University of the Witwatersrand
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EPIGENETIC MODIFICATION AT IMPRINTED LOCI FOLLOWING ALCOHOL EXPOSURE DURING PRENATAL DEVELOPMENT

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Declarations

I, Matshane Lydia Masemola, declare that this thesis is my own, unaided work, unless otherwise specified in the text. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this, or any other University.

Signed this 29th day of February, 2016

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Dedications

To my late grandmother Maryjane Mosadisadi “Tsakatse” Kgwedi

To my parents, siblings, husband and my two boys

Publications and Presentations arising from this study

The following publications and presentations arose from this research study

Publications

- Masemola M.L., van der Merwe L., Lombard Z., Viljoen D., Ramsay M. (2015) Reduced DNA methylation at PEG3 DMR and KvDMR1 loci in children exposed to alcohol in utero: a South African Fetal Alcohol Syndrome cohort study; Frontiers in Genetics, 6: 85.

Poster presentations

- 2010 ISBRA World Congress in Paris, France (13-16 September 2010)
  - Masemola ML, Lombard Z, Ramsay M, DNA methylation profiles of the IGF2/H19 imprinting control region (ICR) in subjects with fetal alcohol syndrome (FAS)
- 2010 WITS Faculty Research day and at the 3rd Cross Faculty Postgraduate Symposium (2010)
  - Masemola ML, Lombard Z, Ramsay M, DNA methylation profiles of the IGF2/H19 imprinting control region (ICR) in subjects with fetal alcohol syndrome (FAS)
- Joint AfSHG and SASHG Conference in Cape Town (6th to 9th March 2011)
  - Masemola ML, Lombard Z, Ramsay M, Epigenetic modification at imprinted loci following alcohol exposure during prenatal development

Abstract

Fetal alcohol syndrome (FAS) is a devastating developmental disorder resulting from alcohol exposure during fetal development. It is a considerable public health problem worldwide, but in several communities in South Africa, specifically in the Western and Northern Cape, it has an exceptionally high prevalence of 68.0 – 89.2 per 1000 children of school going age. FAS is a developmental disorder characterised by facial dysmorphic features, growth retardation and central nervous system abnormalities. Twin concordance studies and animal models suggest that there are genetic and epigenetic susceptibility factors for developing FAS. Imprinted genes are known to play an important role in growth and development and most of them are located in imprinted clusters. The *IGF2/H19*, *DLK1/MEG3 (GTL2)*, *CDKNIC/ KCNQ1OT1* and *PEG3* imprinted loci play a critical role in fetal development. Each of these imprinted loci contain several imprinted genes that are reciprocally imprinted, and their differential expression is controlled by differentially methylated regions (DMR) referred to as imprinting control regions (ICRs). The ICR for *IGF2/H19* is called *H19 ICR* and for *DLK1/MEG3 (GTL2)* is *IG-DMR* and they are both marked with DNA methylation on their paternal allele. *KvDMR1* and *PEG3 DMR* are ICRs for *CDKNIC/KCNQ1OT1* and *PEG3* imprinted loci respectively and they are marked with methylation on their maternal allele. DNA methylation at CpG dinucleotides is an epigenetic modification that is important in regulating gene expression during embryogenesis. It is proposed that alcohol-associated alterations in fetal DNA methylation at the four ICRs may contribute to developmental abnormalities seen in FAS and which persist into adulthood.

The aim of the study was to examine the effect of maternal alcohol consumption during pregnancy on DNA methylation profiles at specific ICRs (*H19 ICR, IG-DMR, KvDMR1* and *PEG3 DMR)* between FAS offspring and unaffected controls. The participants were FAS children and controls from the Western and Northern Cape Province. DNA samples extracted from blood and buccal tissues were bisulphite modified and the ICRs were amplified by PCR. The pyrosequencing method was used to derive a quantitative estimate of methylation at selected CpG dinucleotides. Analyses were done for *H19 ICR* (6 CpG sites; 50 controls and 73 cases); *KvDMR1* (7 CpG sites; 55 controls and 86 cases); *IG-DMR* (10 CpG sites; 56 controls and 84 cases) and PEG3 *DMR* (7 CpG sites; 50 controls and 79 cases).

Age and gender are reported confounders in DNA methylation studies and their effects were investigated in the present study. In this study age was shown to influence methylation at three of the four loci investigated, *IG-DMR*, *KvDMR1* and *PEG3 DMR*. The effect of gender on methylation was shown to be significant at only one locus, *PEG3 DMR*. After adjusting for gender and age, there was a significant difference in methylation (CpG specific and locus averaged) at *KvDMR1* and *PEG3* DMR but not at the *H19 ICR*, with only a small effect on average methylation (0.84% lower in cases; p=0.035) at *IG-DMR*. The two maternally imprinted loci, *KvDMR1* and *PEG3 DMR,* showed significantly lower locus averaged methylation in the FAS cases (1.49%; p<0.001 and 7.09%; p=0.001, respectively). Hypomethylation at the *KvDMR1* was unexpected since reduced methylation at *KvDMR1* has been associated with Beckwith Wiedemann Syndrome, an overgrowth syndrome. The largest effect was observed at the *PEG3 DMR*, which regulates the paternal *PEG3* gene expression in the brain, but we are yet to understand its impact on the FAS phenotype. This study provides supportive evidence for the role of epigenetic modulation as a mechanism for the teratogenic effect of alcohol by altering the methylation profiles of ICRs of imprinted loci in a locus-specific manner.

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Abbreviations

α	Alpha
β	Beta
λ	Lambda
^{o} C	Degrees Celsius
µg	Micrograms(s)
µl	Microlitre(s)
µM	Micromolar
µmol	Micromoles
%	Percentage
A	Absorbance
OD	Optical density
ADP	Adenosine diphosphate
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate
BLAST	Basic Local Alignment Tool
Bp	Base pair
BWS	Beckwith-Weidemann Syndrome
CHIP	C-terminus of heat shock cognate 70 stress protein-interacting protein
CYPE21	Cytochrome P450 E21
cm	Centimetre
CO_2	Carbon dioxide
CTCF	CCCTC-binding factor
Da	Daltons

dH_2O	distilled water
ddH_2O	deionised distilled water
DMR	differentially methylated region
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase protein
dsDNA	Double-stranded DNA
EDTA	Ethylene diamine tetra-acetic acid
EtOH	Ethanol
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorders
g	Gram(s)
g	Gravitational force
GSH	Glutathione
HAT	Histone acetyltransferase
HDAC	Histone deacetylace protein
HMTases	Histone methyltransferase protein
ICR	Imprinting control region
K	Lysine residue
kBp	Kilo base pair
kDa	Kilodaltons
L	Litre
MAT	Methionine adenosyltransferase
MBD	Methyl-CpG binding domain protein
MeCP	Methyl-CpG binding protein
M	Molar
min	Minute(s)

mg	Milligram(s)
ml	Millilitre(s)
mM	Millimolar
mmol	Millimoles
mRNA	Messenger RNA
MS	Methionine synthase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
ng	Nanogram(s)
OCM	One carbon metabolism
PCR	Polymerase Chain Reaction
PGC	Primordial germ cell
pH	-log $[H^+]$
Pi	Inorganic phosphate
PPi	Pyrophosphate
PWS	Prader- Willi Syndrome
RNA	Ribonucleic acid
RSS	Russell-Silver syndrome
RT	Room temperature
rpm	Revolutions per minute
s	Second(s)
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulphate
SRS	Silver-Russell syndrome
Ta ^{o}C	Annealing temperature

TBE buffer	Tris-Borate-EDTA buffer
TBS	Tris buffered saline
TE buffer	Tris-EDTA buffer
TEMED	N,N,N,N-tetramethylenediamine
TET	Ten-eleven translocation family of dioxygenases
Tris	Tris-2-amino-2(hydroxymethyl)-1,3-propandiol
XCI	X-chromosome inactivation
Xist	X-inactive specific transcription pattern mouse gene
U	Units
µl	Microlitre (s)
UV	Ultraviolet
V	Volt

Nucleic Acids	Code
Adenosine	A
Cytosine	C
Guanine	G
Thymine	T
Uracil	U

The nomenclature used the five nucleic acids are listed above as outlined by the International Union of Pure and applied Chemists (IUPAC) and the international Union of Biochemistry and Molecular Biology (IUBMB).

1. Introduction

1.1. Overview/prenatal alcohol exposure

Alcohol consumption during pregnancy results in a wide range of detrimental effects on the individual exposed (Sokol et al., 2003). Alcohol is a teratogen (an agent that is able to cause birth defects) and therefore can have devastating effects on the developing embryo and fetus (Riley et al., 2011). The most profound prenatal alcohol exposure effects are manifested on physical, cognitive and behavioural outcomes with possible lifelong implications (Floyd et al., 2006, Kleiber et al., 2014, Riley and McGee, 2005). Thus the consequences of prenatal alcohol exposure represent a major public health problem worldwide, having a wide range of effects on the economy (Health, Educational and Social Services (May and Gossage, 2001, Riley et al., 2011).

