correcting liver enzyme values for sex and age. Sixteen sex-specific correlations were estimated: five twin correlations (MZM, DZM, MZF, DZF, DOS), three twin-sibling and three sibling-sibling correlations (male-male, male-female, female-female), four parent-offspring correlations (father-son, mother-son, father-daughter, mother-daughter) and one spouse correlation (father-mother). Next, the effects of age and sex were examined on enzyme levels and on the familial correlations. To explore age by genotype effects, we tested whether correlations are lower for pairs of relatives who show a larger age difference, i.e. we compared correlations for DZ twin pairs, sibling pairs, and parent-offspring pairs.

Quantitative sex differences in the correlation structure were tested by equating correlations over sex between all first-degree relatives and for MZ pairs. Qualitative sex differences were examined by equating the opposite-sex offspring correlation to the same-sex offspring correlation. Qualitative sex differences in the parent-offspring correlations were

tested by comparing the correlation for opposite-sex parent-offspring pairs to same-sex parent-offspring pairs.

Finally, spousal resemblance was examined by testing if the correlation between liver enzyme values of spouses was larger than zero. If this was the case, we explored whether correlations could be explained by cohabitation effects by estimating the correlation between the absolute difference in enzyme levels for spouses and the duration of their relationship. If living together influences resemblance between spouses, we expect this correlation to be negative, i.e. smaller differences between spouses the longer they are together. Data for these analyses were available for 984 spouse pairs with information on the duration of their relationship (727 parents of twins and 257 twin-spouse pairs). If no strong evidence was detected for cohabitation effects, it was assumed that phenotypic assortment was the mechanism underlying the spousal correlations, and hence was implemented as such in the genetic model.

To estimate the sources of variance of liver enzyme levels, variance decomposition models were fit to the data (depicted in Figure 1, see the Supplementary Materials to this chapter for an explanation and identification of these models). The variance in liver enzyme levels due to additive genetic influences (A), common environmental influences shared within the offspring generation (C), and non-shared individual-specific environmental influences (E) was estimated in two models. In the ACE model, shared environmental transmission from parents to offspring was taken into account (also known as cultural transmission), whereas in the ACDE model parent-offspring these transmission paths were set to zero (for reasons of model identification) and variance due to non-additive genetic influences was estimated (see Neale and Cardon (1992) (1992) and Posthuma (Posthuma et al. 2003) for details on twin-family variance decomposition models). Constraints in the genetic model were based on the results from saturated models (including those on spousal resemblance and sex differences). A detailed description of all tests performed is given in the Supplementary Materials to this chapter.

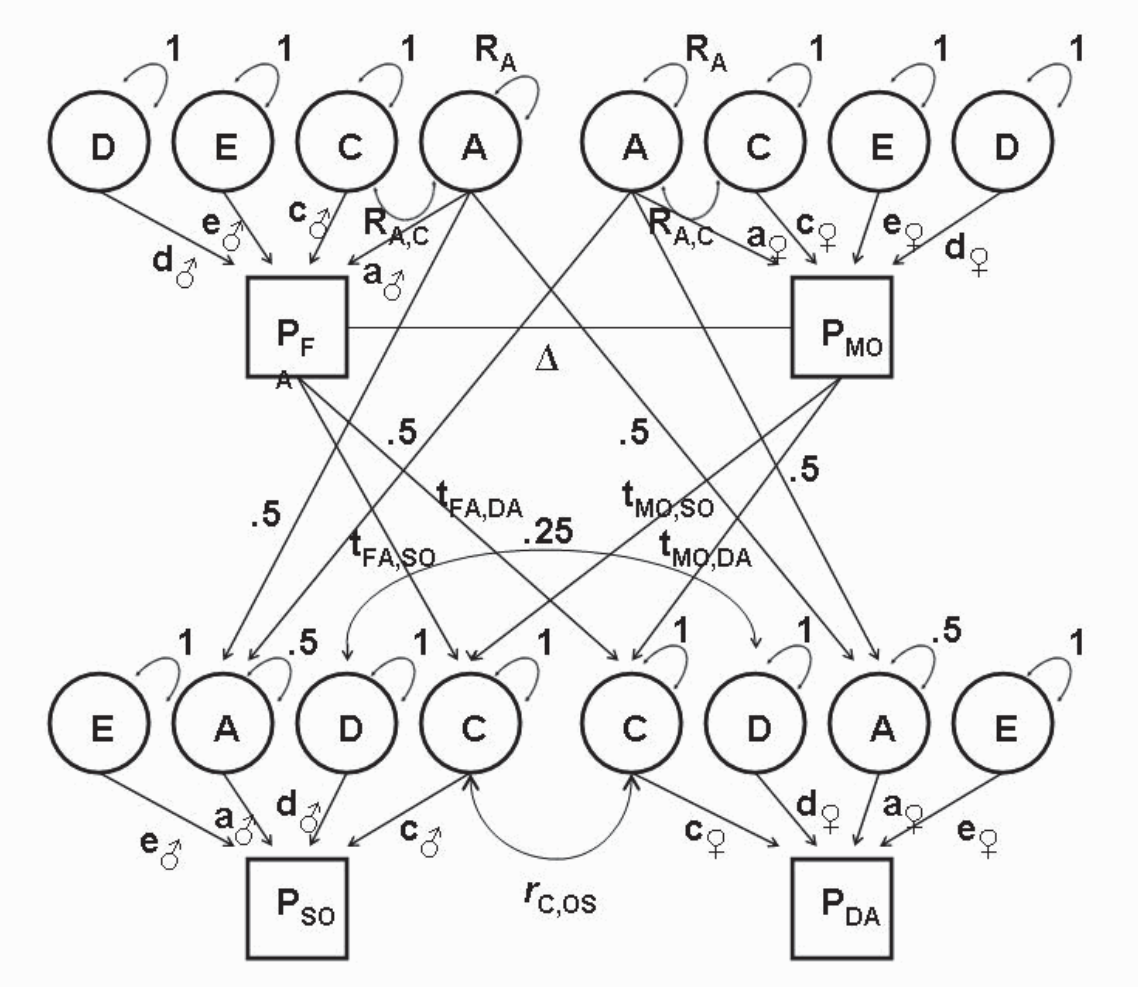


Figure 1 Path diagram for variance decomposition (shown for DOS twin pair with parents)

P_{FA}, P_{MO}, P_{SO}, P_{DA} = phenotype of father, mother, son, daughter

A= additive genetic factor with variance R_A (differs from 1 in the case of phenotypic assortment)

D= non-additive genetic factor

C=shared environmental factor with variance R_C (differs from 1 in the case of shared environmental transmission)

E=individual-specific environmental factor

a,c,d,e= path loadings (d-paths estimated in ACDE model; set to zero in ACE model with shared environmental transmission)

$r_{C,OS}$=correlation between shared environmental factors for opposite-sex

$t_{FA,SO}$, $t_{FA,DA}$, $t_{MO,SO}$, $t_{MO,DA}$ = shared environmental transmission paths (estimated in ACE model with shared environmental transmission; set to zero in ACDE model)

$R_{A,C}$=covariation between A and C (differs from 1 in the case of shared environmental transmission)

Δ =path representing correlations between the latent factors induced by phenotypic assortment

♂=male-specific path loadings

♀=female-specific path loadings

3. Results

For 1,318 (15.7%), 40 (.5%) and 165 (2.0%) individuals, GGT, ALT or AST levels were elevated compared to the reference values. Age and sex affected liver enzyme levels. Table 2 shows the means and medians of GGT, ALT and AST, with SDs, ranges and the correlations with age. The association with age was significant for all three liver enzymes (GGT: $\chi^2(6)=183.29$, $p<.001$; ALT: $\chi^2(6)=68.32$, $p<.001$; AST: $\chi^2(6)=143.86$, $p<.001$). Mean GGT levels were higher among parents than offspring (males: $\chi^2(1)=12.74$, $p<.001$; females: $\chi^2(1)=15.72$, $p<.001$), although ALT and AST levels did not differ for the parental and offspring generation (ALT males: $\chi^2(2)=3.53$, $p=.172$; ALT females: $\chi^2(2)=9.01$, $p=.011$; AST males: $\chi^2(2)=.41$, $p=.816$; AST females: $\chi^2(2)=3.95$, $p=.139$). Means were higher for men than women (GGT parents: $\chi^2(1)=66.03$, $p<.001$; GGT offspring: $\chi^2(1)=401.47$, $p<.001$; ALT: $\chi^2(1)=265.96$, $p<.001$; AST: $\chi^2(1)=316.38$, $p<.001$).

Seasonal effects were present for ALT and AST levels, but not for GGT. Individuals who were visited in spring (April-June) had significantly lower ALT and AST levels than individuals who were visited in other seasons (ALT: $\chi^2(1)=15.17$, $p<.001$; AST: $\chi^2(1)=341.66$, $p<.001$; GGT: $\chi^2(1)=5.66$, $p=.017$). Correcting for the effect of season when individuals were visited, did not change the familial correlations (data available upon request).

Familial correlations for GGT, ALT and AST are displayed in Figures 2a-c respectively. Supplementary Materials Table S1 presents the model fit statistics for all submodels, described below, that tested age and sex effects on the correlations. Furthermore, Supplementary Materials Tables S2a-c give an overview of the parameter constraints that are tested in each submodel, to explain the number of degrees of freedom that are associated with these tests. The amount of variance explained by genetic and environmental factors as estimated in the best fitting genetic models, is presented in Table 3.

Table 2 Descriptive statistics for males and females, separately for twins, siblings and parents

		Males					Females				
		mean [a] (SD)	median [a] (range)	correlations with ALT	AST	age	mean [a] (SD)	median [a] (range)	correlations with ALT	AST	age
twins	GGT	36.1 (34.63)	28 (9-820)	.31	.38	.22[c]	24.3 (24.9)	20 (8-819)	.25	.36	.21[c]
	ALT	12.9 (7.06)	11 (3-75)		.63	-.10[d]	9.1 (5.1)	8 (3-97)		.53	.06[d]
	AST	23.7 (8.09)	22 (11-122)			.05[e]	19.4 (6.7)	18 (9-107)			.20[e]
	age	35.4 (11.0)	32 (18-77)				36.5 (11.0)	33 (18-90)			
siblings	GGT	36.9 (34.53)	29 (13-564)	.34	.34	.20[c]	25.2 (22.1)	20 (8-439)	.31	.38	.18[c]
	ALT	12.3 (6.77)	11 (3-54)		.55	-.11[d]	9.6 (6.2)	8 (3-100)		.52	.07[d]
	AST	23.8 (12.11)	22 (11-220)			.08[e]	19.6 (7.1)	18 (9-142)			.20[e]
	age	40.2 (13.3)	36 (18-82)				42.2 (10.3)	42 (18-84)			
parents	GGT	45.4 (40.45)	34 (14-456)	.27	.40	-.04[c]	33.9 (40.4)	25 (10-867)	.30	.37	.06[c]
	ALT	10.8 (6.35)	10 (3-107)		.52	-.16[d]	9.4 (5.5)	8 (3-89)		.51	-.07[d]
	AST	23.5 (8.32)	22 (7-111)			-.06[e]	21.3 (7.3)	20 (7-128)			.09[e]
	age	61.4 (7.0)	60 (33-89)[f]				60.1 (7.8)	59 (19-90)[f]			
spouses	GGT	41.2 (75.4)	27 (14-917)	.49	.57	.22[c]	24.2 (18.9)	20 (9-181)	.21	.20	.31[c]
	ALT	12.5 (7.2)	11 (3-54)		.65	-.05[d]	8.9 (4.1)	8 (3-28)		.52	.06[d]
	AST	24.4 (9.3)	22 (14-92)			.19[e]	19.2 (5.2)	19 (12-41)			.16[e]
	age	42.5 (10.7)	39 (25-71)				38.7 (12.4)	34 (22-76)			

[a] untransformed values for liver enzyme levels

[b] *ln*-transformed values for liver enzyme levels

[c] in the genetic models for GGT, two betas for the age regression were estimated: fathers -.32, others 1.16, and four means (male/female offspring, fathers, mothers)

[d] in the genetic models for ALT, four betas for the age regression were estimated: male offspring -.52, fathers -1.29, female offspring .56, mothers -.25, and two sex specific means

[e] in the genetic models for AST, two betas for the age regression were estimated: males .02, females .63, and two sex specific means

[f] minimum age for parents who participated with their offspring (aged 18+) is 45 for mothers and 48 for fathers; lower ages in the parental group reflect those of parents of twins who were younger than 18 at the time of assessment

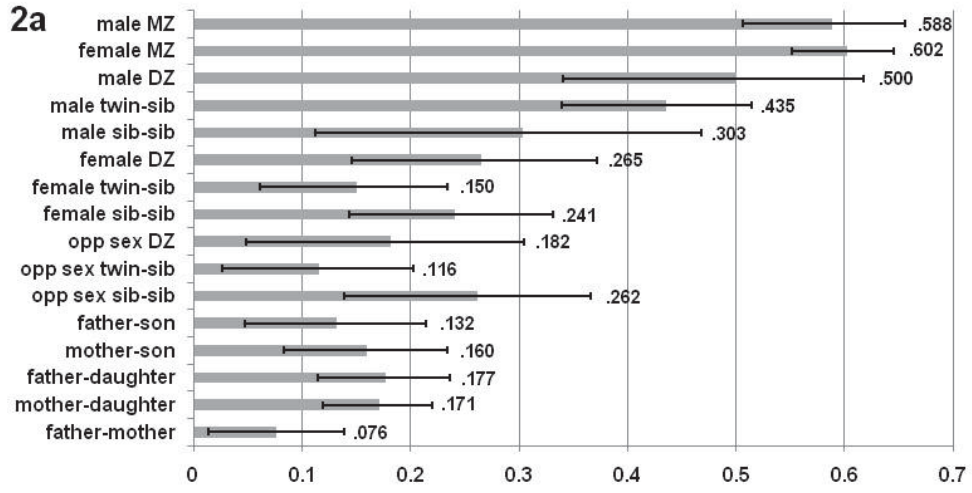


Figure 2a Familial correlations for GGT (with 95% confidence intervals)

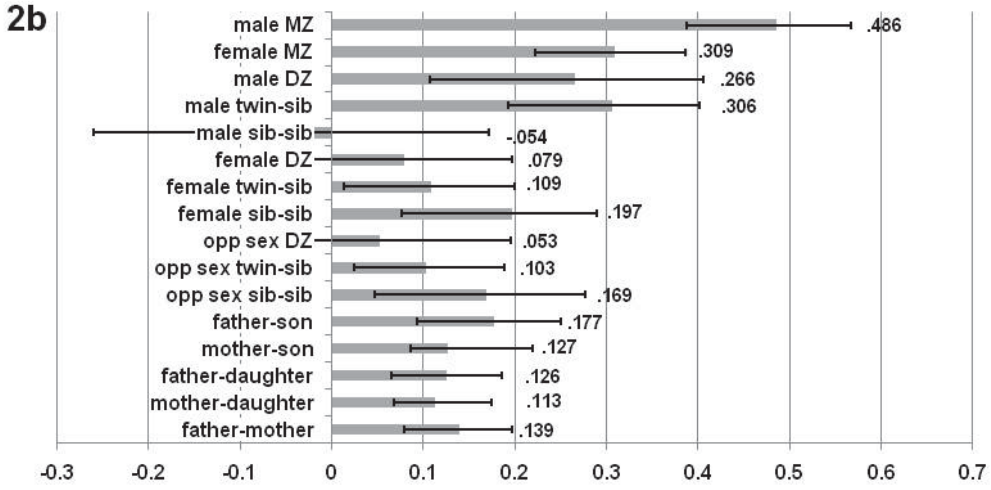


Figure 2b Familial correlations for ALT (with 95% confidence intervals)

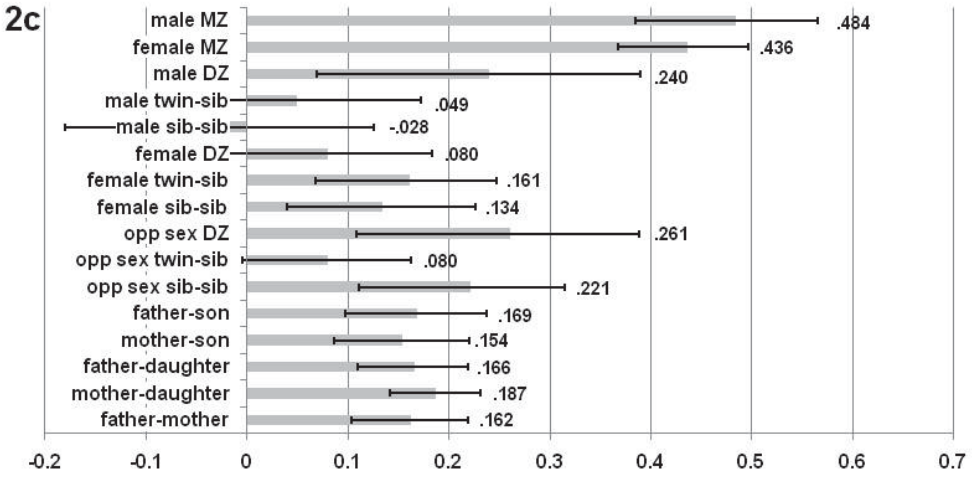


Figure 2c Familial correlations for AST (with 95% confidence intervals)

GGT

For GGT, there were quantitative sex differences in the offspring correlations ($r_{DZM/SIB}$ .43; $r_{DZF/SIB}$ .21), but not in the MZ correlations (r_{MZ} .60). The correlations among opposite-sex family members indicated no qualitative sex differences. The correlations among family members differed as a function of age, especially among men ($\chi^2(3)=32.06$, $p<.001$; r_{DZ} .50; r_{SIBS} .41; $r_{PAR\text{-}OFF}$ .13). Spousal resemblance was not present.

The ACDE model provided a better fit ($\chi^2(8)=16.98$, $p=.030$) than the ACE model with shared environmental transmission ($\chi^2(4)=46.83$, $p<.001$), compared to the saturated model. For females, both additive and non-additive genetic influences were important (drop D: $\chi^2(1)=25.68$, $p<.001$). Shared environmental effects were not present ($\chi^2(1)=.000$, $p=.999$). For men, additive genetic influences explained a significant amount of variance (drop A+D: $\chi^2(2)=74.31$, $p<.001$), whereas non-additive genetic influences did not (drop D: $\chi^2(1)=.33$, $p=.565$). In men, shared environmental factors played a role ($\chi^2(1)=26.82$, $p<.001$), which explains the age differences and quantitative sex differences in the correlation structure noted above. The absence of qualitative sex differences in the correlations (see Supplementary Materials Table S1) indicates that the same genes are expressed in men and women. Additional analyses showed that the age differences in the correlations for GGT among males were quantitative, due to additional environmental effects shared within the offspring generation (C), rather than being due to a different expression of genes across age (qualitative age differences). This was tested by estimating the genetic correlation between fathers and their male offspring and testing whether this correlation was significantly lower than .5. Taking the ACE (males) – ADE (females) model as a reference, allowing the genetic correlation between father and their male offspring to be lower than .5 did not significantly improve model fit, whereas dropping shared environmental effects among males from the model deteriorated model fit (still allowing for a genetic father-son correlation <.5) ($\chi^2(1)=35.44$, $p<.001$).

In the final model, for males, 30% of the variance was explained by additive genetic, 28% by shared environmental and 42% by non-shared individual-specific environmental factors. For females, this was 32% by additive genetic, 28% by non-additive genetic, and 40% by individual-specific environmental factors.

Table 3 Percentage of phenotypic variance in GGT, ALT and AST levels explained by additive (A) and non-additive genetic factors (D), and shared (C) and individual-specific (E) environmental influences as estimated in the best fitting genetic models

					proportion of phenotypic variance explained by			
		SD [a]	spouse corr	additive genetic variance	A	D	C	E
GGT	males	5.46	.00	1.00	29.6%		28.5%	41.9%
	95% CI				19.8% - 40.2%		19.2% - 37.5%	35.6% - 49.1%
	females	5.46	.00	1.00	32.2%	27.6%		40.2%
	95% CI				23.4% - 41.4%	17.8% - 37.5%		35.3% - 45.7%
ALT	males	4.54	.15	1.42	40.4%			59.6%
	95% CI				31.9% - 51.8%			51.8% - 68.0%
	females	4.54	.15	1.42	22.4%			77.6%
	95% CI				16.6% - 28.5%			71.6% - 83.7%
AST	males	2.86	.15	1.47	28.0%	14.7%		57.3%
	95% CI				22.6% - 33.5%	7.1% - 22.3%		51.8% - 63.5%
	females	2.62	.15	1.47	28.0%	14.7%		57.3%
	95% CI				22.6% - 33.5%	7.1% - 22.3%		51.8% - 63.5%

[a] These are SDs for *ln*-transformed liver enzyme values x 10. For GGT, SDs in the offspring generation were smaller than those in the parental generation and differed over sex (male offspring: 4.94; female offspring 4.25; parental generation 5.46).

ALT

For ALT, the correlation structure was similar over age, thus genotype by age interactions were not present. There was an indication for sex by genotype interactions, however. There were quantitative sex differences in the MZ correlations (r_{MZM} .43; r_{MZF} .31), but not in the correlations among first-degree relatives (r .14). Spouses resembled each other in ALT levels (r .14) which was not explained by cohabitation effects, as the correlation between the difference in liver enzyme levels within spouse pairs and the duration of their relationship was not significant (r= -.02, 95% CI: -.08, .04).

The ACDE model fit the data better ($\chi^2(6)=12.92$, p=.044) than the ACE model with shared environmental transmission ($\chi^2(6)=25.50$, p<.001), compared to the saturated model. Analyses of submodels showed that additive genetic factors influenced variation in enzyme levels (drop A+D: $\chi^2(5)=87.38$, p<.001), but non-additive genetic factors (drop D: $\chi^2(2)=5.72$, p=.057), or shared environmental influences ($\chi^2(2)=1.32$, p=.518) did not. This resulted in the AE model as the final model. The amount of variance explained by additive genetic factors and non-shared individual-specific environmental factors was 40% and 60% for males, and 22% and 78% for females respectively.

AST

For AST, the correlation structure was similar over age and sex, thus genotype by age or sex interactions were not present. Spousal resemblance was significant (r .16). The correlation between the difference in liver enzyme levels within spouse pairs and the duration of their relationship was significant ($\chi^2(1)= 6.92$, p=.009) but the effect was small (r= -.09; 95% CI: -.15, -.02) suggesting that cohabitation effects play a minor role. Familial correlations were .45 for MZ pairs and .16 for first-degree relatives, suggesting the presence of non-additive genetic effects rather than shared environmental effects.

The ACE model with shared environmental transmission did not fit the data as well ($\chi^2(9)= 42.55$, p<.001) as the ACDE model ($\chi^2(10)= 28.24$, p=.002), compared to the saturated model. Submodels were evaluated for the ACDE model and showed evidence for both additive and non-additive genetic effects (drop D: $\chi^2(1)=14.32$, p<.001). Shared environmental influences did not explain any variation ($\chi^2(1)=.00$, p=1). According to the final ADE model, 28% of the variance could be explained by additive genetic factors, 15% by non-additive genetic factors and 57% by non-shared (individual-specific) environmental factors (for both men and women).

4. Discussion

Variation in liver enzyme levels is moderately heritable. For GGT and ALT, quantitative sex differences in the genetic architecture were present, but there was no evidence that different genes were expressed in men and women. In addition, there were quantitative age differences in the relative importance of genetic and environmental influences on GGT that are due to environmental effects that were shared within the male offspring generation (c^2 28%), but not among parents and offspring. The results from our sex-specific analyses in which additive and non-additive genetic and shared and non-shared environmental influences could be estimated simultaneously may explain variability in previous heritability estimates (Rahmioglu et al. 2009; Whitfield et al. 2002; Pilia et al. 2006) (studies N>3,000). For GGT in males as well as for ALT (both sexes), only additive genetic influences were important (GGT h^2 males 30%, ALT h^2 males 40%, females 22%). For GGT in females and AST (both sexes), genetic influences consisted of both additive and non-additive effects (h^2 60% and 43% respectively). The different heritability estimates over sex for GGT and ALT correspond to the lower and upper bounds of previous heritability estimates (32%-69% and 22%-44% respectively). Likewise, the narrow-sense heritability estimates for GGT in females and AST (both sexes) (32% and 28% respectively) reflect the lower bound of previous heritability estimates (32% and 21%), whereas the broad-sense heritability estimates (60% and 43%) correspond to the upper bound of these estimates (69% and 40%).

The genetic and environmental influences on liver enzyme levels may derive from the biological effects of factors that are in turn partly genetically and partly environmentally mediated. These include metabolic risk factors (Whitfield et al. 2002; Loomba et al. 2010; Makkonen et al. 2009; Skurtveit and Tverdal 2002), inflammation parameters (Neijts et al. 2013), alcohol use (Whitfield and Martin 1985; Sung et al. 2011; Skurtveit and Tverdal 2002), smoking (Honjo et al. 2001; Skurtveit and Tverdal 2002; Vink et al. 2004a), and coffee consumption (Honjo et al. 2001; Vink et al. 2009; Skurtveit and Tverdal 2002). Shared environmental effects for GGT (28% for males in our sample) have been detected before for both males and females (aged 18-34) (Whitfield and Martin 1985). These effects may reflect shared environmental influences on the above mentioned metabolic and lifestyle factors. In studies that examined the association between liver enzyme levels and metabolic risk factors (Whitfield et al. 2002; Makkonen et al. 2009) or alcohol use (Whitfield and Martin 1985; Sung et al. 2011), shared environmental factors could not explain the covariance among these traits however.

Genome-wide association (GWA) studies have started to elucidate the genetic pathways underlying variation in liver enzyme levels. For GGT, genome-wide significant associations have been found for loci in or near the genes involved in glutathione metabolism (*GSTT2B, GGT1*)

(Chambers et al. 2011; Middelberg et al. 2012; Yuan et al. 2008; Kamatani et al. 2010), biliary transport (*ATP8B1*) (Chambers et al. 2011), alcohol metabolism (*ALDH2*) (Kamatani et al. 2010), lipid metabolism (*HNF1A, CEPT1*), carbohydrate metabolism and insulin signalling (*GCKR, MLXIPL, SLC2A2*) (Chambers et al. 2011; Middelberg et al. 2012; Yuan et al. 2008), inflammation and immunity (*GCKR, STAT4, CDH6, ITGA1, HNF1A, RORA, CD276* (Chambers et al. 2011; Middelberg et al. 2012), glycoprotein biology (*FUT2*) (Chambers et al. 2011), as well as for other genes, including the *C14orf73* gene (Chambers et al. 2011; Middelberg et al. 2012) that is strongly expressed in the liver but of which the function is uncertain (Chambers et al. 2011). Variants in the *PNPLA3* gene, involved in energy utilisation and storage by adipocytes, has been associated with both AST and ALT levels (Chambers et al. 2011; Kollerits et al. 2010; Sookoian and Pirola 2011; Yuan et al. 2008; Kamatani et al. 2010). For AST, a significant variant was detected in a gene implicated in inflammation and immunity (*MRC1*) (Kamatani et al. 2010). Additional loci have been detected for ALT in or near genes involved in glucose and lipid metabolism (*TRIB1, CHUK*) (Chambers et al. 2011; Yuan et al. 2008), inflammation and immunity (*CPN1*) (Chambers et al. 2011; Yuan et al. 2008) and the biogenesis of mitochondria (*SAMM50*) (Yuan et al. 2008).

Spousal resemblance was detected for ALT and AST. It can thus be hypothesized that the seasonal effects that were observed for ALT and AST may underlie this spousal resemblance. If family members are measured closer in time, seasonal effects, if present, will come up as an effect of the shared environment and/or spousal resemblance. Correcting for the effect of season, did not change the familial correlations, rendering this interpretation less likely (data available upon request). The seasonal effects were similar to those found in a large Japanese study (based on almost 300,000 liver function test results). While ALT and AST levels showed seasonal variation, showing peak values in the winter and a nadir in the summer, GGT did not (Miyake et al. 2009). In future research, it will be of interest to further examine the mechanism that underlies this annual variation in liver enzyme levels.

There was some evidence for cohabitation effects (Van Grootheest et al. 2008) on the spousal resemblance for AST. That is, spouses are more similar in their AST levels the longer they are together. The effects were small however. Spousal resemblance for AST and ALT was therefore modelled as resulting from phenotypic assortment, assuming that the largest part of spousal resemblance is due to phenotypic assortment for factors correlated with liver enzyme levels, such as alcohol, cigarette and coffee consumption, metabolic risk factors and physical exercise (Conigrave et al. 2003; Skurtveit and Tverdal 2002). This will not have affected the results for AST to a great extent. Assuming spousal resemblance was absent changed the heritability with only 1.3%, indicating that if spousal resemblance for AST would be due to

cohabitation effects rather than to phenotypic assortment, by assuming it was fully explained by phenotypic assortment, did not drastically change the results.

The effects of sex and age effects on mean liver enzyme levels are in accordance with the literature. A different distribution of levels of GGT, ALT and AST over sex and age has been previously reported (Pilia et al. 2006; Stromme et al. 2004), but the exact mechanisms for these effects are not clear. We detected a positive correlation for GGT with age for females ($r_{DZF/SIB/MOTHER}$=.27) and males in the offspring generation ($r_{DZM/SIB}$ =.21), but little change with age for older men ($r_{FATHERS}$= -.04) in agreement with Middelberg et al. (2012). Lower ALT levels in older men (age>60) have been detected before (Pilia et al. 2006; Stromme et al. 2004), supporting our finding that ALT levels among men were negatively associated with age. For females, lower ALT levels among older women were not reported by earlier studies (Pilia et al. 2006; Stromme et al. 2004). AST levels in females increased with age, whereas those for males remained constant, in accordance with Pilia et al. (2006).

The sex differences in the genetic architecture of GGT and ALT might reflect genetic effects on alcohol use and/or BMI that show different relationships with liver enzyme levels in men and women (Skurtveit and Tverdal 2002), which may partly result from the effect of sex hormones. GGT levels are known to vary with differences in the hormonal balance in women (Sillanaukee et al. 2000a) and some animal studies suggests that estrogens may be important in the development of alcohol-related liver injury. Chronic alcohol consumption results in a different gene expression in female rat livers, although it is not clear whether this is directly caused by hormonal influences (Eagon 2010).

The current study allowed to disentangle additive genetic, non-additive genetic effects, shared environmental and non-shared environmental effects on variation in liver enzyme levels that mark liver injury and other disease. By the inclusion of data on non-twin family members, power has improved (Posthuma and Boomsma 2000) allowing us to test for genotype by sex and genotype by age interactions. Results from these tests show that overall, there was no evidence for qualitative sex or age differences in the heritability, although quantitative sex and age differences were detected for GGT and ALT. It should be noted however that the current study does not have the power to detect the small gene by age (and gene by sex) effects that may be present at the level of individual SNPs. Our results therefore do not imply that these do not exist, as they evidently are present, as demonstrated by Middelberg et al. (2012). It will be interesting to explore whether a possible implication of our results, namely that gene finding studies on liver enzyme levels may benefit from combining data across sex and age, holds true. Although pooling data over sex and age in gene finding studies will introduce noise in the prediction model, because of the sex- or age dependent effects of some SNPs, it is hypothesized that, overall, combining results over sex and age will increase the true association signal to a

greater extent because of the increase in power and the fact that, qualitative sex or age differences in these twin-family based estimates of the heritability were not important.

In summary, genes play a substantial role in explaining differences in plasma levels of GGT, ALT and AST (h^2 22%-60%), which are important markers of liver injury and other disease. Genetic influences on liver enzyme levels include additive and non-additive genetic effects. Genotype by sex interactions (for GGT and ALT) and genotype by age interactions (for GGT in males) were present, but there was no evidence that different genes are expressed across sex or age. This suggests a positive message for genome-wide association studies. Pooling data over sex and age (with appropriate covariates to adjust sex and age mean level differences) may increase the signal-to-noise ratio to detect susceptibility loci because of increased power, without introducing too much heterogeneity that will dilute the association signal.

SUPPLEMENT TO CHAPTER 5

THE GENETIC ARCHITECTURE OF LIVER ENZYME LEVELS: GGT, ALT AND AST

Statistical analysis of the genetic architecture of liver enzyme levels

The genetic analysis of liver enzyme levels was performed with saturated models and structural equation models. In the model we have called the full saturated model, 16 familial correlations were estimated, six means, six beta coefficients for the age regression, and six standard deviations (SDs) (for twins, siblings, and parents, separate for both sexes). This model is referred to as model 1a. In a series of submodels, sex and age differences in means and variances were tested, as well as age and sex effects on the correlation structure. This was done by comparing the model fit results for the constrained models with the full model, based on the likelihood ratio test with a significance level (α) of .01. If model fit deteriorated significantly for the constrained model compared to the full model, the specific parameter constraints were not allowed (e.g. sex-invariant familial correlations), thus informing which effects were present (e.g. sex effects on the correlations).

Fixed effects of age were examined by (a) testing whether mean enzyme levels differed for twins, siblings and parents (within sex), (b) constraining betas for the age regression to zero to see if an effect of age was present, if so, (c) by constraining the betas to be equal within sex (equating betas for twins, siblings and parents) to test whether the age effect was similar for twins, siblings and parents who differ in mean age, (d) by constraining the betas over sex to test whether the age effect was equal over sex. Fixed effects of sex on enzyme levels were tested by equating mean enzyme levels over sex. To test for fixed effects of age and sex on the variance, the standard deviations (SDs) were equated for twins, siblings, and parents within sex and over sex respectively. Model 1b included the constraints on the means, SDs and age regression that were allowed based on the tests described above and provided information on the 16 familial correlations given in Figures 2a-c.

The effect of age on the familial correlations was tested in models 2a-c, and that of sex on the familial correlations in models 3a-e. In model 2a it was tested whether twin-sibling correlations were similar to sibling-sibling correlations. Using this model as a reference, in model 2b it was tested whether dizygotic (DZ) twin correlations could be constrained to sibling correlations. When this was allowed, no evidence was present for a special twin environment. Model 2c tested whether correlations among first-degree relatives could be equated. Since parent-offspring pairs show larger within-pair age differences than offspring and DZ twin pairs, the absence of significant differences in these correlations would indicate that there were no age differences in the heritability. Model 3a and 3b tested for quantitative sex differences in the

correlations. In model 3a it was tested whether the correlation among monozygotic twin pairs could be constrained to be equal. In model 3b this was tested for all first-degree relatives. Model 3c and 3d tested for qualitative sex differences in the correlations. In model 3c, male and female DZ twin and sibling correlations were constrained to be equal. Taking this model as a reference, with model 3d it was examined whether the correlations among opposite-sex DZ twin and sibling pairs could be equated to same-sex DZ twin and sibling pairs. The presence of spousal resemblance was tested in model 4. When significant spousal resemblance was detected (model 4), we explored whether this could be explained by cohabitation effects. This was tested by estimating the correlation between the absolute difference in enzyme levels for spouses and the duration of their relationship in Mx v3.2 (Neale et al. 2006) in two zygosity groups (MZ, DZ). If living together influences resemblance between spouses, this correlation will be negative, i.e. smaller differences between spouses the longer they are together. This test was based on data of 727 parental pairs and 257 twin-spouse pairs with data on relationship duration.

Structural equation modeling was performed to estimate the sources of variance that could explain differences in liver enzyme levels. Factor models were fit to the data by which phenotypic values were regressed on latent factors specifying the genetic and environmental contributions (see Figure 1). In the ACE model with shared environmental transmission, the variation in the phenotypic values for fathers, mothers, sons and daughters (depicted as P_{FA}, P_{MO}, P_{SO}, P_{DA} respectively) was regressed on three latent variables: an additive genetic factor (with factor loadings $a_{♂}$ and $a_{♀}$ for males and females respectively), and a shared environmental (factor loadings $c_{♂}$ and $c_{♀}$) and non-shared environmental factor (factor loadings $e_{♂}$ and $e_{♀}$). If there were sex differences in the correlation structure, the factor loadings were sex-specific (and thus the variance decomposition by $\Lambda\Psi\Lambda'$), otherwise, these were constrained to be equal over sex. MZ twin pairs were modeled to share their entire genetic material (that is, the additive genetic factors (A) correlate 1 within twin pairs), whereas DZ twin pairs/siblings, and parent and offspring are estimated to share half of their genes (A factors were modeled to correlate .5 DZ twin, sibling and parent-offspring pairs). The latent additive genetic variance in the offspring generation of .5 reflects the segregation variance that emerges due to recombination. This within-family additive genetic variance emerges since parents pass alleles, not genotypes, to their offspring and resulting in new genetic variance for every generation. C factors reflecting shared environmental influences were correlated 1 among the offspring generation within each family. This model allowed for shared environmental transmission from parents to offspring, indicated by $t_{FA,SO}$, $t_{MO,SO}$, $t_{FA,DA}$, $t_{MO,DA}$. The presence of both genetic and shared environmental transmission, gives rise to a correlation between the additive genetic and shared environmental factors (A-C correlation). This is represented by $R_{A,C}$. Spousal resemblance was modeled to run via the Δ-path that represents the correlations

between the latent genetic and environmental factors influencing the phenotypes of the parents, that result from phenotypic assortment. The presence of phenotypic assortment will increase the additive genetic variance, represented by RA.

If the familial correlations suggested qualitative sex differences, it could be tested whether these resulted from a (a) different expression of genes over sex, (b) differences in the environmental factors either shared within the offspring generation or (c) resulting from a sex-specific transmission of shared environmental effects from parents to offspring. Whether liver enzyme levels among males and females were affected by different genes could be tested by estimating the genetic correlation between opposite-sex parent-offspring pairs and testing whether this correlation was significantly different from .5. Qualitative sex differences in the environmental factors shared by the offspring generation, could be examined by estimating the correlation between the shared environmental factors ($r_{C,OS}$) in opposite-sex offspring pairs and testing whether this correlation differed significantly from 1. Qualitative sex differences in the shared environmental transmission paths could be explored by testing whether shared environmental transmission differed significantly for opposite-sex parent-offspring pairs and same-sex parent offspring pairs.

In the case familial correlations did not indicate evidence for qualitative sex differences, the tests described above to explore the qualitative sex differences in the genetic model were not performed. Thus if the familial correlations did not suggest qualitative sex differences, the genetic correlation for opposite-sex parent-offspring pairs was fixed to .5; the correlation between the shared environmental factors for opposite-sex twin/sibling pairs to 1; and the path loadings for the shared environmental transmission for opposite-sex parent-offspring pairs equated to those for same-sex parent-offspring pairs (resulting in the estimation of one sex-invariant shared environmental transmission path).

The ACDE model differed from the ACE with shared environmental transmission model in that the shared environmental transmission paths from parents to offspring were set to zero (for reasons of model identification) and additionally, non-additive genetic influences (D) were estimated (with factor loadings d♂ and d♀). D factors correlated 1 among MZ twin pairs and .25 among DZ twins and sibling pairs. By dropping shared environmental transmission from the model, A-C correlation was no longer implicated and set to zero.

If the familial correlations suggested age differences, the genetic models allowed to examine whether these represented quantitative or qualitative age differences in the heritability. Qualitative age differences denote a different expression of genes over age and would be indicated if the genetic correlation between same-sex parent-offspring pairs is significantly lower than .5. Note that with data of twins, siblings and parents, simultaneous estimation of the genetic parent-offspring correlation, and additive genetic, non-additive

genetic, shared environmental and non-shared environmental factors is not possible and one of these has to be set to zero to keep the model identified (a model estimating the genetic correlation between parents and offspring *and* cultural transmission effects from parents to offspring is also not identified). Quantitative age differences do not reflect a different expression of genes over age, but indicate that the heritability differs over age, for instance because of the presence of shared environmental effects or non-additive genetic effects that are shared within the offspring generation but not between parents and offspring.

Table S1 Model fit statistics for tests of age and sex effects on familial correlations for GGT, ALT and AST

		Model	NP	-2LL	*df*	vs	Δχ2	Δ*df*	*p*	Effect on correlations
GGT	1a	full saturated model	34	47724.962	8076					
	1b	saturated model with constraints on means and SDs[a]	25	47742.568	8085	1a	17.606	9	.040	
	2a	twin-sibling=sibling-sibling correlation (within sex)	22	47749.840	8088	1b	7.272	3	.064	equal twin-sibling and sibling-sibling correlations
	2b	DZ twin=sibling correlation (within sex)	19	47752.697	8091	2a	2.857	3	.414	no special twin environment
	2c	DZ twin=sibling=parent-offspring correlation (within sex)	15	47782.640	8095	1b	40.072	10	<.001	age differences in correlations first-degree relatives
	3a	male=female MZ correlation	24	47742.661	8086	1b	.093	1	.761	no quantitative sex diff in MZ corr
	3b	male=female=opposite-sex DZ twin/sib/par-off correlation	13	47789.155	8097	1b	46.587	12	<.001	quantitative sex differences in correlations first-degree relatives
	3c	male=female DZ twin/sib correlation	20	47768.211	8090	1b	25.643	5	<.001	quantitative sex differences in offspring generation
	3d	male=female=opposite-sex DZ twin/sib correlation	17	47779.352	8093	3c	11.141	3	.011	no qualitative sex differences in correlations offspring generation
	3e	same-sex=opposite-sex par-off correlation	23	47742.847	8087	1b	.279	2	.870	no qualitative sex differences in parent-offspring correlations
	4	spouse correlation=0	24	47748.226	8086	1b	5.658	1	.017	no spousal resemblance
ALT	1a	full saturated model	34	45446.979	7761					
	1b	saturated model with constraints on means and SDs[b]	23	45466.019	7772	1a	19.040	11	.060	
	2a	twin-sibling=sibling-sibling correlation (within sex)	20	45475.796	7775	1b	9.777	3	.021	equal twin-sibling and sibling-sibling correlations
	2b	DZ twin=sibling correlation (within sex)	17	45477.593	7778	2a	1.797	3	.616	no special twin environment
	2c	DZ twin=sibling=parent-offspring corr (within sex)	13	45479.039	7782	1b	13.020	10	.223	no age differences in correlations first-degree relatives
	3a	male=female MZ correlation	22	45473.616	7773	1b	7.597	1	.006	quantitative sex diff in MZ corr
	3b	male=female opp sex DZ twin/sib/par-off	11	45511.921	7786	1b	18.037	12	.115	no quantitative sex differences in

		correlation								correlations first-degree relatives
	3c	male=female DZ twin/sib correlation	18	45480.320	7777	1b	14.301	5	.014	no quantitative sex differences in correlations offspring generation
	3d	male=female=opp sex DZ twin/sib correlation	15	45482.886	7780	3c	2.566	3	.463	no qualitative sex differences in correlations offspring generation
	3e	same-sex=opposite-sex par-off correlation	21	45466.937	7774	1b	.918	2	.632	no qualitative sex differences in parent-offspring correlations
	4	spouse correlation=0	22	45486.658	7773	1b	20.640	1	<.001	significant spousal resemblance
AST	1a	full saturated model	34	38520.527	8034					
	1b	saturated model with constraints on means and SDs[c]	22	38549.677	8046	1a	29.150	12	.085	
	2a	twin-sibling=sibling-sibling correlation (within sex)	19	38554.675	8049	1b	4.998	3	.172	equal twin-sibling and sibling-sibling correlations
	2b	DZ twin=sibling correlation (within sex)	16	38563.456	8052	2a	8.781	3	.032	no special twin environment
	2c	DZ twin=sibling=parent-offspring correlation (within sex)	12	38570.088	8056	1b	20.412	10	.026	no age differences in correlations first-degree relatives
	3a	male=female MZ correlation	21	38550.371	8047	1b	.694	1	.405	no quantitative sex diff in MZ corr
	3b	male=female opposite-sex DZ twin/sib/par-off correlation	10	38571.054	8058	1b	21.378	12	.045	no quantitative sex differences in correlations first-degree relatives
	3c	male=female DZ twin/sib correlation	17	38557.833	8051	1b	8.156	5	.148	no quantitative sex differences in correlations offspring generation
	3d	male=female=opposite-sex DZ twin/sib correlation	14	38566.211	8054	3c	8.378	3	.039	no qualitative sex differences in correlations offspring generation
	3e	same-sex=opposite-sex par-off correlation	20	38550.182	8048	1b	.506	2	.777	no qualitative sex differences in parent-offspring generation
	4	spouse correlation=0	21	38578.384	8047	1b	28.708	1	<.001	significant spousal resemblance

NP = number of parameters in statistical model; -2LL =-2 log likelihood, fit function, for model with *df* degrees of freedom

$\Delta \chi^2$ = model fit statistic: difference in -2LL of two nested models approximately distributed as χ^2 with $df = \Delta df$, the difference in NP between the models; p-value was regarded significant when <.01; vs=versus, model that constrained model is compared against; corr=correlation; diff=differences

[a] For GGT constraints included three SDs (male/female offspring, parents), four means (male/female offspring, fathers, mothers), two betas for the age regression (fathers, others)

[b] For ALT constraints included one sex invariant SD, two sex specific means, and four betas for age regression (male/female offspring; fathers, mothers)

[c] For AST constraints included sex specific SDs, means, betas for age regression

Table S2a Parameter constraints (and number of degrees of freedom) associated with tests on saturated models

Model comparison	Test	Phenotype	Δ*df*	Constraints
1b vs 1a	constraints on means and SDs	GGT	9	mean father=mean mother (Δ*df*=1) mean male twin=male sibling (Δ*df* =1) mean female twin=female sibling (Δ*df* =1) SD male twin=male sib (Δ*df* =1) SD female twin=female sib (Δ*df* =1) beta age male twin=male sib=female twin=female sib=mother (Δ*df* =4) *Number of parameters saturated model with constraints: 25* - 4 SDs (male twins/siblings, female twin/siblings, fathers, mothers) - 3 means (male twins/siblings, female twin/siblings, parents) - 2 betas age regression (fathers, others) - 16 familial correlations (see text)
		ALT	11	mean male twin=male sibling=father (Δ*df* =2) mean female twin=female sibling=mother (Δ*df* =2) SD male=female twin/sibling/parent (Δ*df* =5) beta age male twin=male sib (Δ*df* =1) beta age female twin=female sib (Δ*df* =1) *Number of parameters saturated model with constraints: 23* - 1 SDs (sex invariant) - 2 means (males, females) - 4 betas age regression (male twin/sib, female twin/sib, fathers, mothers) - 16 familial correlations (see text)
		AST	12	mean male twin=male sibling=father (Δ*df* =2) mean female twin=female sibling=mother (Δ*df* =2) SD male twin=male sib=father (Δ*df* =2) SD female twin=female sib=mother (Δ*df* =2) beta age male twin=male sib=father (Δ*df* =2) beta age female twin=female sib=mother (Δ=2) *Number of parameters saturated model with constraints: 22* - 2 SDs (males, females) - 2 means (males, females) - 2 betas age regression (males, females) - 16 familial correlations (see text)

Table S2b Parameter constraints (and number of degrees of freedom) associated with tests on correlations

Model comparison	Test	Pheno-type	Δ*df*	Constraints
2a vs 1b	twin-sibling=sibling-sibling correlation (within sex)	ALL	3	male twin-brother=brother-brother correlation (Δ*df* =1) female twin-sister=sister-sister correlation (Δ*df* =1) opposite-sex twin-sibling=brother-sister correlation (Δ*df* =1)
2b vs 2a	DZ twin=sibling correlation (within sex)	ALL	3	male DZ twin=male twin/brother-brother correlation (Δ*df* =1) female DZ twin=female twin/sister-sister correlation (Δ*df* =1) opposite-sex DZ twin=opposite-sex twin/sibling-sibling corr (Δ*df* =1)
2c vs 1b	DZ twin=sibling=parent-offspring correlation (within sex)	ALL	10	male DZ twin=male twin-brother=brother-brother=father-son correlation (Δ*df*=3) female DZ twin=female twin-sister=sister-sister=mother-daughter corr (Δ*df* =3) opposite-sex DZ twin=opposite-sex twin-sibling=brother-sister = mother-son=father-daughter correlation (Δ*df* =4)
3a vs 1b	male=female MZ correlation	ALL	1	male MZ twin=female MZ twin correlation
3b vs 1b	male=female opposite-sex DZ twin/sib/par-offspring correlation	ALL	12	male DZ twin=male twin-brother=brother-brother=father-son= female DZ twin=female twin-sister=sister-sister=mother-daughter= opposite-sex DZ twin=opposite-sex twin-sibling=brother-sister =mother-son=father-daughter correlation (Δ*df* =12)
3d vs 3c	male=female=opposite-sex DZ twin/sibling correlation	ALL	3	same-sex DZ twin/sib=opposite-sex DZ twin correlation (Δ*df* =1) same-sex DZ twin/sib=opposite-sex twin-sibling correlation (Δ*df* =1) same-sex DZ twin/sib=opposite-sex sib-sib correlation (Δ*df* =1)
3e vs 1b	same-sex=opposite-sex par-off correlation	ALL	2	father-son=mother-son correlation (Δ*df* =1) mother-daughter=mother-son correlation (Δ*df* =1)
4 vs 1b	spouse correlation = 0	ALL	1	father-mother correlation=0 (Δ*df* =1)

vs=versus; Δ*df*= the difference in degrees of freedom between the tested model and the full model (is equal to the difference in the number of parameters of the two models)

Table S2c Parameter constraints (and number of degrees of freedom) associated with tests in ACDE model/ ACE model with shared environmental transmission

Model comparison	**Test**	**Phenotype**	**Δ*df***	**Constraints**
ACE with shared environmental transmission vs 1b	Does the ACE model with shared environmental transmission fit data	GGT	8	*Number of parameters saturated model with constraints (model 1b): 25* *Number of parameters ACE model with CT: 17* - 4 SDs (male twins/siblings, female twin/siblings, fathers, mothers) - 3 means (male twins/siblings, female twin/siblings, parents) - 2 betas age regression (fathers, others) - 2 additive genetic (a) path-loadings (males, females) - 2 shared environmental (c) path-loadings (males, females) - 1 shared environmental transmission paths (sex-invariant) - 2 non shared individual specific environmental (e) path loadings - 1 covariance between additive genetic and shared environmental effects ($r_{A,C}$)
		ALT	6	*Number of parameters saturated model with constraints: 23* *Number of parameters ACE model with CT: 17* - 1 SDs (sex invariant) - 2 means (males, females) - 4 betas age regression (male twin/sib, female twin/sib, fathers, mothers) - 2 additive genetic (a) path-loadings (males, females) - 2 shared environmental (c) path-loadings (males, females) - 1 shared environmental transmission paths (sex-invariant) - 2 non shared individual specific environmental (e) path loadings - 1 covariance between additive genetic and shared environmental effects ($r_{A,C}$) - 1 covariance among parents (estimation of delta path to model phenotypic assortment) - 1 additive genetic variance (R_A) (due to phenotypic assortment)
		AST	9	*Number of parameters saturated model with constraints: 22* *Number of parameters ACE model with CT: 13* - 2 SDs (males, females) - 2 means (males, females) - 2 betas age regression (males, females) - 1 additive genetic (a) path-loadings (sex-invariant)

				- 1 shared environmental (c) path-loadings (sex-invariant) - 1 shared environmental transmission paths (parent-offspring, sex-invariant) - 1 non shared individual specific environmental (e) path loadings (sex-invariant) - 1 covariance between additive genetic and shared environmental effects ($r_{A,C}$) - 1 covariance among parents (estimation of delta path to model phenotypic assortment) - 1 additive genetic variance (R_A) (due to presence phenotypic assortment)
ACDE vs 1b	Does the ACDE model fit data	GGT	8	*Number of parameters saturated model with constraints: 25* *Number of parameters ACDE model: 17* - 3 SDs (male offspring, female offspring, parents) - 4 means (male offspring, female offspring, fathers, mothers) - 4 betas age regression (male twin/sib, female twin/sib, fathers, mothers) - 2 additive genetic (a) path-loadings (males, females) - 2 non-additive genetic (d) path-loadings (males, females) - 2 shared environmental (c) path-loadings (males, females) - 2 non shared individual specific environmental (e) path loadings
		ALT	6	*Number of parameters saturated model with constraints: 23* *Number of parameters ACE model with CT: 17* - 1 SDs (sex invariant) - 2 means (males, females) - 4 betas age regression (male twin/sib, female twin/sib, fathers, mothers) - 2 additive genetic (a) path-loadings (males, females) - 2 non-additive genetic (d) path-loadings (males, females) - 2 shared environmental (c) path-loadings (males, females) - 2 non shared individual specific environmental (e) path loadings - 1 covariance among parents (estimation of delta path to model phenotypic assortment) - 1 additive genetic variance (R_A) (due to phenotypic assortment)
		AST	10	*Number of parameters saturated model with constraints: 22* *Number of parameters ACE model with CT: 12* - 2 SDs (males, females) - 2 means (males, females) - 2 betas age regression (males, females)

				- 1 additive genetic (a) path-loadings (sex-invariant) - 1 non-additive genetic (d) path-loadings (sex-invariant) - 1 shared environmental (c) path-loadings (sex-invariant) - 1 non shared individual specific environmental (e) path loadings (sex-invariant) - 1 covariance among parents (estimation of delta path to model phenotypic assortment) - 1 additive genetic variance (R_A) (due to presence phenotypic assortment)
ADE vs ACDE	drop C to zero	ALL both sexes	2	c-path males=c-path females=0 (Δdf =2)
ACE vs ACDE	drop D to zero	ALL both sexes	2	d-path males=d-path females=0 (Δdf =2)
AE vs ACDE	drop A+D to zero	ALL both sexes	5	a-path males=a-path females=0 (Δdf =2) d-path males=d-path females=0 (Δdf =2) additive genetic variance (R_A)=1 (Δdf =1)

vs=versus; Δdf= the difference in degrees of freedom between the tested model and the full model (is equal to the difference in the number of parameters of the two models)

CHAPTER 6

THE ASSOCIATION OF ALCOHOL INTAKE WITH GAMMA-GLUTAMYL TRANSFERASE (GGT) LEVELS: EVIDENCE FOR A CAUSAL EFFECT?[4]

Abstract

Background Blood levels of gamma-glutamyl transferase (GGT) are used as a marker for (heavy) alcohol use. The role of GGT in the anti-oxidant defense mechanism that is part of normal metabolism supposes a causal effect of alcohol intake on GGT. However, there is variability in the response of GGT to alcohol use, which may result from genetic differences between individuals. This study aimed to determine whether the association between alcohol intake and GGT at the population level is causal or reflects the effects of shared genes (e.g. genetic pleiotropy).

