Human Reproduction Open, pp. 1–12, 2017
doi:10.1093/hropen/hox014

human reproduction open

ORIGINAL ARTICLE

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

Heidi Marjonen[1], Hanna Kahila[2], and Nina Kaminen-Ahola[1,*]

[1]Department of Medical and Clinical Genetics, Medicum, University of Helsinki, Helsinki, Finland [2]Department of Obstetrics and Gynecology, Helsinki University Hospital and University of Helsinki, Helsinki, Finland

*Correspondence address. Department of Medical and Clinical Genetics, Biomedicum Helsinki, University of Helsinki, PO Box 63, FI-00014, Finland.
Tel: +358-50-4482768; E-mail: nina.kaminen@helsinki.fi

Submitted on May 9, 2017; resubmitted on August 31, 2017; editorial decision on September 7, 2017; accepted on September 11, 2017

STUDY QUESTION: Does prenatal alcohol exposure (PAE) affect regulation of the *insulin-like growth factor 2 (IGF2)/H19* locus in placenta and the growth-restricted phenotype of newborns?

SUMMARY ANSWER: PAE results in genotype-specific trends in both placental DNA methylation at the *IGF2/H19* locus and head circumference (HC) of newborns.

WHAT IS KNOWN ALREADY: PAE can disturb development of the nervous system and lead to restricted growth of the head, even microcephaly. To clarify the etiology of alcohol-induced growth restriction, we focused on the imprinted *IGF2/H19* locus known to be important for normal placental and embryonic growth. The expression of *IGF2* and a negative growth controller *H19* are regulated by the *H19* imprinting control region (*H19* ICR) with seven-binding sites for the methylation-sensitive zinc-finger regulatory protein CTCF. A single nucleotide polymorphism rs10732516 G/A in the sixth-binding site has shown to associate with genotype-specific DNA methylation profiles at the *H19* ICR.

STUDY DESIGN, SIZE, DURATION: By grouping 39 alcohol-exposed and 100 control samples according to rs10732516 polymorphism we explored alcohol-induced, genotype-specific changes in DNA methylation at the *H19* ICR and the promoter region of *H19* (*H19* differentially methylated region). Also, *IGF2* and *H19* mRNA expression level in placenta as well as the phenotypes of newborns were examined.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We explored alcohol-induced, genotype-specific changes in placental DNA methylation by MassARRAY EpiTYPER and allele-specific changes by bisulphite sequencing. *IGF2* and *H19* expression in placenta were analyzed by quantitative PCR and the HC, birthweight and birth length of newborns were examined using national growth charts.

MAIN RESULTS AND THE ROLE OF CHANCE: We observed a consistent trend in genotype-specific changes in DNA methylation at *H19* ICR in alcohol-exposed placentas. DNA methylation level in the normally highly methylated paternal allele of rs10732516 paternal A/maternal G genotype was decreased in alcohol-exposed placentas. In addition to decreased *IGF2* mRNA expression in alcohol-exposed placentas of this specific genotype ($P = 0.03$), we observed significantly increased expression of *H19* in relation to *IGF2* when comparing all alcohol-exposed placentas to unexposed controls ($P = 0.006$). Furthermore, phenotypic examination showed a significant genotype-specific association between the alcohol exposure and HC of newborns ($P = 0.001$).

LIMITATIONS REASONS FOR CAUTION: Owing to the exceptional character of the alcohol-exposed human samples collected in this study, the sample size is restricted. An increased sample size and functional studies are needed to confirm these data and clarify the biological significance or causality of the observed associations.

WIDER IMPLICATIONS OF THE FINDINGS: Our results suggest that the rs10732516 polymorphism associates with the alcohol-induced alterations in DNA methylation profiles and head growth in a parent-of-origin manner. We also introduce a novel genotype-specific approach for exploring environmental effects on the *IGF2/H19* locus and ultimately on embryonic growth.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Academy of Finland (258304), The Finnish Foundation for Alcohol Studies, Finnish Cultural Foundation, Juho Vainio Foundation, Yrjö Jahnsson Foundation and Arvo and Lea Ylppö Foundation. No competing interests are declared.

Key words: prenatal alcohol exposure / epigenetics / DNA methylation / insulin-like growth factor 2 / H19 / placenta / head circumference / pregnancy / imprinting / rs10732516

WHAT DOES THIS MEAN FOR PATIENTS?

This paper looks at why alcohol consumption in pregnancy impacts on a baby's development. We know that drinking too much alcohol in pregnancy is a key cause of developmental problems. When the effects are severe, the growth of the baby's head may be restricted and this can be an indication that brain development may also be affected.

The researchers wanted to know why some babies seem to be affected more seriously than others by their mother drinking alcohol in pregnancy. To understand more about this they explored whether the changes in gene regulation could play a role. They examined the placentas of newborn babies whose mothers had reported drinking high levels of alcohol in pregnancy. They found that drinking alcohol had an impact on the way DNA is regulated but that this varied from one person to another depending on their individual genetic make-up.

Introduction

Prenatal alcohol exposure (PAE) is a leading cause of preventable developmental disabilities globally and a substantial risk factor for comorbidities (Popova *et al.*, 2016). Alcohol exposure can produce fetal alcohol spectrum disorders (FASD), which is an umbrella term for all alcohol-related neurodevelopmental disorders and birth defects. Fetal alcohol syndrome, with growth restriction, craniofacial dysmorphology and central nervous system defects, represents the most severe end of FASD. The estimated prevalence of FASD ranges from 3 to 5% in Europe and North America to over 10% in South Africa (Roozen *et al.*, 2016), but owing to a low rate of diagnosis it is impossible to determine the exact numbers. Reduction in head circumference (HC) is used in the diagnosis of FASD and considered as an indication of deficient brain growth, and abnormal morphogenesis or abnormal neurophysiology (Hoyme *et al.*, 2016). Previous studies suggest that the amount (Russell *et al.*, 1991) as well as timing and pattern (Feldman *et al.*, 2012) of alcohol exposure *in utero* relate to the reduction of HC. However, despite a positive correlation of HC with brain volume, there is increased variation in this relationship among alcohol-exposed children and also a lack of correlation between HC and cognitive measures (Treit *et al.*, 2016).

Both human (Streissguth and Dehaene, 1993) and animal (Downing *et al.*, 2009) studies have shown that genetic variation is involved in the susceptibility to adverse effects of PAE. There is limited knowledge about the genetic risk factors for alcohol-induced developmental disorders and previous genetic studies have mainly concentrated on enzymes involved in alcohol metabolism (Warren and Li, 2005; Lewis *et al.*, 2012). Owing to the increasing comprehension about environmental effects on gene regulation and embryonic development, the study of epigenetics has become a prominent approach to understanding the molecular mechanisms behind FASD. In a previous study, we showed that early PAE in mice is capable of changing the DNA methylation level at the epigenetically sensitive Agouti viable yellow allele and consequently the coat color of alcohol-exposed offspring (Kaminen-Ahola *et al.*, 2010). The offspring phenotype was reminiscent of human alcohol-induced disorders with craniofacial dysmorphology, postnatal growth restriction (Kaminen-Ahola *et al.*, 2010) and both functional (Sanchez Vega *et al.*, 2013) and structural (Marjonen *et al.*, 2015) changes in central nervous system.

