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Alcohol Consumption among Adolescents

Psychosocial and Genetic influences

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Abstract
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The present thesis is based on four studies focusing on alcohol consumption among Swedish adolescents, and therewith related psychosocial and genetic factors.

One main objective was to study the reasons for drinking alcohol among different population - representative samples of adolescents in order to identify motives for drinking. Relationships between these drinking motives, alcohol consumption, and alcohol - related problems were also investigated. Three motives emerged from this study: social - enhancement, coping and dominance. The association with alcohol consumption and alcohol - related problems was positive for social - enhancement and coping motives, but negative for the dominance motive.

A significant heritability of alcohol use disorders has been demonstrated by family, adoption and twin studies. Environmental influences have also been acknowledged to play an important role in the development of alcohol use disorders. Moreover, the interaction between genetic and environmental factors is likely to influence the risk - resilience for alcohol use disorders. In view of this knowledge, plausible candidate polymorphisms were considered in gene - environment interaction models. An effect of the genetic polymorphisms was only present when a G x E model was considered. A genetic variant of the clock gene *Period2*, in an interaction with sleep problems, was studied in relation to alcohol consumption among adolescents. High alcohol consumption was associated with the AA genotype of the *PER2* SNP10870 polymorphism, in an interaction with several and frequent sleep problems, among adolescent boys. A genetic variant in the *opioid μ receptor 1* gene, in an interaction with alcohol consumption, was studied in relation to depressive symptoms. Depressive symptoms were predicted by the G allele of the *OPRM1* A118G polymorphism, in an interaction with high alcohol consumption, among adolescent girls. Additionally, the *PER2* SNP10870 and the *OPRM1* A118G polymorphisms were studied in a sample of severely alcoholic females.

Furthermore, alcohol consumption was assessed by using different instruments, such as biomarkers and surveys. Comparisons were carried out to identify the most suitable method to assess alcohol consumption among adolescents. Questionnaire and interview seemed more suitable tools than biomarkers in this regard.The results eventually support the importance of psychosocial and genetic influences, and their interaction effect on alcohol consumption among adolescents.

Keywords: adolescents, alcohol, biomarkers, depression, drinking motives, FAEE, gene environment interaction, interview, OPRM1, PER2, PEth, questionnaire, sleep

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Ai miei cari

Back cover: “Nature - nurture interplay: genetic influences on behaviour in different environments and environmental influences on gene expression”, Erika Comasco

Erika Comasco (born 19/11/1982) graduated in 2006 with a M.Sc. in Pharmacy from the University of Pavia, Italy. Since 2005, she has been a research student at the Department of Neuroscience, Uppsala University, Sweden.

List of Papers

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

I Why do adolescents drink? Motivational patterns related to alcohol consumption and alcohol-related problems.
Substance use and misuse (2010) 45:1532–1547 *In press*
Comasco, E., Berglund, K., Oreland, L., Nilsson, K. W.

II The clock gene *PER2*: association with alcohol consumption and sleep problems among adolescents.
Upsala Journal of Medical Science (2010) 115:41-48
Comasco, E., Nordquist, N., Göktürk, C., Åslund, C., Hallman, J., Oreland, L., Nilsson, K. W.

III The Asn40Asp polymorphism of the μ-opioid receptor gene and alcohol: association with psychiatric conditions in severely alcoholic females and with depressive symptoms among adolescents - *Manuscript*
Comasco, E., Göktürk, C., Nordquist, N., Oreland, L., Nilsson, K. W., Hallman, J.

IV Adolescent alcohol consumption: biomarkers PEth and FAEE in relation to interview and questionnaire data.
Journal of studies on alcohol and drugs (2009) 70:797–804
Comasco, E., Nordquist, N., Leppert, J., Oreland, L., Kronstrand, R., Alling, C., Nilsson, K. W.

Contents

Abbreviations

A	Adenine
Asn	Asparagine
Asp	Aspartate
AUDIT	Alcohol use disorders identification test
FAEE	Fatty acid ethyl esters
G	Guanine
G x E	Gene - environment interaction
HPA axis	Hypothalamic - pituitary - adrenal axis
OPRM1	Opioid receptor mu 1
PER2	Period 2
PEth	Phosphatidylethanol
SNP	Single nucleotide polymorphism

Introduction

Alcohol consumption among adolescents

Excessive drinking can cause illness and distress not only to the drinker, but also to the society, resulting in physical and mental harm, social problems, addiction, suicide and early death [1]. Globally, the World Health Organization has reported alcohol as being one of the leading risk factors for morbidity and mortality world-wide, with approximately 1.8 million caused deaths annually, and representing a considerable economic problem for many communities around the world [1, 2]. A substantial proportion of these deaths is the result of injuries caused by hazardous and harmful drinking, such as road traffic injuries and interpersonal violence [2].

World-wide, five per cent of all deaths of individuals between the ages of 15 and 29 have been attributed to alcohol use [3]. Underage drinking increases the risk of developing alcohol use disorders later in life, with an inverse proportional relation to the age of initiation [3]. Alcohol consumption during adolescence is proved to affect brain development, and to have harmful consequences on cognitive and behavioural functions [4]. Among Swedish adolescents, 22% during the early 2000s, and 13% during mid-2000s reported their first episode of drunkenness to be before the age of 14 [5, 6].

Figure 1. Retrospective self-report of age at first episode of drunkenness among 17–18 year old Swedish adolescents (SALVe 2006: ■ boys □ girls).

In Sweden, alcoholic beverages containing more than 3.5 (v/v) % ethanol are only sold in state-controlled outlets ("Systembolag") and restaurants. The minimum age for the purchase of alcohol is 20 years of age in the outlets and 18 years in restaurants. In grocery stores, where beverages containing 3.5 (v/v) % ethanol or less are sold, the minimum age for purchase is 18 years of age. Despite these age limits, Swedish adolescents have easy access to alcohol (81% to beer and cider, and 67% to spirits), usually through older peers, siblings or even parents [6].

During the last few decades, survey data have provided evidence of a convergence in drinking habits among adolescents towards patterns related to youth cultures, and associated in developed countries with episodic heavy drinking, called "binge" drinking. Binge drinking has been described as a pattern of drinking alcohol that corresponds to consuming five or more drinks at one occasion [7], although a cut-off of three or more has been proposed for adolescent females [8]. The ESPAD report from 2007, a cross-national screening study on substance use among 15-16 year-old adolescents, reported average alcohol consumption on the latest occasion to be higher among Swedish adolescents than the European average (5.2 cl. compared with 4.2 cl. 100%). The frequency of alcohol use during the previous 12 months among Swedish adolescents was below the European mean. More than one third (37%) of Swedish adolescents reported drunkenness during the same period, which was almost equal to the European average [6]. Binge drinking episodes in the last month were reported once or twice by 18% and 23%, and three or more episodes by 17% and 16%, of boys and girls, respectively [6]. There is therefore increasing evidence that besides volume of alcohol, the drinking pattern itself is also relevant to study alcohol consumption among adolescents.

Furthermore, in recent decades there has been growing concern about sex differences in drinking behaviour [9]. Sexual equality is a fundamental imperative of Swedish society. A common hypothesis about convergence in drinking patterns is that increased opportunities for females to perform traditionally male roles have also enabled and encouraged women to increase their drinking. However, convergence between the sexes is usually more evident among young adolescents than adults [10]. Differences between males and females regarding patterns of alcohol consumption have become smaller among Swedish adolescents [6, 11]. The proportion of Swedish adolescents reporting having had five or more drinks on one occasion during the last 30 days was 39% among boys and 35% among girls in 2003, while in 2007 it was 36% and 39% respectively [6].

The main focus of this project has been on alcohol use and abuse among adolescents. *Adolescence* is a transitory period extraordinarily rich in physiological, psychological and lifestyle changes that can lead to an escalation of alcohol use, and alcohol-related problems, as well as to an increased vulnerability to the damaging effects of alcohol [4, 12-15]. Adolescents have been shown to be more resistant to negative effects of alcohol (e.g. acute effects such as motor impairment or sedation, and long-term effects such as hangover and anxiety), but more sensitive to the positive effects of ethanol [16]. Brain development continues throughout adolescence, experiencing changes in synaptic plasticity, neural connectivity, and functioning of neurotransmitter and endocrine systems. Structural and functional brain changes are also constantly influenced by environmental stimuli, to which an adaptation response has to follow [4, 15]. Many stressors, as well as alcohol use, can negatively affect several neurobiological systems, as well as the structural and functional development of the brain (e.g. memory tasks)[4]. Moreover, rapid physical growth and hormonal changes occurring with the beginning of puberty lead to changes in adolescent self-image and self-esteem [15]. The establishment of relationships outside the family, an increasingly independent will, and disagreement with parental restrictions often result in the search for an own identity [12, 13]. In summary, adolescence represents a transitional and critical stage of life, and a time linked to alcohol initiation.

Drinking motives

Alcohol use typically begins in early adolescence and increases sharply in late adolescence. Investigations on the role of cognition and emotions, as well as of expectancies associated with alcohol use, have identified different domains (e.g. social reinforcement, tension reduction) [17]. Four major categories of motives for drinking have been found. The distinction regards the valence of the expected effect (i.e. positive and negative), and the source of the motive (i.e. expectancies related to a collective/social experience, and to an introspective/ emotional experience) [17-19].

The majority of adolescents indicates the pleasurable aspects of drinking, such as the enjoyable taste of alcohol, and its ability to make an individual feel good or high, as main reasons for drinking. Additionally, reasons of conformity, such as peer pressure, are addressed [17]. Indeed, the first time adolescents try alcohol is usually with peers, and alcohol use could be the result of peer pressure for the adolescent to be accepted by the group. Adolescents report also enhancement motives [17]. Adolescence is characterized not only by a high level of social interaction, but also by high sensation and novelty seeking, impulsiveness and risk taking behaviour. Enhancement motives might encompass reasons for drinking such as to get high, to try new things, to do something forbidden (minimum legal age law), and to take part in exciting activities that may be dangerous. An alternative set of reasons for drinking is represented by coping motives [17]. Adolescents expect

that alcohol will help them to relax or relieve tension, and to forget about their problems; they are drinking to cope with their problematic situations. Such reasons are usually reported by those adolescents with a disturbed family situation, who have internalizing behaviour, distress, are upset, feel ill or lack confidence, as well as those suffering episodes of bullism and maltreatment. All these circumstances make the adolescent more vulnerable, and at a greater risk for alcohol use disorder [17]. However, adolescents with the same problems might also indicate reasons for drinking that pertain to the enhancement motive sphere, such as drinking to get high and because it gives a pleasant feeling. Furthermore, psychopathological symptoms are often indicated as precursors of alcohol use initiation or misuse, and thus could have an influence on drinking motives. Of importance there are conduct disorders, attention deficit hyperactive disorders, depression and anxiety disorders, personality traits such as impulsiveness, and antisocial, aggressive and disruptive behaviours [12, 13, 20].

Several screening tools have been developed to assess reasons for drinking both among adolescents and adults (e.g. the Drinking Motives Questionnaire, and its Adolescent Version developed by Cooper *et al.* [21, 22]) [17]. These questionnaires usually consist of 10-30 items administered in a random order. The responses are then analyzed to identify hidden structures regarding motives for drinking.

Drinking motives represent the proximal antecedents of behaviours such as alcohol consumption. To investigate why adolescents drink can be useful to understand the reasons promoting and maintaining alcohol consumption, as well as for developing prevention and intervention strategies.

Assessment of alcohol consumption

At clinical levels, the diagnostic criteria indicated by the Diagnostic and Statistical Manual of Mental Disorders [23], or by the International Classification of Disorders [24], are used to diagnose alcohol use disorders. For other purposes, the assessment of alcohol consumption or alcohol use disorders symptoms is based on screening tools. A screening tool is usually developed by comparing it with a reference diagnostic tool, also called the gold-standard. One way to define a test is by using the *sensitivity* and *specificity* parameters [25]. Sensitivity is defined as the percentage of all individuals with a condition of interest (e.g. alcoholism) who are correctly identified by the instrument as having the condition. Specificity is defined as the percentage of all patients without a condition of interest who are correctly identified as not having the condition [25].

There are different forms of surveys, with both advantages and disadvantages, which can be used: self-reports, such as paper questionnaires and web surveys, as well as personal and telephone interviews [26-29]. The basic requirement of a screening tool for alcohol consumption is to provide a defini-

tion of the alcohol content, and the serving size of the beverages corresponding to one drink-unit. There are several screening instruments to identify individuals at potential risk of alcohol use disorders, such as the Alcohol Use Disorders Identification Test, and the Adolescent Alcohol Involvement Scale [30]. Short tests mainly aim to assess average frequency of drinking and average quantity per occasion, in a brief period of time, such as AUDIT-C, FAST, CRAFFT, DSM-IV-2-item scale [31]. The Alcohol Use Disorders Identification Test (AUDIT) is a 10 item questionnaire covering hazardous alcohol use, dependence symptoms and harmful alcohol use [32]. The AUDIT-C includes the first three questions of the AUDIT, and thus measures hazardous alcohol use [32, 33]. These indexes can be used as an ordinal scale, or as dichotomous variables when cut-offs are applied, although official thresholds have not been given [31, 34].

Additionally, a bogus-pipeline procedure can be applied to potentially increase reliability of the answers, and decrease bias due to social desirability [35, 36]. The technique consists in alerting the participant that veracity of their responses will be verified by an objective measurement (e.g. biological samples will be analyzed for the presence of alcohol) [37].

Biomarkers

An alternative way of assessing alcohol consumption is represented by the use of biological markers [38]. Biomarkers for alcohol can be defined as objectively measurable and parametrically quantifiable indicators of presence and quantity of past-alcohol consumed [39]. They are valuable tools to monitor alcohol consumption, to screen and diagnose alcohol use disorder, as well as to give evidence of abstinence [38]. Biomarker measures can be obtained from blood, hair, or urine samples, with time estimates of alcohol consumed that can range from the previous hours to the previous months.

Alcohol is mainly metabolized in an oxidative manner in the liver by alcohol dehydrogenase to acetaldehyde, and further to acetic acid by aldehyde dehydrogenase; a minimum part of ethanol is excreted in the breath and urine, and the rest of ethanol is metabolized in a non-oxidative manner [40]. Biomarkers used to measure alcohol consumption are related to products of the alcohol metabolism, or reflect changes in other compounds, cells, or tissues that result from alcohol exposure [38].

Among the most common markers for alcohol consumption are the blood alcohol concentration (BAC) measured in blood samples, and the air expiration, saliva, or urine tests. However, the short half-life of ethanol is a limiting factor for the usefulness of these tests [40]. At present only one study attempted to assess adolescent alcohol consumption with a theoretical estimation of BAC calculated by using a mathematical formula [8]. Hence, assessment of adolescent alcohol consumption with biomarkers remains to be explored. Among others, there are indirect biomarkers which indicate alcohol-induced liver damage, and thus are mainly suitable to target alcoholism.

Gamma glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes whose levels are elevated by liver enzyme induction. Carbohydrate-deficient transferrin (CDT) comprises a group of glycoprotein isoforms for transporting iron, which, after alcohol consumption, are deficient in carbohydrate chains with sialic acid residues. Mean corpuscular volume (MCV) measures the volume of red blood cells, which increases after alcohol exposure [38].

