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Effects of Prenatal Alcohol Exposure on the Epigenome, Gene Expression and Development

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Suljen silmät,
kuvittelen et lennän,
mun täytyy luottaa,
ja antaa vaan mennä,
vien tän äärirajoille

Jokainen kyynel tekee vahvemman,
siks en niit pelkää vuodattaa.
Ylöspäin yks kerrallaan aina askelman,
tulin voittamaan en anna
minkään seisoo tiellä.

Tää ei tuu poistuu täältä koskaan,
Timantit on ikuisia.

Tänään ihmisen puolikas
on huomenna Leijonakuningas!

-Cheek
-Profeetat
-Ellinoora

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LIST OF ORIGINAL PUBLICATIONS

I. Marjonen H, Sierra A, Nyman A, Rogojin V, Gröhn O, Linden A-M, Hautaniemi S, Kaminen-Ahola N (2015) Early Maternal Alcohol Consumption Alters Hippocampal DNA Methylation, Gene Expression and Volume in a Mouse Model. PLoS ONE 10(5): e0124931.

II. Marjonen H, Toivonen M, Lahti L, Kaminen-Ahola N (2018) Early prenatal alcohol exposure alters imprinted gene expression in placenta and embryo in a mouse model. PLoS ONE 13(5): e0197461.

III. Marjonen H, Kahila H, Kaminen-Ahola N (2017) rs10732516 polymorphism at the IGF2/H19 locus associates with a genotype-specific trend in placental DNA methylation and head circumference of prenatally alcohol-exposed newborns. Human Reproduction Open, pp. 1–12, doi:10.1093/hropen/hox014.

In studies I and II H.M. participated in and/or conducted:
- design of the study
- dissection of different tissues, brain parts, embryos and placentas
- extraction and quantification of DNA, RNA
- design and optimizing quantitative PCR expression analysis
- design and optimizing bisulfite sequencing
- MRI: perfusion of the mice
- statistical analyses by SPSS
- writing the manuscript

In study III H.M. participated in and/or conducted:
- design of the study
- collection of the human samples
- extraction and quantification of DNA, RNA
- genotyping of samples
- design and optimizing quantitative PCR expression analysis
- design and optimizing MassARRAY EpiTYPER methylation analysis
- design and optimizing bisulfite sequencing
- statistical analyses by SPSS
- writing the manuscript

In the text, the publications are referred to by Roman numerals I-III.

ABBREVIATIONS

AUDIT	Alcohol use disorder identification test
A^{vy}	Agouti viable yellow allele
CTCF	zinc-finger protein, CCCTC
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DMR	Differentially methylated region
DOHaD	Developmental origins of health and disease
E9.5	Embryonic day 9.5
FASD	fetal alcohol spectrum disorder
FAS	fetal alcohol syndrome
GD8	Gestational day 8
H19	long non-coding RNA
H2K4me3	Trimethylation of lysine 4 on histone protein H3
H3K36me3	Trimethylation of lysine 36 on histone protein H3
H3K27me3	Trimethylation of lysine 27 on histone protein H3
H3K9me2	Dimethylation of lysine 9 on histone protein H3
His1h2ai	Histone cluster 1 H2ai
HC	Head circumference
ICR	Imprinting control region
Igf2	Insulin-like growth factor 2
Kcnq1	Potassium voltage-gated channel subfamily Q member 1
Kcnqot1	Kcnq1 overlapping transcript 1
mRNA	messenger RNA
miRNA	microRNA
MRI	Magnetic resonance imaging
Olfr601	Olfactory receptor gene 601
P60	Postnatal day 60
PCR	Polymerase chain reaction
Peg3	Paternally expressed gene 3
qPCR	Quantitative PCR
RNA	Ribonucleic acid
Rplp0	Ribosomal protein, large, P0
Rps16	Ribosomal protein S16
SD	Standard deviation
SNP	Single nucleotide polymorphism
Snrpn	Small nuclear ribonucleoprotein polypeptide N
TET	Ten-eleven translocation
Vmn2r64	Vomeronasal receptor gene
Vpreb2	pre-B lymphocyte gene 2
YWHAZ	Tyrosine3-monooxygenase/tryptophan5-monooxygenase activation protein zeta

ABSTRACT

Prenatal alcohol exposure affects the developing fetus and causes a wide range of physical and neurological abnormalities. The phenotype is generally described as fetal alcohol spectrum disorders (FASD) which is used as an umbrella term for all disorders caused by prenatal alcohol exposure. The severest end of the spectrum, characterized by growth restriction, craniofacial dysmorphology and central nervous system defects, is defined as the fetal alcohol syndrome (FAS). To date, the prevalence of FASD ranges from 3-5% in Europe and North America to over 10% in South Africa. However, since the phenotype is difficult to diagnose when physical features are absent, the true prevalence may be even higher.

Even though, a wide range of the phenotypic characteristics have been identified, the underlying molecular mechanisms of FASD have remained unclear. Previous studies have suggested that epigenetic mechanisms represent a link between adverse early-life conditions and health consequences. Since the epigenome has a critical role during embryonic development by mediating the gene-environment interactions, this has raised interest in exploring the epigenetic changes behind FASD. The aim of this thesis was to approach the subject of prenatal alcohol exposure from the epigenetic point of view by exploring the associations between the molecular changes and the phenotypic characteristics.

In our investigations of prenatal alcohol exposure, we exploited both a mouse model and human samples. Our mouse model is based on moderate voluntary maternal consumption of 10% (v/v) ethanol for the first eight days of gestation, equivalent to the first three-four weeks of a human pregnancy. This time frame includes the dynamic epigenetic reprogramming and the beginning of neurulation. We hypothesized that this early alcohol exposure would disrupt the epigenome during the reprogramming and lead to alterations in gene expression and to a disruption of embryonic development.

We started by exploring the hippocampus which is particularly vulnerable to alcohol exposure. Genome-wide gene expression analysis of adolescent male hippocampi revealed altered expression of several genes. Interestingly, the expression of two candidate genes was found to be similarly altered in bone marrow and main olfactory epithelium. Moreover, an MRI study of adult male brains in mice revealed asymmetry in the volume of the hippocampi: the left hippocampus was significantly larger than the right in alcohol-exposed offspring.

Furthermore, to clarify the etiology of alcohol-induced growth restriction, we used our mouse model to investigate epigenetically regulated imprinted genes that are crucial regulators of both embryonic and placental growth during pregnancy. We observed that expression of the well-known growth promoter gene *Insulin-like growth factor 2 (IGF2)* was significantly decreased in alcohol-exposed 16.5 days old placentas, whereas the growth controller gene *H19* was significantly increased in the 9.5 days old embryos.

In our human study, we investigated prenatally alcohol-exposed placentas as well as the newborn phenotype. Similarly, as in our mouse model, we focused on the *Insulin-like growth factor 2 (IGF2)/H19* imprinted locus. Since we detected a single nucleotide polymorphism rs10732516G/A within the imprinting control region, we investigated the locus in a new way. Instead of dividing our samples into two genotypes, the effect of imprinting was taken into account and we grouped the samples into four genotypes. As a result, we detected genotype-specific DNA methylation changes in the imprinting control region in alcohol-exposed placentas. Furthermore, the most significant finding was genotype-specific changes in the head circumference of the alcohol-exposed newborns.

The results obtained with the mouse model underline the vulnerability of the early embryo to alcohol-induced effects. Our human results highlight the importance of taking into account individual's genetic characteristics when examining the background behind the alcohol disorder phenotype.

TIIVISTELMÄ

Raskaudenaikainen alkoholialtistus vaikuttaa sikiön kehitykseen aiheuttaen laajan kirjon niin fyysisiä kuin neurologisia kehityshäiriöitä. Yleisesti sikiövaurioiden kirjoa kuvataan termillä sikiöaikaisen alkoholialtistuksen aiheuttamat oireyhtymät (Fetal Alcohol Spectrum Disorders, FASD). Vaurion vakavinta muotoa, jossa lapsella diagnosoidaan kasvuhäiriö, oireyhtymälle tyypilliset kasvonpiirteet sekä keskushermoston toimintahäiriöitä, kutsutaan fetaalialkoholisyndroomaksi (FAS). FASD-oireyhtymän esiintyvyydeksi Euroopassa ja Pohjois-Amerikassa on arvioitu 3-5% ja Etelä-Afrikassa yli 10%. Koska alkoholialtistuksen seurauksia ei tunneta tarpeeksi hyvin ja oireyhtymän diagnosointi on vaikeaa, on raskaudenaikaisen alkoholialtistuksen vaurioittamien lasten määrä todennäköisesti huomattavasti arvioitua yleisempi.

Vaikka alkoholin tiedetään aiheuttavan muutoksia sikiön normaalissa kehityksessä ja vaikuttavan näin yksilön loppuelämän terveyteen ja hyvinvointiin, niin itse taustalla oleva mekanismi on suurelta osin yhä tuntematon. Tämän hetkisten tutkimustulosten mukaan ympäristö vaikuttaisi geenien toimintaan epigeneettisten muutosten kautta jotka näkyisivät muutoksina sikiön kehityksessä. Koska epigenomilla tiedetään olevan merkittävä rooli alkionkehityksen aikana, ja se toimii myös ympäristön ja geenien välisenä säätelijänä, on epigeneettisiä mekanismeja tutkittu myös FASD-oireyhtymän taustalla. Väitöskirjani tavoitteena oli tarkastella raskaudenaikaisen alkoholialtistuksen vaikutuksia epigeneettisestä näkökulmasta. Tutkimuksissani selvitin mitä muutoksia alkoholi sikiön epigenomissa ja genomissa aiheuttaa ja pyrin löytämään yhtäläisyyksiä näiden muutosten ja oireyhtymän ilmiasun välillä.

Tutkiakseni alkoholialtistuksen vaikutuksia olen käyttänyt apunani hiirimallia ja ihmisiltä kerättyä aineistoa. Hiirimallimme alkoholialtistus tapahtuu aivan raskauden alussa, kahdeksan ensimmäisen raskauspäivän ajan, jonka aikana hiirinaaraat juovat 10-prosenttista etanolia. Tämä ajanjakso vastaa ihmisellä 3-4 ensimmäistä raskausviikkoa. Kyseisen ajanjakson aikana alkio käy läpi epigeneettisen uudelleenohjelmoitumisen ja altistus päättyy hermoston kehityksen alkuvaiheeseen. Hypoteesimme mukaan alkoholi vaikuttaa geenien toimintaa sääteleviin epigeneettisiin mekanismeihin jo kehityksen varhaisessa vaiheessa ja saa näin aikaan laajan kirjon alkoholialtistukseen liittyviä kehityshäiriöitä.

Olemme aloittaneet tutkimalla hiirten hippokampusta, sillä sen on osoitettu olevan herkkä alkoholista aiheutuville muutoksille. Koko genomin laajuinen tutkimus osoitti, että alkoholialtistus muuttaa nuorten uroshiiripoikasten hippokampuksessa useiden geenien

toimintaa. Mielenkiintoista oli, että löysimme myös samansuuntaisia muutoksia geenien ilmenemisessä kahdessa muussa kudoksessa, luuytimessä ja hajuepiteelillä. Tämä löytö tukee hypoteesiamme: alkoholi muuttaa geenien säätelyä jo hyvin varhaisessa vaiheessa, mahdollisesti kantasoluissa, sillä muutokset ovat nähtävissä useissa eri kudoksissa. Lisäksi, aikuisiällä oleville uroshiirille tehdyn aivojen magneettikuvantamisen perusteella havaitsimme epäsymmetrisyyttä oikean ja vasemman aivopuoliskon välillä: vasen hippokampus oli merkittävästi oikeaa suurempi. Koska FASD-oireyhtymän yksi tunnetuimpia piirteitä on kasvun häiriintyminen, tutkimme hiirimallin avulla myös epigeneettisesti säädeltyjä leimautuneita geenejä, joilla on merkittävä rooli sikiön ja istukan kasvun kontrolloinnissa raskauden aikana. Havaitsimme, että alkoholialtistus vähensi tunnetun kasvua edistävän geenin *Insuliininkaltainen kasvutekijä 2* ilmenemistä 16.5 alkiopäivän ikäisessä istukassa, ja lisäsi kasvua kontrolloivan geenin *H19* ilmenemistä 9.5 päivän ikäisessä alkiossa.

Kolmannessa osatyössä tutkimme ihmisaineistomme istukkakudosta ja tarkastelimme vastasyntyneen vauvan ilmiasua. Kuten hiiritutkimuksessa, tutkimme *Insuliinikaltainen kasvutekijä 2 (IGF2) /H19* -leimattua geenilokusta myös ihmisissä. Koska tutkimuksessa havaitsimme yhden emäksen variaation (SNP, single nucleotide polymorphism) rs10732516G/A lokuksen säätelyalueella, tutkimme geenilokusta uudesta näkökulmasta ja jaoimme lapset ryhmiin genotyypin perusteella. Tarkastelemalla alkoholin vaikutusta lokuksen epigeneettisiin merkkeihin, DNA metylaatioihin, havaitsimme, että alkoholin aiheuttamat muutokset säätelyalueen metylaatioprofiilissa riippuivat yksilön genotyypistä. Tämän lisäksi tutkimuksen merkittävin löytö oli alkoholialtistuksen vaikutus päänympärykseen: alkoholille altistuneiden vastasyntyneiden päänympärykset poikkesivat merkittävästi toisistaan riippuen geneettisestä variaatioista.

Tämän väitöskirjan tutkimustulokset hiirimallilla osoittavat, että alkoholialtistus aivan raskauden alussa vaikuttaa merkittävästi sikiön kehitykseen, mikä korostaa alkuraskauden herkkyyttä altistua haitallisille ympäristötekijöille. Ihmisillä tehty tutkimus puolestaan korostaa erityisesti geneettisen variaation merkitystä alkoholivaurioiden taustalla. Tutkimuksemme perusteella olemme löytäneet ensimmäisen alkoholivaurioiden taustalla olevan geneettisen tekijän, joka vaikuttaa oireyhtymän ilmiasuun ja voisi selittää sen miksi jokaiselle ei tule samanlaisia vaurioita äidin runsaasta juomisesta huolimatta. Tätä löytöä voidaan käyttää tulevaisuudessa apuna alkoholivaurioiden diagnosoinnissa.

INTRODUCTION

Prenatal alcohol exposure represents the leading cause of preventable developmental disabilities and therefore should be recognized globally as a major health problem (Popova et al., 2016). Despite the existing guidelines, the diagnosis of FASD remains challenging and the severity of the teratogenic effects depends on the drinking pattern, the amount of alcohol consumed per occasion, timing during pregnancy as well as genetic susceptibility (Hoyme et al., 2016). Persons with FASD often require life-long assistance and extensive access to health care services. In Canada, the costs of one FASD individual have been estimated to be around one million dollars (Popova et al., 2011). In Finland, no economic estimations have been published. Hence, there is an urgent need to develop new tools that could improve the present diagnostic guidelines and facilitate the identification of prenatal alcohol exposure.

The environmental impact on epigenetic regulation has attracted a great deal of research in recent years. It has been suggested that the epigenome provides one way to understand how environmental factors might affect us without changing the DNA sequence, and have a potential association with diseases that cannot be explained by conventional genetic mechanisms (Feil & Fraga, 2012). Recent evidence from both mouse (Kaminen-Ahola et al., 2010a) and human (Portales-Casamar et al., 2016) studies suggest that prenatal alcohol exposure may affect the epigenome during development and be the source of associated phenotypic defects. This has raised the question of whether the altered epigenetic marks could play a role as biological markers for FASD (Lussier et al., 2018).

Usage of an inbred mouse model provides the opportunity to minimise genetic and environmental variation and to focus on the epigenome. In our mouse model, early alcohol exposure induces FASD phenotype-like characteristics and affects the epigenome, thus represents a good model for prenatal alcohol exposure studies (Kaminen-Ahola et al., 2010a; Kaminen-Ahola et al., 2010b; Sanchez Vega et al., 2013). However, since the effects in the central nervous system have not yet been investigated, we started our work from hippocampus, a structure known to be particularly vulnerable to alcohol exposure (Berman & Hannigan, 2000).

Because growth restriction is one of the main characteristics of FASD we have focused on the mammalian genomic imprinting. This unique phenomenon consists of a subset of genes that have a mono-allelic expression in a parent-of-origin manner. These genes are functionally highly active in the placenta and embryo as important regulators of normal development and growth (Ferguson-Smith, 2011). Since the mono-allelic expression

pattern is gained through epigenetic mechanisms, imprinted genes provide a useful tool to study how environmental stimuli impact on epigenome. Findings in both mice and humans suggest that imprinted loci are sensitive to known environmental agents (Tobi et al., 2012; Susiarjo et al., 2013; Nelissen et al., 2013) making them potential genomic sites for studying the effects *in utero* and for later health outcomes. In this study, we collected human placental samples of Finnish origin that had been exposed to alcohol in pregnancy and also used a mouse model to investigate alcohol-induced effects in imprinted genes.

Our results present new information into the alcohol-related epigenetic and genetic research field. We show that alcohol exposure at the very beginning of pregnancy can already cause significant alterations in different brain structures. Moreover, our human work highlights the importance of considering the genetic background in alcohol-induced effects and it also emphasizes the use of genetic information in future diagnostics of prenatal alcohol exposure.

1 REVIEW OF THE LITERATURE

1.1 PRENATAL ALCOHOL EXPOSURE

Alcohol not only harms the drinker herself but since it is a known teratogen, it also harms the developing fetus during pregnancy. Prenatal alcohol exposure is a serious risk factor for adverse pregnancy outcomes such as stillbirth (Kesmodel et al., 2002), spontaneous abortion (Henriksen et al., 2004), premature birth (Albertsen et al., 2004) intrauterine growth restriction (Q. Yang et al., 2001), and low birthweight (Patra et al., 2011). The latest statistics estimate that globally about 10% of women in the general population consume alcohol during their pregnancy (Popova et al., 2017). A recent study in Finland showed that over 50% of pregnant women drink alcohol during pregnancy and nearly one fourth of them consume at least three doses of alcohol on one occasion (Komulainen et al., 2017). It seems that despite the public health guidelines to reduce the consumption of alcohol during pregnancy, in many countries a significant number of pregnant women still continue to consume alcohol, especially in North America and the European countries (Popova et al., 2017). One major concern is unplanned pregnancies; in these cases, there is an apparent risk that the embryo can become exposed to alcohol during the particularly vulnerable stages of fetal development (Feldman et al., 2012).

1.1.1 <u>Alcohol as a teratogen</u>

Teratogenesis refers to birth defects due to environmental causes. Teratogens are environmental substances known to cause severe effects during the critical periods of development, such as the times of differentiation and morphogenesis. Because of the physiochemical properties of ethanol (mildly polar but well soluble in water), it can freely diffuse across the placental barrier. Once it has gained access to the fetus, alcohol spreads throughout the body causing damage to the developing organs (Guerri & Sanchis, 1985). Ethanol-metabolizing enzymes are present at very low concentrations in fetal liver which explains why the fetus is not able to adequately cope with ethanol. Some of the teratogenic effects may be caused by the ethanol metabolite, acetaldehyde, a compound known to be highly reactive towards DNA (Wang et al., 2000). Therefore, the fetus relies on the mother´s metabolism and the only way to eliminate the alcohol from the fetus is diffusion back into mother´s blood circulation through the placenta. However, since this process is rather slow, it may lead to similar or even higher levels of alcohol in the fetus than in the mother´s blood and these elevated levels can last for an extended time (Guerri & Sanchis, 1985; W. Guo et al., 1994).

The severity of the symptoms depends on the pattern and amount of alcohol, the timing during pregnancy as well as the overall maternal health and genetic factors (Pollard, 2007). Animal studies have explored the effects of binge-drinking patterns. This kind of drinking exposes the fetus to high blood alcohol levels for short periods. It has been shown that a binge-drinking pattern is more harmful as compared to a continuous drinking pattern, even if the overall amount of alcohol consumed is smaller (Maier & West, 2001). Even a single binge may be teratogenic and cause significant defects (Goodlett & Eilers, 1997). Long-term human studies have demonstrated that children of binge-drinking mothers exhibit severe cognitive and behavioral disabilities (Streissguth et al., 1994). This is probably due to the presence of a high blood alcohol level during a critical brain development periods (Sulik, 2005). Furthermore, a recent study suggested that even low levels of alcohol consumption can influence the craniofacial development of the fetus emphasizing the significance of the critical first trimester period (Muggli et al., 2017). In fact, no safe time or level of alcohol consumption during pregnancy is known.

1.1.2 Fetal alcohol spectrum disorder

The effects of alcohol exposure were first introduced by Jones and Smith in 1973 (K. L. Jones et al., 1973). They diagnosed children who exhibited central nervous system damage, growth deficiency, characteristic craniofacial dysmorphology and major malformations born to heavy-alcohol-drinking mothers. The term "fetal alcohol syndrome" (FAS) was originally used to describe the cluster of birth defects due to prenatal alcohol exposure (K. L. Jones et al., 1973). However, the term "fetal alcohol spectrum disorders" (FASD) has emerged to describe a broader spectrum of these disabilities. FASD refers to the wide variety of structural, neurocognitive, physiological and behavioral deficits that can occur following prenatal alcohol exposure (Popova et al., 2016). FASD has become an umbrella term to encompass the range of alcohol-induced outcomes including diagnoses of FAS, partial FAS (pFAS), alcohol-related neurodevelopmental disorder (ARND) and alcohol related birth defects (ARBD) (Hoyme et al., 2016).

The severest end of the spectrum is FAS which is usually the consequence of chronic exposure to high levels of alcohol during prenatal life. It has long been recognized as one of the major causes of intellectual disabilities and behavioral problems lasting the entire lifespan (Streissguth et al., 1994; Burden et al., 2005; Kelly et al., 2009). The three main characteristics of the syndrome are: pre- and postnatal growth restriction, facial abnormalities (smooth philtrum, thin vermillion border, short palpebral fissures) and central nervous system abnormalities. Many children with FAS have varying degrees of intellectual disabilities as well as microcephaly. Thus, one of the diagnostic criteria has been

the reduction in head circumference to indicate deficient brain growth as well as abnormal morphogenesis (Hoyme et al., 2016).

The diagnosis of FASD is challenging because it requires confirmation of prenatal alcohol exposure and a medical evaluation with both a dysmorphological and neurodevelopmental assessment performed by a multidisciplinary team (Hoyme et al., 2016). The prevalence of FASD is estimated to range from 3-5% in Europe and North America, but it may be as high as 10% in South Africa (Roozen et al., 2016; May et al., 2018). It has been estimated that every year 1-5% of babies born in Finland show signs of FASD (Autti-Rämö et al., 2011). It is difficult to estimate the true prevalence of prenatal alcohol exposure because the vast majority of the affected children are never diagnosed. This is mainly due to the lack of good methodologies to confirm prenatal alcohol exposure and clinically suspect FASD in children with normal physical appearances who nonetheless display various cognitive and/or neurobehavioral disorders. The difficulties in cognitive performance may not become evident until early adolescence, for example as specific learning disorders (Mattson et al., 1997; Sood et al., 2001). Furthermore, since FASD is very heterogeneous in terms of phenotype and comorbid disorders are common, specific diagnoses such as ADHD, mood disorder and conduct disorder may seem more likely and the underlying cause –prenatal alcohol exposure– is easily overlooked (Popova et al., 2016).

FASD is a lifelong condition. Thus, it is essential to reduce the occurrence of alcohol consumption during pregnancy and to establish a universal screening tool for prenatal alcohol exposure. If there was a reliable tool that could help to identify prenatal alcohol exposure, this would facilitate the early identification of FASD which is crucial if one wishes to intervene early before permanent damage is inflicted. Several potential alcohol biomarkers have been already established: fatty acid ethyl esters (FAEEs), ethyl-glucuronide (EtG) and phosphatidylethanols (PEth) (Ostrea et al., 2006; Bakdash et al., 2010; Stewart et al., 2010). However, the major concern related to these biomarkers is that they only confirm maternal alcohol consumption during pregnancy and do not reflect the severity of the effects caused by alcohol exposure. This has led researchers to investigate the molecular mechanisms behind alcohol-induced teratogenesis. During recent years, several research teams have reported that both the genomic variation as well as epigenetic mechanisms might have an impact on alcohol-induced teratogenic outcomes. For example, a twin study found that the genetic background has a major influence on alcohol teratogenesis by showing that monozygotic twins appear to display more uniform effects of alcohol as compared to dizygotic twins (Streissguth & Dehaene, 1993). Thus, a better understanding of the role of genetic and environmental interactions will help to identify targets for early interventions and possibly even prevent FASD.

1.2 GENOME

In humans, each cell contains 23 pairs of chromosomes of which 22 are known as the autosomes and the 23rd pair as the sex chromosomes (XX for females, XY for males). Chromosomes are made of DNA which carries the genetic information. DNA is a twisted double-helix ladder-like construction. It is coded by four chemical bases: adenine (A), thymine (T), guanine (G) and cytosine (C), which pair up with each other (A-T, G-C) forming units called base pairs.

Genes are fragments of the DNA structure and the functional part of heredity; their length varies from a few hundred DNA bases to more than two million bases. To date, about 20 000 genes have been found in the human genome (Ezkurdia et al., 2014). Every individual has two copies of one gene, one inherited from the father and one from the mother. Hence, the two alternative forms of the same gene are known as alleles. Genetic variation in the alleles may produce differences in the observable features that determine each individual's unique phenotype. The gene pool of a population contains different allele combinations and frequencies resulting in individuals with various genotypes. The allele that occurs most frequently in a population is often called the wild-type allele and this corresponds to a common phenotype. In order to characterize the genetic variation in the human genome, the 1000 Genomes Project (Auton et al., 2015) was the first to sequence the genomes of a large number of people (2,504 individuals); this represents a comprehensive resource of human genetic variants with frequencies of ≥1% in 26 populations. In addition, in Finland, the Sequencing Initiative Suomi (SISu) project (http://www.sisuproject.fi) has collected genetic data from over 10 000 Finnish individuals to examine the attributes and appearances of different variants in Finnish cohorts and their aggregate distribution in Finland.