In this study, we used placentas of 39 alcohol-exposed and 100 control human newborns to examine the effects of alcohol on regulation of the intensively studied epigenetically regulated imprinted *insulin-like growth factor 2 (IGF2)/H19* locus on chromosome 11p15.5. Allele-specific gene expression in this locus is crucial for normal placental and embryonic growth. According to mouse studies, paternally expressed Igf2 is a major driver of growth (DeChiara *et al.*, 1991), whereas the non-coding RNA gene *H19* is a maternally expressed negative growth controller (Gabory *et al.*, 2009). The locus is regulated by allele-specific DNA methylation at the *H19* imprinting control region (*H19* ICR), *H19* promoter region (*H19* DMR) and *IGF2* differentially methylated regions (DMR0, DMR1 and DMR2, Fig. 1). *H19* ICR contains seven-binding sites for methylation-sensitive zinc-finger proteins, CCCTC-binding factors (CTCFs). These binding factors organize chromatin contacts at the imprinted locus and play a critical role in the establishment and maintenance of imprinting (Phillips and Corces, 2009). *H19* ICR is unmethylated on the maternal allele, which enables binding of CTCF proteins. According to mouse studies, the binding of CTCF is required to prevent enhancers from acting on maternal Igf2, thus repressing its

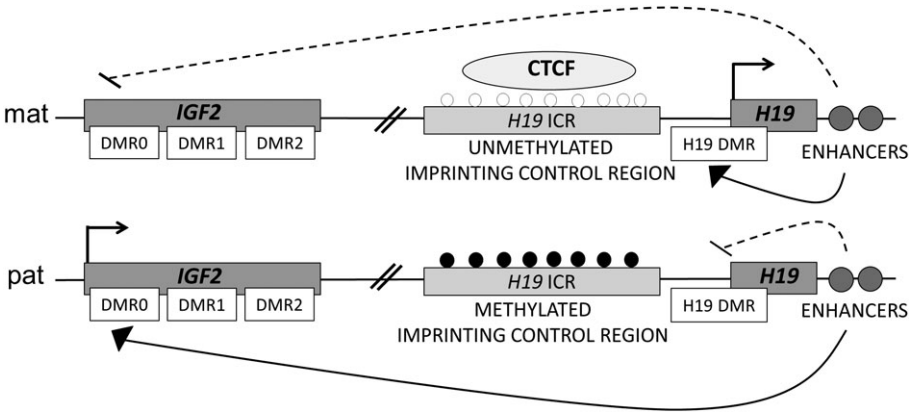

Figure 1 Schematic structure of the *insulin-like growth factor 2 (IGF2)/H19* locus on chromosome 11p15.5. CTCF protein binds to the maternal (mat) unmethylated imprinting control region (*H19* ICR), which blocks the interaction between downstream enhancers and the *IGF2* promoter, and enables the expression of maternal *H19*. The methylation of paternal (pat) *H19* ICR prevents the binding of CTCF protein, allowing access of downstream enhancers to the *IGF2* promoter and provoking the expression of paternal *IGF2*.

expression (Hark *et al.*, 2000). Hypomethylation at *H19* ICR in human is known to cause downregulation of *IGF2* and biallelic expression of *H19*, leading to a growth restriction disorder, Russell–Silver syndrome (Bartholdi *et al.*, 2009). By contrast, hypermethylation of *H19* ICR leads to overexpression of *IGF2* and downregulation of *H19*, causing fetal over-growth, known as Beckwith–Wiedemann syndrome.

The effects of PAE on DNA methylation level at the *IGF2/H19* locus have been already examined in some previous mouse and human studies. Altered DNA methylation at the *H19* ICR has been associated with PAE in mouse placenta (Haycock and Ramsay, 2009) and brain tissue (Laufer *et al.*, 2013). Also genome-wide DNA methylation studies of both mouse embryos exposed to alcohol (Liu *et al.*, 2009) and buccal epithelial cells of children with FASD have identified decreased methylation of *H19* (Portales-Casamar *et al.*, 2016).

Both genetic variation and the environment affect embryonic development, generating phenotypic differences between individuals. In this study we detected a single nucleotide polymorphism rs10732516 G/A in the sixth-binding sequence of CTCF (CTCF6), which associated with the DNA methylation level of *H19* ICR in placenta. Our finding was consistent with earlier studies, where whole blood samples were used (Coolen *et al.*, 2011; Renteria *et al.*, 2013) and encouraged us to explore the effects of alcohol exposure in a genotype-specific manner. Altered DNA methylation level at the CTCF6 showed an association with changes in *IGF2* and *H19* expression in a previous human study (Takai *et al.*, 2001). Therefore, in addition to DNA methylation level of CTCF6 at *H19* ICR and *H19* DMR, we explored gene expression in both alcohol-exposed and control human placentas. Finally, we studied the genotype-specific effects of alcohol on the phenotype of newborns by using new Finnish growth charts (Sankilampi *et al.*, 2013).

Materials and Methods

Study design and sample collection

Placental samples

Pregnant women, admitted from primary care to the special antenatal outpatient clinic in Helsinki University Central Hospital, Finland, because of their abundant alcohol consumption, were recruited to this study (Table I and Supplementary Table SI). The maternal alcohol consumption before pregnancy was registered by using self-reported information: Alcohol Use Disorders Identification Test (AUDIT) or the amount of reported units of alcohol per week (1 unit is 12 g of ethanol). A 10-item screening tool AUDIT, developed by the World Health Organization, assesses alcohol consumption, drinking behavior and alcohol-related problems (Babor *et al.*, 2001). Scores 8–40 indicate harmful or hazardous alcohol use. More than 12–16 alcohol units per week is considered as heavy drinking (Rehm *et al.*, 2015). Seven mothers did not smoke during pregnancy. Of the alcohol-exposed newborns, two were of African origin, one child's mother was Estonian and father Russian, and the others were children of Finnish, Caucasian mothers. The controls were newborns of healthy Finnish, Caucasian mothers who have not used alcohol or smoked cigarettes during the pregnancy, according to their self-reported information. Control samples were collected during the same period as the alcohol-exposed samples (years 2013–15) in Helsinki University Central Hospital, Finland.

The Finnish growth charts used in this study are register data which include 753 036 infants born in 1996–2008 in Finland and it refers to optimal intrauterine growth in the Finnish population. Because of the significant differences in birth size between populations, which probably originate mainly from differences in genetic factors in Western countries, the use of population-based references is beneficial. Measures deviating more than ±2 SDs are commonly considered abnormal.

Placental biopsies (1 cm^3), precisely from the fetal side and within a radius of 2–3 cm from the umbilical cord, were collected immediately after delivery, rinsed in cold 1 × PBS, and stored in RNAlater® (Thermo Fisher Scientific, Vilnius, Lithuania) at −80°C.

Informed consent was obtained from all participants and the study was approved by the Ethics Committee of Helsinki University Central Hospital (386/13/03/03/2012).

Methylation analysis

EpiTYPER

Genomic DNA was extracted from four pieces of placenta per sample by standard phenol-chloroform protocol and pooled. DNA methylation profiles were measured by MassARRAY EpiTYPER (SEQUENOM Inc.) based on matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Supplementary Table SII). Sodium bisulphite

Table I General characteristics of the control and alcohol-exposed newborns and their mothers included in the study.

Newborns	Sex (Male/female)	Gestational age (Weeks+days)	Birthweight (g)	(SD)	Birth length (cm)	(SD)	HC (cm)	(SD)	Placental weight (g)
Controls ($n = 100$) mean (±SD)	52/48%	40 + 4 (±1 +1)	3668 (±412)	0.0 (±0.97)	51 (±2)	0.0 (±1.08)	35.5 (±1.3)	0.2 (±0.98)	627.6 (±125.3)
Alcohol-exposed ($n = 39$) mean (±SD)	38.5/61.5%	39 + 5 (±2 +0)	3309 (±616)	−0.37 (±1.2)	49 (±3)	−0.45 (±1.4)	34.3 (±2.2)	−0.33 (±1.5)	592.4 (±154.9)
Differences between groups *P*-value, Mann–Whitney		0.064	0.003		0.001		0.001		0.122
Differences between groups. Only Caucasian, *P*-value, Mann–Whitney		0.081	0.006		0.002		0.002		0.191
SD differences between groups. Only Caucasians, *P*-value, Mann–Whitney				0.25		0.175		0.065	

Mothers	Maternal age (years)	Parity	BMI kg/m^2
Controls mean (±SD/*n*)	31.6 (±4.6/100)	0.6 (±0.7/100)	22.8 (±3.5/99)
Alcohol consumers mean (±SD/*n*)	29.3 (±7.9/39)	0.4 (±1.0/39)	25.3 (±6.5/33)
Differences between groups *P*-value, Mann–Whitney	0.047	0.039	0.131

Differences in weights, lengths and HCs of newborns have been calculated using both anthropometric measures (separately for all newborns and only Caucasian newborns, excluding two newborns of African origin) and the SD of measures based on national growth references (only Caucasian newborns) adjusted for gestational age at birth, twinning, parity and gender. The mean values (±) SD are presented. *P*-values were calculated using the non-parametric Mann–Whitney test.

conversion of placental DNA (1000 ng) was carried out using the EZ methylation kit (Zymo Research, Irvine, CA, USA). Primers for the *H19* ICR (CTCF6), *H19* DMR and *LINE-1* regions were obtained from previous publications (Ollikainen *et al.* 2010; Wang *et al.* 2010) and optimized (Supplementary Table SIII). PCR was performed in triplicate using the HotStar PCR kit (Qiagen, Valencia, CA, USA) in a 10 µl reactions according to the provider's instructions. Each fragment of both control ($n = 42$) and alcohol-exposed ($n = 33$) samples was analyzed simultaneously in the same PCR plate. Technical replicates that showed >5% difference from the median value of the three replicates were discarded, leaving a duplicate that was used in the analysis.