1.2. History of Fetal Alcohol Syndrome (FAS)

The knowledge of the harmful effect of maternal alcohol consumption on the fetus seems to date back to biblical and ancient times, even though it was not understood it was noted or suspected. A biblical passage in the Old Testament states that an angel spoke to the mother of the legendary hero, Sampson, before he was conceived and said "Thou shall conceive, and bear a son. Now therefore, beware, I pray thee, and drink no wine or strong drink" (Judges, 13: 3 – 4, King James I Holy Bible). Some of the ancient connections can be traced back to the ancient Greek and Roman beliefs that alcohol consumption at the time of procreation results in the birth of a damaged child (Green, 1974). There was also an ancient Carthaginian custom that prohibited bridal couple from drinking on the wedding night in order to prevent the conception of defective children (Jones and Smith, 1973, Calhoun and Warren, 2007). In the 1700s there was a gin epidemic in England after the country lifted restriction on the consumption of spirits, this led to cheap gin being readily available resulting in widespread abuse of gin drinking. Several physician groups during that period described children born to alcoholic women as "weak, feeble and distempered children" (Abel, 2001, Calhoun and Warren, 2007).

Physical and behavioral abnormalities together with medical disorders associated maternal alcohol drinking were first documented in the medical literature in 1968 by French

researchers Lemoine and colleagues who reported these characteristics in over 100 children born to mothers who drank heavily during their pregnancies (Calhoun and Warren, 2007, Jones, 2011, Koren, 2012, Lemoine et al., 1968). Their article described all the characteristics which were later referred to as the features of Fetal Alcohol Syndrome (FAS), however it did not lead to wide recognition of the deleterious effect of alcohol on the fetal development in France or elsewhere in Europe (Calhoun and Warren, 2007, Koren, 2012).

FAS was first specifically labelled as such in 1973 by two pediatric dysmorphologists, Kenneth L Jones and David W Smith, from the University of Washington, Seattle, USA (Jones and Smith, 1973) after they noted aberrant physical features in eight unrelated children of three different ethnic groups (native Americans, black and white), all born to chronic alcoholic mothers. The infants had similar patterns of craniofacial, limb and cardiovascular defects. These children were brought to their attention by a hospital pediatrician Dr Christy N Ulleland after observing growth deficiency and developmental delays in them (Abel, 1995, Jones and Smith, 1973, Jones et al., 1973). The infants were also diagnosed by the child psychologist Ann P Streissguth to have aberrant intellectual, motor and behavioral performances. By labelling the characteristics of FAS in their publication, Jones and his colleagues managed to get the worldwide attention of the public and scientific community, and initiated a new branch of research and clinical practice into the impact of alcohol on the developing fetus (Sanders, 2009, Streissguth et al., 1994)

1.3. Fetal alcohol spectrum disorders (FASD) and Fetal alcohol syndrome (FAS)

As mentioned above prenatal alcohol consumption during pregnancy results in a wide range of effects on the individuals exposed, these effects are collectively known as fetal alcohol spectrum disorders (FASD) (Sokol et al., 2003). FASD is a non-diagnostic term but an umbrella term used to describe the range of effects on an individual due to prenatal alcohol exposure (Riley et al., 2011).

FASD include the following classifications according to the Institute of Medicine (IOM) i.e. Fetal alcohol syndrome (FAS) with or without confirmed maternal alcohol exposure; Partial FAS (pFAS) with confirmed history of maternal alcohol exposure (when there is a confirmed history of prenatal alcohol exposure and some components of the full syndrome but not

enough to establish the diagnosis of FAS)(Stratton et al., 1996); Alcohol Related Birth Defects (ARBD) is used when prenatally exposed children without FAS facial features have other alcohol related physical abnormalities of the skeleton and other organ systems; and Alcohol Related Neurodevelopmental Disorders (ARND) is used when there is serious alcohol induced mental impairment on prenatally exposed children but no characteristic facial defects and growth deficiency seen in FAS (Chudley et al., 2005, Hoyme et al., 2005, Stratton et al., 1996, Welch-Carre, 2005). FAS represents the more severe end of the spectrum.

FAS is one of the leading causes of non-genetic preventable mental retardation and developmental disabilities in the world. It is an international problem that shows no racial boundaries (Clarren and Smith, 1978, Masotti et al., 2006). Children with FAS can be diagnosed by the following characteristics in three distinct areas (Landgraf et al., 2013):

1. Three key facial dysmorphic features i.e. shorter palpebral fissures (abnormally small space between the inner and outer canthus of each eye), smooth philtrum (absence of grooves on the upper lip leading to the nose) and thin vermilion border to the upper lip (abnormally thin upper lip with a distinct border) (Figure 1).
2. Growth retardation (pre and postnatal) e.g. height and weight less than 10^{th} percentile.
3. Central nervous system (CNS) abnormalities e.g. head circumference less than 10^{th} percentile, mental retardation, hyperactivity, learning disabilities and poor social skills.

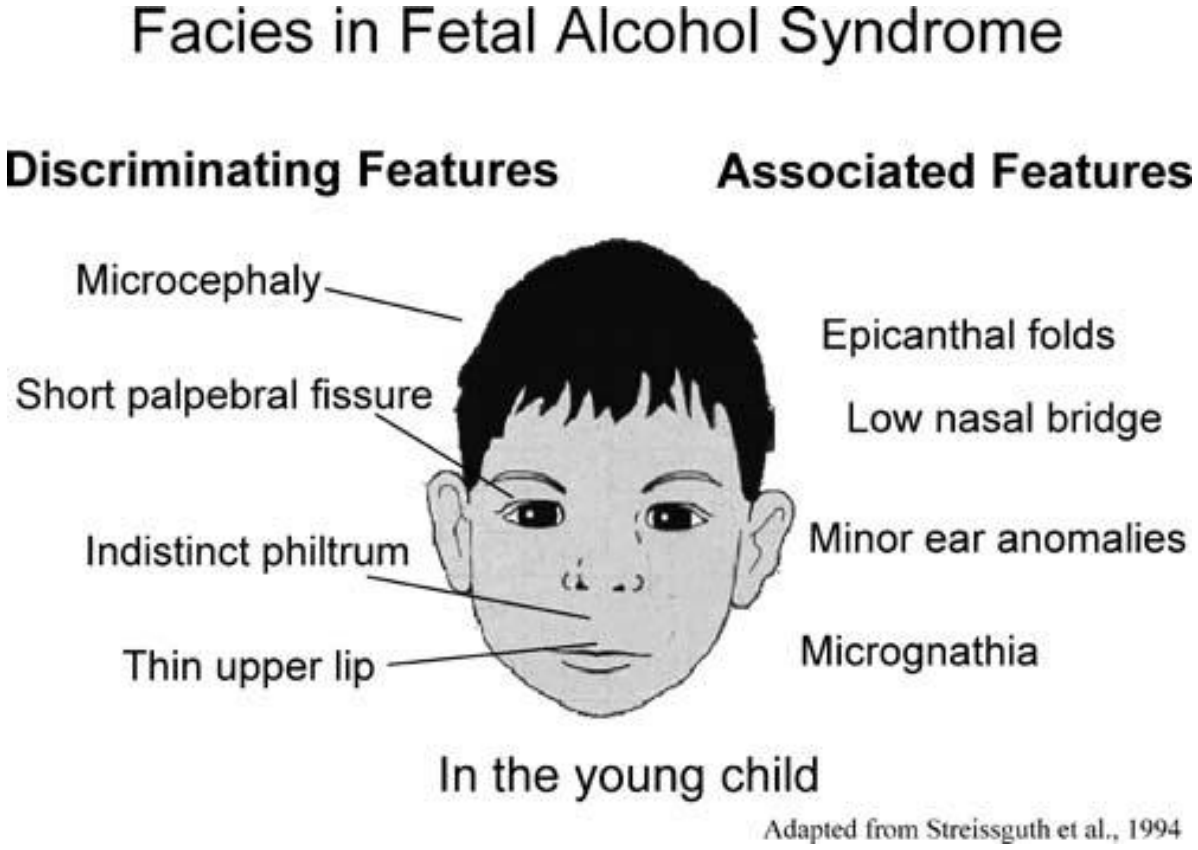


Figure 1: The facial phenotype of FAS

A smooth philtrum, thin vermillion border to the upper lip, and short palpebral fissures are typically used in the diagnosis of FAS, although the other features are common (Riley and McGee, 2005).