Genetic variation refers to the differences between individuals within a population. If the variation occurs in germ cells, it can be inherited by the next generation. Single nucleotide polymorphism (SNP) is a common type of variation in individuals and refers to a replacement of a single nucleotide base in the DNA strand. For example, guanine has been converted into adenine. These polymorphisms can be inherited as a unit of a haplotype, as clusters of sequence variations linked to a chromosome. Most of the SNPs present throughout the human genome are harmless, exerting no influence on health. However, if the SNP happens to occur within a gene, it may affect the function of the gene and, in some situations, may have disease-causing effects. At present, SNPs can be used as biological markers to trace the disease loci as well as the disease inheritance within families (Ipe et al., 2017).

Naturally the phenotype is affected by a complex set of several genes influencing certain characteristics. The input of genes from multiple loci creates a continuous variation across range of phenotypes described as quantitative trait. The specific genes involved in a quantitative trait are known as quantitative trait loci. Although the genes display a major role in defining the genetic background, our personal phenotype is a much more complicated combination of genome, epigenome and environmental influence which together generate even more complex traits (Albert & Kruglyak, 2015).

1.3 EPIGENOME

The structure and function of cells in a multicellular organism depend on differential gene expression. These differences between cells arise during development and remain throughout cell division. The mechanisms causing the differences are known as the epigenome. The concept of epigenetics was first introduced by Conrad Waddington who proposed the canalization theory of dynamic programming of epigenomic profiles of stem cells during development (Waddington, 1942). The epigenome itself refers to modifications of DNA and its regulatory factors such as chromatin and non-coding RNA that alter the gene expression and cellular functions without changing the underlying genomic sequence. The term "*epi*" means "on top of" the genome. The purpose of a unique epigenetic landscape of each cell type is to control cell fate and differentiation (Wolffe & Matzke, 1999).

Epigenetic regulation is established by modifying the chromatin structure through DNA methylation or histone marks (Allis & Jenuwein, 2016) (**Figure 1**). These chromatin changes can either allow or prevent the binding of transcriptional factors affecting the gene transcription. Usually different epigenetic processes act together. The epigenome is influenced by genomic, stochastic and even environmental variation throughout life (Feil & Fraga, 2012). This means that epigenetic modifications may be reversible and be a part of the organism´s normal development. This highlights the major difference between genome and epigenome: mutations in genome are always permanent.

The field of epigenetic research culminated in 2015, when the ENCODE project established a first epigenetic road map to illustrate a human reference epigenome (www.encodeproject.org). The best characterized epigenetic modifications are DNA methylation, histone modifications and non-coding RNAs, which will be described in the next sections in more detail.
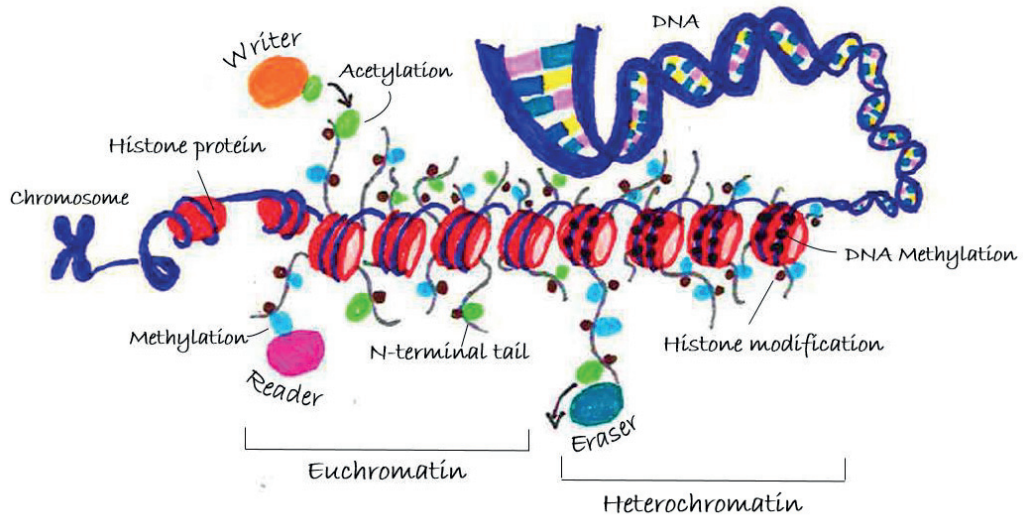
Figure 1. A schematic picture of the chromatin. DNA strand (purple) is folded around histone proteins (red) forming nucleosome particles. The chromatin is usually organized into two different domains, euchromatin or heterochromatin. On the histone proteins, the chemical histone modifications (brown) on the flexible N-terminal tails (gray) are regulated via number of different covalent changes, for example methylation (blue) or acetylation (green). Regulation is undertaken by a network of chromatin-modifying enzymes termed as readers (pink), writers (orange) and erasers (turquoise). DNA methylation on the DNA strand is presented as black dots.

1.3.1 DNA methylation

To date, DNA methylation has been the best characterized epigenetic mechanism that regulates gene expression. In mammals, DNA methylation is a post-replication modification that involves a covalently attached methyl group in the 5´position of cytosine usually found at the cytosine-guanine (CpG) dinucleotide sequence (Z. D. Smith & Meissner, 2013). Although, DNA methylation is mainly restricted to CpG sites, non-CpG methylation at CpA, CpC or CpT sites has been observed in pluripotent stem cells (Ramsahoye et al., 2000; Ziller et al., 2011), oocytes (Tomizawa et al., 2011) and neurons in the brain (J. U. Guo et al., 2014). In the human genome, the estimated CpG content is ~ 29 million of which 60–80% are methylated (Lister et al., 2009). Regions of high CpG density are known as CpG islands. These islands are usually spread out throughout the genome, often either at the promoters of genes or within the gene body. The definition of an island depends on the length (200-500bp), CpG content (50-55%) and the observed:expected frequency of at least 0.6

(Gardiner-Garden & Frommer, 1987; Takai & Jones, 2002). CpG regions that lie within 2kb up- and downstream of the island with a lower CpG density is known as a CpG shore. A region about 2kb outside of the shore is described as CpG shelf (Sandoval et al., 2011). The level of methylation depends on the genomic region: usually there is more unmethylated cytosines closer to the transcription site than a distance from the coding region. Only a small part of the CpG islands next to exonic regions become methylated during the development (P. A. Jones, 2012).

Since both DNA strands are identically methylated at the CpG sites, the similar methylation pattern is always transferred to daughter cells through mitosis. Enzymes responsible for DNA methylation are called as DNA methyltransferases (DNMTs) including DNMT1, DNMT3A, DNMT3B and DNMT3C (Okano et al., 1999; Barau et al., 2016). DNMT1 (DNA methyltransferase 1) enzyme is responsible for maintaining global DNA methylation. Deletion of *Dnmt1* in mice resulted as global demethylation and led to embryonic lethality (E. Li et al., 1992). During the cell division, DNMT1 copies the methylation pattern to the new complementary DNA strand, thus maintaining the methylation (Leonhardt et al., 1992). In contrast, DNMT3A and DNMT3B act as *de novo* methyltransferases by transferring methyl groups to unmethylated cytosine. These methyltransferases form a complex structure with DNMT3L, which interacts with unmethylated histone H3 lysine 4 to recruit DNMTs (Ooi et al., 2007). *De novo* methylation is most active in the gametes and early embryo but has also been shown to occur also in adult somatic cells (T. Chen et al., 2002). However, not all the genomic regions are susceptible to *de novo* methylation and DNMT3B has been shown to induce methylation in specific regions such as repetitive elements and X-chromosome inactivation (Auclair et al., 2014). DNA methylation can be removed by passive or active mechanisms. Active demethylation occurs through enzymatic process where Ten–eleven translocation (TET) family enzymes (TET1, TET2 and TET3) remove the methyl group from cytosine (Kohli & Zhang, 2013).

The main role of DNA methylation is to regulate the gene expression by directly controlling the binding of transcription factors to the gene regulatory region (Deaton & Bird, 2011). It is generally thought that the regulatory regions need to be hypomethylated to allow transcription binding. However, a recent study observed that the specificity of transcription binding is more diverse and there are many transcription factors that prefer methylated regions (Yin et al., 2017). In addition to regulating gene expression, DNA methylation has also been shown to associate with exon-intron boundaries where it regulates alternative transcriptional sites leading to altered mRNA splicing (Maunakea et al., 2013). DNA methylation is a crucial factor during development, since it is involved in cell differentiation and affects genomic imprinting as well as X-chromosome inactivation. In genomic imprinting, the methyl groups usually silence one of the two inherited alleles (maternal or

paternal), while in X-inactivation, methylation randomly inactivates one of the two X-chromosomes in females (T. Mohandas et al., 1981; Ferguson-Smith et al., 1993). DNA methylation also protects against transposon and RNA -retrotransposon elements by silencing them (Rebollo et al., 2012). In addition to DNA methylation, there is also a form of hydroxymethylation (5hmC) that is oxidized through TET-proteins and has a role in regulation of gene expression (Ito et al., 2011). In the gene body, it is suggested to induce gene expression whereas in the promoter region it is believed to suppress the expression. Hydroxymethylation occurs at high levels in pluripotent cells and also in the adult brain indicating it is involved in neurodevelopment and cognitive processes (Santiago et al., 2014).

DNA methylation is sensitive to environmental stimuli, and in adverse conditions, the methylation profile may be deleterious. In many diseases, the methylation profile is disrupted, leading to hypomethylation in genomic regions normally methylated or vice versa. For this reason, DNA methylation is a potential biomarker for predicting the adverse outcomes of environmental exposures (Lussier et al., 2018).

1.3.2 Histone modification

In the nucleus, DNA is wrapped around nucleosomes which are composed of histone protein octamers. Thus, two copies of each H3, H4, H2A and H2B histone protein form the nucleosome core particle. These nucleosome subunits form a structure known as chromatin (Luger et al., 1997) (**Figure 1**). Chromatin is usually organized into two different domains, euchromatin or heterochromatin, which associate with distinct genomic functions. Euchromatin is a more loose structure and typically it allows active transcription, whereas the tighter heterochromatin is transcriptionally repressive (G. Li & Reinberg, 2011). The form of packing of chromatin is influenced by several factors such as the actions of linker histone H1, histone variants, chromatin remodelers, histone chaperones, and small chemical modifications.

The chemical modifications occur via a number of different covalent changes: lysine acetylation, lysine and arginine methylation, arginine citrullination, lysine ubiquitination, lysine sumoylation, ADP-ribosylation, proline isomerization, and serine/threonine/tyrosine phosphorylation (Gardner et al., 2011). The majority of these are established by the flexible N-terminal tails of the histone proteins. The function of the modifications depends on the cellular context and location on the histone tail (Kouzarides, 2007; Bowman & Poirier, 2015). Active chromatin domains are filled with distinct histone marks and located around the transcription region: in enhancers, promoters as well as being present in the gene

bodies (Creyghton et al., 2010; Deckert & Struhl, 2001; G. Liang et al., 2004). These histone modifications regulate transcription by opening the chromatin and recruiting other effectors to mediate the gene expression or by repressing the expression. However not all marks are present at the same time and there is a vast network of chromatin-modifying enzymes called readers, writers and erasers (not described in detail here) that regulate the histone modifications. Hence, the modifications are binding sites for the readers which recognize the modifications and recruit other writers to induce gene expression. All these molecules create a complex layer of post-translational modification on the chromatin.

Modifications of trimethylation of lysine 4 on histone protein H3 (H3K4me3) as well as trimethylation of lysine 36 on histone protein H3 (H3K36me3) are the best characterized marks of active genes. For example, it was recently discovered that in mouse pre-implantation embryos, H3K4me3 occurs rapidly after fertilization, which is consistent with the major wave of zygotic genome activation at the two-cell stage. Furthermore, the breadth of the H3K4me3 domain was shown to have a highly dynamic feature as it associated with higher transcription activity and cell identity not only in pre-implantation development but also in the process of deriving embryonic stem cells from the inner cell mass and trophoblast stem cells from the trophectoderm (X. Liu et al., 2016). In addition, lysine acetylation of H4, which is another regulator of many cellular processes, was observed to be a crucial factor in the regulation of pluripotency in embryonic stem cells (Gonzales-Cope et al., 2016). In contrast, the methylations of H3K27 and H3K9 are modifications of repressive chromatin often found at silent gene loci. Therefore, they associate with the formation of heterochromatin. H3K27me3 is a well-characterized mark in repressed transcription, especially during development (X. Liu et al., 2016). On the other hand, H3K9me2 has been shown to directly mark promoter and gene bodies and act as an important regulator during the post-implantation period (Zylicz et al., 2015). In addition, both H3K27me3 and H3K9me2 have been shown to be present on the inactive X chromosome (F. Yang et al., 2015; Escamilla-Del-Arenal et al., 2013).

1.3.3 Non-coding RNAs

Micro-RNAs (miRNA) are 19-25 nucleotides long and one of the most widely studied class of non-coding RNAs. Their main function is to mediate post-transcriptional gene silencing. To date, about 2000 miRNA genes have been described in the human genome. MiRNAs can derive from intron regions, untranslated protein-coding genes, pseudogenes or inverted repeats. The miRNA biogenesis is a multi-step process including various molecules (RNA polymerase, enzymes DROSHA and DICER) that generate a mature miRNA form. The main task of these molecules is to degrade mRNA or to inhibit the translation. They are also

known to alter the chromatin conformation by recruiting chromatin-modifying complexes to DNA (Y. Lee et al., 2003; Carthew & Sontheimer, 2009). These small non-coding RNAs are usually involved in many processes during development, including proliferation and differentiation of cells. For example, in human blastocysts, miRNA expression has been suggested to associate with the maintenance of pluripotency (Rosenbluth et al., 2013). From the epigenetic point of view, histone acetylation, as well as methylation on the promoter region has been shown to affect the miRNA expression. It is also known that 50% of the miRNA genes associate with CpG islands (Weber et al., 2007). In addition of the epigenetic control, miRNAs themselves can regulate the epigenetic machinery. They have shown to directly target *de novo* DNA methyltransferases *DNMT3A* and *DNMT3B* (Garzon et al., 2009) as well as influencing the enzymes that regulate histone acetylation (Noonan et al., 2009).

Long non-coding RNAs are >200 nucleotide long transcripts. In the human genome, 50 000 long non-coding RNAs have been identified which are known to regulate gene expression at the transcriptional, post-transcriptional, and translational levels in a wide range of biological processes (Iyer et al., 2015). According to their function, long non-coding RNAs can be divided into several groups from which long intergenic non-coding RNAs are most related to epigenetic regulation (Di Gesualdo et al., 2014). They associate with chromatin-modifying complexes to create cell type-specific epigenetic states. During embryonic development, long intergenic non-coding RNAs participate cell lineage-specific gene expression, X-chromosome inactivation and genomic imprinting (Khalil et al., 2009; Sheik Mohamed et al., 2010; Brown et al., 1991; Bartolomei et al., 1991). Since long non-coding RNAs mediate the epigenetic regulation, they might have a potential to be an epigenetic biomarker for therapeutic interventions of complex diseases that involve epigenetic alterations.

1.4 GENOMIC IMPRINTING

1.4.1 Overview

As mentioned above, mammals are diploid organisms having two sets of matched chromosomes, thus two copies of every gene - one inherited from the mother and one from the father. Normally both copies of the genes are expressed equally from the paternal and maternal alleles, resulting in bi-allelic expression. However, a small subset of genes show monoallelic expression in a parent-of-origin-specific manner. This means that only one of the two inherited alleles is expressed either from the paternal or maternal side;

when the paternal allele is expressed, the maternal copy is silenced, or *vice versa*. This unique phenomenon is known as genomic imprinting. It is controlled by epigenetic marks that distinguish the two parental alleles. Imprinted regions are usually marked with imprinting control regions and the specific expression of these genes is driven by several epigenetic processes: DNA methylation, non-coding RNAs and histone modifications (Ferguson-Smith, 2011). Genomic imprinting is known to occur in placental mammals, marsupials and a subset of flowering plants and it plays a major role in maintaining normal embryogenesis, and prenatal and postnatal growth (Cordeiro et al., 2014; G. E. Moore et al., 2015).

Despite having major importance to human health and well-being, imprinted genes were only discovered just over 30 years ago. Genomic imprinting was suggested for the first time in the early 1980s when pronuclear transplantation experiments with mouse embryos showed that both maternal and paternal genomes are needed for the normal development (McGrath & Solter, 1984a; Surani & Barton, 1983; Surani et al., 1984). An improved nuclear transfer technology was used to test the possibility of generating diploid uniparental embryos. A donor female pronucleus was taken from a newly fertilized egg and it was placed inside a host fertilized egg already containing a maternal pronucleus. A diploid embryo was generated but with two maternal (gynogenic) or two paternal (androgenetic) genomes. The experiment confirmed that these embryos failed to survive and the genes on the maternal and paternal copy of chromosome 17 functioned differently during embryonic development, providing strong evidence of genomic imprinting in mammals (McGrath & Solter, 1984b). Furthermore, studies of uniparental disomy (UPD) for specific chromosomes in mice revealed that the parental origin effect was not widespread throughout the genome, but localized to specific chromosomal regions (Cattanach & Kirk, 1985; Searle & Beechey, 1990). The first three imprinted genes: *Insulin-like growth factor 2* (*Igf2*), *Igf2 receptor* and non-coding transcript *H19* were discovered in mice ten years later in 1991 (Barlow et al., 1991; DeChiara et al., 1991; Ferguson-Smith et al., 1991; Bartolomei et al., 1991). Thus far, around 127 imprinted genes in mouse and approximately 100 imprinted genes in human have been identified (Babak et al., 2015; Baran et al., 2015) (www.geneimprint.com) with several of them being conserved in different species (human, mice, pigs, etc.).

The reason for imprinting is still obscure, however there must have been strong selective advantages to maintain this phenomenon. Eutherian mammals such as placental mammals and marsupials have genomic imprinting whereas egg-laying mammals, known as prototherians, seem to lack imprinted genes (Hore et al., 2007). The discriminating factor is the reproductive strategy: embryos of placental mammals, but not those of egg-laying mammals, are directly influenced by the maternal resources that are used for the

embryonic growth. In addition to the egg-laying reproductive strategy, invertebrates and vertebrates can also use parthenogenesis in which a diploid individual is developed without requiring fertilization by a male gamete. These different strategies have raised the question of why the imprinting phenomenon has evolved in only certain organisms.

There are two theories to explain the origin of imprinting. The Kinship theory was first introduced by Moore and Haig (1991). They claimed that during the developmental stages when the fetus is reliant on the mother's resources for nutrition, there is a conflict between the maternal and paternal genes. This means that there are opposite interests of the maternal and paternal genome: while the paternally expressed imprinted genes try to increase the embryonic growth, and maximize the individual´s fitness, the maternally expressed imprinted genes try to suppress fetal growth. Because the mother has limited resources, nutrients are more equally distributed to all offspring, and it is possible to produce a maximum number of viable offspring. It also guarantees that the maternal genome can pass to multiple offspring despite different paternal genomes.

In contrast, the mother–offspring coadaptation theory is considered as another explanation of the evolution of genomic imprinting. This theory claims that the imprinted genes act co-adaptively to optimize fetal development as well as maternal nurturing (Curley et al., 2004). The hypothesis was presented for the first time with an example of *Paternally expressed gene 3* (*Peg3*) which influences food intake and milk release when expressed in the mother, and placental nutrient intake and suckling when expressed in the offspring. This example showed that during development, there is an interaction between the fetus, the placenta and the mother's hypothalamus. This triangle influences fetal growth and brain development, the provision of maternal resources during both prenatal and postnatal stages, as well as regulation of postnatal maternal care. Thus, it was suggested that imprinted genes regulate the parent-fetus interaction through a selection of co-expressed genes in the placenta and the mother's hypothalamus. However, thus far, the theory has only been presented for the paternally expressed gene which is expressed in both the placenta and the hypothalamus.

The theories for the evolution of imprinting are still being vigorously debated, and it has been argued that neither of the theories alone can provide a full explanation (Haig, 2014). Interestingly, imprinted genes have been identified in flowering plants in which the endosperm has a placenta-like function (Satyaki & Gehring, 2017). The endosperm transfers nutrient resources to the embryo, strengthening the hypothesis that genomic imprinting has evolved to regulate nutrient transfer between the parent and offspring.

1.4.2 <u>Function of imprinting</u>

It has become clear that many imprinted genes play a significant role in regulating the growth of the embryo and placenta (G. E. Moore et al., 2015). Furthermore, imprinting has an essential role in ensuring the infant's survival after birth. These genes are involved in feeding, the maintenance of body temperature and the regulation of metabolism, as well as in infant and maternal behaviors (Schaller et al., 2010; Hernandez et al., 2007; Champagne et al., 2009). However, in addition to embryonic development, there is also some evidence that imprinting is related to stem cell maintenance and renewal (Besson et al., 2011; Venkatraman et al., 2013; Barroca et al., 2017) and it has been implicated in a wide range of common diseases such as diabetes mellitus (Bak et al., 2016), psychiatric disorders (Ingason et al., 2011) and cancer (Vennin et al., 2017), as well as intrauterine growth restriction (IUGR) (Iglesias-Platas et al., 2014). Mice have been important models when investigating the role of imprinted genes and unravelling their major effects on prenatal and postnatal development.

1.4.2.1 Pre- and postnatal growth

In general, it is known that paternally expressed genes promote growth (*Insulin-like growth factor 2*, *Paternally expressed gene 1*, *Paternally expressed gene 3*) whereas maternally expressed genes repress growth (*Insulin-like growth factor 2 receptor*, *Cyclin-dependent kinase inhibitor 1c*, Long non-coding RNA *H19*, *Growth factor receptor-bound protein 10*) (Charalambous et al., 2003; Leighton et al., 1995; L. Li et al., 1999; G. E. Moore et al., 2015). There are some genes that are expressed only in placenta. Two genes: *Mash2* (*Achaete-scute family bHLH transcription factor 2*) and *Paternally expressed gene 10* (*Peg10*) play a crucial role in the formation of a viable placenta (Guillemot et al., 1995; Ono et al., 2006). In contrast, other imprinted genes affect the function of the placenta by regulating the nutrient supply either by affecting growth and the structure of the placenta (*Insulin-like growth factor 2*) (Constancia et al., 2002) or by modifying specific transport systems (solute carrier family 22 members: *Slc22a2, Slc22a3*) (Verhaagh et al., 1999). Various imprinted genes are expressed in both placenta and embryo creating a regulatory network of growth and differentiation (Arima et al., 2005; Varrault et al., 2006). These genes include *Insulin-like growth factor 2* (*Igf2*)*, Insulin-like growth factor 2 receptor* (*Igf2r*)*, Delta-like homolog 1* (*Dlk1*)*, Cyclin-dependent kinase inhibitor 1c* (*Cdkn1c*)*, Growth factor receptor-bound protein 10* (*Grb10*)*, Pleiomorphic adenoma gene-like 1* (*Zac1*)*, Paternally expressed gene 1* (*Peg1*) and *Paternally expressed gene 3* (*Peg3*). In the embryo, the expression is appeared in muscle, cartilage and bone, specific brain regions, neuro-endocrine and endocrine tissues and sites of energy storage (Westbury et al., 2001; J. E. Lee et al., 1990). Disrupted

imprinting of these genes can result in either fetal and placental overgrowth or growth restriction (Charalambous et al., 2003; Shiura et al., 2009).

After birth, a newborn faces many challenges associated with independent survival such as maintaining the body temperature, obtaining food and regulating metabolism. The maintenance of body temperature is vital for the survival of newborn mammals and this relies on the process called non-shivering thermogenesis in brown adipose tissue (Cannon & Nedergaard, 2004). Several imprinted genes are reported to affect brown adipose tissue and regulate thermogenesis, such as *Delta-like homolog 1* (*Dlk1*) and *Type III iodothyronine deiodinase gene* (*Dio3*) (Hernandez et al., 2007). The feeding behavior is mainly controlled by *Melanoma antigen, family L, 2* (*Magel2*) and loss of expression resulted in the newborn either failing to attach to the nipple and suckle or showing delayed attachment with weak suckling, leading to neonatal lethality (Schaller et al., 2010). In addition, maternal behavior plays a crucial role in newborn feeding and the loss of *Paternally expressed gene 3* expression results in maternal carelessness and inability to care for her offspring (Champagne et al., 2009).

1.4.3 <u>Mechanism of imprinting</u>

The basic characteristic of imprinted genes is that they function only on one chromosome, thus being so-called *cis*-acting elements. This means that genomic imprinting is dependent on an epigenetic system that modifies one of the two parental chromosomes to induce parental-specific differences in expression. The epigenetic modifications are used to attract transcriptional processing factors to induce the expression on one of the parental alleles. Since the discovery of the first imprinted genes, DNA methylation was proposed to mark the parental identity of an imprinted gene (Ferguson-Smith et al., 1993). These regions are known as differentially methylated regions (DMRs); they are found in most imprinted clusters examined. By using a targeted deletion method for both maternal and paternal germline DMRs in mice, several studies have demonstrated that DMRs are crucial imprinting control regions (ICR) responsible for mono-allelic expression (Wutz et al., 1997; Thorvaldsen et al., 1998; Fitzpatrick et al., 2002; Lin et al., 2003; Williamson et al., 2006). In general, the unmethylated DMR enables expression of a long non-coding RNA whereas it is repressed by methylated DMR. Imprinting control regions can regulate multiple imprinted genes in the gene cluster at some distance from the ICR. Usually the maternally methylated ICR is situated in the promoter area while the paternally methylated ICR is located in the intergenic regions.