Other recently developed biomarkers include direct biomarkers derived from the non-oxidative metabolism of alcohol, still containing the ethyl group [38]. Phosphatidyl ethanol (PEth) is formed after ethanol intake from phosphatidylcholin by the enzyme phospholipase D [41], which normally catalyzes the formation of phosphatidic acid using water and phosphatidylcholin as substrates. PEth has been suggested as a promising marker of recent heavy drinking because of its high specificity and sensitivity [42]. Determination of PEth can be performed in whole blood extracts, mainly in erythrocytes [43]. PEth remains detectable in alcoholic blood for up to two weeks after abstinence [42]. Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are direct metabolites of alcohol formed from the conjugation with glucuronic acid and sulphate, respectively. EtG can be detected in the blood up to 36 hours, in the urine up to one week after heavy alcohol consumption, and in hair for months [38, 44]. Fatty acid ethyl esters (FAEEs) are metabolic products mainly resulting from the interaction between ethanol and fatty acids. They can be measured, both in hair and blood, as a combination of four different esters: ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate [45]. Additionally, other different markers have been considered [38], such as the 5-hydroxytryptophol (5HTOL) a metabolite of serotonin used as a biomarker in urine analyses [46].

Several biomarker methods have thus been studied, with both strengths and weaknesses [38]. The choice of one biomarker instead of another is influenced by the time window of detection of the biomarker, the biological sample available for analyses (blood, urine, hair, breath-test), and the specificity and sensitivity associated with the biomarker [38].

In a complementary way, many studies tend to use more than one biomarker together with survey reports to increase the specificity and sensitivity of alcohol consumption measurement.

Gene-environment interaction

Genetic and environmental interactive influences on human behaviours and psychiatric disorders are widely recognized [47, 48]. A milestone study in this field of research was carried out by Caspi *et al.* in 2002, which demonstrated a significant interaction effect between a genetic variation and psychosocial environment on risk of antisocial behaviour among young males [49]. Subsequently, with the rapid development of molecular technology for genetic analysis, and the extraordinary flow of data from the human genome project, a significant body of literature has confirmed the presence of G x E interactions in psychiatric disorders [50]. However, the neurobiological processes involved in G x E still need to be elucidated [51, 52]. Twins, monozygotic and dizygotic pairs, have been studied in relation to several psychiatric disorders to distinguish between nurture and nature influences (e.g. see [53]). Genetic and environmental contributions to the different phenotypes have been shown to be considerably heterogeneous. Each single genetic variant has only a modest effect on a given phenotype in an interaction with environmental factors, and the interaction effect is likely to differ during development [20, 48, 54, 55].

Alcohol use disorder is a multi-factorial disorder, in which both genetic and environmental factors interact. On the one side, a genetic heritability has been demonstrated to account for ≈50% of the liability to develop alcohol use disorder. On the other side, it has been shown that alcohol use disorders are influenced by psychosocial and cultural factors [52, 56]. Cloninger proposed two forms of alcoholism which can be distinguished as having distinct genetic and environmental causes [57]. "Type 1" alcoholism is characterized by late onset, a low degree of heritability and few social complications. "Type 2" alcoholism is characterized by high heritability, early onset, use and abuse not only of alcohol but also of other drugs, impulsiveness and sensation-seeking behaviour, and with social complications such as a family history of alcoholism and depression [57].

Heritability is the proportion of phenotypic variation in a population that is attributable to genetic components [52]. However, the variance accounted for a certain phenotype is more likely to be given by the interaction between genetic and environmental factors [48]. Twin studies do represent a natural model to estimate heritability and environmental influences on alcohol use disorder, while adoption twin studies provide a natural example to study gene-environment interaction [57-61].

Adolescence is a key developmental period: brain development still occurs, and typical behavioural traits and environmental cues increase the vulnerability to develop alcohol use disorder, as well as other psychopathologies [62]. Adolescent drinking is an area of behavioural research in which there

is a need for a dual approach, involving both genetic and psychosocial variables. This has been demonstrated by findings in twins (see [63]), and in G x E association studies [64-69]. Therefore, genetic and environmental factors, alone and in combination, are of importance for the risk trajectory that ultimately can culminate in alcohol use disorder.

Genetic factors

Research on the genetic basis of alcohol use disorders focuses on the role of genes involved in alcohol metabolism, and in pathways of brain neurotransmission, which are affected directly or indirectly by alcohol. Several candidate gene markers have been studied in relation to neurotransmitter systems such as genes regulating dopamine (DA), serotonin (5HT), glutamate, gamma-aminobutyric acid (GABA), endogenous opioids, corticotrophin releasing factor (CRF), and neuropeptide Y (NPY) [70, 71].

Animal studies, with rodents or non human primates, have been effective to investigate genetic factors related to initiation, craving, reinforcement, and addiction to alcohol (e.g. knock-out, and pharmacological models) [72-74]. The advantage of studies on non human primates is that the animals have a longer period of adolescence in comparison to rodents [75]. To investigate the genetic background of alcohol use disorder in humans, studies have focused on gene expression in the post-mortem brain, on single genetic polymorphisms, or a whole genome scan [52, 74, 76-78]. Nevertheless, not every adolescent having a genetic risk variant will develop alcohol use disorder, indeed factors for resilience have also been shown (see [79]).

The human genome, consisting of DNA, is built up as a code from four nucleotides, called bases (Adenine, Thymine, Cytosine, Guanine), linked in pairs (A-T, C-G) and attached to a phosphate group and deoxyribose sugar [80]. The DNA is arranged as a double strand helix molecule, and then into 22 pairs of chromosomes plus a pair of sex chromosomes. One member of a chromosome pair originates from the mother and one from the father. Each chromosome consist of several genes, which are mainly composed of a regulatory region with transcriptor binding sites upstream of the DNA sequence called 'promoter', non-coding elements called 'introns', coding elements for the proteins called 'exons', and an untranslated downstream region [81].

The nucleotide sequence of DNA differs between individuals by an average of 0.1% [82]. Any locus is made up of two variants called 'alleles'. When the DNA sequence at a given locus varies in the population, the locus is said to show allelic variation. When an allelic variant is found in at least 1% of the population, the variant is called a genetic polymorphism [83]. There are various types of polymorphisms, such as a single nucleotide polymorphism (SNP), and a variable number of tandem repeats (VNTR) [83]. If both alleles at a locus are identical, the individual is referred to as 'homozygous', whereas the individual that has two different allelic forms the individual is referred to as 'heterozygous' for that allele. A pair of alleles for a polymorphism constitutes the 'genotype' for a specific locus. The polymorphic variants can lead to functional differences by affecting transcription binding sites, translation process, protein structure, function, and amount.

Environmental factors

Several environmental factors have been shown to protect / predispose to a number of psychopathologies [84]. Pre-natal exposure to alcohol has been demonstrated to cause physical and cognitive dysfunctionalities in the child, as well as to be associated with increased vulnerability to alcohol-related problems during adolescence [85]. Post-natal environmental stressors are as well strong predictors of alcohol use disorder. Early life stress such as poor parenting, a parental history of psychiatric disorders, inter-parental violence, mental, physical or sexual maltreatment, low socioeconomic status, and peer-influences are all well-known environmental risk factors [20, 56]. Prenatal stress, maternal separation, and social conflict have also been investigated as environmental risk factors in animal models [86, 87]. Nevertheless, not every adolescent exposed to environmental stressors will develop alcohol use disorders, and several factors for resilience have also been found (e.g. a high education level, living with two biological parents) [56, 84].

Adolescence and psychiatric disorders

Adolescence is a critical time during which several psychopathologies have their common onset, such as depression, anxiety disorders, attention deficit hyperactivity disorders, conduct disorders, eating disorders, and personality disorders [20, 88, 89]. Sub-clinical conditions of these disorders, as well as sleep problems, emotional and behavioral problems, are also common but remain undiagnosed, and underestimated by parents, and school-teachers. All these conditions and problems represent potential risk factors which make the adolescent more vulnerable to an early initiation of alcohol use and misuse [20, 89, 90].

Comorbidity is defined as the simultaneous or succeeding presence of two or more disorders within a specified period. Comorbidity between alcohol use disorder and other psychiatric disorders is common among adults [91, 92]. However, the causal relationship between these comorbid disorders, as well as to what extent shared risk factors contribute to the comorbidity, are still poorly understood [53, 93]. Psychiatric conditions or symptoms increase the risk of alcohol use disorders. As opposite, individuals with an addictive disorder, such as alcohol use disorder, are at a higher risk then the general population of developing any other addictive disorder (e.g. pathological gambling, or opiate addiction), and/or other psychopathologies (e.g. affective, personality, or anxiety disorders, or ADHD) [71].

Among adolescents, comorbidity is also common, and not an exception [90]. Differences exist in regard to the age of onset and prevalence of different psychopathologies in relation to hormonal driven changes related to the age of puberty [88]. Debates exist regarding the influence of sex on psychopathologies. It seems that a greater proportion of female subjects with anxiety and mood disorders preceding alcohol dependence, whereas for

males conduct and antisocial disorders seem to occur concomitant or subsequent to alcohol use disorders [91]. Moreover, an early occurrence of psychiatric conditions is generally related to subsequent early initiation of alcohol use [90, 91]. A vicious cycle can thus be started involuntary by the adolescent who could regard drinking alcohol as a remedy to his/her problems. Consequently, exacerbation of the pre-existing disorder could occur, as well as an initiation of alcohol misuse [89].

Sex-specific effects

Sex effects are not negligible for neuroscience and psychology [94-96]. Studies on sex differences have found sex differences related to the gonadal hormones, structural (e.g. amygdala anatomy) and functional (e.g. neurotransmitters) dimorphisms in the brain, sex differences in gene expression (e.g. X chromosome partial inactivation), in epigenetic mechanisms (e.g. genomic imprinting) , sex diversity in response to environmental stimuli (e.g. stress), as well as a dissimilar prevalence and nature of psychopathologies among males and females [95, 96].

Genetic and environmental interaction mechanisms in females and males can differently influence the development of the nervous system, and of psychopathologies. The attention has been drawn on the mechanisms which contribute to sex differences in the heritability and susceptibility of an individual to develop psychiatric disorders [10, 97]. Sex differences have been shown in animal models in relation to stress response and alcohol consumption [72, 98, 99]. Among adolescents, sex differences in regard to G x E have been demonstrated in relation to alcohol consumption and alcohol-related problems [64, 65, 100]. Additionally, sex differences might concern more which genetic and psychosocial factors interact than the actual outcome. Indeed, males and females carrying the same genotype may react differently to similar life experiences, as demonstrated by our group in a study on G x E [101].

Gene-environment interaction in association studies

Association studies are based a priori on a potential etiological relation between a certain genetic variation and a certain phenotype. A large amount of associations analyses between candidate genetic markers and psychiatric conditions have been carried out in the last decade, however the results have often been controversial. Replications have shown both inconsistencies and failures in demonstrating previous association findings [48]. Possible explanations encompass the heterogeneity of the phenotypes, and of the gene-environment interaction. The effect of a single genetic polymorphism typically accounts for a minor percentage of the variance in a phenotype. A single genetic variation in itself is not sufficient, and not necessary to a phenotype, but only contributes in concert with other factors to explain a certain phenotype, or an endo-phenotype related to the phenotype [50]. Additionally, an

adverse environment is likely to be a prerequisite for penetrance of genetic risk factors in developing alcohol use disorder [55].

Association studies on GxE effects on alcohol as outcome have been carried out in humans but need further investigation to be considered robust (see [102]). GxE effect in relation to adolescents' alcohol use and misuse has been studied mainly in a one polymorphism - one trait manner. For instance, a repeat polymorphism in the promoter of the serotonin transporter gene (5HTT-LPR), which affects the release of serotonin back into presynaptic terminals, has been associated with adolescent alcohol consumption, in an interaction with bad family functioning or stressful life events [66-68, 103, 104]. A repeat polymorphism in the enzyme metabolizing monoamines (MAOA-uVNTR) has been associated with alcohol-related problems, in an interaction with maltreatment and bad family functioning, [64, 65]. A SNP in the corticotrophin releasing hormone receptor 1 gene (CRHR1 rs1876831), involved in stress and alcohol response, has been associated with higher alcohol consumption and heavy drinking among adolescents experiencing several negative life events [69]. A SNP in the dopamine d2 receptor gene (DRD2 TaqI A, rs1800497) has been associated with alcohol consumption among adolescents with permissive parenting [105].

Animal studies have demonstrated a link between early life stress, alcohol intake, and the monoaminergic and opioid systems in rodents [106, 107]. Moreover, studies on adolescents non human primates have examined alcohol response and consumption in relation to genetic and environmental factors [75, 108, 109].

Alcohol use disorder encompasses complex phenotypes, with not only a risk allele or a specific environmental stimulus being the etiological factor. By learning more about the interactions between genes and psychosocial factors (nG x nE) in relation to alcohol consumption among adolescents, it would be possible to identify potential pathways involved in alcohol use disorder.

Examples of potential genetic and psychosocial pathways in relation to adolescent alcohol consumption are the followings:

Sleep problems and the circadian clock system

Sleep problems, such as difficulties falling asleep, nightmares, and sleepiness during the day, are common among adolescents [110]. Several measures have been developed to evaluate sleep quality and the quantitative aspects of sleep. Most sleep surveys have been used indistinctly for different population groups (e.g. the Pittsburgh Sleep Quality Index, and the Karolinska Sleep Questionnaire) [111, 112], while specific surveys for adolescents are for instance the School Sleep Habits Survey and the Sleep-Wake Activity Inventory [113].

Sleep problems during childhood and adolescence have an impact on mood, behaviour, and are also a warning sign for psychiatric conditions such as depression and anxiety [114, 115]. Comorbidity between alcohol use disorders and sleep disturbances is well known, though the causal-consequential relation remains debated [116, 117]. Adolescents sleep/wake behaviour and potential sleep problems can be influenced by both environmental (e.g. a change in sleep habits, week/week-end sleep patterns) and biological factors (e.g. circadian rhythms phase changes) [118].

Circadian clock system

Circadian rhythms, from the Latin words *circa* = about and *diem* = a day, are biological rhythms encompassing a period of about 24 hours and being regualted by an internal primary circadian clock. In mammals, this circadian clock is located in the suprachiasmatic nuclei (SCN) in the hypothalamus [119, 120]. The SCN rhythm is self-sustained, but also receives afferent and efferent signals, as well as input from the external environment, which act as zeitgeber. External cue factors, such as food, social cues, and internal cue signals from the rest of the body, can contribute to synchronize the circadian clock, which in response sends output signals to regulate physiological mechanisms, such as metabolism and feeding [119-121]. The photic signal, the major 'zeitgeber', is transmitted from the retina via the retino-hypothalamic tract by the mediating action of glutamate. Two other pathways project afferent signals to regulate the SCN, the serotonergic input from the raphe nuclei, and a third input via the geniculohypothalamic tract by release of NPY, enkephalin, and GABA. Additionally, the SCN interacts with the pineal gland via melatonin in both directions, as well as with other systems via many other receptors present in the SCN (e.g. leptin and ghrelin) (see [122]).

The circadian clock consists of an auto-regulatory feedback loop between clock genes and related-proteins (see [122]). Circadian oscillations have been demonstrated for the clock genes and proteins, both in the brain and peripherally. Several genes participate in the maintenance of the circadian rhythms, with a repressing or activating role in a regulatory loop of their transcription/translation. In the core loop, the Clock and Bmal1 proteins form a complex which enters into the nucleus. The Clock-Bmal1 complex activate the transcription of *PER1*, *PER2*, *PER3*, *CRY1*, and *CRY2* genes, by binding E-box enhancer motifs in the promoter region of these genes. The Clock-Bmal1 complex activates REV-ERBα, which in turn acts as a repressor of BMAL1 and CLOCK genes. In turn, after several hours, Per and Cry proteins form a complex and enter into the nucleus to inhibit the transcription of the CLOCK-BMAL1 complex, thereby inhibiting their own transcription. Post-translationally, protein modification occurs by kinases such as the kinase CKIε [119, 121].