Over the years the diagnosis of FAS has been expanded and refined (Douzgou et al., 2012, Riley et al., 2011) but still includes many anomalies described in the original publications (Jones and Smith, 1973, Jones et al., 1973). Currently there are four commonly used diagnostic schemas (Riley et al., 2011): 4-digit code (Astley and Clarren, 2000); National Task Force/CDC (Bertrand et al., 2005); Canadian Guidelines (Chudley et al., 2005) and Revised IOM (Hoyme et al., 2005). Despite the differences among all four schemas, they still rely on anomalies in three distinct areas i.e. prenatal and/or postnatal growth deficiency, central nervous system (CNS) abnormalities and characteristic facial dysmorphologies. All the three schemas agree on facial characteristics used to define FAS but differ on how many must be present to provide a diagnosis. Growth deficiency for FAS is defined as pre or postnatal weight or height below the 10^{th} percentile in all the schemas. The CNS dysfunction is the

most variable among the schemas because it has a wide range of potential deficits but is most consistently defined as evidence of a structural brain anomaly (Riley et al., 2011).

At birth, children with FAS are recognisable by their apparent growth deficiency and characteristic facial anomalies that tend to become less noticeable and adopt a more normal appearance as the child matures (Chaudhuri, 2000). Therefore less evident at birth, but far more devastating in the FAS children and their families, are the lifelong effects of alcohol-induced damage to the developing brain(Riley and McGee, 2005). The problems associated with the neurodevelopmental and behavioural characteristics related to FAS remain throughout life and change very little (Streissguth et al., 1991, Michaelis and Michaelis, 1994). In addition to a deficit in general intellectual functioning, individuals with FAS often demonstrate difficulties with learning, memory, problem solving and attention, mental health and social interaction (Kodituwakku, 2009).

Not all individuals who are prenatally exposed to high doses of alcohol develop all the diagnostic features of FAS, there are variability in the range of physical and behavioral outcomes. There are a number of predisposition factors that could influence the outcome of prenatal alcohol exposure, such as dose and drinking pattern, timing of exposure, genetic factors, and nutritional factors/status of the mother during pregnancy.

1.3.1. Dose and Drinking pattern

In general the amount of alcohol consumed is correlated with the severity of the outcome (Sood et al., 2001, Streissguth et al., 1989). However patterns of alcohol exposure can often moderate this effect (Bailey et al., 2004). Both the amount of alcohol consumed and drinking patterns of alcohol consumption are predisposition factors in the etiology of FAS. The more alcohol is consumed and the more quickly it is consumed, the higher the blood alcohol levels (BAL). The higher BAL will result in more alcohol passing freely across the placenta and entering the fetus' circulatory system. Since the fetus is compromised in its ability to eliminate the teratogenic alcohol, the more likely the given fetus will reach the threshold for developing FAS (Abel and Hannigan, 1995, Brien et al., 1983, Schneider et al., 2011). All teratogens including alcohol produce their effect within a range of exposures, below one level there may be no noticeable damage to the conceptus, above another level

there may be various anomalies while at very high levels the teratogen may be fatal to the embryo or fetotoxic.

There are drinking behaviours/patterns that have been defined as conferring significant risk for FAS, for example, one drink per day during pregnancy or, in the case of binge drinking, five drinks per episode (Sokol et al., 2003). However there is no threshold for alcohol's harmful effect on the fetus because there have been reports of deleterious outcomes for offspring prenatally exposed to small amounts of alcohol, for example half a drink per day (Sood et al., 2001). Binge drinking is defined as consumption of large amount of alcohol in a short period of time i.e. in one evening or day while chronic drinking is the consumption of one or two drinks everyday over a long period i.e. a week or a month. Binge drinking has been shown to produce more severe brain damage and behavioural changes than chronic drinking in rats (Thomas and Riley, 1998). Animal studies have shown that binge drinking, which causes a high peak of blood alcohol concentration produces more cellular damage and thus severe microencephaly, neural cell loss and behavioural deficit in the offspring. Chronic alcohol consumption produces a continuous but low blood alcohol concentration and causes a less severe phenotype (Bailey et al., 2004, Pierce and West, 1986). Long term studies in humans have confirmed that binge drinking in pregnant women may result in children who have severe cognitive and behavioural deficits (Maier and West, 2001).

1.3.2. Prenatal alcohol exposure during different periods of fetal development (timing of exposure)

Alcohol exposure during different periods of fetal development can greatly influence the pattern and severity of structural and functional abnormalities seen in FAS (Guerri et al., 2009).

The effect of alcohol exposure during different prenatal developmental stages was studied in animal models. High dosage of alcohol exposure in early gestation may results in fetal death, spontaneous abortion and premature birth (Kleiber et al., 2014). Exposure during the pre-differentiation period (which is approximately 6 days between fertilisation and implantation in both mice and humans) was shown to increase blastocyst death (resorption) however, malformations were observed, affecting craniofacial development, eye, urogenital and limb development in live born pups (Padmanabhan and Hameed, 1988). In the period of

embryonic development (period between early germ layer differentiation and completion of organ formation, which begins after implantation, which in humans is between 3-8 weeks of gestation (Figure 2) alcohol exposure was shown to produce craniofacial malformations resembling those seen in FAS, as well as brain anomalies (Sulik, 2005, Lipinski et al., 2012). These malformations are due to the abnormal development of the neural crest and its derivatives. At this stage most cell types appear to be more vulnerable to alcohol induced cell death, more particularly the neural cell populations (Da Lee et al., 2004, Sulik et al., 1986, Sulik et al., 1981, Mantha et al., 2014). During the period of fetal development (interval from end of organogenesis until birth, week 9 to birth in humans (Figure 2) alcohol exposure has been shown to produce histological changes in tissues, inhibit growth and produce subtle damage to the developing CNS (often manifesting as neurobehavioral effects) and other organ systems by interfering with histogenesis, synaptogenesis, and formation of myelin and other biochemical processes (Ikonomidou et al., 2000, Stratton et al., 1996). It should be noted that the CNS develops throughout the gestation period and even up to adolescent stage(in humans), therefore it is constantly vulnerable to the harmful effects of alcohol (Kleiber et al., 2014). In rats reports have shown that heavy alcohol intake during the brain growth spurt during the early postnatal period, corresponding to the third trimester and early infancy in humans, significantly reduces the weight of the forebrain, brain stem and cerebellum (Chen et al., 1998, Maier et al., 1997). Heavy and frequent alcohol consumption during the first trimester has been shown to predominantly affect facial and structural features whereas drinking in the third trimester has adverse effects on growth (May et al., 2004). Therefore maternal alcohol consumption during any time of pregnancy is detrimental to the fetus or embryo.

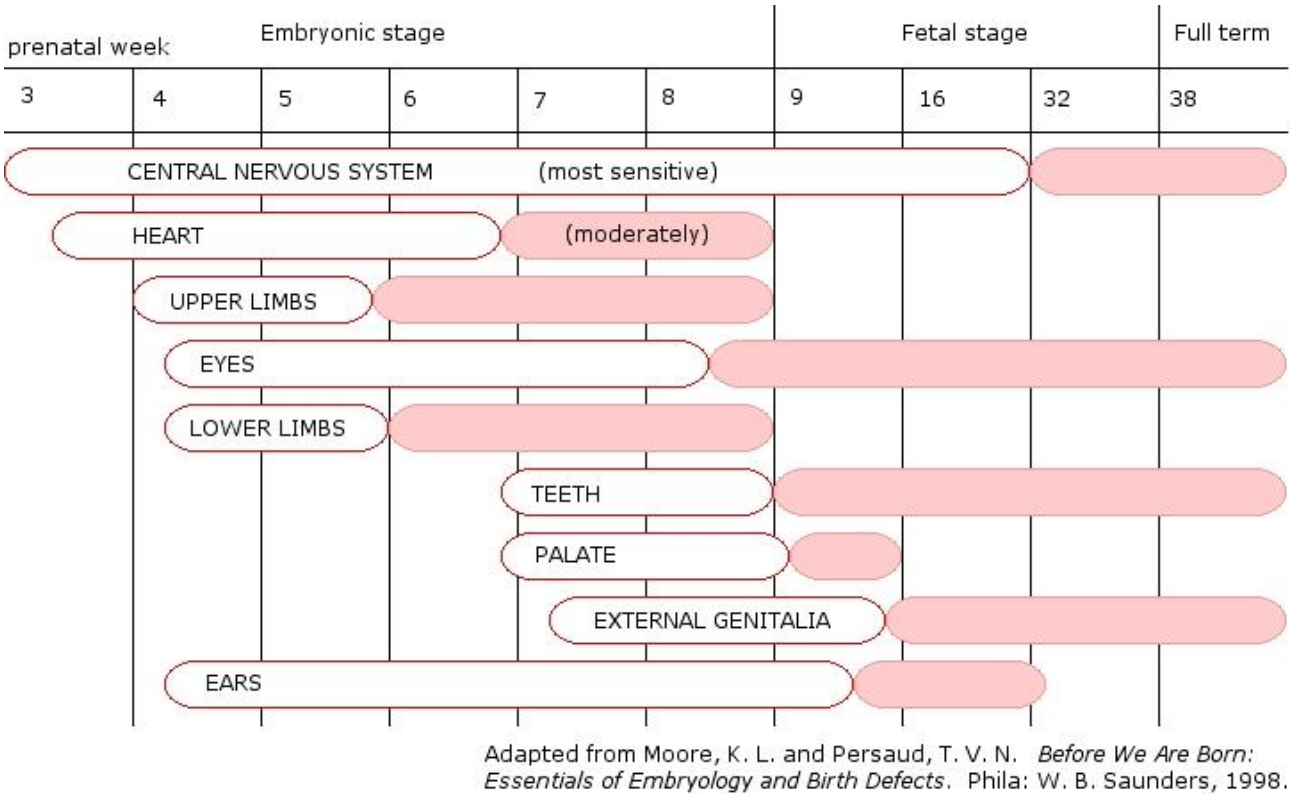


Figure 2: Stages off fetal organ development

Various stages of organ development and critical periods, at which the fetus is most susceptible to birth defects induced by teratogens. Pink indicates the highly sensitive periods where major defects may be produced. White represents the stages that are less sensitive to teratogenic effects where minor defects may be induced (Boeree, 2009).