Thus, more than 80% of the identified imprinted genes are typically located in clusters of 3-12 genes that are spread over 20-3700 Kb of DNA (Edwards & Ferguson-Smith, 2007). These clusters can contain both maternally and paternally expressed imprinted genes encoding both protein-coding and non-coding RNAs. For example, there can be multiple protein-coding genes on one parental chromosome and at least one long non-coding RNA on the opposite chromosome. The promoter of the long non-coding RNA is usually close to or within an intron of the protein-coding gene. To date, two well-defined mechanisms of imprinted gene regulation have been described: the insulator model of *Insulin-like growth factor 2* (*Igf2*)/*H19* and the non-coding RNA model of *Kcnq1* (*Potassium voltage-gated channel subfamily Q member 1*)/*Kcnqot1* (*Kcnq1 overlapping transcript 1*) (J. T. Lee & Bartolomei, 2013).

The insulator model is best illustrated in the *Igf2/H19* locus (**Figure 2**). The *Igf2/H19* is one of the most widely studied and well characterized imprinted regions that has a paternally methylated ICR. These two genes are located 100 kb apart from each other, sharing common sites for regulating expression including enhancer sequences that are located downstream of *H19* (DeChiara et al., 1991; Bartolomei et al., 1991). In this locus, the ICR is located in the intergenic region, about 2kb upstream from the transcriptional start site of *H19* (Jinno et al., 1996; Tremblay et al., 1997). It functions as a transcription insulator and controls the monoallelic expression of *Igf2* and *H19* by insulating the interaction between the *H19* downstream enhancers and the promoter of the genes. The ICR contains multiple binding sites (seven in human, four in mouse) for the chromatin insulator zinc-finger protein, CCCTC (CTCF), which binds only to the unmethylated maternal allele (Frevel, Hornberg, et al., 1999; Frevel, Sowerby, et al., 1999; Bell & Felsenfeld, 2000; Hark et al., 2000). The presence of CTCF on the maternal allele blocks the enhancers from interacting with *Igf2* promoters thus silencing the gene activity. Instead, the enhancers on the maternal allele activate the promoter of *H19* leading to its expression. In contrast, on the methylated paternal allele, the binding of the CTCF is blocked allowing the enhancers to access *Igf2* promoters to drive the gene activity. The exact mechanism through which the insulator acts is still unclear. Such interactions between promoters and enhancers have been investigated in chromatin conformation studies. It has been suggested that CTCF interacts with DNA molecules through the formation of chromatin loops modulated by differential DNA methylation as well as CTCF binding on the two parental chromosomes (Murrell et al., 2004; Kurukuti et al., 2006; Engel et al., 2008; Nativio et al., 2009).
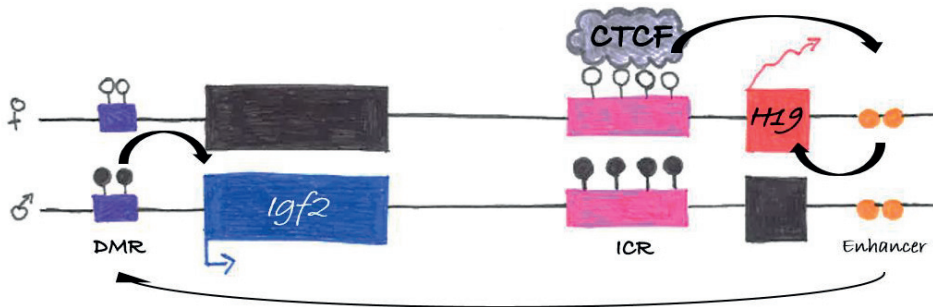
Figure 2. A schematic picture of *Insulin-like growth factor 2* (*Igf2*)/*H19* insulator model. The imprinting control region (ICR) is located in the intergenic region and functions as a transcription insulator controlling the monoallelic expression of *Igf2* and *H19* genes. The ICR contains binding sites for the chromatin insulator zinc-finger protein, CCCTC (CTCF), which binds only to the unmethylated maternal allele. Binding of CTCF triggers the enhancers to activate the promoter of *H19* leading to its expression. In contrast, on the paternal allele, the binding of the CTCF is blocked by methylation allowing the enhancers to access *Igf2* differentially methylated region (DMR) to drive the gene activity.

The other imprinting model is known as the non-coding RNA model where the promoter of a long non-coding RNA is located within the ICR. The ICR is unmethylated on the paternal allele allowing expression of the non-coding RNA, which in turn silences the rest of the genes in the same allele. The methylated maternal allele instead silences the non-coding RNA, thereby allowing activation of proximal genes on the maternal allele. One of the characterized regions controlled by this mechanism is the *Kcnq1/Kcnq1ot1* gene cluster (Mancini-Dinardo et al., 2006). In this locus, the promoter of the long non-coding RNA *Kcnq1ot1* is located in the ICR. On the paternal allele, the ICR is unmethylated, allowing the expression of *Kcnq1ot1*, which in turn silences the genes on the paternal allele. In contrast, on the maternal allele, *Kcnq1ot1* is not expressed because of the methylated ICR, leading to expression of other genes in the cluster (**Figure 3**).
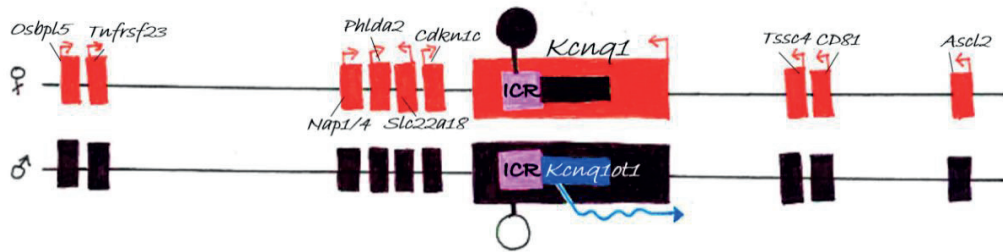
Figure 3. A schematic picture of the *Kcnq1/Kcnq1ot1* non-coding RNA model. The imprinting control region (ICR) is located on the paternal allele and contains a promoter of the long non-coding RNA, *Kcnq1ot1*. Because the ICR is unmethylated on the paternal allele, it allows the expression of the *Kcnq1ot1*, which in turn silences the rest of the genes in the same allele. In contrast, since on the maternal allele, the ICR is methylated, it silences the *Kcnq1ot1*, thereby allowing activation of proximal genes on the maternal allele.

It is not precisely known how non-coding RNAs silence genes but several mechanisms have been proposed. One possibility is that there is the formation of double-stranded RNA, RNA-interference (RNAi), between the mRNA and non-coding RNA (Lennox & Behlke, 2016). This kind of sense-antisense overlap may also be formed together with a promoter or an enhancer affecting the transcription from the mRNA promoter (Pauler et al., 2012). Moreover, models have been postulated where non-coding RNAs attract the machinery that lays down repressive chromatin marks (Murakami et al., 2007; Nagano et al., 2008; Pandey et al., 2008) or prevents the recruitment of RNA polymerase II at promoters (Latos et al., 2012).

1.4.4 Human imprinting disorders

The diploid state of genes increases the protection against the effects of mutations since the gene can be still expressed from the other allele. Because of the monoallelic feature of imprinting, the risk for harmful effects caused by mutations in one of the alleles becomes even greater. Thus, the importance of normal genomic imprinting can be demonstrated by the presence of human imprinting disorders. These diseases are rare, but recognized for their severe clinical phenotypes that involve diverse growth or neurological developmental disorders. They result from disruptions or epimutations of imprinted genes such as uniparental disomy (both chromosomes in a pair are inherited from the same parent), structural chromosome rearrangements and gain or loss of DNA methylation at ICR. Most known disorders are Prader–Willi syndrome and Angelman syndrome as well as Silver-

Russell syndrome and Beckwith-Wiedemann syndrome (more detail information found in: (Soellner et al., 2017)).

1.5 EPIGENETIC REPROGRAMMING

During mammalian development, the genome undergoes dynamic reprogramming of the epigenome (**Figure 4A, B**). This global event occurs twice: immediately after fertilization as well as in primordial germ cells in the early germ line (Reik et al., 2001). Erasure of the cellular DNA methylation memory is required to either reset the epigenome for naive pluripotency or to facilitate gametogenesis. However, some of the genomic regions are protected from the reprogramming due to the strict regulation of erasure and *de novo* methylation. To date, most of the studies have been done with mice, however major progress in the human research field has revealed that despite the presence of minor differences, the epigenetic reprogramming seems to be similar.

1.5.1 Reprogramming after fertilization

Following fertilization, the egg cytoplasm contains both maternal and paternal pronuclei that are epigenetically distinct. Rapidly after fertilization, the distinct pronuclei undergo genome-wide demethylation by different dynamics and methods. The more highly methylated paternal genome is actively demethylated even before the two pronuclei merge. The rapidly lost 5-methylcytosine is replaced with an increase of 5-hydroxymethylcytosine, suggesting that TET-mediated oxidation is responsible for the loss of methylation in the paternal genome. This was confirmed by a knockdown of TET3 in the mouse zygote, revealing it to be one of the key demethylation drivers (Gu et al., 2011). In contrast, the maternal genome becomes gradually demethylated following subsequent cleavage divisions. After the fusion of the pronuclei, both genomes are demethylated passively through replication-dependent processes (Inoue & Zhang, 2011). As a result of the global demethylation, the inner cell mass becomes nearly hypomethylated (blastocyst stage E3.5, mouse).
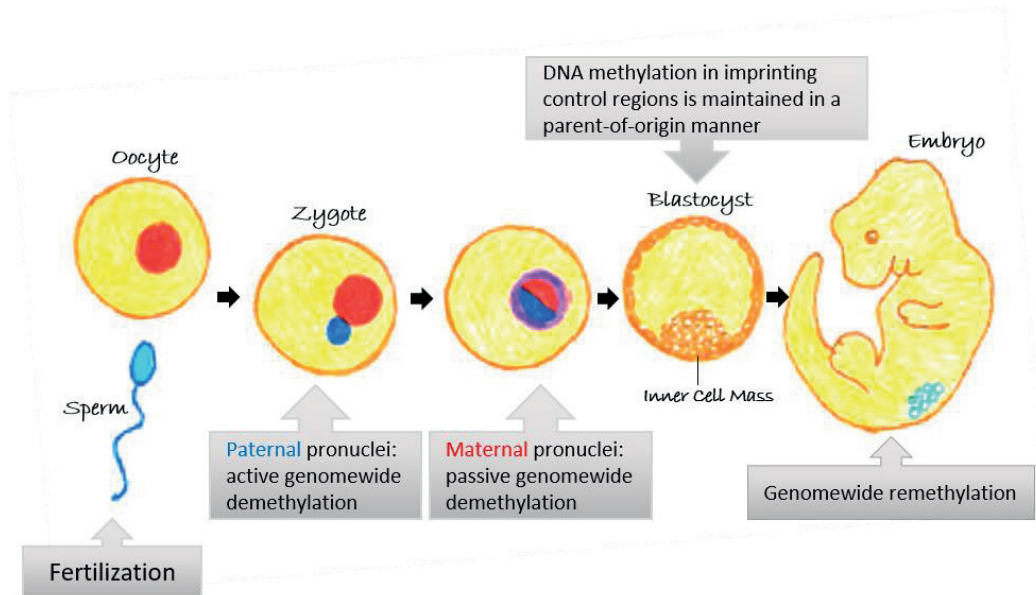
Figure 4A. A schematic picture of epigenetic reprogramming after fertilization.

However, a small proportion of the genome escapes demethylation, including imprinting-associated DMRs and transposons (Z. D. Smith et al., 2012). The maintenance of imprinting is particularly important as these genes are regulators of embryonic development. Since the imprints are maintained through the genome-wide demethylation, they represent a possible mechanism for intergenerational epigenetic inheritance.

A few mechanisms are known to be responsible for the maintenance of DNA methylation early in development. The oocyte-specific isoform DNMT1o of the methyltransferase DNMT1 has been shown to be a one key player in maintaining the imprints during one single cell cycle in pre-implantation development (Howell et al., 2001). Later, the maintenance is carried out by the somatic form of DNMT1 (Kurihara et al., 2008; Hirasawa et al., 2008). It is still unclear how the DNMT1 isoforms find the specific DMRs among many other DNA regions, however it has been suggested that a mammalian-specific region near the amino terminus of DNMT1 is involved (Borowczyk et al., 2009). There are also other proteins which could have a role in the imprint maintenance in early embryos. Zinc finger protein 57 (Zfp57) is a maternal protein in early embryos essential for the maintenance of DNA methylation at several paternally and maternally methylated germline DMRs (X. Li et al., 2008). It has been shown that this protein binds to cofactor KAP1, and then recruits

other epigenetic regulators such as DNMT1 (Quenneville et al., 2011). Another maternal protein, PGC7/STELLA, is also known to protect some germline DMRs from the reprogramming through interactions with H3K9me2 histone modification (Nakamura et al., 2012).

During the late blastocyst stage, increasing expression of *de novo* methyltransferases induces global methylation leading to a hypermethylated genome (Auclair et al., 2014).

1.5.2 <u>Reprogramming in primordial germ cells</u>

During embryonic days E7.5-E13.5 in mice (week 2 from conception in humans), a specific cell population undergoes differentiation and migrates to the genital ridge. These cells are known as primordial germ cells, the precursors of female and male gametes. They become distinguished from the totally hypermethylated post-implantation epiblast and are epigenetically reprogrammed, becoming almost completely hypomethylated (Seisenberger et al., 2012). This genome-wide DNA demethylation event is carried out in two phases.

The first phase begins around E8.0 during the migration. Passive demethylation is established through the inactivation of the methylation maintenance system by downregulating *Uhrf1* (a DNMT1 recruitment factor) as well as *de novo* methyltransferases (DNMT3A, 3B, 3L), and the loss of the histone modification H3K9me2 (Kagiwada et al., 2013; Kurimoto et al., 2008; Seki et al., 2007). During the first demethylation wave, only 30% of the total methylation level is reduced by the time the primordial germ cells reach the genital ridge. The remaining methylation at the CpG islands on the X-chromosome, differentially methylated regions of imprinted genes and some germline-specific genes are removed during the second wave of demethylation around E11.5. This active demethylation is established by TET-enzyme activity. TET1 and TET2 have been suggested to play a critical role, especially for imprints, by converting the 5-methylcytosine into 5-hydroxymethylcytosine (Hackett et al., 2013; Vincent et al., 2013; Yamaguchi et al., 2013).
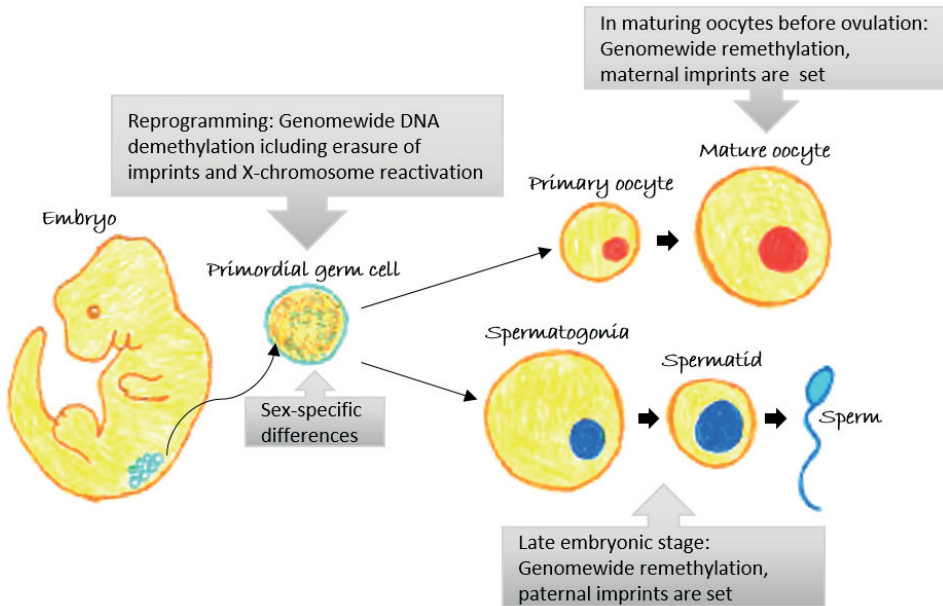
Figure 4B. A schematic picture of epigenetic reprogramming in primordial germ cells.

However, despite the presence of a global demethylation wave, there are a few regions known to be able to avoid the reprogramming: transposon families and variably erased CpG islands (Hackett et al., 2013; Guibert et al., 2012). These "escapees" have raised the question of whether these elements could be the potential carriers of the epigenetic information from one generation to the next or even across multiple generations, particularly in association with metabolic disease phenotypes. How these regions remain methylated is not completely understood. However, DNMT1 has been shown to be highly expressed in these cells, despite the downregulated *Uhrf1*, and it may be responsible for maintaining the methylation (Gkountela et al., 2015; Tang et al., 2015).

Sex-specific *de novo* methylation is established as the primordial germ cells develop into female or male gametes. In the male germline, *de novo* methylation of differentially methylated regions begins in prospermatogonia around E14.5 and is entirely established during late fetal development (Davis et al., 2000; Ueda et al., 2000). In contrast, in the female germ line, maternal specific imprint marks are obtained after birth (postnatally) during the growth phase of oocytes as they are recruited into folliculogenesis for further maturation and ovulation (Obata & Kono, 2002; Lucifero et al., 2004; Hiura et al., 2006). Several factors such as different DNMT isoforms, piwi-RNAs and chromatin markers have been suggested to be involved in the establishment of the new methylation pattern (Smallwood et al., 2011; Kaneda et al., 2004; Kato et al., 2007; Kuramochi-Miyagawa et al., 2008).

1.6 ENVIRONMENTAL EPIGENETICS

Prenatal exposure to diverse environmental factors such as chemicals (Agay-Shay et al., 2015), stress (Weaver et al., 2004) and dietary components (Sinclair et al., 2007) have been shown to impact on the development and health in both mice and humans. It has been suggested that the environment induces changes in the epigenome that alters gene expression and ultimately affects the phenotype (Waterland & Jirtle, 2003; Kaminen-Ahola et al., 2010a). The exact mechanism of how the epigenome associates with early-life exposure remains largely unknown but exploring both humans and experimental models has expanded our understanding of environmental programming on long-term health.

1.6.1 <u>Early life exposure, genetics and the epigenome</u>

In the early 1930s, epidemiological research suggested that early life conditions had as important a role as the genomic background on the prevalence of disease. This led to concern for environmental stressors to interfere with embryonic development (Kermack et al., 2001). It was not until the Second World War that there was an opportunity to explore this in humans with the onset of famine: an exceptional possibility to perform so-called natural experiments. The Second World War exposed thousands of pregnant women to periods of severe undernutrition including the Dutch Winter Famine in 1944-45 (C. A. Smith, 1947; Roseboom et al., 2001). Subsequent research revealed that starvation close to conception induced miscarriage and malformation whereas during the last trimester, it led to reduced birth weight. Many years later, associations were found between gestational undernutrition and metabolic diseases in the young adults who had been exposed to the devastating effects of the famine during their early pregnancy (Ravelli et al., 1976) and even transgenerational effects have been observed (Lumey, 1992). Subsequently, the research field of early life exposure grew rapidly and in the 1980s, the work of epidemiologist David Barker evolved a hypothesis of 'Developmental Origins of Health and Disease' (DOHaD).

The DOHaD field suggests that early life environmental exposure influence the development and physiology of the fetus leading to affected growth and development and a higher risk of chronic disorders later in life. The reason for increased metabolic disease is predicted to arise from adaptation to undernutrition *in utero* followed by postnatal exposure to a nutritionally rich environment (Hales & Barker, 1992). The actual molecular mechanism behind the hypothesis has been poorly understood until recent findings indicated that the epigenome would bridge the gap between the environment and phenotype. It has been hypothesized that environmentally-induced epigenetic alterations take place during the very early stages of embryogenesis and spread out by cell divisions,

hence affecting a large proportion of cells in the growing fetus (Feil & Fraga, 2012). In this way, the epigenetic marks provide a possible molecular 'memory' of the environmental exposure that continuously alters gene expression, and thus triggers the appearance of the phenotype. One classic example of the role of environment on phenotype are evident in monozygotic twins. Even though monozygotic twins share a common genotype, they can exhibit phenotypic discordance, such as a different susceptibility to diseases (Lehtovirta et al., 2010; Dempster et al., 2011; Casey et al., 2017). The epigenome is suggested to be responsible for these differences (N. Mohandas et al., 2018). As an example, Fraga and colleagues (2005) noticed that young epigenetically indistinguishable twins exhibited alterations in different epigenetic patterns that affected the expression of their genes as they grew older.

Furthermore, in addition to the environmentally induced changes in the epigenome, the genetic background seems to have a major impact on the epigenetic regulation. It has been noticed that there are more epigenetic differences between dizygotic twins than between monozygotic twins (Kaminsky et al., 2009; Ollikainen et al., 2010). Furthermore, males and females display differences in DNA methylation levels of many autosomal genes (Tobi et al., 2009) and different inbred mouse strains exhibit various epigenetic changes after exposure to environmental stimuli (Rosenfeld, 2010). Thus, the genetic background seems to have a major impact on epigenetic regulation. However, it is still not clear how broadly the underlying genome influences the epigenome. Single nucleotide polymorphisms have been observed to impact on nearby methylation levels (Gertz et al., 2011; Heijmans et al., 2007). About 10% of the common polymorphisms associate with the allele-specific methylation level (Hellman & Chess, 2010). Furthermore, the epigenetic profile has also been shown to be loci-specific. For example, loci that are CpG-rich regions gain more methylation over time compared to CpG-poor regions that lose methylation (Christensen et al., 2009). According to these observations, both epigenome and genome should be considered when studying early-life environmental exposure.

1.6.2 Experimental models

The field of environmental epigenetics has rapidly expanded during the past ten years with animal models, particularly rodents, providing a valuable resource in this field. Various animal models have been used to describe the link between epigenetics and developmental abnormalities. In addition, there are specific examples where non-mutagenic, environmental insults such as diet and several toxicants: endocrine-disrupting chemicals, alcohol and tobacco can cause unexpected phenotypic changes in the offspring (examples below). In mice, there are well-characterized loci where the methylation pattern

is associated with affected phenotypic outcomes (Duhl et al., 1994; Vasicek et al., 1997). The use of these markers provides a valuable way for studying how maternal environmental exposure alters epigenome of the fetus and reveals the phenotypic responses in her offspring.

The agouti viable yellow A^{vy} mouse is of the best characterized mouse models; in these animals, an epigenetic change in the regulatory region of gene is inherited and leads to well-defined pathological outcomes. The expression of the *Agouti* gene modifies the coat color of these mice. Agouti protein is a paracrine signaling molecule that promotes hair-follicular melanocytes to produce the yellow phaeomelanin pigment in wild type mice (Duhl et al., 1994; D. Lu et al., 1994). The A^{vy} ´viable yellow' mutation of the gene was first described in the 1960s (Dickies, 1962) and has been the most commonly used *Agouti* mutation for epigenetic studies. The A^{vy} allele is the result of the insertion of a murine intracisternal A-particle (IAP) retrotransposon about 100 kb upstream of the transcriptional start site of the *Agouti* gene. *Agouti* expression is controlled by the IAP whose ability to drive expression is correlated with the degree of methylation. Hence, the methylation status can be variable, leading to a range of coat colors from yellow (unmethylation of the A^{vy} IAP) to mottled (yellow with varying degrees of *Agouti* patches) to completely pseudoagouti (full methylation of the A^{vy} IAP). The inappropriate expression of the *Agouti* gene is also known to cause obesity and diabetes (Wolff et al., 1986).

With the help of A^{vy} studies, it has been possible to demonstrate how the maternal environment affects the offspring. The coat color of the mice provides a visual marker that can be assessed after birth to determine the cause of the environmental exposure and their predisposition to diseases later in life. For example, dietary methyl donors and vitamin cofactors fed to mothers, increase the methylation of the IAP promoter site leading to more pseudoagouti offspring and less pups with the yellow-obese syndrome (Cooney et al., 2002; Waterland & Jirtle, 2003). In contrast, exposure to bisphenol A (BPA) decreases the methylation status of IAP shifting the coat color distribution of the offspring toward yellow (Dolinoy et al., 2007). However, ethanol exposure during pregnancy exerts an opposite effect as it increases the methylation, resulting in more mice with a pseudoagouti colored coat (Kaminen-Ahola et al., 2010a). Together, these experiments emphasize the importance of the viable yellow mice as a powerful tool to study the relationship between epigenetics and maternal environment.

1.6.3 <u>Maternal environment</u>

In mammals, the placenta controls the flow of nutrients and oxygen to the fetus and at the same time, the placenta removes waste and acts as a barrier to toxins. The placenta adapts to the maternal environment and plays a major role in ensuring fetal survival during the *in utero* life. Thus, the placenta is sensitive to maternal influences, and changes in the gestational environment can directly impact on fetal health (Dimasuay et al., 2016). Therefore, as a 'temporary' tissue, it offers a major opportunity to investigate the conditions of prenatal life after birth.

The placenta starts to form after the blastocyst has implanted into the maternal endometrium. The implantation is driven by the trophoblast cells involving genes that are regulated by DNA methylation (Rahnama et al., 2006). As mentioned earlier, preimplantation is a critical window of epigenetic reprogramming. DNA methylation levels become re-established in the inner cell mass-derived cells during the implantation whereas the trophoblast cells remain hypomethylated (Santos et al., 2002). In fact, during its growth, the placenta is globally less methylated compared to the embryo, though the reason for this is still obscure (Novakovic et al., 2010; Hon et al., 2013).