Methods Data on alcohol intake (grams alcohol/day) and GGT, originating from twins, their siblings and parents (N=6,465), were analyzed with structural equation models. Bivariate genetic models tested whether genetic and/or environmental influences on alcohol intake and GGT correlated significantly.

Results Phenotypic correlations between alcohol intake and GGT were significant in men (r=.17) and women (r=.09). Genetic influences on alcohol intake and GGT correlated significantly (p<.001), whereas environmental influences did not (p=.041).

Conclusions In this healthy population sample, the association of alcohol intake with GGT is most likely explained by shared genetic effects. Future longitudinal twin studies should determine whether a causal effect of alcohol use on GGT is confined to heavy drinking populations.

[4] This chapter is under revision for publication in Drug and Alcohol Dependence as:
van Beek, JHDA, de Moor, MHM, Geels, LM, Sinke, MRT, de Geus, EJC, Lubke, GH, Kluft, C, Neuteboom, J, Vink, JM, Willemsen, G, Boomsma, DI. The association of alcohol intake with gamma-glutamyl transferase (GGT) levels: evidence for a causal effect?

1. Introduction

Blood levels of the liver enzyme gamma-glutamyl transferase (GGT) are used as a biomarker for heavy drinking (Peterson 2004). GGT is implicated in alcohol use by keeping intracellular glutathione, the body's most abundant anti-oxidant, at adequate levels to protect cells from oxidative stress resulting during metabolism, including that of alcohol (Whitfield 2001). Experimental studies support a causal relation between heavy alcohol use and increased GGT levels, but also in experimental settings there is variability in the response of GGT to alcohol which depends on individual characteristics, such as sex, age, and previous drinking habits (Whitfield 2001). This inter-individual variability in GGT levels in response to alcohol may reflect the effect of genetic differences between individuals. The association of alcohol use and GGT levels at the population level (Conigrave et al. 2003) may then not result from a causal effect of alcohol use on GGT levels but be reflective of genetic effects on alcohol use that are shared with those on GGT (e.g. genetic pleiotropy).

There is evidence that genetic influences on alcohol use and GGT are correlated. Variation in GGT and alcohol use is moderately heritable, with a broad-sense heritability of 30%-70% for GGT (Van Beek et al. 2013a), ~50% for alcohol intake (Van Beek et al. 2013c) and the overlap in genetic risk factors estimated at 8% (Whitfield and Martin 1985). Genetic variants in the ALDH2 gene linked to alcohol metabolism (Wang et al. 2012) have also been associated with levels of GGT (Kamatani et al. 2010). Three studies showed that the effect of these genetic variants on GGT was absent when stratifying individuals according to alcohol use (Kamatani et al. 2010; Nose et al. 2008; Tseng et al. 2008), suggesting that the effect of alcohol use on GGT is causal. In two other studies, the effect was attenuated but still present, with lower GGT levels among carriers of the ALDH2*2 allele (Takeshita et al. 2000; Takeuchi et al. 2011), providing support for the hypothesis of shared genes. These effects though detected among East-Asians may extend to alcohol-related variants of the ALDH1A1 and ALDH1B1 genes that, in contrast to the ALDH2*2 allele, are not invariant in European populations (Hurley and Edenberg 2011). In addition, alcohol use is affected by many more genetic variants (Morozova et al. 2012) that may also influence GGT levels.

The nature of the association between alcohol use and GGT levels can be tested by conducting a bivariate genetic analysis using data from twins and their family members. Twin studies can dissect phenotypic trait variation as well as covariation between traits into effects that can be ascribed to genetic differences between individuals and differences in the environment that individuals were exposed to. Monozygotic (MZ) twins are assumed to share all their genetic material, whereas dizygotic (DZ) twins share about half of their genes. Comparing the phenotypic resemblance among MZ twins versus that of DZ twins thus gives an indication of the importance of genetic and environmental factors (Van Dongen et al. 2012). By

including data of family members other than twins (parents, siblings), the number of individuals is increased and the power to disentangle genetic and environmental effects is augmented (Posthuma and Boomsma 2000). If alcohol use is causally influencing GGT levels, both genetic influences and environmental influences on alcohol use must be correlated with those for GGT. If either genetic influences on alcohol intake and GGT or environmental influences on alcohol intake and GGT are not correlated, a causal effect of alcohol use on GGT levels is less likely. The presence of a correlation between the genetic influences on alcohol intake and GGT but not between the environmental influences suggests that the association emerged due to shared genes (De Moor et al. 2008).

Two twin(-family) studies have investigated whether genetic and environmental effects on alcohol use are correlated with those for GGT (Whitfield and Martin 1985; Sung et al. 2011). In both studies, alcohol use significantly predicted GGT levels among males (r=.19-.39), but not among females (at α=.01), underlining that GGT is a less sensitive marker of alcohol use in women. Regarding the results for men, genetic and environmental effects on GGT were correlated with those for problematic alcohol use in Koreans (N♂=811) (Sung et al. 2011), in line with a causal effect of alcohol use on GGT. In a sample of 199 Australian men, Whitfield and Martin (1985) detected that genetic influences, but not environmental influences, on alcohol intake and GGT were significantly correlated, thus pointing at effects of shared genes. The discrepancy in findings may be explained by differences in sample size (and associated differences in power), ethnicity, and/or phenotype. If the effect of problematic alcohol use on GGT is not a mere reflection of (extreme) alcohol intake, then a different mechanism may be at play with a different etiology.

The aim of this study is to examine the mechanism that underlies the association of alcohol intake with GGT in a healthy Dutch population sample. By modeling data from 6,465 twins and their family members, it is tested whether the association of alcohol use with GGT could be causal in origin or is better explained by shared genes. Sex differences in the mechanism underlying the association are examined.

2. Methods

2.1 Participants

Data on alcohol intake and GGT levels originated from adult twins and their family members registered with the Netherlands Twin Register (NTR) (Boomsma et al. 2002c; Willemsen et al. 2013). Information on GGT levels determined in plasma were present for 8,754 individuals (aged ≥18) who participated in the NTR biobank study conducted between 2004 and 2008 (Willemsen et al. 2010). Data on alcohol intake came from the 2002, 2004 and 2009 surveys of the longitudinal survey study on health, personality and lifestyle. Analyses were

performed on data from 6,465 individuals for whom data on alcohol intake and levels of GGT were present (3,193 twins, 1,304 siblings, and 1,968 parents from 2,815 families). Data of 2,289 individuals were excluded, including data of abstainers (N=121) and individuals with known liver disease (N=11) (see Supplementary Materials for a complete list of excluded individuals). Individuals were categorized into five zygosity by sex groups (see Table 1), based on the zygosity of the twin pair, which for same-sex twins was determined by DNA comparison. Overall, 64.9% was female (year of birth 1915-1988).

Table 1 Number of participants per zygosity by sex group

	Number of participants				
Zygosity by sex group [a]	**twins** [b]	**brothers**	**sisters**	**fathers**	**mothers**
MZM	487 (67.4%)	42	56	94	112
DZM	252 (56.3%)	29	37	65	83
MZF	1,230 (75.0%)	81	124	189	225
DZF	602 (70.8%)	58	76	118	146
DOS	622 (56.3%)	61	89	148	176
Families without twins [c]	0	102	549	267	345
Total	3,193	373	931	881	1,087

[a] MZM: monozygotic males, DZM: dizygotic males, MZF: monozygotic females, DZF: dizygotic females, DOS: dizygotic opposite sex

[b] percentage reflects what proportion of twins is part of a complete twin pair

[c] families in which twins did not participate

2.2 Measures

GGT levels were determined in blood collected between 7.00 and 10.00 a.m. after an overnight fast at the participant's home. Participants were asked to refrain from smoking one hour before the home visit, and to abstain from physical exertion and medication on the day of the home visit, if possible. Blood was collected in heparin plasma tubes that were turned gently 8-10 times immediately after collection to prevent clotting (Willemsen et al. 2010). During transportation, heparin plasma tubes were stored in melting ice. When the samples arrived at the laboratory, plasma was collected and six samples of 500 µL were snap-frozen and stored at -30 °C. Levels of GGT were determined with Vitros assays (Vitros 250, Ortho-Clinical Diagnostics; Johnson & Johnson, Rochester, USA)[5] in units per liter (U/L) (Willemsen et al. 2010).

Self-report data on alcohol use were obtained from surveys 6, 7 and 8 collected between 2002-2012, as part of the longitudinal study on health, lifestyle and personality (Willemsen et

[5] Acceptance criteria: Inter-assay CV <5.0%; Intra-assay CV <3.5%

al. 2013). If alcohol data were available from two or more surveys, the survey was selected for which the time interval to the biobank visit was smallest (absolute time difference 15.1 months on average, SD=13.3). Alcohol intake was measured by the question 'How many glasses a week do you drink on average?'. In survey 6 and 7, response categories were: 'less than 1 glass', '1–5 glasses a week', '6–10 glasses a week', '11–20 glasses a week', '21-40 glasses a week', and 'more than 40 glasses a week'. In survey 8, individuals were asked to report the number of glasses of beer, wine and spirits they drank for each day of the week. These numbers were summed and categorized as in survey 6 and 7. In the analyses described below, alcohol intake was analyzed as the average amount of (grams of) alcohol consumed per day. This was obtained from the question given above by taking the median number of drinks per week for each category (0, 3, 8, 15, 30.5 or 46), multiplied by 14 grams of alcohol per glass, divided by seven (days in the week). The last category ('more than 40 glasses a week') was given the value of 46 based on the median number of drinks among individuals who reported to consume 41 or more drinks per week in survey 8 (in which number of drinks was reported as a continuous measure). Alcohol intake (Van Beek et al. 2013c) and GGT (Van Beek et al. 2013a) were highly skewed and *log*-transformed to approximate normality. Age effects were regressed out prior to the analyses.

2.3 Statistical analyses

The bivariate genetic analyses of alcohol intake with GGT levels were conducted in Mx (v1.54) (Neale et al. 1994; Neale et al. 2006) and performed in five zygosity by sex groups. The analyses consisted of two steps. First, a saturated model (model 1) was fitted that estimated the familial cross-trait correlations as well as the familial within-trait correlations for alcohol intake and GGT. Means and variances were modeled as in Van Beek et al. (2013a,c). For alcohol intake, one variance was estimated (equal over sex) and two means (males, females). For GGT, four means were estimated (for parents and offspring, separately over sex) and three variances (male offspring, female offspring, parents; see also Supplementary Materials). The significance of the cross-trait correlations between alcohol intake and GGT was evaluated in an overall model (model 2) and separately by sex (models 2a, 2b). Model comparison was based on the likelihood ratio test with a significance level of .01 (Bentler and Bonett 1980).

In a second step, by structural equation modeling it was estimated what part of the correlation of alcohol intake with GGT could be ascribed to genetic and what part to environmental correlations. This was done in bivariate genetic factor models (Neale et al. 1994). The amount of the variance and covariance of alcohol intake and GGT that can be ascribed to genetic and environmental factors, can be estimated because family members share their genetic and environmental background to different degrees. MZ twin pairs share (nearly) all of their genetic material, whereas DZ twin and sibling pairs, share half of their segregating genes

on average. Parents and their offspring share exactly 50% of their segregating genes. Non-additive genetic influences that reflect effects of interacting risk alleles due to dominance and/or epistasis (Posthuma et al. 2003), can be estimated because these are correlated 1 in MZ pairs, whereas DZ twins and sibling pairs share on average a quarter (.25) of the non-additive genetic factors. Parent-offspring pairs share none of the non-additive genetic factors. Individual-specific environmental effects are not shared between family members, and are estimated as the remainder of the (co)variance that is not captured by genetic and/or environmental effects that are common to family members. For the covariance structure, a model was specified that included additive genetic (A), non-additive genetic (D) and individual-specific environmental factors (E) (model 3; see Figure 1), informed by the fact that shared environmental influences (C) were not significantly influencing alcohol intake levels (Van Beek et al. 2013c) and thus could not influence the covariance with GGT. The Supplementary Materials offer further details on the bivariate variance-covariance decomposition of alcohol intake and GGT.

The genetic and environmental correlations between alcohol intake and GGT were tested for significance separately over sex (models 4-6). If genetic influences on alcohol intake and GGT were correlated *and* environmental influences on alcohol intake and GGT were correlated, this would be consistent with a causal effect of alcohol intake on GGT. If only the genetic effects were correlated, but the environmental effects were not, the association was better explained by shared genes.

The correlations between genetic and environmental influences on alcohol intake (ALC) and GGT (GGT), were calculated from the genetic and environmental variances (*var*) and covariances (*cov*) for these traits. The additive genetic correlation $r_{a,ALC,GGT}$, non-additive genetic correlation $r_{d,ALC,GGT}$, and individual-specific environmental correlations $r_{e,ALC,GGT}$, can then be expressed as follows:

$$r_{a,ALC,GGT} = \frac{\text{cov}(A_{ALC,GGT})}{\sqrt{\text{var}(A_{ALC}) * \text{var}(A_{GGT})}} \qquad r_{d,ALC,GGT} = \frac{\text{cov}(D_{ALC,GGT})}{\sqrt{\text{var}(D_{ALC}) * \text{var}(D_{GGT})}}$$

$$r_{e,ALC,GGT} = \frac{\text{cov}(E_{ALC,GGT})}{\sqrt{\text{var}(E_{ALC}) * \text{var}(E_{GGT})}}$$

3. Results

3.1 Descriptives

Table 2 shows levels of alcohol intake, GGT, and age separately for males and females. Alcohol intake and GGT were higher in men than women. Inspection of mean GGT levels over drinking categories did not show evidence of a J-shaped function. GGT levels linearly increased with drinking categories (data available upon request).

Table 2 Descriptive statistics[a] for males and females

	Mean (SD)	
	Males	**females**
alcohol grams/day	18.1 (18.4)	8.7 (12.0)
GGT	40.0 (38.4)	26.9 (30.5)
age	46.4 (15.7)	43.5 (13.8)

[a] untransformed values for alcohol in grams/day and GGT

3.2 Genetic analysis of alcohol intake with GGT

Alcohol intake predicted GGT levels. The phenotypic correlation of alcohol intake with GGT was significant in men (r=.17) and women (r=.09) (Table 2). Model fit statistics of the tested models and conclusions that follow from it, are reported in Table 3. Familial cross-trait correlations were significant for MZ pairs (r_{MZM}=.14; r_{MZF}=.07), but not for first-degree relatives (see Table 4). The bivariate genetic model that specified additive (A), non-additive (D) and individual-specific environmental factors (E) affecting the covariation of alcohol intake with GGT (ADE model), fitted well compared to the saturated model. For both males and females, genetic factors correlated between GGT and alcohol, but there was no significant correlation between the environmental factors (at α=.01). This suggest the presence of genetic pleiotropy. Since the environmental correlation was close to significant however (p=.041), a causal effect of alcohol intake on GGT may play a role.

The overlap in genetic influences on alcohol intake and GGT was mainly found for non-additive genetic effects. For females, the correlation between non-additive genetic influences on alcohol intake and GGT (r_d) was .39 (95% confidence interval, CI, .06-.47) with the correlation between additive genetic influences on alcohol intake and GGT (r_a) estimated at .00 (95% CI .00-.15). For males, all non-additive genetic effects on GGT were modeled to be shared with alcohol intake (r_d=1), with r_a again estimated at .00 (95% CI .00-.12). Looking at the specific contribution of these genes, for men 7.6% of the variance in GGT could be explained by non-additive genetic effects that were shared with those for alcohol intake. For women, this was 4.6%.

Table 3 Model fit statistics for genetic models of alcohol intake with GGT

	Model	NP	-2LL	*df*	vs.	Δχ2	Δ*df*	*p*	Conclusions
1	saturated model [a]	40	67929.817	12890					
2	all correlations with GGT set to 0	29	68066.526	12901	1	136.71	11	<.001	Alcohol intake predicts GGT
2a	within male person cross-trait corr set to 0	39	67990.590	12891	1	60.77	1	<.001	Sign correlation alcohol intake and GGT males
2b	within female person cross-trait corr set to 0	39	67964.927	12891	1	35.11	1	<.001	Sign correlation alcohol intake and GGT females
3	ADE model for cross-trait variation	30	67952.666	12900	1	22.85	10	.011	ADE model fits well
4	drop genetic correlation D to 0 i.e. AE model for cross-trait variation	28	67969.542	12902	3	16.88	2	<.001	Sign non-additive genetic correlation alcohol intake and GGT
4a	drop genetic correlation D to 0 males only	29	67963.211	12901	3	10.54	1	.001	Sign non-additive genetic correlation alcohol intake and GGT males
4b	drop genetic correlation D to 0 females only	29	67959.552	12901	3	6.86	1	.009	Sign non-additive genetic correlation alcohol intake and GGT females
5	drop genetic correlation, A+D, to 0 i.e. E model for cross-trait variation	25	67974.150	12905	3	21.48	5	<.001	Sign (additive+non-additive) genetic correlation
5a	drop genetic correlation (A+D) to 0 males only	28	67964.703	12902	4	12.04	2	.002	Sign (additive+non-additive) genetic correlation males
5b	drop genetic correlation (A+D) to 0 females only	28	67962.714	12902	4	10.05	2	.007	Sign (additive+non-additive) genetic correlation females
6	drop environmental correlation (E) to 0 i.e. AD model for cross-trait variation	28	67959.059	12902	4	6.39	2	.041	Environmental corr not significant
6a	drop environmental correlation (E) to 0 males only	29	67956.255	12901	4	3.59	1	.058	Environmental corr not sign males
6b	drop environmental correlation (E) to 0 females only	29	67955.486	12901	4	2.82	1	.093	Environmental corr not sign females

NP=number of parameters in statistical model; -2LL=-2 *log*-likelihood, fit function, for model with *df* degrees of freedom

Δ χ^2=model fit statistic: difference in -2LL of two nested models approximately distributed as χ^2 with *df* = Δ *df*, the difference in NP between the models; *p*-value was regarded significant when < .01; sign=significant (at α=.01); corr=correlation

[a] Constraints included two means (males, females) and one variance (equal for males and females) for alcohol intake and four means (male twins/sibs, female twins/sibs, fathers, mothers) and three variances (male twins/sibs, female twins/sibs, parents) for GGT.

Table 4 Cross-trait correlations for alcohol intake with GGT, for male-male, female-female and opposite-sex pairs

	Cross correlations alcohol intake with GGT (95% confidence intervals)		
	Male-male	**Female-female**	**Opposite sex**
Within-person	0.169 (0.127, 0.210)	0.093 (0.062, 0.123)	
MZ	0.140 (0.064, 0.214)	0.072 (0.025, 0.118)	
DZ/sib	0.007 (-0.071, 0.084)	-0.035 (-0.084, 0.014)	-0.041 (-0.096, 0.015)
Parent-offspring	-0.015 (-0.084, 0.054)	0.005 (-0.040, 0.050)	-0.040 (-0.092, 0.011)[a] -0.041 (-0.103, 0.020)[b]

[a] cross-trait correlation for father-daughter

[b] cross-trait correlation for mother-son

4. Discussion

The current study examined whether the association of alcohol intake with GGT in a Dutch healthy population sample is best explained by a causal effect of alcohol intake on GGT or by shared genes (e.g. genetic pleiotropy). Since there was a significant genetic correlation of alcohol intake with GGT, but an environmental correlation that was not significant at α=.01, in this healthy population sample results seem most consistent with genetic pleiotropy, in line with Whitfield and Martin (1985).

The difference in results between our study and that among Koreans (Sung et al. 2011), may suggest that the mechanism underlying the association of alcohol use with GGT differs over ethnicity. Alternatively, the causal effect of alcohol use on GGT levels is dependent on the level of (heavy) drinking. Although NTR participants are representative to the Dutch population for regular alcohol use (Distel et al. 2007), male NTR participants drank less and not as heavy as the Korean participants (127 grams/week, 16% AUDIT score≥8 versus 181 grams/week, 39% AUDIT score ≥8 respectively) (Sung et al. 2011). It is possible that some sensitization occurs in the liver during heavy drinking that is absent or different from that in normal drinkers. This sensitization may be triggered by factors that differ between normal drinkers and heavy drinkers, such as certain diet specifics (e.g. low carbohydrate, high fat content), the development of fatty liver, iron overload or certain immune reactions (Whitfield 2001). Future longitudinal twin studies should test whether a causal effect of alcohol use on GGT is confined to heavy drinking populations.

This study is the first to show that genes for alcohol intake and GGT are shared among women. Female NTR participants drank more than those in the study by Sung et al. (2011) (62 grams/week versus 53 grams/week), which may explain the higher correlation of alcohol use with GGT among Dutch compared to Korean women. Given that the response of GGT is dependent on previous drinking habits (Whitfield 2001), the higher correlation of alcohol use

with GGT among Dutch women is likely due to the augmented alcohol consumption in this group over the past years (Geels et al. 2013).

The rather low within-person correlations of alcohol intake with GGT (.09-.17) might in part be due to the time difference between the blood draw and the assessment of alcohol intake (15 months on average). Other studies detected higher correlations for alcohol intake with GGT for males (r=.20-.40), although not for females (r=.00-.30) (Conigrave et al. 2003; Whitfield and Martin 1985; Sung et al. 2011). The influence of this time interval on the results is considered to be minor however. First, the surveys inquired about alcohol consumption in the past year and were therefore assumed to capture regular alcohol use, the type of drinking pattern that is most clearly associated with GGT (Conigrave et al. 2003). The stability of alcohol intake over time was high (r=.80 for over a two year period; .67 for over a six year period) and the correlation between alcohol intake and GGT was not dependent on the time difference (data available upon request). Second, the correlation between alcohol intake and GGT was comparable to that in another Dutch sample that assessed liver enzyme levels and alcohol use at the same day (.11-.17 for females-males), as shown in a previous study (Van Beek et al., in preparation). The lower correlations are more likely explained by variation in whether or not participants drank alcohol during the days preceding the home visit. Although participants abstained from food and drinks from 10 p.m. the night before the blood collection, for individuals who had been drinking heavily earlier that evening, GGT levels would have been temporarily increased, leading to noise in the overall prediction (which is not equal to bias). Conversely, it may be argued that our results were biased since by including frequent as well as infrequent drinkers in the sample, not all participants will have been drinking the days preceding blood collection, which may have diluted the association signal. The aim of the study was not to show an increase in GGT after drinking however, but to test whether at the population level, individuals who drink more have higher GGT levels and to explain that association. Nevertheless, to examine whether any dilution of the association signal might have occurred because we included not-frequent drinkers in the sample, we reran the analyses on data restricted to individuals who reported to drink at least several times per week to increase the likelihood of sampling individuals who had been drinking in the days preceding blood collection. Results were similar. The bias resulting from a dilution of the association signal due to sampling time, is therefore considered to be minor.

In the current study that compared the hypothesis of full causality versus full genetic pleiotropy, data were more consistent with the latter, although the environmental correlation between alcohol intake and GGT was close to significant and a causal effect of alcohol intake on GGT could not be ruled out. Whereas the power to detect shared genetic effects (additive and non-additive) was very good (.95 for α=.01) it was lower for environmental effects (.51 for α=.01; .73 for α=.05). The reality may be complex, with a combination of genetic pleiotropy and

causality explaining the association at the population level. The mechanism of association may be dependent on drinking level with genetic pleiotropy explaining the association in a low drinking population and causality that in a heavy drinking population as stated above. This can suggest gene by alcohol interaction, which presents an interesting venue for future research.

The shared genetic effects detected in this study may reflect genetic effects on cardiometabolic traits that are associated with alcohol use and GGT levels. Genetic risk factors for GGT have been linked to cardiometabolic traits such as levels of cholesterol (LDL, HDL), triglycerides, glucose, and insulin resistance (Chambers et al. 2011; Kim et al. 2011; Whitfield et al. 2002). In reverse, the CDH13 gene associated with alcohol dependence (Morozova et al. 2012), has been associated with blood pressure, hypertension (Johnson et al. 2011), and metabolic syndrome (Fava et al. 2011). The DSCAML1 gene linked to alcohol phenotypes in humans and other species (Morozova et al. 2012), has been associated to levels of triglycerides (Pollin et al. 2008). Alcohol use and conditions of the cardiometabolic syndrome have similar effects on liver functioning through induction of oxidative stress via mitochondrial defects (Mantena et al. 2008). Since GGT has been coined a marker for fatty liver (Targher 2009) and oxidative stress (Lee et al. 2004), the shared genetic effects on alcohol intake and GGT may reflect that the genetic etiology of alcohol-induced and obesity-induced fatty liver disease is partly shared causing an association between alcohol use and GGT at the population level.

Despite the shared genetic effects for alcohol use and GGT, genome wide association studies for GGT have not yet detected variants that have been implicated in alcohol use (e.g. see Chambers et al. (2011)), with the exception of (variants in close linkage with) the ALDH2*2 allele in East-Asian populations (Kamatani et al. 2010; Kim et al. 2011; Baik et al. 2011). Based on the current findings it will be interesting to take the substantive non-additive genetic variation underlying the association of alcohol intake and GGT into account by performing gene-finding studies that assume a (2*df*) genotypic model instead of an (1*df*) additive model. In addition, prediction models may be fitted that involve complex interactions among the genetic markers, such as random forests (Molinaro et al. 2011).

To conclude, the current study detected that the association between alcohol intake and GGT at the population level is best explained by shared genes, although an additional causal effect of alcohol intake on GGT cannot be ruled out. The observation that the relation between alcohol intake and GGT is genetically mediated involving mostly non-additive genetic effects warrants further study and calls for gene-finding efforts that take dominant genetic effects and complex gene interactions into account.

SUPPLEMENT TO CHAPTER 6
THE ASSOCIATION OF ALCOHOL INTAKE WITH GAMMA-GLUTAMYL TRANSFERASE (GGT) LEVELS: EVIDENCE FOR A CAUSAL EFFECT?

Number of participants and reasons for exclusion

Information on GGT levels was present for 8,754 individuals (aged ≥18). Analyses were performed on data from 6,465 individuals for whom data on alcohol intake and levels of GGT were present (3,193 twins, 1,304 siblings, and 1,968 parents). Data were excluded for 11 individuals with known liver disease (ICD-10 codes K70-K77), 1523 individuals who did not participate in the 2002-2009 surveys, and 121 individuals who reported to have never drunk any alcohol. For 133 persons who participated in the 2002-2009 surveys, data on alcohol intake were missing, and for 31 individuals excluded during data cleaning (because of extreme/impossible values, see Van Beek et al. (2013c)). For 470 individuals, data were excluded, since they were not biological relatives of the twins (N=26), zygosity was not yet known (N=2), they formed part of a triplet (N=2), were sibling in a family with more than two same sex siblings (N=64; a maximum of two same sex siblings was included per family), or had another relation to the twin than co-twin, sibling or parent (mostly spouse of twin, N=315; other N=61, e.g. child).

Description bivariate genetic analysis

Bivariate genetic analysis was performed with saturated models and structural equation models. The bivariate saturated model estimated 30 familial correlations. Ten familial correlations were estimated for alcohol intake, nine for GGT and eleven cross-trait correlations for the relation of alcohol intake with GGT. For alcohol intake, GGT and the cross-trait covariance of alcohol intake with GGT, these were five offspring correlations (MZM twin pairs, MZF twin pairs, male-male DZ/sibling pairs, female-female DZ/sibling pairs, and opposite sex twin/sibling pairs), and four parent-offspring correlations (father-son, mother-daughter, father-daughter, mother-son) (thus, 27 in total). In addition, two within-person cross-trait correlations (males, females) were estimated for the relation between alcohol intake and GGT, and one spouse correlation for alcohol intake. As spousal resemblance for GGT was negligible (Van Beek et al. 2013a), this was not included, nor was cross-trait spousal resemblance for alcohol intake with GGT.

Structural equation models were informed by the results based on univariate genetic structural equation models performed on alcohol intake (Van Beek et al. 2013c) and GGT (Van

Beek et al. 2013a). Variation in alcohol intake was best explained by effects of additive and non-additive gene action as well as individual-specific environmental effects for both men and women (Van Beek et al. 2013c). Results for GGT differed over sex. Variation in GGT among women was explained by additive genetic, non-additive genetic and non-shared individual-specific environmental factors, whereas for men, non-additive genetic effects were not significant, but shared environmental effects were (Van Beek et al. 2013a). In the structural equation models of this study, based on these previous results, alcohol intake of fathers, mothers, sons and daughters (depicted as ALC_{FA}, ALC_{MO}, ALC_{SO}, ALC_{DA} respectively) was regressed on factors representing additive genetic (A_1), non-additive genetic (D_1), and individual-specific environmental effects (E_1). The factor loadings (a_1, d_1, e_1) were allowed to differ over sex (represented as $a_{1,m}$, $a_{1,f}$, $d_{1,m}$, $d_{1,f}$, $e_{1,m}$, $e_{1,f}$). Variation in GGT levels among females (mothers depicted as GGT_{MO}, daughters as GGT_{DA}) could also be ascribed to additive genetic (A), non-additive (D) genetic and individual-specific environmental effects. If GGT levels would be uncorrelated with levels of alcohol intake, variation in GGT would be solely regressed on factors that were specific to GGT, that is on $A_{2,f}$, $D_{2,f}$ and $E_{2,f}$ (the factors loadings $a_{21,f}$, $d_{21,f}$, and $e_{21,f}$ would be set to zero). As can be seen in Figure 1, GGT levels are assumed to be correlated with alcohol intake and this correlation is specified through the factor loadings $a_{21,f}$, $d_{21,f}$, $e_{21,f}$ that run from the factors for alcohol intake ($A_{1,f}$, $D_{1,f}$, $E_{1,f}$) to GGT. For males, the same principle holds true. The association of alcohol intake with GGT among males was modeled to be influenced by alcohol-effects ($A_{1,m}$, $D_{1,m}$, $E_{1,m}$ with factor loadings $a_{21,m}$, $d_{21,m}$, and $e_{21,m}$ and GGT-specific effects (A_2, C_2, E_2 with factor loadings $a_{2,m}$, $c_{2,m}$, and $e_{2,m}$).

The amount of variance and covariance ascribed to genetic and environmental factors, can be estimated by the fact that family members share their genetic and environmental background to different degrees. MZ twin pairs are assumed to share their entire genetic material (that is, the additive genetic factors (A) were modeled to correlate 1 within twin pairs), whereas DZ twin pairs, siblings, and parent and offspring are estimated to share half of their genes (A factors were modeled to correlate .5 for DZ twin, sibling and parent-offspring pairs). Non-additive genetic influences (D) can be estimated by assuming that MZ twin pairs share all their genetic material with each other including non-additive genetic factors (that is: D factors are correlated 1), DZ twins and sibling pairs share on average a quarter of the non-additive genetic factors (D factors are correlated .25), and parent-offspring pairs share none of their non-additive genetic factors. Shared environmental influences (represented by C factors) on GGT in males were correlated 1 among co-twin/siblings and 0 with their parents. Variance and covariance that was individual-specific (not shared between family members) was estimated as E.

GGT levels were assumed to be independent among parents. Spousal resemblance for alcohol intake was modeled to run via the Δ-path that represents the correlations between the latent genetic and environmental factors influencing the phenotypes of the parents, that result from phenotypic assortment. The increase in the additive genetic variance (RA) that results from the presence of phenotypic assortment for alcohol intake was modeled through the additive genetic variance component. The variance of the additive genetic factors in the offspring generation (.5) reflects the segregation variance that emerges due to recombination. This within-family additive genetic variance emerges since parents pass their alleles, not genotypes, giving rise to new genetic variance in the offspring generation (Keller et al. 2009).

Constraints were equal to those in the univariate models: two means (males/females), one variance (equal over sex) for alcohol intake (Van Beek et al. 2013c) and four means (male twins/siblings, female twins/siblings, fathers, mothers) and three variances (male twin/siblings, female twin/siblings, parents) for GGT (Van Beek et al. 2013a).

CHAPTER 7

ASSOCIATIONS BETWEEN ADH GENE VARIANTS AND ALCOHOL PHENOTYPES IN DUTCH ADULTS [6]

Abstract

Recently, Macgregor et al. (2009) demonstrated significant associations of ADH polymorphisms with reactions to alcohol and alcohol consumption measures in an Australian sample. The aim of the present study was to replicate these findings in a Dutch sample. Survey data on alcohol phenotypes came from 1,754 unrelated individuals registered with the Netherlands Twin Register. SNPs in the ADH gene cluster located on chromosome 4q (*n*=491) were subdivided in seven gene sets: ADH5, ADH4, ADH6, ADH1A, ADH1B, ADH1C and ADH7. Within these sets associations of SNPs with alcohol consumption measures, age at onset variables, reactions to alcohol and problem drinking liability were examined. Of the original 38 SNPs studied by Macgregor et al. (2009), six SNPs were not available in our dataset, because one of them had a minor allele frequency <.01 (rs1229984) and five could not be imputed. The remaining SNP associations with alcohol phenotypes as identified by Macgregor et al. (2009), were not replicated in the Dutch sample, after correcting for multiple genotype and phenotype testing. Significant associations were found however, for reactions to alcohol with a SNP in ADH5 (rs6827292, *p*=.001) and a SNP just upstream of ADH5 (rs6819724, *p*=.0007) that is in strong LD with rs6827292. Furthermore, an association between age at onset of regular alcohol use and a SNP just upstream of ADH7 (rs2654849, *p*=.003) was observed. No significant associations were found for alcohol consumption and problem drinking liability. Although these findings do not replicate the earlier findings at the SNP level, the results confirm the role of the ADH gene cluster in alcohol phenotypes.

[6] This chapter is published as:
van Beek, JHDA, Willemsen, G, de Moor, MHM, Hottenga, JJ, Boomsma, DI (2009) Associations between ADH gene variants and alcohol phenotypes in Dutch adults. Twin Res Hum Genet 13:30-42

1. Introduction

Alcohol is commonly used in Western societies: the vast majority of adults report to have drunk alcohol in the previous 12 months (Ahlstrom et al. 2001; Wilsnack et al. 2000; CBS 2008; Van Laar et al. 2008). There is substantial individual variation in the amounts of alcohol consumed. Based on data from the World Health Organization 15.7% of individuals aged 15 and older are classified as heavy drinkers (males >40g and females >20g pure alcohol per day) (Rehm et al. 2006).

The harmful effects of alcohol on health are well-documented. Increased alcohol use has been linked to over 60 chronic health diseases, ranging from cancers and heart disease to depressive disorders and birth defects (Rehm et al. 2003). Alcohol use disorder is in the top 10 conditions with the highest burden of disease (WHO 2008; Begg et al. 2007; Hilderink and van 't Land 2008). In addition, alcohol takes its toll through motor vehicle accidents and other alcohol related injuries (Rehm et al. 2003; Van Laar et al. 2008). In Western European countries, 6.6% of deaths among males can be attributed to alcohol consumption (Rehm et al. 2006). However, there is also evidence that alcohol can have beneficial effects on health. For example, moderate alcohol use has been associated with a decreased risk of both coronary heart disease and ischaemic stroke (Burger et al. 2004). Given the large impact of alcohol consumption, it is important to understand the causes of individual differences in alcohol use.

Twin studies have shown that individual differences in a range of different alcohol phenotypes are all explained to some degree by differences in genetic makeup (Dick et al. 2009b). Additive genetic influences explain between 30% and 54% of the variation in alcohol consumption among adults (Whitfield et al. 2004; Hansell et al. 2008; Kendler et al. 2008c). Heritability estimates for the risk of alcoholism or abuse in adults range from 30% to 75% (Kendler et al. 1997; Heath et al. 1997; Prescott and Kendler 1999; Knopik et al. 2004; Jang et al. 2000; Sartor et al. 2009; Xian et al. 2008; Walters 2002). Estimates of genetic influences on alcohol initiation are more variable. Although the average heritability is estimated to be around 26% (Dick et al. 2009b), estimates range from 0 to 83% (Poelen et al. 2008; Pagan et al. 2006; Rhee et al. 2003; Rose et al. 2001; Viken et al. 1999; Penninkilampi-Kerola et al. 2005; Han et al. 1999; Fowler et al. 2007a; Koopmans and Boomsma 1996; Stallings et al. 1999; Sartor et al. 2009) . The heritability of age at onset of regular alcohol use has been estimated at 35-43% (Stallings et al. 1999; Liu et al. 2004).

One of the mediating mechanisms by which genes may influence alcohol use, is through alcohol metabolism (Higuchi et al. 2006). Two groups of genes that have received much attention are the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase (ALDH) gene clusters that code, respectively, for the ADH and ALDH enzymes that are involved in the break-down of ethanol. ADH is one of the main enzymatic pathways metabolizing ethanol into

acetaldehyde, a highly toxic by-product. ALDH oxidizes acetaldehyde into a less toxic compound, acetate, which is subsequently converted into water and carbon dioxide. Variants of ADH and ALDH genes encode enzymes with different characteristics that can change the rate by which ethanol is metabolized and hence influence alcohol use and other alcohol-related phenotypes (Edenberg 2007).

ADH enzymes are encoded by 7 different genes, located in a 365 kb region on chromosome 4q, that have been categorized into 5 classes: a) ADH1A, ADH1B and ADH1C that encode the enzymes α-, β-, and γ-ADH; b) ADH4 that encodes μ-ADH, c) ADH5 that codes for χ-ADH, d) ADH6 that encodes an ADH enzyme little is known about and e) ADH7 that encodes σ-ADH. ALDH is encoded by two genes. Gene ALDH1A, located on chromosome 9q21.13, codes for the enzyme ALDH1. The ALDH2 gene located on chromosome 12q24.2, codes for the enzyme ALDH2. Most variants in the ADH and ALDH gene clusters are changes in single nucleotide polymorphisms, also called SNPs (Edenberg 2007).

Macgregor et al. (2009) investigated whether genetic variants in the ADH and ALDH2 genes were associated with alcohol consumption, physical reactions to alcohol and alcohol dependence in an Australian population sample from Western European descent (n=4,597; age range 26-89). They genotyped 38 polymorphisms in the ADH gene cluster and 8 polymorphisms in the ALDH2 gene. Significant associations were found between variants in the ALDH2 gene and measures of alcohol dependency. Variants in the ADH genes were found to be related to alcohol consumption measures and physical reactions and only marginally with alcohol dependency measures. The most significant finding was obtained for the association of marker rs1229984 (ADH1B) with alcohol consumption variables (p=2.7 x 10^{-06} – 8.9 x 10^{-08}) and physical reactions (p=8.2 x 10^{-07}), but significant associations for other SNPs were also reported.

In this paper we sought to replicate Macgregor et al.'s (2009) findings on the association of ADH polymorphisms with alcohol consumption measures, physical reactions to alcohol and alcohol dependency. To this end, the data from 1,754 unrelated individuals registered with the Netherlands Twin Register (NTR) were analyzed. The participants were unselected for alcohol use and comparable to the Australian sample in age and phenotypic assessments. A problem drinking scale was used as a proxy for alcohol dependency. In addition, we examined the associations between ADH polymorphisms and age at onset of alcohol use. The participants were genotyped on 64 SNPs in the ADH gene cluster. To enlarge the coverage of the region, 427 additional SNPs were imputed. Two different methods that correct for multiple SNP testing were used and compared: one method, based on linkage disequilibrium (LD), corrected for the effective number of SNPs tested; another method corrected for multiple testing by permutation.

2. Materials and methods

2.1 Participants

Participants took part in a longitudinal survey on health, lifestyle and personality conducted in adult twins and their family members registered with the Netherlands Twin Register (NTR). Characteristics and recruitment of participants are described in Boomsma et al. (2002c) (Boomsma et al. 2006). Since 1991, every two to four years participants have received surveys with questions about health, lifestyle and personality. Twins were approached in 1991, 1993, 1995, 1997, 2000, 2002 and 2004/2005. Parents were invited to participate at all time points except 1997 and 2000. Siblings were asked to participate from 1995 onwards and spouses since 2000. Questions about alcohol use were included at all time points. In total, 23,173 individuals completed at least one questionnaire. For 1,774 individuals with information on alcohol phenotypes, genome-wide quality controlled SNP data were available as part of a genome wide association study (GWAs) on Major Depressive Disorder (GAIN-MDD study). NTR participants in the MDD study were biologically unrelated, of Western European ancestry and mainly served as healthy controls. Detailed information on the GAIN-MDD study, recruitment and DNA collection can be found in Boomsma et al.(2008). Permission for the GAIN-MDD study was obtained from the Ethics Committee of Research on human subjects of the VU University Medical Center, Amsterdam.

Nearly all individuals with information on alcohol phenotypes had drunk alcohol at least once. Individuals who reported to have never drunk alcohol were excluded (n=20; 1.1%). This resulted in a sample of 1,754 individuals (n=1,112 female; 63.4%). The median year of birth was 1959 (ranging from 1923 to 1986). The majority of individuals (n=1,204; 68.6%) provided data on three to seven surveys, 419 (23.9%) on two and 131 (7.5%) on one survey.

2.2 Phenotyping

For alcohol consumption variables, physical reactions to alcohol and problem drinking liability, reports were only included if the participant was 26 or older at questionnaire completion. For age at onset variables no restriction was set on the age of the respondent, but data were set to missing when no age of onset was reported. The exact number of individuals with genotypic information differs per phenotype, not only because of age restrictions, but also because not all questions about alcohol use were included in all surveys.

2.2.1 Alcohol consumption

Data on alcohol consumption were collected with two questions included at all time points except 1991. The first question was ‘How often do you drink alcohol? Also count the times that you only drank a small amount (e.g. half a pint of beer or a few sips of wine)’. The

response categories were 'I don't drink alcohol', 'once a year or less', 'a few times a year', 'about once a month', 'a few times a month', 'once a week', 'a few times a week' and 'every day'. Based on this question four frequency measures were defined in 1,565 individuals:

- *Daily drinking (last* and *maximum reported)* analyzed as a dichotomous 'yes'/'no' variable.
- *Weekly drinking or more (last* and *maximum reported)* analyzed as a dichotomous 'yes'/'no' variable.

Last reported frequency of alcohol use measures was based on most recent information, for maximum reported frequency the measurement with the highest frequency was taken. For the 327 individuals who participated in only one survey, last reported and maximum reported frequency of alcohol consumption measures were based on the same measurement and thus similar.

The second question was 'How many glasses a week do you drink on average (including the weekend)?'. There were seven response categories: 'less than 1 glass', '1-2 glasses a week', '3-5 glasses a week', '6-10 glasses a week', '11-20 glasses a week', '21-40 glasses a week' and 'more than 40 glasses a week'. Based on this question two quantity measures were defined in 1550 individuals:

- *Number of alcoholic drinks per week (last* and *maximum reported)* with six response categories (the categories '1-2 glasses a week' and '3-5 glasses a week' were combined).

For last reported number of drinks the most recent information was taken. Maximum number of drinks reported was based on the survey with the highest number of drinks reported. For the 353 individuals who participated in the survey only once, last reported and maximum number of drinks reported were based on the same measurement.

2.2.2 Age at onset alcohol use

Information on age at onset of alcohol use was collected for three traits: experimental alcohol use (included in surveys in 1991, 1993, 1995, 1997 and 2000; *n*=1,384), regular alcohol use (included in 1993, 1995, 1997 and 2000; *n*=1,098) and getting drunk or tipsy (included in 1993, 1995 and 1997; *n*=624). For these behaviors the question started with 'If you have ever used any of the substances listed below, please indicate the age at which you used them for the first time' and was followed by 'Tried an alcoholic drink', 'Regularly drank alcohol' and 'Gotten drunk or tipsy'. In 1991 age at onset was a continuous measure. In 1993, 1995 and 1997 there were eight response categories: '11 or younger', '12', '13', '14', '15', '16', '17', and '18 or older'. In 2000 response categories were brought back to five: '11 or younger', '12-13', '14-15', '16-17' and '18 or older'. Based on the three questions three phenotypes were defined:

- *Age at onset of experimental alcohol use* with the same response categories as in 2000.