Mutations in the clock genes lead to alteration or disruption of the circadian rhythms, and have an impact on behaviour and physiological func-

tions [123]. Clock genes can affect sleep/wake rhythms. For instance, a mutation in the phosphorilation site in the PER2 gene has been found to cause a shortening of the rhythm and a phase advance, the so called familial advanced sleep phase syndrome (FASPS) [124]. Many genes are regulated in a circadian manner, and interact with clock genes. Recent findings indicate a link between circadian clock genes and the monoaminergic system. In an animal model, it has been shown that a regulatory activity is exerted by the *PER2* gene on MAOA gene transcription, which results in altered dopamine levels and ultimately relates to mood [125, 126]. Furthermore, *PER2* has been suggested as a candidate gene for alcohol consumption in rodents, with a hyperglutamatergic state in Per2 mutant mice being associated with increased alcohol consumption [127]. In a clinical sample of severe alcoholics a significant association has been shown between alcohol intake and an intronic polymorphism in the *PER2* gene *(SNP* 10870) [127]. The polymorphism consists of an A/G substitution, and is located in an enhancer-like structure, with the genomic sequence around the G allele being rich in potential transcription factor binding motifs [127, 128]. The clock gene *PER2* has also been investigated in regard to other psychiatric disorders all characterized by comorbid sleep problems, such as winter and bipolar depression [128, 129]. Thus, the *PER2* gene is a plausible candidate gene for studies on adolescent alcohol consumption and sleep disturbances.

Internalizing disorders and the endogenous opioid system

Internalizing disorders, such as depression and anxiety, are common alone and in comorbidity, and especially among females. Comorbidity of internalizing disorders with alcohol use disorder is also common, though alcohol use disorders are more common among males [71, 130, 131]. The aetiological relation between the comorbid disorders is still debated, and concerns regard the order of onset, and which are the distinct/shared risk factors [130, 131].

A clinical diagnosis of depressive and anxiety disorders can be done on the base of established criteria [24, 132]. For other purposes, assessment of depressive or anxiety symptoms can be done with screening scales developed for adolescents, such as the depression self-rating scale (DSRS) [133], and the State-Trait Anxiety Inventory for Children and Adolescents (TAICA)[134]. Basic features of depression are the marked and persistent presence of symptoms such as depressed mood, lack of interest or pleasure in activities, weight changes, feelings of worthlessness, thoughts of death, fatigue, sleep problems, and indecisiveness [24, 132]. Typical symptoms of generalized anxiety disorders comprise recurrent apprehension and motor tension [24, 132].

Alcohol use and internalizing disorders are common among adolescents, as well as in comorbidity [135, 136]. An occurrence at an early age of internalizing disorders has been related to an early initiation of alcohol use, and higher risk of developing alcohol use disorders [135, 137, 138]. It has been shown that a greater proportion of females with an anxiety or affective disorder

develops alcohol dependence than males [91]. Animal studies suggest the importance of environmental stressors, as well as of neurobiological pathways related to stress response and alcohol reinforcement, such as the serotonergic and the endogenous opioid systems [75, 106, 107].

Endogenous opioid system

The endogenous opioid peptide/receptor system influences several physiological and neurobiological systems, such as pain perception and the reward system. Activation of the opioid system has broad effects: on appetite, gastrointestinal and respiratory functions, euphoria and analgesia, endocrine functions, learning and memory, stress responsivity and mood [139]. Several opioid receptors have been identified, and all belong to the G-protein coupled receptors family. The most studied receptors are μ, κ, δ, and their subtypes, while ζ, λ and ε have not yet been well characterized. Additionally an opioid receptor-like 1 receptor has been later identified, whereas the σ receptor is no longer considered an opioid receptor.

The opioid receptors can be activated both by endogenous opioid peptides, such as β-endorphin, endomorphin, enkephalins, dynorphin, and nociceptin, and by exogenous opiates, such as morphine extracted from opium of the poppy plant. These peptides derive from different precursors (e.g. β-endorphin is the cleavage product of pro-opiomelanocortin), and all, except for nociceptin, share a common pentapeptide sequence. Each endogenous ligand acts on a preferred specific receptor (e.g. endomorphin has a high affinity and selectivity for the μ receptor) [140]. Opioid receptors, and their endogenous ligands, are widely distributed in the brain, and mainly exert an inhibitory response. Though compensatory effects are activated, the opioid system response encompasses an inhibitory effect on the GABAergic neurons, and consequently an excitatory effect on the dopaminergic neurons [140, 141]. The activation of the reward system by drugs of abuse, such as ethanol and morphine, thus leads to a stimulation of the dopaminergic system, and contributes to the reinforcing effect of these drugs [142]. In the mesocorticolimbic reward pathway the dopaminergic neurons project from the ventral tegmental area (VTA) towards the nucleus accumbens, the amygdala and the frontal cortex.

The endogenous opioid system has been studied in relation to alcohol sue disorder, and pharmacological antagonism of the opioid receptors is used as a treatment for alcoholism (e.g. naltrexone) [70, 143]. Additionally, the interaction between the HPA axis and the endogenous opioid system has been shown to be the link between psychosocial stress and alcohol consumption [139, 144]. Animal and human studies have shown that the μ opioid receptor 1 (*OPRM1*) gene is involved in reward pathway implicated in the acquisition and persistence of, and relapse to alcohol addiction [139, 145]. It has been shown that alcohol modulates the release of endogenous opioids such like the OPRM1 agonist β-endorphin (β-END) [146, 147].

Several polymorphisms have been identified in the *OPRM1* gene. One in particular, a single nucleotide polymorphism (A / G), which causes an amino-acid substitution (Asn40Asp), has gained interest (*OPRM1A118G*). This SNP (rs1799971) has been shown to be functional in *in vitro* studies, although with conflicting results [148-151]. In non human primates having an ortholog gene, the G equivalent allele has been associated with increased alcohol consumption and alcohol preference [152], as well as with higher naltrexone treatment efficacy [153]. In human studies, the association between the *OPRM1A118G* and alcohol use disorder have given conflicting results [154-160]. Treatment response after administration of the μ-preferring opioid antagonist naltrexone has also been studied in relation to the *OPRM1A118G*, with the G allele being associated with a better response to the treatment [161-164]. Among adolescents, the G allele has been associated with alcohol use disorders and enhancement motives for drinking [165]. Hence, the *OPRM1* gene, through its role on the stress response and the reward system, seems to be a potential candidate for studies on adolescent alcohol consumption in relation to internalizing disorders.

In summary, a significant heritability of alcohol use disorder has been demonstrated by family, adoption and twin studies. Environmental influences have also been acknowledged to play an important role in the development of alcohol use disorders. Moreover, the interaction between genetic and environmental factors is likely to influence the risk-resilience for alcohol use disorders. In view of this knowledge, plausible candidate polymorphisms were considered in gene-environment interaction models in relation to alcohol consumption among adolescents.

Aims

This study aimed to investigate alcohol consumption among adolescents, psychosocial and genetic influences on adolescent alcohol consumption, and possible sex differences.

Specific aims:

Paper I

- To investigate reasons for drinking among adolescents and to identify drinking motives.
- To study the relation between drinking motives, alcohol consumption, and alcohol-related problems among adolescents.

Paper II

- To investigate the relation between a variation in the clock gene PER2, sleep problems, and adolescent alcohol consumption.
- To investigate the effect of the PER2 variation in a group of severely alcoholic females.

Paper III

- To investigate the effect of a variation in the μ opioid receptor 1 (OPRM1) gene on alcoholism in a group of severely alcoholic females and the relation with comorbid anxiety and depression
- To investigate the relation between the OPRM1 variation, depressive symptoms, and alcohol consumption among adolescents

Paper IV

- To study the congruence of biomarkers, questionnaires, and interviews as instruments with which to assess adolescent alcohol consumption.

Methods

Participants

Adolescents

The following three studies: SALVe 2001, SALVe 2004, and SALVe 2006 are part of the Survey of Adolescent Life in Västmanland which is carried out biennially to monitor the psychosocial health of the adolescent population of the county of Västmanland. Västmanland is a medium-sized Swedish county, situated about 100 kilometres north-west of Stockholm, and considered to be representative of Sweden as a whole because of its distribution of rural and urban areas, and educational, income and employment levels (SCB, 2008). According to Statistics Sweden, in Västmanland in the year 2005, 91% of the adolescent population continued in secondary education until the age of 19, 4.3% went to other types of school and the remainders were out of school, because of truancy issues or having dropped out.

SALVe 2001 **[**Paper I, III and IV**]**

All secondary school students aged 15-16 and 18-19 years of age were asked to complete a questionnaire during class hours. This was done by a total of 4260 adolescents (82% of the eligible population), of which 2611 were 16 and 1649 19 years of age. All students had an opportunity to give their informed consent to participate in an in-depth interview by giving their full personal identification number on the front page of the questionnaire form. Informed consent was received from 785 students who could be traced afterward with valid names. The participants were then classified by using a risk index and divided into four equally large groups, depending on their weighted-risk behaviours (i.e. alcohol, narcotics, sexual, and offences involving property and violence) reported in the questionnaire. A randomised sample of 400 students matched for age, sex, and weighted-risk behaviours, was drawn from the volunteers to have enough participants from both ends of the deviant behaviour continuum (high 25%, medium 25+25% and low 25% risk groups). When asked a second time, 81 of the boys and 119 of the girls agreed to participate, gave blood and hair samples, and took part in a personal interview. Seventy-eight girls and 57 boys were 16 and 41 girls and 24 boys were 19 years of age. A follow-up study was carried out three

years later with a written questionnaire which included 180 of these adolescents: 114 girls and 66 boys.

SALVe 2004 **[**Paper I**]**

This survey included 5092 adolescents in the county of Västmanland who gave their informed consent for inclusion and completed a questionnaire during class hours in the year 2004. Participation, which was anonymous and voluntary, was generally high, with an overall response rate of 81.5 % of the total population, and a 98.6 % internal (form) response rate. The total sample included 5048 adolescents, 2872 aged 15-16 years old and 2176 aged 17-18 years old (44 did not state their sex).

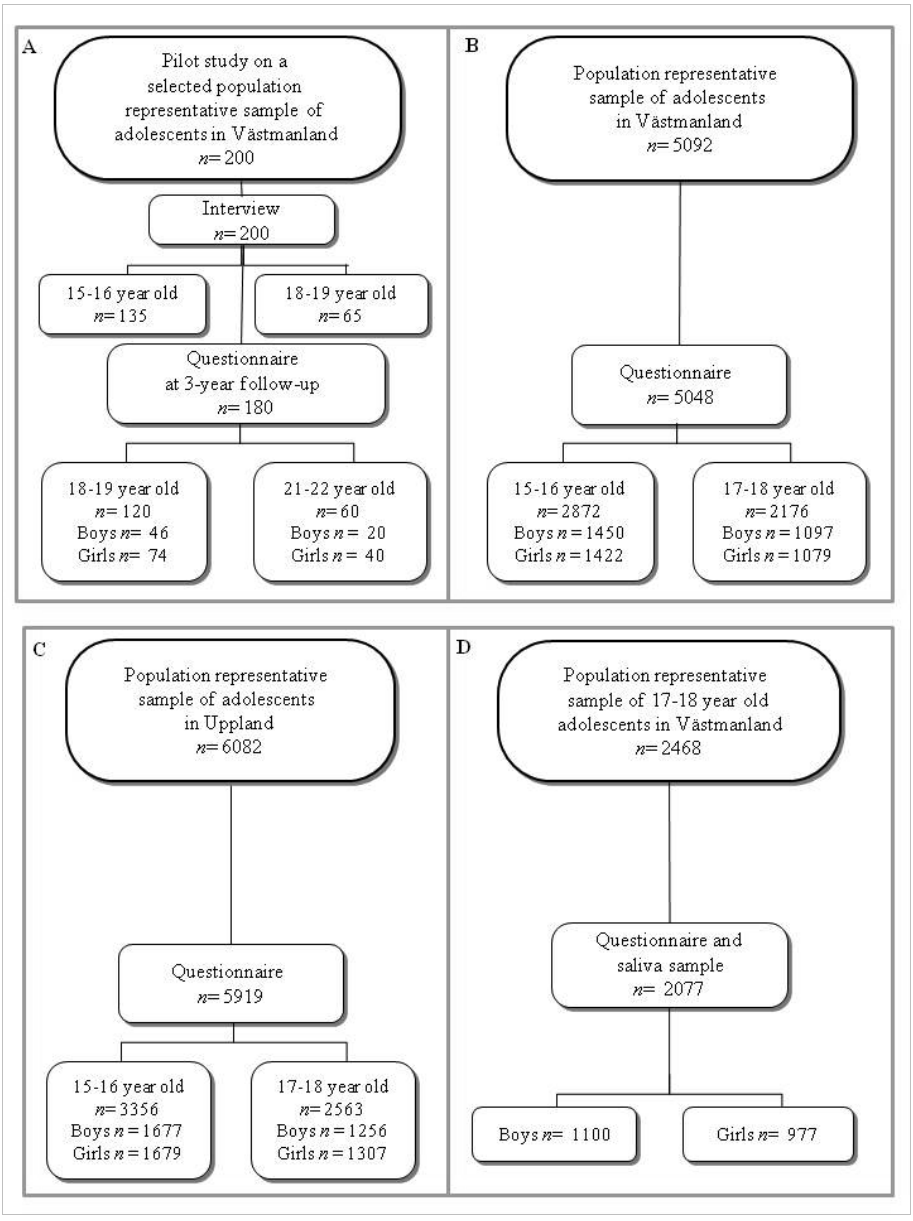
"SALVe 2006" **[**Paper II**]**

This medium/large-scale epidemiological study included a cohort of non-clinical individuals comprising the target population of all 17–18 year old secondary school students in the county (87% of the population for this age group). All participants were asked to complete a questionnaire during class hours and, in addition, were asked to provide a saliva sample for biological analyses. Questionnaires were provided for the teacher to be given to the students not attending class at the time of the study. Participation was anonymous and voluntary. The overall response rate was 77.4% with an internal response rate of 97.7%. A total of 2468 students completed the questionnaire of which 183 late-respondents returned their questionnaires by mail. A saliva sample was provided by 2131 participants, 54 adolescents did not state their sex. Due to problems with DNA extraction, genotype analyses, or missing answers to the questions, the final study sample in the different manuscripts included a different number of individuals.

UPPLAND 2005 **[**Paper I**]**

This study is part of the Survey of Adolescent Life in Uppland carried out to monitor the psychosocial health of the adolescent population of the county of Uppland. The study population included 6082 adolescents in secondary education, of which 5919 (15-16 years old, *n*= 3356; 17-18 years old, *n*= 2563) completed a questionnaire in class time during the year 2005. The remainder, 163 adolescents, did not state their sex. Respondents gave their informed consent to be included in the study by answering the questionnaire. The personal identification numbers were deleted directly and the survey data were thus anonymous. The area investigated covers different municipalities in the county of Uppland which has about one million inhabitants and is located in the central part of Sweden. The response rate was 88.2 % for the 15-16 year old and 76.1 % for the 17-18 year old.

Figure 2. Descriptive chart of the population - representative samples of adolescents A: SALVe 2001; B: SALVe 2004; C: UPPLAND 2005; D: SALVe 2006.