The placental epigenome has a significant role in the regulation of development and function. Various maternal factors have been shown to cause changes in the placental DNA methylation profiles. In humans, maternal nutrition is one of the well-known influences regulating the placental epigenetic profile (Bouchard et al., 2012). Conditions such as gestational diabetes mellitus have been shown to be associated with global hypomethylation (Nomura et al., 2014). Furthermore, increased glucose levels are associated with decreased methylation levels (Desgagne et al., 2014) whereas choline may increase methylation (Jiang et al., 2012). In mice, calorie restriction increased the DNA methylation level which seemed to reduce placental glucose transporter expression, ultimately resulting in smaller offspring (Ganguly et al., 2014). Moreover, maternal smoking during pregnancy alters the structure and function of the placenta in humans. Nicotine affects the nutrient transport flow by reducing the nutrient supply leading to restricted growth as well as increasing the later disease risk (Lambers & Clark, 1996; Jauniaux & Burton, 2007). There is also evidence that smoking alters the placental methylation pattern (Morales et al., 2016).

Since the placenta carries the genetic fingerprint of the fetus, this represents a reason to study the epigenome of the placenta. For example, the epigenetically regulated imprinted genes are vital for normal placental growth and development, thus they can be used as environmental sensors to probe the epigenetic profile in order to reveal novel insights into prenatal conditions.

1.7 EPIGENETIC ALTERATIONS BEHIND PRENATAL ALCOHOL EXPOSURE

Based on the previous studies described above, the dynamic interaction between the epigenome and environment provides an attractive concept where the epigenome connects the genome to environmental signals. Thus, the investigation of epigenetic mechanisms has proved to be an advantageous approach to understanding the molecular mechanisms behind FASD. In the following paragraphs, the current state of FASD-related epigenetic research will be presented by highlighting the role of different epigenetic markers such as DNA methylation, histone modification and non-coding RNA.

1.7.1 <u>DNA methylation</u>

Since DNA methylation is the most extensively studied epigenetic modification, it has been proposed to be altered by prenatal alcohol exposure. In 1991, Garro and colleagues were the first to show alcohol-induced changes in DNA methylation. In their mouse model, embryos were exposed acutely to ethanol during gestational days 9-11 and the methylation level was measured by using *HpaII* methyltransferase. Alcohol caused hypomethylation in the genome probably by inhibiting the DNMT1 activity (Garro et al., 1991). Thereafter, additional evidence showing that alcohol alters the methylation profile was revealed by experiments conducted with the agouti viable (A^{vy}) yellow mouse model. However, the result was opposite to the hypomethylation seen in the previous study. In this mouse model, prenatal alcohol exposure at the beginning of pregnancy increased the methylation level, resulting in more pseudoagouti offspring. This was the first study to demonstrate the actual link between the epigenome and phenotype (Kaminen-Ahola et al., 2010a).

Since the brain seems to be vulnerable to alcohol-induced teratogenesis, studies have focused on different stages of development to identify the DNA methylation alterations in the brain tissue. For instance, alcohol exposure during the neonatal period caused hypermethylation in the hippocampus and prefrontal cortex in rat offspring (Otero et al., 2012). Hypermethylation was also seen in mouse hippocampus as a consequence of continuous alcohol exposure throughout gestation (Y. Chen et al., 2013). In two mouse studies, a genome-wide approach revealed that alcohol exposure at different time points caused significant and widespread changes in DNA methylation in either whole brain (Laufer et al., 2013) or hippocampus (Chater-Diehl et al., 2016). These methylation changes encompassed a relatively large number of gene promoters (Laufer et al., 2013; Chater-Diehl et al., 2016).

Alcohol appears not only to affect global methylation but also specific gene targets, and some genomic regions seem to be differentially sensitive to alcohol exposure, resulting in

more or less methylation (Y. Liu et al., 2009). For example, the differentially methylated region 1 of the *Insulin-like growth factor 2* (*Igf2*) imprinted gene showed increased methylation in the embryo (GD9) after acute alcohol exposure in mice (Downing et al., 2011). In addition, the expression of a serotonin transporter *Slc6a4* gene was decreased due to increased methylation in hippocampus (PD55) in mice exposed to alcohol throughout the pregnancy (Ngai et al., 2015). In contrast, *Methyl CpG Binding Protein 2* gene was upregulated as a result of decreased methylation at the *Mecp2* regulatory elements in neural stem cells exposed to alcohol at three different time points (Liyanage et al., 2015).

In addition to mouse models, recent human studies have described preliminary evidence of the first DNA methylome signature of FASD. Lee et al. studied the effect of periconceptional alcohol exposure at selected loci in Korean newborns. Either paternal or maternal heavy alcohol consumption resulted in a reduced methylation level on the promoter of dopamine (*DAT*) as well as serotonin (*SERT*) transporters in cord blood (B. Y. Lee et al., 2015). In a South Africa cohort of FAS children (median age of 9 years), blood samples were collected and the methylation status was investigated in selected imprinting control regions. It was reported that two maternally imprinted loci, *KvDMR1* and *PEG3 DMR*, showed lower average locus-wide methylation in the FAS cases with *PEG3 DMR* displaying the largest effect (Masemola et al., 2015). One of the limitations of investigating in humans is the lack of tissues that can be accessed. The advantage of using buccal epithelial cells is that they provide an opportunity to examine the methylation status of neuronal cells types such as brain because the cells share an ontogenetic relationship i.e. they are both derived from the ectoderm. In two Canadian studies, buccal epithelial cells were collected from children confirmed with FASD (age between 5-18 in Laufer et al., 2015 and 3-10 in Portales-Casamar et al., 2016) for characterizing the genome wide methylation profile. In both studies, hundreds of differentially methylated CpG-sites were observed and these could have the potential of being biomarkers for prenatal alcohol exposure in human population (Laufer et al., 2015; Portales-Casamar et al., 2016).

To summarize all of these findings, it seems that alcohol exposure affects the methylome, although the mechanism has remained obscure. Alcohol-induced hypomethylation has been suggested to be a consequence of disruptions in the methionine-homocysteine cycle (Halsted et al., 1996; S. C. Lu et al., 2000) (**Figure 5**). Methionine is an amino acid that cannot be synthesized by the human body itself and must be obtained from the diet. Methionine has a critical role as it is a precursor of the compound *S*-adenosylmethionine (SAM). SAM, in turn, serves as a key methyl donor for DNA and histone methylation. In the cycle, methionine is transferred to SAM, and ultimately through various stages, it is converted into homocysteine. The regeneration of methionine occurs through either

folate-dependent or folate-independent methylation of homocysteine. Alcohol exposure has been observed to affect the methionine–homocysteine cycle by disrupting the enzymes required for methionine metabolism (Barak et al., 1993; Lieber, 1988; S. C. Lu et al., 2000). Alcohol inhibits the activity of methionine synthase and decreases the amount of folate. This leads to a reduction in the levels of methionine which in turn reduces the levels of SAM and thus disrupts SAM-dependent methylation reactions and produces hypomethylation (Halsted et al., 1996; Halsted et al., 2002).
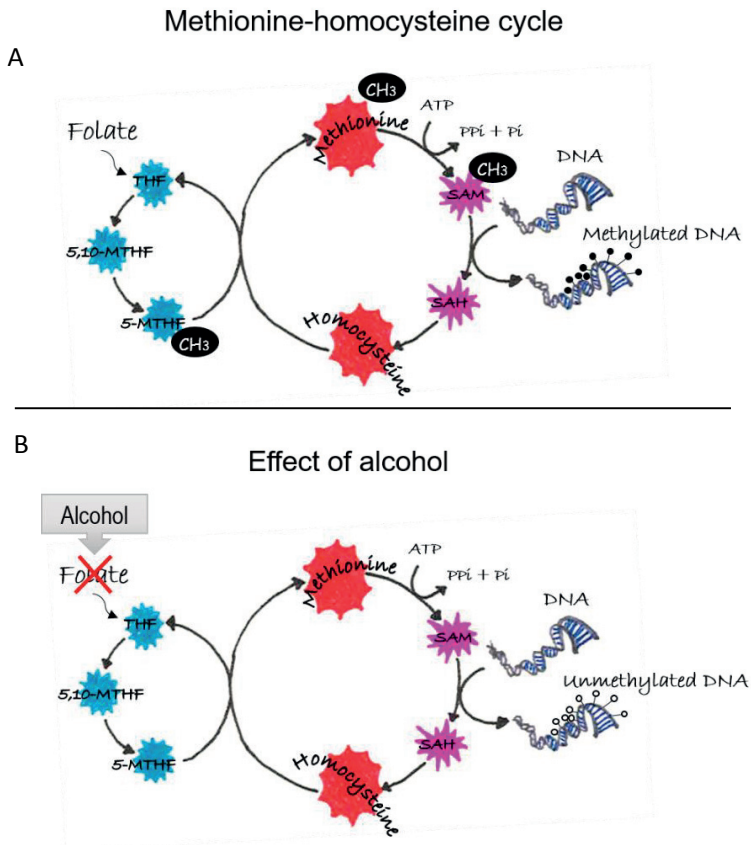
Figure 5. Simplified picture of the methionine-homocysteine cycle. (**A**) In the cycle, methionine is generated from homocysteine through folate-dependent cycle. Homocysteine is methylated by 5-methyltetrahydofolate (5-MTHF) which is converted into tetrahydrofolate (THF) and continues in the folate cycle. Methionine transfers the methyl group to S-adenosylmethionine (SAM) which serves as a methyl group donor for DNA. After donating the methyl group, SAM is transformed into S-adenosylhomocysteine (SAH) and then homocysteine. (**B**) Alcohol decreases the amount of folate which leads to a reduction in the levels of methionine which in turn reduces the levels of SAM and thus disrupts SAM-dependent methylation reactions and produces hypomethylation.

Hence, it has been suggested that adding folate into the maternal diet would directly affect the metabolic pathway and mitigate the outcomes of alcohol exposure. It is known that women who experience folic acid deficiency during pregnancy are more likely to have premature births and to give birth to infants with low birth weights as well as neural tube defects (Molloy et al., 2009). Since ethanol directly inhibits the metabolism of folate (Halsted et al., 2002) and decreases the transport of folic acid to the fetus (Hutson et al., 2012), this has raised the question of whether the neurological outcomes could be actually attributable to the reduced folate level. Serrano and colleagues (2010) showed that by adding folic acid to the diet of the dams could prevent the effects of alcohol exposure allowing normal gene expression and cardiogenesis. Therefore, more work should be done to determine the effect of increasing the amounts of folate in the diet of pregnant women.

1.7.2 <u>Histone modifications</u>

Even though histone modifications are known to play a crucial role in regulating transcriptional activity and the overall chromatin structure (Bannister & Kouzarides, 2011), the effects of alcohol exposure have been less extensively studied. However, there are findings suggesting that alcohol´s impact on different chromatin proteins ultimately can alter chromatin structure and function. Guo et al. (2011) showed that exposure to ethanol during postnatal day 7 (3rd trimester-equivalent of human pregnancy) in a rat model reduces a histone acetyltransferase CREB binding protein levels leading to acetylation of both H3 and H4 histones in the cerebellum. In another rat study, gestational alcohol exposure (GD7-GD21) affected several histone modifications as well as altering mRNA expression of the enzymes that edit these histone modifications in hypothalamus (PD60-65). Interestingly, choline, which is also a part of the one-carbon pathway, normalized the alcohol-induced altered modifications and the mRNA levels (Bekdash et al., 2013).

Alcohol exposure has also been shown to affect histone modifications associated with neuronal apoptosis. Levels of histone modification of γH2AX, a relevant modification in apoptosis, were globally increased in the cerebral cortex following postnatal (PD7) acute ethanol exposure in mice. Immunohistochemical staining revealed significant cell death in several brain regions indicating ethanol-induced neurodegeneration (Goldowitz et al., 2014). Furthermore, mice exposed to acute ethanol exposure during postnatal day 7 induced apoptotic neurodegeneration by activating dimethyltransferase (G9a). This was followed by increased dimethylation of histone H3 lysine 9 (H3K9me2) and 27 (H3K27me2) which led to activation of caspase-3 and finally ethanol-induced neuronal apoptosis in the developing brain (Subbanna et al., 2013). Even low concentrations of ethanol have shown the same effect (Subbanna et al., 2014). However, by inhibiting G9a, the level of

neurodegeneration was decreased (Subbanna & Basavarajappa, 2014). These studies highlight the possible mechanism behind the neurodevelopmental outcomes of prenatal alcohol exposure.

Since it is known that different genes exhibit diverse histone modification profiles, the research has focused on specific target genes (Veazey et al., 2013; Veazey et al., 2015). A study using neural cortical stem cell model showed that the alterations in different histone modifications (H3K9me2, H3K9ac, and H3K27me3) were dependent on the dose of alcohol as well as on the gene under investigation. These changes were associated with alterations in the transcripts of genes involved in neurogenesis. Furthermore, histone modifications were consistent with those modeled *in vivo*, highlighting the value of cell culture models in providing significant data along with *in vivo* models (Veazey et al., 2015). At the genome-wide level, hundreds of H3K4 and H3K27 trimethylation changes in gene promoters were found in the adult mouse hippocampus tissue as a result of ethanol exposure during postnatal days 4 and 7. This indicates that alcohol exposure can cause long-term alterations in histone modifications (Chater-Diehl et al., 2016).

Even though, both genome-wide and locus-specific findings support the effects of alcohol exposure on histone modifications, the causative link between the epigenetic state and gene expression remains unclear. However, this is not surprising when considering the complexity of the histone code. Therefore, more studies are needed to investigate the importance of higher chromatin structure in the interplay of environmental exposure, especially in human populations.

1.7.3 Non-coding RNA

Non-coding RNAs are known to play an important role in the neuronal regulation and brain development (Petri et al., 2014). There is still limited information about their connection to alcohol-induced neurodevelopmental deficits. Recent studies have revealed that disruptions in their expression have been associated with neurological disorders such as autism, schizophrenia and anxiety (Spadaro & Bredy, 2012; Williams et al., 2009; Barry et al., 2014; Spadaro et al., 2015). Because neuronal loss is a prominent etiological factor for fetal alcohol spectrum disorders, the question has been raised – are non-coding RNAs also involved in alcohol-induced neuronal apoptosis?

One of the early studies examining the interactions between microRNAs and the teratogenic effects of ethanol was carried out with a fetal mouse cerebral cortex-derived neuroepithelium cell culture model. It was found that there was suppressed expression of four miRNAs, miR-21, miR-335, miR-9, and miR-153 due to heavy alcohol exposure and

noted their impact for controlling developmentally important genes. These miRNAs were suggested to be ethanol-sensitive but their contribution to cell death remained unclear (Sathyan et al., 2007). In addition, 62 miRNAs were differentially expressed after chronic intermittent exposure of primary neuronal cells to ethanol. Withdrawal of the ethanol exposure induced a distinct expression pattern of these miRNAs, suggesting a notable plasticity in the miRNAs expression due to the effect of ethanol (Y. Guo et al., 2012). In addition, one candidate miRNA, miR-29b, has been suggested to be a specific target of ethanol. A high dose of ethanol suppressed the expression of miR-29b in primary cultures of cerebellar granule neurons and possibly mediated ethanol-induced neuronal apoptosis (Qi et al., 2014). Even long-term alterations of miRNAs have been discovered (Mantha et al., 2014). Because the miRNAs seem to be sensitive to alcohol exposure, they may represent a plausible biomarker for alcohol-induced fetal programming. For example, maternal plasma miRNAs have been suggested to be a useful marker to predict the outcomes of affected infants (Balaraman et al., 2014; Balaraman et al., 2016).

To date, there is only one evidence that a small nucleolar RNA (snoRNA) (Laufer et al., 2013) as well as long non-coding RNA (Veazey et al., 2013) can be altered as a consequence of alcohol exposure. Because of the variety of non-coding RNA species, more studies will be needed to clarify their role in the neural functions involved in the development of FASD. Nonetheless, the above findings add new evidence to our understanding of the role of non-coding RNAs in mediating the effects of alcohol. These molecules seem to play a critical role in the nervous system and it is important to assess their function in the regulatory pathways in the brain. It has already been proposed that there may be candidate non-coding RNAs that associate directly with prenatal alcohol exposure. Findings of non-coding RNAs linked to facial dysmorphisms (Tal et al., 2012), neuroapoptosis and altered neurodevelopment (Pappalardo-Carter et al., 2013) ultimately support the idea of epigenetic impact behind prenatal alcohol exposure.

2 AIMS OF THE STUDY

The aim of my thesis was to study the molecular and phenotypic alterations caused by prenatal alcohol exposure. In the first two studies, I used a mouse model based on voluntary maternal consumption of ethanol provided at the beginning of pregnancy. In the first study, the focus was on the effects of alcohol in the brain tissue of four-week-old offspring. The aim was to explore genome-wide gene expression changes and locus-specific DNA methylation changes in hippocampus, as well as structural changes in the brain. Furthermore, since one of the main characteristics of prenatal alcohol exposure is growth restriction, the aim of the second study was to explore the effects of alcohol on DNA methylation and gene expression in growth-related imprinted genes in mouse embryos and placentas. In the third study, I examined human placental samples to investigate the effects of maternal alcohol consumption on DNA methylation and gene expression in growth-related imprinted gene locus. Furthermore, the alcohol-induced alterations were explored in the phenotype of the newborns.

Specific aims of the studies:

I. To investigate the effects of prenatal alcohol exposure on the genome-wide as well as locus-specific gene expression, DNA methylation and structure of the hippocampus in a mouse model.

II. To study if the prenatal alcohol exposure alters the DNA methylation profile and gene expression of *Insulin-like growth factor* 2 (*Igf2*), *H19, Small nuclear ribonucleoprotein polypeptide N* (*Snrpn*) and *Paternally expressed gene 3* (*Peg3*) imprinted genes in the early embryo and placenta in a mouse model.

III. To explore prenatal alcohol-induced effects on the gene expression and DNA methylation profile at imprinted *Insulin-like growth factor* 2/*H19* locus in human placenta, and to study the phenotypic changes in the newborns.

3 MATERIAL AND METHODS

3.1 MOUSE MODEL FOR FETAL ALCOHOL SPECTRUM DISORDER (studies I, II)

All animals were handled and maintained with good animal practice according to the instructions, orders and ethical principles of EU-directive (European Union: 2010/63/EU, 2007/526/EY). Animal work was approved by the Animal Experiment Board in Finland (ESAVI/3312/04.10.03/2011, ESAVI/976/04.10.07/2013).

The mice in this study were inbred, genetically identical, C57BL/6J Rcc strain (Harlan, Netherlands). The experiments were performed in two animal houses, where all environmental factors (cage type, temperature: 22°C, air humidity: 56%, photoperiod: 12:12) were standardized. The C57BL/6 mouse strain is known to have a strong drinking preference for ethanol over water, making them ideal for this kind of investigation (Belknap et al., 1993). In these studies, voluntary consumption strategy was used for ethanol exposure, instead of intraperitoneal injections or intragastric administration, to avoid additional maternal stress.

This mouse model is based on moderate and chronic maternal consumption of 10 % (v/v) ethanol for the first eight days of gestation, a time frame encompassing implantation to the beginning of neurulation (**Figure 6**). It is equivalent to the developmental stage of early fourth week post-fertilization in a human pregnancy. A female mouse (8–10 weeks old) was caged with a male mouse and the day of plugging was designated as gestational day (GD) 0.5. The male was removed from the cage and the water bottle was replaced with a bottle containing 10% (v/v) ethanol into the female´s cage. The ethanol solution was changed, and consumption was measured every 24 hours. The average daily consumption of 10% ethanol during GD 0.5–8.5 was 3.2±0.6 (mean±SD) ml/mouse/day (or 12g±2.6g ethanol/kg body weight/day). According to previous studies, in female mice, the consumption of 10% (w/v) ethanol or 14 g ethanol/kg body weight/day produces an average peak blood alcohol level of ~120mg/dl (Allan 2003). This kind of 0.12% blood alcohol level can be considered as a realistic alcohol consumption level in humans. Pregnant females had free access to the 10% ethanol bottle and food throughout a period of eight days. Water was not available during the exposure period. The ethanol bottle was replaced with a bottle containing tap water on the final day of exposure (GD8.5). Control females drank tap water throughout the whole procedure.

Study I

After birth, a cross-fostering procedure was used to exclude potential alcohol-induced changes in maternal behavior or care, which could affect the epigenome of offspring. The litter from the alcohol-exposed dam was transferred into the cage of the control dam and

vice versa within one day of birth. The cross-fostered control offspring were not used as controls in our study.

The offspring were left with the dams until weaning at three weeks of age. Only male offspring were used in further investigations. To avoid the potential effects of hierarchy, the offspring were housed individually for a week. Four-week-old (P28) male offspring were sacrificed by cervical dislocation followed by dissection of hippocampi, olfactory bulbs, main olfactory epithelium, and bone marrow from hind-limb bones. Samples were snap frozen in liquid nitrogen at -80°C. Male mice designated for the MRI procedure were housed with male siblings for 5.5 weeks after weaning until being anaesthetized and perfused as adults (P57-P60, mainly P60).

Study II

At embryonic day 9.5 (E9.5) or 16.5 (E16.5) dams were sacrificed by carbon dioxide. Whole embryos and placentas were dissected on embryonic day 9.5. In contrast, on embryonic day E16.5, the placenta was dissected carefully removing the maternal decidua. All the samples were snap frozen in liquid nitrogen at -80°C.

The sex of the embryos was determined from DNA with *Sex-determining region Y* (*Sry*) and *Interleukin 3* (*Il3*) genes by PCR (Lambert et al., 2000). PCR was done by using AmpliTaq Gold® DNA polymerase kit, according to the manufacturer´s protocol (Thermo Fisher, Waltham, MA, USA) and both primer pairs (*Sry, Il3*) were included as a single reaction. As a result, two PCR products were visible for male embryos (*Sry, Il3*) and one for females (*Il3*).
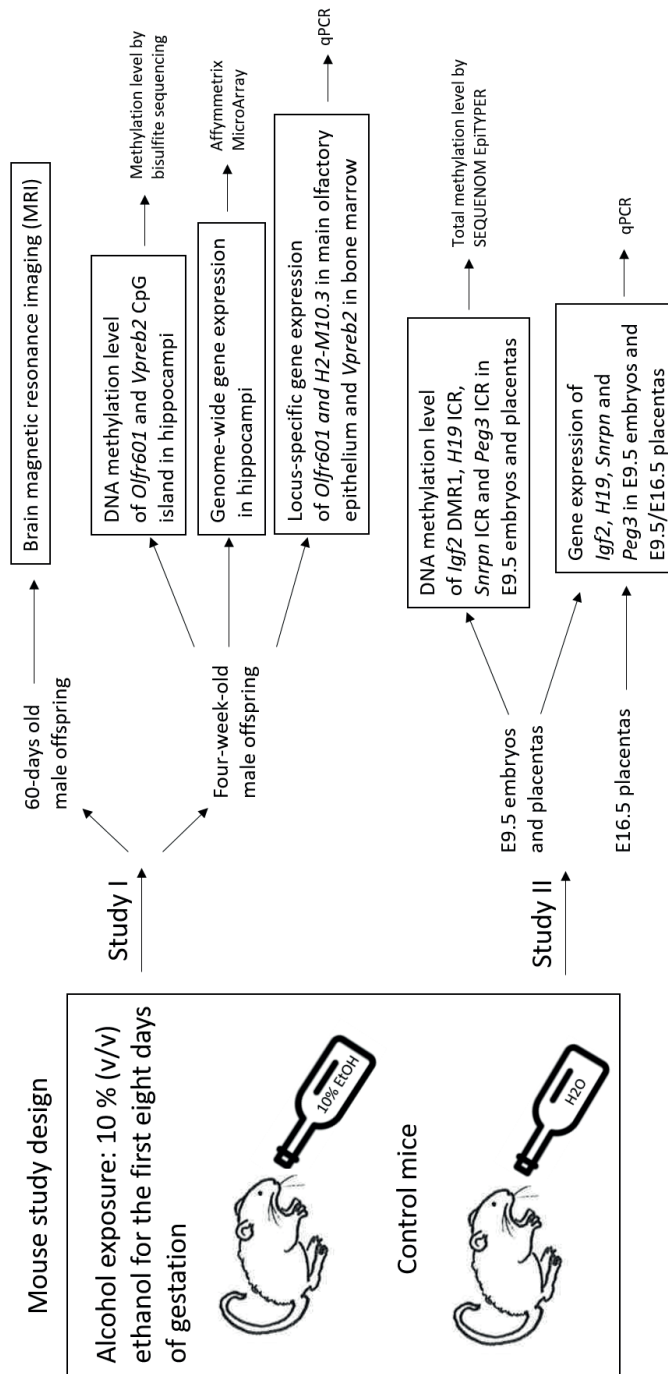
Figure 6. Summary of the material and methods used in the mouse studies I and II.

3.1.1 <u>Expression analysis (studies I, II)</u>

3.1.1.1 Gene expression array (study I)

RNA microarray analysis was used to examine the genome-wide alcohol-induced expression changes in the hippocampal tissue. In the expression array, the hippocampal RNA was extracted by using AllPrep DNA/RNA/Protein Mini Kit or miRNeasy Mini Kit (Qiagen). The quality was confirmed by BioAnalyzer (Agilent RNA 600 Nano, Agilent, Germany) (Biomedicum Functional Genomics Unit, Helsinki, Finland) and only samples with RNA Integrity Numbers (RINs) above 9 were accepted. The Affymetrix Mouse Exon 1.0 ST Array was used to analyze gene expression in the hippocampi of five control and five alcohol-exposed four-week-old male offspring (Biomedicum Functional Genomics Unit, Helsinki, Finland).