- *Age at onset of regular alcohol use,* with the same categories as in 2000.
- *Age at onset of getting drunk* with response categories coded as '17 or younger' and '18 or older'.

Data were set to missing when no information was reported on age at onset. For individuals who provided data on age at onset of experimental alcohol use (*n*=914), regular alcohol use (*n*=592) or getting drunk (*n*=278) on multiple surveys, the lowest reported age at onset was taken.

2.2.3 Physical reactions to alcohol

Information on experiencing unpleasant physical reactions after alcohol use was based on one question included in 1993 and 1995 (*n*=872). This question 'Do you experience unpleasant physical reactions, like flushing of face or body, itching, sleepiness or heart beating after drinking one or two glasses of alcohol?' with response categories 'always', 'sometimes' and 'never' was used to define *Physical reactions to alcohol* with the analyzed categories 'yes' (always; sometimes) and 'no' (never). The 559 individuals who filled out this question in both years, were classified as experiencing reactions to alcohol if in one of the two surveys they had responded 'yes'.

2.2.4 Problem drinking liability

Risk for problem drinking was assessed by the CAGE (Ewing 1984) that was included at all time points except 1991. The CAGE consists of four items: 'Have you ever felt you should cut down on your drinking?', 'Have people annoyed you by criticizing your drinking?', 'Have you ever felt bad or guilty about your drinking?', 'Have you ever had a drink first thing in the morning to steady your nerves or to get rid of a hangover?'. Each item could be answered with 'yes' or 'no'. The number of yes items was used to define *Problem drinking liability* (*n*=1,559). To take into account the possibility of non-random missing data and prevent assigning a low liability score to possible problem users, data of individuals were set to missing in case of two or more missing items. If one item was missing the mean value was imputed. For the 1,226 individuals who filled out the CAGE twice or more, the maximum score was taken.

2.3 Genotyping and imputation

DNA was isolated from whole blood (Boomsma et al. 2008). Genotyping was performed by Perlegen Sciences using high-density oligonucleotide arrays. The 599,156 SNPs on these arrays had been selected to tag common variation in the Hapmap European and Asian panels (Sullivan et al. 2009). Genotyping procedures, genotyping calling rate algorithms and quality control procedures are available in the Supplementary Methods of Sullivan et al. (2009). In the

ADH gene cluster on chromosome 4q successfully genotyped SNPs were selected (*n*=64) ranging from base pair position 100186714 to base pair position 100626045 (build 36, release 24), to cover the same region as Macgregor et al. (2009).

SNPs that were not genotyped in this region, but that were included in the HapMap dataset were imputed. Imputation was done using Impute v0.5 (Marchini et al. 2007), with as a reference set the Hapmap2 CEU panel (build 36) which was obtained from the Impute website https://mathgen.stats.ox.ac.uk/impute/impute.html. Imputed genotypes were set to missing if none of the possible genotypes reached a maximum average genotype posterior probability of .70. Exclusion of SNPs with a minor allele frequency (MAF) <.01 (*n*=152; all imputed) resulted in a final marker set of 491 SNPs of which 64 SNPs were genotyped and 427 imputed. Six of the 38 SNPs analyzed by Macgregor et al. (2009) were not included in the present study. Five of these were not genotyped as part of the GAIN-MDD study nor included in the HapMap2 dataset. One, the ADH1B polymorphism rs1229984, was imputed, but was excluded from further analyses since it had a MAF <.01.

2.4 Statistical analysis

First, to examine the possible occurrence of selection bias, the genotyped sample was compared to a random sample of NTR participants without genotypic data, stratified by sex and age, on alcohol phenotypes. These comparisons were done in SPSS (version 15.0) by chi-squared tests on categorical variables and *t*-tests for independent samples on quantitative variables.

Next, associations between ADH polymorphisms and alcohol phenotypes were investigated by performing regression analyses in PLINK 1.06 (Purcell et al. 2007). Categorical data were analyzed with logistic regression; quantitative data were analyzed with linear regression. We modeled an additive effect and sex was included as covariate in each analysis. Age at completion of the survey was used as an additional covariate for last reported alcohol consumption measures. For maximum reported alcohol consumption variables, age at onset variables, problem drinking liability and reactions to alcohol we used year of birth as a covariate. SNPs in the ADH gene cluster were subdivided and analyzed within seven gene sets: ADH5, ADH4, ADH6, ADH1A, ADH1B, ADH1C and ADH7. These gene sets were defined as all SNPs ranging from the midpoint of one intergenic region until the midpoint of the next intergenic region, thereby including the SNPs in the particular gene. The outer gene sets spanned a larger intergenic region to include the same region as Macgregor et al. (2009): the ADH5 gene set was defined as all SNPs ranging from rs1230210 (in the gene METAP1, flanking the ADH region) to the midpoint of the intergenic region between ADH5 and ADH4; the ADH7

gene set was defined as all SNPs ranging from the midpoint of the intergenic region between ADH1C and ADH7 to rs1583971. The seven gene sets are described in Table 1.

Table 1 Description of gene sets within ADH gene cluster on chromosome 4

Gene set	from SNP (bp[a])	to SNP (bp[a])	Total no. of SNPs	No. SNPs genotyped	No. SNPs imputed	M_{eff}[b]
ADH5	rs1230210 (100186714)	rs1840231 (100246202)	79	6	73	15.38
ADH4	rs6819724 (100247474)	rs10024022 (100307276)	119	11	108	15.88
ADH6	rs6532803 (100316196)	rs7439160 (100380677)	37	4	33	12.41
ADH1A	rs2173199 (100390402)	rs13145277 (100437458)	34	5	29	10.11
ADH1B	rs1229988 (100438024)	rs1789888 (100466593)	24	5	19	11.19
ADH1C	rs1789891 (100469442)	rs10516439 (100522181)	85	9	76	17.53
ADH7	rs2851292 (100522524)	rs1583971 (100626045)	113	24	89	30.34

[a] Base pair position SNP as estimated in Hapmap2, build 36, release 24

[b] Effective number of SNPs in gene set (for explanation see paragraph on Statistical Analyses)

Testing for associations between 491 genetic variants and 11 phenotypes posed the challenge how to deal with the problem of multiple testing. To reduce the type 1 error rate two methods to handle multiple SNP testing were applied and one method to handle multiple phenotype testing. The first method regarding multiple SNP testing was based on the correction of *p*-values for the number of tests performed. Given the fact that the LD structure among SNPs is not independent, adjusting the *p*-value for the actual number of tests would be overly stringent and result in a loss of power (Nyholt 2004). With the first method the *p*-values were therefore adjusted for the estimated number of *independent* SNPs tested. Calculation of the number of independent SNPs (also called the effective number of SNPs; M_{eff}) was based on the number of eigenvalues of the *n* x *n* correlation matrix of allele frequencies of SNPs using equation 5 by Li and Ji (2005). For each gene set M_{eff} was estimated by an interface developed by Nyholt (2004) , available at http://genepi.qimr.edu.au/general/daleN/SNPSpD/. SNPs in perfect LD (r^2=1) were automatically removed to obtain better estimates (Nyholt 2005). With the estimates of M_{eff}, as summarized in Table 1, the asymptotic *p*-value of the most significant SNP per gene set was adjusted for multiple testing by correcting the *p*-value of the SNP for the estimate of M_{eff} of that gene set.

The second method to deal with the problem of testing multiple SNPs was based on permutation. In PLINK 1.06 (Purcell et al. 2007) set-based tests were performed using gene sets

as described in Table 1 to select up to *n* SNPs per gene set that were independently associated with the particular phenotype and derive an empirical *p*-value based on permutation. This method had the advantage of selecting more than one significant SNP (if independent from each other) and could therefore give a better coverage of the association signal of the gene set than was possible with the first method employed. Set-based tests were performed as follows. First, within each gene set LD was calculated for each pair of SNPs. Next, regression analysis with sex and age (at completion or year of birth) as covariates was carried out. Based on these regression analyses, the most significant SNP per gene set with a threshold *p*-value below .05 was selected (--set-p 0.05; default value). Other significant SNPs were selected ($p<.05$) in order of decreasing significance, only if these were relatively independent ($r^2<.5$) from the SNPs already selected (--set-r2 0.5; default value) and if the number of selected SNPs did not exceed 5 (--set-max 5; default value). Since alcohol phenotypes can be seen as complex traits with many variants of small effects involved (O'Dushlaine et al. 2009), a threshold *p*-value of .05 was chosen over a more stringent *p*-value. Subsequently, for each gene set the mean of the test statistics of selected SNPs was calculated. Then, within the dataset, phenotype labels were permuted 10,000 times (--mperm 10000) keeping the LD between SNPs constant. For each permuted dataset SNPs were selected as described above (if $p<.05$; in order of decreasing significance; independent of already selected SNPs) and for each permutation set the mean of test statistics of selected SNPs was calculated. This resulted in a distribution of mean test statistics that was used to calculate an empirical *p*-value for each gene set and phenotype based on the number of mean test statistics from the permuted datasets that exceeded the original mean test statistic of the actual dataset.

Lastly, the fact that multiple phenotypes were tested ($n=11$) was accounted for. This correction was done in addition to the corrections for multiple SNP testing described above. Since the phenotypes tested were correlated, applying a Bonferroni correction by dividing the significance level of .05 by the actual number of phenotypes ($n=11$), would be too conservative and results in a loss of power (Nyholt 2004). Therefore correction for multiple phenotype testing was done by correcting the significance level of .05 for the number of *independent* phenotypes. An estimate of the number of independent phenotypes, derived from the number of eigenvalues of the n x n correlation matrix of phenotypes, was based on equation 5 given by Li and Ji (2005), analogous to the estimation of M_{eff}, and obtained by another interface developed by Nyholt (2004), available at http://gump.qimr.edu.au/general/daleN/matSpD/ . The number of independent phenotypes in this study was estimated to be 7. This resulted in a significance level of .05/7=.007. Therefore, for all analyses conducted, a *p*-value of .007 or less was considered significant.

3. Results

3.1 Comparison of genotyped sample with random sample

Table 2 shows that individuals in the genotyped sample were comparable to individuals in the random NTR sample in last reported alcohol consumption measures, age at onset variables, problem drinking liability and in the frequency of experiencing physical reactions to alcohol. Based on maximum reported alcohol consumption measures however, individuals in the genotyped sample more often reported to have drunk alcohol daily and weekly and reported to have drunk more glasses of alcohol per week than individuals in the random NTR sample (*p*-values <.007).

3.2 Associations of ADH polymorphisms with alcohol phenotypes

For each phenotype Tables 3–6 show the unadjusted asymptotic *p*-values, the *p*-values adjusted for the number of independent SNPs per gene set and the empirical *p*-values for the most significant SNPs per gene set.

Alcohol consumption None of the ADH polymorphisms were significantly associated with alcohol consumption measures after correction for multiple SNP and phenotype testing, for either of the test methods applied (see Table 3).

Age at onset of alcohol use Based on empirical *p*-values derived by permutation, but not on adjusted *p*-values (corrected for the number of independent SNPs), one polymorphism in the ADH7 gene set (rs2654849), located 40.4 kb upstream of the ADH7 gene (base pair position 100615713, build 36) was significantly associated with age at onset of regular alcohol use (empirical *p*-value =.003), as can be seen in Table 4. Individuals who started regular alcohol use at an earlier age more often have the minor G allele at this locus (β=-.10; MAF=.34; major allele=T). Other associations between ADH polymorphisms and age at onset measures were not detected.

Physical reactions to alcohol Table 5 shows that a non-coding genetic variant in ADH5, rs6827292, was significantly associated with experiencing unpleasant physical reactions to alcohol, based on its empirical *p*-value (*p*=.001). Each copy of a C-allele (MAF=.044) conveyed an increased risk for experiencing physical reactions to alcohol, compared to carrying a T-allele (odds ratio(OR)=2.426; base pair position 100212017, build 36). Its *p*-value adjusted for the effective number of SNPs tested, was marginally significant (adjusted *p*-value =.008). The same applied for the genetic variant rs6819724 in the ADH4 gene set. Based on its empirical *p*-value the marker rs6819724 was significantly associated with experiencing physical reactions to alcohol (empirical *p*-value =.0007), but considering its adjusted *p*-value, rs6819724 was only marginally significant (adjusted *p*-value =.008).

Table 2 Description of alcohol-related phenotypes for genotype sample and comparison with random stratified sample

		Genotype sample	Comparison sample	Test statistic
Ever used alcohol	N	1774	1774	$\chi^2(1)=.212$, $p=.645$
	Yes (%)	1754 (98.9)	1751 (98.7)	
Daily drinking (last reported)	N	1565	1750	$\chi^2(1)=3.235$, $p=.072$
	Yes (%)	324 (20.7)	319 (18.2)	
Daily drinking (maximum reported)	N	1565	1750	$\chi^2(1)=14.306$, $p<.001$*
	Yes (%)	404 (25.8)	355 (20.3)	
Weekly drinking or more (last reported)	N	1565	1750	$\chi^2(1)=4.281$, $p=.039$
	Yes (%)	1028 (65.7)	1089 (62.2)	
Weekly drinking or more (maximum reported)	N	1565	1750	$\chi^2(1)=15.830$, $p<.001$*
	Yes (%)	1128 (72.1)	1149 (65.7)	
No. drinks per week (last reported)	N	1550	1736	$\chi^2(5)=9.367$, $p=.095$
	<1 (%)	454 (29.3)	540 (31.1)	
	1-5 (%)	491 (31.7)	606 (34.9)	
	6-10 (%)	281 (18.1)	280 (16.1)	
	11-20 (%)	252 (16.3)	243 (14.0)	
	21-40 (%)	65 (4.2)	61 (3.5)	
	>40 (%)	7 (0.5)	6 (.3)	
No. of drinks per week (maximum reported)	N	1550	1736	$\chi^2(5)=25.746$, $p<.001$*
	<1 (%)	359 (23.2)	485 (27.9)	
	1-5 (%)	484 (31.2)	591 (34.0)	
	6-10 (%)	298 (19.2)	307 (17.7)	
	11-20 (%)	295 (19.0)	270 (15.6)	
	21-40 (%)	103 (6.6)	72 (4.1)	
	>40 (%)	11 (0.7)	11 (.6)	
Age at onset experimental use	N	1384	905	$\chi^2(4)=13.526$, $p=.009$
	<12 (%)	104 (7.5)	38 (4.2)	
	12-13 (%)	177 (12.8)	101 (11.2)	
	14-15 (%)	423 (30.6)	277 (30.6)	
	16-17 (%)	422 (30.5)	300 (33.1)	
	18+	258 (18.6)	189 (20.9)	
Age at onset regular use	N	1098	698	$\chi^2(4)=4.268$, $p=.371$
	<12 (%)	3 (0.3)	5 (.7)	
	12-13 (%)	12 (1.1)	7 (1.0)	
	14-15 (%)	103 (9.4)	55 (7.9)	
	16-17 (%)	311 (28.3)	184 (26.4)	
	18+ (%)	669 (60.9)	447 (64.0)	
Age at onset getting drunk	N	624	351	$\chi^2(1)=4.353$, $p=.037$
	<18 (%)	239 (38.3)	111 (31.6)	
Physical reactions to alcohol	N	872	512	$\chi^2(1)=.890$, $p=.346$
	Yes (%)	225 (25.8)	144 (28.1)	
Problem drinking liability	N	1559	1748	$t(3305)=-.584$, $p=.559$
	M	.476	.459	
	SD	.832	.855	

* = significant at the $\alpha=.007$ level (adjusted for multiple phenotype testing)

Table 3 Most significant SNPs per gene set (based on asymptotic *p*-values adjusted for the effective no. of SNPs and empirical *p*-values) for measures of alcohol consumption

	Drinking daily (*n*=1,565)								**Drinking weekly or more (*n*=1,565)**								**No. of alcoholic drinks per week (*n*=1550)**							
	Last reported				Maximum reported				Last reported				Maximum reported				Last reported				Maximum reported			
Gene set	SNPs	Min. p^b	Adj. p^c	Emp. p^d	SNPs	Min. p^b	Adj. p^c	Emp. p^d	SNPs	Min. p^b	Adj. p^c	Emp. p^d	SNPs	Min. p^b	Adj. p^c	Emp. p^d	SNPs	Min. p^b	Adj. p^c	Emp. p^d	SNPs	Min. p^b	Adj. p^c	Emp. p^d
ADH5	rs1061187	.016	.246	.155	rs1230201	.079	1	1[f]	rs17216887 rs17216446[e] rs1230201[e] rs2924584[e]	.024	.369	.369	rs1230205	.027	.415	.304	rs17216887[i] rs1061187[e] rs1230201[e] rs6827292[e]	.036	.554	.432	rs1312200[i] rs1230201[e]	.024	.369	.389
ADH4	rs17218003	.061	.969	1[f]	rs7694844	.073	1	1[f]	rs7694844	.070	1	1[f]	rs7694844	.088	1	1[f]	rs10026860 rs6819724[e]	.032	.508	.382	rs7694844	.015	.238	.132
ADH6	rs7661441	.160	1	1[f]	rs4699734[g]	.060	.745	1[f]	rs4699734[g]	.123	1	1[f]	rs6532803[j]	.088	1	1[f]	rs4699734[g]	.014	.174	.098	rs4699734[g]	.034	.422	.274
ADH1A	rs904092	.148	1	1[f]	rs12506882	.053	.536	1[f]	rs904092	.076	.768	1[f]	rs904092	.009	.091	.059	rs12506882 rs904092[e]	.015	.152	.133	rs12506882	.036	.364	.281
ADH1B	rs1789882	.191	1	1[f]	rs17033	.058	.649	1[f]	rs1789882	.076	.850	1[f]	rs1789882	.015	.168	.120	rs3811802 rs1789882[e] rs17033[e]	.022	.246	.218	rs17033	.066	.739	1[f]
ADH1C	rs1826907	.036	.631	.456	rs1826907	.052	.912	1[f]	rs3114046[h]	.205	1	1[f]	rs1814125	.129	1	1[f]	rs1229864	.138	1	1[f]	rs283410	.142	1	1[f]
ADH7	rs12505135 rs7667212[e]	.017	.516	.475	rs12505135 rs1372680[e]	.010	.303	.369	rs284787[i] rs894369[e]	.031	.941	.583	rs11933667	.031	.941	.552	rs1154486	.034	1	.628	rs3805331	.094	1	1[f]

[a] Effective number of SNPs in gene set

[b] Asymptotic *p*-value of most significant SNP in gene set, unadjusted for multiple SNP testing

[c] Asymptotic *p*-value of most significant SNP in gene set adjusted for the effective number of SNPs in gene set = Min. $p * M_{eff}$

[d] Empirical *p*-value of selected SNPs (most significant SNP if asymptotic *p*-value <.05 and, if applicable, other SNPs that are independent, r^2<.5) of SNPs already selected)

[e] Additional SNP selected by set-based test (having a *p*-value <.05 and being independent of SNP(s) already selected)

[f] No SNPs were selected by set-based test (*p*-value most significant SNP >.05), therefore empirical *p*-value could not be calculated.

[g] SNP rs4699734 is in LD 1 with SNPs rs7375429; these SNPs have the same *p*-value.

[h] SNP rs3114046 is in LD 1 with SNPs rs1391088; these SNPs have the same *p*-value.

[i] SNP rs284787 is in LD 1 with SNPs rs284784 and rs1827567; these SNPs have the same *p*-value.

[j] SNP rs6532803 is in LD 1 with SNPs rs2213039; these SNPs have the same *p*-value.

Table 4 Most significant SNPs per gene set (based on asymptotic *p*-values adjusted for the effective number of SNPs and empirical *p*-values) for measures of age at onset of alcohol use

	Experimental alcohol use (*n*=1,384)				Regular alcohol use (*n*=1,098)				Getting drunk (*n*=624)			
Gene set	**SNPs**	**Min. *p*[b]**	**Adj. *p*[c]**	**Emp. *p*[d]**	**SNPs**	**Min. *p*[b]**	**Adj. *p*[c]**	**Emp. *p*[d]**	**SNPs**	**Min. *p*[b]**	**Adj. *p*[c]**	**Emp. *p*[d]**
ADH5	rs17028457	.014	.215	.139	rs1230201	.134	1	1[f]	rs10084896	.074	1	1[f]
ADH4	rs6837685[k]	.154	1	1[f]	rs1540053	.146	1	1[f]	rs2156731	.097	1	1[f]
ADH6	rs4699734[g]	.198	1	1[f]	rs2097122	.137	1	1[f]	rs12507078	.149	1	1[f]
ADH1A	rs17028765	.239	1	1[f]	rs1230024	.241	1	1[f]	rs12506882	.206	1	1[f]
ADH1B	rs1229982	.107	1	1[f]	rs4147536	.194	1	1[f]	rs17033	.126	1	1[f]
ADH1C	rs1826907	.259	1	1[f]	rs7661978	.063	1	1[f]	rs4699743	.058	1	1[f]
ADH7	rs7663410	.004	.121	.031	rs2654849	.0007	.021	**.003***	rs1583971	.056	1	1[f]
	rs2584448[e]											

[k] SNP rs6837685 is in LD 1 with SNPs rs4699710; these SNPs have the same *p*-value.

For further legend, see Table 3.

Each copy of a G-allele at this locus (MAF=.045) increased the risk of experiencing physical reactions, compared to carrying an A-allele (OR=2.417). Rs6819724 was located 18.7 kb upstream of the ADH5 gene (base pair position 100247474, build 36). Additional analyses showed that rs6827292 and rs6819724 were in LD 1 with each other. SNP rs6819724, located just intermediate of the genes ADH4 and ADH5, was included in gene set ADH4. Therefore a significant result was obtained in both gene sets.

Problem drinking liability Table 6 summarizes the test results for the associations between ADH polymorphisms and problem drinking liability per gene set. None of the *p*-values reached significance after correction for multiple SNP and phenotype testing by either method.

Table 5 Most significant SNPs per gene set (based on asymptotic *p*-values adjusted for the effective number of SNPs and empirical *p*-values) for physical reactions to alcohol (*n*=872)

Gene set	SNPs	Min. p^b	Adj. p^c	Emp. p^d
ADH5	rs6827292	.0005	.008	**.001***
ADH4	rs6819724	.0005	.008	**.0007***
ADH6	rs7439160	.053	.658	1[f]
ADH1A	rs17028770[f]	.025	.253	.182
ADH1B	rs1693439	.039	.436	.332
ADH1C	rs17586246	.038	.666	.449
ADH7	rs2718682	.003	.091	.151
	rs11933667[e]			
	rs7696921[e]			

For legend, see Table 3.

Table 6 Most significant SNPs per gene set (based on asymptotic *p*-values adjusted for the effective number of SNPs and empirical *p*-values) for problem drinking liability (*n*=1,558)

Gene set	SNPs	Min. p^b	Adj. p^c	Emp. p^d
ADH5	rs1154400[l]	.054	.831	1[f]
ADH4	rs2851247	.053	.842	1[f]
ADH6	rs6830685	.028	.347	.224
ADH1A	rs904092	.022	.222	.169
ADH1B	rs1693457	.021	.235	.202
	rs6810842[e]			
ADH1C	rs2866152	.067	1	1[f]
ADH7	rs12505135	.019	.576	.330

[k] SNP rs1154400 is in LD 1 with SNPs rs1311620; these SNPs have the same *p*-value.

For further legend, see Table 3.

4. Discussion

The aim of the present study was to replicate the associations between polymorphisms in the ADH gene cluster and alcohol consumption variables and physical reactions to alcohol previously demonstrated by Macgregor et al. (2009). Associations between ADH polymorphisms and age at onset of alcohol use and problem drinking liability were also studied. The associations between genetic variants in the ADH1B, ADH1C, ADH4 and ADH5 genes and current alcohol use which were found by Macgregor et al. (2009) were not replicated in the current study. Because of the low MAF of the ADH1B polymorphism rs1229984, the associations of this SNP with alcohol phenotypes could not be tested. Our study does however show that three other polymorphisms in the ADH gene cluster are associated with alcohol phenotypes. The genetic marker rs2654849, located 40.4 kb upstream of ADH7, was found to be associated with age at onset of regular alcohol use. Two other SNPs in high LD with each other were associated with experiencing physical reactions to alcohol: rs6827292, a non-coding genetic variant located in ADH5 and rs6819724 located 18.7 kb upstream of ADH5. At this point it is unknown whether one of these markers is a causal variant itself, or whether they are in LD with the causal variant. Interestingly, Edenberg et al. (2006) reported significant SNPs in the same region between ADH4 and ADH5 for alcohol dependence. The specific significant variants detected in this study have not been reported in the literature before for the alcohol phenotypes that we studied or related phenotypes. Still, the number of studies focusing on variants across the entire ADH region is small. So far, only four studies have been conducted in populations from Western European descent (Macgregor et al. 2009; Luo et al. 2006; Edenberg et al. 2006; Kuo et al. 2008b).

Physical reactions to alcohol were linked to SNPs in different genetic regions than in previous studies. The 'Asian flush' has been related to variants in the ALDH2 gene on chromosome 12 and, to a lesser extent, in the ADH1B gene that we also studied (Shibuya et al. 1989; Takeshita et al. 1996; Chen et al. 1998). Variants in these genes are hypothesized to change the rate at which alcohol is metabolized. The resulting excess of acetaldehyde is considered to give heightened reactions to alcohol (Eng et al. 2007). In the present study, reactions to alcohol were associated with the ADH5 gene polymorphism rs6827292 and the nearby SNP rs6819724. This raises the question whether reactions to alcohol reported in this study are comparable to the Asian flushing response characterized by facial flushing, nausea, and tachycardia (Brooks et al. 2009). In line with the description of the Asian flush given by Brooks et al. (2009) and Chen et al. (1998), the present study inquired whether people experienced reactions as flushing of face or body, itching, sleepiness or heart beating after drinking one or two glasses of alcohol. Experiencing nausea was not included in the question however. Further research is necessary to investigate which regions of the ADH gene cluster are

related to experiencing physical reactions to alcohol in Western populations and by what mechanism.

The genetic variant rs2654849 was associated with age at onset of regular alcohol use. Large studies in adults have demonstrated that age at onset of drinking is associated with alcohol dependence, with people that started drinking early being more likely to experience (symptoms of) alcohol dependence (Hingson et al. 2006; Dawson et al. 2008). Furthermore, twin studies have shown that age at onset of alcohol use and alcohol dependence status are correlated at the genetic level with a genetic correlation of .54-.59 indicating substantial overlap in the genetic factors that affect both traits (Sartor et al. 2009; Grant et al. 2006). In future studies it would therefore be interesting to test whether the SNP rs2654849 is also associated with alcohol dependence.

Variants of ADH genes are assumed to encode enzymes that influence the rate by which ethanol is metabolized (Edenberg 2007). Birley et al. (2009) investigated which genetic variants in the ADH gene cluster were associated with alcohol metabolism in vivo. They found inter-individual variation in the early stages of alcohol metabolism (absorption of ingested alcohol) to be associated with SNPs in or near ADH7 and also with SNPs in the ADH1A, ADH1B, ADH4 genes. Variance in the elimination rate of alcohol was related to markers in the ADH1B and ADH1C genes and to variants located in the intergenic region between ADH1C and ADH7. About 20% of the genetic variance in alcohol metabolism could be explained by the combined effects of these SNPs (Birley et al. 2009). These findings were unlinked to the significant SNPs in the present study. This could indicate that the ADH gene cluster is also related to alcohol use by other mechanisms than the metabolizing pathway. Examples of other pathways that can relate genes to alcohol traits are sensitivity to the effects of alcohol (level of response) or personality traits that increase the risk of substance related problems such as impulsivity, sensation seeking and disinhibition (Schuckit 2009).

Further evidence for the role of the ADH gene cluster in explaining individual differences in alcohol use comes from studies among Western populations of European descent that have looked at alcohol dependency. These studies demonstrated positive associations with alcohol dependence for ADH1A markers (Edenberg et al. 2006), haplotypes (Kuo et al. 2008b) and diplotypes (Luo et al. 2006). A meta-analysis showed variants in the ADH1B to be associated with alcoholism (Zintzaras et al. 2006). They did not find evidence for an association between variants in the ADH1C gene and alcoholism, although a recent study did detect haplotypic association between the ADH1C gene and alcohol dependence (Kuo et al. 2008b). This discrepancy might be explained by the fact that variants in the ADH1B and ADH1C genes are in strong LD, the risk for alcoholism associated with the ADH1C gene possibly being attributable to a variant in the ADH1B gene (Chen et al. 1999; Osier et al. 1999). Positive

associations between alcohol dependence and ADH gene variation have also been detected for ADH4 genotypes (Luo et al. 2006) and haplotypes (Edenberg et al. 2006), for ADH5 genotypes (Luo et al. 2006) and haplotypes (Kuo et al. 2008b)and variants in the ADH7 gene (Edenberg et al. 2006). Whether these variants are specific to alcohol dependency or also influence alcohol use, awaits further study.

Testing 491 ADH polymorphisms provided a detailed picture of the association signal in the ADH region. Yet it had the drawback of increasing the multiple testing problem. This was dealt with in two ways. The first method corrected for multiple testing by adjusting the asymptotic *p*-value of the most significant SNP in a gene set for the effective number of SNPs in that set. The second method was based on empirical *p*-values derived by permutation. Both adjusted and empirical *p*-values were subjected to a significance-level corrected for testing of multiple phenotypes as well. When comparing the performance of the two methods to correct for multiple testing, adjusting *p*-values for the effective number of SNPs seems a slightly more conservative approach than correction based on permutation. Three SNPs were significant considering their empirical *p*-values, but not (rs2654849) or only marginally significant (rs6827292 and rs6819724) when considering the *p*-values adjusted for the effective number of SNPs in the gene set. This difference in *p*-values might be due to an overestimation of M_{eff} resulting from the fact that higher-order SNP correlations are not captured in the pairwise LD estimates on which the M_{eff} is based (Nyholt 2005). In line with the observed differences in *p*-values in this study, the literature considers using empirical *p*-values based on permutation one of the best ways to correct for multiple testing (Knight et al. 2008).

A possible limitation of the current study is that most individuals in the genotyped sample were selected as having a low liability for MDD to serve as control in the GAIN-MDD study. This might have caused a difference in the comparability between our sample of hyper controls and the Australian sample. The comparison between the genotyped NTR sample and random NTR sample however shows few differences for alcohol phenotypes, in spite of reported associations between alcohol consumption and depression (Graham et al. 2007). Individuals in the genotyped sample more often reported to have drunk alcohol daily and weekly and reported higher quantities of alcohol use, but not more problem drinking than in the random NTR sample. This finding is in line with the existing research that moderate drinking is associated with less mental health problems compared to light or heavier drinking (Chen 2006). The comparability of this sample to the Australian sample is further strengthened by the agreement in frequencies of alcohol phenotypes, such as alcohol use ever and alcohol use in the previous 12 months.

A further limitation is that the sample size of the replication study was smaller than that of the Australian discovery sample. Obviously, this influenced the power to detect effects of the

size found by Macgregor et al. (2009). We carried out ad-hoc power analyses for phenotypes with different prevalence. For phenotypes for which the prevalence was low, the power to detect significant effects after correcting for multiple SNP testing (within gene sets) and multiple phenotype testing was low regardless of relative risk (RR). For phenotypes with a higher prevalence (around 65%; e.g. weekly drinking) power was still relatively low for a RR of 1.1 (between .25 and .55 for SNPs with a MAF ranging from .20 to .40). For a RR of 1.2 or higher the power was very good (> .95). Thus, for most phenotypes we had sufficient power to detect associations with modest to large effect sizes (RR>1.2), but our power to detect very small effect sizes was low (RR~1.1).

In order to maximize coverage of the genetic variation in the ADH gene cluster, we imputed all SNPs in HapMap2 that were not genotyped. This is a sensible route to undertake given that the LD structure in the present study is similar on a genome-wide level to the HapMap reference set (Pardo et al. 2009). Still, six genetic markers could not be analyzed. The top SNP in Macgregor et al. (2009), rs1229984, was imputed, but had to be discarded from the analyses, since its MAF was <.01 (based on the HapMap2-CEU sample). Five other markers analyzed by Macgregor et al. (2009) could not be imputed, as they were not included in the HapMap2-CEU reference set. A total of 491 SNPs was measured or imputed across the ADH region. In our analyses we divided the region into seven gene sets, which corresponded with the known genes in this region. Corrections for multiple testing were carried out within gene sets. It could be argued that the entire ADH region should be considered as one gene set. In that case the association between age at onset of regular alcohol use and SNP rs2654849 still is significant (empirical *p*-value =.004). The associations of physical reactions with rs6827292 and rs6819724, for which data on 872 individuals were available, would however no longer be significant (empirical *p*-value =.127).

In conclusion, the present study contributes to the existing evidence that the ADH gene cluster is important for understanding the genetics of alcohol use.

CHAPTER 8

HERITABILITY OF GGT, ALT AND AST LEVELS ESTIMATED FROM GENOME-WIDE SNP DATA AND ITS GENETIC COVARIANCE WITH ALCOHOL USE[7]

Abstract

Plasma levels of liver enzymes can mark liver injury and increase with heavy drinking. Twin-family studies suggest that variation in liver enzyme levels is moderately heritable, but genome-wide association (GWA) consortia have traced less than 2% of the variance back to effects of single nucleotide polymorphisms (SNPs). In the current study, we estimated the heritability of the levels of three liver enzymes (GGT, ALT en AST) using genome-wide SNP data in 5,084 unrelated individuals of the NTR/NESDA sample and using meta-analysis results from GWA consortia on GGT and ALT. The heritability was estimated by two different methods, one based on genetic relatedness and the other on density estimation. Secondly, we test whether part of the genetic variance due to SNPs is shared with that for alcohol intake. For this we (a) compared heritability estimates with and without regressing out effects of alcohol intake, (b) calculated the correlation between additive genetic SNP effects on liver enzyme levels and additive SNP effects on alcohol intake in a bivariate analysis based on genetic relatedness, and (c) tested whether polygenic scores based on the GWA meta-analysis results predicted alcohol intake levels.

Depending on the method, 15-17%, 2-15% and 13% of the variance in GGT, ALT, and AST was explained by the joint effect of genome-wide SNPs respectively. These estimates correspond to 50-57%, 7-52% and 46% of the narrow-sense heritability estimates for these liver enzymes from twin-family studies. Heritability estimates using meta-analysis results were more than halved (GGT 8%; ALT 5%). Alcohol intake correlated with GGT levels in both sexes (r=.18-.27), but not consistently with ALT and AST. The question regarding shared variance with alcohol intake was therefore examined for GGT only. Regressing out the effect of alcohol intake did not affect heritability estimates, nor were additive SNP effects on GGT and alcohol intake levels significantly correlated. However, polygenic risk scores based on the GWA meta-analysis results for GGT explained 0.25% of the variance in alcohol intake.

[7] Part of this chapter is submitted as:
Van Beek, JHDA, Lubke, GH, de Moor, MHM, Willemsen, G, de Geus, EJC, Hottenga, JJ, Walters, RK, Smit, JH, Penninx, BWJH, Boomsma, DI. Heritability of GGT, ALT and AST levels estimated from genome-wide SNP data.
.

To conclude, currently used genome-wide SNP platforms contain substantial information regarding the underlying genetic variation in liver enzyme levels, indicating that a substantial part of this genetic variation is not missing but undetected. Using polygenic risk scores the association of alcohol intake with GGT can be traced back to SNPs. Given adequate sample sizes, this information may lead to the detection of new susceptibility loci for alcohol use, liver enzyme levels and their association.

1. Introduction

Blood levels of the liver enzymes γ-glutamyl transferase (GGT), alanine and aspartate aminotransferase (ALT, AST) predict liver disease and all-cause mortality (Kazemi-Shirazi et al. 2007; Hyeon et al. 2004). Clinically, these enzymes are used as markers for liver injury (Pratt and Kaplan 2000). Risk of liver disease is increased by heavy drinking (Mann et al. 2003) and epidemiological studies show increased levels of GGT, ALT and AST among heavy drinkers (>40 grams alcohol/day) compared to abstainers (Arndt et al. 1998; Alatalo et al. 2009b). In most studies, GGT is also increased among moderate drinkers, whereas AST and ALT are not or not consistently elevated (Alatalo et al. 2009b; Arndt et al. 1998; Liangpunsakul et al. 2010).

Variation in liver enzyme levels and the association with alcohol use can be partly explained by genetic differences among individuals. Broad-sense heritability estimates for liver enzyme levels from twin-family studies range from 22% to 60% (Van Beek et al. 2013a). Although genetic influences on liver enzyme levels are substantial, most of the genes underlying the variation are still unidentified. For GGT, the combined effect of all genome-wide significant single nucleotide polymorphisms (SNPs with *p*-values $<5x10^{-8}$) explains 2% of the variation, for ALT and AST this is less than 1% (Chambers et al. 2011; Kamatani et al. 2010). Several explanations for this so-called 'missing heritability' (Maher 2008) have been put forward. The sample sizes of current genome-wide association (GWA) studies might be too small to detect the effects of individual SNPs under the stringent significance thresholds that are used to correct for multiple testing (Purcell et al. 2009). Alternatively, genetic variation may be due to effects other than those captured by SNPs on currently used platforms (e.g. rare variants or copy number variants) (Stranger et al. 2011). To gain insight in the genetic architecture of liver enzyme levels and optimize the success of future gene finding studies, it is important to know to what extent genetic variation underlying liver enzyme levels is not yet detected because of inadequate power to find small SNP effects and to what extent it is not captured by the SNPs that have been analyzed (either measured or imputed).

By examining the proportion of the variation in liver enzyme levels that can be explained by the joint effect of all measured and imputed genome-wide SNPs it can be tested to what extent the heritability is hidden instead of missing, and secondly, whether part of this variation is shared with that for alcohol intake. The degree to which this estimate is higher than the proportion of variance that is currently explained by genome-wide *significant* SNPs, most likely reflects associations that have not yet been detected due to the multiple testing burden.

Two methods have been proposed to study the aggregate effect of SNPs on phenotypic variability. The first method, implemented in the software package genome-wide complex trait analysis (GCTA) (Yang et al. 2010; Yang et al. 2011a), is a two-step procedure where the first step consists of estimating the genetic relationship matrix (GRM) between all pairs of subjects.

This pair-wise genetic relatedness is calculated as a correlation between two individuals using all SNPs. In the second step, the pair-wise genetic relatedness is used as a random effect in a linear mixed model to estimate the proportion of variance attributable to additive genetic effects. To avoid confounding additive SNP effects with genetic effects shared between family members that are not captured by SNPs, genetic relatedness is calculated for 'unrelated' individuals only. The method relies on the fact that 'unrelated' individuals are still distantly related, which can be traced back to the correlation structure in the genome. Distantly more related individuals have more alleles in common than distantly less related individuals. Applying the method to unrelated individuals also circumvents the problem that phenotypic similarity among individuals can be due to shared environmental effects (Yang et al. 2010). The GRM method provides an unbiased estimate of the variance that can be explained by the joint additive effects of all SNPs if the causal SNPs are perfectly correlated with the measured/imputed SNPs (i.e., if linkage disequilibrium, LD, is 1). In the case of imperfect LD, for instance because the distribution of minor allele frequencies (MAF) differs for the causal and measured/imputed SNPs, estimates are biased downwards.

The density estimation (DE) method proposed by So, Li and Sham (2011) provides an alternative approach to estimate the proportion of variance that can be ascribed to SNP effects. The DE method compares the distribution of observed effect sizes of SNPs that resulted from a GWA study (or meta-analysis of GWA studies) to the distribution expected under the null hypothesis of zero effects. The extent to which the distribution of observed effect sizes has thicker tails than the distribution under the null reflects the proportion of phenotypic variance that is captured by SNPs. Specifically, the proportion of phenotypic variance explained is estimated from 'true' effect sizes computed using a correction for sampling variation suggested by Efron (2009). To avoid inflated estimates due to SNPs with non-zero effects that are in LD, the SNP data need to be pruned to obtain independent SNP signals. The phenotypic variance of continuous phenotypes due to SNPs is calculated using a sums of squares approach similar to ANOVA (So et al. 2011).

The current study has two aims. The first aim is to apply the GRM and DE methods to estimate the genetic variance due to all SNPs separately for GGT, ALT and AST levels. Secondly, we examine whether part of the variation in liver enzyme levels that can be ascribed to SNPs is shared with that for alcohol intake. The latter is accomplished by testing whether (a) regressing out effects of alcohol intake lowers the variance components for liver enzyme levels attributable to additive SNP effects, (b) the genetic correlation between liver enzyme levels and alcohol intake differs significantly from zero in a bivariate GRM analysis, and (c) polygenic risk scores based on GWA meta-analysis results for liver enzyme levels predict alcohol intake levels (Lee et al. 2012b).

Data originate from (a) participants of the Netherlands Twin Register (NTR; N=3,057 unrelated subjects) (Boomsma et al. 2002c), (b) participants of the Netherlands Study on Depression and Anxiety (NESDA; N=2,027 unrelated subjects) (Penninx et al. 2008) and (c) the available summary statistics from a meta-analysis on GGT and ALT (N≈55,000) by an international consortium (Chambers et al. 2011). To compare the performance of the two methods, the GRM and DE approaches were also applied to data on BMI and height in the NTR/NESDA samples as well as to the available summary statistics from two large meta-analysis on BMI and height by the GIANT consortium (Speliotes et al. 2010; Lango Allen et al. 2010). BMI and height serve as bench mark traits for which the additive genetic variance explained by SNPs has been studied before (Lubke et al. 2012; Yang et al. 2011b).

2. Materials and Methods

2.1 Participants

For 5,797 subjects who participated in the NTR biobank study (Willemsen et al. 2010), genotype data and data on liver enzyme levels were available. Data were excluded for six individuals with known liver disease (ICD-10 codes K70-K77) and 164 individuals from non-European descent. Data were selected from unrelated individuals, which resulted in a sample of 3,057 NTR participants (60.7% females; year of birth 1915-1987). For 2,213 individuals who participated in the NESDA biobank study (Penninx et al. 2008), liver enzyme levels and genotype data were available. Data were excluded for 115 individuals from non-European descent and for 71 persons who were related to other NESDA or NTR participants, resulting in 2,027 unrelated individuals whose data were included in the analyses (66.6% females; year of birth 1939-1988). The total NTR/NESDA sample thus consisted of 5,084 unrelated individuals (3,057 + 2,027). For 4,842 individuals, data on alcohol intake in grams per day were available (NTR: 2,832; NESDA: 2,010). Permission for the biobank studies was obtained from the Central Ethics Committee on Research Involving Human Subjects of the VU University Medical Center Amsterdam, and informed consent was obtained from all participants (Willemsen et al. 2010; Penninx et al. 2008).

Meta-analysis summary statistics (*z*-scores and *p*-values) for GGT and ALT levels originated from a large meta-analysis on data from 52,350 individuals with Caucasian ethnicity (including 1,721 NTR and 1,724 NESDA participants; <5% of the total sample size), as well as from 8,739 participants with an Indian-Asian background (Chambers et al. 2011).

For comparison purposes, analyses were also performed on BMI and body height. For nearly all NTR/NESDA participants, data on BMI (N=5,065) and body height (N=5,071) were assessed at the same time as their liver enzyme data. Meta-analysis summary statistics (*p*-values) from large GWA studies on BMI (N=249,796, including 3,516 participants from

NTR/NESDA) (Speliotes et al. 2010) and height (N=183,727, including 3,522 NTR/NESDA participants) (Lango Allen et al. 2010) were downloaded from http://www.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files . Table 1 gives a summary of all data that were available for each phenotype, with the respective numbers of participants.

2.2 Genotyping and quality control

Genotyping in the combined NTR/NESDA sample was performed on five platforms: Affymetrix 6.0, Affymetrix 5.0-Perlegen (Affymetrix, Santa Clara California), Illumina 660, Illumina Omni Express 1M and Illumina 370 (Illumina, San Diego, California). SNP data quality control (QC) was performed in two steps. A first round of QC was platform-specific. SNPs were aligned to the Hapmap 3 build 37 reference set (Altshuler et al. 2010a). SNPs were removed if the number of alleles was >2, if SNPs matched to none or multiple positions, if SNP missingness was >5%, allele frequencies deviated from Hardy-Weinberg equilibrium (HWE; p<.000001), Mendelian error rate was >1%, double samples typed error rate was >1%, MAF was <.01 and/or if alleles were C/G or A/T and MAF >.35. SNPs were then aligned to the 1000 Genomes reference set (Altshuler et al. 2010b) and SNPs were excluded if allele frequencies differed >.20 compared to the reference set. Samples were excluded if missing rate was >10%, heterozygosity <-.10 or >.10, principal component (PC) values indicated batch effects, or sex status did not match genotypic sex. Imputation was performed with Impute2 (Howie et al. 2009) using the 1000 Genomes CEU sample as reference set (Altshuler et al. 2010b). A second round of quality control was performed on the imputed set. SNPs were excluded if allele frequencies differed from HWE (p<.000001), differed >.15 compared to the reference set, SNPs showed large platform-specific case-control differences (p<.00001), Mendelian error rate >2%, if imputation quality was low (r^2_hat <.3, proper info <.3, SNP info <.3) or MAF <.001. Overall, 29,311,591 SNPs were excluded in these QC steps, resulting in a final dataset of 8,115,142 autosomal SNPs.

2.3 Phenotypes

Liver enzymes were determined in heparin plasma tubes that were collected after overnight fasting. Before the start of the blood sample collection, the NTR and NESDA biobank protocols for processing and storage of blood samples were harmonized (Boomsma et al. 2008). For the NTR biobank project, blood samples were collected in the morning at the participant's home or, if preferred, at his/her work. and processing took place at one central laboratory within six hours (average transport time 196 minutes). Blood was collected in two heparin plasma tubes, which were stored in melting ice during transportation.

Table 1 Number of participants with SNP data and information on liver enzyme levels, alcohol intake, BMI and height, and methods applied to these phenotypes/ samples

	Phenotypes						Methods		
	GGT	**ALT**	**AST**	**Alcohol intake**	**BMI**	**Height**	**GRM-based**	**DE-based**	**Polygenic risk scores** [a]
NTR	3,056	2,974	3,041	2,832	3,040	3,046	X	X	
with alcohol data	2,832	2,760	2,820				X	X	
NESDA	2,000	2,027	2,027	2,005	2,025	2,025	X	X	
with alcohol data	1,978	2,005	2,005				X	X	
NTR+NESDA	5,056	5,001	5,068	4,837	5,065	5,071	X	X	
with alcohol data	4,810	4,765	4,825				X	X	X
Meta-analysis summary statistics consortia [b]	57,690	55,486			~123,912	~133,859		X	

[a] Polygenic risk scores were based on the GWA meta-analysis summary statistics (Chambers et al. 2011).

[b] For all markers, data was available for a subset of the individuals. These numbers reflect the maximum number of observations that was available for a marker.

When the samples arrived at the laboratory, plasma was collected and six samples of 500 µL were snap-frozen and stored at -30 °C. Liver enzyme levels were determined with Vitros assays (Vitros 250, Ortho-Clinical Diagnostics; Johnson & Johnson, Rochester, USA) in units per liter (U/L) (Willemsen et al. 2010). The average time between blood sample collection and liver enzyme determination was 37.0 months (SD=11.9). For NESDA, blood sampling took place during the NESDA baseline assessment at seven field sites that were within walking distance of a laboratory. At the laboratory, processing of blood samples and determination of liver enzyme levels was performed within the hour (Boomsma et al. 2008; Penninx et al. 2008). Liver enzyme levels were determined with Roche Diagnostic assays (Chambers et al. 2011).

Information on alcohol intake was collected through self-report questionnaires and analyzed as grams of alcohol consumed per day. For NTR participants, data on alcohol intake came from multiple surveys collected between 2002-2011 as part of the longitudinal studies on health, lifestyle and personality (Boomsma et al. 2002c; Willemsen et al. 2013). Alcohol intake was measured by the question 'How many glasses a week do you drink on average?' with response categories: '< 1 glass', '1–2 glasses, '3–5 glasses', '6–10 glasses', '11–20 glasses', '21–40 glasses' and '> 40 glasses' per week, or with the option to report the number of glasses of beer, wine and spirits per day of the week. The average intake in grams per day was calculated by taking the midpoint number of glasses per week for each drinking category divided by 7, and multiplied by 14 (grams of alcohol per glass). If alcohol data were available from multiple surveys, the survey closest in time to the biobank visit was selected. The average time between the biobank visit and measurement of alcohol use was 15 months (SD=12.4).