Bisulphite sequencing

To find out allele-specific methylation levels 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 bisulphite sequenced (Supplementary Table SIV). Due to the presence of heterozygosity and imprinting it was straightforward to discern paternal and maternal alleles. Two bisulphite conversions (EZ methylation kit, Zymo Research, Irvine, CA, USA) and three independent PCR reactions (HotStar PCR kit, Qiagen, Valencia, CA, USA) 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) (Supplementary Table SIII) and primer sequences were revised to allow for potential polymorphisms. PCR fragments were gel-isolated (NucleoSpin Gel and PCR Clean-up kit, Macherey-Nagel, Düren, Germany) and the three PCR products were pooled. The products were subcloned into the pGEM®-T Vector system I (Promega, Madison, WI, USA), and cloning was performed by a standard protocol. On average, 48 clones per individual sample were sequenced. The sequences were analyzed by BiQ Analyzer (Bock *et al.* 2005). Any clones with lower than 90% conversion rate were excluded from the dataset.

Genotype analysis

Two EpiTYPER primer pairs for *H19* ICR were used to genotype samples (Supplementary Table SIII). Depending on the primer pair used 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 ~0.80, in G/G 0.30, in patA/matG 0.02, and in A/A there was no value. Altogether, 60 samples were analyzed by traditional bisulphite sequencing; 21 samples were genotyped and 39 genotypes determined by EpiTYPER were confirmed by this traditional method.

Gene expression analysis

Total RNA from four placental pieces per sample was extracted by TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) in the Biomedicum Functional Genomics Unit (FuGU, Helsinki, Finland). Two to four pieces (on average three) were analyzed individually and RNA was DNAse treated (RQ1 RNase-Free DNase, Promega, Madison, WI, USA) followed by cDNA synthesis (iScript™ cDNA Synthesis Kit, Bio-Rad Laboratories, Hercules, CA, USA). Reaction conditions were as specified by SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). *Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta*, (*YWHAZ*), which showed unaffected expression by alcohol in 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 (Rancourt *et al.* 2012; Nelissen *et al.* 2013). *IGF2*, *H19* and *YWHAZ* for a single sample were analyzed on the same plate. The plates were normalized using one same sample on each plate. Samples were analyzed in triplicates and the expression fold changes of *IGF2* and *H19* were obtained by normalizing

the Ct-values to the housekeeping gene according to the ΔΔCt method (Livak and Schmittgen, 2001). The *H19:IGF2* ratio in an individual placental sample was calculated from *H19* and *IGF2* fold change values. Details regarding the primers and protocols of methylation and expression studies are shown in Supplementary Table SIII.

Statistical analysis

The measured birthweight, length and HC were compared to the new Finnish population-based charts (Sankilampi *et al.* 2013). Two newborns of African origin were excluded from the phenotypic analysis.

Statistical analyses were conducted using either SPSS software for windows version 22.0 (NY, USA) or GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are expressed as the mean ± SD for a normal distribution of variables or as the interquartile range for a skewed distribution of variables. Samples were divided into four groups according to the genotype and the *chi*-square test was used to compare the prevalence of the rs1072516 in the control and alcohol samples. The non-parametric Mann–Whitney test was used to compare the characteristics of the mothers and newborns, the methylation level of chosen CpG sites as well as gene expression between the alcohol and control samples. In the methylation analysis, nominal *P*-value were considered significant when <0.05 and Bonferroni correction was used for multiple testing correction. Effect size for the gene expression was calculated with Cohen's *d* test. One-way ANOVA, followed by Bonferroni *post hoc* test when significant, was used to identify the differences among the genotype groups. Correlations were calculated using Pearson or Spearman's rank correlation.

Results

Participant characteristics

The age and parity of the mother who had consumed alcohol at least in the beginning of the pregnancy differed significantly from the control mothers (Table I). Also the weight (g), length (cm) and HC (cm) of the alcohol-exposed newborns (all or only Caucasian newborns) differed significantly from the controls. The measured birthweight, length and HC were also compared to the new Finnish population-based charts (Sankilampi *et al.* 2013), in which the gestational age at birth, twinning, parity and gender have been considered when calculating the SD (*z*-score) of birth measures. Unexpectedly, according to the national growth charts, there were no significant differences in SDs of HC, weight or length between control and alcohol-exposed Caucasian newborns (Table I). Two newborns of African origin were excluded from these calculations.

DNA methylation profiles at *H19* ICR, *H19* DMR and *LINE-1*

EpiTYPER

We assessed the DNA methylation level of CTCF6 at *H19* ICR and *H19* DMR and *LINE-1* in both alcohol-exposed and control placentas as Sequenom MassARRAY EpiTYPER units, which contain one or more binding sites for methyl groups (CpGs). We did not see significant differences in the methylation levels of CTCF6 at *H19* ICR or *H19* DMR between these two groups (Supplementary Table SII). Interestingly, the rs10732516 G/A polymorphism in the CTCF6 binding sequence at *H19* ICR showed genotype-specific, significantly different DNA methylation levels at 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) (Fig. 2A). Owing to these clearly distinct methylation profiles, we divided all our samples according to the genotype: rs10732516 G/G, paternal G/maternal A (patG/matA), paternal A/maternal G (patA/matG) and A/A, and explored potential genotype-specific effects of alcohol exposure. 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).

To determine alcohol-induced alterations in the global methylation level in placenta, we examined methylation in *Long interspersed nuclear elements* (*LINE-1*) in the alcohol-exposed and control placentas. However, no significant changes in the methylation levels were observed (Supplementary Table SII).

Genotype-specific DNA methylation profiles at *H19* ICR and *H19* DMR

EpiTYPER

Despite our genotype-specific approach, we did not observe any alcohol-induced changes in the methylation level of *H19* ICR by using EpiTYPER (Supplementary Table SII). However, we detected decreased methylation levels at units CpG6 and CpG16 at *H19* DMR in G/G genotype in the alcohol-exposed placentas compared to controls (nominal *P*-value P = 0.01 and P = 0.04, respectively, Mann–Whitney) (Fig. 2B). Nevertheless, changes in DNA methylation levels were not significant after multiple testing correction (P = 0.28, P = 1.12, respectively, Bonferroni correction). Furthermore, genotype-specific methylation levels at *H19* DMR were detected in the alcohol-exposed samples: the methylation level of A/A was lower compared to patG/matA and patA/matG at unit CpG6 ($F_{3,19}$ = 5.7, P = 0.006, one-way ANOVA).

Bisulphite sequencing

We confirmed the genotype-specific EpiTYPER results of CTCF6 at *H19* ICR by traditional bisulphite sequencing for heterozygous control and alcohol-exposed samples (patG/matA and patA/matG). We first counted the average methylation percentages separately for the paternal and maternal alleles, and then calculated the total methylation levels for each CpG site (CpG1–CpG27) by weighing both of the alleles equally (Supplementary Table SIV). By using a different primer pair we attempted to exclude any potential primer-specific bias. The traditional method showed a similar trend of DNA methylation levels in both genotypes as were seen by EpiTYPER and in a previous study with whole blood cells (Coolen *et al.* 2011): patG/matA genotype has a higher methylation level in the end of the sequence 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*-value CpG1: P = 0.015, CpG25: P = 0.002 and CpG26 P = 0.001, Mann–Whitney) placentas (Fig. 3, Supplementary Tables SIV and SV). However, changes in methylation levels were not significant after multiple testing correction either in control (CpG25: P = 0.47, CpG26: P = 0.10, Bonferroni correction) or alcohol-exposed placentas (CpG1: P = 0.78, CpG25: P = 0.10, CpG26: P = 0.05, Bonferroni correction). The overall methylation levels observed were higher using bisulphite sequencing, where both the alleles