3.1.1.2 Quantitative real-time PCR (studies I, II)

(*Study I*)

Quantitative PCR was used to confirm the microarray results in the hippocampus and to determine whether similar gene expression changes of three genes could be detected in other tissues. RNA from hippocampus, main olfactory epithelium, and bone marrow for TaqMan procedure was extracted by AllPrep DNA/RNA/Protein Mini Kit (Qiagen) or NucleoSpin RNA II kit (Macherey-Nagel) (hippocampus), Allprep DNA/RNA Mini Kit (Qiagen) (main olfactory epithelium), and TRIzol Reagent (Ambion) (bone marrow). After DNAse treatment (RQ1 RNase-Free DNase, Promega), cDNA synthesis was performed by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories).

TaqMan for *Olfactory receptor 601 (Olfr601)*, *Histocompatibility 2, M region locus 10.3* (*H2-M10.3*) and pre-B lymphocyte gene 2 (*Vpreb2*) genes was performed by using TaqMan Gene Expression Assays (Applied Biosystems) and iTaq Universal Probes Supermix kit (Bio-Rad Laboratories) according to the manufacturer´s protocol (Applied Biosystems) (**Table 1**). Housekeeping gene *Ribosomal protein S16* (*Rps16*) was used as a reference gene. According to our experiments and previous alcohol studies, Rps16 was utilized as a convenient reference gene in this study (Carnahan et al., 2013).

Table 1. The table presents the TaqMan Gene Expression Assay probe codes for studied genes, the tissue in which the gene was studied with the number of samples.

Studied gene	TaqMan Gene Expression Assay	Tissue	n=Control samples	n=Alcohol-exposed samples
Olfr601	Mm01280848_s1	Main olfactory epithelium	10	10
H2-M10.3	Mm01277728_g1	Main olfactory epithelium	9	9
Vpreb2	Mm00785621_s1	Bone marrow	5	5

(*Study II*)

Quantitative PCR was also used to study the alcohol-induced effects on gene expression of imprinted genes *Insulin-like growth factor 2* (*Igf2*), *H19*, *Small nuclear ribonucleoprotein polypeptide N* (*Snrpn*) and *Paternally expressed gene 3* (*Peg3*), in E9.5 embryos and placentas, and E16.5 placentas. The E9.5 embryos and placentas were studied from the same individuals, except for one control sample in which embryo and placenta were from different origins.

Total RNA was extracted by Allprep DNA/RNA Mini kit (kit_1) (8 E9.5 embryos and placentas, E16.5 placentas: 4 ethanol-exposed and 4 controls) or Allprep DNA/RNA/miRNA kit (kit_2) (12 E9.5 embryos and placentas, E16.5 placentas: 6 ethanol-exposed and 6 controls) (Qiagen). RNA was DNAse treated (RQ1 RNase-Free DNase, Promega) and transcribed to cDNA by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The qPCR reaction conditions were as specified by SYBR® Green PCR Master Mix according to the manufacturer´s protocol (Applied Biosystems). *Rplp0* (Ribosomal protein, large, P0) was used as a housekeeping gene. Primers were chosen from previous publications (**Table 2**).

Table 2. The table presents the studied gene and the reference publications for the primers as well as number of samples.

Studied gene	Primers from prevous publications	n=Control samples	n=Alcohol-exposed samples
Igf2	Zhang et al. 2009	10	10
H19	Wang et al 2010	10	10
Snrpn	Wu et al. 2006	10	10
Peg3	Morita et al. 2014	10	10
Rplp0	Verona et al. 2008	10	10

Furthermore, to rule out the possible contamination of the maternal decidua in E16.5 placentas, the expressions of *Tissue factor pathway inhibitor 2* (*Tfpi2*) and *Adenosine monophosphate deaminase 3* (*Ampd3*) genes were examined by qPCR prior to the expression studies. These genes show a high expression in the placental cells of maternal side (Okae et al., 2012).

(Studies I, II)

In the qPCR studies, all the samples were analyzed in triplicate and the individual samples (both control and alcohol-exposed) were normalized by adding one same control sample on each plate. If the Ct-value was <0.5 between the triplicates, all of them were accepted for further analysis. The relative expression of studied genes was obtained by normalizing the Ct-values of the gene of interest to the Ct-values of the housekeeping gene. The control samples were used as a calibrator to alcohol-exposed samples to calculate the ΔΔCt and fold changes were calculated as $2^{-\Delta\Delta Ct}$ according to the Livak & Schmittgen (2001) method. To assure the effectiveness and reliability of each qPCR plate, a standard curve was generated. Outliers were tested with Grubb´s test and excluded from the further analysis.

3.1.2 Methylation analysis (studies I, II)
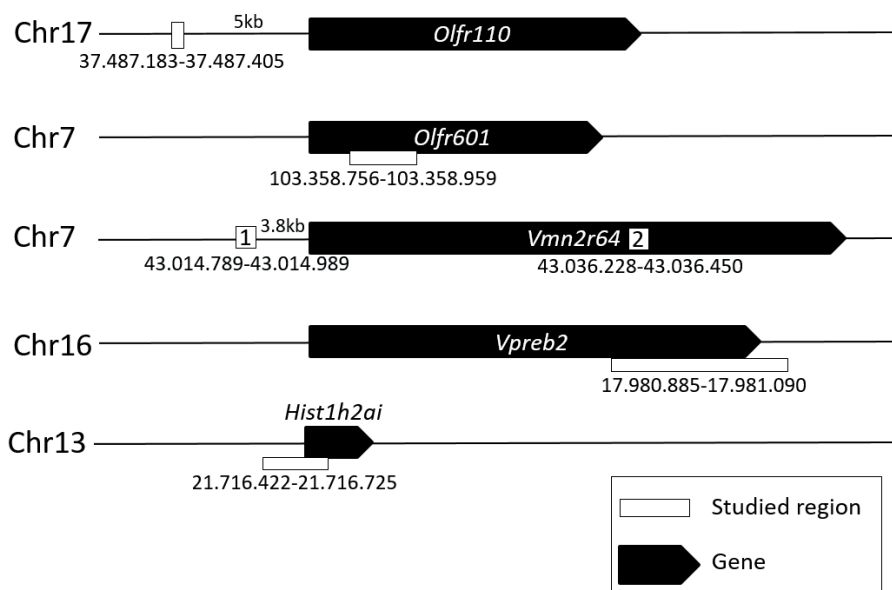
3.1.2.1 Bisulfite sequencing (study I)

Genomic DNA of hippocampus and main olfactory epithelium samples for bisulfite sequencing was extracted using commercial kits (AllPrep DNA/RNA/Protein Mini Kit and Allprep DNA/RNA Mini Kit, Qiagen and NucleoSpin RNA II kit, Macherey-Nagel) and standard phenol-chloroform protocol. Prior to bisulfite conversion, some hippocampal DNA samples, extracted by commercial kits, were cleaned by Genomic DNA Clean and Concentrator kit (Zymo Research). The main olfactory epithelium DNA was cleaned by Proteinase K (Macherey-Nagel) and Genomic DNA Clean and Concentrator kit (Zymo Research).

Sodium bisulfite conversion of hippocampus and main olfactory epithelium DNA (500 ng) was carried out using the EZ methylation kit (Zymo Research). Bisulfite converts the unmethylated cytosine to uracil whereas methylated cytosine (5mC) remain as cytosine. For each mouse, one to two bisulfite conversions as well as one to two independent PCR reactions were performed. PCR primers (Sigma-Aldrich) were designed by using the MethPrim program (The Li Lab, Department of Urology, UCSF) and the AmpliTaq Gold PCR kit (Applied Biosystems) was used according to the manufacturer´s protocol. PCR fragments

were gel-isolated with NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel) and ligated into the pGEM-T Vector system I (Promega).

Transformation of *E. coli* was performed by using the standard heat shock protocol with 100 µl of DH5α competent cells (Invitrogen) on ampicillin (100 µg/ml) LB-plates equilibrated with IPTG (Bioline) and X-Gal (Promega). White bacterial colonies were expanded in 2 ml of LB with 20 µl of ampicillin (100 µg/ml) overnight. Plasmids were extracted by using NucleoSpin Plasmid and Plasmid EasyPure kits (Macherey-Nagel) and sequenced in the Institute for Molecular Medicine Finland (Helsinki, Finland).

Six CpG islands of five differentially expressed candidate genes in alcohol-exposed offspring were bisulfite sequenced (**Figure 7, Table 3**). The CpG island of *Olfr601* was also sequenced in the main olfactory epithelium. The information of CpG islands was based on the data of the National Center for Biotechnology (NCBI). The CpG content in the islands was about 50% or above. Two additional CpG sites for *Vmn2r64* (2) were found (CpG9 and CpG10). *Hist1h2ai* has a long CpG island covering the whole gene-body and promoter region, from which was investigated 43 CpG sites.

Figure 7. The figure presents the five candidate genes with six CpG islands which were chosen for bisulfite sequencing: Olfactory receptor 110 (*Olfr110*) Olfactory receptor 601 (*Olfr601*), Vomeronasal type 2 receptor 64 (*Vmn2r64*, 2 CpG islands), Pre-B lymphocyte gene 2 (*Vpreb2*), and Histone cluster 1 H2ai (*Hist1h2ai*). Exons are in black and studied CpG islands in white.

Table 3. The table presents the tissue in which the candidate genes were studied. The columns in the middle represent the number of samples and how many different litters were used. The average amount of clones per mouse is in the last column.

Tissue	Studied gene	Number of control samples/litters	Number of alcohol samples/litters	Average amount of clones/mouse
Hippocampus	*Olfr 110*	7/5	7/6	9
	Olfr 601	10/4	8/4	10
	Vmn2r64(1)	8/4	8/4	10
	Vmn2r64(2)	7/5	7/6	9
	Vpreb2	6/3	6/4	9
	Hist1h2ai	6/4	5/3	7
Main olfactory epithelium				
	Olfr 601	5/3	5/3	9

Sequences were analyzed with BiQ Analyzer (Bock et al., 2005). Any clones with a lower than 90% conversion rate were excluded from the dataset.

3.1.2.2 MassARRAY EpiTYPER (study II)

The MassARRAY EpiTYPER (SEQUENOM Inc.) technique was used to identify alcohol-induced changes in the methylation profile of the imprinted genes. This quantitative methylation analysis method is based on MALDI-TOF (matrix-assisted laser desorption/ionization Time-of-flight) and MassCLEAVE (base-specific cleavage of RNA) technology. DNA is first bisulfite converted and then amplified with T7 promoter-tagged reverse primers, as this is needed for the *in vitro* transcription of the DNA to RNA in the next phase. RNA is finally cut into fragments by a base-specific (T or C) cleavage. These different size cleavage products lead to mass-specific differences which are determined by the mass spectrometer. The MALDI-TOF determines the relative amount of methylation in the RNA fragments (containing one or several CpG units) by a distinct signal pattern that results from the methylated or unmethylated target sequence.

In the methylation studies, genomic DNA was extracted by traditional phenol-chloroform protocol or by Allprep DNA/RNA/miRNA kit (Qiagen). Methylation analysis was performed on 10 E9.5 day old whole embryos (five controls and five ethanol-exposed) and placentas (five controls and five ethanol-exposed). Embryos and placentas were chosen from same individuals, except for one control and one alcohol-exposed samples in which embryo and placenta were from different origin. Two to three individuals represent the same litter.

The amplicons were designed to cover a small part of the imprinting control region or the differentially methylated region of the studied imprinted genes. Thus, the candidate

methylation profiles were: *Igf2/H19* ICR (CTCF1/CTCF2), *Igf2* DMR1, *Snrpn* ICR and *Peg3* ICR (**Figure 8**). We also examined methylation in long interspersed nuclear elements (Line-1) in E9.5 placentas to determine global alcohol-induced alterations. All the primer sequences were designed with EpiDesigner software (SEQUENOM: http://www.epidesigner.com/; T-reaction) except for Line-1 (Chang et al., 2013). The EpiTYPER analysis was done in triplicate for amplicons: *Igf2* DMR1, *Peg3* and Line-1, and the three PCR products were pooled for amplicons: *Igf2/H19* ICR and *Snrpn*. Prior to analysis, all CpG-units that expressed too low or too high mass or a silent peak overlap of the units, were discarded. The CpG-units, which could not be analysed separately due to their close location, were analysed together as a mean methylation value. Technical replicates showing > 5% difference from the median value were discarded and only two successful replicates were analysed.
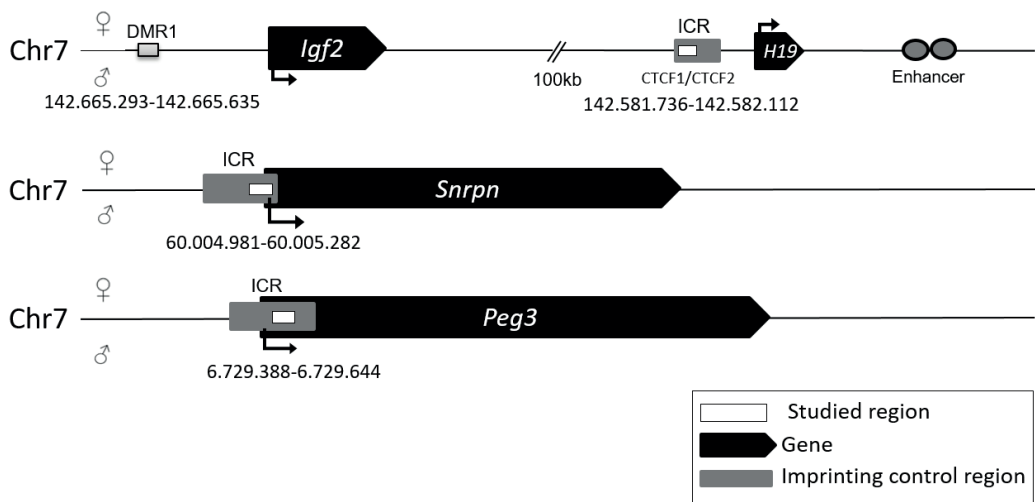
Figure 8. The figure illustrates the four imprinted genes which were studied by EpiTYPER: *Igf2/H19* ICR (CTCF1/CTCF2), *Igf2* DMR1, *Snrpn* ICR and *Peg3* ICR. Exons are in black and studied regions of imprinting control regions in white.

More detailed information regarding to all primers and protocols of both gene expression and methylation analysis are found in original publications I and II (Marjonen et al., 2015, Marjonen et al., 2018).

3.1.3 Magnetic resonance imaging (MRI) (study I)

In order to determine long-term alcohol-induced effects in the offspring brain, magnetic resonance imaging was performed for adult male offspring to observe changes in the volumes of different brain structures.

Briefly, eight control and 13 ethanol-exposed mice (P60) were first anaesthetized following transcardial perfusion. The brain was removed from the skull and post-fixed. Before ex vivo MRI, all the brains were immersed in perfluoropolyether (Galden HS240, Ausimont, Milano, Italy) to avoid signal from the solution. MRI experiments were carried out in a vertical 9.4 T magnet (Oxford Instruments PLC, Abingdon, UK) interfaced to a Varian DirectDrive console (Varian Inc, Palo Alto, CA) using a quadrature volume RF-coil (diameter 20 mm, Rapid Biomedical GmbH, Rimpar, Germany) for transmitting and receiving. The total scan time was 15 hours. The total brain volume including cerebrum (olfactory bulbs, basal ganglia, limbic system and cortex), thalamus, midbrain and cerebellum was measured. The lateral ventricles, hemispheres, hippocampi, olfactory bulbs, and cerebellum were assessed as selected brain areas. Their volumes were normalized to the total brain volume. Body weight, as well as whole brain and left/right hemispheres volumes, was used as such in the calculations.

3.2 HUMAN STUDY DESIGN (study III)

In collaboration with a special antenatal out-patient clinic in Helsinki University Central Hospital, we recruited 39 pregnant women who consumed alcohol during pregnancy (**Table 4**). Pregnant women (n=23) reported their alcohol consumption habits they had before pregnancy in the 10-item screening tool of Alcohol Use Disorders Identification Test (AUDIT). This tool has been developed by the World Health Organization to assess alcohol consumption, drinking behaviour and alcohol-related problems (Babor et al., 2001). Scores of 8-40 indicate harmful or hazardous alcohol use. Alcohol consumption was also measured by the amount of reported alcohol units per week (i.e. 1 unit is 12 grams of ethanol) (n=10). More than 12-16 alcohol units per week is considered as heavy drinking (Rehm et al., 2015). For six mothers, the alcohol usage was unknown. Seven mothers did not smoke during pregnancy. All the newborns were Finnish, except for two newborns who were of African origin and one of newborns´ mother was Estonian and father Russian.

Controls (n=100) were healthy Finnish newborns with Caucasian mothers who had not used alcohol or smoked cigarettes during the pregnancy according to their self-reported information and were recruited during the same period as the alcohol-exposed samples (years 2013-2015) in Helsinki University Central Hospital. This study was approved by the Ethics Committee of Helsinki University Central Hospital (386/13/03/03/2012).
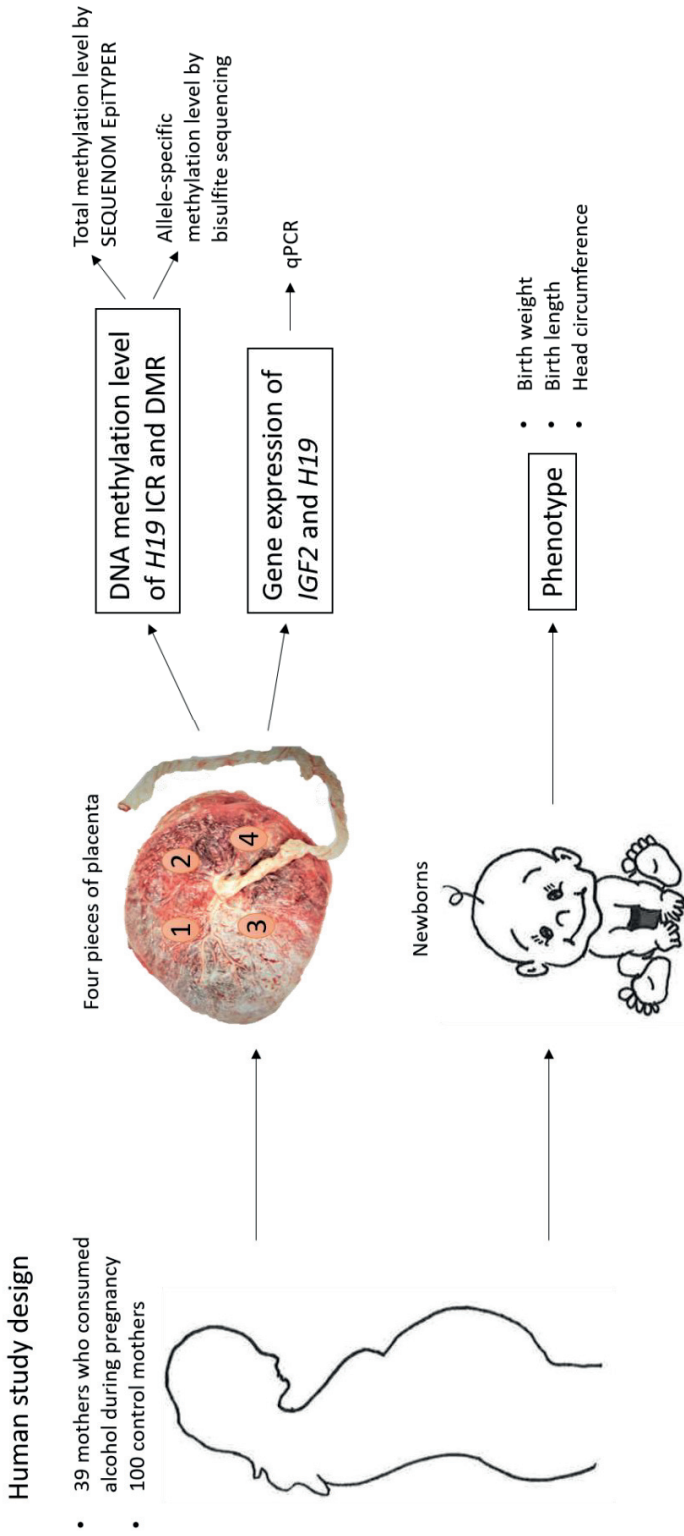
Figure 9 summarises the material and methods used in the human study.

Four placental biopsies (1 cm^3), from the fetal side and within a radius of 2-3 cm from the umbilical cord, were collected from all pregnancies (n=39 alcohol-exposed, n=100 controls) immediately after delivery, rinsed in cold 1xPBS, and stored in RNAlater® at -80°C.

Table 4. General information about alcohol-exposed newborns and their mothers.

Genotype	Sample ID	GW	Duration of drinking	Alcohol consumption	HC SD	Weight SD	Length SD	Placental weight (g)	Apgar 5 min	Sex	Smoking	Section (e=elektive)	Other
G/G	1	36+0	whole pregnancy	detoxification at 35 GW for ten days	-5,8	-5,2	-5,7	335	5	F	N/A		African origin
	2	41+1	whole pregnancy		-1	-0,2	-0,5	590	9	F	N/A	yes	African origin
	3	38+6	H12	audit 13	0,6	-1,1	-0,6	760	10	F	H6		
	4	42+0	H8	audit 14	0,1	0,4	0	802	9	M	H12		
	5	41+0	H6	12-16 ad	-1,2	-0,3	-0,3	480	9	F	H20		SSRI medication till 36 gestational weeks. GDM: diet only, no need for medication
	6	41+5	H6	audit 13/16-60 ad	-2,2	-0,2	0,1	525	9	M	yes		
	7	38+6	H5	24-39 ad	-0,5	1,3	0,8	980	9	F	yes		GDM, insulin and metformin medication, SSRI, antipsychotic medication (quetiapine)
	8	41+1	H5	audit 8/40 ad	0,9	-0,3	1,2	570	10	M	yes	yes. e	
	9	41+4	H4-5	audit 21	-0,8	0,1	-0,5	620	10	F	yes		first trimester use of SSRI, diazepam and melatonin
	10	41+2	H4-5	audit 23/30-40 ad	-1,2	-1,9	-0,9	383	10	M	no	yes	CS after trial of labor, induction of labor due to hypertension
pat G/mat A	11	41+0	whole pregnancy	audit 28	-2,7	-1,4	-1,4	650	10	M	yes		cleft lip
	12	36+6	H19	audit 16	-1,1	0,6	1,5	462	9	F	yes		
	13	37+2	H12	6 ad	-1,7	-2	-2,1	385	9	F	yes		Estonian mother, Russian father
	14	39+2	H7+	audit 23	-1,1	-2,4	-2,6	408	9	F	yes		CS after trial of labor, induction of labor due to prolonged pregnancy, cannabis use before pregnancy, labetalol medication for hypertension, aciclovir for genital herpes suppressive therapy
	15	42+3	H6+	audit 8	0,7	0,3	-0,1	820	9	M	no	yes	cleft lip, PPROM
	16	35+4	H6	audit 37	-2,1	0,1	1,7	700	9	F	yes		SSRI till 5+4 gestational weeks
	17	41+0	H5+4	14-27 ad	-0,4	-0,6	-0,2	550	9	M	no		
	18	40+2	H5+3	audit 31/10 ad	-0,9	-0,4	-0,1	427	9	F	no		SSRI till 17 gestational weeks
	19	39+2	H5	audit 21	-0,3	-1,2	-2	495	9	F	yes		
pat A/mat G	20	37+6	H27	audit 10	0,1	-0,7	-0,2	490	9	F	yes		
	21	40+4	H23	8 ad	-1,4	-0,7	-1,3	620	10	F	yes		
	22	41+0	H21	audit 17	0,4	-0,5	-0,3	675	10	M	yes	yes	CS after trial of labor due to suspected chorioamnionitis
	23	41+0	H20		1,1	0,2	-1,1	735	4	F	yes		cleft lip
	24	39+0	H9	audit 31	-1,4	-1,3	-1,9	360	9	F	H9		
	25	42+0	H7		-0,2	0,3	0,5	605	9	F	N/A		
	26	40+6	H7	2xweek strong drunkenness	-0,7	-0,5	0,4	590	7	M	H7		
	27	39+4	H5-6	audit 9	0,1	1	1,4	700	10	F	no		
	28	39+5	H5	audit 14	0,7	1,1	0,2	695	9	M	H5		
	29	42+1	H4	audit 22	-0,2	-0,4	-1,3	560	10	M	H4		diazepam medication for insomnia
	30	39+5	H4	audit 17	-0,4	-0,2	0,2	510	10	F	yes		first trimester use of SSRI
A/A	31	32+5	H34+	5-10 ad	1,4	1,1	0,7	476	9	F	yes		
	32	39+4	H33	audit 10/4-6 ad	0,5	-1,4	-0,9	433	7	F	no	yes. e	GDM, labetalol medication for hypertension, two thumbs in right hand
	33	39+4	H29	20 ad	1,5	-0,4	-1,4	655	10	M	yes		
	34	40+3	H10+5	24-40 ad	-1,2	-1,5	-2,1	605	10	F	yes		
	35	39+2	H8	4-10 ad	0	0	0,4	470	9	M	H20		GDM, insulin medication
	36	41+4	H5+	audit 22	2,6	0,8	-0,4	804	9	M	yes		
	37	35+5	H5	3xweek strong drunkenness	1,9	1,2	0,7	944	8	F	yes	yes	CS due to PPROM and chorioamnionitis, SSRI and antipsychotic medication (quetiapine)
	38	39+6	H4+3	audit 30	1,5	0,8	-0,4	694	7	M	yes	yes. e	hepatitis C, oxazepam medication occasionally, SSRI, GDM, metformin medication started at 30 gestational weeks
	39	40+5	H47	12-24 ad	1,4	0,9	1	542	9	F	no		

GW is gestational weeks (+days), letter H in "duration of drinking" column represents pregnancy week when mother has stopped drinking according to her self-reported information, audit: alcohol use disorders identification test, ad: alcohol units per week (more information about audit scores and alcohol units can be found in the Materials and Methods section),
GDM: gestational diabetes mellitus, SSRI: selective serotonin reuptake inhibitors, a class of drugs that are used as antidepressants,
CS: Caesarean section and PPROM: preterm premature rupture of membranes.