For NESDA participants, the level of alcohol intake was calculated from the first two items of the AUDIT questionnaire that was collected at the day of the blood sample collection. Item 1 assessed drinking frequency: 'How often do you have a drink containing alcohol?' with response categories 'never', 'monthly or less', 'two to four times a month', 'two to three times a week', 'four or more times a week'. Item 2 assessed drinking quantity at a drinking day: 'How many drinks containing alcohol do you have on a typical day when you are drinking? with response categories '1 or 2', '3 or 4', '5 or 6', '7, 8 or 9', '10 or more'. To calculate alcohol intake levels, (each category of) drinking frequency was expressed as the number of drinking days per year and (each category of) drinking quantity as the midpoint number of glasses consumed at a drinking day. The last category of drinking quantity was given the midpoint value of 11 based on the median number of drinks per day reported by NTR participants who drank more than 10 glasses per day. Alcohol intake in grams per day was calculated by dividing the number of drinking days per year by 365 (days in the year), multiplied by the midpoint number of drinks per drinking day, multiplied by 14 (grams of alcohol per glass). If individuals reported to 'never' drink alcohol on the first AUDIT item, they were assigned an alcohol intake level of 0 grams of

alcohol per day. For subsequent analyses, liver enzyme values and alcohol intake in grams per day were *log*-transformed to approximate normality.

2.4 Statistical analyses

2.4.1 Preparatory analyses

Fixed effects of source and sex Differences in liver enzyme levels were examined with respect to source (NTR, NESDA), and sex (male, female) by ANOVA. Correlations between liver enzyme levels and alcohol intake (grams/day) were estimated as a function of source and sex. Based on these analyses, regression analyses were carried out with source (NTR/NESDA), age (for all phenotypes but height), year of birth (for height), sex, time in months between blood draw and liver enzyme assessment, two PCs representing platform effects, three PCs representing population stratification and lab effects[8] as covariates. All regression analyses were performed in SPSS 20.0 (IBM Corp. 2011). The residuals from these regression analyses were used in all subsequent analyses.

GWA In the NESDA/NTR dataset SNP associations were tested in a linear model assuming additive SNP effects using Plink (v1.07) (Purcell et al. 2007). GWA results are the input for the methods to estimate heritability, and were therefore inspected by quantile-quantile (QQ) and Manhattan plots. QQ plots depict the observed distribution of *p*-values against the expected *p*-values. Manhattan plots show the *p*-values of the individual association tests for each SNP. Thresholds for suggestive and genome-wide significance were $1x10^{-5}$ and $5x10^{-8}$ respectively.

Effect of level of pruning on GRM and DE method The performance of the GRM method was explored by comparing the standard errors around the GRM-based estimates under four scenarios that differed in levels of LD-based SNP and relatedness pruning: (a) no LD-based SNP pruning, no relatedness pruning, (b) no LD-based SNP pruning, relatedness pruning ≤.025, (c) LD-based SNP pruning at r^2 ≤.95, no relatedness pruning, (d) LD-based SNP pruning at r^2 ≤.95, relatedness pruning ≤.025. To apply the DE method on the NTR/NESDA SNP dataset the recommendation of So, Li and Sham (2011) to prune at a level of r^2 .25 may not be valid, since this recommendation was based on datasets containing .1-2.6 million SNPs and the NTR/NESDA dataset contained ~8 million SNPs. The effect of LD-based SNP pruning was therefore explored by estimating DE-based heritability estimates under four levels of pruning: r^2 ≤.25, r^2 ≤.10, r^2 ≤.05, r^2 .00. For each level of pruning, a 100-SNP sliding window was used, proceeding by 25 SNPs in each step (Plink options e.g. --indep-pairwise 100 25 0.00).

[8] Lab effects (among NESDA participants) were (significant and) included as covariate for AST levels only, not for GGT and ALT levels.

2.4.2 Main analyses

Estimation of SNP-based heritability with GRM method The genetic relatedness matrix was estimated for all individuals (option --make-grm) using the free software package GCTA (v1.04) (Yang et al. 2011a). Then, a linear mixed model with the genetic relationships as a random coefficient was used to estimate the phenotypic variance that is due to genetic relatedness. Estimation was performed using restricted maximum likelihood (REML) (option --reml). In additional analyses, the variance that can be explained by SNPs on each individual chromosome was estimated by genetic relatedness matrices that were estimated for each chromosome separately.

Estimation of SNP-based heritability with DE method Analyses with the DE method were performed in R2.15.1 (R Development Core Team 2012) with the script for continuous traits obtained from the developer's website: https://sites.google.com/site/honcheongso/software/total-vg The method takes a vector with *z*-statistics as input that correspond to the effect sizes of SNPs obtained in a GWA study. Under a standard normal probability distribution, *z*-statistics correspond to specific *p*-values and vice versa (ignoring the sign of the *z*-statistics), with *z*-statistics defined as the standardized deviation of a value *x* to the mean $\bar{x}$, that is $z = \frac{x - \bar{x}}{sd(x)}$, with sd($x$)=1. Using this relation, *z*-statistics were extracted from GWA *p*-values or obtained as part of the downloaded GWA meta-analysis. The sign of the *z*-statistics was permuted 100 times, in order to avoid incorrect estimates in case all observed effects are in the same direction. So, Li and Sham (2011) described two different estimates, based on conditional and unconditional kernel estimation respectively, but noted that unconditional Kernel estimation is more accurate and stable with pruned data than conditional Kernel estimation. This was in line with our preliminary results. Therefore reported results were based on unconditional Kernel density estimation. To avoid inflation of the variance explained by SNPs by redundant SNP effects, SNPs in high LD with other SNPs were removed by pruning. Note that pruning was independent of the trait associations. In the GWA meta-analysis data on liver enzymes, BMI and height, raw SNP data were not available, and pruning was based on the LD pattern among SNPs as observed in the Hapmap 2 CEU reference set (release 22, hapmap-ceu-all.zip) that had been used as a reference set for imputation for the individual cohorts included in the meta-analyses, and which was downloaded from the Plink website http://pngu.mgh.harvard.edu/~purcell/plink/res.shtml#hapmap. For the GWA meta-analysis data, pruning was based on a r^2 threshold of .25, a 100-SNP sliding window and proceeding by 25 SNPs in each step (Plink options --indep-pairwise 100 25 0.25) as suggested by So, Li and Sham (2011). For the NTR/NESDA dataset, final estimates were based on truly independent

SNPs (corresponding to a r^2 level of .00; --indep-pairwise 100 25 0.00; left ~50,000 SNPs in pruned set, see Supplementary Table S2). This level of pruning would serve as a lower bound of the variance explained by all SNPs. To obtain an indication of the stability/variability of the heritability estimates, the Hapmap and NTR/NESDA dataset were pruned 10 times. The analysis was carried out on each pruned set, and results were then averaged.

Tests for shared SNP effects on liver enzyme levels and alcohol intake Whether part of the variance in liver enzyme levels ascribed to SNPs was shared with that for alcohol intake was tested (a) by comparing variance components due to SNPs with and without regressing out effects of alcohol intake, (b) by calculating the genetic correlation between liver enzyme levels and alcohol intake in a bivariate GRM analysis, and (c) with polygenic scores.

Applying the GRM and DE methods, in univariate analyses on the NTR/NESDA data it was tested whether including alcohol intake as fixed effect in the GRM or DE analysis changed the amount of variance explained by SNPs. The GRM method, based on the same genetic relatedness matrix as described above, included alcohol intake as fixed effect in a linear mixed model. For the DE method, GWA analyses were performed for NTR/NESDA participants with and without including alcohol intake as additional fixed effect. If SNP effects on liver enzyme levels reflect SNP effects on alcohol use, then regressing out the variance in liver enzyme levels due to alcohol use, will result in a lower estimate of variance explained by SNPs.

A bivariate GRM analysis (Lee et al. 2012b) estimated the correlation between the additive SNP effects on alcohol intake and liver enzyme levels in a linear mixed model, based on the genetic relatedness matrix described above. This correlation, here referred to as the genetic correlation r_a between alcohol intake and liver enzyme levels, is defined as the standardized genetic covariance between alcohol intake and liver enzyme levels:

$$r_{a,ALC,LIV} = \frac{cov(A_{ALC,LIV})}{\sqrt{var(A_{ALC}) * var(A_{LIV})}}$$

That is, the genetic correlation r_a between alcohol intake (ALC) and liver enzyme levels (LIV) is defined as the additive genetic covariance between alcohol intake and liver enzyme levels due to SNPs $cov(A_{ALC,LIV})$, divided by the square root of the product of the additive genetic variance component for alcohol intake due to SNPs ($var(A_{ALC})$) and the additive genetic variance component for liver enzyme levels ($var(A_{LIV})$).

With the polygenic score approach (Purcell et al. 2009), sets of SNPs are selected that are associated with the trait of interest in a discovery sample for different levels of association which are then used for prediction of a trait of interest in a validation sample. GWA meta-analysis summary statistics (Chambers et al. 2011) served as the discovery set and alcohol

intake data of NTR/NESDA individuals as target set. Eleven sets of SNPs were created that contained SNPs with meta-analysis *p*-values ≤.01, ≤.1, ≤.2, ≤.3, ≤.4, ≤.5, ≤.6, ≤.7, ≤.8, ≤.9 and ≤1. For each individual in the target set, based on each set of SNPs, polygenic risk scores were calculated by multiplying the meta-analysis *z*-score of the selected SNP with the number of effect alleles for the individuals in the target set (Plink option --score). The polygenic scores were tested for prediction of alcohol intake levels by performing a linear regression analysis in R.

An overview of all analyses performed on the datasets is included in Table 1. All statistical tests were considered to be significant if *p*-values <.01, which roughly corresponds to estimates that are larger than 2.5 times their standard error/estimate of variability.

3. Results

3.1 Preparatory analyses

Fixed effects of source and sex Table 2 summarizes the mean and median liver enzyme levels (with SD and range) for NTR and NESDA, separately over sex. Mean liver enzyme levels and alcohol intake were higher for men than women. For both sexes, GGT and alcohol intake levels were higher in NTR than in NESDA participants, whereas for ALT and AST the reverse was observed (see Table 2). Table 3 shows the correlations among liver enzyme levels and alcohol intake, by source and sex. There were positive correlations among the three liver enzyme levels ranging from .26 to .67. The correlation between GGT and ALT was higher for NESDA than NTR participants (.54-.56 versus .26-.31). Correlations between AST and GGT and AST and ALT were similar in NTR and NESDA (∼.38 and ∼.59 respectively). Alcohol intake was significantly correlated with GGT levels in both sexes (.18-.27), whereas it was not consistently associated with ALT and AST levels (Table 3). These results were similar when partialling out the effect of age. Hence, the overlap in variance of liver enzyme levels due to SNPs with that for alcohol intake, was tested for GGT only.

GWA Supplementary Figures S1.1-6.1 show the QQ plots with *p*-values for the SNP associations for liver enzyme levels, alcohol intake, BMI and height that resulted from GWA analyses performed on the NTR/NESDA datasets. Figures S1.1a-S6.1a show the QQ plots for the NTR dataset, Figures S1.1b-S6.1b for the NESDA dataset and Figures S1.1c-S6.1c for NTR/NESDA combined. QQ plots are also given for the downloaded meta-analysis results for liver enzymes, BMI and height (Figures S1.1d-6.1d). In line with the published results, these show that the observed *p*-values strongly deviate from the line with expected *p*-values, indicating large polygenic variation for GGT, ALT (Figures S1.1d, S2.1d), BMI and height (Figures S5.1d, S6.1d). For the NTR/NESDA data, observed *p*-values showed a much weaker deviation from the line with expected *p*-values as can be seen in Figures S1.1c-6.1c. For ALT and to a

lesser extent for alcohol intake, the observed *p*-values for the combined NTR/NESDA data fell below the line with expected *p*-values (Figures S2.1c, S4.1c), whereas this was not the case when the NTR and NESDA data were analyzed separately (Figures S2.1a-b, S4.1a-b). This may indicate phenotypic or genetic heterogeneity. For subsequent analyses we therefore reported results for NTR and NESDA separately as well as combined. Manhattan plots for liver enzyme levels, alcohol intake, BMI and height for the NTR/NESDA datasets and the GWA meta-analysis data are shown in Supplementary Figures S1.2-6.2.

Effect of level of pruning on GRM and DE method Standard errors around the GRM-based estimates were lowest when no pruning was performed on either SNPs or the relatedness among individuals (see Supplementary Table S1). For subsequent analyses, the GRM method was therefore applied without SNP or relatedness pruning. DE-based estimates on the *proportions* of variance explained by SNPs reached impossible values (>1) for at least one of the phenotypes when performing LD-based SNP pruning at a level of r^2 >.00 (see Supplementary Table S2) for the NTR/NESDA data. For subsequent analyses on the NTR/NESDA data, the DE method was therefore performed on truly independent SNPs (corresponding to pruning at r^2 .00).

3.2 Main analyses

SNP-based heritability based on GRM and DE methods Table 4 shows the GRM- and DE-based estimates for the variance explained by SNPs for liver enzyme levels, alcohol intake, BMI and height. For both methods, the variability of the variance estimates became smaller as the sample size increased. Note that GRM-based estimates for BMI and height were significantly different from zero (estimates >2.5 times estimate of variability), whereas those for liver enzyme levels and alcohol intake were not.

Both methods agreed very well on the point estimate of heritability for GGT (15-17%) and AST (13%). The estimates for ALT were more variable. Based on the DE method, 15% of the variance could be explained by SNPs, whereas the GRM-based estimate was only 2%. Note that for ALT and to a lesser degree for AST, GRM-based estimates on the combined NTR/NESDA data were lower than those for the separate sets, whereas for GGT, alcohol intake and BMI, GRM-based estimates for the combined NTR/NESDA set were in between those for the separate NTR and NESDA sets. This may reflect the phenotypic/genetic heterogeneity that was observed for ALT (see Figure S2.1c as compared to Figures S2.1a-b), although it should be noted that estimates of variability were large, and differences between ALT and AST estimates were not significant. In total, 15% of the variance in alcohol intake could be explained by the combined effect of SNPs (based on the NTR/NESDA data).

Table 2 Means (with standard deviations), medians (with range) for liver enzyme levels, alcohol intake, BMI, height and age, separately for NTR and NESDA, split over sex

	NTR				NESDA			
	Males		Females		Males		Females	
	mean (SD)	median (range)	mean (SD)	median (range)	mean (SD)	median (range)	mean (SD)	median (range)
GGT[abh]	41.0 (42.7)	31 (10-917)	27.5 (31.5)	21 (9-867)	37.2 (37.8)	26 (8-285)	20.7 (25.0)	15 (2-563)
ALT[aci]	11.9 (7.1)	11 (3-107)	9.2 (5.4)	8 (3-89)	31.6 (19.5)	27 (1-218)	19.9 (12.8)	17 (4-248)
AST[adj]	23.6 (8.8)	22 (7-122)	20.0 (7.1)	19 (7-128)	29.6 (12.0)	27 (10-112)	23.6 (8.4)	22 (10-94)
alcohol[aek]	18.5 (18.7)	16 (0-92)	8.7 (11.8)	6 (0-92)	20.8 (25.1)	15.2 (0-121)	11.1 (15.6)	4.8 (0-121)
BMI[fl]	25.8 (3.5)	25.6 (17.3-10.2)	25.0 (4.3)	24.2 (15.7-48.9)	26.5 (4.6)	26.0 (16.0-50.2)	25.3 (5.2)	24.1 (14.7-53.3)
height[gm]	181.3 (7.1)	182 (160-206)	168.8 (6.4)	168 (150-196)	181.5 (7.2)	181 (159-210)	169.1 (6.5)	169 (143-190)
age	49.2 (14.8)	54 (18-89)	45.1 (14.0)	44.5 (19-89)	44.3 (12.3)	46 (18-64)	41.6 (12.8)	43 (18-65)

[a] untransformed levels; statistical analyses were performed on *log*-transformed levels of liver enzymes and alcohol intake

[b] mean GGT levels higher for NTR than NESDA (males: $F(1,1867)=23.1$, $p<.001$; females: $F(1,3183)=282.1$, $p<.001$)

[c] mean ALT levels lower for NTR than NESDA (males: $F(1,1852)=1550.3$, $p<.001$; females: $F(1,3143)=2323.4$ $p<.001$)

[d] mean AST levels lower for NTR than NESDA (males: $F(1,1864)=191.0$, $p<.001$; females: $F(1,3198)=288.1$, $p<.001$)

[e] mean alcohol intake levels comparable among NTR and NESDA males ($F(1,1771)=.62$, $p=.43$), but lower among NTR than NESDA females ($F(1,3060)=22.3$, $p<.001$).

[f] mean BMI lower for NTR than NESDA ($F(1,1870)=27.1$, $p<.001$; females: $F(1,3189)=12.3$, $p<.001$)

[g] no differences in mean height between NTR and NESDA (males: $F(1,1872)=2.6$, $p=.108$; females: $F(1,3193)=.18$, $p=.670$)

[h] mean GGT levels higher in men than women (NTR: $F(1,3053)=357.9$, $p<.001$; NESDA: $F(1,1997)=331.0$, $p<.001$)

[i] mean ALT levels higher in men than women (NTR: $F(1,2971)=208.2$, $p<.001$; NESDA: $F(1,2024)=349.2$, $p<.001$)

[j] mean AST levels higher in men than women (NTR: $F(1,3038)=210.6$, $p<.001$; NESDA: $F(1,2024)=186.2$, $p<.001$)

[k] mean alcohol intake higher in men than women (NTR: $F(1,2829)=285.3$, $p<.001$; NESDA: $F(1,2002)=84.7$, $p<.001$)

[l] mean BMI levels higher in men than women (NTR: $F(1,3034)=10.5$, $p=.001$; NESDA: $F(1,2022)=13.7$, $p<.001$)

[m] mean height higher in men than women (NTR: $F(1,3043)=2949.1$, $p<.001$; NESDA: $F(1,2022)=1708.3$, $p<.001$)

Table 3 Correlations between liver enzyme levels, alcohol intake (*log*-transformed), BMI and age separately for NTR and NESDA, split over sex [correlations significant at α=.01 shown in bold]

	NTR										**NESDA**									
	Males					**Females**					**Males**					**Females**				
	ALT	**AST**	**ALC**	**BMI**	**age**	**ALT**	**AST**	**ALC**	**BMI**	**age**	**ALT**	**AST**	**ALC**	**BMI**	**age**	**ALT**	**AST**	**ALC**	**BMI**	**age**
GGT [a]	**.26**	**.40**	**.20**	**.30**	**.16**	**.31**	**.39**	**.13**	**.26**	**.28**	**.56**	**.37**	**.17**	**.37**	**.30**	**.54**	**.36**	**.11**	**.27**	**.22**
ALT		**.56**	-.04	**.12**	**-.22**		**.53**	.03	**.10**	**.07**		**.67**	.01	**.37**	.09		**.60**	.00	**.32**	**.23**
AST			.04	**.16**	-.02			**.09**	.06	**.23**			**.10**	**.17**	.02			-.02	**.13**	**.17**
ALC [b]				.01	**.10**				**-.04**	**.18**				.02	**.10**				**-.10**	**.13**
BMI					**.28**					**.30**					**.29**					**.23**

[a] In the GWA meta-analysis (Chambers et al. 2011), the correlation between *log*-transformed values of ALT and GGT was .64

[b] In the SNP-based heritability analyses, *log*-transformed levels of alcohol intake were analyzed, which are given in Table 3. Correlations with untransformed levels of alcohol intake were higher. For NTR males, NTR females, NESDA males, NESDA females, correlations with GGT were .23, .20, .27, .18; with ALT -.05, .04, .12, .03; and with AST .04, .12, .15, .04 respectively.

Interestingly, a significant part of the SNP effects were attributable to two chromosomes (4, and 15 explaining ~4.5%, of the variance each) according to chromosome-specific GRM analyses (see Supplementary Table S3). For liver enzyme levels none of the chromosomes separately explained a significant part of the variance.

For BMI, the total variance due to SNPs was 17-22%. The estimates for height differed significantly between 23% and 58%, according to the DE and GRM method respectively (confidence intervals of +/-2.5 times the estimate of variability did not overlap). Note that the estimate of 23% should be considered as a lower bound on the variance explained by SNPs, namely that is due to all measured/imputed SNPs. This estimate will differ from the true variance since measured/imputed SNPs are in imperfect LD with the causal variants (So et al. 2011). Comparing the GRM- and DE-based estimates to the amount of additive genetic variance for these phenotypes as estimated with a extended twin-family design (see Table 4) shows that about half of the additive genetic variation was explained by additive SNP effects. That is, 50-57% of the additive genetic variance of GGT, 7-52% for ALT, 46% for AST, 65% for alcohol intake, 42-55% for BMI, and 30-76% for height.

Comparison of DE-based estimates using the NTR/NESDA GWA results and the GWA meta-analysis results The DE-based estimates for the GWA meta-analysis results for GGT, ALT and BMI were much lower than those based on the NTR/NESDA GWA results. This was surprising given the large polygenic variation that was suggested by the QQ plots for the meta-analysis (see Supplementary Figures S1.1d-S6.1d). Note that the DE estimates based on the GWA meta-analysis results for liver enzymes are within the confidence intervals of the GRM estimates for GGT and ALT. According to the DE estimates, ~8% of the variance in GGT and ~5% of the variance in ALT was explained by SNP effects, corresponding to ~27% and ~17% of the narrow-sense heritability of GGT and ALT respectively. The DE estimates based on the GWA meta-analysis results for BMI and height were 10% and 26% respectively, corresponding to 25% and 34% of the narrow-sense heritability (Table 4). A potential cause for the low estimates using meta-analysis *p*-values in the DE method is heterogeneity among the individual cohorts in the GWA meta-analysis.

Table 4 GRM- and DE-based estimates on the proportion variance explained by all SNPs (with estimates of variability) for liver enzyme levels, alcohol intake, BMI and height

	GGT		ALT		AST		Alcohol		BMI		Height	
	GRM	**DE**	**GRM**	**DE**	**GRM**	**DE**	**GRM**	**DE**	**GRM**	**DE**	**GRM**	**DE**
NTR [ab]	.116	.261	.115	.374	.244	.246	.146	.287	.165	.288	.507 [d]	.263
	(.115)	(.035)	(.120)	(.042)	(.120)	(.031)	(.133)	(.033)	(.126)	(.027)	(.127)	(.028)
NESDA [ab]	.350	.313	.209	.410	.318	.325	.000	.397	.416	.355	.432 [d]	.411
	(.188)	(.054)	(.188)	(.037)	(.187)	(.048)	(.187)	(.070)	(.194)	(.029)	(.182)	(.035)
NTR+NESDA [ab]	.149	.168	.024	.152	.127	.130	.149	.148	.217 [d]	.169	.579 [d]	.226
	(.076)	(.022)	(.073)	(.017)	(.074)	(.031)	(.080)	(.024)	(.078)	(.023)	(.077)	(.023)
Consortium meta-analysis [cd]		.079 [e]		.047 [e]						.099 [e]		.258 [e]
		(.003)		(.006)						(.002)		(.004)
% variance explained by GWAs	2% [f]		<1% [f]		<1% [g]		[h]		1.5% [i]		10% [j]	
Narrow-sense h^2 twin-family study	.30 (.24-.37) [k]		.29 (.24-.33) [k]		.28 (.23-.34) [k]		.23 (.19-.28) [l]		.40 (.37-.43) [m]		.76 (.74-.80) [m]	

[a] estimates based on residuals, after regressing out effects due to source (NTR/NESDA) and age (for liver enzymes, alcohol and BMI), sex, year of birth (for height), time difference between blood draw and liver enzyme determination in months (for liver enzymes), lab effects (for AST levels of NESDA participants), PCs representing effects of platform and population stratification.

[b] To obtain DE-based estimates, NTR/NESDA SNP dataset was pruned at r^2 level of .00, using a 100 SNP sliding window, proceeding by 25 SNPs in a step (see Supplementary Table S2 and text for details)

[c] To obtain DE-based estimates, Hapmap SNP dataset was pruned at r^2 level of .25, using a 100 SNP sliding window, proceeding by 25 SNPs in a step (see text for details)

[d] *p*-values were 'uncorrected' for overall genomic control inflation factor, not for the study-specific genomic inflation factors

[e] GRM-based estimates significant at α=.01

[f] estimate based on Chambers et al. (2011)

[g] estimate based on Kamatani et al. (2010)

[h] no overall estimate of variance explained by genome-wide significant SNPs available, although several genome-wide significant SNPs have been reported (see Morozova et al. (2012))

[i] estimate based on Speliotes et al. (2010)

[j] estimate based on Lango Allen et al. (2010)

[k] heritability due to additive genetic effects only (based on the NTR biobank sample). For AST in both sexes and GGT in females, non-additive genetic effects were also present. In addition, quantitative, but not qualitative, sex differences were present in the heritability of GGT and ALT (Van Beek et al. 2013a).

[l] heritability due to additive genetic effects only (based on NTR participants). Non-additive genetic effects were also present (Van Beek et al. 2013c)

[m] heritability due to additive genetic effects only (based on NTR participants). Non-additive genetic effects were also present for BMI and height and there were quantitative sex differences in the heritability of BMI (J. van Dongen, personal communication)

Shared SNP effects for GGT and alcohol intake Including alcohol intake as an additional fixed effect did not significantly decrease GRM or DE estimates (Table 5). According to the bivariate GRM analysis, the genetic correlation between GGT and alcohol intake was .35, but this correlation was not significantly different from zero (s.e. .35). Polygenic scores based on the meta-analysis for GGT (Chambers et al. 2011) significantly predicted alcohol intake levels for all *p*-value thresholds from ≤.1 to ≤1 however, although explained variance did not exceed 0.25%. Hence, the overlap in SNP effects indicated by the polygenic risk score approach was not confirmed by the GRM and DE method.

Table 5 GRM-based and DE-based estimates (estimate of variability) for GGT with/without alcohol intake as additional fixed effect

	GRM-based		**DE-based**[a]	
	without alcohol as fixed effect	**with alcohol as fixed effect**	**without alcohol as fixed effect**	**with alcohol as fixed effect**
NTR	.036 (.123)	.051 (.123)	.269 (.044)	.341 (.028)
NESDA	.309 (.188)	.297 (.188)	.374 (.036)	.408 (.060)
NTR+NESDA	.150 (.080)	.140 (.080)	.173 (.016)	.189 (.020)

[a] To obtain DE-based estimates, NTR/NESDA SNP dataset was pruned at r^2 level of .00, using a 100 SNP sliding window, proceeding by 25 SNPs in a step (see Supplementary Table S2 and text for details)

4. Discussion

The current study aimed at estimating the proportion of variance of liver enzyme levels that can be explained by the joint effect of all SNPs, and at testing whether part of this variance was shared with that for alcohol intake. The study also addressed a series of methodological issues that arise when applying different methods for estimating SNP-based heritability.

4.1 SNP-based heritability based on GRM and DE methods

For GGT and AST, ~16% and ~13% of the phenotypic variance can be explained by additive SNP effects respectively. For alcohol intake this was 15% with a significant fraction explained by chromosomes 4 and 15. Estimates for ALT ranged from 2% to 15%. With these estimates about half of the additive genetic variance for these phenotypes can be explained by genome-wide assessed SNPs. Given that current GWA studies have explained less than 2% of the phenotypic variation in liver enzyme levels by actual SNP effects (Kamatani et al. 2010; Chambers et al. 2011), we can conclude that currently used SNP platforms contain relevant, but undiscovered, information with regard to the underlying genetic variation in liver enzyme levels.

The low GRM-based estimate for ALT is consistent with the observation that Chambers et al. (2011) did not detect more than four significant SNPs in the combined data from >55,000

individuals. This may be explained by genetic or phenotypic heterogeneity among the NTR and NESDA sample that was suggested by the QQ plots for ALT in which the observed *p*-values showed a much larger deviation from the line from expected *p*-values when data from NTR and NESDA were analyzed separately than when data from NTR and NESDA were combined. Alternatively, polygenic variation underlying ALT can be truly close to zero, which is however contradicted by the significant heritability estimate (due to additive genetic effects) resulting from a twin-family study (Table 4).

4.2 Association alcohol intake with liver enzyme levels and shared SNP effects

Alcohol intake was associated with GGT levels in both sexes, whereas ALT and AST were not consistently associated with alcohol consumption. This is most likely explained the fact that the NTR/NESDA sample contained relatively few heavy drinkers. Among the NTR/NESDA participants, 9.6% of men drank heavily (>21 glasses alcohol/week; >42 grams alcohol/day) and 6.4% of women (>14 glasses alcohol/week; >28 grams alcohol/day). Whereas levels of GGT, ALT and AST are elevated among heavy drinkers, most studies show that among moderate drinkers, only GGT is increased and not ALT or AST (Alatalo et al. 2009b; Arndt et al. 1998; Liangpunsakul et al. 2010).

With the polygenic risk score approach, there was evidence for shared SNP effects for GGT and alcohol intake levels. The amount of variance explained was small, however, and a lack of power of the GRM and DE methods to detect such a small effect may be the reason why these methods could not confirm this common SNP association. Otherwise it can be argued that the lack of common SNP variation may be reflective of the fact that alcohol intake and GGT are causally related to each other, not only through a common genetic pathway. If individuals prone to (heavy) alcohol use show elevated GGT levels as a consequence of their drinking, regressing out variance of GGT due to alcohol use does not necessarily lead to a decrease in variance explained by SNPs, but may instead result in a similar or even higher proportion of variance explained. However, the bivariate GRM analysis could not detect significant overlap in SNP effects on alcohol intake and GGT either, although it did not rely on the assumption that the association between alcohol intake and GGT must be due to common genetic effects, not to a causal effect of alcohol use on GGT. Futhermore, in a bivariate genetic analysis on alcohol intake with GGT in a sample of 6,465 twins and their family members we did not detect evidence for a causal effect of alcohol use on GGT. Instead, the association between alcohol intake and GGT was best explained by shared genetic effects that acted in a non-additive manner: interacting risk alleles that influence alcohol intake as well as GGT (Van Beek et al. 2013b). To test whether we did not detect common SNP variation because the methods that were applied assumed that additive SNP effects were underlying the association of alcohol intake with GGT, whereas the

true model implied non-additive SNP effects, we ran a GWA analysis for GGT under a model of dominance (Plink options --linear --genotypic), and applied the DE method. Since the DE method relies on summary statistics, it is not restricted to testing additive SNP effects alone. The amount of variance ascribed to dominant gene effects did not differ significantly when including alcohol intake as fixed effect (.14, with estimate of variability .02 versus .16 estimate of variability .02). Hence, the lack of common SNP variation is not explained by having applied a model assuming additive SNP effects, whereas the correct model implied dominant gene action.

4.3 Comparison of liver enzyme results to BMI and height

To examine the performance of the GRM- and DE-based methods, analyses were performed for BMI and height, two phenotypes for which the amount of SNP variation has been studied previously (Yang et al. 2011b; Lubke et al. 2012; Kutalik et al. 2011). Estimates for BMI (17- 22%) agreed very well with GRM-based estimates in another study (15-17%) (Yang et al. 2011b) and correspond to about half of the additive genetic variation in BMI. For height, results were less consistent. According to the GRM method, a large proportion of the variance of height can be ascribed to additive SNP effects (58%; 76% of the narrow-sense h^2). The DE-based estimate did not fall in the confidence interval (CI) of the GRM-based estimate however (+/-2.5x s.e.). That is, according to the DE method, the amount of variance explained by SNPs is much lower (23%; 30% of the narrow-sense h^2). This DE estimate was not significantly different from the proportion of variance explained by SNPs in a previous study among NTR/NESDA participants however that was imputed against the Hapmap 2 reference set and pruned at a level of r^2 .25 (29%; CI 21.2%-37.8% based 2.5x s.e.) (Lubke et al. 2012).

4.4 Methodological considerations

The present study raised several methodological considerations.

Pruning Since some concerns had been expressed about the robustness of the GRM method when dealing with strong LD between SNPs (Speed et al. 2012), the effect of relatedness and/or LD-based SNP-pruning was explored. With regard to the GRM method, pruning was associated with larger standard errors around the estimates and was therefore not employed. For the NTR/NESDA sample, the DE method was applied to truly independent SNPs (r^2 .00), since less stringent SNP pruning resulted in impossible estimates of proportions of variance explained by SNPs for AST. Given that the amount of variance that could be ascribed to SNP effects for height was much lower based on the DE method (23%) than that based on the GRM method (58%), whereas estimates agreed for GGT, AST, alcohol intake and BMI, it may be argued that the optimal level of LD-based SNP pruning is dependent on the specific genetic architecture of the phenotype. That is, it may depend on how many SNPs have an effect and how

many of those SNPs happen to be in high or moderate LD with other SNPs. The DE method is introduced as a method to uncover that part of the heritability that is captured by measured/imputed SNPs. The variance explained by SNPs in the pruned set < variance explained by all measured/imputed SNPs in the dataset < all additive genetic variance in the genome (So et al. 2011). The DE estimate of 23% thus gives a lower bound of the variance that can be explained by SNPs for height. The estimate was not significantly different from that in a previous study among NTR/NESDA participants (29%, s.e. 3.5), although that dataset was imputed against the Hapmap 2 reference set and pruned at r^2 .25 (Lubke et al. 2012). It is therefore argued that LD-based SNP pruning has not been too stringent.

Difference in DE-based estimates from the NTR/NESDA GWA and the GWA meta-analysis results DE estimates based on the meta-analysis results were lower than those for the NTR/NESDA GWA results (with the exception of height). Given that DE estimates for the NTR/NESDA sample agreed very well with those based on the GRM-method (for GGT, AST and alcohol intake), the estimates based on the meta-analysis results likely represent underestimates of the variance explained by SNPs rather than that the DE estimates for the NTR/NESDA sample were being overestimated. A first explanation for the low estimates for the meta-analysis results may be heterogeneity among the samples that went into the meta-analysis. If not taken into account sufficiently this will lead to a lower amount of variance that can be explained by SNPs. Take for example the situation in which SNP effects from two equal sized samples are combined through inverse variance meta-analysis. In the case of genetic heterogeneity, if SNP *x* has an effect in sample 1 (*log*(Odds Ratio, OR)=1) but not in sample 2 (*log*(OR)=0), the meta-analysis effect size of this SNP is halved (*log*(OR)=0.5), but its explained variance reduced to one quarter, since explained variance is proportional to *log*(OR) squared. That is, a quarter of the variance due to SNP *x* is lost when combining effects through inverse variance meta-analysis as compared to the average variance explained weighted by sample size (the square of $\{[log(OR_1)*N_1 + log(OR_2)*N_2]/N_1+N_2\}$) (P.C. Sham, personal communication). It might be argued that the large polygenic variation that is evident from the QQ plots for the meta-analysis data argues against a loss of variance explained (Figures S1.1d-6.1d). The deviation of observed *p*-values is not only reflecting true association signals however, but also false positives. Since the DE method corrects observed effects for sampling fluctuation to get 'true' effect sizes, the deviation of observed *p*-values that is evident in the QQ plots will only to some extent be picked up by the DE method.

By means of simulation studies it was tested whether the DE method was dependent on sample size. This was not the case. When the true population was simulated to consist of 30,000 individuals, drawing samples of 3,000 individuals each did not result in overestimated proportions of variance explained by SNPs. The lower DE estimates based on the meta-analysis

results could not be explained either by the DE method being sensitive to the distribution of effect sizes. Simulating data under the assumption that the distribution of SNP effect sizes was exponential, that is small effects for SNPs that are relatively common and large effects for SNPs with low MAF, did not lead to distorted DE-based estimates (R.W., unpublished data). Given the results from this simulation study, the low DE-based estimates for the meta-analysis as well as the lower DE-based estimates for the combined NTR/NESDA dataset compared to the separate datasets, are strongly suggestive of effects of genetic/phenotypic heterogeneity.

Second, the DE estimates based on meta-analysis results may be underestimated if genomic control correction has affected the SNP associations. In most meta-analyses *p*-values are corrected for the genomic control inflation factor (λ_{GC}), and often double corrected (e.g. see Lango Allen et al. (2010) and Speliotes et al. (2010)). First, the study specific *p*-values are corrected for the study-specific genomic inflation factor. Next meta-analysis *p*-values are corrected for the overall genomic inflation factor. Since *p*-values are the direct input for the DE method, any correction of the *p*-values will result in lower DE estimates. For the current study, the DE estimates based on the meta-analysis results are based on *p*-values that were 'uncorrected' for the overall genomic inflation factor correction (by transforming the *p*-values into χ^2 statistics, multiplying the χ^2 statistics by the genomic inflation factor and then calculating new *p*-values from these new χ^2 statistics). Nevertheless, to the extent that the first study-specific genomic control correction has affected the SNP associations, the DE estimates for meta-analysis data will be underestimated.

4.5 Future research

More research is needed to explore the merits and limitations of the GRM and DE methods. In future research it will be interesting to explore whether the performance of the DE method can be further improved when the optimal level of pruning is considered to be a meta parameter whose value needs to be set through cross validation. The basic idea is that the choice of the level of pruning is guided by the prediction error, with the preferred pruning level having the lowest prediction error. For the DE method this may be implemented by pruning the SNP dataset *x* times, each time for a different level of SNP pruning, then drawing *k* random samples for each pruned set *x*, calculating the DE estimate for each of the *k* samples, and calculating the standard error over all *k* samples for each level of pruning. Then, for the level of SNP pruning that is associated with the lowest prediction error (expressed as variability around the mean), the actual amount of variance can be estimated in fresh data (that is, a random draw of the data that was set aside beforehand). The performance of the DE and GRM method can then be further compared to newly developed methods to estimate the amount of variance explained by SNPs, such as those that propose improvements on current methods (e.g. the GRM

method) (Speed et al. 2012), other means to estimate and sum 'true' effect sizes for SNPs in pruned SNP sets (Kutalik et al. 2011) and/or those that derive from other statistical (e.g. Bayesian) backgrounds (Stahl et al. 2012).

In addition, work needs to be done on computing standard errors which are are not automatically obtained for the DE method. In this study an estimate of the variability of the DE estimates was obtained by repeating the DE method on 10 different pruned sets. To the extent that SNPs in different pruned sets reflect the same information because they are in considerable LD, these estimates of variability may have underestimated the true variability around the estimates. Future studies could focus on ways to derive standard errors analytically, numerically or through bootstrapping.

To conclude, about half of the additive genetic variation in liver enzyme levels and alcohol intake can be explained by SNP effects as captured on currently used SNP platforms. Based on polygenic risk scores, the association of alcohol intake with GGT can be traced back to genetic polymorphisms. Given adequate sample sizes, this information may lead to the detection of new susceptibility loci that may elucidate new biological pathways of alcohol use, liver enzyme levels and their association.

SUPPLEMENT TO CHAPTER 8

HERITABILITY OF GGT, ALT AND AST LEVELS ESTIMATED FROM GENOME-WIDE SNP DATA AND ITS GENETIC COVARIANCE WITH ALCOHOL USE

Table S1 Proportions of variance explained (s.e.) by GRM method as implemented in GCTA for different levels of LD and relatedness pruning, for NTR and NESDA combined

Pruning		Phenotypes					
LD SNPs	**relatedness**	**GGT**	**ALT**	**AST**	**ALCOHOL**	**BMI**	**HEIGHT**
none	no cut-off[a]	.149 (.076)	.024 (.073)	.127 (.074)	.149 (.080)	.217 (.078)	.579 (.077)
	cut-off <.025	.138 (.080)	.000 (.077)	.138 (.078)	.105 (.083)	.147 (.082)	.557 (.082)
r^2 <.95	no cut-off	.283 (.114)	.000 (.111)	.049 (.110)	.259 (.121)	.354 (.116)	.719 (.114)
	cut-off <.025	.271 (.120)	.000 (.118)	.025 (.116)	.222 (.127)	.249 (.120)	.710 (.119)

[a] standard errors are lowest for the scenario without LD (among SNPs) nor relatedness (among individuals) pruning

Table S2 Proportions of variance explained [ab] (estimate of variability) by the DE method as proposed by So et al. (2011) for different levels of LD pruning, for NTR and NESDA combined

Pruning		Phenotypes					
LD SNPs	**# SNPs [c]**	**GGT**	**ALT**	**AST**	**ALCOHOL**	**BMI**	**HEIGHT**
r^2 .25	1,426,563	1.121	1.264	1.580	1.201	1.154	1.559
r^2 .10	963,706	.932	1.058	1.088	.991	.882	1.265
r^2 .05	694,659	.721	.832	1.046	.845	.790	.784
r^2 .00	50,037	.195	.111	.161	.164	.139	.242

[a] proportions >1 constitute impossible values

[b] pruning at an threshold of r^2 .00 (using a 100 SNP sliding window, proceeding by 25 SNPs in a step) results in possible values for all phenotypes (that is, proportions ≤1)

[c] number of SNPs in pruned set for given pruning threshold; as a reference, for summary statistics of GWA meta analysis, after pruning (at r^2 .25) ~190,000 SNP associations were left for BMI, height and ~215,000 SNP associations for ALT, GGT.

Table S3 Proportions of variance explained (s.e.) by GRM method as implemented in GCTA per chromosome, for NTR and NESDA combined [estimates significant at α=.01 shown in bold]

	GRM-based estimates per chromosome (chr)																					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
GGT	.036	.000	.016	.006	.005	.017	.007	.000	.013	.029	.000	.029	.020	.016	.000	.000	.000	.000	.000	.023	.000	.013
	(.023)	(.022)	(.020)	(.020)	(.020)	(.019)	(.018)	(.017)	(.018)	(.019)	(.016)	(.019)	(.015)	(.014)	(.013)	(.016)	(.014)	(.015)	(.011)	(.015)	(.011)	(.011)
ALT	.007	.000	.000	.000	.010	.000	.017	.023	.000	.009	.020	.002	.013	.000	.000	.014	.000	.000	.000	.000	.000	.000
	(.021)	(.022)	(.019)	(.019)	(.020)	(.018)	(.019)	(.018)	(.018)	(.017)	(.017)	(.017)	(.016)	(.015)	(.015)	(.016)	(.015)	(.012)	(.011)	(.014)	(.011)	(.010)
AST	.026	.000	.012	.000	.019	.037 [c]	.027	.000	.000	.000	.003	.000	.014	.001	.004	.000	.000	.017	.010	.004	.000	.006
	(.023)	(.023)	(.019)	(.019)	(.020)	(.018)	(.018)	(.016)	(.017)	(.017)	(.012)	(.017)	(.015)	(.014)	(.014)	(.014)	(.015)	(.016)	(.011)	(.013)	(.011)	(.011)
ALC[a]	.002	.012	.000	**.047** [d]	.022	.000	.000	.002	.022	.000	.015	.000	.030	.017	**.044** [d]	.013	.000	.001	.004	.000	.006	.000
	(.023)	(.024)	(.020)	**(.022)**	(.021)	(.018)	(.019)	(.018)	(.019)	(.017)	(.016)	(.017)	(.017)	(.016)	**(.017)**	(.017)	(.014)	(.015)	(.012)	(.015)	(.012)	(.011)
BMI	.008	.035	.000	.000	.008	.021	**.056** [e]	.027	.031	.001	.000	.013	.009	.013	.000	.030	.007	**.035** [e]	.016	.005	.000	.000
	(.022)	(.023)	(.020)	(.020)	(.019)	(.018)	**(.022)**	(.019)	(.018)	(.017)	(.017)	(.017)	(.016)	(.015)	(.015)	(.017)	(.015)	**(.017)**	(.013)	(.013)	(.010)	(.010)
HGT[b]	**.048**	**.070**	.030	.014	.020	**.060**	.022	**.049**	**.040**	**.053**	.015	.026	.000	.018	.022	.031	**.040**	.000	.014	.014	.009	.026
	(.023)	**(.024)**	(.020)	(.020)	(.020)	**(.021)**	(.019)	**(.020)**	**(.019)**	**(.020)**	(.016)	(.018)	(.015)	(.015)	(.015)	(.016)	**(.016)**	(.015)	(.011)	(.014)	(.011)	(.012)

[a] alcohol intake

[b] height

[c] chr6, p=.02.

[d] chr4, p=.01; chr15, p=.002.

[e] chr7, p=.004; chr18, p=.01.

[f] chr1, p=.01; chr2, p<.001; chr6, p<.001; chr8, p=.005; chr9, p=.01; chr10, p=.003; chr17, p=.002.