Severely alcoholic females [Paper II and III]

A sample of 110 Caucasian inpatients, all fulfilling the criteria for alcohol and/or substance dependence according to the ICD-10 diagnostic criteria, was included in the study. The patients were recruited between July 2001 and July 2006 from a long-term inpatient treatment facility for female patients suffering from alcoholism and drug abuse. Although there are possibilities for patients to initiate contact with the institution themselves, most of the patients were sent to the facility following a court order by social authorities from all over Sweden (107/110). Usually the social authorities are alerted by a physician that there is a substantial risk for the patient in question of endangering her own psychic or physical health or of endangering the wellbeing of others. This system, in the presence of a court order, allows the requested physician to immediately contact social authorities and the social authorities to send the patient to a long-term drug abuse treatment facility in accordance with the law (The Swedish Substance Abuse Treatment Act). The age of the patients in the study ranged between 18 and 75 years. All patients were examined by a physician and a specialist in psychiatry for psychic and somatic parameters. Patients were asked to have their expiratory air alcohol concentration measured, and a urine sample test for drug screening was collected. Ten patients did not comply with alcohol and drug testing. A blood sample was collected on day 1 and was included in the clinical routines, which included genetic tests, biomarkers tests for alcohol consumption, and a screening test for viral hepatitis. The patients were examined for the severity of alcohol toxicity and hepatic damage. Recruited patients were asked to fill in a questionnaire on past and present somatic and psychic illness, past and present substance use, and social factors such as employment and marital status, family history concerning alcohol and drug use, criminality and psychic illness. Data on behaviours leading to arrests, sentences, prison and overt aggressiveness, were acquired from the criminal reports. As 107 out of the 110 patients were admitted due to a court order, all relevant information was available. Nursing staff at the treatment facility completed the questionnaire with information about vital parameters, withdrawal symptoms and medication on day 1. The lifetime diagnoses of co-morbid disorders such as anxiety disorders and depression were made by a trained specialist in psychiatry who was also responsible for the treatment of the patients. The diagnoses were made by means of the ICD-10 diagnostic criteria for research together with all available information, including interview data, and clinical records.

Material

Questionnaires or interviews were used to investigate the lifestyle of adolescents. The aim of the survey was to investigate health status, lifestyle factors and living conditions such as weight and height, country of origin, socio-economic conditions, family relations, critical life events, experience of belittlement, sex experiences, social cohesion, school environment, physical activity, smoking habits, and alcohol consumption habits (SALVe 2001, SALVe 2004, SALVe 2006, and UPPLAND 2005).

Interview [Paper I and IV]

An in-depth interview was the method used in the sub-group of adolescents selected from the study SALVe 2001 and took the form of a meeting between interviewer (KWN) and interviewee (Ip). The interview was semi-structured and based on a previously designed interview guide regarding the topics or the structure of the interview. Data were tape-recorded. The questions were not defined in advance; open questions were adapted to the situation, reasoned and reflected in a qualitative dimension in order to reflect the interviewee statements. Special attention was made to use as few pre-conceptions as possible and avoid directing the answers of the interviewee (Ip). A reflective "mirror image" from the interviewer was anchored in time and place (e.g. last weekend with your peers or during mid-summer with your sister and her friends). Example of dialogue: KWN: Why do you drink alcohol? Ip: Because it gives me pleasure…; KWN: It gives you pleasure…? Ip: Yes, I enjoy being drunk….; KWN: You enjoy being drunk….? etc. The interviewee's responses were afterward transformed from a qualitative to a quantitative format by by fitting them into a structured template.

Questionnaire [Paper I, II, III and IV]

The written questionnaire was administered in classrooms during a one hour session by teachers and a research assistant to subjects included in the studies SALVe 2001, SALVe 2004, SALVe 2006, and UPPLAND 2005. The questionnaire included different types of questions. Close-ended questions limit the respondent´s ability to choose from a pre-existing set of survey answers. The types used were the following: dichotomous yes/no answers; multiple-choice with a list of possible answers, scaled questions, and ranking scale regarding to which degree the respondent agrees with a certain statement or rating scales which reflect the perceived level of a certain status. Short open-ended questions for data completion were used to collect data about age, weight, height, hours of sleep, age of eating problems, age at which sexual intercourse was first experienced, age of having been caught by the police for the first time, etc.

Ethical Approval

The studies were approved by the different regional ethical review boards and the human ethical committee of the medical faculty at Uppsala University.

Measures

Alcohol consumption

The adolescents received an explanatory table to refer to when answering the questions. A standard drink refers to a glass/bottle/can of beer (ca 50 cl), a glass/bottle/can of cider (ca 50 cl), two glasses/bottles of alcopops (ca 50 cl), a glass of wine (ca 15 cl), or a glass of spirits (ca 5 cl).

AUDIT-C

[Paper I, II and III (SALVe 2001, SALVe 2004, UPPLAND 2005, SALVe 2006)]

The Alcohol Use Disorders Identification Test-Consumption (AUDIT-C) has been developed from the Alcohol Use Disorders Identification Test (AUDIT), by including only the first three questions of the AUDIT which measure the domain of hazardous alcohol use [33]. The three items cover the frequency of drinking, and the typical quantity and frequency of heavy drinking. The possible answers were scored as: [Question I] How often have you had a drink containing alcohol in the last 12 months? (0) never [pass over questions II and III]; (1) every other month or once a month; (2) 2-4 times a month; (3) 2-3 times a week; (4) 4, or more, times a week. [Question II] How many drinks containing alcohol do you have on a typical day when you are drinking? (0) 1-2 glasses; (1) 3-4 glasses; (2) 5-6 glasses; (3) 7-9 glasses; (4) 10 or more glasses. [Question III] How often do you have six or more drinks on one occasion? (0) never; (1) every other month or more seldom; (2) about once a month; (3) 2-4 times a month; (4) 2-3 times a week. The scores of the three items were summed to produce an alcohol consumption index "AUDIT-C" (range 0-12).

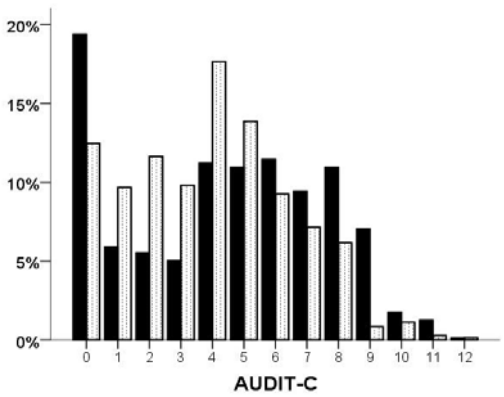


Figure 3. Alcohol Use Disorder Identification Test - Consumption among 17-18 years old Swedish adolescents (SALVe 2006: ■ boys; □ girls).

Alcohol-related problems

[Paper I (SALVe 2001, SALVe 2004, UPPLAND 2005)**]**

Alcohol-related problems were assessed as the number of occasions on which an adolescent had experienced any problems related to his/her alcohol use. The list of problems has been based on the items used in The European School Survey Project on Alcohol and Other Drugs (ESPAD) [166]. The component score was summed to produce an "Alcohol-related problems index". The alcohol-related problems included items covering four subgroups: personal problems such as "poor performance at school or at work", "accident or injury", relationship problems such as "serious problems with either friends or parents", sexual problems such as "engagement in sexual intercourse that you regretted the next day", "engagement in sexual intercourse without a condom", delinquency problems such as "physical fighting", "victimisation by robbery or theft" and "troubles with the police".

High-low alcohol consumption **[**Paper IV (SALVe 2001)]

Questions about the amount and frequency of alcohol intake were used in the questionnaire to define high-low alcohol consumption. The frequency of alcohol consumption was calculated by the addition of frequencies of consumption for each of the alcoholic beverages, ranging from 0 to 365 times a year. The frequency of recurring intoxication was measured using the question: "How often do you get drunk when you consume alcohol?" On a six-point scale, possible answers included the following: [1] I do not drink alcohol, [2] never, [3] seldom, [4] occasionally, [5] almost always, and [6] always. A new variable for high versus low alcohol consumption was created by combining the following two variables: (1) the frequency of alcohol consumption, and (2) the frequency of recurring intoxication. This new variable was dichotomized as follows: those who consumed alcohol twice a month or more, and always or almost always got drunk ("high alcohol consumption"), and those who consumed alcohol less frequently and never, seldom, or occasionally became intoxicated, or intermediate-frequency alcohol consumers who never or seldom became intoxicated ("low alcohol consumption").

The semi-structured interview was adapted to the situation regarding formulation, sequence, and extent to reflect alcohol habits in a qualitative dimension. The interview answers were used to code the amount and frequency of different alcoholic beverages. Those adolescents who drank twice or more per month, and became drunk always, or almost always, were classified as "high alcohol consumers". Those individuals who consumed alcohol less frequently or frequently, but who were never or seldom drunk were classified as "low alcohol consumers".

Alcohol intake (gr / year) [Paper III (SALVe 2001)]

The semi - structured interview contained questions for assessing alcohol consumption which were adapted to the situation to elicit the personal behaviour of the participants and to "capture" their drinking history from the first occasion all the way to the interview day. The interview answers were used to code the amount and frequency of different alcohol beverages and were transformed into grams of alcohol per year (the "alcohol intake" index).

Reasons for drinking

[Paper II (SALVe 2001, SALVe 2004, UPPLAND 2005)

The reasons for drinking were assessed using a self-administered questionnaire including seventeen reasons for drinking which have been previously identified by interview in a pilot study. Adolescents could indicate if any or several alternatives were appropriate for them, by answering yes or no to each item. The reasons given as answer to the question "Why do you drink?" were: to feel good; to relax; to think on something else/to forget my worries, my problems; to become calm/reduce tension; to enjoy to be drunk; to establish contacts easily; to dare more/to reduce inhibitions; to feel the pleasure/outgoing; to meet more people/to be sociable, friendly; to party; to create a better atmosphere; to get drunk; to become cool; to increase aggressiveness; to have others´ respect; to put a person down /to influence others to do as wanted; because others do it".

Sleep problems [Paper II (SALVe 2006)]

Sleep complaints were assessed with 18 questions based on the Karolinska Sleep Questionnaire [112], which inquires about frequency of sleep disturbances and subjective sleep quality. The questions were: "During the last three months, how often have you experienced: difficulties falling asleep, difficulties awaking, repeated awakenings with difficulty going back to sleep, heavy snoring (according to surroundings), insufficient sleep, light and superficial sleep, breathing interruptions during sleep (according to surroundings), nightmares, not having been thoroughly rested, a premature (final) awakening, disturbed sleep, feelings of being exhausted at awakening, sleepiness at school /work, sleepiness during your spare time, drowsiness/prolonged fatigue, having involuntary naps (until your evening meal) at school or work, having involuntary naps (until your evening meal) during your spare time, having to fight off sleep to be able to stay awake". The possible answers were scored as: [1] never; [2] seldom, occasional moments; [3] sometimes, a few times per month; [4] often, 1-2 times per week; [5] mostly, 3-4 times a week; [6] almost always, five times per week or more.

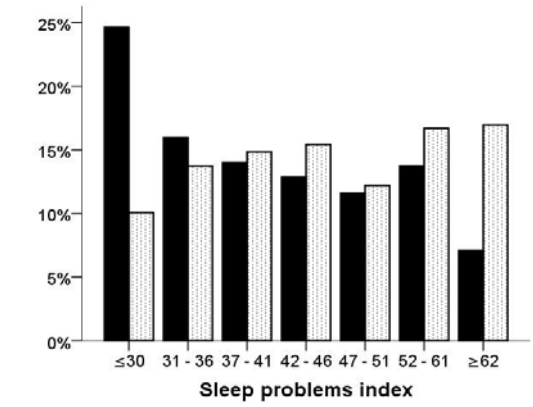


Figure 4. Sleep problem index among 17-18 year old Swedish adolescents (SALVe 2006: ■ boys; □ girls).

The component score was summed to produce a "sleep problem index" (range 1-108). The cut-off to dichotomize the variable was based on the mean score. Individuals who scored >44 (sleep problem index mean = 44) were considered to have "several and frequent sleep problems", while individuals who scored ≤44 were considered to have "few and seldom sleep problems".

Depressive symptoms [Paper III (SALVe 2001)]

The Depression Self-Rating Scale (DSRS), based on the DSM-IV-TR A criterion for major depression, was used to estimate symptoms of depression [23, 167, 168]. Subjects answered 15 questions on depressive symptoms occurred during the last two weeks, with answer alternatives being "yes" [1], or "no" [0]. There are several items that measured the same symptom twice. One symptom was only counted once, such as in case of both significant weight loss and gain during the previous two weeks, the participants could only score once. Additionally one item about irritable mood was added, because it is of special importance among adolescents. The 9 symptoms were: sadness or irritability, absence of interest for everything, weight changes, sleep disturbances, feelings of worthlessness or guilt, fatigue or loss of energy, thoughts of suicide, concentration problems, or psychomotor agitation or retardation [23]. The depression index was calculated as a summation of the symptoms ("depressive symptoms" index, range 0-9).

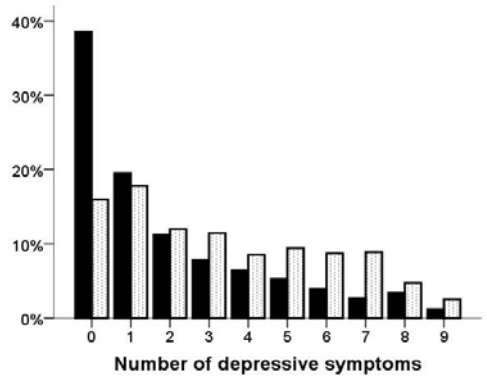


Figure 5. Number of depressive symptoms among 19-21 years old Swedish adolescents (SALVe 2001: ■ boys; □ girls).

Laboratory work

DNA extraction

For the DNA source in paper II (SALVe 2006), exfoliated buccal epithelial cells and other cells found in saliva were used. Genomic DNA was extracted by a modification of the SDS-KAc protocol [169] to enable the processing of a large number of samples, requiring a minimal workload and providing a sufficient quantity of DNA for subsequent analyses.
As a DNA source we used blood samples and a QIAamp 96 DNA Blood Kit QIAGEN®) to extract the DNA.
The average concentration was ~32 ng/µl, with A_{260}/A_{280} ratio ~2.0.

Genotyping [Paper II and III (SALVe 2001, SALVe 2006, EKEBYLUND)]

Genotyping assays of candidate polymorphic variants were run by using optimized protocols. SNPs were analyzed by allele discrimination polymerase chain reactions. Genotypes were scored both manually and automatically by using software. As a control material, a random number of samples were analyzed and genotyped twice, and inconsistencies were identified.

SNP primers design

Primer pairs for *OPRM1* SNP were selected from aTaqMan® Pre-Designed SNP Genotyping Assay which contains sequence-specific forward and reverse primers for a certain polymorphism together with two TaqMan® MGB probes each labelled with a FAM® or VIC® reporter dye at the 5´end of and a non-fluorescent quencher at the 3´end of each (Applied Biosystem®, USA)**.** Primers for the *PER2* SNP 10870 were not available and therefore they were manually designed on the basis of the DNA sequence published at www.ensemble.org, by using the TaqMan® Custom SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA)**.**

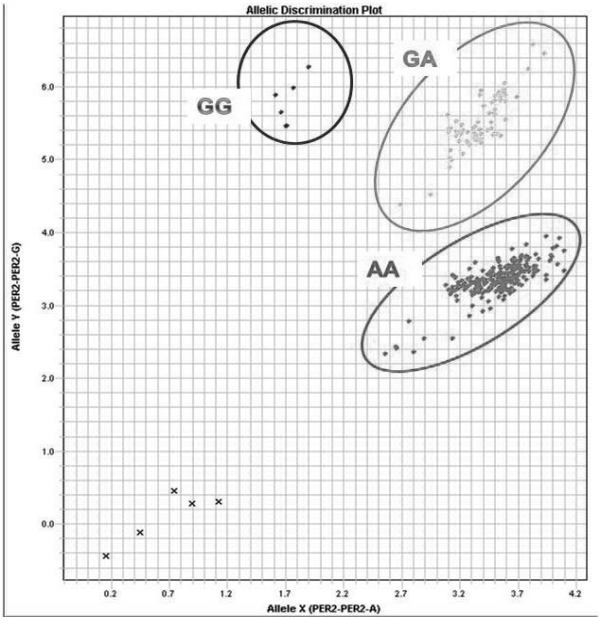
Allele Discrimination PCR for SNPs

Allele Discrimination PCR was used to detect SNPs, PER2 SNP 10870 and OPRM1 SNP rs1799971. The reactions were performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems®) in a 5µl reaction mixture containing ~20ng Genomic DNA, plus 0,125µl of 40x Pre-Designed or Custom TaqMan®SNP Genotyping Assay Mix. This contains sequence-specific forward and reverse primers, and two TaqMan® MGB probes each labelled with a FAM™ or VIC® reporter dye at the 5´end and a non-fluorescent quencher at the 3´end of each, plus 2,5µl of TaqMan®Universal PCR Master Mix (Applied Biosystem®).