Figure 9. Summary of the material and methods used in the human study.

3.2.1 Methylation analysis (study III)

3.2.1.1 MassARRAY EpiTYPER

In the examination of alcohol-induced alterations on the DNA methylation profile, DNA methylation levels of CTCF6 at *H19* ICR and *H19* DMR (**Figure 10**) were measured by MassARRAY EpiTYPER (SEQUENOM Inc.), as described in the "mouse model: *MassARRAY EpiTYPER (II)*". In addition, alterations in global methylation level were studied with LINE-1 retrotransposable element.

Genomic DNA was extracted from four pieces of placenta per sample by standard phenol-chloroform protocol and pooled. Sodium bisulfite conversion of placental DNA (1000 ng) was carried out using the EZ methylation kit (Zymo Research). Primers for the *H19* ICR (CTCF6), *H19* DMR and LINE-1 regions were obtained from previous publications (Ollikainen et al., 2010; L. Wang et al., 2010) and optimized. PCR was carried out in triplicate using the HotStar PCR kit (Qiagen). Each fragment for both control (n=42) and alcohol-exposed (n=33) samples was analysed simultaneously in the same plate. Technical replicates that showed >5% difference from the median value of the three replicates were discarded leaving a duplicate that was used in analysis.
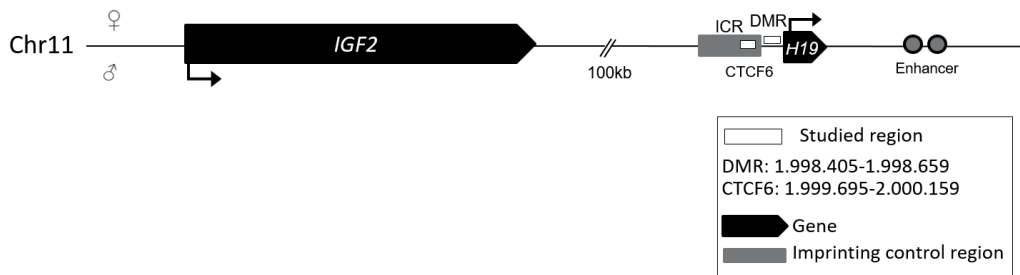
Figure 10. The figure illustrates the *Insulin-like growth factor 2/H19* imprinted locus which was studied by EpiTYPER. Exons of *Igf2* and *H19* are in black and the studied region of the imprinting control region (CTCF6) and *H19* DMR in white.

3.2.1.2 Bisulfite sequencing

In order to determine allele-specific methylation levels on *H19* ICR CTCF6, as well as to confirm the EpiTYPER result, 28 rs10732516 heterozygous samples (6 control and 8 alcohol-exposed placentas from both patG/matA and patA/matG genotypes) were bisulfite sequenced. Due to the heterozygosity and imprinting, it was straightforward to discern

paternal and maternal alleles. Two bisulfite conversions (EZ methylation kit, Zymo Research) and three independent PCR reactions (HotStar PCR kit, Qiagen) were performed for each sample. To exclude any primer-specific bias, a different pair of PCR primers was used in this traditional method compared to EpiTYPER (Coolen et al., 2011) and primer sequences were revised for potential polymorphisms. PCR fragments were gel-isolated (NucleoSpin Gel and PCR Clean-up kit, Macherey-Nagel) and the three PCR products were pooled. The products were subcloned into the pGEM®-T Vector system I (Promega), and cloning was performed with a standard protocol (as described in the "mouse model: bisulfite sequencing"). On average, 48 clones per individual samples were sequenced. The sequences were analysed by the BiQ Analyzer (Bock et al., 2005). Any clones with a lower than 90% conversion rate were excluded from the dataset.

3.2.2 Genotype analysis (study III)

All the samples were genotyped for the rs10732516 polymorphism. Genotype analysis was done by using either EpiTYPER or bisulfite sequencing primer pairs for *H19* ICR CTCF6. Depending on the used primer pairs and the fragmentation in EpiTYPER, the genotype was detected in unit CpG17,18,19,20 (Coolen et al., 2007) or in unit CpG10 (Ollikainen et al., 2010) as "genotypic" methylation levels: in patG/matA approximately 0.80, in G/G 0.30, in patA/matG 0.02, and in A/A there was no value.

3.2.3 Gene expression analysis (study III)

Quantitative PCR was used to analyse the alcohol-induced expression changes of *IGF2* and *H19* genes. Total RNA from four pieces per placenta was extracted by TRIzol® Reagent (Invitrogen). RNA quality was evaluated with Agilent 2100 Bioanalyzer (Agilent Technologies) by the Biomedicum Functional Genomics Unit (FuGU, Helsinki, Finland). To study the changes in expression, 2-4 pieces (on average 3) from each placenta was used individually. RNA was DNAse treated (RQ1 RNase-Free DNase, Promega) followed by cDNA synthesis (iScript™ cDNA Synthesis Kit, Bio-Rad Laboratories). The qPCR reaction conditions were as specified by SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories). *Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*YWHAZ)*, which showed unaltered expression after exposure to alcohol in the differentiation of trophectoderm stem cells *in vitro* (Carnahan et al., 2013) and an optimal expression level in placenta, was used as a housekeeping gene. Primers for *YWHAZ*, *IGF2* and *H19* were chosen from previous publications (Nelissen et al., 2013; Rancourt et al., 2012). cDNA prepared from these pieces was analysed individually in triplicates on the

qPCR plate. All the three genes (*IGF2*, *H19* and *YWHAZ)* were analysed on the same plate for a single sample. To normalize the individual samples (both control and alcohol) between separate qPCR plates, one identical control sample was used on each plate. First, the mean Ct value between the 2-4 individual pieces was calculated. Thereafter, the expression fold changes of *IGF2* and *H19* were obtained by using the Livak & Schmittgen method similarly as described in "mouse model: Expression analysis *(I,II)*". *H19:IGF2* ratio in an individual placental sample was calculated from *H19* and *IGF2* fold change values.

Details regarding all the primers and protocols of methylation and expression studies are found in the supplementary material in the original publication of Marjonen et al. 2017.

3.3 STATISTICAL ANALYSIS (studies I, II, III)

Microarray data was analyzed with the MEAP algorithm (Brazma et al., 2001; Dalma-Weiszhausz et al., 2006; P. Chen et al., 2011) and the further analysis in the freely available Anduril computational framework (Ovaska et al., 2010). In order to correct the nominal *p*-values, multiple testing correction was performed.

Statistical analyses were conducted using either SPSS (IBM SPSS Statistics 22 and 24) or GraphPad Prism 7. All data is expressed as the mean $\pm$ standard deviation for a normal distribution of variables or as the interquartile range for a skewed distribution of variables. Depending on the distribution of the data calculations were done by either parametric or nonparametric tests. Because of the small sample size in most analysis, Shapiro-Wilk test was used to test the normality of the data.

Student's t-test or Mann-Whitney U test was used to observe the statistically significant differences between the means of two independent groups and Wilcoxon test to measure means of two dependent groups. One-way ANOVA, followed by Bonferroni *post hoc* test when significant, was used to identify the differences among multiple independent groups. Two-way ANOVA was used to eliminate the possible interaction effect. The effect size was calculated by Cohen´s *d*. Correlations were calculated using Pearson or Spearman´s rank correlation. *Chi*-square test was used to indicate the differences between the groups according to the prevalence of the rs1072516 polymorphism. Two-tailed *p*-values were considered significant if *p*<0.05. Bonferroni correction was used as multiple testing correction for nominal *p*-values. Grubbs test was used to detect outliers of the data.

Finnish population-based charts (Sankilampi et al., 2013), in which the gestational age at birth, twinning, parity and gender have been considered, were used to calculate the standard deviation (z-score) in the anthropometric birth measures. Measures deviating

more than ±2 standard deviations are commonly considered as abnormal. The Finnish growth chart is a birth register data based on 753,036 infants born in 1996-2008 in Finland that refers to optimal intrauterine growth in the Finnish population. Thus, because of the significant differences in birth size between populations, originating mainly from differences in genetic factors, two newborns of African origin were excluded from these calculations.

4 RESULTS

4.1 MOUSE MODEL (studies I, II)

4.1.1 <u>Early alcohol exposure alters hippocampal gene expression (study I)</u>

We started our study by characterizing potential alcohol-induced gene expression changes in hippocampi by performing a genome-wide expression array in five alcohol-exposed and five control four-week-old (P28) male mice. Since no significant gene expression differences between alcohol-exposed and control mice were observed after correction for multiple testing, genes with nominal p-value less than 0.05 and fold-change (log2-space) more than 1.5 were considered to have altered expression. Thus, gene expressions of 23 genes and three microRNAs were potentially altered in the hippocampi of alcohol-exposed offspring. **Table 5** presents the most interesting findings noted with the array.

Table 5. Altered gene expression of 15 genes and two microRNAs in alcohol-exposed hippocampus. The first column describes the altered direction of genes, the second column lists the nominal p-values < 0.05 and the third column has the fold change ≥ 1.5.

Upregulated genes	p-value	fold change
Cxcr1	0.012	1.6
Defb14	0.017	1.7
Krtap6-1	0.038	2.1
Tas2r124	0.006	1.6
Mir290	0.01	1.9
Mir138-2	0.023	1.5
Olfr937	0.048	1.6
Olfr601	0.005	1.7
Olfr553	0.01	1.7
Olfr1305	0.0003	1.7
Vpreb2	0.013	1.6
H2-M10.3	0.03	1.5
Downregulated genes	**p-value**	**fold change**
Hist1h2ai	0.023	0.6
Vmn2r64	0.032	0.4
Olfr51	0.012	0.6
Olfr967	0.018	0.6
Olfr110	0.034	0.6

To verify the genome-wide array result we used quantitative PCR. However, the expression levels of the genes of interest in hippocampus were too low to be detected by qPCR. For this reason, we chose three genes from our array gene list that were highly expressed in other tissues. We investigated if similar upregulated expression of *Olfactory receptor 601 (Olfr601)* and *Histocompatibility 2, M region locus 10.3* (*H2-M10.3*) could be seen in both

the hippocampus and main olfactory epithelium, and pre-B lymphocyte gene 2 (*Vpreb2*) in hippocampus and bone marrow. The expression array result was supported by our qPCR study. We observed significantly increased expression in two of the three genes in the tested tissues of alcohol-exposed offspring, *Olfr601* in main olfactory epithelium and *Vpreb2* in bone marrow (p = 0.04 and p < 0.001, respectively, one-tailed Student's t-test). No significant difference was observed in *H2-M10.3* expression. (**Figure 11**).
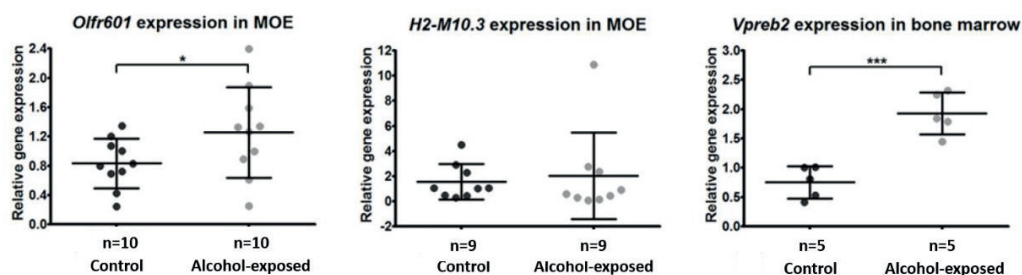
Figure 11. Gene expression of *Olfr601* and *H2-M10.3* in main olfactory epithelium (MOE) and *Vpreb2* in bone marrow. Figure shows relative expression values measured by qPCR. Each dot is an individual four-week-old (P28) male mouse. Control mice are in black and alcohol-exposed in grey. Bars are mean ± SD and n represents the number of samples. One-tailed Student's t-test: *p<0.05 and ***p<0.001.

4.1.2 Altered expression was not caused by DNA methylation changes (study I)

To clarify whether the altered gene expression was caused by alcohol-induced epigenetic gene regulation, we explored the hippocampal DNA methylation profile of the CpG island located in gene body in both *Olfr601* and *Vpreb2* genes. Since *Olfr601* was upregulated in hippocampus and in main olfactory epithelium in alcohol-exposed offspring, we examined the methylation profile in both tissues. Since no changes in DNA methylation were detected at *Olfr601* or *Vpreb2*, this suggests that the gene expression change is not caused by methylation.

We also evaluated if the methylation profile in other candidate genes, which had a CpG island either in the promoter region or in the gene body, had been altered. *Olfactory receptor 110* has a CpG island upstream of the transcription region, *Vomeronasal type 2 receptor 64* (*Vmn2r64*) has two CpG islands (upstream and gene body) and *Histone cluster 1, H2ai* (*Hist1h2ai*) has a long CpG island covering the whole gene-body and promoter region, from which we investigated only a part (**Figure 7**).

We observed that the CpG islands of all the genes except *Hist1h2ai* were highly methylated in both experimental groups. However, there were no significant differences in the total methylation level between alcohol-exposed and control offspring. Since there is earlier evidence of site-specific DNA methylation associating with decreased transcription (Martinowich et al., 2003; B. Jones & Chen, 2006), we also calculated the CpG site-specific DNA methylation in each CpG island. However, we did not observe any significant site-specific alterations in the *Vmn2r64* and *Olfr110* CpG islands. (Data shown in Marjonen et al., 2015).

4.1.3 Alcohol exposure caused permanent brain structure abnormalities (study I)

Previously, this mouse model has demonstrated alcohol-induced effect on the skull (Kaminen-Ahola et al., 2010a), but the impact on different brain structures has remained unclear. We performed a magnetic resonance imaging (MRI) for adult male offspring to determine the alcohol-induced alterations in the volumes of different brain structures.

The alcohol-exposed offspring showed a reduced body weight compared to controls (Cohen´s d = -1.42). In addition, a small decrease in total brain volume was detected (Cohen´s d = -0.29). We measured the effect size of alcohol on the hippocampus, olfactory bulb, ventricles and cerebellum by normalizing them to the total brain volume. The volume of the hippocampi (Cohen´s d = 0.84) as well as lateral ventricular volume (Cohen´s d = 0.65) were increased and olfactory bulbs (Cohen´s d = -0.85) decreased in alcohol-exposed offspring. Most interestingly, these regions showed asymmetric changes between hemispheres: the left hemisphere exhibited larger changes, such as an increase in volume of the left hippocampus (Cohen´s d = 1.32) and left ventricle (Cohen´s d = 0.83) and a decrease in left olfactory bulb (Cohen´s d = -1.40). No differences were seen in the volume of cerebellum (Cohen´s d = -0.02).

The most interesting finding of the MRI was the trend towards asymmetry of the brain structures (**Figure 12**). The left hippocampal volume was significantly larger in alcohol-exposed mice compared to control mice (p < 0.05, Mann-Whitney U test) as well as when compared to the right hippocampus within the mice (p < 0.001, Wilcoxon test) (**Figure 12A**). In contrast, the left olfactory bulb volume was found to be significantly smaller in the ethanol-exposed as compared control mice (p < 0.05, Mann-Whitney U test) (**Figure 12B**). Although, the ventricular volumes of both hemispheres appeared slightly larger in ethanol-exposed mice, no significant differences were found (**Figure 12C**).
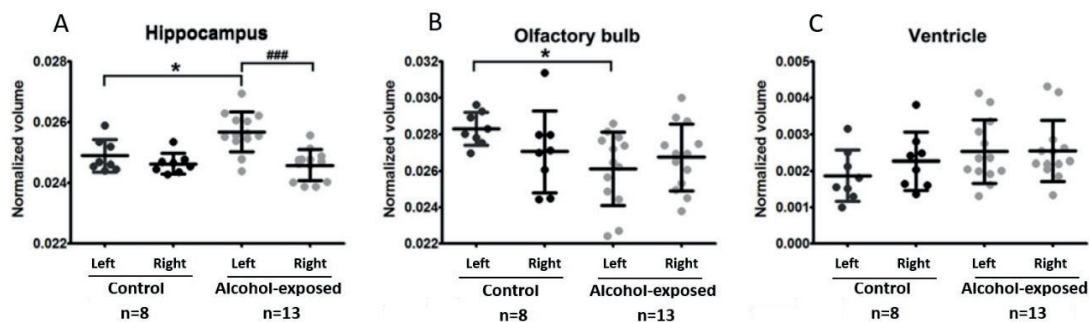
Figure 12. Result of MRI. Volumes of left and right hippocampus (A), olfactory bulbs (B), and lateral ventricles (C) in control and alcohol-exposed adult male mice (P60). Volumes of the different structures are normalized to the total brain volume. Dots represent the normalized value of the volume from each individual. Control mice are in black and alcohol-exposed in grey. Bars are mean ± SD and n represents the number of samples. Significance between left and right side of the brain is calculated by Wilcoxon test (###$p<0.001$) and significance between alcohol-exposed and control mice by Mann-Whitney U test (*$p<0.05$).

4.1.4 Alcohol exposure altered imprinted gene expression in embryo and placenta (study II)

Since one of the main consequences of prenatal alcohol exposure is growth restriction, we determined if this kind of moderate alcohol exposure in our mouse model would alter growth-related imprinted genes. We used quantitative RT-PCR to assess the expression changes in *Igf2, H19*, *Snrpn* and *Peg3* imprinted genes in alcohol-exposed mouse whole embryos (E9.5) and placentas (E9.5, E16.5). Because two different RNA extraction methods were used in the study (see materials and methods), we eliminated the possible interaction effect of the extraction kit by using a two-way ANOVA. Furthermore, we explored DNA methylation changes at the differentially methylated or imprinting control regions by MassARRAY EpiTYPER.

4.1.4.1 Insulin-like growth factor 2/H19 locus

The gene expression study showed a significant upregulation of *H19* in the alcohol-exposed embryos (E9.5). However, since there was an interaction effect with the extraction kit (p = 0.004, two-way ANOVA), we divided the samples into two groups according to the kits. Thereafter, a significant difference between alcohol-exposed and control samples was detected only in the group of extraction kit_1. *H19* expression was 18-fold higher in

alcohol-exposed samples compared to controls (p < 0.001, two-tailed Student´s t-test) (**Figure 13**). No expression change of *Igf2* was observed in E9.5 embryos.

In addition to embryos, we explored alcohol-induced expression changes in the E9.5 placental tissue. However, no expression change of either *Igf2* or *H19* was observed (**Figure 13**). Furthermore, we also explored the gene expression in E16.5 placentas. Interestingly, *Igf2* showed 0.85-fold and *H19* 0.61-fold decrease in E16.5 alcohol-exposed placentas (p = 0.04, p = 0.007, respectively, two-way ANOVA) (**Figure 13**).
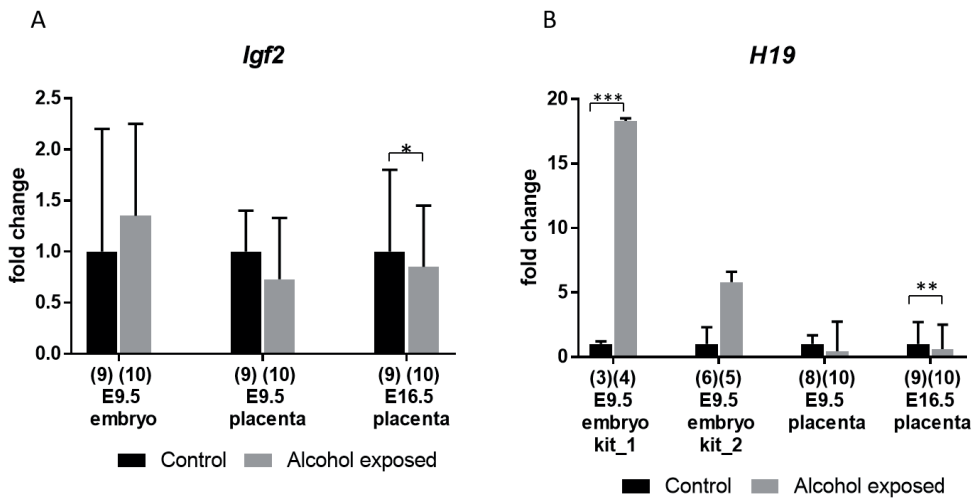
Figure 13. Gene expression of *Igf2* (**A**) and *H19* (**B**) in control and alcohol-exposed E9.5 embryos and E9.5/E16.5 placentas by qPCR. Figure presents the fold change values. Error bars denote the mean with SD. The number of samples is presented in brackets. Control mice are in black and alcohol-exposed in grey. The difference between control and alcohol-exposed samples was evaluated by two-way ANOVA (*p<0.05, **p<0.01, ***p≤0.001).

*4.1.4.2 Small nuclear ribonucleoprotein polypeptide N*

We observed a significant increase of *Snrpn* expression in alcohol-exposed E9.5 embryos. Since there was an interaction effect with the extraction kit (p = 0.01, two-way ANOVA), we divided the samples into two groups according to the kits. A significant 6.2-fold difference between alcohol-exposed and control samples was detected only in the group of extraction kit_1 (p < 0.002, two-tailed Student´s t-test). No changes were observed in either E9.5 or E16.5 placentas (**Figure 14**).
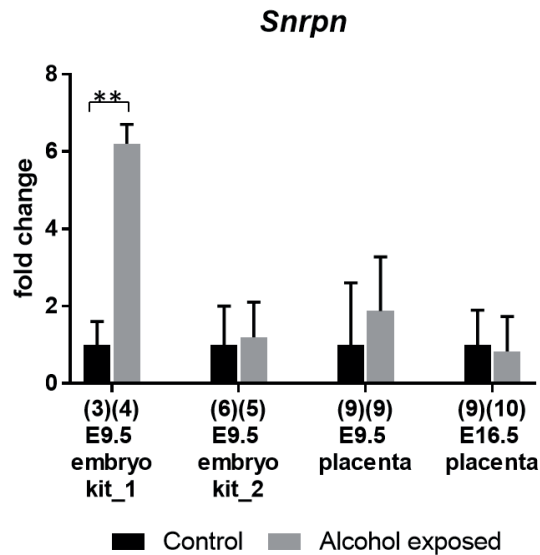
Figure 14. Gene expression of *Snrpn* in control and alcohol-exposed E9.5 embryos and E9.5/E16.5 placentas by qPCR. The figure presents the fold change values. Error bars denote the mean with SD. Number of samples is presented in brackets. Control mice are in black and alcohol-exposed in grey. Difference between control and alcohol-exposed samples by two-way ANOVA (**p<0.01).

4.1.4.3 Paternally expressed gene 3

We did not observe any *Peg3* gene expression changes (**Figure 15**) between alcohol-exposed and control E9.5 embryos or E9.5/E16.5 placentas.
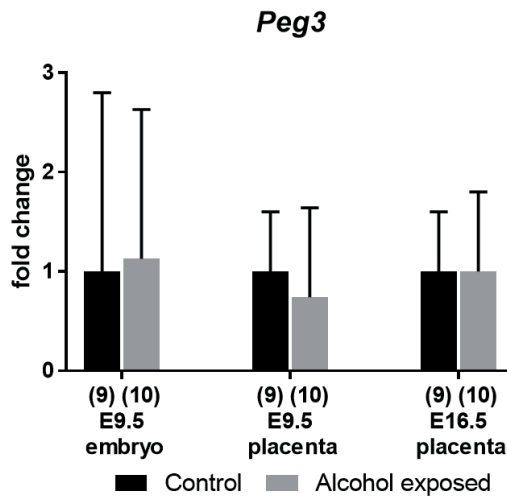
Figure 15. Gene expression of *Peg3* in control and alcohol-exposed E9.5 embryos and E9.5/E16.5 placentas by qPCR. The figure presents the fold change values. Error bars denote the mean with SD. Number of samples is presented in brackets. Control mice are in black and alcohol-exposed in grey.

4.1.5 No DNA methylation changes were observed (study II)

To explore whether the expression changes associate with changed DNA methylation level, we investigated the differentially methylated region (DMR1) at *Igf2* and the imprinting control region at *Igf2/H19* locus, *Snrpn* and *Peg3*. However, no methylation changes were observed in alcohol-exposed E9.5 embryos or placentas. Furthermore, in order to detect alcohol-induced alterations in the global methylation level, we examined DNA methylation in the long interspersed nuclear elements (Line-1) only in E9.5 placentas. However, no significant methylation changes were observed in the alcohol-exposed samples.

4.1.6 No sex-specific effects were observed (study II)

We also explored sex-specific effects since previous studies have shown that environmental factors can affect DNA methylation as well as gene expression in a sex-specific manner (White et al., 2018; Masemola et al., 2015). Due to the small sample size (five controls and five alcohol-exposed) in DNA methylation analysis, we explored sex-specific effects only on gene expression in alcohol-exposed samples. However, no differences between males and females were observed. (Data shown in Marjonen et al., 2018, supplementary material Table S5.)

4.2 HUMAN STUDY (study III)

4.2.1 Differences between the study groups

To observe differences between the control mothers and the mothers who consumed alcohol during pregnancy, we gathered the following basic information: maternal age at birth, parity and BMI. The age and parity of the mothers who consumed alcohol differed significantly from the control mothers ($p = 0.047$; $p = 0.039$, Mann-Whitney U test, respectively, Table I in the original publication of Marjonen et al., 2017).