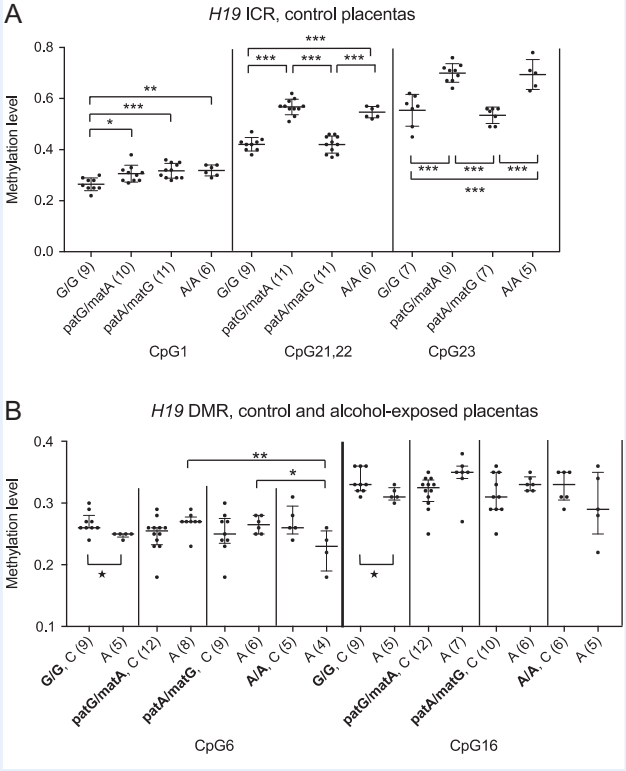

Figure 2 Genotype-specific DNA methylation levels of CpG units at the *IGF2/H19* locus in control and alcohol-exposed placentas measured by Sequenom EpiTYPER. (**A**) Genotype-specific DNA methylation levels in units CpG1, CpG21,22, and CpG23 at *H19* ICR (CTCF6) in control placentas ($P = 0.001$; $P < 0.0001$, $P < 0.0001$ respectively, one-way ANOVA). (**B**) Genotype-specific DNA methylation levels in units CpG6 and CpG16 at the *H19* DMR. Genotype-specific methylation levels were compared between control (C) and alcohol-exposed (A) placentas (units CpG6 and CpG16 in G/G genotype, a star (★) illustrates nominal *P*-value < 0.05, Mann–Whitney) and genotype-specific methylation levels in alcohol-exposed placentas (unit CpG6, $P = 0.006$, one-way ANOVA). Error bars denote the median with interquartile range. The numbers of samples are in brackets. Bonferroni *post hoc* test for one-way ANOVA: *$P < 0.05$, **$P < 0.01$, ***$P \leq 0.001$.

are weighed as equals, which indicates that the alleles were not equally amplified in EpiTYPER.

When comparing the alcohol-exposed placentas to controls within genotypes the methylation levels in patA/matG genotype were lower in sites CpG1 and CpG14 in alcohol-exposed samples (nominal *P*-value $P = 0.05$ and $P = 0.02$, respectively, Mann–Whitney) (Fig. 3D). This trend of hypomethylation was similar to the EpiTYPER results (site CpG14 corresponds to unit CpG11,12 in EpiTYPER, Supplementary Table SV). We did not observe a similar systematic trend of alcohol-associated hypomethylation in the patG/matA genotype, which indicates genotype-specific effects of alcohol exposure (Fig. 3A, Supplementary Table SIV). However, differences in DNA methylation levels were not significant after multiple testing correction (CpG1: $P = 2.6$, CpG14: $P = 1.04$, Bonferroni correction).

Allele-specific DNA methylation profiles at *H19* ICR

Bisulphite sequencing: We next wanted to assess potential allele-specific effects of alcohol exposure on CTCF6 at *H19* ICR using traditional bisulphite sequencing (Fig. 3B, C, E and 3F, Supplementary Table SIV). By comparing the methylation levels of paternal and maternal alleles separately in control and alcohol-exposed placentas we observed an obvious and consistent trend of alcohol-associated decreased average methylation in the paternal allele of patA/matG genotype (Fig. 3E). Two CpG sites, CpG7 and CpG14, were notably less methylated in the alcohol-exposed placentas compared to controls (nominal *P*-values $P = 0.02$ and $P = 0.007$, respectively, Mann–Whitney; after multiple testing correction $P = 2.08$, $P = 0.73$, respectively, Bonferroni correction). This decreased methylation in the paternal allele is consistent with an earlier mouse study, where alcohol-associated allele-specific methylation levels in placenta were studied (Haycock and Ramsay, 2009). In the maternal allele, despite of a trend of alcohol-associated increased methylation in the end of the sequenced clones, we did not observe any significant site-specific alterations.

We did not observe a consistently decreased methylation level in the paternal allele of patG/matA genotype in alcohol-exposed placentas. Instead of hypomethylation, we detected increased site-specific methylation in the paternal allele of the patG/matA genotype in the alcohol-exposed placentas compared to control placentas (nominal *P*-value CpG9: $P = 0.02$, Mann–Whitney; after multiple testing correction $P = 2.08$, Bonferroni correction) (Fig. 3B, Supplementary Table SIV), which is contrary to the hypomethylated paternal site CpG9 of the patA/matG genotype in the alcohol-exposed placentas. This, together with the demethylated methylation profile of the patA/matG genotype, suggests allele-specific effects of the alcohol exposure.

Genotype-specific gene expression of *IGF2* and *H19*

We next examined whether PAE affects gene expression at the *IGF2/H19* locus. The mRNA expression level of *IGF2* and *H19* was highly variable and we did not see significant changes in *IGF2* and *H19* expression when we compared all alcohol-exposed placentas to controls ($P = 0.82$, $P = 0.1$, respectively, Mann–Whitney) (Fig. 4A, 4B). Upregulation of *IGF2* mRNA expression in alcohol-exposed placentas is consistent with an earlier study where alcohol-exposed human trophoblast cell lines and placental tissue were used, but the expression of *H19* was not studied (Joya *et al.* 2015). We did not see any genotype-specific trends in gene expression (*IGF2*: $F_{3,58} = 0.21$, $P = 0.89$; *H19*: $F_{3,49} = 1.16$, $P = 0.34$ in control placentas and *IGF2*: $F_{3,25} = 1.44$, $P = 0.26$; *H19*: $F_{3,24} = 1.38$, $P = 0.27$ in alcohol-exposed placentas, one-way ANOVA) (Fig. 3D, E). The only exception was the patA/matG genotype: the expression of both *IGF2* ($P = 0.03$, Mann–Whitney) and *H19* was consistently repressed in the alcohol-exposed placentas compared to controls in this specific genotype, where a hypomethylated paternal allele was detected by traditional bisulphite sequencing.

Previous Russell–Silver and Beckwith–Wiedemann syndrome studies (Bartholdi *et al.* 2009) have shown that hypermethylation at *H19* ICR leads to overexpression of *IGF2* and downregulation of *H19*, and controversely, hypomethylation of *H19* ICR leads to downregulation of *IGF2* and biallelic expression of *H19*. Therefore, we decided to compare the

