GENETICS OF ALCOHOL USE AND LIVER ENZYMES: 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 risk of problematic drinking, 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.