1.4. Prevalence of FAS

The worldwide average prevalence of FAS in the high income countries is estimated at 0.97 per 1000 live births (Abel, 1995, May and Gossage, 2001, McKinstry, 2005). In a developed country like the United States (US) the rate of FAS was reported to be 0.5-2/1000 live births (May and Gossage, 2001). Prevalence rates among selected American Indian reservation communities group in the US, who were considered at high-risk, did not exceed 10/1000 live births (May et al., 2000, Viljoen et al., 2005). Recently May et al., (2009), reported the prevalence of FAS as 2-7 per 1000 school children in the US. It appears that FAS is on the increase, but this could partly be due to an increased awareness of the condition and a correlated increase in the diagnosis rate (May et al., 2009).

The prevalence of FAS in South Africa is one of the highest reported in the world, especially in the populations of the Western Cape (a wine producing areas) and Northern Cape where many of the FAS studies have been done. In the year 2000, the rates were reported at 40.5-46.4/1000 first grade children in regions of the Western Cape (May et al., 2000) and two follow up studies conducted in the same area has reported an increasing prevalence to 65.2-74.2/1000 first grade children (Viljoen et al., 2005) and 68-89.2/1000 first grade children (May et al., 2007). In a study done in the Northern Cape in primary school going children (De Aar-sheep farming area and Upington-wine farming area), the overall FAS rate of 67.2/1000 was reported for both areas, with the FAS rate being higher in De Aar than in Upington (Urban et al., 2008). This higher rate of FAS in a sheep farming area (De Aar) compared to a wine producing area (e.g. Upington), shows that high rates of FAS are not limited to wine farming areas. Another study supporting this fact was conducted in four areas of Johannesburg, Gauteng province in South Africa, where wine production is absent, where the reported FAS prevalence was 19/1000 children. Although the value is low compared to those of the Western Cape, it is still high compared to the rates worldwide (Viljoen et al., 2003). The most recent study by Urban et al., (2015), reported the prevalence of FAS to be 63/1000 first grade learners in predominantly mixed ancestry population of Roodepan, Northern Cape; and 52/1000 first grade learners in predominantly black population of Galeshewe, Northern Cape (Urban et al., 2015).

1.4.1. FAS and the Economy

Considering the high prevalence of FAS reported in the Western Cape and Northern Cape, it is clear that FAS is a considerable public health problem in these communities. Throughout the world FAS poses a huge economic burden on the health care system and also on the families of children having the condition, with a greater percentage of financial costs being incurred by the need for special schooling, home schooling and medical treatment (Popova et al., 2012, Stade et al., 2009). According to a study by Crede et al., (2011) children with FAS/PFAS from the Western Cape utilise the healthcare system on average 3 times more than those without the condition, indicating a significant financial burden that this condition puts on the health care system's budget. Again another burden was indicated on the social development system because children with FAS/PFAS are likely to be given foster care

grants and if they are suffering from severe or permanent disability would qualify for care dependency-grants throughout their lives (Crede et al., 2011).

1.4.2. Factors that influence the high prevalence of FAS

The reasons for the high prevalence of FAS in specific communities in the Western Cape and Northern Cape, South Africa, is not known or understood, but contributing factors may be multifaceted, as described below.

1.4.2.1. Maternal binge drinking and a history of alcohol abuse

Regular binge drinking (a heavy episodic drinking of five to more units of alcohol per occasion) is a drinking pattern that poses a high risk for FAS (Jacobson et al., 1998), and binge drinking is a well recognised problem drinking pattern in some South African communities (Bulletin, 2011, Marais et al., 2011). Binge drinking was reported to be high among women attending an antenatal clinic in the Western Cape (Croxford and Viljoen, 1999, Katwan et al., 2011), and 50% of mothers of FAS children reported drinking more heavily during their pregnancies (May et al., 2000, McKinstry, 2005). The mothers of FAS children in the Western Cape communities have been reported to come from families with a long history of generations of alcohol abuse and heavy drinking, this factor may be one influencing factor to maternal heavy drinking (Viljoen et al., 2002). This destructive behaviour can be blamed partly on the 'Dop' system.

1.4.2.2. Dop system

Dop or 'tot' system can be described as a historical practice of paying farm workers in part with alcohol, and was developed in the 1700s by colonial farmers in the Cape (London, 2000). Initially the payment consisted of bread, tobacco and wine and the tradition became an institutionalised element of the farming practice and played a key role in recruitment and retention of coastal people as farm workers. It was also used to get rid of low grade wine, which was sold to the farm workers for close to nothing (London, 1999). Even though the dop system was made illegal in 1961, alcohol abuse still prevails (as it is still favoured by many people and valued) in this population; this is due to the fact that the dop system entrenched a bad culture of alcohol abuse and a high prevalence of excessive alcohol consumption amongst the farm workers of the Western and Northern Cape (Gossage et al.,

2014), therefore the dop system can be partly blamed for the high prevalence of FAS in these populations.

1.4.2.3. Abundance of Shebeens (informal bars)

A Shebeen is a form of illegal informal bar where cheap, and often inferior alcohol, is sold (May et al., 2000). It is illegal because the owner usually does not have a license to sell alcohol. It is not regulated, and therefore alcohol can be accessed anytime of the day and night. According to Dennis Viljoen there are about 27 sheebens in a population of 28 000 in De Aar, Northern Cape Province (Viljoen, 2011) and 37 000 shebeens have been reported to exist in the Western Cape Province (Bulletin, 2011). Therefore the high number of shebeens in these communities contributes considerably to the alcohol abuse and maternal drinking in this population because alcohol is highly accessible at any time.

1.4.2.4. Poverty

The prevalence of FAS varies depending on the poverty of the population, the poorer the population, the higher the risk of developing FAS. The populations most at risk for FAS are overwhelmingly poverty stricken (Abel and Hannigan, 1995, Viljoen et al., 2002). Poverty is a factor of low socioeconomic status and it goes together with malnutrition or an unbalanced diet. A balanced diet is a requirement for normal pregnancy outcome and for normal fetal/embryonic development and growth and also for maintaining maternal health (Dreosti, 1993). Severe malnutrition has been shown to increase the risk of abortion, intrauterine growth retardation and impaired fetal brain development (Gabr, 1987) therefore it can be speculated that malnutrition with deficiencies of essential nutrients, as a resultant of poverty, may be an enhancing risk factor for the development of FAS (Keen et al., 2010). Maternal malnutrition with deficiencies of essential nutrients such as folate; coupled with maternal alcohol consumption during pregnancy may exacerbate the risk of developing FAS (Abel et al., 1995) and this may mediated through the disturbance of OCM.

OCM is critical pathway responsible for the production of universal methyl donor and other compound that are vital for DNA methylation and DNA synthesis respectively; and therefore it is important for normal fetal development (Bailey et al., 2012). Folate is a coenzyme that plays an important role as a source of methyl group in the OCM. Since humans cannot synthesise folate, dietary deficiencies due to poverty will lead to reduced availability of

methyl groups therefore leading to disturbances in the OCM and then DNA methylation (Wani et al., 2012). Alcohol intake on the other hand may cause folate deficiency through poor diet and eating less (as seen in alcoholics) and also by interfering with folate absorption and renal re-absorption (Hamid et al., 2009). Therefore maternal malnutrition in the presence of alcohol abuse during pregnancy will lead to unavailability or critically low levels of the essential nutrients like folate and other methyl groups, with the resultant of fetal malformation and FAS development.