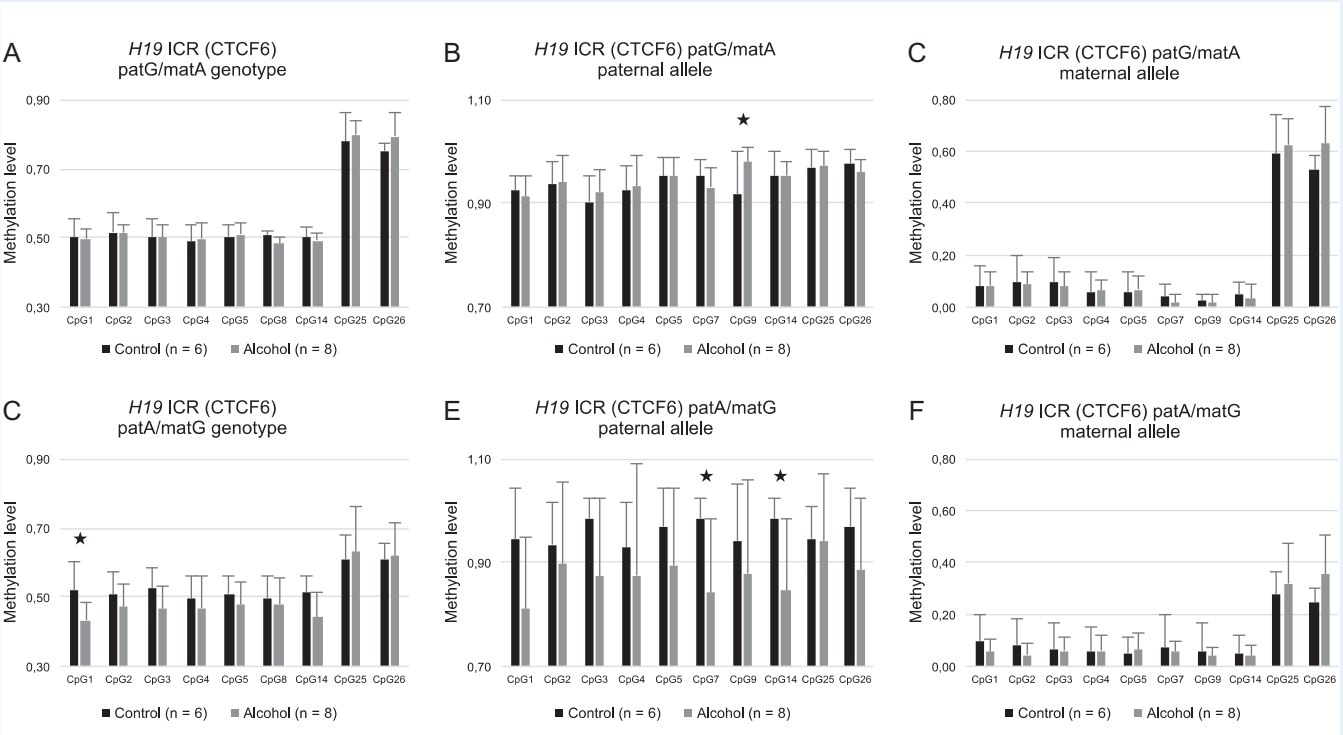

Figure 3 Genotype- and allele-specific DNA methylation levels of CpG sites at *H19* ICR (CTCF6) in control and alcohol-exposed placentas measured by bisulphite sequencing. Methylation levels of selected CpG sites in the (**A**) patG/matA genotype, (**B**) paternal allele of patG/matA genotype, (**C**) maternal allele of patG/matA genotype, (**D**) patA/matG genotype (**E**) paternal allele of patA/matG genotype and (**F**) maternal allele of patA/matG genotype. Error bars denote the SD. The numbers of samples are in brackets. A star (★) illustrates nominal *P*-value < 0.05, Mann–Whitney.

expression ratios of *H19* and *IGF2* in each placenta. The relative *H19* expression was significantly increased when we compared all the alcohol-exposed placentas to unexposed controls ($P = 0.006$, Mann–Whitney, effect size: 0.6, Cohen's *d*) (Fig. 4C), which indicates alcohol-induced changes in the regulation of these two genes. However, when the samples were divided according to the genotype, increased exposure-associated expression of *H19* compared to *IGF2* was observed only in the genotypes with a paternal rs10732516 G allele (G/G: $P = 0.03$ and patG/matA: $P = 0.02$, Mann–Whitney) (Fig. 4D).

Genotype-specific phenotypes of alcohol-exposed newborns

We explored genotype-specific changes in the alcohol-exposed newborns' phenotype by comparing birth anthropometry to the new Finnish population-based growth charts (Sankilampi *et al.* 2013). According to the charts, in which the gestational age at birth, twinning, parity and gender have been considered, there are no significant phenotypic differences between control and alcohol-exposed Caucasian newborns (Table I). However, genotype-specific comparison surprisingly revealed a significant association between the rs10732516 polymorphism and the HC of alcohol-exposed newborns ($F_{3,33} = 7.5$, $P = 0.001$, one-way ANOVA) (Fig. 5A). Alcohol-exposed newborns' HCs were significantly smaller in the patG/matA genotype than control HCs ($P = 0.004$, Mann–Whitney) and the largest in the A/A genotype. The HCs of alcohol-exposed newborns ($n = 37$) correlate with the SD of birthweight ($r = 0.538$, $P = 0.001$, Pearson correlation) and placental weight ($r = 0.422$, $P = 0.009$), but not with the birth length ($r = 0.228$, $P = 0.176$) (Fig. 4). The HCs of controls ($n = 100$) correlate with the SD of birthweight ($r = 0.501$, $P < 0.001$), length ($r = 0.292$, $P = 0.003$) and placental weight ($r = 0.247$, $P = 0.013$).

There was a negative correlation between the AUDIT scores (Babor *et al.* 2001) and HCs of alcohol-exposed newborns ($r = -0.487$, $P = 0.018$, $n = 23$, Spearman's rank correlation), which indicates that the reported AUDIT scores to some extent represent the degree of alcohol consumption. This concerns other genotypes except A/A, of which the majority of AUDIT data are missing. We did not observe a correlation between HC and the amount of reported alcohol units per week ($r = 0.129$, $P = 0.659$, $n = 14$), HC and duration of alcohol exposure ($r = -0.041$, $P = 0.812$, $n = 36$) or HC and number of cigarettes smoked per day ($r = 0.109$, $P = 0.611$ $n = 24$). Furthermore, we did not observe a correlation between the HCs of alcohol-exposed newborns and methylation of any CpG site (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 a genotype-specific manner.

Discussion

We have explored effects of PAE on regulation of the *IGF2/H19* locus by analyzing our samples according to the rs10732516 polymorphism and the distinct epigenetic profiles associated with it. This genotype-specific approach is supported by recent Icelandic research where a