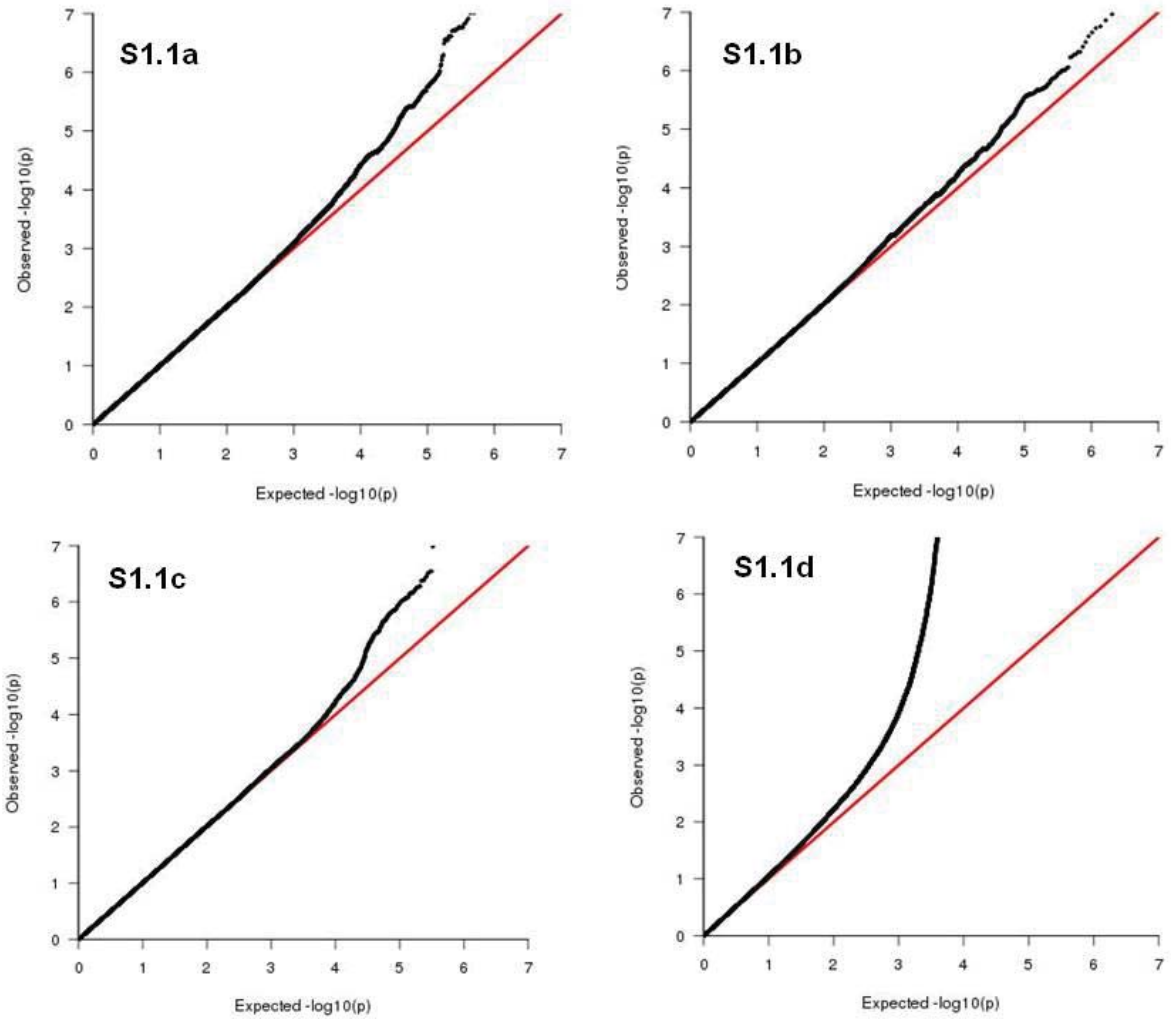


Figure S1.1a-S1.1d QQ plots for GGT

S1.1a: NTR sample; S1.1b: NESDA sample; S1.1c: combined NTR/NESDA sample; S1.1d: meta-analysis sample (Chambers et al. 2011)

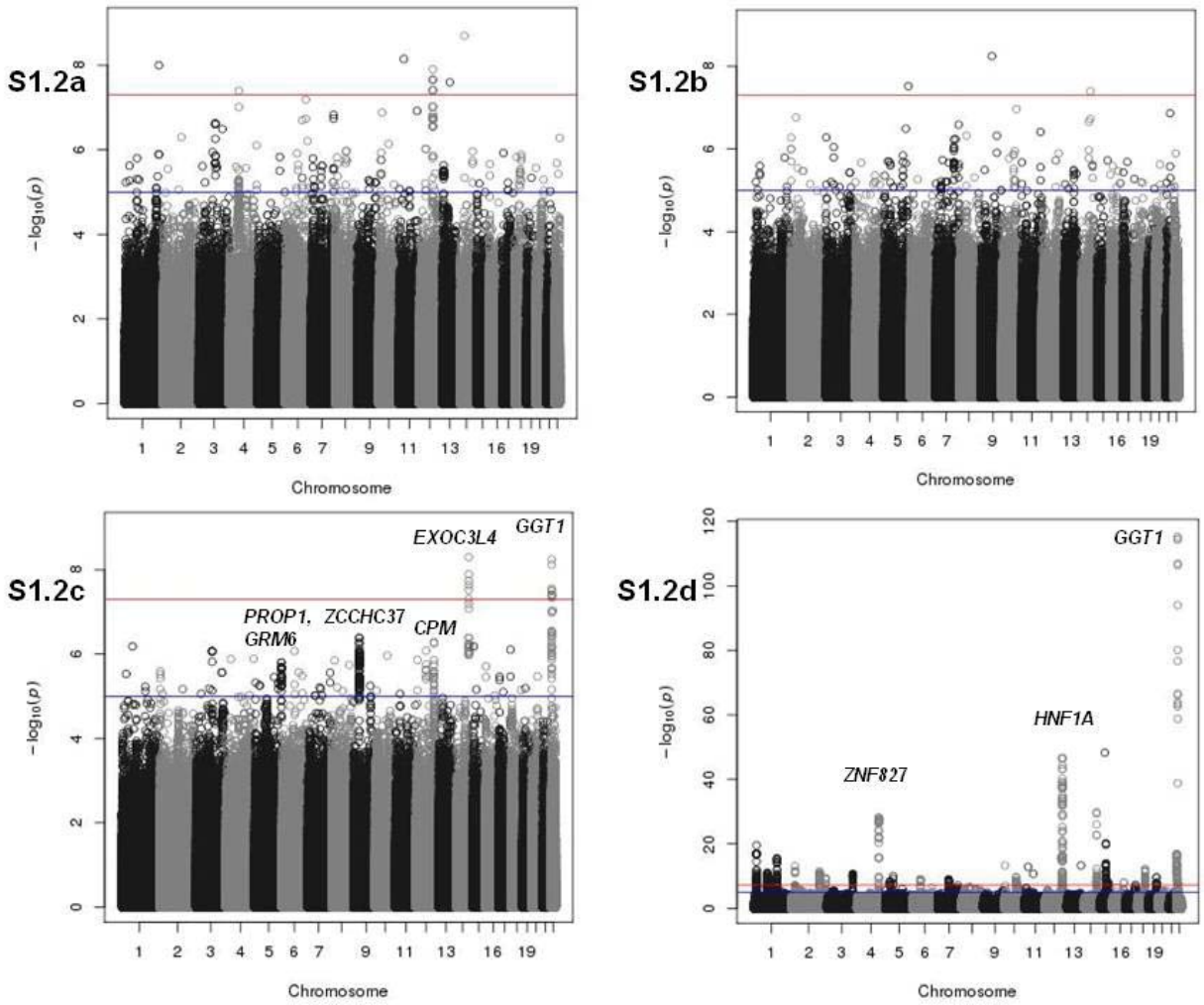


Figure S1.2a-S1.2d Manhattan plots for GGT

S1.2a: NTR sample; S1.2b: NESDA sample; S1.2c: combined NTR/NESDA sample; S1.2d: meta-analysis sample (Chambers et al. 2011)

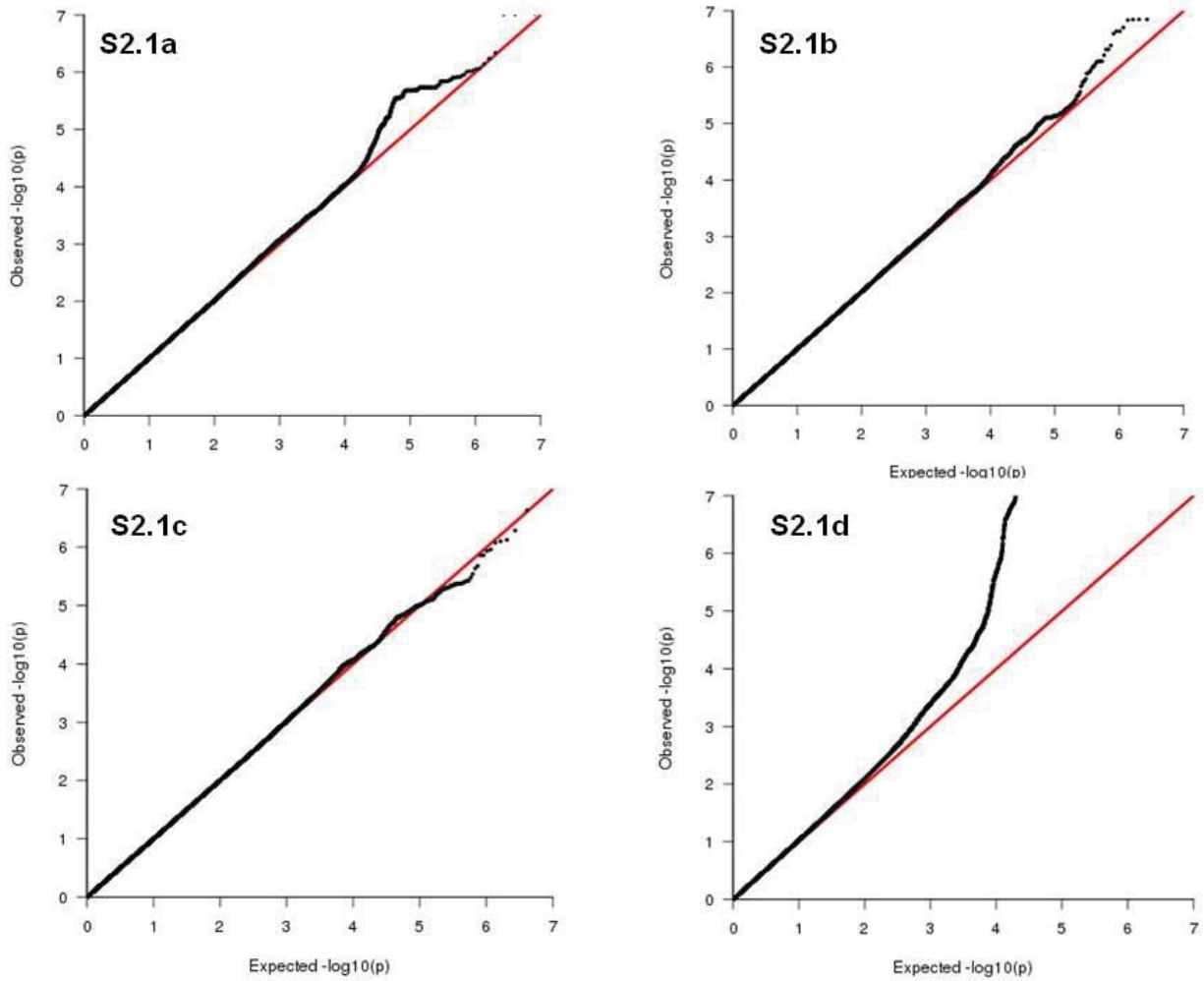


Figure S2.1a-S2.1d QQ plots for ALT

S2.1a: NTR sample; S2.1b: NESDA sample; S2.1c: combined NTR/NESDA sample; S2.1d: meta-analysis sample (Chambers et al. 2011)

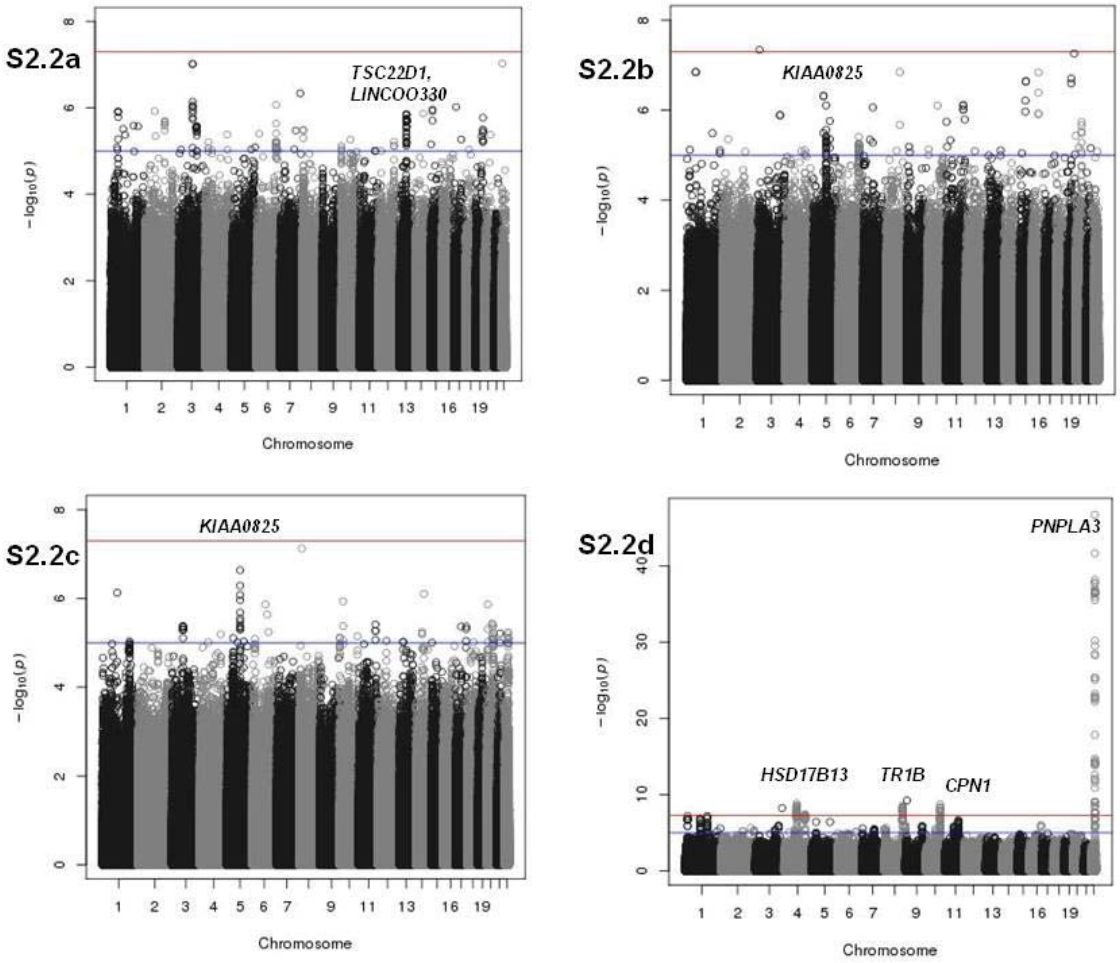


Figure S2.2a-S2.2d Manhattan plots for ALT

S2.2a: NTR sample; S2.2b: NESDA sample; S2.2c: combined NTR/NESDA sample; S2.2d: meta-analysis sample (Chambers et al. 2011)

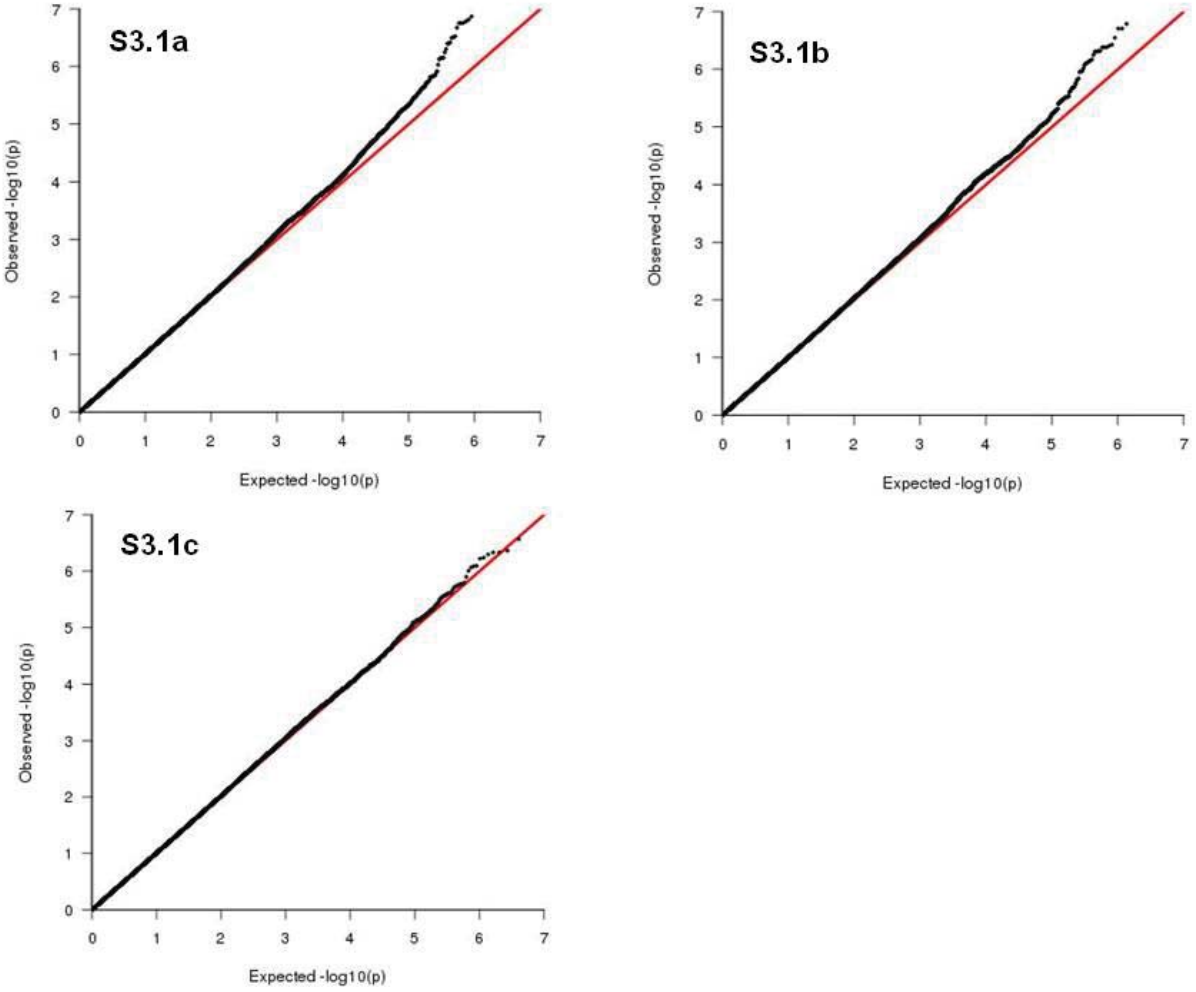


Figure S3.1a-S3.1c QQ plots for AST

S3.1a: NTR sample; S3.1b: NESDA sample; S3.1c: combined NTR/NESDA sample

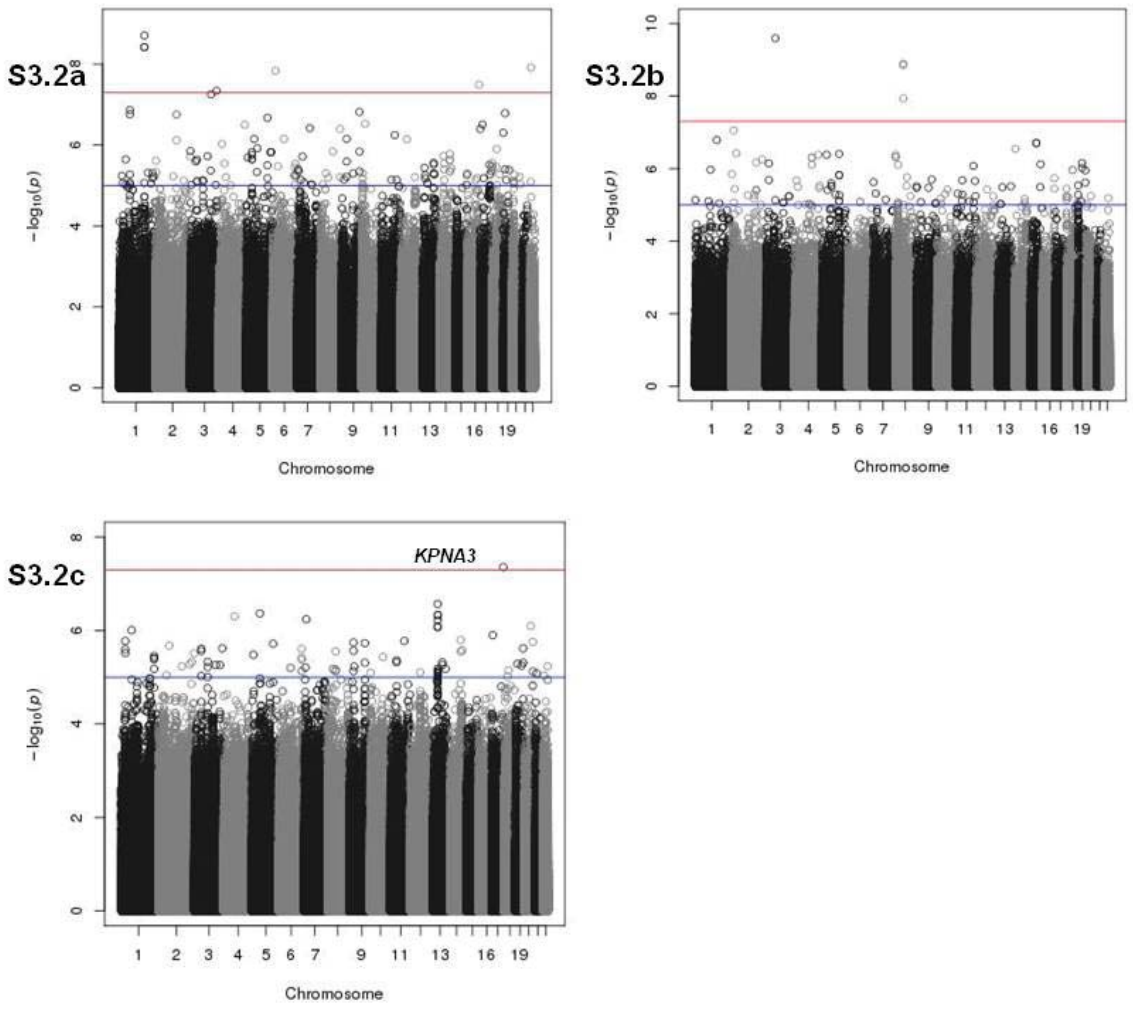
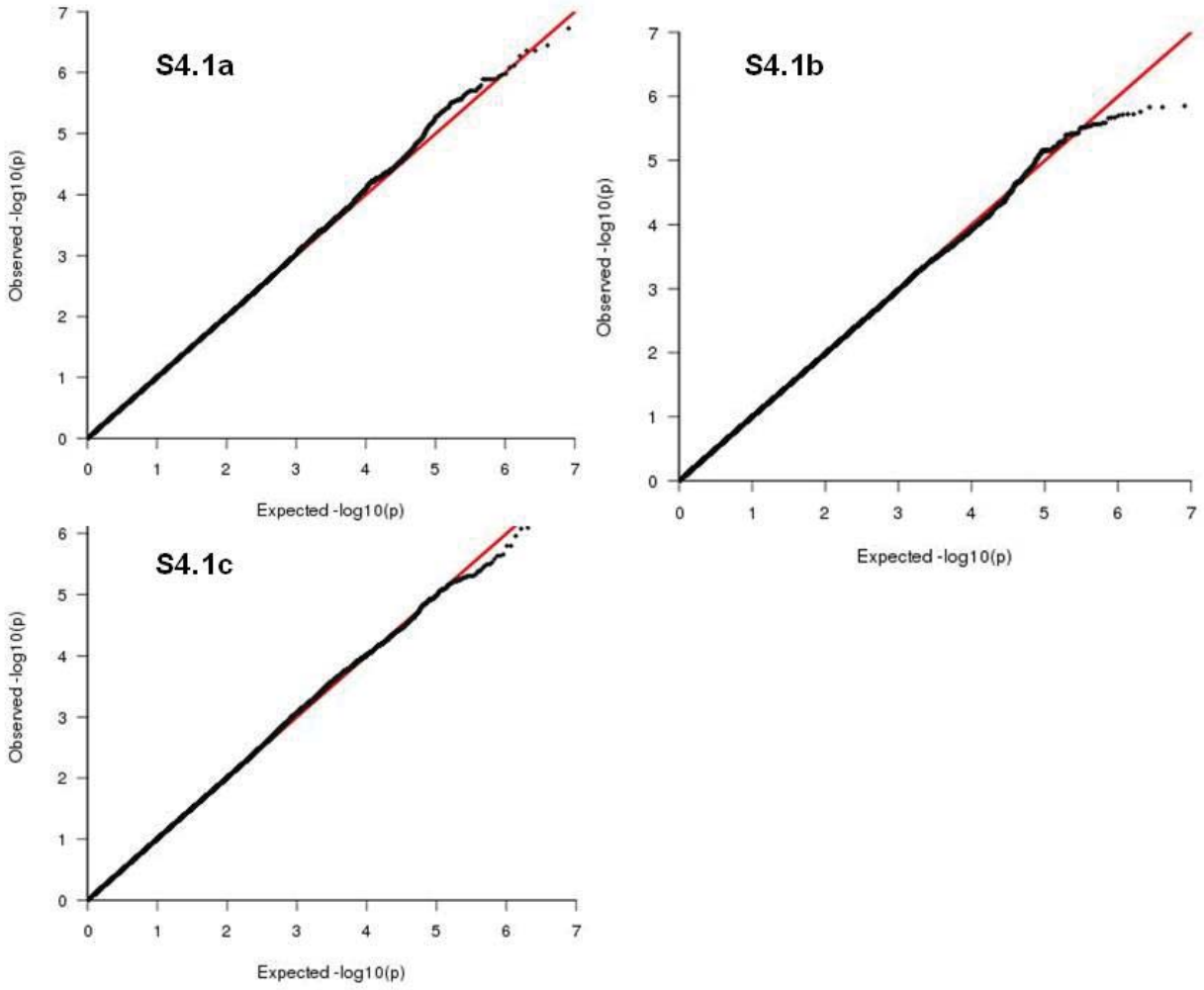


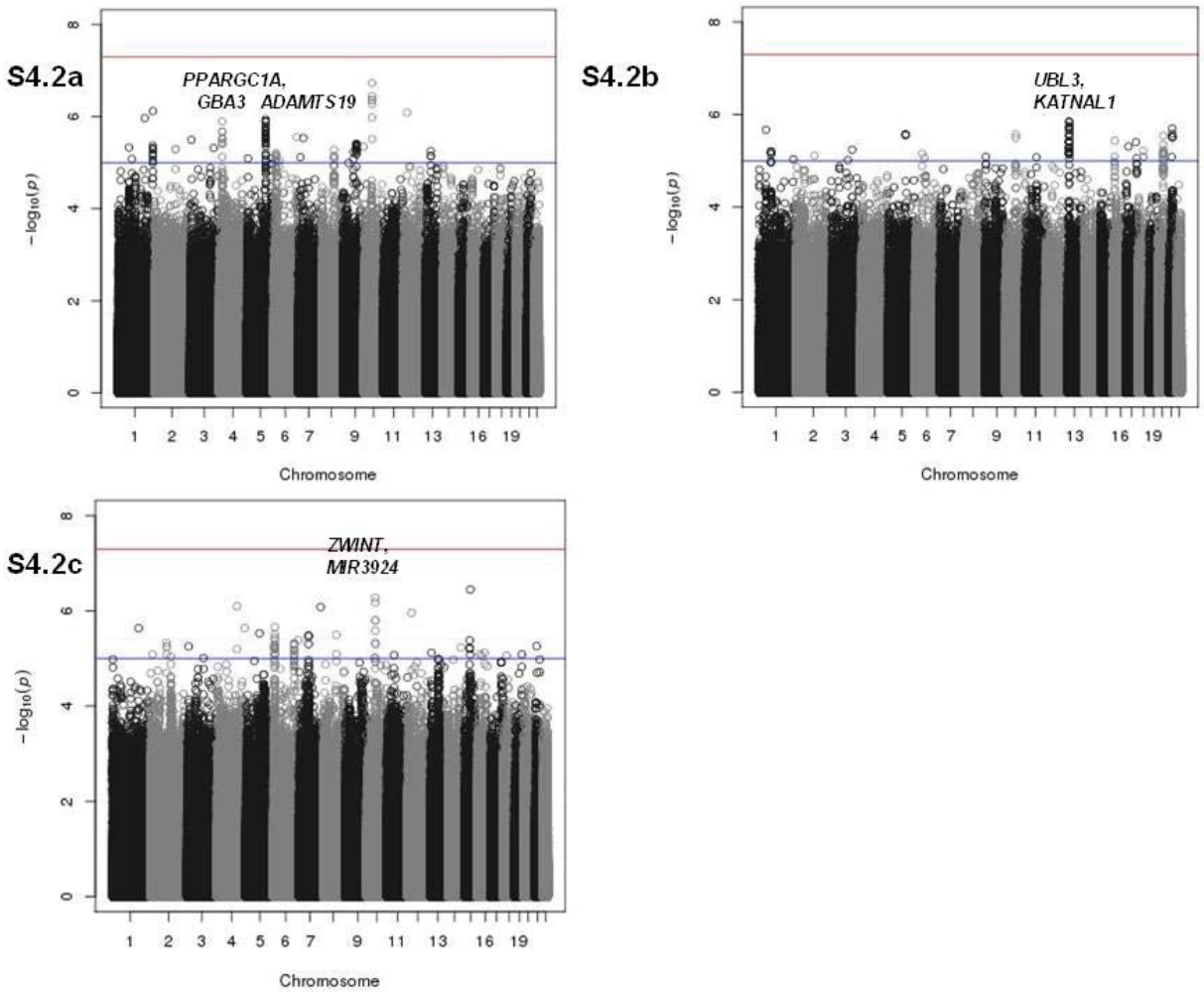
Figure S3.2a-S3.2c Manhattan plots for AST

S3.2a: NTR sample; S3.2b: NESDA sample; S3.2c: combined NTR/NESDA sample



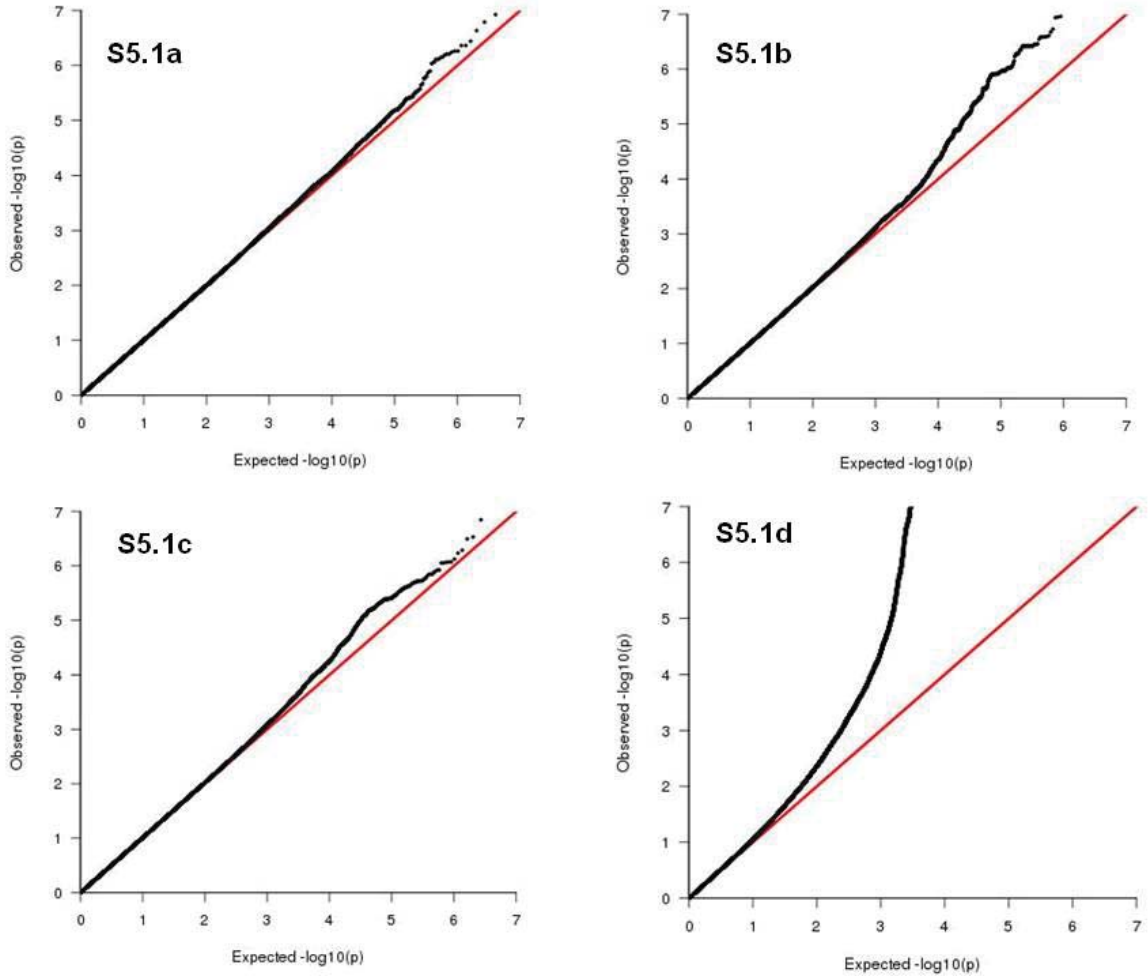
Figures S4.1a-S4.1c QQ plots for alcohol intake

S4.1a: NTR sample; S4.1b: NESDA sample; S4.1c: combined NTR/NESDA sample



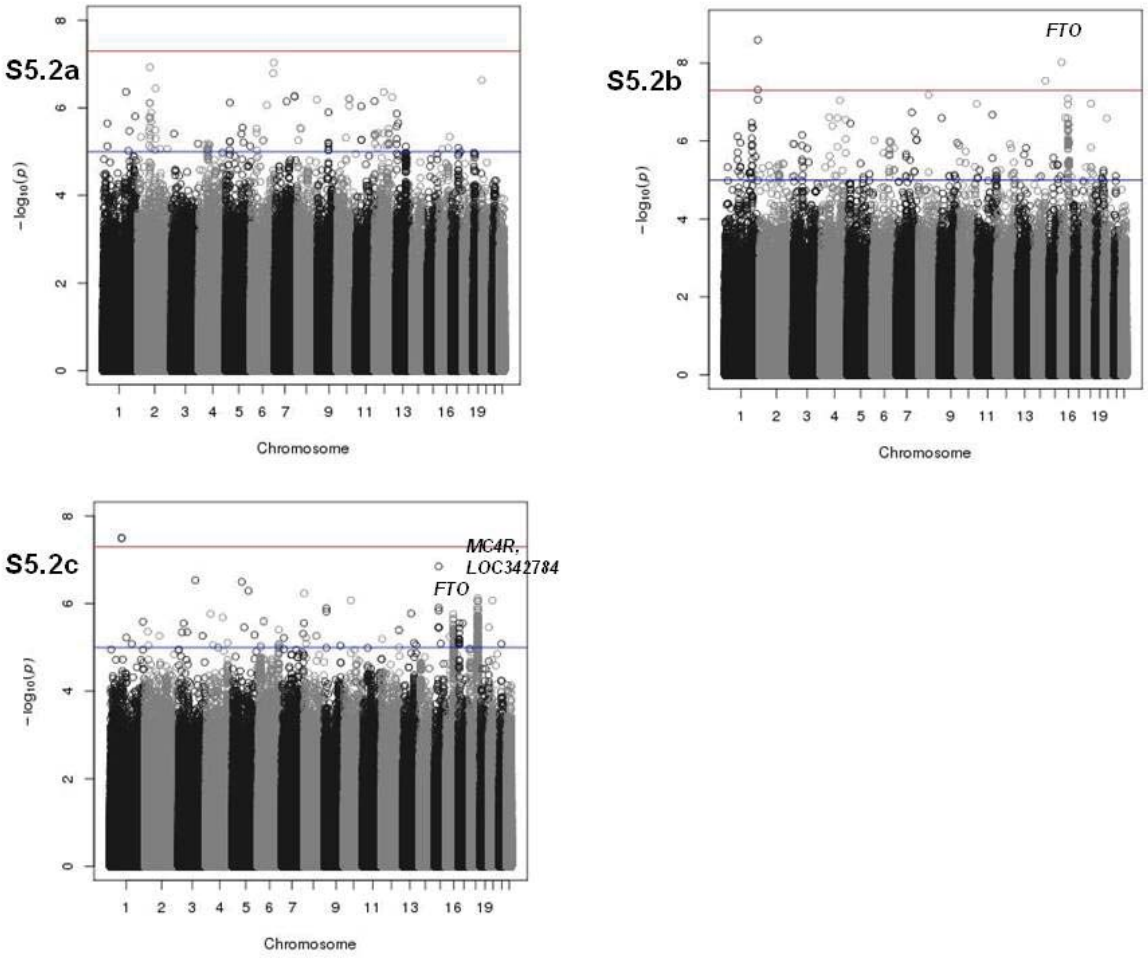
Figures S4.2a-S4.2c: Manhattan plots for alcohol intake

S4.2a: NTR sample; S4.2b: NESDA sample; S4.2c: combined NTR/NESDA sample



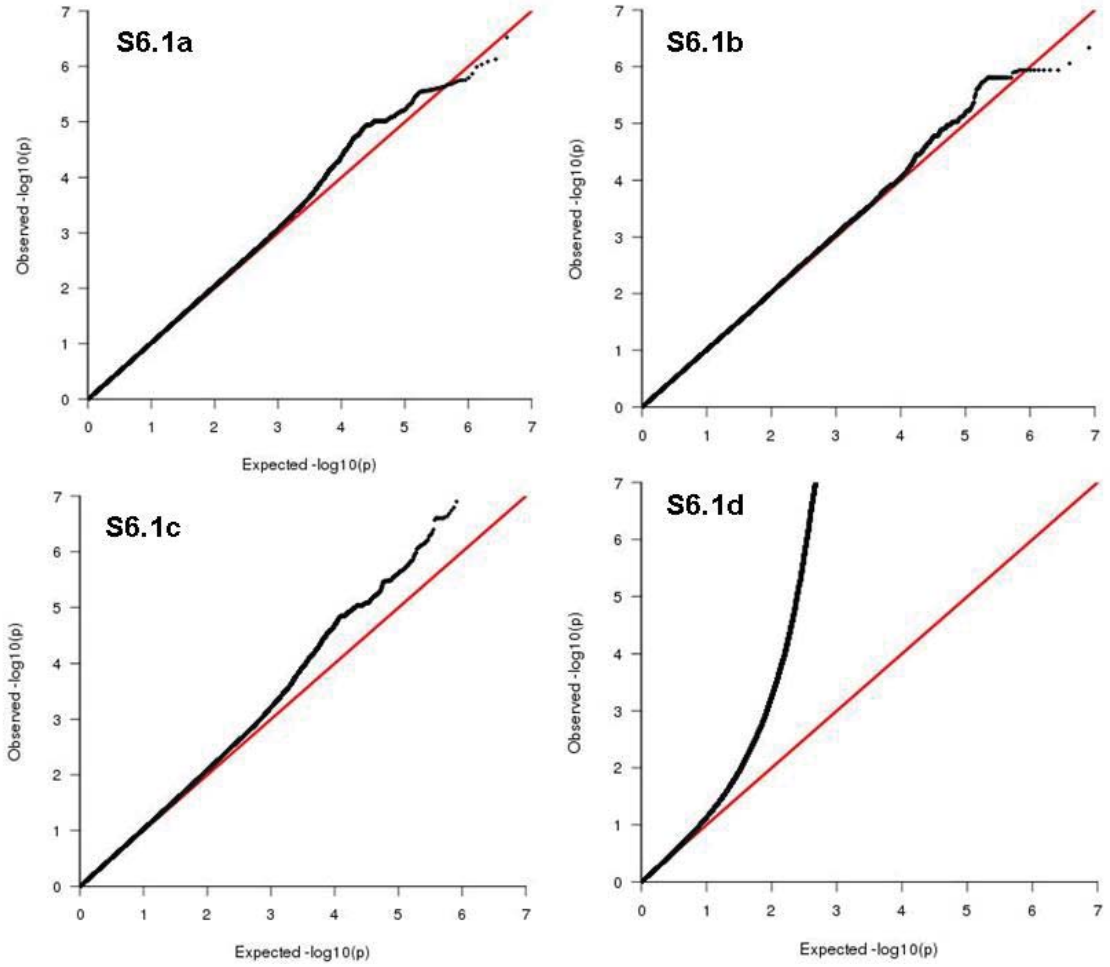
Figures S5.1a-S5.1d QQ plots for BMI

S5.1a: NTR sample; S5.1b: NESDA sample; S5.1c: combined NTR/NESDA sample; S5.1d: meta-analysis sample (Speliotes et al. 2010)



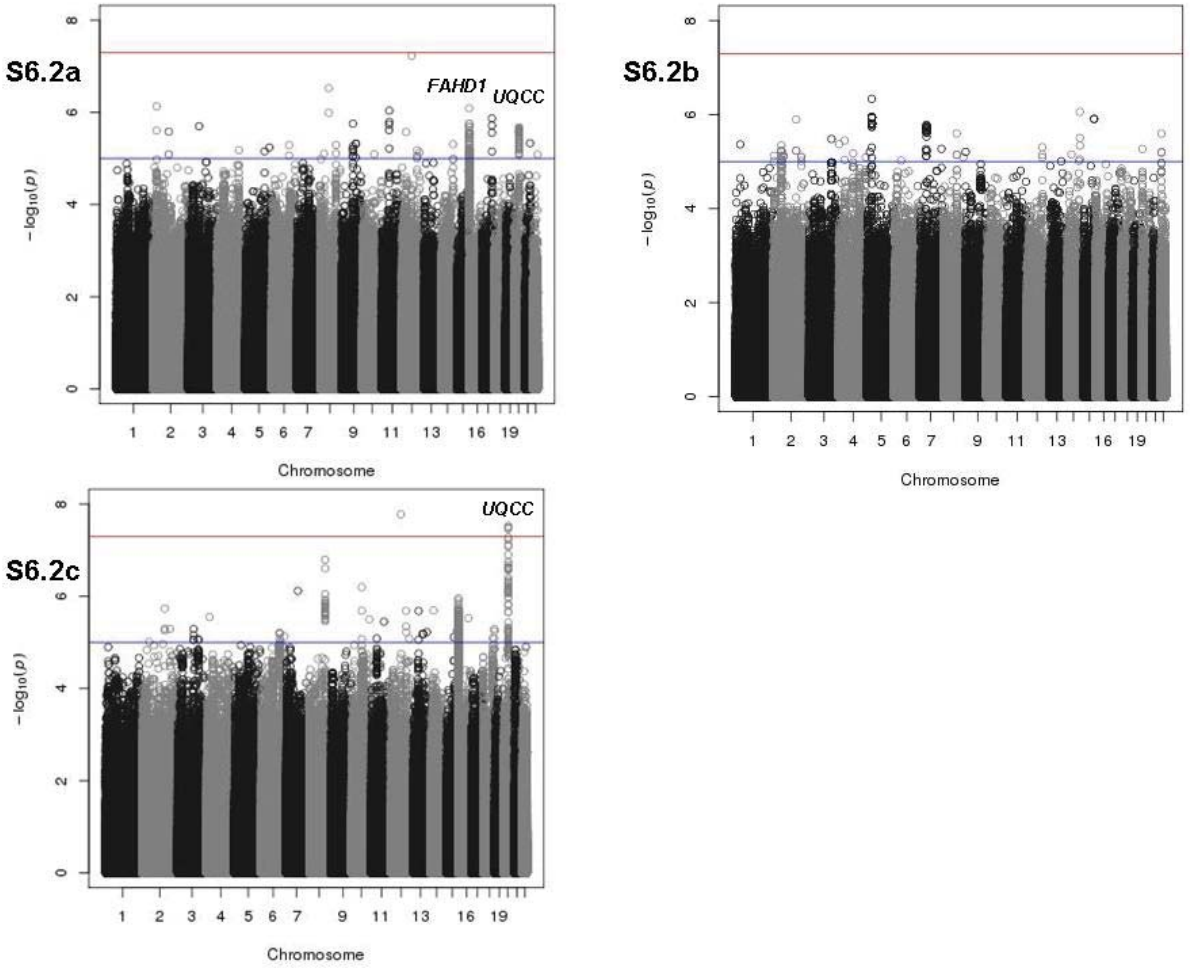
Figures S5.2a-S5.2c Manhattan plots for BMI

S5.2a: NTR sample; S5.2b: NESDA sample; S5.2c: combined NTR/NESDA sample



Figures S6.1a-S6.1d QQ plots for height

S6.1a: NTR sample; S6.1b: NESDA sample; S6.1c: combined NTR/NESDA sample; S6.1d: meta-analysis sample (Lango Allen et al. 2010)



Figures S6.2a-S6.2c Manhattan plots for height

S6.2a: NTR sample; S6.2b: NESDA sample; S6.2c: combined NTR/NESDA sample

Chapter 9

Summary and General Discussion

The studies described in this thesis aimed to unravel the genetic architecture of variation in alcohol use and blood levels of three liver enzymes, gamma-glutamyl transferase, alanine and aspartate aminotransferase (GGT, ALT and AST). As these three liver enzymes are markers of liver injury, and the risk of liver injury increases with heavy drinking, genetic factors underlying the association between alcohol use and liver enzymes received special attention.

1. Summary

Chapter 1 outlined definitions of (problematic) alcohol use, and gave a short literature overview of the relation between alcohol use and liver injury. **Chapter 2** provided an overview of the data that were analyzed in this thesis, describing the measurement of alcohol use and the assessment of liver enzyme levels. This chapter also introduced the statistical methods that were applied to estimate heritability based on twin-family designs and genetic effects from genome-wide marker data. With twin-family designs the heritability of traits is estimated based on the known genetic relatedness among mono- and dizygotic twin pairs, their parents and their siblings. Methods that employ genetic marker data can determine what part of the family-based heritability estimates of traits is attributable to effects of measured/imputed genetic variants (here single nucleotide polymorphisms, SNPs) as well as locate important risk variants associated with alcohol use and/or GGT. The data that are analyzed in this thesis came from adolescent and adult participants of the Netherlands Twin Register (Adult NTR; ANTR) (Boomsma et al. 2002c; Willemsen et al. 2013) and the Netherlands Study of Depression and Anxiety (NESDA) (Penninx et al. 2008).

Using methods that rely on the known genetic relatedness among family members, Chapters 3, 4 and 5 showed that variation in alcohol use and liver enzyme levels is substantially heritable. **Chapter 3** estimated the broad-sense heritability of alcohol intake (grams per day) among adults at 53%, with a considerable proportion of the variation due to genetic influences that act in a non-additive way (30%) (see Table 1). Although men report higher levels of alcohol intake than women, variation in alcohol intake is equally heritable among men and women, with the same genes expressed in both sexes. Genetic influences are also similar across the different ages. Parent-offspring pairs (who show a larger within-pair age difference than twin/sibling pairs), are equally correlated in their alcohol intake levels. Spouses resemble each other in alcohol intake levels (correlation between spouses: 0.39, 95% confidence interval, CI, .34-.44). Cohabitation effects were not significant (at α=.01; p=.011), suggesting that spousal

resemblance mainly comes from phenotypic assortment. That is, individuals choose their spouse (partly) based on (factors related to) his or her alcohol intake level.

In **Chapter 4**, the development of symptoms of alcohol abuse and dependence (AAD) was studied from adolescence into young adulthood, an important time of life in which drinking habits are commonly formed. In this longitudinal study, data of twins were categorized into six age groups of three years each, from 15-17 years until 30-32 years, according to age at assessment of AAD symptoms. The frequency of symptoms of AAD was substantially higher in males than females. Sex differences in the heritability for the risk of AAD symptoms were not detected. The development in risk of having one or more symptoms of AAD for across this age period between 15 and 32 years was explained by one genetic factor, representing a set of genes that increased in importance over age. At ages 15-17 years, genetic effects explained 28% of the variance, and environmental factors that were shared among twins were more prominent. Genetic influences readily increased in importance when subjects became older, whereas the importance of shared environmental influences declined. At ages 21-23, the heritability of risk of AAD symptoms increased to 58% and stayed high at 52-56% at ages 24-32. The increase in heritability over age was not due to genetic innovation but due to an augmentation of genetic influences that were already present in adolescence.

Chapter 5 indicated that variation in liver enzyme levels was moderately heritable. There were quantitative sex differences in the heritability, with heritability sometimes being higher in men and sometimes higher in women (see Table 1). The broad-sense heritability of GGT was 30% in males and 60% in females. For ALT, the broad-sense heritability was 40% and 22% for males and females respectively. For AST, 43% could be explained by genetic influences in both sexes. Non-additive genetic factors played a role for AST (both sexes 15%) and GGT in females (28%), but not for ALT (both sexes) and GGT in males.

Opposite-sex and same-sex pairs resembled each other to the same degree in liver enzyme levels, indicating that the genes underlying variation in liver enzyme levels are similar for men and women. In other words, there is evidence for quantitative, but not for qualitative sex differences. Parent-offspring pairs and sibling pairs, despite larger within-pair age differences for the first group than for the second, resembled each other to the same extent in ALT and AST levels, indicating that the same genes were expressed over age. There were quantitative age differences in the relative importance of genetic and environmental effects on GGT that were due to environmental effects that were shared within the male offspring generation (c^2 28%), but not among parents and offspring. The shared environmental effects on GGT could not be accounted for by seasonal effects or spousal resemblance. These were present for ALT and AST (r_{SPOUSE}=.15), but not for GGT.

Chapter 6 focused on the association of alcohol intake with GGT at the population level and how this association could be explained. I examined whether increased GGT levels co-occur with high levels of alcohol intake, because alcohol intake causally influences GGT levels, or whether both are explained by the same genetic factors that induce a correlation between alcohol intake and GGT at the population level (genetic pleiotropy). Higher levels of alcohol intake predicted higher GGT levels in men (r=.17) and women (r=.09). Genetic effects on alcohol intake were correlated with those on GGT levels (p<.001), whereas the correlation between environmental effects on alcohol intake and GGT was not significant (at α=.01; p=.041). These findings are most consistent with an effect of shared genes. That is, in this healthy population sample the association of alcohol intake with GGT is induced by genes that affect alcohol intake as well as GGT, although a causal effect could not be ruled out. Interestingly, the genetic effects on alcohol intake and GGT mainly acted in non-additive manner, possibly reflecting effects of interacting risk alleles (due to genetic dominance or epistasis). Looking at the specific contribution of these genes, for men 7.6% of the variance in GGT could be explained by non-additive genetic effects that were shared with those for alcohol intake. For women, this was 4.6%.

Chapter 7 described a candidate gene study on the association of SNPs in the ADH gene cluster with measures of alcohol use (alcohol consumption, reactions to alcohol use, symptoms of AAD and age at onset of alcohol use). Significant associations (at α=.007) were found for reactions to alcohol with a SNP in ADH5 (rs6827292) and a SNP just upstream of ADH5 (rs6819724) that was in strong linkage disequilibrium (LD) with rs6827292. Furthermore, an association between age at onset of regular alcohol use and a SNP just upstream of ADH7 (rs2654849) was observed. No significant associations were found for alcohol consumption and symptoms of AAD. SNP associations that were detected in a previous study (Macgregor et al. 2009) were not replicated. Nevertheless, the detection of the three novel SNPs highlights the importance of the ADH gene cluster in explaining variation in alcohol phenotypes.

In **Chapter 8** it was estimated what proportion of the variance of liver enzyme levels could be explained by the joint effect of all SNPs and whether part of this variance was shared with that for alcohol intake. Two relatively novel methods were applied. With the Genetic Relatedness Matrix (GRM) method, the genetic relatedness among pairs of individuals was estimated based on SNP data. This pair-wise genetic relatedness was included as random effect in a linear mixed model, implemented in the software package genome-wide complex trait analysis (GCTA) (Yang et al. 2011a), to estimate the variance attributable to additive SNP effects. The density estimation (DE) method proposed by So et al. (2011) was based on comparing the effect sizes of SNP associations resulting from GWA or GWA meta-analysis studies to the expected distribution of effect sizes under the null hypothesis of no effect. Data came from three

sources: (a) unrelated participants of the NTR, (b) unrelated participants of the NESDA study (Penninx et al. 2008), and (c) a large consortium from which GWA meta-analysis summary statistics on SNP associations for GGT and ALT were available (Chambers et al. 2011). Around 15-17% of the variance in GGT, 2-15% of the variance in ALT and 13% of the variance in AST could be explained by the joint effect of all SNPs, based on the NTR/NESDA data. For alcohol intake, this was 15%, with a significant proportion of the variation explained by chromosomes 4 and 15 (each ~4.5%). These estimates indicate that ~50% of the narrow-sense-heritability of liver enzyme levels and alcohol intake is attributable to effects of measured/ imputed SNPs. Applying the DE method to GWA meta-analysis results for GGT and ALT, resulted in much lower estimates than based on the NTR/NESDA data (GGT 8%; ALT 5%).