Most of the FAS cases reported in the United State by epidemiological studies were diagnosed predominantly in the African - American population, uniformly characterised by poverty (2.29 cases per 1000 live births), compared to sites where the population was primarily Caucasian and middle class (0.26 per 1000)(Abel, 1995). The 11 FAS children originally diagnosed by Smith and Jones (Jones and Smith, 1973) were racially divergent e.g. Caucasians, African American and Native Americans, but what was similar in all the mothers was that they were all living on welfare. Therefore FAS does occur in people of all races but it occurs predominantly in people of low socioeconomic status (SES) regardless of race (Abel and Hannigan, 1995). The rural populations of the Western and Northern Cape are faced with high poverty levels like most of the rural areas in South Africa. The majority of them are farm workers and they are faced with the difficulties of having to live with extremely low wages (London, 2003) and hence this is a factor contributing to the high prevalence of FAS.

1.4.2.5. Paternal contribution to FAS

Most of mothers of FAS children from the Western Cape have alcoholic partners. Mothers have been reported to consume about 97% of their alcohol intake over the weekend, which is when they spend a significant time with their partners (Viljoen et al., 2002). It has been previously reported that 75% of children with FAS had heavy drinking or alcoholic biological fathers (Abel et al., 1983). This observation lead to the view that a number of FAS cases thought to be contributed by maternal alcohol abuse may have resulted due excessive paternal drinking (Abel, 2004). Animal models have also shown that paternal preconception alcohol abuse had a significant effect on neurobehaviour of subsequent offspring and congenital malformation, low birth weight, growth retardation and neonatal mortality were also reported (Friedler, 1996, Jamerson et al., 2004). Therefore since it has been reported in

the Western Cape that mothers of FAS children have partners that are heavy drinkers or alcoholics, it may be plausible that paternal contribution to FAS is a contributing factor to high prevalence of FAS in this community. Possible mechanism for preconception paternal alcohol consumption to the development of FAS may be through the disruption of epigenetic modifications in the male sperm DNA by alcohol. Therefore transmission of the abnormal sperm DNA epigenetic changes to the offspring may results in development of FAS phenotype. In a study on the sperm of males who consume alcohol, it was shown that epigenetic changes in sperm DNA were correlated with the amount of alcohol consumed (Ouko et al., 2009). A mouse model study by Knezovich and colleagues reported a significant reduction in DNA methylation at the *H19 ICR* in offspring of ethanol-treated sires which corresponded to reduced weight (Knezovich and Ramsay, 2012). Another mouse study also reported increase in hearing loss in offspring of chronic ethanol-exposed sires (Liang et al., 2015). Alcohol has been associated with diminished sperm quality, which in turn is correlated with altered epigenetic changes in specific regions of the sperm DNA (Marques et al., 2008).

1.5. Alcohol metabolism and genetic predisposition to FAS

It is well established that maternal alcohol consumption during pregnancy is the primary cause of FAS development. Both alcohol and its metabolites are teratogenic and therefore may directly cause damage to the fetus. When a woman drinks alcohol during pregnancy, ethanol is metabolised in the maternal liver by cytosolic alcohol dehydrogenase (ADH) to acetaldehyde, acetaldehyde is further oxidised to acetate and water by mitochondrial aldehyde dehydrogenase (ALDH) (Figure 3). Another enzyme, cytochrome P450 E21 (CYPE21), can also metabolise alcohol to acetaldehyde with the release of oxygen derived free radicals (reactive oxygen species-ROS) as a byproduct (Cederbaum et al., 2001). CYPE21 shows a much lower affinity for ethanol than ADH at moderate doses of alcohol. Excessive alcohol exposure and long term alcohol intake induces CYPE21 expression, and thus the rate of ethanol clearance (Howard et al., 2003). CYPE21 is expressed in the placenta, fetal liver and brain during organogenesis where the CYPE21-catalyzed ethanol oxidation may cause oxidative stress (Gemma et al., 2007). Both alcohol and acetaldehyde can freely cross the placental barrier to enter the fetal circulation. Due to its water solubility, ethanol is readily and uniformly distributed to the body water space of the mother and the fetus (Norberg et

al., 2003). ADH activity is low in human fetal liver throughout intrauterine life, far less so than in the adult. Alcohol is cleared more slowly from the fetal than the maternal circulation, its rate of elimination is far less than half that observed in the mother (Kaufman, 1997). Therefore the low activity of fetal ADH may be one factor contributing to fetal alcohol toxicity because it results in persistent high fetal blood alcohol concentration.

FAS is a complex multifactorial disease, even though prenatal alcohol exposure is the primary trigger for the presentation of FAS, twin concordance studies and animal models suggest that there may be genetic susceptibility to the development of FAS (Becker et al., 1996, Streissguth and Dehaene, 1993).

The first indication that genetic factors may underlie the vulnerability to prenatal alcohol induced adverse pregnancy outcome was by a single case report of significant discordance between a dizygotic twin pair born to an alcoholic mother. One twin was severely affected by FAS at birth while the other was minimally affected. The minimally affected twin would not have been recognised in the neonatal period if his other twin had not been so severely affected (Christoffel and Salafsky, 1975). Twin studies undertaken by Streissguth and Dehaene (1993) have demonstrated higher concordance for FAS among monozygous twins when compared to dizygous twins. Among 16 twin pairs (5 monozygotic and 11 dizygotic twin pairs) exposed to high levels of alcohol during gestation, all individuals of five monozygotic twin pairs and seven of the dizygotic twin pairs were equally affected. The remaining four dizygotic twin pairs showed discordance for FAS, suggesting that fetal genotype also may play a role in development of FAS (Streissguth and Dehaene, 1993). These monozygotic and dizygotic twin studies reflect the modulating influence of genes in the expression of the teratogenic effect of alcohol.

Another genetic factor that may influence vulnerability to FAS includes genetic variation in enzymes that metabolise alcohol, thus regulating the blood concentration of alcohol. The metabolic activity of the mothers' alcohol metabolising enzymes is one of the determinants of how much alcohol (or its metabolites) the fetus would be exposed to and thus the predisposition to FAS development (Gemma et al., 2007). There is a significant variation in human alcohol metabolism rates, of which 50% may be genetically determined. Polymorphism at the *ADH1B* allele has been proposed as one of the major determinants to

account for this variability (Arfsten et al., 2004). Allelic variants of *ADH1B* are *ADH1B*1*, *ADH1B*2* and *ADH1B*3* and their respectively encoded isoenzymes show different rates of alcohol metabolism (Warren and Li, 2005, Hurley and Edenberg, 2012). Isoenzymes encoded by *ADH1B*2* and *ADH1B*3* have higher rates of alcohol oxidation than those encoded by *ADH1B*1*, therefore individuals carrying these alleles (*ADH1B*2* and *ADH1B*3*) will have a faster and more efficient alcohol clearance than those carrying the *ADH1B*1* allele (Arfsten et al., 2004). Some *CYPE21* variants are associated with enhanced alcohol metabolic capacity and may modulate the risk for developing FAS (Gemma et al., 2007).

Several studies have evaluated the impact of genetic polymorphism of the *ADH* gene family on the risk of FAS in humans. Viljoen et al., (2001) found that the *ADH1B*2* allele (a rapid metaboliser of alcohol) was significantly more common in the control mothers, than in mothers of FAS affected children and FAS children in the population of the Western Cape (Viljoen et al., 2001). This suggests that the *ADH1B*2* allele may have a protective effect against FAS. In two different studies involving African American populations, the *ADH1B*3* allele (a rapid metaboliser of alcohol) was more highly represented in control mothers than in mothers of children with neurobehavioral abnormalities seen in association with prenatal alcohol exposure (Jacobson et al., 2006, McCarver et al., 1997). There are multiple reasons why the presence of *ADH1B*2* and *ADH1B*3* variants in the mother is associated with reduced risk for FAS development in children, this may be because the mothers drink less or that the alcohol is cleared rapidly and thus it is not present in the blood for longer period leading to less fetal exposure.

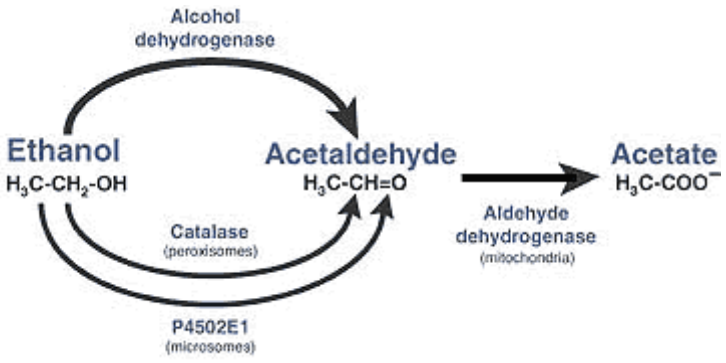


Figure 3: Alcohol Metabolism

ADH oxidises alcohol to acetaldehyde, and ALDH oxidises acetaldehyde to acetate and eventually to CO_2 and water to be eliminated by the body. Another enzyme, CYPE21, can also metabolise alcohol to acetaldehyde with the release of oxygen derived free radicals as a byproduct (U.S. Department of Health and Human Services, 2007)

1.6. Epigenetics as a mechanism of FAS

There is growing understanding that patterns of gene expression controlled by epigenetic imprints are vital for normal development and perturbation of these imprints underlies many states of pathology. Inappropriate or altered levels of gene expression have also been shown to play a role in cancers and other specific abnormalities (Laird and Jaenisch, 1996). It is for these reasons that the role of epigenetic modification as a mechanism for alcohol related effects is investigated for this study.