This is a molecular biology in vitro technique to exponentially amplify a small segment of the DNA molecule. The PCR reaction was carried out in a

thermal cycler and the procedure consisted of: i) the initial heating step at 95°C for 10 minutes; ii) a series of 40 cycles each one made of two steps: 92 ºC for 15 seconds, and 60 ºC for 60 seconds. An electropherogram was visible during the analyses, which is a graph of relative dye concentration as a function of time, plotted for each dye. The instrument is connected to a detector and data analyzer.

The PCR products were analyzed and allelic variants were determined using SDS 2.3 (Applied Biosystems®, Foster City, CA, USA ®). Standard parameters have been applied to the analyses. The call of the alleles was done automatically, together with a quality control, and genotypes were manually checked on chromatograms in cases of low quality of the data.



OPRM1 rs1799971

The SNP consists of an A/G substitution at exon 1 of the OPRM1 gene, resulting in an amino acid change Asn/Asp at codon 40, with the G allele being the most common [148].

PER2 SNP 10870

The SNP consists of an A/G substitution at intron 3 of the *PER2* gene, with the A allele being the most common [127].

Biomarkers [Paper IV (SALVe 2001)]

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [39]. Two biomarkers were analysed, one in blood and one in hair, to test if and how much alcohol an adolescent consumes.

Phosphatidylethanol (PEth)

A whole blood sample, previously stored at -80°C, was used to analyze the biomarker PEth. Lipid extraction from whole blood was followed by high-performance liquid chromatography (HPLC) (Water Alliance 2690 system, Milford, MA, USA) and evaporative light-scattering detection (ELSD) (PL-ELS 2100 Polymer Laboratories, Shrosphire, UK) [170].
The precision of the method was determined by measuring the coefficient of variation (CV) for peak areas with phosphatidybuthanol added as an internal standard at two levels, 0.92 µmol/l and 3.2 µmol/l. The PEth limit of quantification, defined as the lowest concentration at which quantitative results can be reported with a high degree of confidence, was 0.25 µmol/l of blood.

Fatty acid ethyl esters (FAEEs)

Three hair samples were collected from the posterior, lateral and frontal part of the head by cutting as close as possible to the scalp to obtain the proximal 0-6 cm segments, following the samples were then wrapped in tinfoil and saved in dry storage at room temperature. No hair sample was obtained from bald individuals. The hair, previously washed to decontaminate it and then cut to millimetric pieces, was then used to analyze FAEEs by headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) (Hewlett-Packard GmbH, Waldbronn, Germany) [171]. The internal standards used were deuterated standards for the corresponding FAEEs and the limit of quantification was between 0.04 – 0.12 ng/mg. The amount of FAEEs was measured in ng/mg by summing the concentration of four FAEES (ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate). Cut-offs used were: ≥0.40 ng/mg and ≥0.67 ng/mg [45, 172].

Statistics

Statistical analyses were performed using version 17.0 of the software Statistical Package for the Social Sciences (SPSS Inc., Chicago IL, USA).

Significance

The significance level, expressed as a p-value, is the probability of wrongly rejecting the null hypothesis, if it is in fact true. The p-value was compared with the actual significance level of the test and, if it was smaller, the result was significant. The null hypothesis was rejected at the 5% significance level, which means a minimum significance level of $p < 0.05$.

Chi-square

The Pearson chi-square test (χ^2) examines group differences on dichotomous measures by comparing the differences between expected frequencies assuming there was no relationship between the two variables and observed values for each category through a cross-tabulation. It does not require the sample data to be normally distributed and the number of degrees of freedom (df) is a restriction on an model done by considering the number of parameters on which the estimate is based.

Factor Analysis [Paper I]

Factor analysis with Varimax rotation with Kaiser Normalisation criteria was used for structure deduction. A principal factor analysis extracts the underlying interrelation between the variables, to obtain a smaller number of variables, which are underlying dimensions called factors. The factors extracted are the ones which account for most of the variance in the variables. The first component accounts for the largest possible amount of the variance, and consecutive components account for less and less of the variability remaining. No specific assumptions regarding the underlying structure of the variables were set, such as the number of factors to be obtained.

A screen-test based on a visual examination of the plotted Eigenvalues showed the rotated factor matrix of the variables, items having a factor loading greater than 0.4 were identified as significant for that component. The components also had to have three or more variables with loadings over 0.4 to be counted as a component.

Varimax rotation, variance maximizing rotation, is a method used to minimize the complexity of the components. The factors/components are linear relationships between the variables, and represent an orthogonal dimension. The rotation is orthogonal (i.e. a linear combination) and searches for a rotation of the original factors such that the variance of the loadings is maximized, by making the large loadings larger and the small loadings smaller within each component. It clearly marks the factors defined by high loadings for some variables and low loadings for others, to obtain a clear pattern of

loadings on each factor that is as diverse as possible, lending itself to easier interpretation of the factors. The components are not correlated with one other, leading to easier interpretation of the factors.

Eigenvalues represent the variance explained by the components / factors, which are Eigenvectors of a matrix that is given by the variables and the factors.

The Kaiser Normalisation criterion implies that only components with Eigenvalues greater than one are considered, which means that a factor extracts at least as much as the equivalent of one original variable..

Hardy-Weinberg equilibrium **[**Paper II and III**]**

A principle of population genetics is the Hardy - Weinberg equilibrium (HWE), a genetic feature indicative of randomly breeding populations. The HWE test was used to determine if the observed genotype frequencies matched the expected genotype frequencies, in order to exclude the possibility of genotyping errors or sampling bias from the population.

In the case of a biallelic locus, the two alleles X and x, with allele frequencies of p and q, respectively, will have the genotypic frequencies as follows, for the XX homozygous p^2, the Xx heterozygous 2pq and the other xx homozygous q^2 and $(p + q)^2 = 1$.

The frequency of the two alleles was calculated as follows: fX= (2·XX + Xx) / 2N fx= (2 xx + Xx) / 2N. Allele frequency can be used to determine the expected genotype frequency, as the following demonstrated: expXX= $(fX)^2$·N; expxx= $(fx)^2$·N; expXx= fX·fx·N. Generalization for more than two alleles can be carried out.

Kolmogorov - Smirnov test **[**Paper II and III**]**

Kolmogorov - Smirnoff one-sample test (Z) assesses for normal distribution of a variable, by comparing the theoretical versus the observed cumulative distribution function of the variable. A significant result indicates that data for the chosen variable do not fit into a normal distribution.

Mann-Whitney test **[**Paper II and III**]**

The Mann-Whitney test (U) determines the difference between ranks of wo independent groups on continuous variables which are not normally distributed and measured at an ordinal scale. It is a non - parametric test based on median values, resulting in the same *P* - value for test significance as the Kruskal - Wallis test, however the nominal variable which distinguishes the groups is limited to two groups.

Binary logistic regression [Paper II]

Binary logistic regression analysis was used to test if a dichotomous dependent variable can be predicted on the basis of values of a set of predictor variables and their interaction effect, without making any assumptions about the distributions of the predictor variables. Wald values indicate the unique contribution of each predictor, in the context of the other predictors; the regression coefficients (B) as negative or positive parameter estimates of the explanatory variable and odd ratios (OR) measure how much greater/less the odds are for subjects having the risk factor to be in one of the categories of the dependent variable.

General linear model univariate analysis [Paper II and III]

The general linear model (GLM) provides regression analysis and analysis of variance to determine the nature of relationship between one dependent variable, and one or more factors and/or variables, and it estimates the value of the dependent variable corresponding to a given value of the independent variable. This procedure tests the null hypothesis about the effects of other variables on the means of various groups of a single dependent variable. One can investigate the effects of individual factors and interactions between factors, as well as the effects of covariates and covariate interactions with factors. The dependent variable and covariates are quantitative measures as scales, while factor variables are usually categorical.

The F-test indicates if the means of the groups formed by values of the independent variable are different enough not to have occurred by chance. The squared adjusted correlation-coefficient indicates the proportion of variance in the dependent variable accounted for by the independent variables after adjustment for bias. Additionally, after an overall F-test has shown significance, one can use post hoc tests to evaluate differences among specific means and profile interaction plots of the means to visualize some of the relationships. The assumption for this test is that data should be normally distributed.

R-Squared is a statistical measure of how well a regression line approximates real data points and measures the relative prediction power of the model; an R^2 of 1.0 (100%) indicates a perfect fit. Adjusted R-squared adjusts for the degrees of freedom in the model, and penalizes a complex linear model.

Öhrvik test [Paper IV]

The Öhrvik non-parametric test is based on the use of aligned ranks as suggested by the Hodges-Lehmann estimator, which is the median of the pair wise means of the observations. The estimation is done after removing the individual effects of each independent factor, thus this test estimates only the interaction effect [173]. The basic methodology is for example: if a person

A who has sleep problems scores 8 in AUDIT-C score, and the mean-value for AUDIT-C in the sleep problems group is 7, his/her sleep problems effect-score will be 8-7=1. A person B who scores 11 in AUDIT-C, thus his/her sleep problems effect-score will be 11 - 7 = 4. If there is no interaction effect all groups will end up with zero in AUDIT-C scores.

Bivariate correlation [Paper I, II, III and IV]

The bivariate correlation test, with Spearman´s rho coefficient (r), is a non-parametric bivariate measure of association between two variables. It varies from 0 to - 1 for a negative linear relation, and from 0 to + 1 for a positive linear relation. It measures the strength and direction of correlation between the rank - orders of two scale variables regardless of their distribution, not distinguishing between dependent and independent variable and with minimal influence by outliers.

Inter-rate reliability [Paper IV]

Cohen´s kappa coefficient (κ) measures the concordance between two categorical dichotomous variables rating the same object. It examines the inter-rate agreement with a non-parametric approach.

Sensitivity and specificity [Paper IV]

Sensitivity represents the percentage of occurrences correctly identified [true positives / (true positives + false negatives)].Specificity represents the percentage of non occurrences correctly identified [true negatives / (true negatives + false positives)]. Both sensitivity and specificity measure the performance of a method in comparison to a gold standard method used as a reference. True positives are predicted occurrences which are correct while True negatives are predicted non occurrences which are correct. False positives are predicted occurrences which are incorrect while False negatives are predicted non occurrences which are incorrect.

Positive predictive value & Negative predictive value [Paper IV]

The positive predictive value is a ratio indicating the likelihood that a positive result will be correct when restricted to those who test positive [True Positives / (True Positives + False Positives)]. The negative predictive value indicates the likelihood that a negative result will be correct when restricted to those who test negative [True Negatives / (True Negatives + False Negatives)].

Results

Paper I

Adolescents answered to the question "Why do you drink?". The reasons for drinking could be clustered into three distinct motives: 1) drinking to increase social interactions or to enhance positive mood (social-enhancement: internal/external positive reasons), 2) drinking to attenuate negative emotions and reduce the tension (coping: internal negative reasons), and 3) drinking to increase personal power and aggressiveness (dominance: external negative reasons). Social drinking reasons accounted for most of the explained variance. Furthermore, the motivational patterns were differently correlated to alcohol consumption and alcohol-related problems. Social-enhancement and coping motives positively correlated with alcohol consumption and alcohol-related problems, while dominance motives negatively correlated with alcohol consumption and alcohol-related problems. No age or sex differences were observed regarding drinking motives, alcohol consumption, or alcohol-related problems.

Paper II

The SNP 10870 polymorphism in the *Period 2* gene was investigated in relation to alcohol consumption and sleep problems among adolescents. Adolescent boys reported higher alcohol consumption, while sleep problems were reported more by girls. Alcohol consumption and sleep problems were positively correlated in both sexes. The findings supported an association between alcohol consumption and the *PER2* SNP 10870 only in an interaction with sleep problems among boys. The AA genotype, in the presence of several and frequent sleep problems, was associated with high alcohol consumption. Similar results were obtained when adolescents who stated that they did not drink alcohol were excluded from the analyses. Among girls, only sleep problems had an impact in relation to alcohol consumption, whereas no gene effect was present.

Additionally, the *PER2* SNP 10870 was investigated in a sample of severely alcoholic women. A non significant trend was observed, with the G allele being overrepresented in comparison to the control females.

Paper III

The *OPRM1A118G* polymorphism in the μ-opioid receptor gene was investigated in relation to alcohol consumption and depressive symptoms among adolescents. Adolescent boys reported higher alcohol consumption, while adolescent girls reported more depressive symptoms. With regard to age, depressive symptoms were more common among young adolescent girls. Our findings supported an association between depressive symptoms and the *OPRM1A118G* only in an interaction with earlier alcohol consumption, though with sex and age differences. The interaction was present among girls, and if stratified by age, among young adolescents. Presence of the G allele was associated with high alcohol consumption and depressive symptoms. Furthermore, the *OPRM1A118G* polymorphism and alcohol consumption in relation to depressive symptoms was also investigated in a large population-representative sample of adolescents participating in a cross-sectional study. No interaction effect was found between the *OPRM1A118G* and alcohol consumption in association with depressive symptoms.

Additionally, the *OPRM1A118G* polymorphism was studied in a sample of severely alcoholic women. The G allele was significantly overrepresented among severely alcoholic females, predominantly in comorbidity with psychiatric illness, such as anxiety or affective disorders. Parental psychopathology factors, such as alcoholism or depression, made the genetic association obscured and limited only to the cases who had not been exposed to these negative parental psychopathological factors.

Paper IV

Different methods to assess adolescent alcohol consumption were studied: biomarkers PEth and FAEEs, interview and questionnaire. High alcohol consumption in the questionnaire was underreported compared with the interview data. The higher self-reporting of high alcohol consumption in interviews rather than questionnaires could partly be due to the influence of a pipeline procedure, as blood and hair samples were collected to check for substance use. With regard to biomarkers, PEth and FAEE analyses were in poor agreement with self-report data, and were poorly intra-correlated. Specificity and sensitivity of the biomarkers were measured in comparison with interview data. The PEth blood test was the most specific but the least sensitive. The FAEE hair test revealed lower specificity and higher sensitivity than PEth, with changes related to the cut-off used. The results suggest that these biomarkers are not suitable as screening tools for alcohol consumption among adolescents.

Discussion

Psychosocial and biological pathways to adolescent alcohol consumption

Several factors interplay and contribute to an early-age initiation of alcohol consumption, and to a susceptibility of developing alcohol use disorders. Genetic and environmental factors are important for risk or resilience to adolescent alcohol use and misuse, as supported by findings in studies on non human primates [72]. Gene and environment interplay in a bidirectional manner, with genetically influenced behaviours in exposure to different environments, and environmental influences exerted on genetic expression [174]. Cognitive and personality factors are of relevance to understanding the development of behaviours, such as adolescent alcohol consumption. These factors are presumed to mediate the relation between G x E and alcohol consumption, as well as other comorbid disorders [175]. One way to increase the understanding of adolescent alcohol consumption is to investigate drinking motives.