Higher levels of alcohol intake correlated significantly with increased GGT in both sexes, whereas associations with ALT and AST were not consistent. Common SNP variation was therefore examined for alcohol intake with GGT only. The GRM and DE method did not detect evidence for common SNP variation. Polygenic risk scores based on GWA meta-analysis results for GGT significantly predicted alcohol intake levels in the NTR/NESDA set, although the amount of explained variance did not exceed 0.25%.

2. General discussion

2.1 Genetic risk for alcohol intake levels

About 50% of individual differences in alcohol intake levels and symptoms of AAD are attributable to genetic effects, as indicated in Chapters 3 and 4. Chapter 8 showed that 15% of the variation in alcohol intake can be ascribed to effects of measured/imputed SNPs, with chromosomes 4 and 15 contributing significantly to this variation (explaining ~4.5% each).

Chromosome 4 and 15 harbor well known candidate genes for alcohol phenotypes that are involved in the two broad pathways by which genes are hypothesized to impact variation in alcohol use (Kendler et al. 2012). Genetic effects specific to alcohol use include those on alcohol metabolism (Hurley and Edenberg 2011) and sensitivity to the response to alcohol (Schuckit 2009; Heath et al. 1999). A second pathway through which genes can have their effect on alcohol use is by personality characteristics such as impulsivity, disinhibition, sensation seeking (Schuckit 2009) and externalizing psychopathology (Kendler et al. 2011a; Krueger 1999), traits that influence risk for substance use in general (Kendler et al. 2012).

Table 1 Estimates on the proportion of variance of alcohol intake and liver enzyme levels explained by additive genetic (A), non-additive genetic (D), shared environmental (C) and non-shared individual-specific (E) environmental effects (with 95% confidence intervals)

			Proportion of phenotypic variance explained by			
		spouse correlation	A	D	C	E
Alcohol intake	both sexes	.39	23.4% (19.1 - 27.5)	29.9% (23.9 - 36.0)		46.7% (43.1 - 50.7)
GGT	males	.00	29.6% (19.8 - 40.2)		28.5% (19.2 - 37.5)	41.9% (35.6 - 49.1)
	females	.00	32.2% (23.4 - 41.4)	27.7% (17.8 - 37.5)		40.2% (35.3 - 45.7)
ALT	males	.15	40.4% (31.9 - 51.8)			59.6% (51.8 - 68.0)
	females	.15	22.4% (16.6 - 28.5)			77.6% (71.6 - 83.7)
AST	both sexes	.15	28.0% (22.6 - 33.5)	14.7% (7.1 - 22.3)		57.3% (51.8 - 63.5)

Chromosome 4 contains the ADH gene cluster involved in alcohol metabolism that include the ADH1B and ADH1C genes that are known to influence the rate of alcohol metabolism (rs1229984 and rs1693482 present two functional variants in these genes) (Hurley and Edenberg 2011). The SNP associations detected for regular alcohol use (rs2654849 just upstream of ADH7) and reactions to alcohol use (rs6827292 in and rs6819724 just upstream of ADH5) in Chapter 7, highlight the importance of the ADH gene cluster for alcohol-related phenotypes. Although SNPs detected in Chapter 7 do not cause changes in gene products, they may affect expression of coding genes as suggested by Hurley and Edenberg (2011). In additional analyses, it was explored whether SNPs in the ADH gene cluster were associated with alcohol intake levels in the GWA analyses in Chapter 8. The three SNPs detected in Chapter 7 were not significantly associated with alcohol intake levels in the NTR/NESDA sample (*p*-values >.6). However, seven other SNPs met the study-wide significance level as calculated in Chapter 7 (rs190421768 in ADH5; rs145110520 in ADH6; rs138542405 in between ADH6 and ADH1A; rs138244919 in ADH1C; rs114714597 in between ADH1C and ADH7; *p*-values <.007). For neither of these SNPs significant associations have been reported before. Replication of these associations is therefore needed before strong conclusions can be drawn.

GABAA receptor coding genes located on chromosome 4 and 15 are implicated in the mesolimbic dopamine reward system that is involved in the development of alcoholism (Enoch 2008) and are suggested to be related to personality characteristics related to addiction. The GABRA2 gene in the GABAA receptor chromosome 4 gene cluster has been associated with externalizing behavior (Dick et al. 2009a) and impulsivity by questionnaire responses and insula activity activation measured in a fMRI study (Villafuerte et al. 2011). The GABRG3 gene in the GABAA receptor chromosome 15 gene cluster is hypothesized to be associated with externalizing behavior and disinhibition (Dick et al. 2006). In the GWA results for the NTR/NESDA sample, however, SNPs in the GABRA2 and GABRG3 genes did not meet the levels of genome-wide nor suggestive significance (*p*-values>$5x10^{-8}$ and >$1x10^{-5}$).

In line with the substantive impact of non-additive genetic effects on alcohol intake levels (30%; see Table 1) as described in Chapter 3, it is interesting to find that non-additive genetic effects have been implicated for traits related to the two general pathways by which genes are hypothesized to impact variation in alcohol use. That is, non-additive genetic effects have been detected for alcohol metabolism (Chen et al. 1999; Kuo et al. 2008a) and for personality traits such as novelty seeking (Keller et al. 2005).

The non-additive genetic effects on alcohol intake described in Chapter 3 may reflect effects of genetic dominance or epistasis. Epistasis represents the effects of interacting risk alleles from different loci, which are assumed to be shared to the same degree for all first-degree relatives. In the presence of additive-by-additive epistasis, all first-degree relatives are

assumed to share 25% of the non-additive genetic variation. In the case of higher-order epistasis, the amount of non-additive genetic variation is less frequently shared among first-degree relatives. Dominant gene action, refers to effects of interacting risk alleles at the same locus and thus requires that individuals share both alleles at a locus. This is assumed to be the case for one quarter of the DZ twin-sibling pairs, but not for parent-offspring pairs, because parents transmit only one of their alleles to their children. MZ pairs are perfectly correlated for all non-additive genetic factors, regardless of whether effects are due to dominance or epistasis (Heath et al. 1984). Based on similar parent-offspring correlations and offspring correlations (Chapter 3), it may be argued that dominant gene action does not likely explain a large part of the non-additive genetic variation. Along the same line, higher-order epistasis can be considered as being less likely, since setting the correlation between non-additive genetic factors for DZ twins and siblings from .25 to .20 or .15 did not improve model fit (Keller et al. 2005). Additive-by-additive genetic epistasis would then be the most likely source of non-additive genetic variation underlying alcohol intake. However, comparing parent-offspring and offspring does not allow to estimate the correlation between the non-additive genetic values among the offspring generation. Without information on the actual genetic variants it is almost impossible to estimate the correlation between non-additive genetic values among the offspring generation that would give an indication of epistasis (Keller and Coventry 2005). Nonetheless, regardless of the precise underlying mechanism the evidence for genetic non-additivity is clear. Gene finding studies for measures of alcohol use may benefit if they would take these substantive non-additive genetic effects into account.

2.2 Genetic risk for increased liver enzyme levels

The genetic influences on liver enzyme levels described in Chapter 5 may reflect genetic effects on factors that influence liver enzyme levels, including alcohol use (see Chapter 6), smoking (Conigrave et al. 2003; Broms et al. 2006; Vink et al. 2004a; Broms et al. 2006; Swan et al. 1996), coffee consumption (Cadden et al. 2007; Laitala et al. 2008; Vink et al. 2009), cardiometabolic risk factors (Loomba et al. 2010; Whitfield et al. 2002; Makkonen et al. 2009; Van Dongen 2013) and inflammation parameters (Neijts et al. 2013).

Genetic pathways underlying variation in liver enzyme levels have been further elucidated by genome-wide association (GWA) studies. For GGT, genome-wide significant associations have been found for loci in or near genes involved in glutathione metabolism (*GSTT2B*, *GGT1*) (Chambers et al. 2011; Middelberg et al. 2012; Yuan et al. 2008; Kamatani et al. 2010), biliary transport (*ATP8B1*) (Chambers et al. 2011), alcohol metabolism (*ALDH2*) (Kamatani et al. 2010), lipid metabolism (*HNF1A*, *CEPT1*), carbohydrate metabolism and insulin signalling (*GCKR*, *MLXIPL*, *SLC2A2*) (Chambers et al. 2011; Middelberg et al. 2012; Yuan et al.

2008), inflammation and immunity (*GCKR*, *STAT4*, *CDH6*, *ITGA1*, *HNF1A*, *RORA*, *CD276* (Chambers et al. 2011; Middelberg et al. 2012), glycoprotein biology (*FUT2*) (Chambers et al. 2011), as well as for genes with unknown or uncertain function (Chambers et al. 2011), including the *C14orf73* gene (Chambers et al. 2011; Middelberg et al. 2012) that is strongly expressed in the liver. Variants in the *PNPLA3* gene, involved in energy utilisation and storage by adipocytes, have been associated with both AST and ALT levels (Kollerits et al. 2010; Yuan et al. 2008; Sookoian and Pirola 2011; Chambers et al. 2011; Kamatani et al. 2010). For AST, in addition a significant variant was detected in a gene implicated in inflammation and immunity (*MRC1*) (Kamatani et al. 2010). For ALT, additional loci have been detected in or near genes involved in glucose and lipid metabolism (*TRIB1*, *CHUK*) (Chambers et al. 2011; Yuan et al. 2008), inflammation and immunity (*CPN1*) (Yuan et al. 2008; Chambers et al. 2011), alcohol metabolism (*ALDH2*) (Kamatani et al. 2010) and the biogenesis of mitochondria (*SAMM50*) (Yuan et al. 2008).

2.3 Association of alcohol intake with liver enzyme levels

Whereas alcohol intake significantly predicted GGT levels (males: r=.17; females=.09; Chapter 6), alcohol intake was not consistently associated with AST and ALT levels (as described in Chapter 8). AST and ALT levels are known to increase with heavy drinking, but not so much with moderate drinking (Arndt et al. 1998; Alatalo et al. 2009b; Liangpunsakul et al. 2010), whereas GGT increases with moderate drinking as well as heavy drinking. Overall, the number of heavy drinkers was small in the NTR/NESDA sample. Among men, 9.6% drank heavily (>21 glasses alcohol/week; >42 grams alcohol/day) and 6.4% of women (>14 glasses alcohol/week; >28 grams alcohol/day). Alcohol intake levels were predictive of AST levels in male NESDA participants and female NTR participants (r=.09-.10), but not in female NESDA participants, nor among male NTR participants. The increased AST levels among male NESDA participants may be explained by the finding that problematic alcohol use was more frequent among male NESDA participants than among female NESDA participants and NTR participants. Problematic alcohol use, as indicated by an AUDIT score ≥8 was observed among 35% of the male participants in the NESDA study, 17% of the female NESDA participants, 11% of the male NTR participants and 4% of the female NTR participants (see Chapter 2). This does not explain the significant correlation between alcohol intake and AST levels among female NTR participants however (r=.09) .

2.4 Genetic overlap for alcohol intake with GGT

Chapter 6 showed that the association of alcohol intake with GGT at the population level was best explained by an effect of genetic pleiotropy. This finding does not necessarily contradict that alcohol use increases GGT levels in experimental settings (Conigrave et al. 2003), but suggests that in relatively healthy population samples, genes that influence alcohol use, also affect variation in liver enzyme levels, resulting in a correlation between alcohol drinking and increased liver enzyme levels at the population level. The genetic effects on alcohol intake and GGT were largely non-additive, possibly reflecting effects of interacting risk alleles due to genetic dominance or epistasis. For females, non-additive genetic influences on alcohol intake and GGT correlated (r_d) .39 (95% CI .06-.47), whereas the correlation between additive genetic effects (r_a) was estimated at .00 (95% CI .00-.15). For males, all non-additive genetic effects were modeled to run via alcohol intake to GGT only (r_d=1), as non-additive genetic effects unique to GGT were not significant among males (described in Chapter 5). Additive genetic effects on alcohol intake and GGT for males correlated .00 (95% CI .00-.12).

Chapter 8 tested whether this common genetic variation could be traced back to measured SNP effects. Assuming an additive model underlying SNP effects on GGT and the association with alcohol intake, the GRM and DE method could not replicate the common genetic variation that was detected in Chapter 6. Based on a different approach, polygenic risk scores based on GWA meta-analysis summary statistics from a large consortium for GGT significantly predicted alcohol intake levels in the NTR/NESDA sample, although the amount of variance explained did not exceed 0.25%. There are several reasons that may explain the null findings of the GRM and DE method with regard to the common genetic variation underlying alcohol intake and GGT.

First, the power of the GRM and DE methods may be too low to pick up the rather low amount of shared genetic variance underlying alcohol intake levels and GGT. The correlation between alcohol intake and GGT was rather low, which is likely due to the fact that respondents were asked to report on their level of alcohol intake during the past year, and not whether they had been drinking during the days before the blood collection. Based on the relation between alcohol use and GGT in our sample, it is reasonable to expect a decrease of ~1.7% in the SNP-based heritability of the covariance of alcohol intake and GGT. If for males, 7.6% of the variance in GGT and for females 4.6% of the variance in GGT is due to genetic effects shared with those for alcohol intake (Chapter 6), then overall, that is for males and females together, 5.6% of the variance in GGT can be ascribed to genetic effects on alcohol intake that are shared with those for GGT (weighting the results for males and females by the 1:2 ratio in number of male versus female participants). This estimate of 5.6% represents the broad-sense heritability. Assuming that the proportion of the broad-sense heritability of the covariance of alcohol intake and GGT

that can be ascribed to SNP effects is equal to the proportion of the broad-sense heritability of alcohol intake and GGT attributable to SNP effects (for alcohol intake 15%/53%; for GGT 15-17%/30% for males; for GGT 15-17%/60% for females), would suggest that ~30% of the covariance could be ascribed to SNP effects. Under this scenario, a decrease of ~1.7% in SNP-based heritability is expected. The GRM and DE estimates are characterized by large confidence intervals however in which a change of 1.7% would easily go undetected (i.e. the estimate of variability for the GRM method was 8%; for the DE method 1.6%; see Chapter 8, Table 5). That is, even when regressing out variance due to alcohol intake truly decreases the heritability of GGT due to SNPs by 1.7%, this change would not be significant. The polygenic risk score approach did not suffer from this problem as was evident from the fact that the 0.25% explained variance was significant.

If the association between alcohol intake and GGT is causal instead of best explained by genetic pleiotropy, then regressing out variance due to alcohol intake does not necessarily result in a lower heritability. Since under a causal model not only genetic effects on alcohol intake and GGT are correlated, but also the environmental effects, regressing out variance due to alcohol use may lower the total variance which may result in a higher heritability (given that heritability is calculated as the proportion of genetic variance divided by the total variance). In contrast, regardless of whether the association between alcohol intake and GGT is best explained by a causal effect or by shared genes the bivariate GRM analysis and the polygenic risk score approach are assumed to work. The reason for this is that under both scenarios the genetic risk factors underlying alcohol intake and GGT are correlated, which will result in a significant genetic correlation in the bivariate GRM analysis and a significant amount of phenotypic variance explained by genetic risk scores based on SNP associations for alcohol use *or* SNP associations for GGT (given adequate power). GRM-based and DE-based point estimates for variance components attributable to SNP effects seemed indeed to increase somewhat (instead of decrease) after regressing out variance due to alcohol intake (Chapter 8, Table 5), but the change of the increase was not significant. Thus in line with Chapter 6, findings on the association of alcohol intake with GGT are most consistent with an effect of shared genes, although a causal effect cannot definitely be ruled out.

Alternatively, the null-findings of the GRM and DE method may have resulted from applying the wrong underlying model of risk. The GRM and DE method assumed that additive SNP effects underlie variation in liver enzyme levels and its association with alcohol intake, whereas Chapter 5 showed that GGT levels in females were influenced by substantial non-additive gene action and Chapter 6 had indicated that that the overlap of alcohol intake with GGT was due to non-additive rather than additive genetic effects. It was therefore tested whether by assuming that dominant SNP effects would underlie the variation in GGT levels and

the association with alcohol intake, the DE method could detect significant common SNP variation. The DE method only needs *p*-values as input and is thus not restricted to fitting an additive model. Regressing out variance due to alcohol intake did not significantly lower the amount of variance in GGT that could be explained by dominant SNP effects. Hence, the null findings are not likely explained by the fact that an additive model was assumed to underlie variation in liver enzyme levels and the association with alcohol intake, whereas the 'true' underlying model of risk would involve genetic dominance. Further research is needed to test whether SNP-based heritability methods that include epistatic effects can significantly detect common SNP variation for the association of alcohol intake with GGT.

Fourth, the non-additive genetic variation underlying the variation of alcohol intake and GGT (Chapter 6) may not reflect effects of genetic dominance or epistasis, but gene by age interaction. In the genetic model fitted in Chapter 6, non-additive genetic variation was modeled to be shared among the offspring generation, not between parents and offspring. Since the within-age difference is smaller for offspring pairs than for parent-offspring pairs, gene by age interaction will show up as effects shared within the offspring generation, that is, as non-additive genetic effects (Eaves et al. 1978). Gene by age interaction for GGT has been demonstrated for genetic loci at the *GGT1* locus on chromosome 22. For one group of SNPs, including one that affects expression of GGT1, GGT2 and GGTLA4 in the human liver, having the minor allele was associated with a decrease in GGT levels in adults, whereas it increased GGT levels in adolescents. Another group of SNPs (located between *GGT1* and *PIWIL3*) affected GGT levels in adolescents, but not in adults. Additional heterogeneity for adults and adolescents was observed for SNPs in the *CELF2* gene that codes for transcription factors that may have trans-acting effects on GGT expression (Middelberg et al. 2012). With regard to the association of alcohol use with GGT, gene by age interaction effects may result from fluctuation in sex hormone levels which are thought to underlie variation in GGT levels, at least for women (Sillanaukee et al. 2000a). GGT levels are higher among postmenopausal women, women who take hormone treatment to increase fertility, and those who use oral contraceptives (Sillanaukee et al. 2000a). Given the link of GGT and alcohol use with immunity and inflammation (Sierksma et al. 2004; Sierksma et al. 2002; Reuter et al. 2009), here the association between age-related fluctuations in estrogen among women and associated differences in immunity is also of interest (Gameiro et al. 2010). It should be noted however that the cross-trait correlations in Chapter 6 were not fully congruent with gene by age interaction. Gene by age interaction effects predict higher offspring cross-trait correlations than parent-offspring cross-trait correlations (Eaves et al. 1978). The finding that DZ/sibling cross-trait correlations were not higher than parent-offspring correlations is thus not consistent with this

interpretation. Further research is necessary to explore possible effects of gene by age interaction in the explanation of the association of alcohol intake with GGT.

2.5 Methodological considerations regarding methods to estimate variance due to SNPs

Chapter 8 aimed to estimate the amount of variance of liver enzyme levels and alcohol intake that could be explained by SNPs. Two relatively novel methods were applied: a GRM method as implemented in GCTA (Yang et al. 2011a) and the DE method as proposed by So et al. (2011). Several methodological considerations, mainly with regard to the DE method, were addressed.

First, DE estimates based on the GWA meta-analysis data were lower than the estimates based on the NTR/NESDA dataset, which was not specific to liver enzyme levels, but also present for BMI which was used as a bench mark trait (not for height, see the discussion below). Given that simulation studies showed that the DE method was not dependent on sample size nor sensitive to violations of the assumption of normally distributed effect sizes (see Chapter 8), possible explanations of the lower meta-analysis-based SNP-based heritability estimates are the correction of *p*-values for the genomic inflation factor in GWA meta-analysis, and heterogeneity between the samples that are included in the meta-analysis. Since effect sizes based on *p*-values are the direct input for the DE method, correcting *p*-values based on the genomic control inflation factor will lower the amount of variance that can be explained by SNPs. The possible impact of heterogeneity not only applies to DE estimates, but also to GRM-based estimates. In the case of genetic heterogeneity, when combining SNP effects, a substantial proportion of the variance may be lost. If for example SNP *x* has an effect in study 1 (*log*(Odds Ratio, OR)=1) but not in study 2 (*log*(OR)=0), the meta-analysis effect size of this SNP (based on inverse variance meta-analysis) is halved (*log*(OR)=0.5), but its explained variance is reduced to one quarter, since explained variance is proportional to *log*(OR) squared. That is, a quarter of the variance due to SNP *x* is lost as compared to the average variance explained weighted by sample size (P.C. Sham, personal communication).

Second, decisions needed to be taken with regard to the level of SNP pruning that was performed (i.e. the removal of one of each pair of SNPs that is in LD above a certain threshold; LD-based SNP pruning) and relatedness pruning (the removal of one of each pair of individuals who are too closely related). For the GRM method, inclusion of related individuals in the GRM-based analysis should be avoided to ensure that heritability estimates do not reflect more than just the variance captured by the measured/imputed SNPs, and do not become confounded with shared environmental effects among individuals. To ensure relatedness and population stratification are maximally reduced, relatedness pruning is suggested by removing individuals whose pairwise genetic relatedness >.025 (Yang et al. 2010) (corresponding to cousins two to

three times removed). In addition, some concern had been expressed about the robustness of the GRM method in the presence of strong LD between SNPs (Speed et al. 2012). In Chapter 8, the precision (i.e. standard errors) of the heritability estimates was therefore examined under different scenarios of LD-based SNP pruning and relatedness pruning. Relatedness pruning (exclusion of individuals with relatedness >.025) and SNP pruning (removing SNPs in extreme high LD, r^2>.95) yielded higher standard errors, probably due to the usage of less information (when pruning out SNPs in high LD) and/or restriction of range (when excluding individuals based on their pairwise relatedness). Analyses were therefore performed without performing SNP or relatedness pruning.

With regard to the DE method, So et al. (2011) recommended LD-based SNP pruning at a level of r^2>.25, based on SNP data sets containing .1-2.6 million SNPs. These numbers of SNPs correspond well to the number of SNPs when datasets are imputed against the Hapmap reference set. In the current study, this recommendation could not be followed. SNP data were analyzed that were imputed against the 1000 Genomes reference set (~8 million SNPs). Applying the recommended level of pruning (r^2>.25) resulted in (impossible) proportions of variance that exceeded 1. For the analyses in Chapter 8, only 'truly' independent SNPs were selected (corresponding to r^2>.00, using a 100 SNP sliding window, taking steps by 25 SNPs at a time).

As a benchmark, the DE method was also applied to GWA meta-analysis results of BMI and height. For BMI, the DE-based estimate on the variance explained by SNPs agreed well with that based on the GRM method (22% versus 17% respectively), but for height, the amount of variance that could be ascribed to SNP effects based on the DE method was much lower (23%) than that based on the GRM method (58%). It may be possible that the optimal level of LD-based SNP pruning is dependent on the specific genetic architecture of the phenotype. That is, in addition to the appropriate level of SNP pruning being dependent on the SNP density of the dataset, it may depend on the genetic architecture of a trait, including how many SNPs have an effect and how many of those SNPs happen to be in high or moderate LD with other SNPs. Future work should address how to determine the optimal level of SNP pruning for different phenotypes.

The performance of the GRM and DE method can be further compared to other newly developed methods to estimate the amount of variance explained by SNPs, such as those that propose improvements on current methods (e.g. on the estimation of the GRM matrix) (Speed et al. 2012), those that use other means to estimate and sum true effect sizes for SNPs in pruned SNP sets (Kutalik et al. 2011) and/or derive from other statistical (e.g. Bayesian) backgrounds (Stahl et al. 2012). In addition, the performance of the DE method to estimate heritability based on GWA meta-analysis data can be compared to the variance that can be explained by the joint

effect of all SNPs by performing a joint GWA analysis of meta-analysis data. With a recently added feature in the GCTA software package (Yang et al. 2012), it is possible to perform a joint GWA analysis on GWA meta-analysis summary statistics by using the LD structure between SNPs as observed in one of the meta-analysis samples. Note that this joint GWA analysis on meta-analysis statistics is equivalent to a multiple regression analysis on the raw genotype and phenotype data. In contrast to the DE method, observed effect sizes are not corrected to their 'true' effect size in this method however. In addition, betas with standard errors (or the *log*(OR) for categorical data) are needed as input, which are obtained from inverse variance meta-analysis (or from single GWA analyses), whereas the DE method can be applied to the GWA meta-analysis *p*-values alone. For GGT and ALT levels *p*-value based meta-analysis was performed weighted by sample size (Chambers et al. 2011) and betas and standard errors were therefore not available.

2.6 Implications of findings

2.6.1 Recovering the missing heritability for alcohol use and liver enzyme levels

Although the heritability of alcohol use and liver enzyme levels is substantial (Table 1), GWA studies have tracked less than 2% of the variance back to effects of SNPs (Chapter 8, Table 4). To examine the so-called missing heritability (Maher 2008) two relatively novel methods were applied to estimate the SNP-based heritability of alcohol intake levels and liver enzyme levels. When focusing on the NTR/NESDA data, around 15-17% of the variance in GGT, 2-15% of the variance in ALT, 13% of the variance in AST and 15% of the variance in alcohol intake could be explained by the joint effect of all SNPs. Given that less than 2% of the variation in liver enzyme levels has been explained by effects of SNPs (see Chapter 8, Table 4), ~12% of the variance in liver enzyme levels and alcohol intake is explained by SNPs having effect sizes that are too small individually to reach the genome-wide significance thresholds typically used to correct for multiple testing (14% minus 2%). With these estimates, around 50% of the narrow-sense heritability would be explained. Thus, currently used SNP platforms contain substantial information on the underlying genetic variation in alcohol intake and liver enzyme levels.

These estimates leave another ~50% open for additional explanations for the missing (narrow-sense) heritability. These include (a) underestimated effect sizes of associated SNPs because of incomplete LD between the measured/imputed SNPs and causal variants, (b) the specific contribution of rare alleles (MAF <.001), as well as structural variation, if poorly tagged by the current available SNPs, and (c) overestimated heritability estimates if epigenetics or gene-environment interactions contribute to the narrow-sense heritability (Stranger et al. 2011; Manolio et al. 2009). Given that non-additive genetic variation underlying alcohol intake and AST and GGT levels was substantial, further research should also focus on the performance of

SNP-based heritability methods to also capture the non-additive genetic effects on trait variation. Whereas the GRM method currently tests for additive SNP effects only, the DE method can be applied to (*p*-values of) (dominant as well as) epistatic SNP effects as well.

2.6.2 Directions for therapeutic interventions

This thesis describes the genetic architecture of variation in alcohol use and blood levels of three liver enzymes, GGT, ALT and AST that are used as marker for liver injury. By understanding more of the biological processes that influence variation in alcohol use and its association with liver enzyme levels, new information might be obtained on pathways in disease causation (Fugger et al. 2012). By following up the research on the shared genetic risk factors for alcohol use and GGT, biological pathways may be implicated in alcohol use, liver enzyme levels and their association, that may lead to the subsequent identification of new targets for treatment of, for example, alcohol-induced fatty liver disease.

With heritabilities explaining up to 50% (Table 1), substantial room is also left for environmental influences, even if part of the non-genetic influences represents noise and measurement error in the data. With regard to risk of developing symptoms of AAD, genetic influences were shown to present stable risk factors, whereas non-shared individual-specific environmental influences were largely age-specific (Chapter 4). This presents interesting information from a clinical perspective. A particular treatment targeting symptoms of AAD may be very effective at one point, but wane with age. Given that genetic risk is stable over age, treatments should focus on providing individuals with the tools to handle this continuing genetic risk.

It should be emphasized that genetic effects explaining differences in a trait on a population level, does not preclude that environmental interventions are effective. A disease that is completely genetic in origin, may well be directed by an environmental intervention (Fugger et al. 2012). The classic example here is phenylketonuria (PKU), an autosomal recessive genetic disorder due to a mutation in the hepatic enzyme phenylalanine hydrooxylase. If untreated, individuals with this condition suffer from mental retardation, seizures and other medical problems. If patients maintain a life-long PHE-restricted diet, they will have a nearly normal development (Poustie et al. 2009). That is, to determine the influence of behavioral interventions for prevention or treatment of problematic alcohol use, experimental studies are needed, preferably in genetically informative samples.

2.7 Future research

2.7.1 Moderation of association between alcohol use and liver enzyme levels

Future research should investigate whether the effect of alcohol use on liver enzyme levels is dependent on the level of drinking and/or other factors that are correlated with liver enzyme levels, such as BMI, smoking, coffee consumption, medication use (Conigrave et al. 2003; Skurtveit and Tverdal 2002), and (sex) hormone levels (Sillanaukee et al. 2000a). As described in Chapter 6, the association of alcohol use with GGT was best explained by genetic pleiotropy. Given that earlier research pointed at a causal effect of alcohol use on GGT for a heavy drinking population (Sung et al. 2011), the mechanism of association of alcohol intake with GGT may be dependent on the level of drinking, that is, there may be gene by alcohol interaction. Complex relationships between alcohol use, liver enzyme levels and other factors such as obesity or smoking are proposed by the so-called two-hit hypothesis, that states that alcohol-related liver injury will develop as a consequence of continued heavy drinking in combination with so-called second hit factors (obesity, smoking) (Mantena et al. 2008). In two studies, the effect of smoking on GGT was found to be dependent on the level of drinking. Among heavy drinkers, smoking increased GGT levels, whereas in the absence of heavy drinking, the effect of smoking on GGT was either not significant (Breitling et al. 2011), or no longer significant when the effects of inflammation parameters were taken into account (Wannamethee and Shaper 2010).

2.7.2 Relation of alcohol use with cardiovascular/metabolic traits

Light to moderate alcohol consumption has been associated with a reduced risk of cardiovascular disease and type 2 diabetes. Protective mechanisms of moderate alcohol include healthy changes in lipid profiles (increased HDL levels), blood clotting factors (lower levels of fibrinogen), inflammation (e.g. lower levels of CRP, IL-6), and/or specific constituents of wine and beer (e.g. polyphenols) (Di Castelnuovo et al. 2010; Brien et al. 2011). Increased insulin sensitivity after moderate alcohol consumption has been proposed as an additional biological mechanism to explain reduced risk for type 2 diabetes (Rehm et al. 2010). For both alcohol use and GGT, genetic risk factors have been linked to levels of cardiovascular/metabolic traits. That is, genetic influences on GGT variation have been associated with cholesterol (LDL, HDL), triglycerides, glucose, and insulin resistance (Chambers et al. 2011; Kim et al. 2011; Whitfield et al. 2002). Genes for alcohol dependence (e.g. the CDH13 gene and DSCAML1 gene) (Morozova et al. 2012) have been associated with triglycerides (Pollin et al. 2008), blood pressure, hypertension (Johnson et al. 2011) and the metabolic syndrome (Fava et al. 2011). Further understanding of the association of alcohol intake with GGT may thus be gained by examining

the relation of alcohol intake, drinking frequency and GGT with cardiovascular/metabolic traits and to explore the role of the immune system in these relations.

Inflammation is a key element in alcoholic liver injury (An et al. 2012). Chronic alcohol consumption can cause leakiness of the digestive tract, which can result in the release of endotoxins in the blood that activate Kupffer cells that reside in the liver. These Kupffer cells activate nuclear factor κB (NF-κB) which stimulate the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) as well as other cytokines with anti-inflammatory properties such as IL-6 and IL-10 that play a role in reducing alcoholic liver injury by induction of STAT 3 (Signal Transducer and Activator of Transcription) (An et al. 2012). Moderate alcohol consumption has been shown to suppress NF-κB activity (Blanco-Colio et al. 2000), resulting in a J-shaped function between alcohol use and inflammation parameters. That is, light to moderate alcohol consumption among non-alcoholic individuals has been associated with lower IL-6 and CRP levels than abstinence or heavy drinking (Volpato et al. 2004; Marques-Vidal et al. 2012; Wannamethee et al. 2007; Imhof et al. 2004; Imhof et al. 2001; Stewart et al. 2002; Albert et al. 2003). Increases in IL-6, IL-1β, IL-8,IL-10, IL-12 and TNF-α have been detected for alcoholics without liver disease (Nicolaou et al. 2004; Laso et al. 2007; Gonzalez-Quintela et al. 2008).

Inflammation and oxidative stress stimulate each other (creating an vicious cycle) via several factors including NF-κB in which the anti-oxidant glutathione also plays a role (e.g. in protecting the cell from endoplasmatic reticulum stress that can be induced by alcohol) (Kulinsky and Kolesnichenko 2009). GGT, involved in keeping intracellular glutathione at adequate levels to protect the cell from oxidative stress (Whitfield 2001) is thus also closely involved in the inflammatory response. A first look at the correlations between alcohol intake and GGT with inflammation parameters, lipids and metabolic traits, collected in the NTR biobank study, showed that alcohol intake correlated .18 with HDL, but not significantly with other lipids or inflammation parameters, which could not be accounted for by a J-shaped relation between alcohol use and inflammation parameters. GGT correlated in between .2-.3 with triglycerides, cholesterol, insulin and CRP, had correlations between .1 and .2 with LDL, glucose, fibrinogen and IL-6, between 0 and .1 with TNF-α and between 0 and -.1 with HDL (taking effects of sex and age into account).

The relation between alcohol use, GGT and cardiovascular/metabolic traits could be further explored by taking specific drinking patterns into account. Cardioprotective effects have only been observed if the pattern of light to moderate drinking did not include heavy drinking episodes (Rehm et al. 2010). The association with GGT is highest for regular heavy drinkers (Conigrave et al. 2003), rendering it important to disentangle the association of alcohol use and GGT for frequent and infrequent drinkers. Furthermore, associations of alcohol intake with GGT

may be different for different kinds of alcohol beverages. Alcoholic beverage preference is moderately heritable (see Appendix), making it important to take genetic effects on the association of alcohol use, GGT and alcoholic beverage preference into account. Moderate drinking levels of wine and beer, but not spirits, have been associated with a lower risk for cardiovascular diseases, whereas increased levels of drinking (beer, wine, spirits) are associated with a higher risk for cardiovascular disease (Costanzo et al. 2011).

2.7.3 The mechanism underlying the disease associations with GGT not explained by alcohol use

Finally, more research can be performed on the specific role of GGT as a marker of disease, that cannot be accounted for by effects of alcohol alone. There are strong associations with type 2 diabetes, cardiovascular disease, chronic kidney disease and cancer, even when alcohol intake is controlled for (Targher 2009).

GGT has both anti-oxidant as well as pro-oxidant properties. Through its role in the metabolism of extracellular glutathione, intracellular glutathione can be formed that is needed to protect the cell against free radicals. GGT may thus be reflective of levels of oxidative stress that are related to risk of disease. However, in the presence of iron or other metals, GGT itself has been suggested to generate free radicals (Lee et al. 2004). Hence, GGT may play a causal role in disease itself as well. The pro-oxidant effects of GGT cannot explain that GGT is already predictive of disease within the normal reference range however, since at low levels of GGT, the pro-oxidant effects of GGT must be low. Neither can all disease associations be explained by GGT being a marker for fatty liver (Lee and Jacobs 2009). ALT levels are more closely related to levels of hepatic fat than GGT (Targher 2009), whereas GGT is related to a wider range of disease than ALT which is mainly associated with liver disease and type 2 diabetes (Fraser et al. 2007; Fraser et al. 2009; Hyeon et al. 2004). GGT has therefore also been proposed to reflect levels of environmental pollutants. Since glutathione plays an important role in the excretion of harmful materials such as heavy metals and given that GGT plays a role in regulating intracellular levels of gluthathione, GGT may be induced through exposure to xenobiotics (Lee and Jacobs 2009).

3. Conclusions

Different indicators of alcohol use, including intake and symptoms of alcohol abuse and dependence, as well as liver enzyme levels are substantially heritable in the Dutch population. These findings come from twin-family studies and from SNP-based heritability estimates that describe the joint effect of genome-wide measured SNPs on alcohol use and liver enzyme levels. The last findings indicate that currently used SNP platforms contain substantive information on the underlying genetic risk alleles for alcohol intake and liver enzyme levels. Further

methodological research needs to focus on SNP-based heritability methods that also capture non-additive genetic effects on trait variation. The association of alcohol intake with one particular liver enzyme, GGT, is on the population level most likely due to effects of shared genes, although a causal effect cannot be ruled out entirely. Future research can further explore the genetic basis of the relation between alcohol use and liver enzyme levels in combination with related factors such as inflammation and cardiometabolic traits. This information may lead to the detection of new biological pathways implicated in alcohol use, liver enzyme levels and their association.

CHAPTER 10

NEDERLANDSE SAMENVATTING: GENETICA VAN ALCOHOLGEBRUIK EN LEVERENZYMEN (DUTCH SUMMARY)

1. Alcoholgebruik en gezondheid

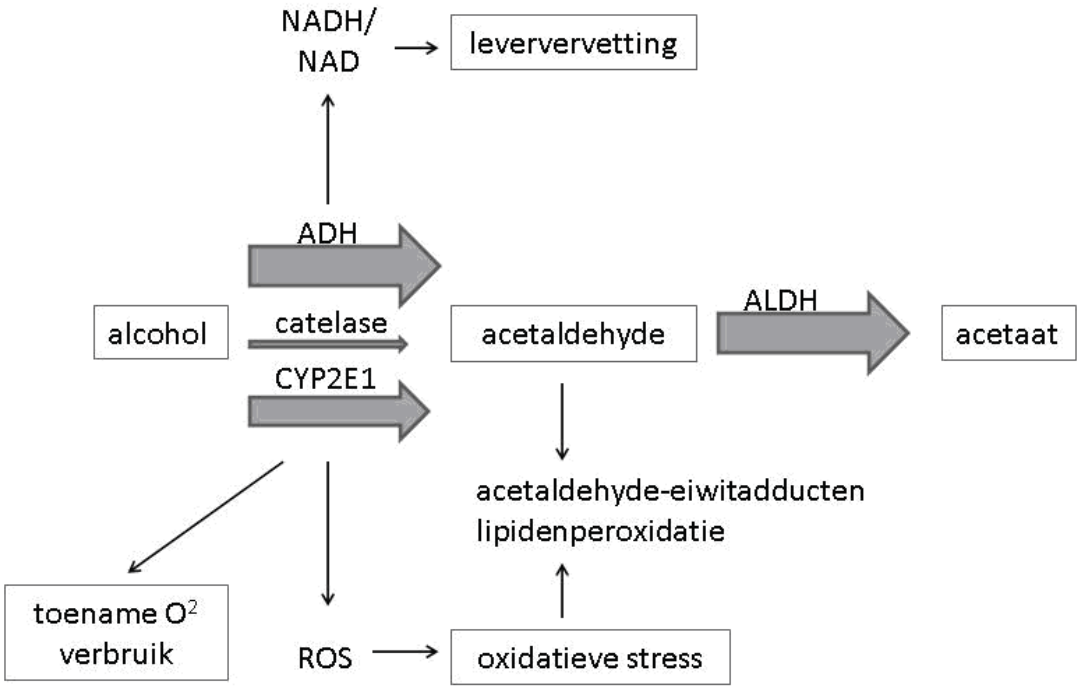
Alcoholgebruik vormt een risico voor de gezondheid. In Westerse landen kan bij mannen 8-12% van de ziektelast ('burden of disease') worden toegeschreven aan effecten van alcoholgebruik (berekend in DALY's, disability-adjusted life years). Bij vrouwen is dit 0,5-3% (Rehm et al. 2003). De meest voorkomende doodsoorzaken waarbij alcohol een rol speelt, zijn kanker, cardiovasculaire stoornissen, levercirrose, diabetes en letselschade (bijv. vanwege rijden onder invloed) (Rehm et al. 2003). Inname van 35-45 gram alcohol per dag (~3 glazen) is al geassocieerd met een verhoogd sterfterisico. Een vergroot risico voor de gezondheid begint bij zo'n 25 gram alcohol per dag (voor aandoeningen als hoge bloeddruk, levercirrose, chronische alvleesklierontsteking, kanker), hoewel het risico op sommige aandoeningen zoals coronaire hartziekte (CHD) en type 2 diabetes, juist lager ligt voor mensen die licht tot matig alcohol gebruiken en het gezondheidsrisico pas bij een veel hogere alcoholinname ontstaat (>60 gram/dag voor type 2 diabetes, beroerte; >89 gram/dag voor CHD; d.w.z. bij >4 en >6 glazen) (Dawson 2011). Naast somatische gezondheidsrisico's, kan problematisch alcoholgebruik leiden tot verslaving, vermindering van arbeidsproductiviteit, agressie, geweld en het in aanraking komen met justitie (NIAAA 2000).

Ondanks de hierboven genoemde (gezondheids)risico's drinkt de meerderheid van de Nederlandse bevolking in meer of mindere mate alcohol. Dit is waarschijnlijk te verklaren vanuit het positieve effect van alcoholgebruik op het reduceren van spanning, het verhogen van de gemoedstoestand en het vergemakkelijken van het sociaal contact (NIAAA 2000). Volgens de Wereldgezondheidsraad (WHO) heeft 82% van de Nederlandse mannen en 64% van de Nederlandse vrouwen van 15 jaar of ouder wel eens alcohol gedronken in het afgelopen jaar. Gegevens van het Nederlands Tweelingen Register (NTR) geven aan dat 9-17% van de mannen en 3-11% van de vrouwen zwaar drinkt (respectievelijk >21 en >14 glazen per week voor personen ≥18 jaar) (Geels et al. 2013). Gezien deze prevalentiecijfers in combinatie met de hierboven genoemde gezondheidsrisico's is kennis van de oorzaken van individuele verschillen in (problematisch) alcoholgebruik belangrijk.

Effecten van alcoholgebruik op de gezondheid kunnen, althans gedeeltelijk, worden toegeschreven aan de effecten van alcoholafbraak in de lever. Alcoholmetabolisme verloopt bij

mensen via verschillende routes, waarvan die via het enzym alcoholdehydrogenase (ADH) het belangrijkst is. De oxidatie van alcohol door middel van ADH resulteert in de vorming van acetaldehyde, een zeer reactief en toxisch bijproduct dat met behulp van het enzym aldehyde dehydrogenase (ALDH) in de mitochondria kan worden omgezet in acetaat (Zakhari 2006).

De afbraak van alcohol kan op verschillende manieren leiden tot leverschade. Enerzijds is het tussentijdse afbraakproduct acetaldehyde toxisch, wat bijvoorbeeld de capaciteit van de mitochondria om acetaldehyde om te zetten in acetaat vermindert, zodat het niveau van acetaldehyde in de mitochondria verhoogt en alcoholgerelateerde weefselschade kan ontstaan (Caballería 2003). Anderzijds is alcoholmetabolisme geassocieerd met metabole veranderingen in de lever zoals een verhoogd zuurstofverbruik, oxidatieve stress en leververvetting die op zichzelf weer kunnen leiden tot leverschade. Figuur 1 geeft een samenvattend overzicht van de metabole veranderingen in de lever als gevolg van alcoholmetabolisme (overgenomen van Callallería, 2003).



Figuur 1: Alcoholmetabolisme en metabole veranderingen in de lever (overgenomen van Caballería, 2003)

Allereerst gaat alcoholoxidatie gepaard met een toename in het zuurstofverbruik (Zakhari 2006). Als levercellen die dicht bij de verse bloedtoevoer zitten teveel zuurstof opnemen voor de afbraak van alcohol, kan dit leiden tot een zuurstoftekort voor levercellen die later op de route

van zuurstof worden voorzien, waardoor deze cellen in een staat van hypoxie kunnen raken en kunnen afsterven (Caballería 2003; Cunningham en Van Horn 2003).

De oxidatie van ethanol en acetaldehyde resulteert in de vorming van reactieve zuurstofdeeltjes (radicalen; de zogenaamde reactive oxidative species, ROS). Deze deeltjes zijn toxisch omdat ze reageren met macromoleculen zoals eiwitten, lipiden en DNA en deze daardoor kunnen beschadigen. Verschillende enzymen en (niet-enzymatische) antioxidanten kunnen de cel beschermen tegen ROS. Wanneer de hoeveelheid ROS-genererende factoren echter groter is dan de hoeveelheid beschermende factoren raakt de cel in een staat van oxidatieve stress en kan leverschade ontstaan (Wu and Cederbaum 2003).

De afbraak van alcohol gaat gepaard met een verandering in de redox (*red*uctie-*ox*idatie) balans in het cytosol en de mitochondria (Zakhari 2006) wat kan leiden tot leververvetting, een overmaat van triglyceriden in de lever, via complexe mechanismen die de synthese en opslag van vetzuren in de lever bevorderen en de oxidatie van vetzuren verminderen (Caballería, 2003). Deze verschillende metabole veranderingen in de lever kunnen vervolgens weer reacties van het immuunsysteem ontlokken die kunnen resulteren in leverschade (Wang et al. 2010).

2. Leverenzymen als markers voor leverschade

Dit proefschrift richt zich op de oorzaken van variatie in alcoholgebruik en in bloedwaarden van drie enzymen die een indicatie geven van schade aan de lever: gamma-glutamyltransferase (GGT), alanine aminotransferase (ALT) en aspartaat aminotransferase (AST). GGT is betrokken bij het instandhouden van een optimaal intracellulair niveau van glutathion, een van de belangrijkste lichaamseigen antioxidanten. GGT is daarmee van invloed op de bescherming van de cel tegen oxidanten die vrijkomen bij de afbraak van ethanol (Whitfield 2001). Bloedwaarden van AST en ALT vormen markers van leverschade die stijgen wanneer het celmembraam beschadigd raakt, waardoor een verhoogde hoeveelheid AST en ALT in de bloedsomloop terechtkomt (Pratt en Kaplan 2000). ALT is aanwezig in verschillende organen en spieren, maar vooral in de lever en is daarom meer specifiek voor leverschade dan AST dat niet alleen wordt gevonden in de lever en skeletspier (Hannuksela et al. 2007), maar ook in het hart en de nieren (Pratt en Kaplan 2000). Bloedwaarden van GGT, ALT en AST zijn sterke voorspellers van leverziekte (Ruhl en Everhart 2009; Kazemi-Shirazi et al. 2007; Hyeon et al. 2004; Lee et al. 2008). Naast leverziekte zijn GGT en ALT ook met andere aandoeningen geassocieerd. GGT en ALT zijn beiden geassocieerd met type 2 diabetes (Fraser et al. 2009) terwijl GGT ook is gerelateerd aan hart- en vaatziekten (Targher 2009; Fraser et al. 2007), kanker en chronische nierziekte (Targher 2009). Om hun rol in deze andere aandoeningen te verklaren zijn GGT en ALT voorgesteld als surrogaat markers van leververvetting (Targher

2009; Schindhelm et al. 2006) en GGT daarnaast als marker van oxidatieve stress (Lee et al. 2004).

3. Alcoholgebruik en de relatie tot leverenzymen

De rol van GGT bij het voorkomen van oxidatieve stress bij alcoholmetabolisme (metabole stress) en de relatie van AST en ALT tot leverschade veronderstelt een positief verband tussen alcoholgebruik en leverenzymwaarden. Matig alcoholgebruik hangt inderdaad samen met verhoogde bloedwaarden voor GGT (Lee et al. 2001; Nakanishi et al. 2000; Higashikawa et al. 2005. Arndt et al. 1998; Liangpunsakul et al. 2010). De relatie tussen matig alcoholgebruik met de aminotransferases (AST en ALT) is echter zwak (Alatalo et al. 2009b; Liangpunsakul et al. 2010) of zelfs afwezig (Lee et al. 2001; Steffensen et al. 1997; Arndt et al. 1998). Bij zwaar alcoholgebruik is er wel een duidelijke verhoging te zien in GGT-bloedwaarden en lijken ook AST en ALT beïnvloed te worden (Conigrave et al. 2002; Arndt et al. 1998; Alatalo et al. 2009b). Ondanks dat bloedwaarden van GGT, ALT en AST oorspronkelijk waren geïntroduceerd als marker in de detectie van zwaar drinken (Whitfield 2001) is hun waarde hierin beperkt. De reden hiertoe is dat een toename van leverenzymen niet specifiek is voor zwaar drinken, en omgekeerd, dat niet bij iedere zware drinker de leverenzymwaarden zijn verhoogd (Peterson 2004).

Samenvattend lijkt GGT geassocieerd te zijn met zowel matig als zwaar alcoholgebruik en heeft zwaar alcoholgebruik ook een invloed op AST en ALT. Verschillende mechanismen zouden deze samenhang kunnen verklaren. Gezien de relatie van AST en ALT met leverschade en de rol van GGT bij het beschermen van de cel tegen metabole stress, zou er sprake kunnen zijn van een causaal effect van alcoholgebruik op leverenzymwaarden. Ook is het mogelijk dat de samenhang tussen alcoholgebruik en leverenzymwaarden kan worden verklaard door een set genen die zowel een effect uitoefent op alcoholgebruik als op leverenzymwaarden.

4. Onderzoeksvragen

In dit proefschrift wordt een aantal vragen onderzocht die volgen uit de hierboven geschetste relatie tussen alcoholgebruik en leverenzymen. Eerst worden de oorzaken in variatie in alcoholinname onderzocht. Vervolgens kijk ik naar de oorzaken van variatie in symptomen van alcoholmisbruik. Daarna wordt de genetica van leverenzymen belicht, en wordt de relatie tussen alcoholgebruik en de relatie met GGT-bloedwaarden gemodelleerd als een functie van pleiotrope genetische effecten die deze samenhang zouden kunnen verklaren. De bovenstaande vragen worden beantwoord door gebruik te maken van statistische technieken uit de genetische epidemiologie. De laatste twee hoofdstukken onderzoeken het belang van direct gemeten genetische varianten.