The Greek prefix “epi-“ in epigenetics implies features that are “on top of” or “in addition to” genetics, therefore epigenetics in fact means to act on “top of” or “in addition” to genetics (Meissner, 2009). A developmental biologist, Conrad Waddington, first coined the term epigenetics in 1938, which is derived from the Aristotelian word 'epigenesis’. He defined it as “the science concerned with causal analysis of development” (Jablonka and Lamb, 2002, Waddington, 1952). During that period there was no evidence that supported a genetic component of development, as it is presently understood. However Waddington’s statements and pictures certainly support that he understood development in terms of what is today called differential gene expression and regulation (Jablonka and Lamb, 2002). Over the years the definition of epigenetics has evolved and been refined. Robin Holliday broadly described epigentics as the “unfolding of genetic program for development” (Holliday, 2006). Later Adrian Bird defined epigenetics as “the structural adaptation of chromosomal regions, so as to register signals and perpetuate altered activity states” (Bird, 2007).

Therefore epigenetics can be defined as the study of heritable changes in gene expression patterns that are not caused by changes in the nucleotide sequence of the genetic code itself (Groom et al., 2011, Tost, 2009). There are three epigenetic mechanisms i.e. DNA methylation (biochemical alteration of DNA), modification of histones that package the DNA and non-coding or interfering RNAs, including micro RNAs. These mechanisms work together to produce a unique, and reversible epigenetic signature that regulates gene expression through chromatin remodelling.

1.6.1. DNA methylation

The most widely investigated epigenetic mechanism is DNA methylation, and therefore it is the best understood (Groom et al., 2011). DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes, DNA methylation provides a way to protect the host cells from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation has a function in regulating gene expression (Costello and Plass, 2001). Robin Holiday and John Pugh, suggested in 1975, that DNA methylation controls gene expression, therefore contributing to developmental changes by controlling gene activity (Holliday and Pugh, 1975). It was also suggested that this modification can serve as heritable epigenetic modification for cellular memory (Riggs, 1975). DNA methylation is a mitotically stable epigenetic mark that regulates gene expression, and together with chromatin structure, has been implicated in important processes like embryogenesis, genomic imprinting, X-chromosome inactivation, cell type specific gene expression and silencing of repetitive elements (Li, 2002).

DNA methylation involves the biochemical modification of the DNA; whereby there is addition of an methyl group to the fifth carbon position of the cytosine base pyrimidine ring, in the presence of DNA methyltransferases to form 5-methyl cytosine (Adams, 1995). The majority of DNA methylation in mammals occurs only in the context of CpG dinucleotides, where a cytosine residue is followed by guanine residue (5'...CpG...3'dinucleotides). These locations are referred to as CpG sites, the "p" indicating the phosphate group between the di-nucleotide pair.

The mammalian genome is globally depleted of CpGs, except at short DNA stretches called CpG islands (CGIs) (Siegfried and Simon, 2010). CGIs are defined as being longer than 500bp and having a GC content greater than 55% and observed CpG /expected CpG ratio of 0.65 (Takai and Jones, 2002). CGIs are often, but not always, found in promoter regions of genes. These include the 5' end of the promoters, 5' untranslated region and exon 1 (Jones and Baylin, 2002). The rest of the genome, such as the intergenic and intronic regions, is considered to be CpG poor. In healthy cells CpG sites in CpG poor regions are often methylated while the CpGs in CGIs are generally hypomethylated. Most CpGs in promoters are protected from methylation in somatic tissues (Schneider et al., 2010). Promoter

methylation leads to stable gene silencing during development /differentiation and may be involved in disease processes (Feinberg, 2007, Rollins et al., 2006, Weber et al., 2007). For example, the pluripotency genes are switched from a demethylated and transcriptionally active state in embryonic stem cells, to a fully methylated repressed state in somatic cells (Okita et al., 2007, Reik, 2007). On the other hand, tumor suppressor genes are demethylated and active in somatic cells, where the ectopic methylation begins early in cancer development (Esteller et al., 2000, Ting et al., 2006).

DNA methylation is generally associated with a repressed chromatin state and inhibition of promoter activity. DNA methylation usually conducts its transcriptional control in two ways: first, cytosine methylation can directly prevent binding of some transcription factors to their target sequences; secondly, DNA methylation can affect chromatin states indirectly by recruitment of methyl-CpG-binding protein (MeCP2) and methyl CpG binding domain protein (MBDs 1-4) and their associated repressive chromatin remodelling activities (Jaenisch and Bird, 2003, Klose and Bird, 2006). The MeCP2 and MBDs proteins recognise methylation sites on DNA then bind the methylated DNA and thus regulate genes by blocking the binding of RNA polymerase to the promoter (Baylin and Herman, 2000). Together these methyl-binding proteins function as transcriptional silencers. They recruit transcriptional co-repressors such as histone deacetylating complexes, polycomp proteins and chromatin remodelling complexes (Ballestar and Wolffe, 2001). Methylated DNA can also be bound by zinc finger proteins like Kaiso which are able to repress transcription in a methylation dependent manner (Tost, 2009).

1.6.2. Role of DNA methyltransferases (DNMTs) in DNA methylation

The activities of DNA methyltransferases (DNMTS) facilitate the epigenetic control of gene expression by cytosine methylation. They recognise the CpGs within the double stranded DNA as a substrate. DNMTs catalyse the transmethylation of cytosine by transferring the methyl group (CH_3) from the methyl donor S-adenosylmethionine (SAM) to position 5 of the pyrimidine ring, (Figure 4) (Hitchler and Domann, 2007). The reaction results in the production of 5-methyl cytosine (5-MeC) and cofactor S-adenosylhomocysteine (SAH). High concentrations of SAH inhibit the activities of methyltransferases. Mammalian one carbon

metabolism provides all methyl groups for all biological methylation reactions through synthesis of universal methyl donor, SAM (Ulrey et al., 2005).

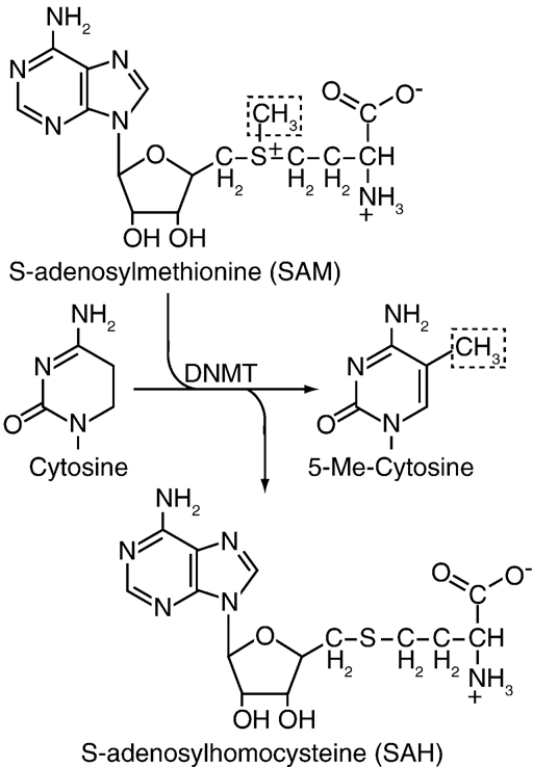


Figure 4: The transmethylation reaction catalysed by DNA transmethylases (DNMTs)

The DNMTs catalyse the transfer of methyl donor groups (dashed box) from SAM to 5 position of cytosine within CpG dinuceotides. The reaction results in the production of 5-Me-cytosine and SAH (Hitchler and Domann, 2007).

In mammals, five DNA methyltranferases (DNMTs) have been characterised and classified according to similarities found in their C-terminal catalytic domain. The enzymes are DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L (Goll and Bestor, 2005). Only DNMT1, 3a and 3b are catalytically active in methylating *in vivo*.

DNMT1 is the major methyltransferase in somatic tissue and has high preference for hemimethylated DNA (when only one strand of the double stranded DNA contains methylated CpGs). It maintains DNA methylation during replication by copying the DNA methylation of the old DNA strand onto the newly synthesised strand, and this occurs rapidly following DNA replication. It is localised to the replication fork during cellular division

(S phase of cell cycle) (Beaulieu et al., 2002). This interaction puts it in close proximity to the newly synthesised hemimethylated CpG dinucleotides (Leonhardt et al., 1992). It plays a primary critical role in maintenance of DNA methylation patterns during replication of DNA, passing on the DNA methylation mark and thus epigenetic control of gene expression to daughter cells (Kautiainen and Jones, 1985).