Drinking motives

Reasons for drinking can be manifold, and several models have attempted to determine the reasons why adolescents drink alcohol [17]. Endorsement of reasons for drinking encompasses expectancies that the adolescents have from drinking alcohol, which might be alcohol-related (e.g. a previous direct experience with alcohol, learned and held in memory), or psychological processes influenced by other factors (e.g. experience of depressive symptoms increasing coping or enhancement motives to drink, and personality traits) [175]. To date, reasons for drinking have been grouped into few major motives which have been identified regarding the valence and the source of the outcome that the individual expects to achieve by drinking. One group relates to positive internal or external reasons (i.e. enhancement and social motives), and one to negative internal or external reasons (i.e. coping and conformity motives) [17].

Three main drinking motives emerged in our study from 17 reasons for drinking: social - enhancement, coping, and dominance (Paper I). No sex or age differences were observed in our study, wherefore all the results were presented considering the total samples of adolescents.

Additionally, correlation between these motives for drinking and alcohol consumption, and alcohol-related problems, was investigated.

A qualitative approach

The methodological approach used in the first part of the study (Paper I) was based on the general principles of the Grounded Theory, which aims to generate a theory by collecting data about an issue in contrast to previous theoretical orientation, and free from assumptions [176]. For example, a qualitative approach allowed the emergence of new reasons for drinking. Furthermore, it provide with an up-to-date description of the phenomenon, as certain reasons could have developed over the previous decades. Thus, a pilot study with a relatively large sample, for a qualitative approach, was carried out among adolescents to identify the most endorsed reasons for drinking.

The instrument used was a semi-structured interview, which, compared with a questionnaire, has the advantage of better capturing personal experience related to the use of alcohol. An example of dialogue between the interviewer (KWN), and the interviewee (Ip) is as follows: "KWN: Why do you drink alcohol? Ip: Because it gives me pleasure…; KWN: It gives you pleasure…? Ip: Yes, I enjoy being drunk….; KWN: You enjoy being drunk….? etc". The interviewee's responses were subsequently transformed from a qualitative into a quantitative format. The resulting answers were classified into categories of commonly indicated reasons. Subsequently, in the second part of the study, 17 reasons for drinking were included in a questionnaire as answers to the question "Why do you drink?". This was submitted to the same cohort three years later, and to two other independent large cohorts of Swedish adolescents (Paper I). Factor analysis was used as the statistical method, without any pre-established structure, to identify clusters of reasons for drinking that would represent different drinking motives. Replication of the results was obtained in the sample of the pilot study at a follow-up, and in two large cohorts of Swedish adolescents, thus the validity of the drinking motive presented was confirmed. However, our "reasons for drinking questionnaire" is not validated against any another measure of assessment.

A limitation of the study is the difficulty in comparing the obtained results with previous studies, because of the heterogeneity of the reasons for drinking considered by each method of assessment. Moreover, a motive might have been described with the same name, but might contains different reasons, as a reason for drinking could have been classified under different motive dimensions (e.g. drinking to relax could be considered both as a coping and an enhancement reason for drinking) [17].

Social-enhancement motives

The social - enhancement motives, such as to enjoy a party and to get drunk, included most of the reasons for drinking reported by the adolescents and explained 28-35% of variance of the motivational model. However, according to Cooper's classification of drinking motives, two distinct positive motives can be identified, a social and an enhancement motive [21]. It is likely that in our study these two motives were constrained in one. Reasons for drinking such as to party and to be sociable were pertaining to the social motive sphere, and reasons for drinking such as expectation of pleasure and to get drunk were pertaining to the enhancement motive sphere. Another common drinking reason during adolescence is represented by the facilitation of sexual contacts, which implies a wish to overcome personal inhibitions [177]. However, this reason did not emerge in our study. Social motives are often regarded as normal, with drinking behaviour often occurring among groups of peers, and are associated with moderate drinking [17]. Of interest with regard to social influences on behaviour are some studies on rodents. As in humans, socially mediated learning of alcohol consumption has been investigated in animal models. The social contact with moderately intoxicated peer has been shown to potentially enhance subsequent alcohol consumption in adolescent rats [178, 179]. Enhancement motives have been positively correlated to alcohol consumption and alcohol-related problems [17], to heavy episodic weekend drinking [180], as well as to high novelty seeking and high extraversion personality traits. These in turn have been shown to be associated with deviant behaviour and increased risk for addictive disorders [181]. In our study, the social-enhancement motive was associated with alcohol consumption and problems related to it, thus this motive could encompass a future risk of alcohol use disorders. Regarding the correlation between drinking motives and alcohol consumption or alcohol-related problems, the incongruence with previous studies might be due to the fact that social and enhancement motives are not distinct motives in our study, but included in one motive category. Additionally, Cooper's questionnaire of reasons for drinking includes a group of reasons related to conformity motives (e.g. to be liked, to fit in with a group, to not be mocked by others for for not drinking) [21]. Evidence indicate that drinking motives of peers are correlated with alcohol consumption via the mediator role of individual drinking motives among adolescents [182]. However, in our study the reason directly related to peer pressure (i.e. because others drink) did not reach the threshold to be represented by a factor-motive. Some of the reasons for drinking included in other motives, such as to party or to be cool, might encompass a certain degree of motives of conformity which did not emerge in our study.

Coping motives

A second drinking motive, named coping motive, was represented by reasons for drinking such as the wish to relax, to become calm, and to think of something else. Coping motives explained 12-16% of variance of the motivational model. A very similar number of reasons (e.g. to reduce stress, overcome feelings of anxiety, and to forget about problems) has also been reported by Cooper [21]. Individuals who address coping reasons for drinking tend to perceive the use of alcohol as a kind of self-medication, a way to regulate negative emotions, and to cope with their problems. In our and many other studies, coping motives have been associated with high alcohol consumption and alcohol-related problems [17, 181]. It has been shown that heavy drinking and stress exposure mediate the association between early initiation of alcohol use, drunkenness and the use of alcohol to cope with unpleasant emotions [183]. Moreover, personality traits, such as high neuroticism, and low agreeableness, which are mediators of the impact of stressors, have been linked to coping motives for drinking [18, 181]. Further understanding of phenotypes associated with vulnerability to stress comes from animal studies on several species which investigate coping styles, and potential neurobiological mechanisms [184]. Neuroendocrine reactivity as well as different neurotransmitters systems (e.g. the serotonergic system) have been shown to influence the outcome phenotype (e.g. proactive and reactive coping styles) [185]. A recent study revised the Cooper's Drinking Motives Questionnaire and defined a five-factor model which distinguishes between coping-anxiety and coping-depression motives [186]. Coping reasons might reveal the presence of other psychopathologies, and in particular internalizing disorders, such as depression or anxiety. Increased vulnerability to alcohol drinking behaviour has been demonstrated in animal models of anxiety [187], and depression [188]. In an animal model, female rats with a depression-like behaviour showed higher alcohol drinking than males [188]. Different drinking motives have been shown to mediate emotional and behavioural responses to stressors between males and females. Among females, coping motives seems to mediate the relation between childhood maltreatment and alcohol consumption [189]. Additionally, the prevalence of internalizing disorders is higher among females. However, controversial results have been reported with regard to sex and age, with coping motive and alcohol-related problems being more reported by females during early adolescence [181]. Of interest to be explored further would be the following questions: are there specific factors generally predisposing for co-occurring disorders? Do symptoms of a disorder enhance the influence of risk factors, such as personality and expectancies, on drinking motives? Does sex play a mediating role between drinking motives and response to stressors?

Dominance motives

A third drinking motive emerged in our study, and referred to reasons for drinking such as to have other's respect, to have influence/power over others, and to increase one's aggressiveness. This dominance motive explained 8-10% of variance of the motivational model. Contrary to the other motives, it was negatively correlated with alcohol consumption and alcohol-related problems. This could be explained by the desire of not losing self-control, and to be able to dominate the situation. A study on older students reported the reason for drinking to increase aggressiveness as being of minor importance [190]. Another study reported the dominance power motive to be a relevant drinking motive above all among males, and to be related to negative outcomes [191]. Interestingly, dominance/subordinate status has been investigated in relation to alcohol consumption in animals. Social dominance in male monkeys was inversely correlated to alcohol intake, however, the explanation given was that high-ranked animals rarely have to defend their position [192]. The lack of research among human adolescents on this motive makes the interpretation speculative.

Methodological features

One concern when examining reasons for drinking to identify major drinking motives regards the statistical methodology applied (Paper I). The rotation in the factor analysis can be seen as a way to determine the best solution, which is characterized by a number of items in a factor giving the maximum explained variance. In our study, we used the orthogonal Varimax rotation, which assumes that the factors/motives are uncorrelated as much as possible to each other, maximizing the contribution of one reason to one motive, and minimizing the contribution of those reasons to the other motives. This method was chosen to obtain an easier interpretation of the results, but can be criticized as an artifact since it is less realistic than the oblique rotation which assumes that different degrees of correlation can be observed among the factors. In reality different motives are correlated to each other, such as in the case of an adolescent who drinks for both coping, and enhancement reasons (e.g. to drink to improve a bad mood, and because it gives a pleasant feeling). Therefore, an alternative would be to extract overlapping motives for drinking, though the understanding of the outcome would be more complicated. A strength in our study, based on the principles of the Grounded Theory, is that our theory/results were extracted from data collected with a qualitative approach. The factor analyses aimed to identify different factors in which different reasons for drinking clustered, without presuming a numbers of factors to be obtained. On the basis of previous studies, the extracted factors were afterwards identified as different drinking motives [17, 21].

Drinking motives and psycho-biological pathways

Two major motives for drinking have been recognized in the social-enhancement and coping motive domains, which seem to differ with regard to precursors, and consequences [17, 181]. The different motivational-anticipatory factors involved in initiation or maintenance of the drinking behaviour could be related to several neurobiological systems (Paper I). As adapted from a three-pathway model proposed for craving, the social-enhancement motive could be related to the dopaminergic/opioidergic systems which are involved in the reward and reinforcement pathways, the dominance motive with the monoaminergic system involved in modulating aggressivity, and the coping motive with the serotonergic/gamma-aminobutyric acid (GABA)-ergic/glutamatergic systems involved in mood and stress regulation [193]. Interestingly, Cloninger's psychobiological model proposes that the three basic personality dimensions for temperament (novelty seeking, harm avoidance, and reward dependence) are related to the dopamine, serotonin, and norepinephrine neurotransmitter systems, respectively [194]. According to Cloninger's theory, personality traits have high heritability (temperaments) but also undergo changes by the environment during adulthood (characters) [195]. Previous studies demonstrate a role of drinking motives (i.e. enhancement and coping motives) in mediating the relation between alcohol use disorder and certain personality disorders (e.g. antisocial disorder) characterized by personality traits such as neuroticism and impulsiveness [196, 197]. Therefore, in concert with motives for drinking, a critical role in vulnerability to alcohol use disorders is played by personality traits. For a future study, one approach would be to investigate, in a longitudinal manner, the relation between drinking motives, personality traits and genetic variations. The results of the present studies (Paper II and III) could be viewed within the picture of "motivational patterns". Of interest, in relation to our results of Paper III, a recent study on adolescents alcohol use disorder found evidence that the association between a polymorphic variant in an opioid receptor and alcohol-related problems is mediated by enhancement motives [165]. In our study, adolescents with depressive symptoms might have used alcohol to cope with their problems, thus the association between depressive symptoms and the *OPRM1A118G* polymorphic variant in interaction with alcohol consumption is likely to be related to the coping motives. This might be related to the neuroendocrine response to stress, which also involves the stress-induced response of the endogenous opioid system. An alternative would be that adolescents with depressive symptoms would drink to enhance their mood. This might be related to the involvement of the endogenous opioid system in the reinforcing effect of alcohol. Moreover, it would be interesting to assess reasons for not drinking, their relation with personality dimensions, related biological and environmental factors for risk/resilience to stress [79].

Gene-environment interactions among adolescents

Drinking motives are mediators and indicators of risk of alcohol use disorders. A study on drinking motives among adolescent twins found that monozygotic and dizygotic pairs do differ with regard to drinking expectancies after they had experience with alcohol, however, their expectancies did not differ before to begin drinking. Therefore, it seems that environmental factors, such as family and peer influences, have a higher impact before the experience of drinking, while genetic differences emerge after the experience of drinking [53]. As mentioned with regard to coping motives, psychiatric symptoms or disorders can contribute to early initiation of alcohol use, and to the trajectory which leads to alcohol use disorders. Papers II and III aimed to investigate the interaction effect between polymorphic variants in genes of interest for alcohol use disorders, and psychosocial factors, such as sleep problems and depressive symptoms, in relation to alcohol consumption among adolescents. Sex-specific effects on G x E were also investigated.

Circadian clock system and sleep problems

Paper II presented a novel association between alcohol consumption among adolescents and a polymorphic variant of the clock gene *PER2* in an interaction with sleep problems. The study presented in Paper II has to be considered exploratory, and results need to be replicated in independent samples. A population-representative group of adolescents has been genotyped for *PER2* SNP10870. Sleep problems were assessed by 18 questions on frequency of different sleep disturbances [112], and alcohol consumption by the first three question of the AUDIT instrument relative to consumption (AUDIT-C) [33]. A statistically significant main effect for the *PER2* SNP10870 polymorphism was only present in a G x E model.

The *PER2* gene, together with other clock genes, participates in the regulation of the circadian clock system [198], which is of importance for many brain and physiological functions, such as sleep regulation, and behaviour [122, 199]. Disruption of the circadian clock has been investigated in several psychiatric conditions, including addictive, affective and sleep disorders [123, 198]. For example, a mutation in the *PER2* gene is associated with a type of the familial advanced sleep phase syndrome, an autosomal dominant circadian phenotype [124]. Sleep disturbances, substance misuse, and comorbid conditions, such as mood disorders, are common among adolescents [117]. In our study on adolescents, alcohol consumption and sleep problems were moderately correlated, however, alcohol consumption was higher among boys, while sleep problems were reported more by girls.

Many psychiatric conditions show seasonal exacerbation, such as patients having an episode of depression during specific times of the year, usually during the winter, as well as in comorbidity with alcohol use disorder [200]. Seasonal variations implicate influence of light on melatonin levels, and on

the circadian behaviours [201], as well as on the functioning of the serotoninergic system [202]. Additionally, i*n vitro* and animal studies suggest the role of glutamate in the circadian-clock phase resetting, in interaction with light inputs, to be likely mediated by the brain-derived neurotrophic factor (*BDNF)* [203, 204]. Moreover, a link between circadian clock genes and the monoaminergic theory of depression is suggested by the regulatory activity that the *PER2* gene has been shown to exert on the monoamine oxidase (*MAOA)* gene transcription in mice. As a consequence, dopamine levels and the reward system are altered, with effects on mood status [125, 126]. Indeed, the monoaminergic hypothesis has been investigated in relation to behaviours and several psychiatric disorders [205]. Variations in the *PER2* gene have been associated to depression vulnerability in a Swedish sample [129]. Interestingly with regard to antidepressant treatment, is the mechanism of action of lithium which might be modulated by the circadian system [122].