5. Onderzoeksmethodologie

Hoofdstuk 2 geeft een overzicht van de gegevens over alcoholgebruik en leverenzymwaarden die in dit proefschrift zijn geanalyseerd. Gegevens over alcoholgebruik en leverenzymwaarden die binnen dit proefschrift werden geanalyseerd zijn afkomstig van volwassen deelnemers van het Nederlands Tweelingen Register (Adult NTR; ANTR) (Boomsma et al. 2002c; Willemsen et al. 2013) en de Nederlandse Studie naar Depressie en Angst (NESDA) (Penninx et al. 2008). Dit hoofdstuk introduceert ook de statistische methoden die werden toegepast om de erfelijkheid van alcoholgebruik en leverenzymwaarden te bepalen op basis van tweelingfamilie- en genetische markerdata. Met het tweelingfamiliedesign is de erfelijkheid van eigenschappen geschat op basis van de genetische verwantschap tussen een-eiige tweelingen, twee-eiige tweelingen en hun familieleden (die verschilt tussen één-eiige tweelingen en twee-eiige tweelingen, hun ouders en hun broers en zussen). Vervolgens is met genetische markerdata (hier single nucleotide polymorphisms, SNPs) geschat welk deel van deze erfelijkheidsschatting zoals op basis van het tweelingfamiliedesign is bepaald, kan worden toegeschreven aan de effecten van gemeten en geïmputeerde genetische varianten. Daarnaast is met genetische markerdata (SNPs) in een genetische associatiestudie getracht specifieke risicovarianten voor alcoholgebruik te lokaliseren.

6. Genetische invloeden op alcoholgebruik en leverenzymwaarden op basis van tweeling(familie)studies

In **Hoofdstuk 3** is onderzocht in welke mate genetische en omgevingsinvloeden individuele verschillen in alcoholgebruik onder volwassenen verklaren. De erfelijkheid van alcoholgebruik (uitgedrukt in aantal grammen alcohol per dag) wordt geschat op 53%, waarbij een aanzienlijk deel van de verschillen in alcoholgebruik wordt verklaard door nonadditieve genetische invloeden (30%) (zie tabel 1). Nonadditieve genetische invloeden beschrijven effecten die niet simpelweg bestaan uit een optelsom van elk van de afzonderlijke effecten van de genen, maar uit effecten van genen die onderling interacteren.

Naast effecten van genetische transmissie, werden effecten van culturele transmissie onderzocht van ouder op kind. Hierbij wordt gekeken of na controle voor de genetische samenhang tussen ouders en kinderen, het drinkgedrag van de ouders nog steeds van invloed is op het drinkgedrag van de kinderen, bijvoorbeeld via social modelling. Uit een eerdere studie was bekend dat effecten van culturele transmissie van alcoholgebruik aanwezig zijn in de adolescentie (Koopmans and Boomsma 1996). Uit de huidige studie blijkt dat effecten van culturele transmissie bij alcoholgebruik onder volwassenen geen rol (meer) spelen.

Hoewel mannen meer alcohol drinken dan vrouwen, is de relatieve invloed van genetische effecten op alcoholgebruik voor mannen en vrouwen gelijk. Deze relatieve invloed

van genen is ook vergelijkbaar over verschillende leeftijdsgroepen. Ouder-kindparen lijken even sterk in hun alcoholgebruik op elkaar als tweeling-/broer- en/of zusparen (hoewel de eerste gekenmerkt worden door een groter leeftijdverschil dan de laatste). Ook partners lijken op elkaar in hun niveau van alcoholgebruik (correlatie tussen echtgenoten: 0.39, 95% betrouwbaarheidsinterval, CI, 0,34-0,44). Deze gelijkenis is echter niet gecorreleerd met de duur van hun relatie (bij α=0,01; p=0,011), wat suggereert dat de gelijkenis tussen partners vooral komt door een fenomeen wat fenotypische selectie ('phenotypic assortment') wordt genoemd. Hierbij kiezen mensen hun partner uit (mede) op basis van (factoren die verband houden met) zijn of haar fenotype, zoals alcoholgebruik, of een eigenschap die samenhangt met alcoholgebruik.

Hoofdstuk 4 beschrijft een studie naar de ontwikkeling van de symptomen van alcoholmisbruik en afhankelijkheid (AAD) gedurende de adolescentie en jongvolwassenheid, een belangrijke periode voor de ontwikkeling van alcoholgebruik, en hoe deze ontwikkeling kan worden verklaard. Als maat voor de frequentie van symptomen van AAD zijn vragenlijstgegevens van de CAGE (Ewing 1984) geanalyseerd. De CAGE, een acroniem, bestaat uit vier vragen die informeren naar vier symptomen van AAD: het gevoel te moeten stoppen met drinken (**C**utdown), lastiggevallen worden met kritiek op het drinkgedrag (**A**nnoyed), zich slecht of schuldig voelen over het drinkgedrag (**G**uilty), en 's ochtends drinken tegen de zenuwen of kater (**E**ye Opener). De CAGE-data zijn verzameld bij tweelingen die toen tussen de 15-32 jaar oud waren en zijn geanalyseerd in zes leeftijdscategorieën (van elk drie jaar, van 15-17 tot 30-32 jaar, naar de leeftijd waarop de tweelingen de vragenlijst hadden ingevuld). Hierbij is onderzocht wat de relatieve bijdrage is van genetische en omgevingseffecten op de ontwikkeling van symptomen van AAD in de adolescentie en jonge volwassenheid. Daarnaast is onderzocht of deze ontwikkeling kan worden verklaard door één set van genen of dat er nieuwe genen tot expressie komen gedurende de adolescentie en jongvolwassenheid (genetische innovatie), bijvoorbeeld rond de periode waarin adolescenten gewoonlijk het ouderlijk huis verlaten.

AAD-symptomen worden aanzienlijk vaker door mannen dan vrouwen gerapporteerd, maar er zijn geen sekseverschillen in de erfelijkheid. De ontwikkeling in het risico op het hebben van een of meer AAD symptomen gedurende de adolescentie en jongvolwassenheid wordt verklaard door één set genen, waarvan de invloed groter wordt naarmate personen ouder worden. Er is dus geen sprake van genetische innovatie, maar van een vergroting van genetische invloeden die reeds aanwezig zijn in de adolescentie.

Op de leeftijd van 15-17 jaar zijn omgevingsinvloeden die worden gedeeld tussen tweelingen belangrijker dan genetische effecten die 28% van de variantie verklaren. Naarmate individuen ouder worden, nemen genetische invloeden in belang toe terwijl de relatieve invloed van gedeelde omgevingseffecten daalt. Op 21-23 jarige leeftijd, wordt de invloed van genetische

effecten op het risico op het hebben van een of meer AAD-symptomen geschat op 58%. In de jaren daarna blijft de invloed van genen hoog (52-56% gedurende de leeftijd van 24-32 jaar).

Hoofdstuk 5 beschrijft in hoeverre individuele verschillen in bloedwaarden van de leverenzymen GGT, ALT en AST aan genetische effecten kunnen worden toegeschreven. Er worden kwantitatieve sekseverschillen gevonden in de erfelijkheid van GGT en ALT (maar niet van AST), waarbij de erfelijkheid van ALT hoger is bij mannen en die van GGT hoger is bij vrouwen (zie tabel 1). De erfelijkheidsschatting (gebaseerd op additieve + nonadditieve genetische effecten) voor GGT is 30% voor mannen en 60% voor vrouwen. Voor ALT is de erfelijkheidschatting 40% en 22% voor mannen en vrouwen respectievelijk. Wat betreft AST kan 43% van de variantie worden verklaard door genetische invloeden (voor zowel mannen als vrouwen). Niet-additieve genetische effecten speleen een rol bij AST (in zowel mannen en vrouwen 15%) en bij GGT in vrouwen (28%), maar niet bij ALT (beide geslachten) en bij GGT in mannen.

De correlatie tussen leverenzymwaarden van familieleden van gelijke sekse is even sterk als die tussen leverenzymwaarden van familieleden van ongelijk geslacht, wat aangeeft dat er geen kwalitatieve sekseverschillen zijn in de genetica van leverenzymen. De correlatie tussen ALT- en AST-niveaus van ouder- en kindparen en broer-/zusparen is even groot, wat aangeeft dat voor deze leverenzymen, de genen die bijvoorbeeld op 20-jarige leeftijd van invloed zijn op ALT- en AST-niveaus grotendeels dezelfde zijn als die op 50-jarige leeftijd. Voor GGT waren er kwantitatieve leeftijdsverschillen in het relatieve belang van genetische en omgevingseffecten. Dit was het gevolg van omgevingsinvloeden op GGT die gedeeld werden tussen (tweeling)broers (c^2 28%), maar niet tussen ouders en hun kinderen. Deze gezamenlijke omgevingseffecten konden niet worden verklaard vanuit bijvoorbeeld seizoensinvloeden die weliswaar aanwezig waren voor ALT en AST maar niet voor GGT.

7. Associatie tussen alcoholgebruik en leverenzymwaarden

Een toename in alcoholgebruik is voorspellend voor een toename in GGT (mannen: r=0,17; vrouwen: r=0,09; **Hoofdstuk 6**). Alcoholgebruik was niet significant geassocieerd met ALT. De associatie tussen alcoholgebruik en AST was inconsistent. Bij NTR-deelnemers was alcoholgebruik geassocieerd met AST bij vrouwen, maar niet bij mannen, terwijl bij NESDA-deelnemers het verband andersom was: alcoholgebruik was geassocieerd met AST-niveaus bij mannen, maar niet bij vrouwen (**Hoofdstuk 8**). Dit is waarschijnlijk te verklaren vanuit het feit dat het NTR/NESDA-sample relatief gezond is qua alcoholgebruik. Onder de mannen kan 9,6% gecategoriseerd worden als zware drinker (>21 glazen alcohol/week;> 42 gram alcohol/dag). Onder de vrouwen is dit 6,4% (>14 glazen alcohol/week;> 28 gram alcohol/dag).

Tabel 1 Percentage van de variantie in alcoholinname en leverenzymwaarden die kan worden toegeschreven aan genetische effecten: additief (A) of nonadditief (genetische dominantie) (D) en omgevingseffecten: gedeeld tussen tweelingen/broers/zussen (C) en/of individu-specifiek (E) (met 95% betrouwbaarheidsintervallen)

			Proportie van fenotypische variantie verklaard door			
		Correlatie tussen partners[a]	A	D	C	E
Alcohol-inname	mannen+ vrouwen	,39	23,4% (19,1 – 27,5)	29.9% (23,9 – 36,0)		46,7% (43,1 – 50,7)
GGT	mannen	,00	29,6% (19,8 – 40,2)		28,5% (19,2 – 37,5)	41,9% (35,6 – 49,1)
	vrouwen	,00	32.2% (23,4 – 41,4)	27,7% (17,8 – 37,5)		40,2% (35,3 – 45,7)
ALT	mannen	,15	40,4% (31,9 – 51,8)			59,6% (51,8 – 68,0)
	vrouwen	,15	22,4% (16,6 – 28,5)			77,6% (71,6 – 83,7)
AST	mannen+ vrouwen	,15	28,0% (22,6 – 33,5)	14,7% (7,1 – 22,3)		57,3% (51,8 – 63,5)

[a] gelijkenis tussen partners in alcoholinname of leverenzymwaarden

Dit bevestigt de bevinding uit eerdere studies dat AST en ALT-waarden verhoogd zijn onder zware drinkers, maar niet of nauwelijks onder matige drinkers. GGT laat daarentegen een toename zien bij zowel matig drinken als zwaar drinken (e.g. Arndt et al. 1998; Alatalo et al. 2009b; Liangpunsakul et al. 2010).

In **Hoofdstuk 6** is verder onderzocht hoe de associatie tussen alcoholinname en GGT het beste kan worden verklaard. Hebben individuen verhoogde GGT-waarden omdat ze meer alcohol drinken of is er een gemeenschappelijke oorzaak die de associatie tussen alcoholgebruik en GGT op populatieniveau kan verklaren, bijv. gedeelde genetische factoren die van invloed zijn op zowel alcoholgebruik als variatie in GGT (genetische pleiotropie)? Gegevens over alcoholinname en GGT bij tweelingen en hun familieleden kunnen hierin inzicht geven.

De associatie van alcoholgebruik met GGT op populatieniveau kan het beste worden verklaard door een effect van genetische pleiotropie. Genetische invloeden op alcoholgebruik zijn gecorreleerd met die op GGT (p<0,001), terwijl de correlatie tussen omgevingseffecten op alcoholinname en GGT niet significant was (bij α=0,01, p=0,041), wat het meest in overeenstemming is met een effect van gedeelde genen, hoewel een causaal effect van alcoholgebruik op GGT niet kan worden uitgesloten. Deze bevinding is niet noodzakelijk in tegenspraak met de bevinding dat alcoholgebruik GGT verhoogt in experimentele settings (Conigrave et al. 2003), maar stelt dat in deze relatief gezonde populatie de genen die alcoholgebruik beïnvloeden, ook variatie in leverenzymen veroorzaken, waardoor een correlatie tussen alcohol drinken en verhoogde leverenzymen op populatieniveau ontstaat. Genen die een rol spelen bij alcoholgebruik, verklaren 7,6% van de variantie in GGT bij mannen en 4,6% van variantie bij vrouwen. Interessant is dat de genetische effecten op alcoholgebruik en GGT voornamelijk niet-additief op elkaar inwerken, wat kan wijzen op interacterende risicoallelen (vanuit genetische dominantie of epistase).

8. Genetische invloeden op alcoholgebruik, leverenzymwaarden en hun samenhang op basis van genetische markerdata

Hoofdstuk 7 en 8 presenteren studies die gebruikmaken van SNP-data. SNPs zijn genetische varianten waarbij het DNA op één nucleotide (adenine, A; cytosine, C; thymine, T; guanine, G) kan verschillen van het DNA uit de referentieset (van een populatie).

In **Hoofdstuk 7** is een kandidaatgenstudie uitgevoerd naar de associatie van SNPs in het ADH-gencluster en verschillende maten van alcoholgebruik. Het ADH-gencluster bestaat uit zeven genen, waarvan de ADH1B en ADH1C genen functionele varianten herbergen die in eerder onderzoek zijn geassocieerd met het risico op alcoholisme (Edenberg 2007). Aangezien relatief weinig bekend was over genetische varianten in de andere ADH-genen en hoe deze betrekking hebben op andere maten van alcoholgebruik dan alcoholisme, is in hoofdstuk 7 onderzocht of

SNPs binnen dit gencluster samenhangen met frequentie en mate van alcoholgebruik, lichamelijke reacties na alcoholgebruik, symptomen van AAD en leeftijd bij aanvang van het alcoholgebruik. Er zijn significante associaties gevonden (α=0,007) voor reacties na alcoholgebruik met een SNP in ADH5 (rs6827292) en een SNP net stroomopwaarts van ADH5 (rs6819724), dat in een sterke samenhang (linkage disequilibrium, LD) verkeert met SNP rs6827292. Daarnaast is de associatie tussen leeftijd bij aanvang van regelmatig alcoholgebruik en een SNP net stroomopwaarts van ADH7 (rs2654849) significant. Er zijn geen significante associaties gevonden voor alcoholgebruik en symptomen van AAD. Hoewel SNP-associaties uit een eerdere studie (Macgregor et al. 2009) niet werden gerepliceerd, wijzen de drie nieuwe SNP-associaties op het belang van het ADH-gencluster in het verklaren van verschillen in maten van alcoholgebruik.

In **Hoofdstuk 8** is geschat welk deel van de variantie in leverenzymwaarden kan worden verklaard door het gezamenlijke effect van alle SNPs en of een deel van deze variantie wordt gedeeld met die van alcoholgebruik. Hiertoe zijn twee relatief nieuwe methoden toegepast. Met de genetische verwantschap Matrix (GRM) methode, is de paarsgewijze genetische verwantschap tussen individuen geschat op basis van SNP-data. Deze paarsgewijze verwantschap is opgenomen als random effect in een linear mixed model, geïmplementeerd in het softwarepakket genome-wide complex trait analysis (GCTA) (Yang et al. 2011a). Hiermee werd de variantie geschat op basis van additieve SNP-effecten. De dichtheidsschatting (DE) methode, voorgesteld door So et al. (2011), is gebaseerd op het vergelijken van de effectgroottes van SNP-associaties afkomstig uit GWA- of GWA meta-analyse studies met de verwachte verdeling van effectgrootten onder de nulhypothese van geen effect. Data waren afkomstig uit drie bronnen: (a) ongerelateerde deelnemers aan het NTR-onderzoek, (b) ongerelateerde deelnemers aan de NESDA-studie (Penninx et al. 2008), en (c) een groot consortium van waaruit GWA meta-analyseresultaten beschikbaar waren over SNP-associaties voor GGT en ALT (Chambers et al. 2011).

Op basis van de NTR/NESDA data kan ongeveer 15-17% van de variantie in GGT, 2-15% van de variantie in ALT en 13% van de variantie in AST worden verklaard door het gezamenlijke effect van alle SNPs. Voor alcoholgebruik is dit 15%, waarbij een aanzienlijk deel van de variatie wordt verklaard door chromosomen 4 en 15 (elk ~4,5%). Deze schattingen geven aan dat ~50% van de erfelijkheidsschattingen (op basis van additief genetische effecten) van leverenzymen en alcoholgebruik verklaard kan worden door de effecten van de gemeten en geïmputeerde SNPs. Deze bevindingen wijzen erop dat SNPs inderdaad informatie bevatten aangaande de onderliggende genetische risicoallelen voor alcoholinname en leverenzymen. Verder methodologisch onderzoek moet zich concentreren op SNP-gebaseerde erfelijkheidschattingsmethoden die ook niet-additieve genetische effecten op de variatie

meenemen. Opvallend is dat de DE-methode in veel lagere erfelijkheidsschattingen van GGT en ALT resulteert wanneer deze worden gebaseerd op de GWA meta-analyseresultaten (GGT 8%, ALT 5%) dan gebaseerd op de NTR/NESDA data. Dit kan mogelijk worden verklaard vanuit heterogeniteit in effectgroottes van SNPs tussen de verschillende samples uit de meta-analyse.

Tenslotte is onderzocht of de variatie in GGT die kan worden toegeschreven aan SNP-effecten, gedeeld wordt met SNP-effecten voor alcoholgebruik. Zowel met de GRM- als met de DE-methode is geen evidentie gevonden voor gemeenschappelijke SNP-variatie. Dit kan mogelijk worden verklaard door een lage power van de SNP-gebaseerde methoden of dat de relatie tussen alcoholinname en GGT door een ander ziektemodel wordt beschreven dan zoals in de studie verondersteld (een causaal effect van alcoholinname op GGT i.p.v. gedeelde genen en/of nonadditief genetische effecten en/of leeftijdsinteractie i.p.v. additief genetische effecten). Met polygenetische risicoscores op basis van de GWA meta-analyseresultaten kan alcoholinname in de NTR/NESDA-set wel worden voorspeld, maar de hoeveelheid verklaarde variantie is niet hoger dan 0,25%.

9. Conclusies

Een aanzienlijk deel van de individuele verschillen in alcoholgebruik (o.a. alcoholinname, symptomen van alcoholmisbruik en -afhankelijkheid) en leverenzymwaarden in de Nederlandse bevolking kan worden toegeschreven aan genetische variatie, zoals bepaald met het tweeling(familie)design en met nieuwere technieken als de GRM- en DE-methode. Met de laatste technieken is een deel van de genetische basis van alcoholgebruik en leverenzymwaarden herleid tot variatie in de DNA-sequentie (SNPs). Wat betreft de relatie tussen alcoholinname en leverenzymwaarden, blijkt alcoholinname voorspellend voor GGT-niveaus, maar geen robuuste voorspeller van ALT- of AST-niveaus. Met het tweelingfamiliedesign kan worden vastgesteld dat de associatie tussen alcoholinname en GGT op populatieniveau in elk geval gedeeltelijk kan worden toegeschreven aan een effect van gedeelde genen.

De bevinding dat verschillen in alcoholgebruik en leverenzymwaarden gedeeltelijk zijn toe te schrijven aan SNP-effecten, geeft aan dat de huidige SNP-platforms informatie bevatten over risicoallelen die ten grondslag liggen aan verhoogd alcoholgebruik en leverenzymwaarden. Aangezien de invloed van nonadditieve genetische effecten op alcoholgebruik en leverenzymwaarden aanzienlijk is, zal toekomstig onderzoek zich moeten richten op methoden die ook nonadditieve genetische effecten meenemen in de SNP-gebaseerde erfelijkheidsschattingen. Deze informatie kan leiden tot het opsporen van nieuwe biologische paden die ten grondslag liggen aan alcoholgebruik, leverenzymen en hun samenhang.

References

Addolorato G, Leggio L, Ojetti V, Capristo E, Gasbarrini G, Gasbarrini A (2008) Effects of short-term moderate alcohol administration on oxidative stress and nutritional status in healthy males. Appetite 50:50-56

Aertgeerts B, Buntinx F, Ansoms S, Fevery J (2001) Screening properties of questionnaires and laboratory tests for the detection of alcohol abuse or dependence in a general practice population. Br J Gen Pract 51:206

Aertgeerts B, Buntinx F, Kester A (2004) The value of the CAGE in screening for alcohol abuse and alcohol dependence in general clinical populations: a diagnostic meta-analysis. J Clin Epidemiol 57:30-39

Agrawal A, Heath AC, Grant JD, Pergadia ML, Statham DJ, Bucholz KK et al. (2006) Assortative mating for cigarette smoking and for alcohol consumption in female Australian twins and their spouses. Behav Genet 36:553-566

Agrawal A, Hinrichs AL, Dunn G, Bertelsen S, Dick DM, Saccone SF et al. (2008) Linkage scan for quantitative traits identifies new regions of interest for substance dependence in the Collaborative Study on the Genetics of Alcoholism (COGA) sample. Drug Alcohol Depend 93:12-20

Ahlstrom S, Bloomfield K, Knibbe R (2001) Gender differences in drinking patterns in nine European countries: descriptive findings. Subst Abuse 22:69-85

Alatalo PI, Koivisto HM, Hietala JP, Bloigu RS, Niemela OJ (2009a) Gender-dependent impacts of body mass index and moderate alcohol consumption on serum uric acid: an index of oxidant stress status? Free Rad Biol Med 46:1233-1238

Alatalo PI, Koivisto HM, Hietala JP, Puukka KS, Bloigu RS (2008) Effect of moderate alcohol consumption on liver enzymes increases with increasing body mass index. Am J Clin Nutr 88:1097

Alatalo PI, Koivisto HM, Puukka K, Hietala J, Anttila P, Bloigu RS et al. (2009b) Biomarkers of liver status in heavy drinkers, moderate drinkers and abstainers. Alcohol Alcohol 44:199-203

Albert MA, Glynn RJ, Ridker PM (2003) Alcohol consumption and plasma concentration of C-reactive protein. Circulation 107:443-447

Allen JP, Litten RZ, Fertig JB, Sillanaukee P (2000) Carbohydrate-deficient transferrin, gamma-glutamyltransferase, and macrocytic volume as biomarkers of alcohol problems in women. Alcohol Clin Exp Res 24:492-496

Allen M, Donohue WA, Griffin A, Ryan D, Turner MMM (2003) Comparing the influence of parents and peers on the choice to use drugs: A meta-analytic summary of the literature. Crim Justice Behav 30:163-186

Alte D, Luedemann J, Rose HJ, John U (2004) Laboratory markers carbohydrate-deficient transferrin, gamma-glutamyltransferase, and mean corpuscular volume are not useful as screening tools for high‐risk drinking in the general population: Results from the study of health in Pomerania (SHIP). Alcohol Clin Exp Res 28:931-940

Altshuler DM, Gibbs RA, Peltonen L, Dermitzakis E, Schaffner SF, Yu F et al. (2010a) Integrating common and rare genetic variation in diverse human populations. Nature 467:52-58

Altshuler DM, Lander ES, Ambrogio L, Bloom T, Cibulskis K, Fennell TJ et al. (2010b) A map of human genome variation from population scale sequencing. Nature 467:1061-1073

American Psychiatric Association (2000) Diagnostic and statistical manual of mental disorders: DSM-IV-TR. Washington, DC: American Psychiatric Association

An L, Wang X, Cederbaum AI (2012) Cytokines in alcoholic liver disease. Arch Toxicol 86:1337-1348

Arndt V, Brenner H, Rothenbacher D, Zschenderlein B, Fraisse E, Fliedner TM (1998) Elevated liver enzyme activity in construction workers: Prevalence and impact on early retirement and all-cause mortality. Int Arch Occup Environ Health 71:405-412

Ask H, Rognmo K, Torvik FA, Roysamb E, Tambs K (2012) Non-random mating and convergence over time for alcohol consumption, smoking, and exercise: The Nord-Trondelag Health Study. Behav Genet 42:354-365

Baik I, Cho NH, Kim SH, Han BG, Shin C (2011) Genome-wide association studies identify genetic loci related to alcohol consumption in Korean men. Am J Clin Nutr 93:809-816

Baker J, Maes HH, Kendler KS (2012) Shared environmental contributions to substance use. Behav Genet 42:345-353

Balding DJ (2006) A tutorial on statistical methods for population association studies. Nat Rev Genet 7:781-791

Bandura, A. McClelland, D. C. (1977) Social learning theory. Englewood Cliffs, NJ: Prentice-Hall.

Bartels M, Van Beijsterveldt CEM, Derks EM, Stroet TM, Polderman TJC, Hudziak JJ et al. (2007) Young Netherlands Twin Register (Y-NTR): a longitudinal multiple informant study of problem behavior. Twin Res Hum Genet 10:3-11

Bathum L, Petersen HC, Rosholm JU, Hyltoft Petersen P, Vaupel J, Christensen K (2001) Evidence for a substantial genetic influence on biochemical liver function tests: results from a population-based Danish twin study. Clin Chem 47:81-87

Bedogni G, Miglioli L, Masutti F, Castiglione A, Croce LS, Tiribelli C et al. (2007) Incidence and natural course of fatty liver in the general population: The Dionysos study. Hepatology 46:1387-1391

Begg, S, Vos, T, Barker, B, Stevenson, C, Stanley, L, Lopez, A (2007) Burden of disease and injury in Australia 2003 (Rep. No. PHE 82) Canberra: AIHW

Bellantani S, Costa G, Saccoccio G (1997) Drinking habits as cofactors of risk for alcohol-induced liver damage. Gut 41:845-850

Bentler PM, Bonett DG (1980) Significance tests and goodness of fit in the analysis of covariance structures. Psychol Bull 88:588-606

Bergen SE, Gardner CO, Kendler KS (2007) Age-related changes in heritability of behavioral phenotypes over adolescence and young adulthood: a meta-analysis. Twin Res Hum Genet 10:423-433

Berks J, McCormick R (2008) Screening for alcohol misuse in elderly primary care patients: A systematic literature review. Int Psychogeriatr 20:1090-1103

Bierut LJ, Agrawal A, Bucholz KK, Doheny KF, Laurie C, Pugh E et al. (2010) A genome-wide association study of alcohol dependence. Proc Natl Acad Sci 107:5082-5087

Birley AJ, James MR, Dickson PA, Montgomery GW, Heath AC, Martin NG et al. (2009) ADH single nucleotide polymorphism associations with alcohol metabolism in vivo. Human Mol Genet 18:1533-1542

Blanco-Colio LM, Valderrama M, varez-Sala LA, Bustos C, Ortego M, Hernandez-Presa MA et al. (2000) Red wine intake prevents nuclear factor-kB activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia. Circulation 102:1020-1026

Bo S, Gambino R, Durazzo M, Guidi S, Tiozzo E, Ghione F et al. (2005) Associations between gamma-glutamyl transferase, metabolic abnormalities and inflammation in healthy subjects from a population-based cohort: A possible implication for oxidative stress. World J Gastroenterol 11:7109-7117

Boker SM, Neale MC, Maes HH, Wilde M, Spiegel M, Brick T et al. (2011) OpenMx: An open source extended structural equation modeling framework. Psychometrika 76:306-317

Boomsma DI, Busjahn A, Peltonen L (2002a) Classical twin studies and beyond. Nat Rev Genet 3:872-882

Boomsma DI, de Geus EJC, Vink JM, Stubbe JH, Distel MA, Hottenga JJ et al. (2006) Netherlands Twin Register: From twins to twin families. Twin Res Hum Genet 9:849-857

Boomsma, DI, Martin, NG (2002b) Gene-environment interactions. In HAHD'Haenen, JA den Boer (Eds), Biological psychiatry (pp. 181-187). London: Wiley

Boomsma DI, Martin NG, Molenaar PCM (1989) Factor and simplex models for repeated measures: Application to two psychomotor measures of alcohol sensitivity in twins. Behav Genet 19:79-96

Boomsma DI, Molenaar PCM (1987) The genetic analysis of repeated measures. I. Simplex models. Behav Genet 17:111-123

Boomsma DI, Vink JM, Van Beijsterveldt CEM, de Geus EJC, Beem AL, Mulder EJCM et al. (2002c) Netherlands Twin Register: A focus on longitudinal research. Twin Res Hum Genet 5:401-406

Boomsma DI, Willemsen G, Sullivan PF, Heutink P, Meijer P, Sondervan D et al. (2008) Genome-wide association of major depression: Description of samples for the GAIN Major Depressive Disorder study: NTR and NESDA biobank projects. Eur J Hum Gen 16:335-342

Bowling A (2005) Mode of questionnaire administration can have serious effects on data quality. J Public Health 27:281-291

Breitling LP, Arndt V, Drath C, Brenner H (2011) Liver enzymes: Interaction analysis of smoking with alcohol consumption or BMI, comparing AST and ALT to GGT. PloS ONE 6:e27951

Brien SE, Ronksley PE, Turner BJ, Mukamal KJ, Ghali WA (2011) Effect of alcohol consumption on biological markers associated with risk of coronary heart disease: systematic review and meta-analysis of interventional studies. BMJ 342:d636

Broms U, Silventoinen K, Madden PAF, Heath AC, Kaprio J (2006) Genetic architecture of smoking behavior: a study of Finnish adult twins. Twin Res Hum Genet 9:64-72

Brooks PJ, Enoch MA, Goldman D, Li TK, Yokoyama A (2009) The alcohol flushing response: an unrecognized risk factor for esophageal cancer from alcohol consumption. PLoS medicine 6:e1000050

Brouwers MCGJ, Cantor RM, Kono N (2006) Heritability and genetic loci of fatty liver in familial combined hyperlipidemia. J Lipid Res 47:2799-2807

Buchsbaum DG, Buchanan RG, Welsh J, Centor RM (1992) Screening for drinking disorders in the elderly using the CAGE questionnaire. J Am Geriatr Soc 40:662-665

Burger M, Bronstrup A, Pietrzik K (2004) Derivation of tolerable upper alcohol intake levels in Germany: a systematic review of risks and benefits of moderate alcohol consumption. Prev Med 39:111-127

Caballeria J (2003) Current concepts in alcohol metabolism. Ann Hepatol 2:60-68

Cadden ISH, Partovi N, Yoshida EM (2007) Review article: possible beneficial effects of coffee on liver disease and function. Aliment Pharmacol Ther 26:1-8

CBS (2008) Gezondheid en zorg in cijfers 2008 Den Haag/Heerlen: CBS

CBS (2011) Particuliere huishoudens naar grootte, 2011-2060. CBS Statline [On-line]. Available: http://statline.cbs.nl/StatWeb/publication/?DM=SLNL&PA=80981NED&D1=a&D2=1-9&D3=0,4,9,14,19,24,29,34,39,44,l&HDR=T&STB=G1,G2&VW=T

Cerda M, Sagdeo A, Johnson J, Galea S (2010) Genetic and environmental influences on psychiatric comorbidity: a systematic review. J Affect Disord 126:14-38

Chambers JC, Zhang W, Sehmi J, Li X, Wass MN, Van der Harst P et al. (2011) Genome-wide association study identifies loci influencing concentrations of liver enzymes in plasma. Nat Genet 43:1131-1138

Chen CC, Lu RB, Chen YC, Wang MF, Chang YC, Li TK et al. (1999) Interaction between the functional polymorphisms of the alcohol-metabolism genes in protection against alcoholism. Am J Hum Genet 65:795-807

Chen, C. M. (2006) Alcohol use and alcohol use disorders in the United States: Main findings from the 2001-2002 National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) Bethesda, MD: NIAAA

Chen J, Conigrave KM, Macaskill P, Whitfield JB, Irwig L (2003) Combining carbohydrate-deficient transferrin and gamma-glutamyltransferase to increase diagnostic accuracy for problem drinking. Alcohol Alcohol 38:574-582

Chen QK, Chen HY, Huang KH, Zhong YQ, Han JA, Zhu ZH et al. (2004) Clinical features and risk factors of patients with fatty liver in Guangzhou area. World J Gastroenterol 10:899-902

Chen WJ, Chen CC, Yu JM, Cheng ATA (1998) Self-reported flushing and genotypes of ALDH2, ADH2, and ADH3 among Taiwanese Han. Alcohol Clin Exp Res 22:1048-1052

Cloninger CR, Bohman M, Sigvardsson S (1981) Inheritance of alcohol abuse: Cross-fostering analysis of adopted men. Arch Gen Psychiatry 38:861-868

Conigrave KM, Davies P, Haber P, Whitfield JB (2003) Traditional markers of excessive alcohol use. Addiction 98:31-43

Conigrave KM, Degenhardt LJ, Whitfield JB, Saunders JB, Helander A, Tabakoff B (2002) CDT, GGT, and AST as markers of alcohol use: the WHO/ISBRA collaborative project. Alcohol Clin Exp Res 26:332-339

Costanzo S, Di Castelnuovo A, Donati MB, Iacoviello L, de Gaetano G (2011) Wine, beer or spirit drinking in relation to fatal and non-fatal cardiovascular events: a meta-analysis. Eur J Epid 26:833-850

Cunningham CC, Van Horn CG (2003) Energy availability and alcohol-related liver pathology. Alcohol Res Health 27:291-299

Dawson DA (2011) Defining risk drinking. Alcohol Res Health 34:144-156

Dawson DA, Goldstein RB, Patricia Chou S, June Ruan W, Grant BF (2008) Age at first drink and the first incidence of adult‐onset DSM-IV alcohol use disorders. Alcohol Clin Exp Res 32:2149-2160

De Moor MHM, Boomsma DI, Stubbe JH, Willemsen G, de Geus EJC (2008) Testing causality in the association between regular exercise and symptoms of anxiety and depression. Arch Gen Psychiatry 65:897-905

Demirkan A, Penninx BWJH, Hek K, Wray NR, Amin N, Aulchenko YS et al. (2010) Genetic risk profiles for depression and anxiety in adult and elderly cohorts. Mol Psychiatry 16:773-783

Dhalla S, Kopec JA (2007) The CAGE questionnaire for alcohol misuse: A review of reliability and validity studies. Clin Invest Med 30:33-41

Di Castelnuovo A, Costanzo S, Donati MB, Iacoviello L, de Gaetano G (2010) Prevention of cardiovascular risk by moderate alcohol consumption: epidemiologic evidence and plausible mechanisms. Intern Emerg Med 5:291-297

Dick DM, Edenberg HJ, Xuei X, Goate AM, Kuperman S, Schuckit MA et al. (2006) Association of GABRG3 with alcohol dependence. Alcohol Clin Exp Res 28:4-9

Dick DM, Latendresse SJ, Lansford JE, Budde JP, Goate AM, Dodge KA et al. (2009a) Role of GABRA2 in trajectories of externalizing behavior across development and evidence of moderation by parental monitoring. Arch Gen Psychiatry 66:649-657

Dick DM, Meyers JL, Rose RJ, Kaprio J, Kendler KS (2011) Measures of current alcohol consumption and problems: two independent twin studies suggest a complex genetic architecture. Alcohol Clin Exp Res 35:2152-2161

Dick, DM, Prescott, CA, McGue, M (2009b) The genetics of substance use and substance use disorders. In Y-K Kim (Ed.), Handbook of behavior genetics (pp. 433-453). New York: Springer

Distel MA, Ligthart L, Willemsen G, Nyholt DR, Trull TJ, Boomsma DI (2007) Personality, health and lifestyle in a questionnaire family study: A comparison between highly cooperative and less cooperative families. Twin Res Hum Genet 10:348-353

Eagon PK (2010) Alcoholic liver injury: influence of gender and hormones. World J Gastroenterol 16:1377-1384

Eaves LJ (1976) The effect of cultural transmission on continuous variation. Heredity 37:41-57

Eaves LJ, Gale JS (1974) A method for analyzing the genetic basis of covariation. Behav Genet 4:253-267

Eaves LJ, Last KA, Young PA, Martin NG (1978) Model-fitting approaches to the analysis of human behaviour. Heredity 41:249-320

Edenberg HJ (2007) The genetics of alcohol metabolism: Role of alcohol dehydrogenase and aldehyde dehydrogenase variants. Alcohol Res Health 30:5-13

Edenberg HJ, Koller DL, Xuei X, Wetherill L, McClintick JN, Almasy L et al. (2010) Genome-wide association study of alcohol dependence implicates a region on chromosome 11. Alcohol Clin Exp Res 34:840-852

Edenberg HJ, Xuei X, Chen HJ, Tian H, Wetherill LF, Dick DM et al. (2006) Association of alcohol dehydrogenase genes with alcohol dependence: a comprehensive analysis. Human Mol Genet 15:1539-1549

Efron B (2009) Empirical Bayes estimates for large-scale prediction problems. J Am Stat Assoc 104:1015-1028

Eijnden R, Mheen D, Vet R, Vermulst A (2011) Alcohol-specific parenting and adolescents' alcohol-related problems: the interacting role of alcohol availability at home and parental rules. J Stud Alcohol Drugs 72:408-417

ENCODE Project Consortium (2012) An integrated encyclopedia of DNA elements in the human genome. Nature 489:57-74

Eng MY, Luczak SE, Wall TL (2007) ALDH2, ADH1B, and ADH1C genotypes in Asians: a literature review. Alcohol Res Health 30:22-27

Engels, RCME, Bot, SM (2006) Social influences on adolescent substance use: Insights into how parents and peers affect adolescent's smoking and drinking behavior. In D de Ridder, J de Wit (Eds.), New perspectives on health behavior: Self-regulation in health behavior (pp. 71-95). New York: Wiley

Enoch MA (2008) The role of GABAA receptors in the development of alcoholism. Pharmacol Biochem Behav 90:95-104

Ewing JA (1984) Detecting alcoholism: The CAGE questionnaire. JAMA 252:1905-1907

Falconer, D. S. (1996) Introduction to quantitative genetics. (4 ed.) Essex, UK: Pearson

Fan JG, Zhu J, Li XJ, Chen L, Li L, Dai F et al. (2005) Prevalence of and risk factors for fatty liver in a general population of Shanghai, China. J Hepatol 43:508-514

Fava C, Danese E, Montagnana M, Sjogren M, Almgren P, Guidi GC et al. (2011) A variant upstream of the CDH13 Adiponectin Receptor gene and metabolic syndrome in Swedes. Am J Cardiol 108:1432-1437

Felson, J. (2009) What can we learn from twin studies? A comprehensive evaluation of the equal environments assumption. The Pennsylvania State University

Fiellin DA, Reid MC, O'Connor PG (2000) Screening for alcohol problems in primary care: A systematic review. Arch Intern Med 160:1977

Fisher RA (1918) XV The correlation between relatives on the supposition of Mendelian inheritance. Trans R Soc Edin 52:399-433

Fowler T, Lifford K, Shelton K, Rice F, Thapar A, Neale MC et al. (2007a) Exploring the relationship between genetic and environmental influences on initiation and progression of substance use. Addiction 102:413-422

Fowler T, Shelton K, Lifford K, Rice F, McBride A, Nikolov I et al. (2007b) Genetic and environmental influences on the relationship between peer alcohol use and own alcohol use in adolescents. Addiction 102:894-903

Fraser A, Harris R, Sattar N, Ebrahim S, Smith GD, Lawlor DA (2007) Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake. Arterioscler Thromb Vasc Biol 27:2729-2735

Fraser A, Harris R, Sattar N, Ebrahim S, vey Smith G, Lawlor DA (2009) Alanine aminotransferase, gamma-glutamyltransferase, and incident diabetes. Diabetes Care 32:741-750

Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, Gibbs RA et al. (2007) A second generation human haplotype map of over 3.1 million SNPs. Nature 449:851-861

Frazer KA, Murray SS, Schork NJ, Topol EJ (2009) Human genetic variation and its contribution to complex traits. Nat Rev Genet 10:241-251

Fugger L, McVean G, Bell JI (2012) Genomewide association studies and common disease--realizing clinical utility. N Engl J Med 367:2370-2371

Gameiro CM, Romao F, Castelo-Branco C (2010) Menopause and aging: Changes in the immune system - a review. Maturitas 67:316-320

Geels LM, Bartels M, Van Beijsterveldt CEM, Willemsen G, van der Aa N, Boomsma DI et al. (2012) Trends in adolescent alcohol use: effects of age, sex and cohort on prevalence and heritability. Addiction 107:518-527

Geels LM, Vink JM, Van Beek JHDA, Bartels M, Willemsen G, Boomsma DI (2013) Increases in alcohol consumption in women and elderly groups: Evidence from an epidemiological study. BMC Public Health (in press)

Gillespie NA, Kendler KS, Prescott CA, Aggen SH, Gardner CO, Jacobson K et al. (2007) Longitudinal modeling of genetic and environmental influences on self-reported availability of psychoactive substances: alcohol, cigarettes, marijuana, cocaine and stimulants. Psychol Med 37:947-959

Gonzalez-Quintela A, Campos J, Loidi L, Quinteiro C, Perez LF, Gude F (2008) Serum TNF-alpha levels in relation to alcohol consumption and common TNF gene polymorphisms. Alcohol 42:513-518

Goodwin DW, Schulsinger F, Hermansen L, Guze SB, Winokur G (1973) Alcohol problems in adoptees raised apart from alcoholic biological parents. Arch Gen Psychiatry 28:238-243

Graham K, Massak A, Demers A, Rehm J (2007) Does the association between alcohol consumption and depression depend on how they are measured? Alcohol Clin Exp Res 31:78-88

Grant JD, Agrawal A, Bucholz KK, Madden PAF, Pergadia ML, Nelson EC et al. (2009) Alcohol consumption indices of genetic risk for alcohol dependence. Biol Psychiatry 66:795-800

Grant JD, Heath AC, Bucholz KK, Madden PAF, Agrawal A, Statham DJ et al. (2007) Spousal concordance for alcohol dependence: evidence for assortative mating or spousal interaction effects? Alcohol Clin Exp Res 31:717-728

Grant JD, Scherrer JF, Lynskey MT, Lyons MJ, Eisen SA, Tsuang MT et al. (2006) Adolescent alcohol use is a risk factor for adult alcohol and drug dependence: evidence from a twin design. Psychol Med 36:109-118

Gunji T, Matsuhashi N, Sato H, Fujibayashi K, Okumura M, Sasabe N et al. (2009) Light and moderate alcohol consumption significantly reduces the prevalence of fatty liver in the Japanese male population. Am J Gastroenterol 104:2189-2195

Gutmann, L. (1955) A new approach to factor analysis: The radex. In P.F.Lazarsfeld (Ed.), Mathematical thinking in the social sciences (2 ed., pp. 258-348). Glencoe, Illinois, USA: The Free Press

Hamaguchi M, Kojima T, Ohbora A, Takeda N, Fukui M, Kato T (2012) Protective effect of alcohol consumption for fatty liver but not metabolic syndrome. World J Gastroenterol 18:156-167

Han C, McGue MK, Iacono WG (1999) Lifetime tobacco, alcohol and other substance use in adolescent Minnesota twins: univariate and multivariate behavioral genetic analyses. Addiction 94:981-993

Hannuksela ML, Liisanantti MK, Nissinen AET, Savolainen MJ (2007) Biochemical markers of alcoholism. Clin Chem Lab Med 45:953-961

Hansell NK, Agrawal A, Whitfield JB, Morley KI, Zhu G, Lind PA et al. (2008) Long-term stability and heritability of telephone interview measures of alcohol consumption and dependence. Twin Res Hum Genet 11:287-305

Harford TC, Grant BF, Yi H, Chen CM (2006) Patterns of DSM-IV alcohol abuse and dependence criteria among adolescents and adults: Results from the 2001 National Household Survey on drug abuse. Alcohol Clin Exp Res 29:810-828

Health Counsel of the Netherlands (2012) Richtlijnen goede voeding 2006: Achtergronddocument Den Haag: Health Counsel

Heath AC, Bucholz KK, Madden PAF, Dinwiddie SH, Slutske WS, Bierut LJ et al. (1997) Genetic and environmental contributions to alcohol dependence risk in a national twin sample: consistency of findings in women and men. Psychol Med 27:1381-1396

Heath AC, Eaves LJ (1985) Resolving the effects of phenotype and social background on mate selection. Behav Genet 15:15-30

Heath AC, Madden PAF, Bucholz KK, Dinwiddie SH, Slutske WS, Bierut LJ et al. (1999) Genetic differences in alcohol sensitivity and the inheritance of alcoholism risk. Psychol Med 29:1069-1081

Heath AC, Martin NG, Eaves LJ, Loesch D (1984) Evidence for polygenic epistatic interactions in man? Genetics 106:719-727

Herbeth B, Samara A, Ndiaye C, Marteau JB, Berrahmoune H, Siest G et al. (2010) Metabolic syndrome-related composite factors over 5 years in the STANISLAS Family Study: genetic heritability and common environmental influences. Clin Chim Acta 411:833-839

Hettema JM, Corey LA, Kendler KS (1999) A multivariate genetic analysis of the use of tobacco, alcohol, and caffeine in a population based sample of male and female twins. Drug Alcohol Depend 57:69-78

Hettema JM, Neale MC, Kendler KS (1995) Physical similarity and the equal-environment assumption in twin studies of psychiatric disorders. Behav Genet 25:327-335

Higashikawa A, Suwazono Y, Okubo Y, Uetani M, Kobayashi E, Kido T et al. (2005) Association of working conditions and lifestyle with increased serum gamma-glutamyltransferase: A follow-up study. Arch Med Res 36:567-573

Higuchi S, Matsushita S, Kashima H (2006) New findings on the genetic influences on alcohol use and dependence. Curr Opin Psychiatry 19:253-265

Hilderink, I, van 't Land, H (2008) GGZ in tabellen 2007 Utrecht: Trimbos Institute

Hingson RW, Heeren T, Winter MR (2006) Age at drinking onset and alcohol dependence: age at onset, duration, and severity. Arch Pediatr Adolesc Med 160:739-746

Hiramine Y, Imamura Y, Uto H, Koriyama C, Horiuchi M, Oketani M et al. (2011) Alcohol drinking patterns and the risk of fatty liver in Japanese men. J Gastroenterol 46:519-528

Hirschhorn JN, Daly MJ (2005) Genome-wide association studies for common diseases and complex traits. Nat Rev Genet 6:95-108

Holmila M, Raitasalo K (2005) Gender differences in drinking: why do they still exist? Addiction 100:1763-1769

Honjo S, Kono S, Coleman MP, Shinchi K, Sakurai Y, Todoroki I et al. (2001) Coffee consumption and serum aminotransferases in middle-aged Japanese men. J Clin Epid 54:823-829

Hopfer CJ, Crowley TJ, Hewitt JK (2003) Review of twin and adoption studies of adolescent substance use. J Am Acad Child Adolesc Psychiatry 42:710-719

Horwitz AV, Videon TM, Schmitz MF, Davis D (2003) Rethinking twins and environments: possible social sources for assumed genetic influences in twin research. J Health Soc Behav 44:111-129

Howie BN, Donnelly P, Marchini J (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS Gen 5:e1000529

Hurley TD, Edenberg HJ (2011) Genes encoding enzymes involved in ethanol metabolism. Alcohol Res Curr Rev 34:339-344

Hyeon CK, Chung MN, Sun HJ, Kwang HH, Kyu Oh D, Suh I (2004) Normal serum aminotransferase concentration and risk of mortality from liver diseases: Prospective cohort study. BMJ 328:983

IBM Corp. (2011) IBM SPSS Statistics for Windows (Version 20.0) [Computer software]. Armonk, NY: IBM Corp

Imhof A, Froehlich M, Brenner H, Boeing H, Pepys MB, Koenig W (2001) Effect of alcohol consumption on systemic markers of inflammation. The Lancet 357:763-767

Imhof A, Woodward M, Doering A, Helbecque N, Loewel H, Amouyel P et al. (2004) Overall alcohol intake, beer, wine, and systemic markers of inflammation in western Europe: Results from three MONICA samples (Augsburg, Glasgow, Lille). Eur Heart J 25:2092-2100