The weight (g), length (cm) and head circumference (HC) (cm) of the alcohol-exposed newborns differed significantly from the control newborns. However, when compared to the national growth charts, in which the gestational age at birth, twinning, parity and gender have been taken into account (see material and methods) (Sankilampi et al., 2013), no significant differences in SDs of weight, length or HC between control and alcohol-exposed Caucasian newborns were observed (Table I in original publication Marjonen et al., 2017). This underlines the significance of normalizing the anthropometric measures to population charts to avoid false conclusions.
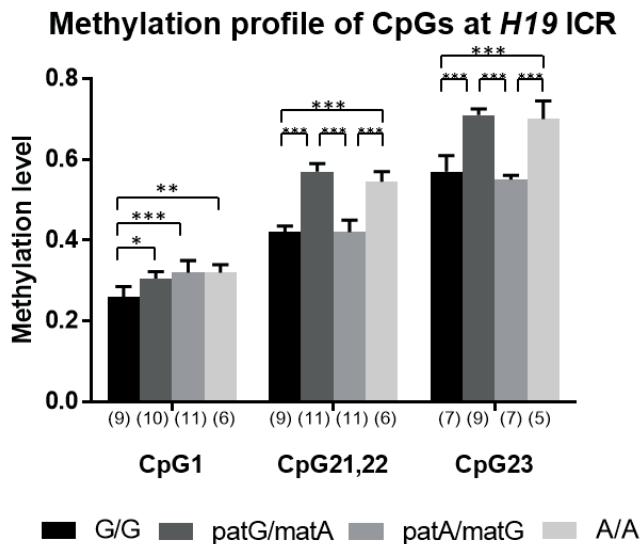
4.2.2 Genotype-specific DNA methylation profile at *IGF2/H19* imprinted locus

We used MassARRAY EpiTYPER to detect alcohol-induced changes in the DNA methylation level of CTCF6 at *H19* imprinting control region (ICR) and *H19* differentially methylated promoter region (DMR) in placenta.

When comparing all control samples to alcohol-exposed samples, no differences in the methylation levels of CTCF6 at *H19* ICR or *H19* DMR between these two groups were observed. However, we detected a known single nucleotide polymorphism, rs10732516 G/A in the binding sequence of CTCF6 at *H19* ICR. The allele frequencies of this polymorphism are nearly equal in the Finnish population (G = 0.47, A = 0.53) (Auton et al., 2015) and there were no differences in the prevalence of rs10732516 genotypes between control and alcohol-exposed groups ($X^2(3) = 0.96$, $p = 0.81$, *Chi*-square test).

Interestingly, when dividing all the samples into four genotype groups (G/G, paternal G/maternal A (patG/matA), paternal A/maternal G (patA/matG) and A/A) according to the polymorphism we observed significantly different genotype-specific DNA methylation levels at CpG units CpG1, CpG21,22 and CpG23 between control samples ($F_{3,32} = 7.1$, $p = 0.001$; $F_{3,33} = 69.8$, $p < 0.0001$; $F_{3,24} = 24.7$, $p < 0.0001$, respectively, one-way ANOVA) (**Figure 16**). A similar result has been observed by Coolen et al. (2011) with whole blood cells.


Figure 16. EpiTYPER result: genotype-specific DNA methylation levels in CpG1, CpG21,22 and CpG23 units at *H19* CTCF6 in control samples. Error bars denote the median with interquartile range. Number of samples is presented in brackets in the columns. Bonferroni *post hoc* test for one-way ANOVA: *P<0.05, **P<0.01, ***P≤0.001.

Furthermore, we also detected genotype-specific methylation levels at *H19* DMR in the alcohol-exposed samples: the methylation level of A/A was lower compared to patG/matA and patA/matG at unit CpG6 (p = 0.006, one-way ANOVA) (figure shown in Marjonen et al., 2017). However, despite our genotype-specific approach, we did not observe any changes in the methylation level between alcohol-exposed and control samples at *H19* ICR or *H19* DMR.

To determine alcohol-induced alterations in global methylation level in placenta, we examined the methylation level of long interspersed nuclear elements (LINE-1) in the alcohol-exposed and control samples. However, no significant changes in the methylation levels were observed.

In order to confirm the genotype-specific results of CTCF6 at *H19* ICR, we bisulfite sequenced heterozygous control and alcohol-exposed samples (patG/matA and patA/matG), since it was easy to determine the maternal and paternal allele according to the polymorphism. The average methylation percentages were calculated separately for the paternal and maternal alleles, and then the total methylation levels for each CpG site (CpG1-CpG27) by weighing both of the alleles equally due to the different amount of clones.

A similar trend of DNA methylation levels was observed in both genotypes as was seen by EpiTYPER: patG/matA genotype was more methylated throughout the studied *H19* CTCF6 region compared to patA/matG genotype in both control (nominal p-values CpG25: p = 0.009 and CpG26: p = 0.002, Mann–Whitney) and alcohol-exposed (nominal p-values CpG1: p = 0.015, CpG25: p = 0.002 and CpG26: p = 0.001, Mann–Whitney) samples. Although this genotype-specific difference was not significant after Bonferroni multiple testing correction (control: CpG25: p = 0.47 and CpG26: p = 0.10; alcohol: CpG1: p = 0.78, CpG25: p = 0.10, CpG26: p = 0.05). **Figure 17** shows the difference in the methylation level between the two genotypes in alcohol-exposed samples.

Genotype-specific methylation level comparison in alcohol-exposed placentas

* rs10732516

■ patG/matA (n=8)   ▢ patA/matG (n=8)
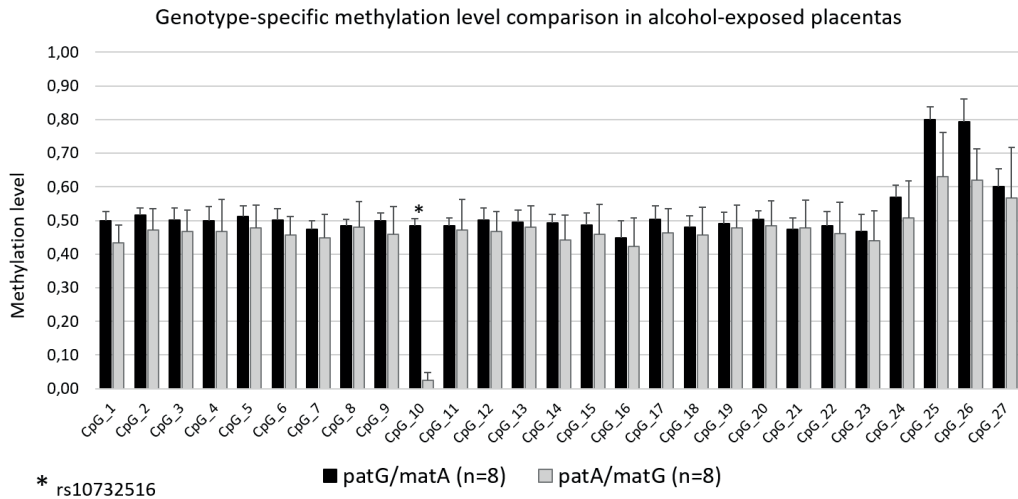
Figure 17. DNA methylation level of 27 CpG sites at *H19* CTCF6 by bisulfite sequencing. Figure illustrates the genotype-specific different methylation pattern between patG/matA and patA/matG genotypes in alcohol-exposed placentas. The patG/matA genotype (black) is more methylated compared to the patA/matG genotype (gray) throughout the studied *H19* CTCF6 region. Error bars denote median with SD. The number of samples is presented in brackets.
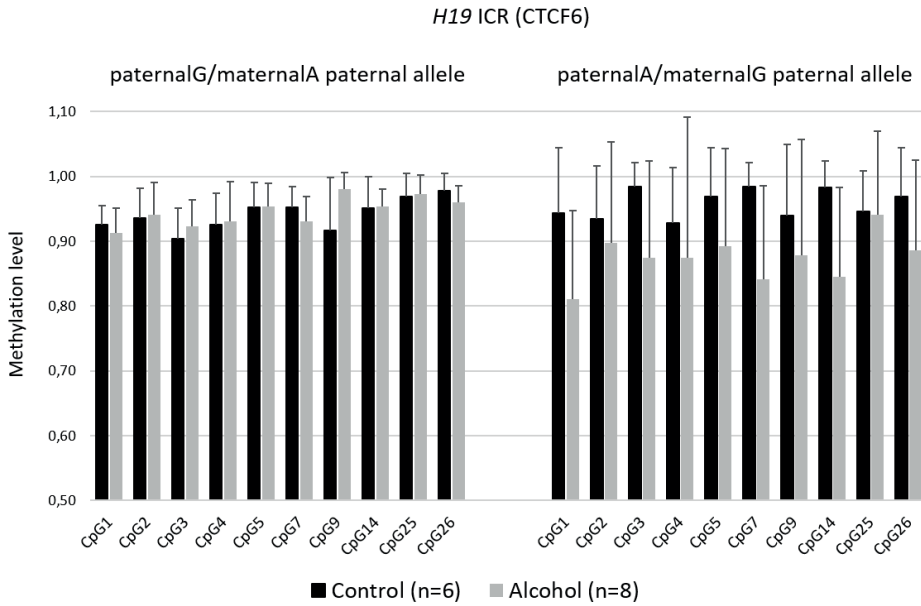
When alcohol-exposed samples were compared to controls within genotypes, we observed a trend of hypomethylation in the patA/matG genotype in the alcohol-exposed samples. However, a similar systematic trend of alcohol-associated hypomethylation was not detected in the patG/matA genotype, indicating potential genotype-specific effects of alcohol exposure (**Figure 18**).
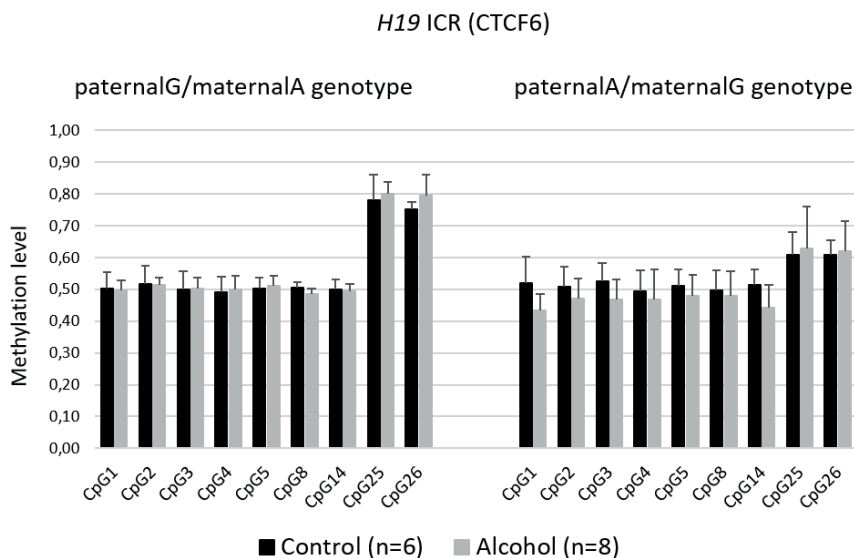
Figure 18. DNA methylation level of 9 CpG sites at *H19* CTCF6 by bisulfite sequencing. Alcohol-exposed samples compared to controls within the two genotypes. A trend of hypomethylation can be seen in the patA/matG (right) but not in the patG/matA genotype (left) in alcohol-exposed samples. Error bars denote median with SD. The number of samples is presented in brackets.

4.2.3 Altered allele-specific DNA methylation profile at *H19* imprinting control region (CTCF6)

We next assessed potential allele-specific effects. By comparing the methylation levels of paternal and maternal alleles separately in control and alcohol-exposed samples, we observed a consistent trend of alcohol-associated decreased methylation pattern in the paternal allele of patA/matG genotype (nominal p-values CpG7: p = 0.02 and CpG14: p = 0.007, Mann–Whitney; after multiple testing correction p = 2.08, p = 0.73, respectively). (**Figure 19**). This result possibly explains the hypomethylation seen in the alcohol-exposed samples in the total genotype comparison (**Figure 18**). Instead of hypomethylation, we observed a trend towards hypermethylation in the paternal allele of patG/matA genotype in alcohol-exposed samples (nominal p-value CpG9: p = 0.02, Mann–Whitney; after multiple testing correction p = 2.08) (**Figure 19**). However, neither of the allele-specific differences were significant after Bonferroni multiple testing correction. In contrast, we did not observe any alterations in the maternal allele in either of the genotypes.

Figure 19. Allele comparison between alcohol-exposed and control samples within the two genotypes. A trend towards hypomethylation can be seen in the paternal allele of the patA/matG genotype in alcohol-exposed samples (right), compared to the paternal allele in the patG/matA genotype (left). Error bars denote median with SD. The number of samples is presented in brackets.

4.2.4 <u>Alcohol-induced effects on the *IGF2* and *H19* gene expression</u>

Since there was a trend of genotype-specific changes in the methylation level at the *H19* imprinting control region, which regulates the function of the locus, we examined whether the changes would affect the gene expression in the placenta. We first explored the gene expression in all samples. However, the expressions of growth promotor gene *IGF2* and negative growth controller gene *H19* were highly variable. Thus, no changes in *IGF2* and *H19* expressions were detected (**Figure 20**).
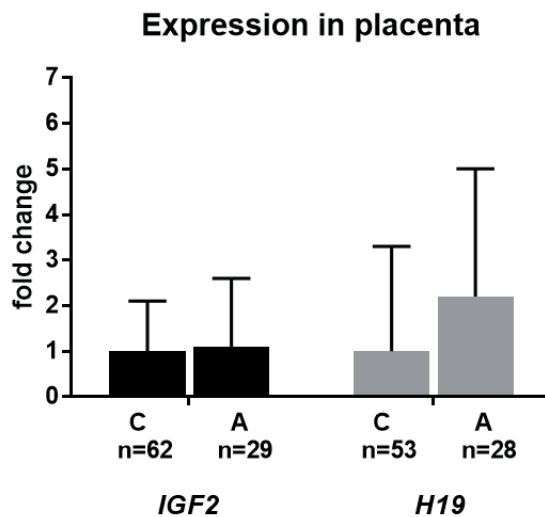

Figure 20. Gene expression levels of *IGF2* (black) and *H19* (grey) in all control (C) and alcohol-exposed (A) samples. The figure presents the fold change values. Error bars denote the mean with SD. The number of samples is presented below the columns.

We also explored genotype-specific gene expression changes. Significantly decreased *IGF2* expression was detected only in the patA/matG genotype of alcohol-exposed samples (0.45-fold decrease, p = 0.03, Mann-Whitney U test) (**Figure 21**).

Genotype-specific expression in placenta
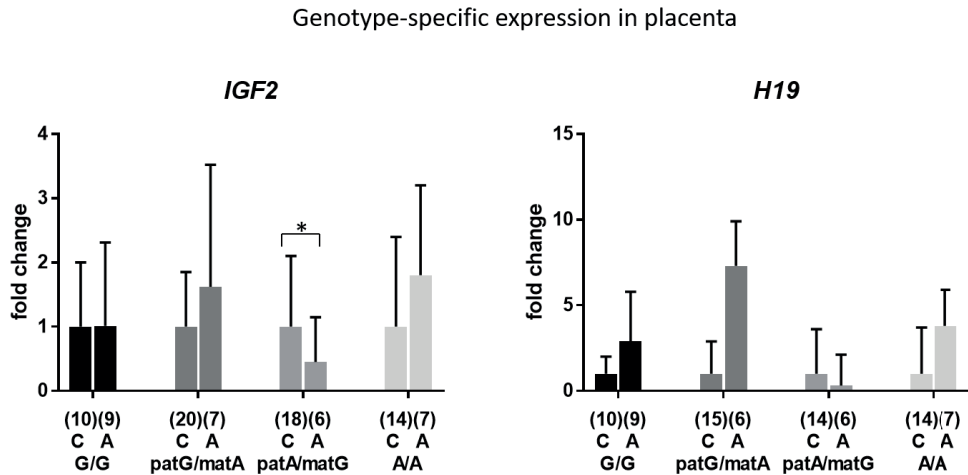
Figure 21. Genotype-specific gene expression levels of *IGF2* and *H19* in control (C) and alcohol-exposed (A) samples. Figure presents the fold change values. Error bars denote the mean with SD. The number of samples is presented in brackets below the columns. Mann-Whitney U test, *p<0.05.

Earlier studies have revealed that the expression level of these two genes in the locus are strongly linked. Hypermethylation at *H19* imprinting control region has been shown to increase expression of *IGF2* and decrease expression of *H19* (Soejima & Higashimoto, 2013), whereas hypomethylation at the *H19* imprinting control region leads to decreased *IGF2* and biallelic expression of *H19* (Gicquel et al., 2005). Therefore, we compared the expression of *H19* in relation to *IGF2* expression in each placenta. We observed a significantly increased *H19* expression in relation to *IGF2* in all alcohol-exposed samples (p = 0.006, Mann-Whitney U test), indicating alcohol-induced changes in the regulation of these two genes (**Figure 22A**). However, the genotype-specific observation revealed that the increased *H19:IGF2* ratio could be only seen in the genotypes with paternal rs10732516 G allele (G/G: p = 0.03 and patG/matA: p = 0.02, Mann-Whitney U test) (**Figure 22B**).
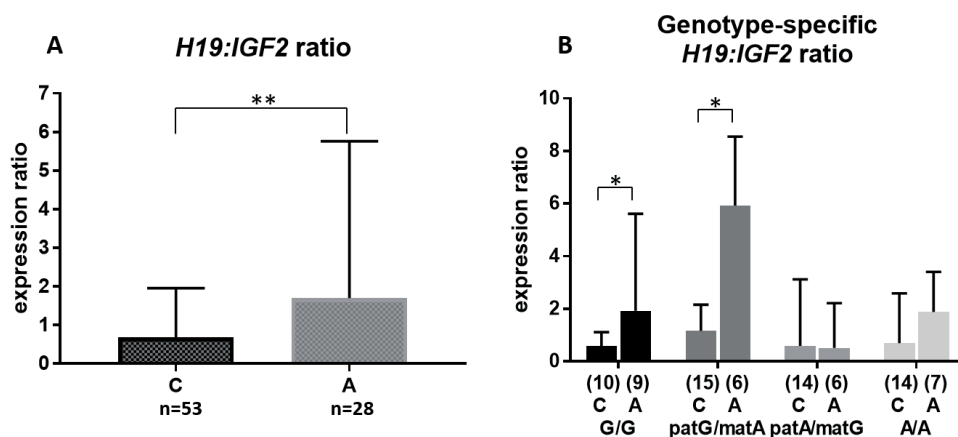
Figure 22. Expression ratio of *H19* and *IGF2*. (**A**) Ratio in all control (C) and alcohol-exposed (A) samples. Error bars denote the median with the interquartile range. The number of samples is presented below the columns. (**B**) Genotype-specific ratio in control (C) and alcohol-exposed (A) samples. Error bars denote the median with the interquartile range. The number of samples is presented in brackets below the columns. Mann-Whitney U test, *p<0.05, **p<0.01.

4.2.5 Genotype-specific phenotype of alcohol-exposed newborns

Because of the interesting findings in the DNA methylation and gene expression analysis of the *IGF2/H19* locus, we also explored changes in the phenotypes of alcohol-exposed newborns by using new Finnish population-based growth charts (as in SDs) (Sankilampi et al., 2013). As mentioned previously, when first comparing all the alcohol-exposed newborns to controls, no differences in the phenotype were observed (for example in the head circumference **Figure 23A**). Surprisingly however, genotype-specific comparison revealed a significant association between the rs10732516 polymorphism and the head circumference of alcohol-exposed newborns ($F_{3,33}$ = 7.5, p = 0.001, one-way ANOVA) (**Figure 23B**). Newborns with A/A polymorphism clearly differed from the other heterozygous G/A and homozygous G/G newborns. Moreover, the head circumferences of alcohol-exposed newborns were significantly smaller in the patG/matA genotype compared to controls (p = 0.004, Mann-Whitney U test) and the largest in the A/A genotype. Even though the weight SD showed a similar pattern, the changes were not statistically significant, nor were the changes observed in length or placental weight.
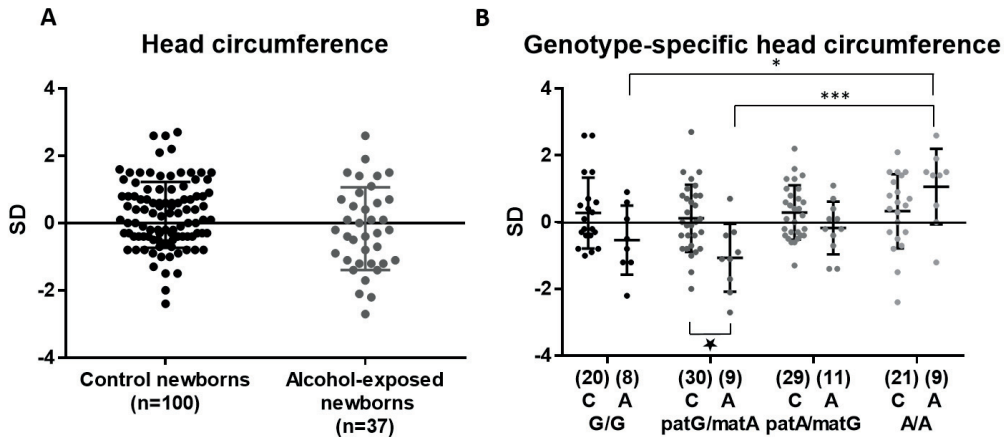
Figure 23. Phenotype comparison between alcohol-exposed and control newborns. (**A**) Head circumferences of all control newborns compared to all alcohol-exposed newborns. Error bars denote the mean with SD. The number of samples is presented in brackets. (**B**) Genotype-specific variation of head circumferences in control (C) and alcohol-exposed (A) newborns. Error bars denote the mean with SD. The number of samples is presented in brackets below the columns. Bonferroni *post hoc* for one-way ANOVA, *p<0.05, ***p≤0.001. Mann-Whitney U test, ⋆p<0.01.

4.2.6 <u>Other observations of the newborns</u>

There were 52 males and 48 females in the control group and 15 males and 24 females in the alcohol-exposed group. We did not observe any sex-specific effects on DNA-methylation, gene expression or phenotype, when all alcohol-exposed newborns were compared to controls, or in the genotype-specific manner.

We explored correlations between SD head circumference, SD weight, SD length and placental weight with Pearson correlation test. Head circumference of alcohol-exposed newborns (n = 37) correlated with weight (r = 0.538, p = 0.001) and placental weight (r = 0.422, p = 0.009), but not with birth length (r = 0.228, p = 0.176). Head circumference of controls (n = 100) correlated with weight (r = 0.501, p < 0.001), length (r = 0.292, p = 0.003) and placental weight (r = 0.247, p = 0.013).

There was a negative correlation between the Alcohol Use Disorders Identification Test (AUDIT) scores (Babor et al., 2011) and head circumference of alcohol-exposed newborns (r = -0.487, p = 0.018, n = 23, Spearman's rank correlation), indicating that the reported AUDIT scores reliably represent the degree of alcohol consumption. This was detected in

the three other genotypes except A/A, in which the majority of AUDIT data was missing. There were no correlations detected between head circumference and the amount of reported alcohol units per week (ads) (r = 0.129, p = 0.659, n = 14) or duration of alcohol exposure (r = -0.041, p = 0.812, n = 36). Furthermore, there was no correlation between the head circumference of alcohol-exposed newborns and methylation of any CpG sites (CTCF6 at *H19* ICR or *H19* DMR) or gene expression (*IGF2* or *H19*). Due to the small number of samples, the analyses were not performed in genotype-specific manner.

We were not able to explore comorbidities and other lifestyle factors that could affect the newborns. Gestational smoking is known to have an impact on the fetus, which can be seen as a smaller birth weight and head circumference (Lindley et al., 2000; Kallen, 2000). We did not observe any correlation between head circumference and smoking (r = 0.109, p = 0.611 n = 24). In addition, genome-wide studies have shown that smoking affects the methylation levels in placenta, but changes at the *IGF2/H19* locus have not been reported (Suter et al., 2011; Maccani et al., 2013). We did not observe any association between smoking and DNA methylation in this study.

5 DISCUSSION

5.1 EFFECTS OF PRENATAL ALCOHOL EXPOSURE ON HIPPOCAMPUS IN A MOUSE MODEL (study I)

Our results highlight the critical period at the beginning of pregnancy. In humans, the central nervous system starts to develop during the third week after conception, often prior to pregnancy awareness. This same time-frame is covered by our mouse model. Our results emphasize that even moderate alcohol consumption can cause significant changes in the developing brain and suggest that the effects not only occur in the mature neurons but possibly even in their stem cells.

We started by investigating the alcohol-induced genome-wide gene expression changes in four-week-old male mice hippocampus. However, we did not detect any significant gene expression differences between the alcohol-exposed and control mice. The multiple correction testing controls the probability of making false positive calls, but it can also be too strict if one has a relatively small sample size. Thus, we continued with the candidate genes that showed nominal p-values < 0.05. But, it is possible that the inaccurate results could be one reason for the lack of correlation between the array and qPCR.

Interestingly, we observed altered expression of genes which belonged into gene families that have been associated with prenatal alcohol exposure in a previous mouse study. The transcript levels of *Cxcr3*, *Defb15*, *Defb30*, *Krtap4-7*, *Tas2r126*, and *Vmn2r54* have been reported to increase in the whole brain of C57BL/6J adult (P70) male mice that were exposed to maternal 10% ethanol consumption before fertilization, throughout gestation, and ten days after birth (P10) (Kleiber et al., 2012). Despite the different timings of ethanol exposure, the altered expression of our candidate genes *Cxcr1*, *Defb14*, *Krtap6-1*, *Tas2r124*, *Hist1h2ai* was consistent. Only *Vmn2r64*, was expressed in the opposite direction (downregulation), when compared to its family member *Vmn2r54* (upregulation) in the earlier study (Kleiber et al., 2012).