Drugs of addiction are linked to the circadian rhythm, disruption of sleep/awake cycles, and to the presence of mood disorders related to circadian rhythms (e.g. seasonal affective disorders) [123, 198]. The *PER2* gene has become an interesting candidate gene for studies on alcohol consumption [123, 198]. Spanagel *et al.* showed that *Per2* mutant mice exhibit decreased expression of the glutamate transporter GLAST, a consequent hyper-glutamatergic state, and increased alcohol intake. Extracellular glutamate levels and alcohol intake in mice were shown to be reduced after acamprosate treatment, a drug used for alcohol dependence and preventing of relapse [127]. Of interest, the G allele of the *PER2* SNP 10870 has been associated with an increased transcription of the *PER2* gene [127, 128], thus with potential implications on the regulation of the circadian clock system. This polymorphism has been investigated in relation to alcohol dependence, with the genotype AA associated with high alcohol intake [127]. In our study, in a G x E model with the alcohol consumption as the outcome variable, the results showed a main effect of the *PER2* SNP10870 polymorphism and sleep problems, and a significant interaction effect between *PER2* SNP10870 and sleep problem among adolescent boys. Carriers of the AA genotype were scoring higher in alcohol consumption in the presence of several and frequent sleep problems. This result was in line with the study by Spanagel *et al.* in which the sample included a majority of severely alcoholic males [127]. Among adolescent females, there was only a significant main effect of sleep problems, which accounted for the variance of the G x E model in relation to alcohol consumption. Frequent and several sleep problems were more common among adolescent girls, and the relation between sleep problems and alcohol consumption was stronger in girls than in boys, thus a potential genetic effect among girls might have been covered by this relation.

In the investigated Swedish region notable seasonal changes occur, with only ~5 daylight hours during the darkest and ~19 daylight hours during the lightest time. Therefore, it would be of interest to investigate on adolescent

alcohol consumption and *PER2* SNP 10870 in relation to seasonal changes and mood disorders.

The endogenous opioid system and depressive symptoms

Paper III presented an association between depressive symptoms among adolescents and a polymorphic variant of the *OPRM1* gene in an interaction with alcohol consumption. A population-representative group of adolescents was genotyped for the *OPRM1A118G* SNP. Depressive symptoms were assessed by 15 questions related to the 9 items used in the A criterion of the DSM-IV-TR to assess depressive disorders [23, 206], and alcohol consumption by the first three question of the AUDIT instrument relative to consumption (AUDIT-C) [33]. A significant main effect for the *OPRM1A118G* polymorphism was only present in a G x E model.

The endogenous opioid system plays a role in the rewarding and reinforcing effects of alcohol, through the endogenous opioid β-endorphin, and interaction with the dopaminergic mesolimbic system [139, 143, 146]. Involvement of the endogenous opioid system has also been demonstrated in mood regulation and other psychiatric conditions [139], with β-END playing a role of in anxiety and depressive disorders through a regulatory effect on the HPA axis activity [207]. Dysregulation of the HPA axis is a central feature of alcohol use disorder, as well as of other disorders such as depression. The role of μ opioid receptor in stress reactivity and emotional response is mediated by the hypothalamic-pituitary-adrenal (HPA) neuroendocrine response [208]. Of several polymorphisms investigated in the *OPRM1* gene, the *OPRM1A118G* has been one of the most studied in relation to alcohol use disorder. The polymorphism consists of an A/G substitution, resulting in an amino acid change of asparagine into aspartate at codon 40 (rs1799971) [148, 158]. The effectiveness of drugs for alcohol dependence has also been shown to be influenced by this polymorphic variation. Carriers of the G allele respond better to naltrexone treatment [161-164]. However, controversial results are presented with regard to the change in functionality related to this polymorphism [148-151, 162]. Nevertheless animal studies with non human primates provide support for a role of the orthologous *OPRM1A118G* polymorphism on alcohol intake, on naltrexone treatment efficacy [153], and attachment after maternal separation [209]. In humans, the HPA axis response to stress and alcohol consumption has also been associated with the OPRM1, but with controversial results [144, 210, 211]. The G allele has been associated with an increased HPA axis activity in response to opioid receptor blockade [212, 213]. Additionally, differences in the neurotransmission mediated by the μ opioid receptor have been found in female cases of depression in comparison to healthy controls [208]. However, no association between the *OPRM1A118G* polymorphism and anxiety-related traits was found in two large Australian cohorts of adults and adolescents [214]. Thus, a rationale for investigating the *OPRM1A118G* polymorphism, depressive symptoms and alcohol consumption seems well supported. An interaction effect was found in our study only in young girls,

with adolescent carriers of the G allele reporting higher alcohol consumption and an increased number of depressive symptoms three years later. Thus, carriers of the G allele might show an altered function of physiological systems, such as the HPA axis and the reward system, modulated by the endogenous opioid system. In our study, a significant relation between the *OPRM1A118G* polymorphism and alcohol consumption was not present, probably because of the study sample which is population - representative. However, initial evidence for an association between the OPRM1 G allele and adolescent alcohol use disorders, as well as with alcohol-related problems mediated by enhancement motives for drinking, has been demonstrated in a recent study [165]. In our study model (Paper I), coping motives could mediate the association between depressive symptoms and alcohol consumption. However enhancement motives could also be of importance for those adolescent with depressive symptoms who drink for positive internal reasons. Depressive symptoms alone were more common among young girls, while alcohol consumption was higher among boys. The GxE model analyzed in our study showed a sex-specific effect also at different ages. A potential explanation could imply pubertal changes which occur later in boys than girls, as well as increasing alcohol consumption in parallel with increasing age (5th grade of compulsory school vs. 3rd grade of high school).

Additionally, the association between alcohol consumption and the *OPRM1A118G* polymorphism in interaction with depressive symptoms has been investigated in an older sample of adolescents (2nd grade of high school) participating in a cross-sectional study. No G x E effect was present. The strength of this study is that it includes a large population-representative sample of adolescents, however, it does not allow investigations in a prospective manner and measures were based on self-reports.

In a comparison between the two studies including the adolescents (Paper III), few concerns have to be mentioned. In the SALVe 2001 study, the assessment of the alcohol intake was based on interview data, for which data on each type of beverage were available. On the contrary, alcohol consumption (AUDIT-C) was assessed in both studies by questionnaire. We have shown in the same study sample (SALVe 2001) that adolescents self-report their alcohol consumption in a different manner when different methods of assessment are used [29]. Moreover, the SALVe 2001 sample was part of a large-population representative sample of adolescents, however, the final sample used in this study was selected with particular attention in including an equal number of adolescents representing the extreme group with regard to several risk behaviour indexes (e.g. alcohol use). Thus the SALVe 2001 study might have more power to detect G x E interaction in relation to alcohol consumption since sufficient individuals from both ends of the deviant behaviour continuum are included.

The opioid receptor system also appear to interact with the circadian clock system [122], and opioid agonists have been shown to regulate the *PER2* gene expression [215]. Thus, the link between the opioid and the circadian

clock system might represent an interesting study subject. Moreover, several brain regions have been related to depressive and anxiety disorders, among which the hippocampus and the amygdala. These brain regions are involved in the process of emotional memory, reward, stress and fear stimuli, and exert a modulatory effect on the HPA axis. A study based on brain imaging techniques would be a complementary approach of great interest.

Sex differences

The results of papers II and III showed a sex-specific effect, and plausible explanations might be related to several development mechanisms [94-96]. Sex differences in the expression of affective disorders have been attributed to gonadal hormonal influence, psychosocial, and genetic factors [216]. Indeed it is known that hormones influence a wide array of behaviours, and that sexual dimorphisms occurs in neurotransmitter systems [95], in brain anatomy and functioning [95, 96], and can be modulated by epigenetic mechanisms [217]. It is important to consider that the interaction effect of the *PER2* SNP10870 with sleep problems in boys, or of the *OPRM1A118G* with depressive symptoms in girls, might reflect sex differences in stress reactivity which in turn affect alcohol consumption, and an increased susceptibility to different types of psychiatric disorders. The prevalence of several psychiatric disorders differs between the sexes (e.g. depressive and anxiety disorders occur more often in females, while childhood attention-deficit hyperactivity disorder and drug dependence are more frequent among males). Sex-differences have also been reported in alcohol consumption in response to childhood stress between female and male rats [98]. Of interest, studies from our group showed that adolescents boys and girls present different G x E effects in relation to alcohol consumption [64, 65]. However, caution on the interpretation of these sex differences should be used until other replications are demonstrated.

Specific limitations

Adolescence is a developmental period during which drinking patterns start to emerge, and are influenced by several factors [135]. Comorbidity between alcoholism and other psychiatric disorders is common [91], but inconsistent results exist regarding the aetiological factors involved in case of comorbidity and/or shared between the disorders. Internalizing symptomatology are usually preceding alcohol use disorder, and are shown to increase the vulnerability to early initiation of alcohol use and alcohol use disorder [135, 137]. A limitation of our studies (Papers II and III) is the lack of information on which risk factor is antecedent, or the causal relationship between the different variables considered (e.g. sleep problems could be risk factors or symptoms of alcohol use disorder). A longitudinal approach from early childhood could address, and provide more information about this question.

A limitation regards the SALVe 2006 sample for which the data were collected by questionnaire, however self-reports are valid instrument to be used

in large scale epidemiological studies [5, 6, 218]. The study sample SALVe 2001 has been investigated by using assessment methods with high validity (e.g. personal interviews) which allow to exclude bias such as unserious self-reported data (e.g. 250 cm of height), and by collecting blood samples from each individual excluding bias due to inter-changed samples between individuals. Furthermore, our studies included adolescents randomly selected from the population, thus the samples can be described as population-representative. However, bias could have been caused by non-random sampling.

A strength is that our studies focused on single polymorphisms previously associated with alcohol use disorder. However, other environmental factors such as stressful life events or psychosocial factors could have been taken into account. Furthermore, in Paper II and III, the inclusion of questions regarding the presence of sleep problems, or depressive symptoms, during periods of time of no drinking, might have helped the understanding of such problems during adolescence.

Association studies have sometimes failed to replicate previous results, probably due to one or more of the following reasons: small sample size, population stratification, and imprecise measures of the phenotypes. When comparing different studies, these factors represent a problem in drawing robust conclusions.

Severely alcoholic females

A sample of severely alcoholic females was collected over several years, and included as a study sample in Papers II and III. A major limitation of this study sample regards the relatively small size of the severely alcoholic women sample, on the other hand the individuals included in the study represent an extreme group among alcoholics which have been collected along many years. Moreover, relatively few studies on alcoholism have included women. At present about 70 more severely alcoholic females, for which the data have been collected during the past few years, are going to be included in the study sample.

In Paper II, on the basis of the results presented by Spanagel *et al*., an exploratory analysis on *PER2* SNP10870 was carried out on a sample of severely alcoholic women. In a comparison with a control sample of females, a non significant trend was observed, with the G allele being overrepresented among the severely alcoholic females. The result did not reach significance, however, the *PER2* SNP 10870 has been associated with winter depression, with the G allele as a risk variant in a sample with a majority of Swedish women [128] . The study of Spanagel *et al.* included a sample of severe alcoholics with the majority being males [127], thus the results cannot be generalized for both males and females, and replication in a larger sample of females is needed. Moreover, the severely alcoholic females with other comorbid psychiatric disorders were excluded, and, although many studies do so,

this is arguable; however there was no information available on which disorder occurred first.

With regard to the association between *OPRM1A118G* and alcohol use disorder in human adults, controversial results have been found [155, 156, 219]. In Paper III, we investigated this polymorphism among severely alcoholic females. Our results showed a significant overrepresentation of carriers of the G allele among the severely alcoholic females compared with a control sample of females. Furthermore, alcoholics with comorbid conditions, such as anxiety or affective disorders, showed a higher frequency of the G allele than alcoholic women without these disorders.

Moreover, we investigated if the *OPRM1A118G* genotype frequency was associated with any presence of psychopathologies (i.e. alcoholism and depression) among the parents of the severely alcoholic females compared with the genotype frequency of a control sample of females. As in a previous study [220], the genetic effect was evident only in the absence of psychopathologies among the parents. One reason for this could be the stronger impact of negative psychosocial factors, such as parental influences, on future risk of alcohol use disorders which obscures the effect of genetic factors. A limitation regards the data on parental psychopathologies, which do not rely on a diagnosis, but on self-reported information by the patients. Furthermore, the small sample size and the absence of a sample of severely alcoholic men with which to investigate possible sex differences are the major limitations of this study.

Statistical concerns

Regarding the statistical methods used, a double approach was applied: both parametrical and non-parametrical methods were used in our studies. A complementary approach had the purpose to increase reliability of the obtained results, demonstrating that the same or similar results can be obtained independently from the statistical method used. Furthermore, the prerequisites needed to use a certain statistical method have not always been present, thus allowing doubts on the robustness of the results. As an assumption, parametric studies require that the variable used should be normally distributed. Alcohol consumption is for example a variable which is rarely normally distributed, however, according to the central limit theorem, the variance approaches a normal distribution with an increasing sample size. It can be assumed that the distribution of the standard error tend to be normal and subsequently that the variable tends to be normally distributed, and thus parametric tests can be used. Additionally, different methods use the selected dependent variable in a different manner, which might not match the research question (e.g. AUDIT-C as a scale or as a dichotomous variable). A continuous measure seems more suited to investigate dimensional variables such as alcohol consumption, while the use of cut-offs reduces the statistical power, and for many variables there are no clearly defined thresholds. More-

over, the univariate analysis considers only one variable, it has a more descriptive purpose, and allows easier interpretations, while the use of multivariate analysis make it possible to take into consideration more variables, and the interrelation between them, the interpretation thus becomes more complicated, but also more realistic.

Concluding notes

Potential mechanisms underlying adolescent alcohol consumption are likely to include adaptive changes in neuronal circuits, such as the endogenous opioid or the circadian clock system. These pathways needs to adapt in response to the interaction effect between genetic variants and environmental stressors [50], to epigenetic mechanisms [221], and of neurotransmitter-mediated effects on neurogenesis during development and adulthood [222, 223].

Alcohol consumption is a complex behaviour, and adolescence is a developmental time of physical and behavioral changes. The findings of the present thesis suggested potential pathways involved in alcohol consumption, and the need to consider the interplay between genetic and psychosocial factors to study alcohol consumption among adolescents. By learning more about gene-environment interactions, we might be able to identify factors that contribute to the vulnerability to develop alcohol use disorders, the identification of new therapeutic targets, and prevention strategies.