Ioannou GN, Boyko EJ, Lee SP (2006) The prevalence and predictors of elevated serum aminotransferase activity in the United States in 1999-2002. Am J Gastroenterol 101:76-82

Jang KL, Vernon PA, Livesley WJ (2000) Personality disorder traits, family environment, and alcohol misuse: A multivariate behavioural genetic analysis. Addiction 95:873-888

Jinks JL, Fulker DW (1970) Comparison of the biometrical genetical, MAVA, and classical approaches to the analysis of the human behavior. Psychol Bull 73:311-349

Johnson AD, Newton-Cheh C, Chasman DI, Ehret GB, Johnson T, Rose L et al. (2011) Association of hypertension drug target genes with blood pressure and hypertension in 86 588 individuals. Hypertension 57:903-910

Kamatani Y, Matsuda K, Okada Y, Kubo M, Hosono N, Daigo Y et al. (2010) Genome-wide association study of hematological and biochemical traits in a Japanese population. Nat Genet 42:210-215

Kazemi-Shirazi L, Endler G, Winkler S, Schickbauer T, Wagner O, Marsik C (2007) Gamma glutamyltransferase and long-term survival: is it just the liver? Clin Chem 53:940-946

Keller MC, Coventry WL (2005) Quantifying and addressing parameter indeterminacy in the classical twin design. Twin Res Hum Genet 8:201-213

Keller MC, Coventry WL, Heath AC, Martin NG (2005) Widespread evidence for non-additive genetic variation in Cloninger's and Eysenck's personality dimensions using a twin plus sibling design. Behav Genet 35:707-721

Keller MC, Medland SE, Duncan LE (2010) Are extended twin family designs worth the trouble? A comparison of the bias, precision, and accuracy of parameters estimated in four twin family models. Behav Genet 40:377-393

Keller MC, Medland SE, Duncan LE, Hatemi PK, Neale MC, Maes HH et al. (2009) Modeling extended twin family data I: Description of the Cascade model. Twin Res Hum Genet 12:8-18

Kendler KS, Aggen SH, Knudsen GP, Roysamb E, Neale MC, Reichborn-Kjennerud T (2011a) The structure of genetic and environmental risk factors for syndromal and subsyndromal common DSM-IV axis I and all axis II disorders. Am J Psychiatry 168:29-39

Kendler KS, Chen X, Dick DM, Maes HH, Gillespie NA, Neale MC et al. (2012) Recent advances in the genetic epidemiology and molecular genetics of substance use disorders. Nature Neurosci 15:181-189

Kendler KS, Gardner CO (1998) Twin studies of adult psychiatric and substance dependence disorders: are they biased by differences in the environmental experiences of monozygotic and dizygotic twins in childhood and adolescence? Psychol Med 28:625-633

Kendler KS, Gardner CO, Annas P, Neale MC, Eaves LJ, Lichtenstein P (2008a) A longitudinal twin study of fears from middle childhood to early adulthood: evidence for a developmentally dynamic genome. Arch Gen Psychiatry 65:421-429

Kendler KS, Gardner CO, Lichtenstein P (2008b) A developmental twin study of symptoms of anxiety and depression: evidence for genetic innovation and attenuation. Psychol Med 38:1567-1575

Kendler KS, Heath AC, Neale MC, Kessler RC, Eaves LJ (1992) A population-based twin study of alcoholism in women. JAMA 268:1877-1882

Kendler KS, Kalsi G, Holmans PA, Sanders AR, Aggen SH, Dick DM et al. (2011b) Genomewide association analysis of symptoms of alcohol dependence in the molecular genetics of schizophrenia (MGS2) control sample. Alcohol Clin Exp Res 35:963-975

Kendler KS, Myers J (2009) A developmental twin study of church attendance and alcohol and nicotine consumption: a model for analyzing the changing impact of genes and environment. Am J Psychiatry 166:1150-1155

Kendler KS, Myers J, Dick D, Prescott CA (2010) The relationship between genetic influences on alcohol dependence and on patterns of alcohol consumption. Alcohol Clin Exp Res 34:1058-1065

Kendler KS, Myers J, Prescott CA (2007) Specificity of genetic and environmental risk factors for symptoms of cannabis, cocaine, alcohol, caffeine, and nicotine dependence. Arch Gen Psychiatry 64:1313-1320

Kendler KS, Neale MC, Heath AC, Kessler RC, Eaves LJ (1994) A twin-family study of alcoholism in women. Am J Psychiatry 151:707-715

Kendler KS, Neale MC, Kessler RC, Heath AC, Eaves LJ (1993) A test of the equal-environment assumption in twin studies of psychiatric illness. Behav Genet 23:21-27

Kendler KS, Prescott CA, Neale MC, Pedersen NL (1997) Temperance board registration for alcohol abuse in a national sample of Swedish male twins, born 1902 to 1949. Arch Gen Psychiatry 54:178-184

Kendler KS, Schmitt E, Aggen SH, Prescott CA (2008c) Genetic and environmental influences on alcohol, caffeine, cannabis, and nicotine use from early adolescence to middle adulthood. Arch Gen Psychiatry 65:674-682

Kerner A, Avizohar O, Sella R, Bartha P, Zinder O, Markiewicz W et al. (2005) Association between elevated liver enzymes and C-reactive protein. Arterioscler Thromb Vasc Biol 25:193-197

Keyes KM, Li G, Hasin DS (2011) Birth cohort effects and gender differences in alcohol epidemiology: a review and synthesis. Alcohol Clin Exp Res 35:2101-2112

Kim HC, Nam CM, Jee SH, Kan KH, Oh DK, Suh I (2004) Normal serum aminotransferase concentration and risk of mortality from liver diseases: Prospective cohort study. BMJ 328:983

Kim YJ, Go MJ, Hu C, Hong CB, Kim YK, Lee JY et al. (2011) Large-scale genome-wide association studies in East Asians identify new genetic loci influencing metabolic traits. Nat Genet 43:990-995

Knight, J, Sham, PC, Purcell, S (2008) Regional multilocus association models. In BM Neale, MAR Ferreira, SE Medland, D Posthuma (Eds.), Statistical genetics: gene mapping through linkage and association (pp. 423-449). New York: Taylor & Francis

Knopik VS, Heath AC, Madden PAF, Bucholz KK, Slutske WS, Nelson EC et al. (2004) Genetic effects on alcohol dependence risk: re-evaluating the importance of psychiatric and other heritable risk factors. Psychol Med 34:1519-1530

Kollerits B, Coassin S, Kiechl S, Hunt SC, Paulweber B, Willeit J et al. (2010) A common variant in the adiponutrin gene influences liver enzyme values. J Med Genet 47:116-119

Koopmans JR, Boomsma DI (1996) Familial resemblances in alcohol use: Genetic or cultural transmission? J Stud Alcohol Drugs 57:19-28

Koppes LLJ, Kemper HCG, Post GB, Snel J, Twisk JWR (2000) Development and stability of alcohol consumption from adolescence into adulthood: the Amsterdam Growth and Health Longitudinal Study. Eur Addiction Res 6:183-188

Kovacs EJ, Messingham KAN (2002) Influence of alcohol and gender on immune response. Alcohol Res Health 26:257-263

Krueger RF (1999) The structure of common mental disorders. Arch Gen Psychiatry 56:921-926

Kulinsky VI, Kolesnichenko LS (2009) The glutathione system. II. Other enzymes, thiol-disulfide metabolism, inflammation, and immunity, functions. Biochem Suppl sB: Biomed Chem 3:211-220

Kuo PH, Kalsi G, Prescott CA, Hodgkinson CA, Goldman D, Van Den Oord EJ et al. (2008a) Association of ADH and ALDH genes with alcohol dependence in the Irish Affected Sib Pair Study of alcohol dependence (IASPSAD) sample. Alcohol Clin Exp Res 32:785-795

Kuo PH, Kalsi G, Prescott CA, Hodgkinson CA, Goldman D, Van Den Oord EJ et al. (2008b) Association of ADH and ALDH genes with alcohol dependence in the Irish Affected Sib Pair Study of Alcohol Dependence (IASPSAD) sample. Alcohol Clin Exp Res 32:785-795

Kutalik Z, Whittaker J, Waterworth D, Beckmann JS, Bergmann S (2011) Novel method to estimate the phenotypic variation explained by genome-wide association studies reveals large fraction of the missing heritability. Genet Epidemiol 35:341-349

Kwan, SH, Purcell, S, Sham, PC (2007) Introduction to biometrical genetics. In Statistical genetics: gene mapping through linkage and association (pp. 17-42). New York, USA: Taylor & Francis

Laitala VS, Kaprio J, Silventoinen K (2008) Genetics of coffee consumption and its stability. Addiction 103:2054-2061

Lango Allen H, Estrada K, Lettre G, Berndt SI, Weedon MN, Rivadeneira F et al. (2010) Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature 467:832-838

Laso FJ, Vaquero JM, Almeida J, Marcos M, Orfao A (2007) Chronic alcohol consumption is associated with changes in the distribution, immunophenotype, and the inflammatory cytokine secretion profile of circulating dendritic cells. Alcohol Clin Exp Res 31:846-854

Latendresse SJ, Rose RJ, Viken RJ, Pulkkinen L, Kaprio J, Dick DM (2010) Examining the etiology of associations between perceived parenting and adolescents' alcohol use: common genetic and/or environmental liabilities? J Stud Alcohol Drugs 71:313-325

Lee DH, Blomhoff R, Jacobs DR (2004) Review: Is serum gamma glutamyltransferase a marker of oxidative stress? Free Rad Res 38:535-539

Lee DH, Ha MH, Christiani DC (2001) Body weight, alcohol consumption and liver enzyme activity: a 4-year follow-up study. Int J Epid 30:766-770

Lee DH, Jacobs DR (2009) Serum gamma-glutamyltransferase: new insights about an old enzyme. J Epidemiol Community Health 63:884-886

Lee SH, Goddard ME, Wray NR, Visscher PM (2012a) A better coefficient of determination for genetic profile analysis. Genet Epidemiol 36:214-224

Lee SH, Wray NR, Goddard ME, Visscher PM (2011) Estimating missing heritability for disease from genome-wide association studies. Am J Hum Genet 88:294-305

Lee SH, Yang J, Goddard ME, Visscher PM, Wray NR (2012b) Estimation of pleiotropy between complex diseases using SNP-derived genomic relationships and restricted maximum likelihood. Bioinformatics 28:2540-2542

Lee TH, Kim W, Benson JT, Therneau TM, Melton III LJ (2008) Serum aminotransferase activity and mortality risk in a United States community. Hepatology 47:880-887

Leonard KE, Eiden RD (2007) Marital and family processes in the context of alcohol use and alcohol disorders. Annu Rev Clin Psychol 3:285-310

Li J, Ji L (2005) Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity 95:221-227

Liangpunsakul S, Qi R, Crabb DW, Witzmann F (2010) Relationship between alcohol drinking and aspartate aminotransferase: alanine aminotransferase (AST: ALT) ratio, mean corpuscular volume (MCV), gamma-glutamyl transpeptidase (GGT), and apolipoprotein A1 and B in the US population. J Stud Alcohol Drugs 71:249-252

Lim JS, Yang JH, Chun BY, Kam S, Jacobs Jr DR, Lee DH (2004) Is serum gamma-glutamyltransferase inversely associated with serum antioxidants as a marker of oxidative stress? Free Rad Biol Med 37:1018-1023

Lin JP, O'Donnell CJ, Fox CS, Cupples LA (2009) Heritability of serum glutamyltransferase level: genetic analysis from the Framingham Offspring Study [Letter]. Liver Int 29:776-777

Lind PA, Macgregor S, Vink JM, Pergadia ML, Hansell NK, De Moor MHM et al. (2010) A genomewide association study of nicotine and alcohol dependence in Australian and Dutch populations. Twin Res Hum Genet 13:10-29

Liu I, Blacker DL, Xu R, Fitzmaurice G, Lyons MJ, Tsuang MT (2004) Genetic and environmental contributions to the development of alcohol dependence in male twins. Arch Gen Psychiatry 61:897-903

Long AD, Grote MN, Langley CH (1997) Genetic analysis of complex diseases. Science 275:1328

Loomba R, Rao F, Zhang L, Khandrika S, Ziegler MG, Brenner DA et al. (2010) Genetic covariance between gamma-glutamyl transpeptidase and fatty liver risk factors: Role of beta 2-adrenergic receptor genetic variation in twins. Gastroenterology 139:836-845

Lubke GH, Hottenga JJ, Walters R, Laurin C, de Geus EJC, Willemsen G et al. (2012) Estimating the genetic variance of major depressive disorder due to all single nucleotide polymorphisms. Biol Psychiatry 72:707-709

Luo X, Kranzler HR, Zuo L, Lappalainen J, Yang B, Gelernter J (2006) ADH4 gene variation is associated with alcohol dependence and drug dependence in European Americans:

results from HWD tests and case-control association studies. Neuropsychopharmacology 31:1085-1095

Lykken DT, McGue M, Bouchard TJ, Tellegen A (1990) Does contact lead to similarity or similarity to contact? Behav Genet 20:547-561

Lynch, M, Walsh, B (1998) Genetics and analysis of quantitative traits. (24 ed.) Sunderland, MA: Sinauer

Macgregor S, Lind PA, Bucholz KK, Hansell NK, Madden PAF, Richter MM et al. (2009) Associations of ADH and ALDH2 gene variation with self report alcohol reactions, consumption and dependence: an integrated analysis. Human Mol Genet 18:580-593

Maes HH, Neale MC, Kendler KS, Hewitt JK, Silberg JL, Foley DL et al. (1998) Assortative mating for major psychiatric diagnoses in two population-based samples. Psychol Med 28:1389-1401

Maes HH, Neale MC, Martin NG, Heath AH, Eaves LJ (1999) Religious attendance and frequency of alcohol use: same genes or same environments: a bivariate extended twin kinship model. Twin Res 2:169-179

Maher B (2008) The case of the missing heritability. Nature 456:18-21

Maisto SA, Saitz R (2003) Alcohol use disorders: screening and diagnosis. Am J Addict 12:s12-s25

Makkonen J, Pietiläinen KH, Rissanen A, Kaprio J, Yki-Järvinen H (2009) Genetic factors contribute to variation in serum alanine aminotransferase activity independent of obesity and alcohol: a study in monozygotic and dizygotic twins. J Hepatol 50:1035-1042

Malone SM, Taylor J, Marmorstein NR, McGue M, Iacono WG (2004) Genetic and environmental influences on antisocial behavior and alcohol dependence from adolescence to early adulthood. Dev Psychopathol 16:943-966

Mann RE, Smart RG, Govoni R (2003) The epidemiology of alcoholic liver disease. Alcohol Res Health 27:209-219

Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ et al. (2009) Finding the missing heritability of complex diseases. Nature 461:747-753

Mantena SK, King AL, Andringa KK, Eccleston HB, Bailey SM (2008) Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol-and obesity-induced fatty liver diseases. Free Rad Biol Med 44:1259-1272

Marchini J, Howie BN, Myers S, McVean G, Donnelly P (2007) A new multipoint method for genome-wide association studies by imputation of genotypes. Nat Genet 39:906-913

Marques-Vidal P, Bochud M, Bastardot F, von Kanel R, Ferrero F, Gaspoz JM et al. (2012) Associations between alcohol consumption and selected cytokines in a Swiss population-based sample (CoLaus study). Atherosclerosis 222:245-250

Martin NG, Eaves LJ (1977) The genetical analysis of covariance structure. Heredity 38:79-95

McGue, M. (1999) Behavioral genetic models of alcoholism and drinking. In Psychological theories of drinking and alcoholism (pp. 372-421)

McGue M, Sharma A, Benson P (1996) Parent and sibling influences on adolescent alcohol use and misuse: Evidence from a US adoption cohort. J Stud Alcohol Drugs 57:8-18

Midanik LT, Clark WB (1994) The demographic distribution of US drinking patterns in 1990: description and trends from 1984. Am J Public Health 84:1218-1222

Middelberg RP, Benyamin B, De Moor MHM, Warrington NM, Gordon S, Henders AK et al. (2012) Loci affecting gamma-glutamyl transferase in adults and adolescents show age x SNP interaction and cardiometabolic disease associations. Human Mol Genet 21:446-455

Minica CC, Boomsma DI, van der Sluis S, Dolan CV (2010) Genetic association in multivariate phenotypic data: power in five models. Twin Res Hum Genet 13:525-543

Miyake K, Miyake N, Kondo S, Tabe Y, Ohsaka A, Miida T (2009) Seasonal variation in liver function tests: a time-series analysis of outpatient data. Annals Clin Biochemistry 46:377-384

Molenaar, PCM, Boomsma, DI, Dolan, CV (1991) Genetic and environmental factors in a developmental perspective. In Problems and methods in longitudinal research: Stability and change (pp. 250-273). Cambridge, UK: Cambridge University Press

Molina PE, Happel KI, Zhang P, Kolls JK, Nelson S (2010) Focus On: Alcohol and the Immune System. Alcohol Health & Research World 33:97-108

Molinaro AM, Carriero N, Bjornson R, Hartge P, Rothman N, Chatterjee N (2011) Power of data mining methods to detect genetic associations and interactions. Hum Hered 72:85-97

Moore AA, Gould R, Reuben DB, Greendale GA, Carter MK, Zhou K et al. (2005) Longitudinal patterns and predictors of alcohol consumption in the United States. Am J Public Health 95:458-464

Morozova TV, Goldman D, Mackay TFC, Anholt RRH (2012) The genetic basis of alcoholism: multiple phenotypes, many genes, complex networks. Genome Biol 13:239

Munafo MR, Matheson IJ, Flint J (2007) Association of the DRD2 gene Taq1A polymorphism and alcoholism: a meta-analysis of case-control studies and evidence of publication bias. Mol Psychiatry 12:454-461

Muthen LK, Muthen BO (2007) Mplus user's guide. 5th. Los Angeles, CA: Muthen & Muthen

Nakanishi N, Nakamura K, Suzuki K, Tatara K (2000) Lifestyle and serum gamma-glutamyltransferase: A study of middle-aged Japanese men. Occup Med 50:115-120

Neale, MC, Boker, SM, Xie, G, Maes, HH (2006) Mx: Statistical modeling. (7 ed.) Richmond: Department of Psychiatry, VCU

Neale, MC, Cardon, LR (1992) Methodology for genetic studies of twins and families. Dordrecht: Kluwer

Neale MC, Walters EE, Eaves LJ, Maes HH, Kendler KS (1994) Multivariate genetic analysis of twin-family data on fears: Mx models. Behav Genet 24:119-139

Neijts M, Van Dongen J, Kluft C, Boomsma DI, Willemsen G, de Geus EJC (2013) Genetic architecture of the pro-inflammatory state in an extended twin family design (submitted)

Neiss M, Rowe DC, Rodgers JL (2002) Does education mediate the relationship between IQ and age of first birth? A behavioural genetic analysis. J Biosoc Sci 34:259-276

Newlin DB, Miles DR, Bree M, Gupman AE, Pickens RW (2006) Environmental transmission of DSM-IV Substance Use Disorders in adoptive and step families. Alcohol Clin Exp Res 24:1785-1794

NIAAA (2000) Health risks and benefits of alcohol consumption. Alcohol Res Health 24:5-11

Nicolaou C, Chatzipanagiotou S, Tzivos D, Tzavellas EO, Boufidou F, Liappas IA (2004) Serum cytokine concentrations in alcohol-dependent individuals without liver disease. Alcohol 32:243-247

Nilsson SE, Read S, Berg S, Johansson B (2009) Heritabilities for fifteen routine biochemical values: findings in 215 Swedish twin pairs 82 years of age or older. Scand J Clin Lab Invest 69:562-569

Nose J, Saito A, Kamatani N (2008) Statistical analysis of the associations between polymorphisms within aldehyde dehydrogenase 2 (ALDH2), and quantitative and qualitative traits extracted from a large-scale database of Japanese single-nucleotide polymorphisms (SNPs). J Hum Genet 53:425-438

Nyblom H, Berggren U, Balldin J, Olsson R (2004) High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. Alcohol Alcohol 39:336-339

Nyholt DR (2004) A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet 74:765-769

Nyholt DR (2005) Evaluation of Nyholt's procedure for multiple testing correction-author's reply. Hum Hered 60:19-25

O'Dushlaine C, Kenny E, Heron EA, Segurado R, Gill M, Morris DW et al. (2009) The SNP ratio test: pathway analysis of genome-wide association datasets. Bioinformatics 25:2762-2763

Oliveira A, Rodriguez-Artalejo F, Lopes C (2010) Alcohol intake and systemic markers of inflammation: Shape of the association according to sex and body mass index. Alcohol Alcohol 45:119-125

Omagari K, Morikawa S, Nagaoka S, Sadakane Y, Sato M, Hamasaki M et al. (2009) Predictive factors for the development or regression of fatty liver in Japanese adults. J Clin Biochem Nutr 45:56-67

Osier M, Pakstis AJ, Kidd JR, Lee JF, Yin SJ, Ko HC et al. (1999) Linkage disequilibrium at the ADH2 and ADH3 loci and risk of alcoholism. Am J Hum Genet 64:1147-1157

Pagan JL, Rose RJ, Viken RJ, Pulkkinen L, Kaprio J, Dick DM (2006) Genetic and environmental influences on stages of alcohol use across adolescence and into young adulthood. Behav Genet 36:483-497

Pardo L, Bochdanovits Z, de Geus EJC, Hottenga JJ, Sullivan PF, Posthuma D et al. (2009) Global similarity with local differences in linkage disequilibrium between the Dutch and HapMap-CEU populations. Eur J Hum Gen 17:802-810

Pares A, Tresserras R, Nunez I, Cerralbo M, Plana P, Pujol FJ et al. (2000) Prevalence and factors associated to the presence of fatty liver in apparently healthy adult men. Med Clin 114:561-565

Pedersen NL, McClearn GE, Plomin R, Nesselroade JR (1992) Effects of early rearing environment on twin similarity in the last half of the life span. Br J Dev Psychol 10:255-267

Penninkilampi-Kerola V, Kaprio J, Moilanen I, Rose RJ (2005) Co-twin dependence modifies heritability of abstinence and alcohol use: a population-based study of Finnish twins. Twin Res Hum Genet 8:232-244

Penninx BWJH, Beekman ATF, Smit JH, Zitman FG, Nolen WA, Spinhoven P et al. (2008) The Netherlands Study of Depression and Anxiety (NESDA): Rationale, objectives and methods. Int J Methods Psychiatr Res 17:121-140

Peterson K (2004) Biomarkers for alcohol use and abuse: A summary. Alcohol Res Health 28:30-37

Petrie J, Bunn F, Byrne G (2007) Parenting programmes for preventing tobacco, alcohol or drugs misuse in children< 18: a systematic review. Health Educ Res 22:177-191

Pickens RW, Svikis DS, McGue M, Lykken DT, Heston LL, Clayton PJ (1991) Heterogeneity in the inheritance of alcoholism: a study of male and female twins. Arch Gen Psychiatry 48:19-28

Pilia G, Chen WM, Scuteri A, Orru M, Albai G, Dei M et al. (2006) Heritability of cardiovascular and personality traits in 6,148 Sardinians. PLoS Gen 2:e132

Plomin R, DeFries JC, Loehlin JC (1977) Genotype-environment interaction and correlation in the analysis of human behavior. Psychol Bull 84:309-322

Poelen EAP, Derks EM, Engels RCME, Van Leeuwe JFJ, Scholte RHJ, Willemsen G et al. (2008) The relative contribution of genes and environment to alcohol use in early adolescents: are

similar factors related to initiation of alcohol use and frequency of drinking? Alcohol Clin Exp Res 32:975-982

Poelen EAP, Scholte RHJ, Engels RCME, Boomsma DI, Willemsen G (2005) Prevalence and trends of alcohol use and misuse among adolescents and young adults in the Netherlands from 1993 to 2000. Drug Alcohol Depend 79:413-422

Poelen EAP, Scholte RHJ, Willemsen G, Boomsma DI, Engels RCME (2007) Drinking by parents, siblings, and friends as predictors of regular alcohol use in adolescents and young adults: a longitudinal twin-family study. Alcohol Alcohol 42:362-369

Pollin TI, Damcott CM, Shen H, Ott SH, Shelton J, Horenstein RB et al. (2008) A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection. Science 322:1702-1705

Posthuma D, Beem AL, de Geus EJC, van Baal GCM, von Hjelmborg JB, Iachine I et al. (2003) Theory and practice in quantitative genetics. Twin Res Hum Genet 6:361-376

Posthuma D, Boomsma DI (2000) A note on the statistical power in extended twin designs. Behav Genet 30:147-158

Poustie VJ, Wildgoose J, Rutherford P (2009) Dietary interventions for phenylketonuria (Review). Cochrane Database Syst Rev

Pratt DS, Kaplan MM (2000) Evaluation of abnormal liver-enzyme results in asymptomatic patients. N Eng J Med 342:1266-1271

Prescott CA, Kendler KS (1999) Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. Am J Psychiatry 156:34-40

Purcell S (2002) Variance components models for gene-environment interaction in twin analysis. Twin Res 5:554-571

Purcell S, Neale BM, Todd-Brown K, Thomas L, Ferreira MAR, Bender D et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81:559-575

Purcell SM, Wray NR, Stone JL, Visscher PM, O'Donovan MC, Sullivan PF et al. (2009) Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. Nature 460:748-752

Quigley BM, Collins RL (1999) The modeling of alcohol consumption: a meta-analytic review. J Stud Alcohol Drugs 60:90-98

R Development Core Team (2012) R: a language and environment for statistical computing [Computer software]. Vienna: R Foundation for Statistical Computing

Rahav G, Wilsnack R, Bloomfield K, Gmel G, Kuntsche S (2006) The influence of societal level factors on men's and women's alcohol consumption and alcohol problems. Alcohol Alcohol 41:i47-i55

Rahmioglu N, Andrew T, Cherkas L, Surdulescu G, Swaminathan R, Spector TD et al. (2009) Epidemiology and genetic epidemiology of the liver function test proteins. PloS ONE 4:e4435

Rebollo I, De Moor MHM, Dolan CV, Boomsma DI (2006) Phenotypic factor analysis of family data: correction of the bias due to dependency. Twin Res Hum Genet 9:367-376

Reed T, Page WF, Viken RJ, Christian JC (1996) Genetic predisposition to organ□specific endpoints of alcoholism. Alcohol Clin Exp Res 20:1528-1533

Rehm J, Baliunas D, Borges GLG, Graham K, Irving H, Kehoe T et al. (2010) The relation between different dimensions of alcohol consumption and burden of disease: an overview. Addiction 105:817-843

Rehm J, Room R, Graham K, Monteiro M, Gmel G, Sempos CT (2003) The relationship of average volume of alcohol consumption and patterns of drinking to burden of disease: An overview. Addiction 98:1209-1228

Rehm J, Taylor B, Patra J (2006) Volume of alcohol consumption, patterns of drinking and burden of disease in the European region 2002. Addiction 101:1086-1095

Reinert DF, Allen JP (2007) The alcohol use disorders identification test: An update of research findings. Alcohol Clin Exp Res 31:185-199

Reuter S, Schnekenburger M, Cristofanon S, Buck I, Teiten MH, Daubeuf S et al. (2009) Tumor necrosis factor-α induces γ-glutamyltransferase expression via nuclear factor-κB in cooperation with Sp1. Biochem Pharmacol 77:397-411

Reynolds CA, Barlow T, Pedersen NL (2006) Alcohol, tobacco and caffeine use: Spouse similarity processes. Behav Genet 36:201-215

Rhee SH, Hewitt JK, Young SE, Corley RP, Crowley TJ, Stallings MC (2003) Genetic and environmental influences on substance initiation, use, and problem use in adolescents. Arch Gen Psychiatry 60:1256-1264

Rijsdijk FV, Sham PC (2002) Analytic approaches to twin data using structural equation models. Brief Bioinform 3:119-133

Rodgers JL, Kohler HP, McGue M, Behrman JR, Petersen I, Bingley P et al. (2008) Education and cognitive ability as direct, mediating, or spurious influences on female age at first birth: behavior genetic models fit to Danish twin data. AJS 114:S202-S232

Rose RJ, Dick DM, Viken RJ, Pulkkinen L, Kaprio J (2001) Drinking or abstaining at age 14? A genetic epidemiological study. Alcohol Clin Exp Res 25:1594-1604

Rose RJ, Kaprio J, Williams CJ, Viken R, Obremski K (1990) Social contact and sibling similarity: Facts, issues, and red herrings. Behav Genet 20:763-778

Ruhl CE, Everhart JE (2009) Elevated serum alanine aminotransferase and gamma-glutamyltransferase and mortality in the United States population. Gastroenterology 136:477-485

Salaspuro M (1999) Carbohydrate-deficient transferrin as compared to other markers of alcoholism: A systematic review. Alcohol 19:261-271

Saraceno L, Munafo M, Heron J, Craddock N, Van Den Bree M (2009) Genetic and non‐genetic influences on the development of co‐occurring alcohol problem use and internalizing symptomatology in adolescence: a review. Addiction 104:1100-1121

Sartor CE, Agrawal A, Lynskey MT, Bucholz KK, Heath AC (2008) Genetic and environmental influences on the rate of progression to alcohol dependence in young women. Alcohol Clin Exp Res 32:632-638

Sartor CE, Grant JD, Bucholz KK, Madden PAF, Heath AC, Agrawal A et al. (2010) Common genetic contributions to alcohol and cannabis use and dependence symptomatology. Alcohol Clin Exp Res 34:545-554

Sartor CE, Lynskey MT, Bucholz KK, Madden PAF, Martin NG, Heath AC (2009) Timing of first alcohol use and alcohol dependence: evidence of common genetic influences. Addiction 104:1512-1518

Saunders JB, Aasland OG, Babor TF, Fuente JR, Grant M (1993) Development of the alcohol use disorders identification test (AUDIT): WHO collaborative project on early detection of persons with harmful alcohol consumption. Addiction 88:791-804

Scarr S (1968) Environmental bias in twin studies. Eugen Q 15:34-40

Schermelleh-Engel K, Moosbrugger H, Muller H (2003) Evaluating the fit of structural equation models: Tests of significance and descriptive goodness-of-fit measures. Methods Psychol Res Online 8:23-74

Schindhelm RK, Diamant M, Dekker JM, Tushuizen ME, Teerlink T, Heine RJ (2006) Alanine aminotransferase as a marker of non-alcoholic fatty liver disease in relation to type 2 diabetes mellitus and cardiovascular disease. Diabetes Metab Res Rev 22:437-443

Schmitz S, Cherny SS, Fulker DW (1998) Increase in power through multivariate analyses. Behav Genet 28:357-363

Schuckit MA (2009) An overview of genetic influences in alcoholism. J Subst Abuse Treat 36:S5-S14

Schuckit MA, Daeppen JB, Tipp JE, Hesselbrock M, Bucholz KK (1998) The clinical course of alcohol-related problems in alcohol dependent and nonalcohol dependent drinking women and men. J Stud Alcohol Drugs 59:581-590

Schuckit MA, Smith TL, Kalmijn J (2004) The search for genes contributing to the low level of response to alcohol: patterns of findings across studies. Alcohol Clin Exp Res 28:1449-1458

Schumann G, Coin LJ, Lourdusamy A, Charoen P, Berger KH, Stacey D et al. (2011) Genome-wide association and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. Proc Natl Acad Sci 108:7119-7124

Scouller K, Conigrave KM, Macaskill P, Irwig L, Whitfield JB (2000) Should we use carbohydrate-deficient transferrin instead of gamma-glutamyltransferase for detecting problem drinkers? A systematic review and metaanalysis. Clin Chem Lab Med 46:1894-1902

Segal, NL, Johnson, W (2009) Twin studies of general mental ability. In Y.-K.Kim (Ed.), Handbook of behavior genetics (pp. 81-99). New York: Springer

Shibuya A, Yasunami M, Yoshida A (1989) Genotypes of alcohol dehydrogenase and aldehyde dehydrogenase loci in Japanese alcohol flushers and nonflushers. Hum Genet 82:14-16

Sierksma A, Patel H, Ouchi N, Kihara S, Funahashi T, Heine RJ et al. (2004) Effect of moderate alcohol consumption on adiponectin, tumor necrosis factor-α, and insulin sensitivity. Diabetes Care 27:184-189

Sierksma A, Van Der Gaag MS, Kluft C, Hendriks HFJ (2002) Moderate alcohol consumption reduces plasma C-reactive protein and fibrinogen levels: a randomized diet-controlled intervention study. Eur J Clin Nutr 56:1130-1136

Sillanaukee P, Alho H, Strid N, Jousilahti P, Vartiainen E, Olsson U et al. (2000a) Effect of hormone balance on carbohydrate‐deficient transferrin and gamma-glutamyltransferase in female social drinkers. Alcohol Clin Exp Res 24:1505-1509

Sillanaukee P, Massot N, Jousilahti P, Vartiainen E, Sundvall J, Olsson U et al. (2000b) Dose response of laboratory markers to alcohol consumption in a general population. Am J Epid 152:747-751

Sillanaukee P, Olsson U (2001) Improved diagnostic classification of alcohol abusers by combining carbohydrate-deficient transferrin and gamma-glutamyltransferase. Clin Chem Lab Med 47:681-685

Skurtveit S, Tverdal A (2002) Sex differences in gamma-glutamyltransferase in people aged 40-42 years in two Norwegian counties. Drug Alcohol Depend 67:95-98

Slof-Op't Landt MCT, van Furth EF, Rebollo-Mesa I, Bartels M, Van Beijsterveldt CEM, Slagboom PE et al. (2009) Sex differences in sum scores may be hard to interpret: The importance of measurement invariance. Assessment 16:415-423

Slutske WS, D'Onofrio BM, Turkheimer E, Emery RE, Harden KP, Heath AC et al. (2008) Searching for an environmental effect of parental alcoholism on offspring alcohol use

disorder: A genetically informed study of children of alcoholics. J Abnorm Psychol 117:534-551

Smit E, Verdurmen J, Monshouwer K, Smit F (2008) Family interventions and their effect on adolescent alcohol use in general populations: A meta-analysis of randomized controlled trials. Drug Alcohol Depend 97:195-206

Smith L, Watson M, Gates S, Ball D, Foxcroft D (2008) Meta-analysis of the association of the Taq1A polymorphism with the risk of alcohol dependency: a HuGE gene-disease association review. Am J Epid 167:125-138

So HC, Li M, Sham PC (2011) Uncovering the total heritability explained by all true susceptibility variants in a genome-wide association study. Genet Epidemiol 35:447-456

Sookoian S, Pirola CJ (2011) Meta-Analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. Hepatology 53:1883-1894

Sorbi D, Boynton J, Lindor KD (1999) The ratio of aspartate aminotransferase to alanine aminotransferase: potential value in differentiating nonalcoholic steatohepatitis from alcoholic liver disease. Am J Gastroenterol 94:1018-1022

Speed D, Hemani G, Johnson M, Balding DJ (2012) Improved heritability estimation from genome-wide SNPs. Am J Hum Genet 91:1011-1021

Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU et al. (2010) Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet 42:937-948

Stahl EA, Wegmann D, Trynka G, Gutierrez-Achury J, Do R, Voight BF et al. (2012) Bayesian inference analyses of the polygenic architecture of rheumatoid arthritis. Nat Genet 44:483-489

Stallings MC, Hewitt JK, Beresford T, Heath AC, Eaves LJ (1999) A twin study of drinking and smoking onset and latencies from first use to regular use. Behav Genet 29:409-421

Steffensen FH, Sorensen HT, Brock A, Vilstrup H, Lauritzen T (1997) Alcohol consumption and serum liver-derived enzymes in a Danish population aged 30-50 years. Int J Epid 26:92-99

Stewart SH, Mainous AG, Gilbert G (2002) Relation between alcohol consumption and C-reactive protein levels in the adult US population. J Am Board Fam Pract 15:437-442

Stranger BE, Stahl EA, Raj T (2011) Progress and promise of genome-wide association studies for human complex trait genetics. Genetics 187:367-383

Stromme JH, Rustad P, Steensland H, Theodorsen L, Urdal P (2004) Reference intervals for eight enzymes in blood of adult females and males measured in accordance with the

International Federation of Clinical Chemistry reference system at 37 C: part of the Nordic Reference Interval Project. Scand J Clin Lab Invest 64:371-384

Sugiura M, Nakamura M, Ikoma Y, Yano M, Ogawa K, Matsumoto H et al. (2005) High serum carotenoids are inversely associated with serum gamma-glutamyl transferase in alcohol drinkers within normal liver function. J Epid 15:180-186

Sullivan PF, de Geus EJC, Willemsen G, James MR, Smit JH, Zandbelt T et al. (2009) Genome-wide association for major depressive disorder: a possible role for the presynaptic protein piccolo. Mol Psychiatry 14:359-375

Sung J, Lee K, Song YM (2011) Heritabilities of Alcohol Use Disorders Identification Test (AUDIT) scores and alcohol biomarkers in Koreans: The KoGES (Korean Genome Epi Study) and Healthy Twin Study. Drug Alcohol Depend 113:104-109

Swan GE, Carmelli D, Cardon LR (1996) The consumption of tobacco, alcohol, and coffee in Caucasian male twins: a multivariate genetic analysis. J Subst Abuse 8:19-31

Takeshita T, Mao XQ, Morimoto K (1996) The contribution of polymorphism in the alcohol dehydrogenase beta subunit to alcohol sensitivity in a Japanese population. Hum Genet 97:409-413

Takeshita T, Yang X, Morimoto K (2000) The ALDH2 genotype, alcohol intake, and liver-function biomarkers among Japanese male workers. Hum Genet 106:589-593

Takeuchi F, Isono M, Nabika T, Katsuya T, Sugiyama T, Yamaguchi S et al. (2011) Confirmation of ALDH2 as a major locus of drinking behavior and of its variants regulating multiple metabolic phenotypes in a Japanese population. Circ J 75:911-918

Targher G (2009) Elevated serum gamma-glutamyltransferase activity is associated with increased risk of mortality, incident type 2 diabetes, cardiovascular events, chronic kidney disease and cancer: A narrative review. Clin Chem Lab Med 48:147-157

Tipping S, Hope S, Pickering K, Erens B, Roth MA, Mindell JS (2010) The effect of mode and context on survey results: Analysis of data from the Health Survey for England 2006 and the Boost Survey for London. BMC Med Res Methodol 10:84

Treutlein J, Cichon S, Ridinger M, Wodarz N, Soyka M, Zill P et al. (2009) Genome-wide association study of alcohol dependence. Arch Gen Psychiatry 66:773-784

Trimbos Institute (2010) National Drug Monitor 2009 Utrecht: Trimbos Institute.

True WR, Xian H, Scherrer JF, Madden PAF, Bucholz KK, Heath AC et al. (1999) Common genetic vulnerability for nicotine and alcohol dependence in men. Arch Gen Psychiatry 56:655-661

Tseng YM, Jin YR, Chen IJ, Huang FD, Wu SH, Ma H et al. (2008) Roles of the genetic variation of alcohol-metabolizing enzymes on biomarkers in trauma patients with excessive alcohol intake at emergency department. Clin Chim Acta 389:14-18

Van Beek JHDA, de Moor MHM, de Geus EJC, Lubke GH, Vink JM, Willemsen G, Boomsma DI (2013a) The genetic architecture of liver enzyme levels: GGT, ALT and AST. Behav Genet 43:329-339

Van Beek JHDA, de Moor MHM, Geels LM, Sinke MRT, Geus EJC, Lubke, GH, Kluft C, Neuteboom J, Vink JM, Willemsen G, Boomsma DI (2013b) The association of alcohol intake with gamma-glutamyl transferase (GGT) levels: evidence for a causal effect? (under review)

Van Beek JHDA, de Moor MHM, Geels LM, Willemsen G, Boomsma DI (2013c) Explaining differences in alcohol intake: evidence for genetic and cultural transmission? (under review)

Van Beek JHDA, Lubke GH, de Moor MHM, Willemsen G, de Geus EJC, Hottenga JJ, Walters RK, Smit JH, Penninx BWJH, Boomsma DI. Heritability of GGT, ALT and AST levels estimated from genome-wide SNP data and its genetic covariance with alcohol use (in preparation)

Van Beek JHDA, Willemsen G, de Moor MHM, Hottenga JJ, Boomsma DI (2010) Associations between ADH gene variants and alcohol phenotypes in Dutch adults. Twin Res Hum Genet 13:30-42

van Beijsterveldt CEM, Groen-Blokhuis M, Hottenga JJ, Franic S, Hudziak JJ, Lamb D et al. (2012) The Young Netherlands Twin Register (YNTR): Longitudinal twin and family studies in over 70,000 children. Twin Res Hum Genet FirstView:1-16

Van den Brink, W (2010) Geschiedenis en classificatie. In I Franken, W van den Brink (Eds.), Handboek verslaving (pp. 13-25). Utrecht: De Tijdstroom

Van Dongen J et al. The heritability of metabolic syndrome traits in a large population-based sample (in preparation).

Van Dongen J, Draisma HHM, Martin NG, Boomsma DI (2012) The continuing value of twin studies in the omics era. Nat Rev Genet 13:640-653

Van Grootheest DS, van den Berg SM, Cath DC, Willemsen G, Boomsma DI (2008) Marital resemblance for obsessive□compulsive, anxious and depressive symptoms in a population-based sample. Psychol Med 38:1731-1740

Van Laar, MW, Cruts, AAN, Verdurmen, JEE, van Ooyen-Houben, MMJ, Meijer, RF (2008) The Netherlands drug monitor: Annual report 2007 Utrecht: Trimbos Institute

Vernon G, Baranova A, Younossi ZM (2011) Systematic review: The epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 34:274-285

Vidali M, Stewart SF, Albano E (2008) Interplay between oxidative stress and immunity in the progression of alcohol-mediated liver injury. Trends Mol Med 14:63-71

Viken RJ, Kaprio J, Koskenvuo M, Rose RJ (1999) Longitudinal analyses of the determinants of drinking and of drinking to intoxication in adolescent twins. Behav Genet 29:455-461

Villafuerte S, Heitzeg MM, Foley S, Yau WYW, Majczenko K, Zubieta JK et al. (2011) Impulsiveness and insula activation during reward anticipation are associated with genetic variants in GABRA2 in a family sample enriched for alcoholism. Mol Psychiatry 17:511-519

Vink JM, Beem AL, Posthuma D, Neale MC, Willemsen G, Kendler KS et al. (2004a) Linkage analysis of smoking initiation and quantity in Dutch sibling pairs. Pharmacogenomics J 4:274-282

Vink JM, Staphorsius AS, Boomsma DI (2009) A genetic analysis of coffee consumption in a sample of Dutch twins. Twin Res Hum Genet 12:127-131

Vink JM, Willemsen G, Stubbe JH, Middeldorp CM, Ligthart L, Baas KD et al. (2004b) Estimating non-response bias in family studies: application to mental health and lifestyle. Eur J Epid 19:623-630

Visscher PM (2010) A commentary on 'Common SNPs explain a large proportion of the heritability for human height' by Yang et al. (2010) Twin Res Hum Genet 13:517-524

Visscher PM, Brown MA, McCarthy MI, Yang J (2012) Five years of GWAS discovery. Am J Hum Genet 90:7-24

Volpato S, Pahor M, Ferrucci L, Simonsick EM, Guralnik JM, Kritchevsky SB et al. (2004) Relationship of alcohol intake with inflammatory markers and plasminogen activator inhibitior-1 in well-functioning older adults. Circulation 109:607-612

Walters GD (2002) The heritability of alcohol abuse and dependence: a meta-analysis of behavior genetic research. The American journal of drug and alcohol abuse 28:557-584

Wang HJ, Zakhari S, Jung MK (2010) Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development. World J Gastroenterol 16:1304-1313

Wang JC, Kapoor M, Goate AM (2012) The genetics of substance dependence. Annu Rev Genomics Hum Genet 13:241-261

Wannamethee SG, Shaper AG (2010) Cigarette smoking and serum liver enzymes: the role of alcohol and inflammation. Ann Clin Biochem 47:321-326

Wannamethee SG, Whincup PH, Rumley A, Lowe GDO (2007) Inter-relationships of interleukin-6, cardiovascular risk factors and the metabolic syndrome among older men. J Thromb Heamostat 5:1637-1643

Whitfield JB (2001) Gamma glutamyl transferase. Crit Rev Clin Lab Sci 38:263-355

Whitfield JB, Martin NG (1985) Individual differences in plasma ALT, AST and GGT: contributions of genetic and environmental factors, including alcohol consumption. Enzyme 33:61-69

Whitfield JB, Zhu G, Madden PA, Neale MC, Heath AC, Martin NG (2004) The genetics of alcohol intake and of alcohol dependence. Alcohol Clin Exp Res 28:1153-1160

Whitfield JB, Zhu G, Nestler JE, Heath AC, Martin NG (2002) Genetic covariation between serum γ-glutamyltransferase activity and cardiovascular risk factors. Clin Chem 48:1426-1431

WHO (2008) The global burden of disease 2004 update. Geneva: WHO press.

Wichers M, Gardner C, Maes HH, Lichtenstein P, Larsson H, Kendler KS (2013) Genetic innovation and stability in externalizing problem behavior across development: A multi-informant twin-study. Behav Genet 43:191-201

Willemsen G, de Geus EJC, Bartels M, Van Beijsterveldt CEM, Brooks AI, Estourgie-van Burk GF et al. (2010) The Netherlands Twin Register biobank: A resource for genetic epidemiological studies. Twin Res Hum Genet 13:231-245

Willemsen G, Posthuma D, Boomsma DI (2005) Environmental factors determine where the Dutch live: results from the Netherlands twin register. Twin Res Hum Genet 8:312-317

Willemsen G, Vink JM, Abdellaoui A, Den Braber A, Van Beek JHDA, Draisma HHM et al. (2013) The Adult Netherlands Twin Register: 25 years of survey and biological data collection. Twin Res Hum Genet 16:271-281

Wilsnack RW, Vogeltanz ND, Wilsnack SC, Harris TR (2000) Gender differences in alcohol consumption and adverse drinking consequences: cross□cultural patterns. Addiction 95:251-265

World Health Organization (1992) International classification of mental and behavioural disorders (ICD-10)

World Health Organization (2011) Global Status Report on Alcohol and Health 2011. http://www.who.int/substance_abuse/publications/global_alcohol_report/en/index.html [On-line]

Wray NR, Goddard ME, Visscher PM (2008) Prediction of individual genetic risk of complex disease. Curr Opin Genet Dev 18:257-263

Wu D, Cederbaum AI (2003) Alcohol, oxidative stress, and free radical damage. Alcohol Res Health 27:277-284

Wu D, Zhai Q, Shi X (2006) Alcohol-induced oxidative stress and cell responses. J Gastroenterol Hepatol 21:S26-S29

Xian H, Scherrer JF, Eisen SA, True WR, Heath AC, Goldberg J et al. (2000) Self-reported zygosity and the equal-environments assumption for psychiatric disorders in the Vietnam Era Twin Registry. Behav Genet 30:303-310

Xian H, Scherrer JF, Grant JD, Eisen SA, True WR, Jacob T et al. (2008) Genetic and environmental contributions to nicotine, alcohol and cannabis dependence in male twins. Addiction 103:1391-1398

Yamada J, Tomiyama H, Yambe M, Koji Y, Motobe K, Shiina K et al. (2006) Elevated serum levels of alanine aminotransferase and gamma glutamyltransferase are markers of

inflammation and oxidative stress independent of the metabolic syndrome. Atherosclerosis 189:198-205

Yamada T, Fukatsu M, Suzuki S, Yoshida T, Tokudome S, Joh T (2010) Alcohol drinking may not be a major risk factor for fatty liver in Japanese undergoing a health checkup. Dig Dis Sci 55:176-182

Yang J, Benyamin B, McEvoy BP, Gordon S, Henders AK, Nyholt DR et al. (2010) Common SNPs explain a large proportion of the heritability for human height. Nat Genet 42:565-569

Yang J, Ferreira T, Morris AP, Medland SE, Madden PAF, Heath AC et al. (2012) Conditional and joint multiple-SNP analysis of GWAS summary statistics identifies additional variants influencing complex traits. Nat Genet 44:369-375

Yang J, Lee SH, Goddard ME, Visscher PM (2011a) GCTA: a tool for genome-wide complex trait analysis. Am J Hum Genet 88:76-82

Yang J, Manolio TA, Pasquale LR, Boerwinkle E, Caporaso N, Cunningham JM et al. (2011b) Genome partitioning of genetic variation for complex traits using common SNPs. Nat Genet 43:519-525

Yuan X, Waterworth D, Perry JRB, Lim N, Song K, Chambers JC et al. (2008) Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. Am J Hum Genet 83:520-528

Zakhari S (2006) Overview: How is alcohol metabolized by the body? Alcohol Res Health 29:245-254

Zintzaras E, Stefanidis I, Santos M, Vidal F (2006) Do alcohol-metabolizing enzyme gene polymorphisms increase the risk of alcoholism and alcoholic liver disease? Hepatology 43:352-361