DNMT3A and DNMT3B are responsible for *de novo* methylation of DNA, because they are able to target the unmethylated CpG sites. The two enzymes have high affinity for unmethylated CpG dinucleotides which is critical for their role in *de novo* methylation(Okano et al., 1998). They are primarily responsible for initiating new epigenetic marks that regulate gene expression that can be passed on during cell division(Okano et al., 1999). They are also reported to work together with DNMT1 to propagate methylation patterns during cell divisions (Jia et al., 2007, Liang et al., 2002). *De novo* methylation can occur anytime following DNA replication to initiate new epigenetic events that can be passed on during future cell divisions. Again DNMT3a has been reported to have preference in methylating CpGs that are packed together while DNMT3b is more efficient at methylating isolated CpGs (Gowher and Jeltsch, 2002). These enzymes for maintenance of DNA methylation and *de novo* DNA methylation are important in generating and perpetuating epigenetic control of gene expression during development, gametogenesis and imprinting.

DNMT3L (accessory protein DNMT 3-like protein) lacks the functional catalytic domains and forms a heterotetramer with DNMT3a or DNMT3b (Jia et al., 2007, Kelsey, 2011). It potentiates DNA methylation through its interaction with DNMT3a and DNMT3b (Okano et al., 1999). It has been reported to stimulate the activities of its *de novo* partners, or guiding the recognition of DNA targets with particular periodicity of CpG sites (Jia et al., 2007). In mice Dnmt3a and Dnmt3l are key regulators of DNA methylation, they co-operate to *de novo* methylate DNA in the germ line. Dnmt3l functions to activate Dnmt3a and recognises the target sequence based on nucleosome modification and CpG spacing (Chedin et al., 2002). Female mice that lack either Dnmt3a or Dnmt3l are fertile but their heterozygous progeny lack the maternal imprint and the mice die before mid-gestation while male mice that lack these methylases are infertile and oligospermic (Kaneda et al., 2004). The last

methyltransferase, Dnmt2, has been shown to have a weak methylation activity *in vitro*, however it has been suggested to function as an RNA transferase *in vivo* (Goll et al., 2006).

DNA methylation facilitated by DNMTs activities is essential for mammalian development, and this has been shown by the lethality, developmental delays and abnormalities in various DNMTs deficient mice (Li, 2002, Li et al., 1999, Okano et al., 1999). Therefore through the normal activities of DNMTs, DNA methylation provides a mechanism for maintaining a specific state of gene expression and genome stability during development (Robertson, 2005).

DNA methylation and other forms of epigenetic modifications, like histone modification, do not act independently of one another, for example, methylation of specific regions can act to recruit histone deacytelation, changing the chromatin state and leading to gene silencing (Jones et al., 1998, Nan et al., 1998). DNA methylation can alter the structure and stability of chromatin relevant for transcriptional control of genes (Hashimshony et al., 2003).

1.7. Genomic imprinting

Genomic imprinting is an epigenetic phenomenon resulting in expression of one parental allele, while the other one is silenced. It is the mono-allelic expression of genes depending on the parental origin of the allele (Ulaner et al., 2003). Genes expressed in this manner are called imprinted genes. Imprinted genes are functionally haploid since they are expressed from only the maternal or paternal allele but not both, making them more vulnerable to negative effects of mutations which otherwise would be recessive (Jirtle et al., 2000). Their function can be changed by environmentally induced changes to the epigenome, which may affect their expression in time and in space (Jirtle and Skinner, 2007). Genomic imprinting is mediated by epigenetic modifications such as DNA methylation and histone modification and is important for development and growth (Rodenhiser and Mann, 2006).

Almost all imprinted genes contain homologous sequence regions with differences in DNA methylation (differential methylation) between the parental alleles; the regions are called differentially methylated regions (DMRs). The DMR serve as a mark that differentiates the paternal allele from the maternal allele. Methylation of the CpG dinucleotides of the DMR is the primary mechanism of imprinting. There are two types of DMRs. Primary DMRs (also

called ICRs or germline DMRs) with imprinting methylation marks established in the gametes and then maintained in somatic tissues of offspring throughout development, also referred to as regions that control genomic imprinting (Smallwood and Kelsey, 2012). Secondary DMRs (also called post-zygotic DMRs) have their imprinting marks established after fertilisation during the genome wide wave of demethylation and *de novo* methylation in the zygote (Geuns et al., 2007a). The establishment of secondary DMRs is dependent on primary DMRs in the cluster in which they reside (Lewis and Reik, 2006). The imprinting marks in both the primary and secondary DMRs are erased in the primordial germ cells before new parental specific methylation is established (Lewis and Reik, 2006).

About 1% of autosomal genes are imprinted genes (Jirtle and Skinner, 2007), and approximately 150 imprinted genes have been identified so far(Barlow and Bartolomei, 2014). One of the hallmarks of imprinted genes is that the majority are found in clusters with other imprinted genes in the same region of the genome, which they sometimes share with common developmental and tissue-specific patterns of gene expression (Lopes et al., 2003, Verona et al., 2003). A few lone or singly positioned imprinted genes (e.g. *Inpp5f_v2*) have been reported (Choi et al., 2005). Imprinted clusters contain two or more imprinted genes over a region that can span 1Mb or more. The clusters generally contain several protein coding genes and at least one non-coding RNA (ncRNA) gene. Each cluster is under the control of single major cis-acting element called the imprinting control region (ICR) even though other elements may modulate the function of the ICR (Edwards et al., 2007). ICRs are able to control the imprinting of all the genes within a cluster because they can affect activity and repression over a large region. Deletion of an ICRs has been shown to result in the loss of imprinting of multiple genes within the cluster, usually with major phenotypic effects (Fitzpatrick et al., 2002). Knockouts of ICRs in mice often result in lethality or severe growth defects while mutations in human ICRs lead to imprinting disorders (Lewis and Reik, 2006).

With singleton imprinted genes, their differentially methylated promoters serve as their ICRs (Choi et al., 2005). An ICR is differentially methylated between the two parental alleles and the imprints are acquired or set up in the germ line at the time when the genomes are in their distinct compartments. After fertilisation the parental imprints must survive the reprogramming that takes place in the preimplantation embryo including DNA

demethylation, protamine-histone exchange and changes in histone modification (Reik and Walter, 2001). ICRs can function as insulators and also serve as promoters for ncRNA (Ideraabdullah et al., 2008). Imprinting clusters can be divided into two categories: maternally methylated ICR whose methylation was acquired during oogenesis on their maternally inherited chromosome and paternally methylated ICR whose methylation was acquired during spermatogenesis on the paternally inherited chromosome (Edwards et al., 2007). In addition to differential DNA methylation, ICRs also show allelic differences in chromatin structure, i.e. histone tail modifications. Several studies have shown that repressive histone modifications such as methylation at histone H3 lysine 9 and lysine 27 are found at ICRs on the methylated allele, whereas the activating histone modifications such as (H3 and H4 acetylations) and H3 lysine 4 are found on the unmethylated allele (Grandjean et al., 2001, Lewis and Reik, 2006, Pedone et al., 1999). A number of imprinted genes remain imprinted throughout the life of an organism, however many genes are imprinted in a tissue or in a temporal specific way. Epigenetic abnormalities at imprinted loci have been observed in cloned mammals and their disruptions have been reported in human developmental disorders and cancers (Wood and Oakey, 2006).

1.7.1. Evolution of imprinting

There are several theories that have been put forward to explain the evolution of imprinting, the most common being the ovarian time bomb hypothesis and the conflict hypothesis.

The ovarian time bomb hypothesis states that imprinting occurs to prevent the problem of an unfertilised oocyte that develops into an individual, a phenomenon called parthenogenesis, which can lead to malignant trophoblastic disease (Wood and Oakey, 2006). Imprinting is believed to protect women against germ cell tumours by guarding against excess placental growth (Jirtle and Skinner, 2007). The ovarian time bomb hypothesis predicts that only a small number of genes vital for early embryonic development would be imprinted, and cannot readily explain the involvement of imprinted genes in postnatal traits (Wood and Oakey, 2006). However it remains possible that the action of a small number of imprinted genes in preventing parthenogenesis has been advantageous to mammalian development.

The most widely accepted theory for the evolution of imprinting is the conflict hypothesis, commonly called kinship theory (Haig, 2000, Haig and Graham, 1991, Jirtle and Skinner, 2007, Moore and Haig, 1991). According to the theory, imprinting evolved because of the genetic tug-of-war over the amount of nutrients extracted from the mother by her offspring. It predicts that paternally expressed genes promote placental growth and thus prenatal growth. On the other hand, maternally expressed genes promote fetal development, but suppress offspring growth in order to maximise the mother's reproductive potential over her reproductive lifespan. The function of most oppositely imprinted genes in a cluster supports this theory, and shows opposite roles in fetal growth, depending on the parental origin of their imprint (DeChiara et al., 1991).