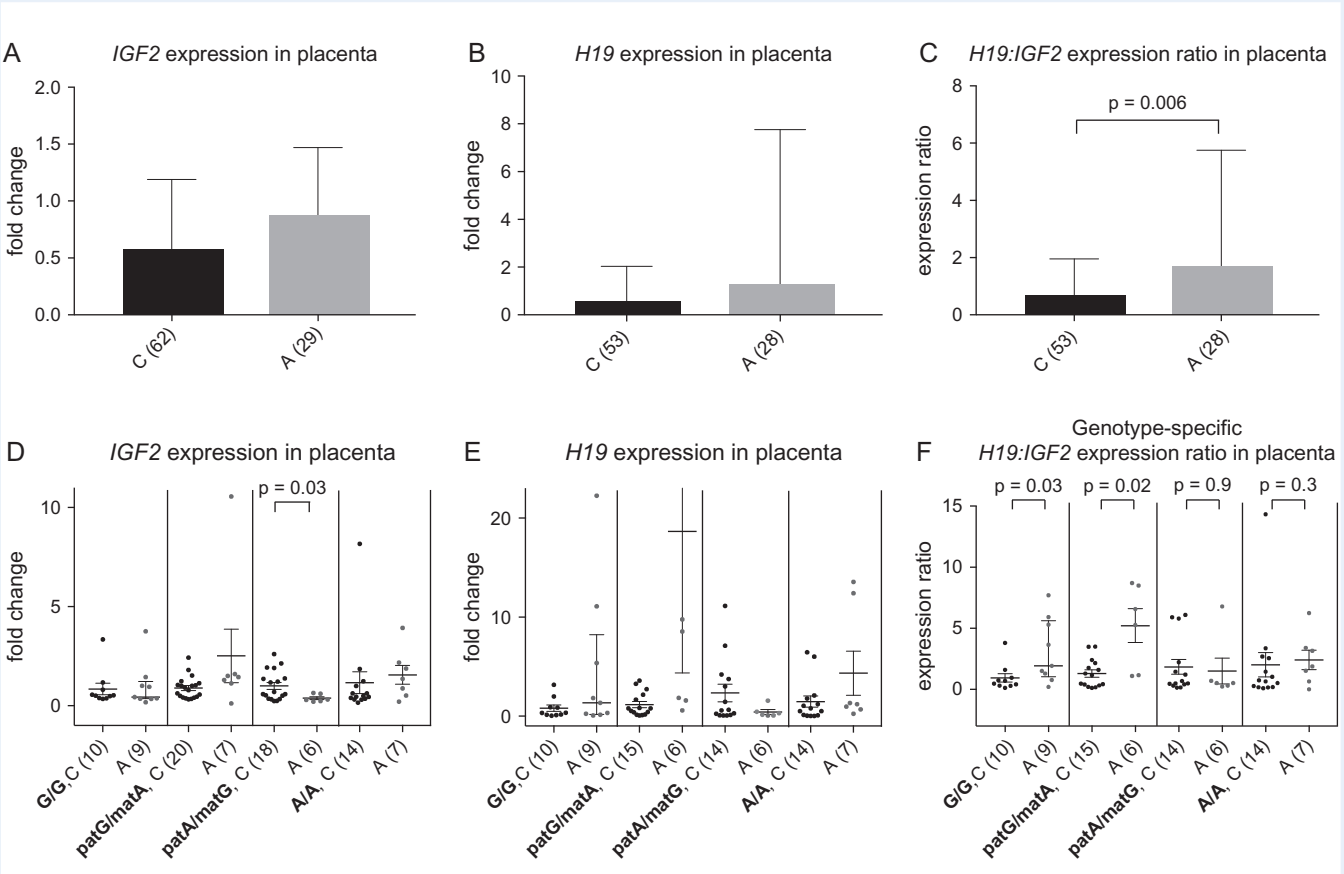

Figure 4 Expression levels of *IGF2* and *H19* mRNAs in control and alcohol-exposed placentas. (**A**) *IGF2* mRNA expression in control (C) and alcohol-exposed (A) placentas. (**B**) *H19* expression in control and alcohol-exposed placentas. (**C**) Increased expression ratio of *H19:IGF2* in alcohol-exposed placentas compared to controls ($P = 0.006$, Mann–Whitney). (**D**) Decreased genotype-specific *IGF2* expression in alcohol-exposed placentas with patA/matG genotype compared to controls ($P = 0.03$, Mann–Whitney). (**E**) Genotype-specific *H19* expression in placenta. The fold change of one data point (89,62) for an alcohol-exposed placenta in genotype patG/matA is outside the axis limit. (**F**) Increased genotype-specific expression ratio of *H19:IGF2* in genotypes with paternal rs10732516 G genotypes G/G and patG/matA compared to controls ($P = 0.03$, $P = 0.02$, respectively, Mann–Whitney). Error bars denote the median with interquartile range. The numbers of samples are in brackets.

parent-of-origin-dependent effect of a polymorphism rs147239461 at the *IGF2/H19* locus on newborn and adult height was discovered (Benonisdottir *et al.* 2016). Previous studies indicate that also rs10732516 associates with growth in a parent-of-origin manner: a polymorphism rs4929984 in the same block of strong linkage disequilibrium with rs10732516 has been associated with parent-of-origin effects on newborn birthweight in independent populations (Adkins *et al.*, 2010). Furthermore, the fetal rs2071094 allele, a polymorphism which is also in linkage with rs10732516, has been associated with increased birthweight, length and HC when inherited from the mother (Petry *et al.*, 2011). rs10732516 is not necessarily the functional polymorphism in the haplotype, but it is a plausible candidate because of its location on the binding sequence of CTCF protein, where allele A deletes a CpG binding site for a methyl group. Considering a relatively weak effect of the polymorphisms on birth measurements in previous studies, rs10732516 could slightly affect the binding efficiency of CTCF protein and consequently change the DNA methylation profile as well as the regulation of the locus depending on the genotype.

Both genetic variation and environmental impacts, such as nutrition in the beginning of pregnancy (Heijmans *et al.*, 2007; Tobi *et al.* 2012), IVF treatments (Nelissen *et al.*, 2013; Loke *et al.* 2015; Castillo-Fernandez *et al.* 2017) and PAE (Haycock and Ramsay, 2009; Liu *et al.* 2009; Laufer *et al.*, 2013; Portales-Casamar *et al.* 2016), have been associated with different DNA methylation levels at the *IGF2/H19* locus in previous studies. In this study we observed decreased DNA methylation level in the paternal allele at the *H19* ICR in alcohol-exposed human placentas, which is consistent with an earlier mouse study (Haycock and Ramsay, 2009). This decreased methylation was seen only in the rs10732516 patA/matG genotype, which suggests that the effect of alcohol exposure on the methylation profile depends on the genotype. The observed changes in the methylation level are relatively small and we have only 14 samples with this genotype. According to a power calculation, at least 44 samples would be needed to reach a significant *P*-value after multiple testing correction. However, the lower DNA methylation level of the alcohol-exposed paternal allele in a patA/matG genotype is consistent, which decreases the likelihood of randomness. This systematic trend of hypomethylation led us to explore if there are any genotype-specific alcohol-induced changes in gene expression or in the newborns' phenotype.

We observed increased *IGF2* mRNA expression in alcohol-exposed placentas, which is consistent with an earlier human study (Joya *et al.*

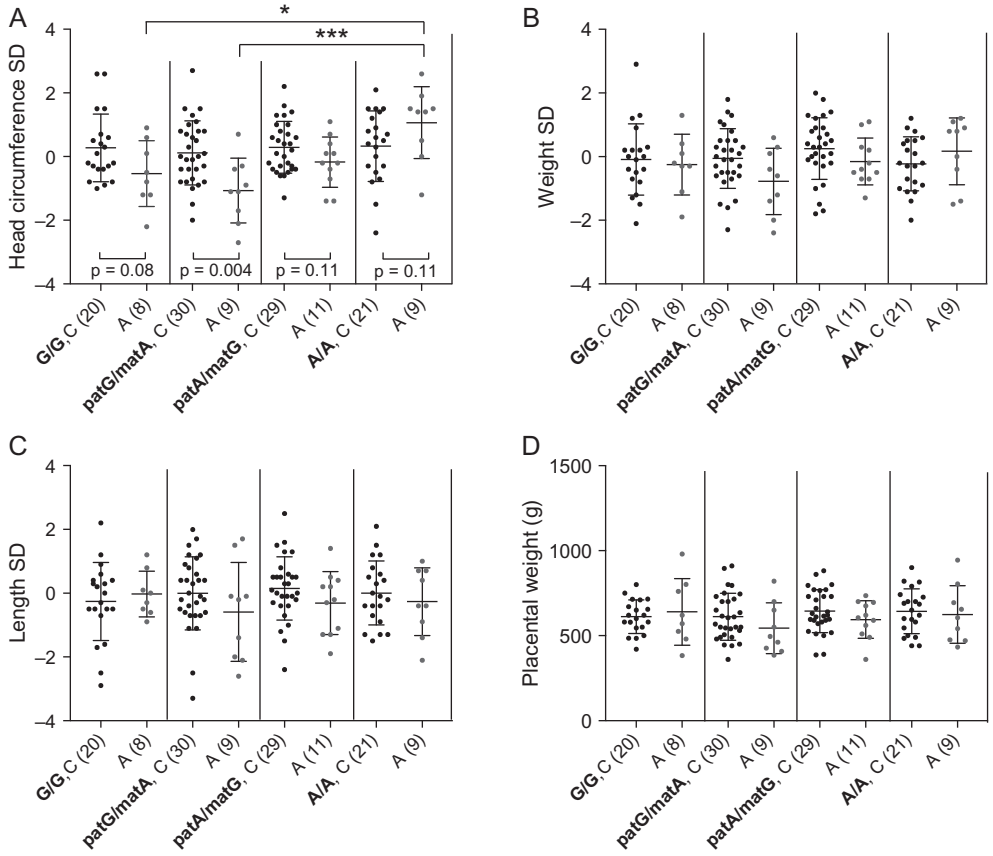

Figure 5 Genotype-specific HCs, weights, lengths and placental weights of control and alcohol-exposed Caucasian newborns. (**A)** Genotype-specific variation in SDs of control (C) and alcohol-exposed (A) newborns' HCs ($P = 0.001$, One-way ANOVA). The SDs of weight and length are shown in panels **B** and **C**, respectively. The genotype-specific weights of control and alcohol-exposed placentas are shown in panel **D**. Error bars denote the SD. The numbers of samples are in brackets. Bonferroni post hoc test for one-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P \leq 0.001$.