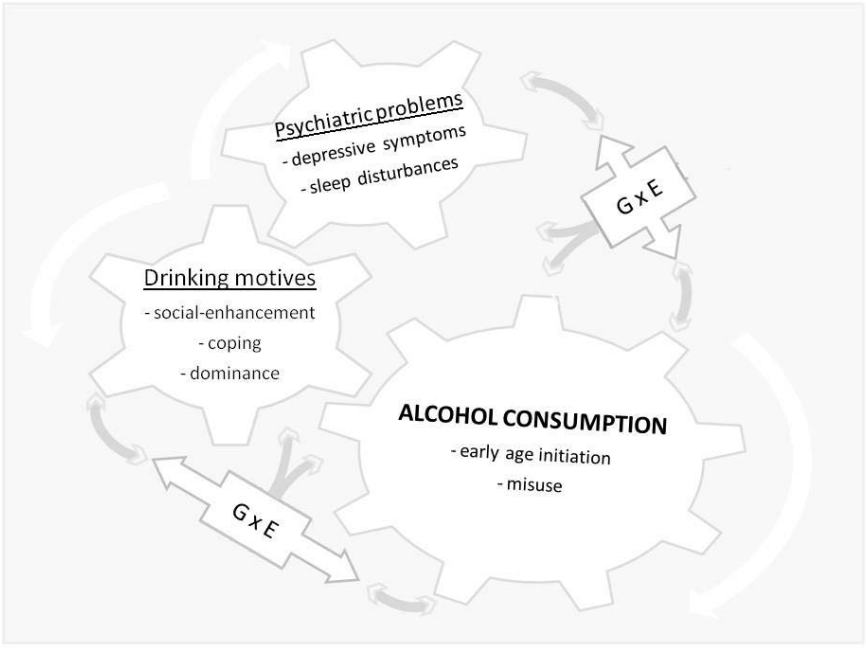


Figure 6. Alcohol consumption among adolescents: a simplified model of the complex interplay between motivational, genetic, and psychosocial factors

Alcohol consumption measures

Survey validity

A major concern in survey research, and particularly in the self-reporting of sensitive behaviours such as substance use, is *validity* [218]. Validity is defined as the degree to which the survey measures those aspects of alcohol consumption, or other behaviours of interest, that are intended to be measured. A *bogus pipeline procedure* has been used in previous studies to test the validity of self-reports, and whether certain behaviours are under or over-reported [36, 37]. In some of our studies biological specimens (i.e. blood, hair) were collected for biological analyses at the same time as the survey was carried out, and thus might have had an influence in self-reported alcohol consumption. It is important for the adolescents to perceive the survey as anonymous, and to be confident that reporting behaviours which are not socially accepted will not imply any negative consequences for them [218]. Both interview and questionnaire can lead the adolescent to a social desirability bias in answering the surveys [218]. *Social desirability*, i.e. the tendency of respondents to give answers that a person believes will show themselves in a good light in the eyes of others, is an important issue in surveys on behaviour which is not society acceptable (e.g. underage binge drinking). Another crucial factor is *reliability*, i.e. the extent to which repeated measurements made under the same conditions produce the same result [218]. Data from a few questions in the questionnaire have been used to measure reliability with regard to inconsistency in relation to lifetime use. For instance, the questionnaire for the SALVe 2006 study contained questions about the use of many substances of abuse. A later set of questions dealt with age at first use of these various substances of abuse. These questions included the response alternative "never", which makes the comparison between these two sets of questions possible (e.g. 3 adolescents stated to have never drank alcohol, however, when they were asked about their age at first use of alcohol they indicated an age, thus giving a contradictory answer: reliability on 152/155 adolescents in the SALVe 2006 study). Test-retest reliability controls would have been possible only if the studies were not anonymous, and the same measurement would have been repeated within a close range of time. Concerns regarding the *representativeness* of the samples, and the generalisability of the results, are also of importance. Samplings were based on schools, and similar consumption patterns to our studies were found in other data collections in Sweden, and other countries [5, 6]. The comparability of the actual results across countries is of vital importance to any survey project. The questions regarding alcohol were based on the AUDIT [32], which is a world-wide validated assessment tool, and the AUDIT-C, which has been validated among teenagers against other alcohol measures, showing considerably high specificity and sensitivity [31, 33]. The question on alcohol related

problem was based on the one used in the ESPAD report which is carried out among European teenagers [65, 166]. On the contrary, the questions on reasons for drinking have not been previously validated (Paper I), but the purpose of the study was to collect data on the topic without any pre-assumptions. The measures of sleep problems and depressive symptoms have not been thoroughly validated among adolescents, however the tests have been developed following previous qualitative studies carried out in our group [100, 101] and were based on validated screening tools [23, 112]. In summary, these measures represent suitable screening tools for adolescents, and allow the detection of so called "diagnostic orphans", that is symptomatic individuals who would not have been considered if criteria for a clinical diagnosis would have been applied [224]. The *response rate*, defined as the proportion of students who completed the questionnaire out of all students in participating classes, was 77.4% in the SALVe 2006 study, which is lower compared with 84% of the ESPAD reports [5, 166, 218, 225]. Many students who were ill the day of the investigation, or absent from class for other reasons, did not return the questionnaire later by mail. It also has to be mentioned that those adolescents with most behavioural and psychological problems are more likely to be absent from school. Therefore it is possible that the missing data regarding these adolescents might have biased our results. The *internal response rate* was however quite high (e.g. 99.7% for the AUDIT-C in the SALVe 2006 study). The combined use of different screening tools could thus be the optimal choice.

Alcohol consumption measures

Paper IV highlighted the interest on which instrument can be used to assess alcohol consumption among adolescents. There is a lack of studies on biochemical measures to assess alcohol consumption among adolescents, who can be heavy but episodic alcohol users. On one hand, direct biomarkers have lately been preferred to indirect ones because of their higher specificity and sensitivity [38]. Indirect biomarkers, such as carbohydrate-deficient transferrin, mainly reflect the organ damage and response to ethanol exposure, which is not usually the case in adolescents. On the contrary, direct biomarkers, such as phosphatidyl ethanol, and fatty acid ethyl esters, are products of the ethanol metabolism still containing an ethyl group; thus, these biomarkers seem more suitable to assess adolescent alcohol consumption.

Interview and questionnaire

In Paper IV, PEth and FAEEs biomarkers were analyzed in blood and hair samples, respectively, to detect alcohol consumption among adolescents, and then compared to questionnaire and interview measures of alcohol consumption. An important issue in these types of study is to identify the so-called gold-standard, which means a reliable measurement tool used as reference

when comparing other measurement methods. Sensitivity and specificity have thus been key concepts of this study as they embody the 'gold standard' or reference measurement. To compare different methods for measuring alcohol consumption, the interview was taken as the reference method. One reason for this choice was the concurrent use of a pipeline procedure. A *bogus pipeline procedure* has been reported to potentially increase the validity of the answers, although with controversial results [35-37]. In our study, the pipeline procedure consisted in alerting the adolescent before the interview of the future control of their answers against biochemical analyses on a sample of their blood and hair for drug use. This procedure has been employed to more accurately estimate alcohol consumption since individuals normally tend to under-report behaviours, such as underage alcohol consumption. Topics that are sensitive might cause feelings of shame and guilt, therefore respondents might feel less intimidated to answer a paper questionnaire than being confronted with a stranger asking questions in a face-to-face interview [226]. However, confidentiality was promised before the interview to encourage the adolescent to give the most sincere answers. On the other hand, the use of questionnaires was providing a more anonymous environment for the respondents by letting them feel more comfortable in reporting personal behaviours. However, participants could have been tempted to under-report alcohol consumption in the questionnaire when not facing and reporting verbally to an adult [226]. The rate of congruence between questionnaire and interview in classifying adolescents as high alcohol consumer was 89% (Paper IV), however it cannot be assumed that self-reports were necessarily the less valid measure in our study.

Questionnaires, especially if simultaneously administered to a group, allow the gathering of data from several individuals in one session, and ensuring a considerably high response rate compared with questionnaires sent via mail. In all our studies with questionnaires, the majority of the questions was in the form of closed-ended questions (UPPLAND and SALVe projects). Closed-ended questions can be more specific while open questions allow respondents to use their own words, and make the comparison between different individuals difficult [218]. Closed-ended questions are easily analyzed by given a number to the answer so that a statistical interpretation can be assessed. They also consume less time for both the participant and the researcher and have an increased response rate compared with surveys that use open-ended questions. Misunderstanding or inattention can contribute to distortion or a lack of reporting information in questionnaire screenings. On the other hand a face-to-face interview can ensure a full qualitative approach. The interview makes it possible to adapt to each individual situation, and to obtain much more information when the personal situation requires it. An interview gives the possibility of catching personal experiences with alcohol and of obtaining more detailed information about type of drinks, frequency, and quantity of consumption. Interviewers try to converse with

the participants without any suggestive effect on their answers, and are able to explain potentially ambiguous questions. Contrary to questionnaires, interviews usually make use of open questions which are not very suitable for computer analyses. If open questions are analyzed quantitatively, the qualitative information is reduced to coding, and answers tend to lose some of their initial meaning. As an alternative, a semi-structured interview was used in our SALVe 2001 study to facilitate the use of the data with a quantitative approach (Papers I, III and IV). Advantages and disadvantages regarding time and costs include the pre-planning of the study design, training for the interviewers, and the delivery and re-collection of the surveys.

In a comparison between written questionnaires and face-to face interviews both strength and limitation are therefore present for each method. Concluding, self-reporting methods (e.g. questionnaires) seem to be reliable and valid instrument to assess alcohol consumption, and to be used in large-scale epidemiological studies [5, 6, 218]. Indeed, a questionnaire could have also been taken as a reference measurement in our study (Paper IV) to measure the sensitivity and specificity of the biomarkers. The congruence rate between questionnaire and biomarkers has, however, been reported, and the resulted smaller than with the interview. Furthermore, data on alcohol consumption from the interview considered the quantity and frequency of different type of beverages, and thus provided with more detailed information on alcohol consumption. No gold-standard or reference method has been clearly identified by previous studies, thus misclassifications of the reference test, which was in our case the interview, could have translated into biased assessments of sensitivity and specificity of the biomarkers and questionnaires.

Biomarkers and limitations

Regarding biomarkers, the use of both PEth and FAEE tests in our study presented several limitations. First of all was the consideration that a typical drinking pattern among adolescents is binge drinking, which is defined as heavy but episodic drinking. Thus, the biomarkers could not have been taken as reference methods as these tests have been developed to detect heavy and frequent alcohol consumption. Another limitation to the use of these biomarkers is the lack of reliable information regarding cut-off values which differentiate between abstinence, moderate drinking and alcoholism, as well as regarding the correlation between reported value and the amount of alcohol consumed [38]. Furthermore, over the last years there has been progress in the methodology used to analyse these biomarkers.

PEth biomarker

In our study (Paper IV), the biomarker PEth, when compared with interview, scored the highest in specificity which corresponded to detecting 187 (93,5%) adolescents as non high alcohol consumers. This result agreed poor-

ly with the 96 (48%) adolescents described as non high alcohol consumers by the interview. On the other hand, the 13 (6.5%) who were identified positive for PEth (0.25-0.7 μmol/L) are probably at risk of developing hazardous drinking, and therefore could be the target of prevention/intervention strategies. Additionally, PEth concentration levels above a certain cut-off have been demonstrated to correlate to the amount of alcohol consumption [227], however, subject variability in the relationship of PEth concentration to the grams of alcohol ingested has been reported [228]. In our study, PEth levels did not reach the clinical threshold for alcohol use disorder used in Sweden (0.7 μmol/L) [229]. Recently, a more sensitive and specific analytical method for the quantification of individual sub-species of PEth has been developed. This method can detect even lower drinking levels (quantitation <0.1 μmol/L versus <0.25 μmol/L in our study) [229].

The main advantage of the PEth biomarker is its specificity as it can be formed only in the presence of ethanol, therefore no false positives can be detected by analyzing PEth. Second, PEth has high sensitivity but requires a heavy and frequent alcohol consumption of ≈ 50 grams per day during the previous few weeks to be detected [170]. Thus, PEth is suitable mainly in the case of regular heavy drinking, which is obviously not applicable to adolescents who usually report heavy but episodic drinking mainly during the week-end. In our case, the PEth test could have resulted in a false negative result. Among the disadvantages of using PEth there is its relatively short time window of detection which is around two weeks, with a half-life of about 4 days [43, 170]. In our study an adolescent could have used or misused alcohol twice or more times during the previous month, but have not got drunk in the previous two weeks, because of, for example not going to parties due to school examinations. A further concern regards the influence of storage conditions on the measurement of PEth (Paper IV). The blood samples in our study were collected during the year 2001, frozen, and analyzed for PEth in 2007. No information is present to indicate PEth stability over years, though its stability was proven to be up to 144 hours when frozen in liquid nitrogen and stored at -80 ºC [230, 231].

FAEE biomarker

FAEEs were used as alcohol biomarker in hair in our study (Paper IV). It has been demonstrated that FAEEs are sensitive and specific long-term markers for alcohol intake [171]. An advantage of analyzing FAEEs in hair is that the method is non-invasive. The hair grows about 1 cm/month, thus the time window of assessment depends on the length of the hair specimen [232], and can be large enough to retrospectively detect the drinking history of an individual in term of months, which represents one of the main advantages of this method [171]. However, in our study (Paper IV), the retrospective history of alcohol consumption was not examined, despite the fact that proximal hair samples of up to 6 cm were taken.

A concern regards false positive results. Different mechanisms have been proposed with regard to the incorporation of FAEEs in hair root via blood, or via sebum after esterification in the sebaceous glands [232]. With regards to FAEEs in hair, they are end-products of the ethanol metabolism, and therefore should be present only after alcohol consumption. However, low quantities of FAEEs have been reported among life-long abstainers [232, 233]. An explanation for this could be the potential interference of cosmetics, and hair care products, containing ethanol which could lead to false positives. On the other hand, some hair treatments could lead to false negative [233]. The adolescent use of hair cosmetics (e.g. gels, sprays, lotions, shampoos, and dying treatments) is common, and thus could partially explain some of the false positive and false negative results in our study.

The cut-offs proposed by previous studies to distinguish between teetotalers, moderate social drinkers, and heavy drinkers partially overlap, and do not clearly provide information on the correlation between the quantity of FAEEs and amount of alcohol consumed [45, 172, 234]. In our study, the use of different cut-off points ($\geq$ 0.67ng/mg; $\geq$0.40ng/mg) changed the sensitivity (12% and 30%) and specificity (85% and 61%) of the test in compared with interview, as well as the correlation ratio between FAEEs and the questionnaire. No significant correlation between the two biomarkers was found. Moreover, the analytical determination of FAEE in hair needs specific expertise and time consuming procedures from sample collection to extraction. A recent study, however, proposed a new method which reduces the time for analysis from 15 to 1 hour, decreases the detection limit, and increases selectivity [235]. A further concern regards the influence of storage conditions on the measurement of FAEEs. In our study the hair samples were collected during the year 2001, stored at room temperature in aluminium foil, and analysed in 2004 for FAEEs. Hair can uptake ethanol from the atmosphere if present [233], though this can mainly be excluded from our study, there is, however, no information regarding their stability over years.

Concluding remarks

Being aware of the strengths and weaknesses of each method, a combined approach by analyzing different biomarkers, and using several surveys would increase the probability to correctly identify both true positives and true negative cases [236]. For future investigations on adolescents, a hypothetical study would make use of the biomarkers analyzed using today's analytical methods, and internet or telephone self-reports to detect alcohol habits with higher frequency. The study would be based on a questionnaire via e-mail at baseline, a telephone interview after two weeks, and an interview and the collection of biological samples to analyze two direct biomarkers from two different sources (e.g. PEth in blood, and EtG in urine) in the third week. The instrument used to evaluate alcohol consumption would be the AUDIT-C.

Conclusions and perspectives

Alcohol use and misuse are widespread among Swedish adolescents. The present thesis focuses on alcohol consumption among Swedish adolescents (16-22 years of age), and therewith related psychosocial and genetic factors.

One main finding was that adolescents drink for different reasons, and that drinking motives are associated with different consequences, such as alcohol misuse and alcohol-related problems. Reasons for drinking are likely to encompass several psychosocial factors, such as depressive symptoms, which should be further investigated in relation to alcohol consumption in a longitudinal manner. Furthermore, the results showed an effect of plausible candidate polymorphisms only when considered in the context of a GxE model. A single genetic variation in itself is not sufficient, and not necessary to a phenotype. Therefore, studies on alcohol consumption among normal populations of adolescents should focus on the interaction between genetic and environmental factors. Sex differences are likely to modulate the interplay between genes and environment. Drinking motives and psychiatric symptoms should be investigated to identify risk/resilience factors for early initiation of alcohol use and alcohol use disorders. Moreover, a complementary use of different methods, such as self-reports and biomarkers, seems to be the most appropriate way to assess alcohol consumption among adolescents.

This thesis aims to provide the reader not only with a summary, but also with a review of the included works. Being aware that the studies could have been done and interpreted in different ways, this thesis aims to represent a starting point for discussion. The present findings have thus to be interpreted in view of the limitations and strengths of the studies, and call for further replications.

The importance of the studies: although single genotype-environment interactions account for only small proportions of the total variance of a phenotype, each uncovered association promotes the understanding of a disorder, and opens new approaches for therapy and prevention, as well as new inputs for further research on the psychosocial and biological mechanisms involved in such interactions. Further investigation on G x E effect in relation to alcohol consumption will be carried out in the next future among the presented samples of adolescents.

Potential future studies would focus on the early adolescence time, and would investigate how risk and resilience factors combine over development to influence alcohol use disorders, and comorbid disorders.

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