Given the potential for alcohol-induced changes in epigenetic marks such as histone modifications and miRNAs (Chater-Diehl et al., 2016; Y. Guo et al., 2012), it was interesting to detect differential expression of histone cluster 1 H2ai (*Hist1h2ai*) and two microRNAs: miR290 and miR138-2. *Hist1h2ai* is a linker histone that interacts with linker DNA between the nucleosomes, affecting the compaction of chromatin structure. In addition, in a previous study, it was reported that *Hist1h3a*, *Hist1h4i*, and *Hist3h2a* were all downregulated in embryos cultured and treated with alcohol for 46 hours at early neurulation (E8.25) (Zhou et al., 2011). *Hist1h2ai* was also downregulated on our array. Moreover, miR290 has been shown to associate with gene regulation in the early embryo

and the maintenance of the pluripotent cell state (Houbaviy et al., 2003; Tata et al., 2011). Furthermore, miR138, the mature form of miR138-2, is associated with the size of dendritic spines in rat hippocampal neurons (Siegel et al., 2009). Both microRNAs were upregulated which could be an indication of abnormal neuronal function.

Our working hypothesis was that environmentally-induced alterations would take place in the very early stages of embryogenesis and then spread out by cell divisions affecting a large proportion of cells in the embryo; this was supported by the results emerging from our qPCR expression study. Thus, in addition to hippocampus, we explored if similar expression changes could be observed in other tissues as well. We decided to compare hippocampus to tissues which also contain actively proliferating stem cells: main olfactory epithelium (peripheral nervous system) and hematopoietic stem cells in bone marrow. Surprisingly, *Olfactory receptor 601* was upregulated in both the hippocampus and main olfactory epithelium and *Vpreb2* was upregulated in the hippocampus and in bone marrow. Hence, regardless of the different cell types and epigenetic profiles of hippocampus, main olfactory epithelium and bone marrow, we could detect similar gene expression changes in all three tissues. This result indicates that the alterations could have already taken place during the stem cell stage in the beginning of early development.

The most remarkable finding of this study was the structural alterations that we saw in hippocampi, olfactory bulbs and ventricles. Interestingly, these brain structures have also been the most severely affected in earlier studies (Akers et al., 2011; Parnell et al., 2009). Our finding of altered hippocampal volume is supported by a human study. Adolescent males with a family history of alcoholism had a larger left hippocampus as compared to adolescent males without any family history of alcoholism (Hanson et al., 2010). Enlarged hippocampi have also been observed in autistic children (Sparks et al., 2002), and it has been suggested to be related to the disturbed memory function in the disorder (Schumann et al., 2004). Autism characteristics have also been related to FASD phenotype (Stevens et al., 2013). Furthermore, a rat model of autism showed that increased hippocampal cell density led to improved performance in the Morris water-maze learning paradigm (Edalatmanesh et al., 2013). Alcohol exposure in our mouse model is also associated with improved spatial memory observed by the Morris water-maze test (Sanchez Vega et al., 2013). Clarifying whether it is related to a larger left hippocampus observed in this study will require further research.

Reduced volume of olfactory bulb in alcohol-exposed offspring (P60) as well as impaired odor discrimination, have been observed in a mouse model, with the same 10% ethanol-exposure. However, this exposure was initiated before fertilization and gradually

decreased after birth, thus continuing throughout pregnancy (Akers et al., 2011). We observed smaller olfactory bulbs in our alcohol-exposed offspring, indicating that even early alcohol exposure can reduce olfactory bulb volume. In another study, a high intraperitoneal ethanol dose delivered twice at GD8 (an early neurulation stage) led to the development of smaller olfactory bulbs in GD17 embryos (Parnell et al., 2009). However, this volume change, as well as other structural changes in hippocampus, was detected on the right side of the brain.

Since the olfactory bulbs relate to hippocampus via the formation of the odor memory, the altered volume of olfactory bulbs could induce molecular changes that could be reflected also in hippocampus. Thus, an altered gene expression was detected in several olfactory receptor genes (*Olfr937*, *Olfr601*, *Olfr553*, *Olfr1305*, *Olfr51*, *Olfr967*, *Olfr110*) in hippocampus. Olfactory receptor genes detect volatile chemicals leading to the initial perception of smell in the brain. The members of this large gene family normally show a monoallelic expression fashion which is regulated in a specific epigenetic manner. This has been compared to phenomena such as genomic imprinting or X-chromosome inactivation. Even though these genes are normally expressed in main olfactory epithelium, they are known to be expressed in the brain, but their function remains still unclear (Kang & Koo, 2012). However, because the expression array assay was carried out in combined right and left hippocampi, we can only predict that changed expression of genes may result from the altered left hippocampus. This result underlines the importance of exploring the left and right hippocampus separately in the future.

In addition to altered volume of hippocampus and olfactory bulb, our MRI revealed enlargement of lateral ventricles in the alcohol-exposed offspring. Similar results have been observed in other FASD animal models (Godin et al., 2010; Zhou et al., 2003; O'Leary-Moore et al., 2010). Alcohol exposure lasting throughout pregnancy decreased the number of neural precursor cells in the subependymal zone of the lateral ventricles (a region of adult neural stem cells), suggesting a possible reason for the extended volume of ventricles (Akers et al., 2011). In addition, in humans, the clinical studies of prenatal alcohol exposure have shown to cause ventrigulomegaly (Johnson et al., 1996; Swayze et al., 1997).

Above all, our study demonstrates that early chronic and moderate prenatal alcohol exposure can alter the development of the brain. Since in humans, magnetic resonance imaging studies are challenging and usage of brain tissue is impossible, this study emphasizes the importance of exploiting a mouse model which displays symptoms similar to FASD.

5.2 EARLY PRENATAL ALCOHOL EXPOSURE ALTERS THE EXPRESSION BUT NOT THE METHYLATION OF IMPRINTED GENES IN MOUSE EMBRYO AND PLACENTA (study II)

Since growth restriction is one of the major consequences of prenatal alcohol exposure, we focused on growth-related imprinted genes. Even though the *Igf2/H19* imprinted region plays a crucial role in regulating normal growth in both embryo and placenta, according to previous studies, this locus has shown to be sensitive to environmental exposure. Prenatal alcohol exposure in both mice and humans (Laufer et al., 2013; Haycock & Ramsay, 2009; Portales-Casamar et al., 2016), as well as in vitro fertilization (Nelissen et al., 2013) and nutrition (Tobi et al., 2012) in humans have either changed the methylation profile on the ICR or altered the expression of the genes.

In our study, the negative growth controller *H19* was upregulated in alcohol-exposed embryos when compared to controls. Although, the precise biological role of *H19* is unknown, it has been postulated that the microRNA 675 encoded by *H19*, has a role in restricting growth or cell proliferation by downregulating *Insulin-like growth factor 1 receptor* expression (Keniry et al., 2012; Tarnowski et al., 2015). However, the expression level of mir-675 was not measured in this study, thus we can only speculate that the increased expression level of *H19* could explain the growth restricted phenotype of offspring. In addition to *H19* expression, our finding of downregulated *Igf2* is similar as seen before. An acute alcohol exposure during the GD9 caused a significant decrease of *Igf2* expression in both embryo and placenta (Downing 2011).

In previous studies, alcohol-induced methylation changes have been found at the ICR region. In mice, a decreased methylation level at *H19* CTCF1/CTCF2 binding sites was observed in the paternal allele followed by a high-dose acute alcohol exposure (Haycock & Ramsay, 2009). However, this was only seen in the placental tissue. In addition, a small decrease in methylation level was found at four CpG sites in the *Igf2* DMR1 region (Downing et al., 2011). Although, this change was only observed in embryos. Moreover, alcohol exposure during GD10-18 decreased the methylation level at *H19* CTCF2 binding site in sperm in the F1 generation as well as in newborn whole brain tissue in the F2 generation (Stouder et al., 2011). Similarly, a slightly decreased methylation level at *H19* ICR has been observed in cerebral cortex of offspring exposed to paternal alcohol exposure (F. Liang et al., 2014). In a genome-wide methylation study of the largest cohort of FASD children to date, decreased methylation was also detected at the *H19* (Portales-Casamar et al., 2016). In contrast to previous studies, voluntary maternal consumption of 10% ethanol increased the methylation level at the *H19* promoter region in the adult mouse brain tissue (Laufer et al., 2013). Nevertheless, despite the observed expression change in our mouse study, no

clear methylation changes were found in the two CTCF binding sites (CTCF1/CTCF2) or *Igf2* DMR1 in E9.5 embryos or placentas.

There are not many studies which have examined alcohol-induced alterations in the function of the maternally imprinted gene *Snrpn,* even though it has been related to the nervous system. It is usually linked to a well-known imprinting disorder, Prader-Willi syndrome which includes mild intellectual disability, low birth weight as well as behavioral and learning problems (Leff et al., 1992). Similar features are also known to occur in FASD children (Popova et al., 2016). Even though we did not observe methylation changes at *Snrpn* ICR, *Snrpn* was upregulated in E9.5 embryos. A similar result has been described before. Paternal exposure to a high dose of ethanol increased the *Snrpn* expression in the cerebral cortex of F1 offspring, but also decreased methylation at *Snrpn* ICR (F. Liang et al., 2014). Nonetheless, as in our study, a low dose of alcohol administration did not affect the *Snrpn* ICR methylation profile in any tissue (Stouder et al., 2011).

Paternally expressed gene 3 is maternally imprinted and shown to be transcribed into a DNA-binding zinc-finger protein that binds to many promoters and enhancers where it regulates the gene expression (Thiaville et al., 2013; S. Lee et al., 2015). It is usually highly expressed in both placenta and brain in which it has a critical role in controlling maternal-caring behaviors such as milk provision and fetal growth. It has been demonstrated that the absence of *Peg3* promotes defects in milk-suckling resulting in weaker offspring due to an insufficient uptake of milk (L. Li et al., 1999; Kim et al., 2013) as well as growth restriction (Curley et al., 2004). Environmental insults such as toxic compounds and obesity have been shown to alter the methylation profile at the *Peg3* (Vidal et al., 2015; Soubry et al., 2015). Altered placental expression of *Peg3* was also associated with prenatal maternal depression in humans (Janssen et al., 2016) and maternal anxiety in mice (McNamara et al., 2017).

In our study, we did not detect altered *Peg3* expression in alcohol-exposed embryos or placentas. Furthermore, similarly as in the study of Stouder and colleagues (2011), we did not observe any changes in the methylation levels at *Peg3* ICR. However, it has been reported that *Peg3* has been influenced by alcohol exposure. In a South African fetal alcohol syndrome cohort study, the methylation level of the *Peg3* ICR was decreased in FAS children compared to control children (Masemola et al., 2015). In contrast, paternal alcohol exposure increased the methylation level in the cerebral cortex of F1 offspring (F. Liang et al., 2014).

To conclude, according to the previously published data, there are no clear results of alcohol´s effect on the imprinting in mice. However, as already mentioned, imprinted genes are important regulators of embryonic development and our study highlights that they can

be susceptible to harmful environmental impacts. This can be seen especially at the *Igf2/H19* locus. Even though prenatal alcohol exposure did not affect the methylation status in any of the regulatory regions, notable expression changes occurred in *Igf2*, *H19* and *Snrpn* genes.

The reason why clear alcohol-induced effects were not detected could be that the alcohol-exposure was too moderate, or the timing of exposure was inappropriate in this mouse model. The moderate exposure causes a highly variable phenotype in the offspring, hence the small sample size used in the methylation analysis could explain the difficulty in detecting subtle changes in the methylation level. In addition, it might be that this early exposure is incapable of changing this specific level of epigenetic regulation. Because the methylation of ICRs is established already in the germline and protected by several factors involved in the epigenetic reprogramming after fertilization, the methylation level of ICRs could be more resistant to environmental factors compared to DMRs.

With respect to the methods used in this study, one could argue that the MassARRAY EpiTYPER is perhaps not the best tool to investigate DNA methylation alterations in imprinted genes. EpiTYPER measures the total methylation level, thus it does not reveal the allele-specific information. For example, in our human study (Marjonen et al., 2017), we detected allele-specific DNA methylation changes after using the traditional bisulfite sequencing. Allele-specific alteration was observed also in the study of Haycock and Ramsey (2009) with bisulfite sequencing. Hence, in the future, two different mouse strains should be used to detect possible allele-specific methylation changes by bisulfite sequencing.

Furthermore, the combination of two sample sets, extracted with two different extraction kits, is most probably the reason for the high standard deviation of the gene expression in all of the studied genes. The direction of the gene expression was similar, hence increased or decreased, with both kits, however, for some unknown reason, the extraction kit_1 gave higher relative expression values. For example, even though the expression of *H19* and *Snrpn* was increased in both extraction kits in alcohol-exposed samples, it was more extensively increased in the kit_1 group. This result was unexpected. Thus, in the future studies, usage of only one extraction kit is highly recommended.

5.3 PRENATAL ALCOHOL EXPOSURE INDUCES GENOTYPE-SPECIFIC CHANGES AT THE IMPRINTED *IGF2/H19* LOCUS IN HUMANS (study III)

Our result suggests that, in humans, prenatal alcohol exposure changes the DNA methylation pattern in a genotype-specific manner. At the *H19* DMR, we observed notable differences between genotypes in alcohol-exposed samples compared to controls, in which the methylation level seemed to be stable in different genotypes. At the *H19* ICR CTCF6, alcohol exposure induced a trend of hypomethylation in the patA/matG genotype. Even though the change was consistent, it was not statistically significant after multiple testing correction. The hypomethylation seen in the patA/matG genotype in alcohol-exposed samples was caused by decreased DNA methylation level in the paternal allele. This result is consistent with an earlier mouse study, where alcohol-associated allele-specific methylation levels in placenta were studied (Haycock & Ramsay, 2009).

Not only alcohol, but also other environmental factors have been demonstrated to alter the DNA methylation level at the *IGF2/H19* locus in humans. Prenatal famine, caused a lower methylation level at *IGF2* DMRs (Tobi et al., 2012) and interestingly, assisted reproductive technology known as *in vitro fertilization* treatments have been shown to cause similar hypomethylation at the *H19* ICR CTCF6 (Nelissen et al., 2013; Loke et al., 2015; Castillo-Fernandez et al., 2017). However, the results of prenatal alcohol exposure have been inconsistent. Genome wide studies have revealed hypomethylation at *H19* ICR CTCF6 in buccal epithelial cells of Canadian FASD children (Portales-Casamar et al., 2016) but not in blood samples of South African FAS children (Masemola et al., 2015) nor in the cord blood of newborns in six general population based cohorts (Sharp et al., 2018).

One explanation of the variable findings could be cell specific methylation profiles in each tissue. However, there is also previous evidence of genetic impact to DNA methylation level. Heijmans et al. (2007) showed that the DNA methylation pattern in *IGF2/H19* locus is mainly determined by heritable factors such as single nucleotide polymorphisms. Thus, our results could explain the inconsistency of the former results by suggesting that the effect of alcohol exposure on the methylation profile depends on the genotype. This finding emphasizes the usage of genotype-specific approach in future studies.

Gene expression analysis revealed upregulated expression of both *IGF2* and *H19* in alcohol-exposed samples. The increased expression of *IGF2* is consistent with an earlier study where alcohol-exposed human trophoblast cell lines and placental tissue were used (Joya et al., 2015). Although upregulated *IGF2* suggests increased growth and thus competes against the growth-restricted phenotype of prenatal alcohol exposure. However, after exploring the *H19:IGF2* expression ratio in each placenta, the *H19* expression was significantly increased in relation to *IGF2* when comparing all alcohol-exposed placentas to

controls. Furthermore, the genotype-specific observation showed clear differences between the alcohol-exposed samples. Both *IGF2* and *H19* were downregulated in the patA/matG genotype. It is worthwhile emphasizing that according to the allele-specific methylation, the hypomethylated paternal allele was detected also in this genotype in the alcohol-exposed samples. Thus, the significantly decreased expression of *IGF2* could be explained by this hypomethylation. Moreover, the increased expression ratio of *H19:IGF2* was detected only in G/G and patG/matA genotypes. Hence, these findings again highlight the importance of genotype-specific approach.

The most significant finding of the study was the phenotypic alteration in newborns. Even though growth restriction has been related to prenatal alcohol exposure, a small head circumference has been considered as one of the main diagnostic criteria for FASD (Hoyme et al., 2016). We observed that the smallest head circumferences of alcohol-exposed newborns were those children with the patG/matA genotype. Interestingly, the expression of the negative growth controller *H19* (in *H19:IGF2* ratio) was most extensively increased in this specific genotype. In contrast, the largest head circumferences were observed in the alcohol-exposed newborns with the A/A genotype. This same genotype A/A has been associated with the strongest growth phenotype in infantile hemangiomas (Schultz et al., 2015). These results suggest that the effect of alcohol exposure on head circumference depends to some extent on genotype. Moreover, this genotype-based finding could explain the observations in previous studies that have failed to detect any correlation between the newborns' head circumference and maternal alcohol consumption (O'Callaghan et al., 2003) as well as the unimpressive correlation between head circumference and the brain volume of alcohol-exposed children (Treit et al., 2016).

The usage of genetic information in future diagnostics of alcohol-induced disorders would be crucial. This kind of genotype-specific approach has previously revealed associations between single nucleotide polymorphisms and anthropometric measures. An Icelandic study discovered a parent-of-origin-dependent effect with respect to a polymorphism rs147239461 at *IGF2/H19* locus on newborn and adult height (Benonisdottir et al., 2016). The rs4929984 polymorphism associated with parent-of-origin effects on newborn birth weight in independent populations (Adkins et al., 2010) and the rs2071094 allele was claimed to associate with increased birth weight, length and head circumference when inherited from the mother (Petry et al., 2011). These two polymorphisms (rs4929984, rs2071094) are in linkage with the rs10732516 polymorphism explored in our study. Hence, our finding strongly supports the use of the rs10732516 polymorphism to monitor genotype-specific changes in the phenotype caused by prenatal alcohol exposure.

6 CONCLUSION

We hypothesized that prenatal alcohol exposure would alter the epigenetic reprogramming of early embryo leading to alterations in gene regulation and development. The aim of this thesis was to search molecular mechanism of prenatal alcohol exposure and to understand the role of environmental impact on fetal development. Our findings in both mouse and human studies extend the knowledge of environmental effects on embryonic development and will act as a stimulus to continue the research in this field.

6.1 HIGHLIGHTS OF THE MOUSE MODEL STUDIES

The results emerging from our mouse model underline the vulnerability of the embryo to environmental insults during a very early period in the pregnancy. It was interesting to examine the effects on the expression level of the crucial *Igf2/H19* imprinted locus which controls the intrauterine growth. The decreased expression of the growth promotor *Igf2* in alcohol-exposed placentas as well as increased expression of the negative growth controller *H19* in alcohol-exposed embryos, were significant findings and could explain the growth-restricted phenotype of the offspring. Furthermore, we observed alcohol-induced long-lasting alterations in the adolescent brain and we also detected a similar gene expression change in two different tissues. This result strengthens our hypothesis of the early origin of alcohol-induced disorders: changes in gene regulation may have already taken place in embryonic stem cells and therefore can be seen in different tissue types in the adult organism. Moreover, the structural changes seen in the adult hippocampus and olfactory bulbs highlight the sensitivity of the nervous system to the effects of alcohol. Clearly the left side of the brain showed more dramatic effects and this phenomenon will need further investigation in the future, for example with histological procedures and RNA sequencing.

However, the epigenetic gene regulation remains complex. Even though we found alcohol-induced expression changes in both mouse studies, we did not observe any changes in DNA methylation. The expression changes that we detected in the Microarray analysis can result from alterations in multilevel pathways in which they are involved and not necessarily from alterations in their own regulatory regions. Hence, because the *Igf2/H19* locus has a more complicated regulatory system in addition to the differentially methylated regions, and furthermore since we investigated minor sites of the whole regulatory region, some of the alcohol-induced methylation changes could have escaped our attention. Moreover, we only focused on DNA methylation, thus we cannot rule out the possible contribution of other epigenetic mechanisms such as histone modifications.

Even though our mouse model displays many of the characteristics of the FASD phenotype, it seems to be a challenging model for use in epigenetic studies. The reason for utilizing this kind of model was to examine chronic and moderate alcohol exposure at the very beginning of pregnancy. It could be that the early exposure is incapable of changing this specific level of epigenetic regulation in the regions that were analyzed or the alcohol exposure could be too mild to evoke changes in the methylome. Moreover, despite the fact that genetically identical mice were used in the study, alcohol exposure in this model leads to a highly variable phenotype in the offspring. This can also be the reason why no alterations at the methylation level were observed. In the future, a larger sample size or a higher alcohol level may probably be needed to reveal the subtle changes at the level of DNA methylation.

6.2 HIGHLIGHTS OF THE HUMAN STUDY

In addition to the mouse model, human samples were used to investigate the effects of prenatal alcohol exposure. We exploited a new approach for exploring the well-known and intensively studied *IGF2/H19* locus. A polymorphism in the sixth CTCF binding sequence on the imprinting control region revealed four different genotypes that associated with the methylation profile of the region. This had been discovered before with normal control blood cells, but changes in placenta or alcohol-induced effects had not been described. Interestingly, alcohol decreased the methylation level on the paternal allele of the rs10732516/A genotype in placenta. A similar alcohol-induced decrease in the degree of methylation on the paternal allele has been also discovered in mice which supports our finding. Since the decreased methylation was detected only in the rs10732516/A genotype, this suggests that the alteration in the methylation profile is genotype-specific.

Both alleles (G=0.47, A=0.53) are common in the Finnish population. In the rs10732516/A genotype, the mutation to an adenine base affects the methylation of the CpG site by deleting it. Even though it has not been verified, this kind of polymorphism could affect the binding of CTCF binding factor and thus it would be functionally important and could influence on the phenotype. Thus far, previous studies have shown associations between anthropometric measures and single nucleotide polymorphisms (rs4929984, rs2071094) which are in linkage with rs10732516. Therefore, our results emphasize that under suboptimal circumstances, the effect of the rs10732516 polymorphism can be significant.

The most significant finding suggests that this polymorphism associates with the alcohol-induced defects in head growth in a parent-of-origin manner. This finding reveals that we have identified the first genetic factor involved in alcohol-induced disorders. Alcohol-exposed newborns in the G/G and paternalG/maternalA genotype had the smallest head

circumferences whereas those in the A/A genotype exhibited the largest. Interestingly, in the placenta, the expression of growth controller *H19* was increased in relation to *IGF2* in these two paternal rs10732516/G genotypes.

Our human study highlights the significance of taking the genetic background into account when investigating alcohol-induced disorders. Since each genotype presented with different alterations, our finding could explain why the severity of the symptoms differs between the children exposed to heavy maternal alcohol consumption during pregnancy. However, our finding does not pinpoint which of the genotypes could be more predisposing or protective against maternal alcohol consumption during pregnancy, since we cannot draw conclusions about the brain size or defects in the nervous system. Longitudinal follow-up studies of the children would reveal more information of the genotype-specific effects.

6.3 FUTURE PROSPECTS

To conclude, it is challenging to adopt an epigenetic approach when investigating the effects of prenatal alcohol exposure. The epigenome itself is influenced both by the genome and by environmental stimuli that originate from both inside and outside of the cell. It is constantly under stochastic changes which also means that some of the modifications can be reversible. Moreover, the variable and often small environmentally-induced epigenetic changes among individuals pose major challenges for research. Thus, despite the knowledge gained this far, it is still not clear how the environment modifies the epigenome.

The research of epigenetic patterns associated with prenatal alcohol exposure has grown rapidly. However, many studies have yielded inconsistent results which has raised concerns, although this should not be surprising in view of the complexity of the FASD phenotype. Perhaps, one of the major reasons for this discrepancy is that most of the studies are based on the correlation between the exposure and consequences rather than true causality of the results. Several investigations have attempted to find associations between the epigenetic patterns and changes at the transcription level omitting information on whether the results are reversal. This would in some part indicate the causality. Findings of single locus analysis as well as genome wide analysis performed in cell cultures do not necessarily reflect the actual functional implications caused by the environmental exposure. These issues must be considered in future studies.

To fully assess the role of the epigenetic machinery behind prenatal alcohol exposure, the future studies will need to integrate DNA methylation, chromatin alterations and non-coding RNAs and to construct a continuum between these processes in order to link them

to gene expression and ultimately to phenotype. Because the epigenome is mainly responsible for the specific cellular composition, the different cell types must be considered. To date, the advanced single cell techniques now make it possible to screen epigenetic map at a greater depth at the cellular level. Furthermore, due to improvements of genetic manipulation techniques, such as CRISPR/Cas9 biotechnology (Cong et al., 2013; Day, 2014; Rienecker et al., 2016), it should be possible to perform functional experiments to reveal direct causation between epigenetic patterns, gene expression and the altered phenotype. This highlights the fact that model organisms are still crucial in alcohol studies as they allow the manipulation of their biological systems in a controlled environment. Different animal models enable us to explore different time points, control the amount of consumed alcohol and use different tissues to create FASD phenotypic outcomes. By studying animals together with humans, we can obtain a better understanding of the alcohol-induced effects and an innovative way to reveal new epigenetic biomarkers.

It is crucial to devise early biomarkers which would evaluate the risk for outcomes of the children since some of the features of FASD (e.g. cognitive defects) only become evident after a long time-scale. However, the heterogeneous human population combined with the various phenotypic characteristics associated with FASD poses a huge challenge. As our human results indicate, the genetic background has a significant effect on the alterations. Hence, in the future, more population based studies such as those examining cohorts of children with the same ethnicities are needed. In addition, longitudinal studies will be required to reveal the potential consequences of altered epigenetic programming in the phenotype of adolescents and adults. Recent studies, including the results presented in this thesis, emphasize that genetic and epigenetic biomarkers might be potential diagnostic tools in the future. They could be utilized as early personalized support in the care of affected children.

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