2015), but contradicts the growth-restricted phenotype of PAE. However, when we compared *IGF2* and *H19* expressions in each placenta, we detected significantly increased *H19* expression compared to *IGF2* in the alcohol-exposed placentas, which could explain the retarded growth. Genotype-specific examination revealed decreased *IGF2* expression in alcohol-exposed placentas in the patA/matG genotype, which could be a consequence of the hypomethylated paternal allele observed in the alcohol-exposed placentas in this genotype. However, also *H19* expression is decreased in this specific genotype indicating that functional studies should be performed to clarify the effects of PAE on gene expression at this complex locus.

When analyzing the newborns' phenotype we observed a genotype-specific association between alcohol exposure and the relative HC of newborns. The smallest HCs are in the genotype patG/matA and, interestingly, in this particular genotype the expression of the negative growth controller *H19* was the most increased when compared to *IGF2* expression in placenta. The alcohol-exposed newborns with an A/A genotype have the largest HCs and only one HC was below population average. This same A/A genotype has been associated with the strongest growth phenotype in infantile hemangiomas in a recent study (Schultz *et al.* 2015). Our finding could explain the observations in previous studies: a lack of correlation between the newborns' HC and maternal alcohol consumption (O'Callaghan *et al.* 2003) or the decreased correlation between HC and brain volume of alcohol-exposed children (Treit *et al.* 2016). The results suggest that the effect of alcohol exposure on HC depends to some extent on genotype, advocating the use of genetic information in future diagnostics of alcohol-induced disorders.

This work suggests that there are genotype-specific alcohol-induced effects on regulation of the *IGF2/H19* locus, which could disturb the balance of gene expression. Although both of the rs10732516 alleles are common in many populations (Auton *et al.* 2015), the polymorphism could prove meaningful under suboptimal developmental circumstances. Interestingly, mutations in *CTCF* gene, which encodes a protein that is pivotal for function of the *IGF2/H19* locus, have been identified in individuals with mild intellectual disability, growth retardation, microcephaly, cleft palate and congenital heart defects (Gregor *et al.* 2013), which all are characteristics of alcohol-induced defects as well. However, despite the interesting associations that we have found, this study does not clarify the molecular mechanisms behind the effects of alcohol exposure on this complex locus or demonstrate causality for observed alterations. Although we did observe genotype-

specific changes in DNA methylation profile, we have explored only a very restricted genomic region with relatively small alterations, and thus the biological significance of these particular alterations are obscure. On the basis of this study, we suggest considering the rs10732516 polymorphism in future studies of environmental effects on the function of *IGF2/H19* locus.

We are aware of some limitations in this study. We have been able to concentrate only on maternal alcohol consumption, although previous studies suggest that also paternal alcohol consumption can alter the methylation level of *H19* ICR in sperm (Ouko *et al.* 2009). We have attempted to exclude multiple substance abusers from this study, but common factors such as smoking and antidepressants (Supplementary Table SI) can also affect embryonic development. Previous genome-wide studies have shown that smoking affects the methylation level in placenta, but changes at the *IGF2/H19* locus have not been reported (Suter *et al.* 2011; Maccani *et al.* 2013). Furthermore, we did not observe an association between smoking and specific changes in DNA methylation in this study. We need to also consider that the amount and the timing of consumed alcohol is self-reported by mothers and there can be considerable inaccuracy in this personal evaluation (Lange *et al.* 2014) (Supplementary Table SI). All the mothers have had serious problems with alcohol consumption at least in the beginning of pregnancy and according to the AUDIT scores (score 15 or above (Babor *et al.*, 2001)) or amount of reported alcohol units (over 12 units per week (Rehm *et al.*, 2015)), there are heavily alcohol-exposed placentas and newborns in each genotype. However, the advantages of performing a genetic study in the Finnish population are the relatively subtle genetic, socioeconomic and environmental differences among participants. This, together with the new national growth charts, may have benefited us when discerning genotype-specific alcohol-induced effects, even with a relatively small sample size.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

Acknowledgements

We thank all participants for their invaluable contributions to the study. We thank Annalaura Jokiniemi and Anni Moilanen for technical assistance, Arttu Ahola for proofreading the manuscript and research nurse Teija Karkkulainen.

Authors' roles

H.M. and N.K.-A. collected the biological samples, designed the study and performed laboratory experiments as well as the statistical analysis of the data. H.K. was responsible for examining the case mothers and recruiting mothers to the study. H.K. collected the clinical data. H.M. and N.K.-A. wrote the manuscript. All authors have read, commented and approved the final manuscript.

Funding

The Academy of Finland (258304) (N.K.-A.), The Finnish Foundation for Alcohol Studies (N.K.-A. and H.M.), Finnish Cultural Foundation (N.K.-A. and H.M.), Juho Vainio Foundation (N.K.-A.), Yrjö Jahnsson Foundation (N.K.-A.) and Arvo and Lea Ylppö Foundation (N.K.-A.).

Conflict of interest

None declared.

References

Adkins RM, Somes G, Morrison JC, Hill JB, Watson EM, Magann EF, Krushkal J. Association of birth weight with polymorphisms in the IGF2, H19, and IGF2R genes. *Pediatr Res* 2010;**68**:429–434.

Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA *et al.* Genomes Project C: a global reference for human genetic variation. *Nature* 2015;**526**:68–74.

Babor TF, Higgins-Biddle JC, Saunders JB, Monteiro MGWorld Health Organization: AUDIT The Alcohol Use Disorders Identification Test, Guidelines for Use in Primary Care. 2001.

Bartholdi D, Krajewska-Walasek M, Ounap K, Gaspar H, Chrzanowska KH, Ilyana H, Kayserili H, Lurie IW, Schinzel A, Baumer A. Epigenetic mutations of the imprinted IGF2-H19 domain in Silver-Russell syndrome (SRS): results from a large cohort of patients with SRS and SRS-like phenotypes. *J Med Genet* 2009;**46**:192–197.

Benonisdottir S, Oddsson A, Helgason A, Kristjansson RP, Sveinbjornsson G, Oskarsdottir A, Thorleifsson G, Davidsson OB, Arnadottir GA, Sulem G *et al.* Epigenetic and genetic components of height regulation. *Nat Commun* 2016;**7**:13490.

Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulphite sequencing. *Bioinformatics* 2005;**21**:4067–4068.

Carnahan MN, Veazey KJ, Muller D, Tingling JD, Miranda RC, Golding MC. Identification of cell-specific patterns of reference gene stability in quantitative reverse-transcriptase polymerase chain reaction studies of embryonic, placental and neural stem models of prenatal ethanol exposure. *Alcohol* 2013;**47**:109–120.

Castillo-Fernandez JE, Loke YJ, Bass-Stringer S, Gao F, Xia Y, Wu H, Lu H, Liu Y, Wang J, Spector TD *et al.* DNA methylation changes at infertility genes in newborn twins conceived by in vitro fertilisation. *Genome Med* 2017;**9**:1–15.

Coolen MW, Statham AL, Gardiner-Garden M, Clark SJ. Genomic profiling of CpG methylation and allelic specificity using quantitative high-throughput mass spectrometry: critical evaluation and improvements. *Nucleic Acids Res* 2007;**35**:e119.

Coolen MW, Statham AL, Qu W, Campbell MJ, Henders AK, Montgomery GW, Martin NG, Clark SJ. Impact of the genome on the epigenome is manifested in DNA methylation patterns of imprinted regions in monozygotic and dizygotic twins. *PLoS One* 2011;**6**:e25590.

DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 1991;**64**:849–859.

Downing C, Balderrama-Durbin C, Broncucia H, Gilliam D, Johnson TE. Ethanol teratogenesis in five inbred strains of mice. *Alcohol Clin Exp Res* 2009;**33**:1238–1245.

Feldman HS, Jones KL, Lindsay S, Slymen D, Klonoff-Cohen H, Kao K, Rao S, Chambers C. Prenatal alcohol exposure patterns and alcohol-related birth defects and growth deficiencies: a prospective study. *Alcohol Clin Exp Res* 2012;**36**:670–676.

Gabory A, Ripoche MA, Le Digarcher A, Watrin F, Ziyyat A, Forne T, Jammes H, Ainscough JF, Surani MA, Journot L *et al.* H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development* 2009;**136**:3413–3421.

Gregor A, Oti M, Kouwenhoven EN, Hoyer J, Sticht H, Ekici AB, Kjaergaard S, Rauch A, Stunnenberg HG, Uebe S *et al*. De novo mutations in the genome organizer CTCF cause intellectual disability. *Am J Hum Genet* 2013;**93**:124–131.

Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 2000;**405**:486–489.

Haycock PC, Ramsay M. Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region. *Biol Reprod* 2009;**81**:618–627.

Heijmans BT, Kremer D, Tobi EW, Boomsma DI, Slagboom PE. Heritable rather than age-related environmental and stochastic factors dominate variation in DNA methylation of the human IGF2/H19 locus. *Hum Mol Genet* 2007;**16**:547–554.

Hoyme HE, Kalberg WO, Elliott AJ, Blankenship J, Buckley D, Marais AS, Manning MA, Robinson LK, Adam MP, Abdul-Rahman O *et al*. Updated clinical guidelines for diagnosing fetal alcohol spectrum disorders. *Pediatrics* 2016:138:e20154256.

Joya X, Salat-Batlle J, Velezmoro-Jauregui G, Clave S, Garcia-Algar O, Vall O. Prenatal ethanol exposure and placental hCG and IGF2 expression. *Placenta* 2015;**36**:854–862.

Kaminen-Ahola N, Ahola A, Maga M, Mallitt KA, Fahey P, Cox TC, Whitelaw E, Chong S. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet* 2010;**6**:e1000811.

Lange S, Shield K, Koren G, Rehm J, Popova S. A comparison of the prevalence of prenatal alcohol exposure obtained via maternal self-reports versus meconium testing: a systematic literature review and meta-analysis. *BMC Pregnancy Childbirth* 2014;**14**:127.

Laufer BI, Mantha K, Kleiber ML, Diehl EJ, Addison SM, Singh SM. Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. *Dis Model Mech* 2013;**6**: 977–992.

Lewis SJ, Zuccolo L, Davey Smith G, Macleod J, Rodriguez S, Draper ES, Barrow M, Alati R, Sayal K, Ring S *et al*. Fetal alcohol exposure and IQ at age 8: evidence from a population-based birth-cohort study. *PLoS One* 2012;**7**:e49407.

Liu Y, Balaraman Y, Wang G, Nephew KP, Zhou FC. Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 2009;**4**:500–511.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402–408.

Loke YJ, Galati JC, Saffery R, Craig JM. Association of in vitro fertilization with global and IGF2/H19 methylation variation in newborn twins. *J Dev Orig Health Dis* 2015;**6**:115–124.

Maccani JZJ, Koestler DC, Houseman EA, Marsit CJ, Kelsey KT. Placental DNA methylation alterations associated with maternal tobacco smoking at RUNX3 gene are also associated with gestational age. *Epigenomics* 2013;**5**:619–630.

Marjonen H, Sierra A, Nyman A, Rogojin V, Grohn O, Linden AM, Hautaniemi S, Kaminen-Ahola N. Early maternal alcohol consumption alters hippocampal DNA methylation, gene expression and volume in a mouse model. *PLoS One* 2015;**10**:e0124931.

Nelissen EC, Dumoulin JC, Daunay A, Evers JL, Tost J, van Montfoort AP. Placentas from pregnancies conceived by IVF/ICSI have a reduced DNA methylation level at the H19 and MEST differentially methylated regions. *Hum Reprod* 2013;**28**:1117–1126.

O'Callaghan FV, O'Callaghan M, Najman JM, Williams GM, Bor W. Maternal alcohol consumption during pregnancy and physical outcomes up to 5 years of age: a longitudinal study. *Early Hum Dev* 2003; **71**:137–148.

Ollikainen M, Smith KR, Joo EJ, Ng HK, Andronikos R, Novakovic B, Abdul Aziz NK, Carlin JB, Morley R, Saffery R *et al*. DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. *Hum Mol Genet* 2010;**19**:4176–4188.

Ouko LA, Shantikumar K, Knezovich J, Haycock P, Schnugh DJ, Ramsay M. Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: implications for fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2009;**33**:1615–1627.

Petry CJ, Seear RV, Wingate DL, Acerini CL, Ong KK, Hughes IA, Dunger DB. Maternally transmitted foetal H19 variants and associations with birth weight. *Hum Genet* 2011;**130**:663–670.

Phillips JE, Corces VG. CTCF: master weaver of the genome. *Cell* 2009; **137**:1194–1211.

Popova S, Lange S, Shield K, Mihic A, Chudley AE, Mukherjee RA, Bekmuradov D, Rehm J. Comorbidity of fetal alcohol spectrum disorder: a systematic review and meta-analysis. *Lancet* 2016;**387**:978–987.

Portales-Casamar E, Lussier AA, Jones MJ, MacIsaac JL, Edgar RD, Mah SM, Barhdadi A, Provost S, Lemieux-Perreault LP, Cynader MS *et al*. DNA methylation signature of human fetal alcohol spectrum disorder. *Epigenetics Chromatin* 2016;**9**:25.

Rancourt RC, Harris HR, Michels KB. Methylation levels at imprinting control regions are not altered with ovulation induction or in vitro fertilization in a birth cohort. *Hum Reprod* 2012;**27**:2208–2216.

Rehm J, Gmel G, Probst C, Shield KD. *Lifetime-Risk of Alcohol-Attributable Mortality Based on Different Levels of Alcohol Consumption In Seven European Countries. Implications for Low-Risk Drinking Guidelines*. Toronto, On, Canada: Centre for Addiction and Mental Health, 2015.

Renteria ME, Coolen MW, Statham AL, Choi RS, Qu W, Campbell MJ, Smith S, Henders AK, Montgomery GW, Clark SJ *et al*. GWAS of DNA methylation variation within imprinting control regions suggests parent-of-origin association. *Twin Res Hum Genet* 2013;**16**:767–781.

Roozen S, Peters GJ, Kok G, Townend D, Nijhuis J, Curfs L. Worldwide prevalence of fetal alcohol spectrum disorders: a systematic literature review including meta-analysis. *Alcohol Clin Exp Res* 2016;**40**:18–32.

Russell M, Czarnecki DM, Cowan R, McPherson E, Mudar PJ. Measures of maternal alcohol use as predictors of development in early childhood. *Alcohol Clin Exp Res* 1991;**15**:991–1000.

Sanchez Vega MC, Chong S, Burne TH. Early gestational exposure to moderate concentrations of ethanol alters adult behaviour in C57BL/6J mice. *Behav Brain Res* 2013;**252**:326–333.

Sankilampi U, Hannila ML, Saari A, Gissler M, Dunkel L. New population-based references for birth weight, length, and head circumference in singletons and twins from 23 to 43 gestation weeks. *Ann Med* 2013;**45**: 446–454.

Schultz B, Yao X, Deng Y, Waner M, Spock C, Tom L, Persing J, Narayan D. A common polymorphism within the IGF2 imprinting control region is associated with parent of origin specific effects in infantile hemangiomas. *PLoS One* 2015;**10**:e0113168.

Streissguth AP, Dehaene P. Fetal alcohol syndrome in twins of alcoholic mothers: concordance of diagnosis and IQ. *Am J Med Genet* 1993;**47**: 857–861.

Suter M, Ma J, Harris A, Patterson L, Brown KA, Shope C, Showalter L, Abramovici A, Aagaard-Tillery KM. Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression. *Epigenetics* 2011;**6**:1284–1294.

Takai D, Gonzales FA, Tsai YC, Thayer MJ, Jones PA. Large scale mapping of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer. *Hum Mol Genet* 2001;**10**:2619–2626.

Tobi EW, Slagboom PE, van Dongen J, Kremer D, Stein AD, Putter H, Heijmans BT, Lumey LH. Prenatal famine and genetic variation are

independently and additively associated with DNA methylation at regulatory loci within IGF2/H19. *PLoS One* 2012;**7**:e37933.

Treit S, Zhou D, Chudley AE, Andrew G, Rasmussen C, Nikkel SM, Samdup D, Hanlon-Dearman A, Loock C, Beaulieu C. Relationships between head circumference, brain volume and cognition in children with prenatal alcohol exposure. *PLoS One* 2016;**11**:e0150370.

Wang L, Wang F, Guan J, Le J, Wu L, Zou J, Zhao H, Pei L, Zheng X, Zhang T. Relation between hypomethylation of long interspersed nucleotide elements and risk of neural tube defects. *Am J Clin Nutr* 2010; **91**:1359–1367.

Warren KR, Li TK. Genetic polymorphisms: impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Res A Clin Mol Teratol* 2005;**73**:195–203.