1.7.2. Epigenetic reprogramming

In mammals epigenetic reprogramming refers to the remodelling of epigenetic marks during germ cell development and following fertilisation in the early embryo. There are three important periods in the life of an imprinted gene: firstly during gametogenesis, when imprints must be reset according to the sex of transmitting parent; secondly during the preimplantation period when there is genome-wide demethylation in the zygote and thirdly during gastrulation period when there is genome-wide *de novo* methylation where secondary imprints arise with important roles in differentiation and proliferation in late gestation (Weinstein et al., 2002) (illustrated in Figure 5).

Soon after the onset of gastrulation in the mouse embryo, precursors of germ cells or primordial germ cells (PGCs) emerge from the epiblast by embryonic day 7.25 (*E7.25*). *E7.25* refers to the 7.25 day of the mouse embryonic gestation, *E* is embryonic day and the number depicts the actual day. They proliferate, migrate to and colonise the genital ridge from which the gonads develop (*E10.5-E11.5*). Because PGCs originate from embryonic cells that have started to adopt the somatic fate, extensive remodelling of DNA methylation and histone modification marks towards the requirement of a germ cell is essential (Feng et al., 2010, Hajkova, 2010). Preexisting DNA methylation patterns are comprehensively erased (including the imprinted regions and non-imprinted loci) during PGCs migration (Figure 5), such that by *E13.5* their overall methylation is <10% as compared to >70% methylation in the entire embryo (Popp et al., 2010, Smallwood and Kelsey, 2012). The consequence of

across-the-board DNA methylation erasure is that the *de novo* DNA methylation during germ cell development takes place on a clean slate. Depending on the sex of the embryo (*E12.5*), the new DNA methylation pattern is established differently in male and female germ cells (Figure 5), resulting in distinct methylation profiles of the mature oocyte and sperm. This asymmetry is related to the fact that *de novo* DNA methylation takes place in distinct cellular contexts in male and female germ cells (Feil, 2009). In the female, germ cell methylation takes place during the postnatal growth phase in oocytes arrested in meiotic phase I. In the male germ cells, methylation initiates before birth in mitotically arrested prospermatogonia, before the onset of meiosis (Smallwood and Kelsey, 2012). The reprogramming at this stage is needed to reset the imprints in each generation and to remove any epigenetic changes that have accumulated in the previous generation (Piedrahita, 2011) .

Epigenetic reprogramming does also occur after fertilisation, resulting in another wave of remodelling and erasure of DNA methylation marks (Figure 5). The demethylation at this stage does not affect the two parental alleles in the same way. Paternally acquired DNA methylation marks are erased quickly in the zygote, by an active demethylation mechanism, which may involve oxidation of methylated cytosine by the ten-eleven translocation family of dioxygenases (TET) protein (Gu et al., 2011). Maternally acquired germ line methylation marks are erased passively, which is thought to be due to the lack of DNA methylation maintenance at replication, resulting in progressive loss of methylation at each cell division. This second wave of demethylation does not affect all regions of the genome, the methylation at primary DMRs of imprinted genes is not affected and it is faithfully maintained after fertilisation as a lifelong memory of parental origin of the allele in the new generation leading to monoallelic expression of the associated imprinted genes. Maximal demethylation is reached at the morula stage of embryonic development. Once the blastocyst starts to develop, a new wave of global methylation is initiated. However the inner cell mass is methylated to a different extend than the trophectoderm of the blastocyst. These differences in DNA methylation levels are maintained post implantation and throughout development, therefore the fetus proper has a higher level of methylation than the placenta (Piedrahita, 2011). The DNA methylation landscape has to be properly remodelled during the epigenetic reprogramming, which is a vurnable period for external

insults and therefore any environmental disturbances to the process will results in abnormal embryonic development.

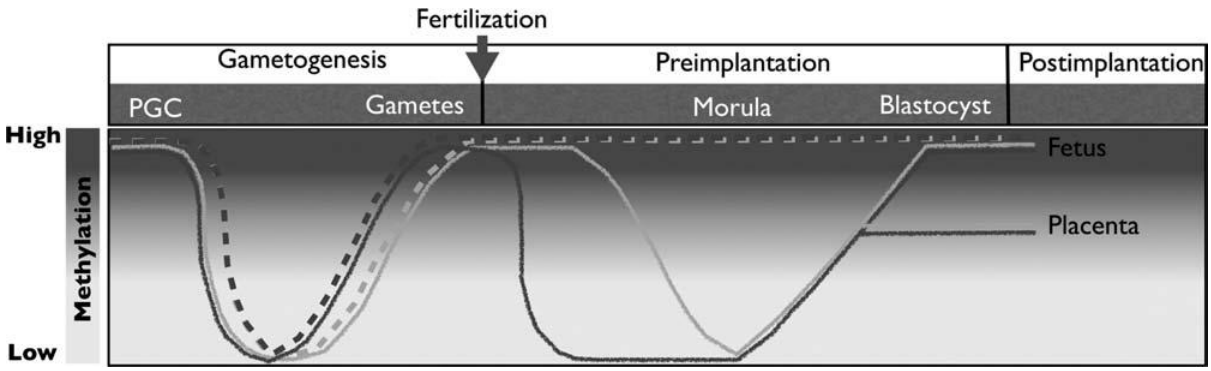


Figure 5: Epigenetic Reprogramming

During fetal development germ cells destined to become PGCs are demethylated. Global methylation (solid lines) and imprinted gene methylation (broken line) occur, as shown. After complete erasure, the new imprints are added. The timing for adding paternal imprints (dark lines) during spermatogenesis differ from maternal imprints (light lines) added during oogenesis. Upon completion of gametogenesis global and imprinted DNA methylation are at their highest. After fertilisation there is a wave of both active and passive demethylation. This demethylation affects global methylation but does not affect imprinting markings. Maximal demethylation is reached at the morula stage of embryonic development. Once the blastocyst starts to develop, a new wave of global methylation is initiated. However the inner cell mass is methylated to a different extend than the trophectoderm of the blastocyst. These differences in DNA methylation levels are maintained post implantation and throughout development, therefore the fetus proper has a higher level of methylation than the placenta (Piedrahita, 2011).

1.7.3. Imprinting clusters

1.7.3.1. H19/IGF2 imprinting cluster

This cluster resides at chromosome 11p15.5 in humans and on distal chromosome 7 in mice. It contains the reciprocally imprinted genes, maternally expressed *H19* and paternally expressed *insulin like-growth factor 2* (*IGF2*) (Bartolomei et al., 1991). Even though most of the studies on *H19* and *IGF2* have been done on mouse models, many characteristics of these genes (gene expression profiles and regulatory mechanism) are similar to their homologues in humans (Ideraabdullah et al., 2008). *H19* and *IGF2* gene are widely expressed during embryonic development and postnatally down-regulated in many tissues in both human and mouse. *H19* encodes for a fully processed 2.3kb non-coding RNA, a putative tumour suppressor (Hao et al., 1993, Yoshimizu et al., 2008). However it has also been

shown to have oncogenic properties (Cui et al., 2002). *IGF2* encodes a potent growth factor protein that plays an important role in promoting embryonic and placental development (DeChiara et al., 1990, Ohlsson et al., 1993).

H19 and *IGF2* imprinting and differential expression is regulated by a germ line DMR, that is methylated on the paternal allele and unmethylated on the maternal allele (Lewis and Reik, 2006, Morgan et al., 2005), located between the two genes, about 2 kb upstream of *H19* gene in humans. The ICR is also called ICR1 or *H19 ICR* (Jinno et al., 1996, Leighton et al., 1995). The region is approximately 5 kb long in humans and 2 kb long in mice. The ICR displays a parent of origin dependent methylation profile in both humans and mice, such that it is methylated on the paternal allele and unmethylated on the maternal allele (Vu et al., 2000). *H19* and *IGF2* promoters share enhancers that lie downstream of *H19,* and the ICR region maintains differential expression of the two genes by regulating the interaction between the *IGF2* and *H19* promoters and their shared enhancers. Deletion of the ICR has been shown to result in loss of imprinting (LOI) at *H19* and *IGF2* (Thorvaldsen et al., 1998). The ICR contains the binding site sequence for an insulator protein, zinc-finger CCCTC-binding factor (CTCF), and therefore it is a biological target for the CTCF. The sequence is conserved in the mouse and human ICR (Frevel et al., 1999). The mouse *H19 ICR* contains 4 CTCF binding sites of which the first three are differentially methylated, while the human ICR contains 7 (Hark et al., 2000). In humans, the sixth CTCF binding site has been shown to be differentially methylated, while the other CTCF binding sites appeared to be methylated irrespective of the parent of origin of the allele (Takai et al., 2001). Therefore the sixth CTCF binding is the key regulatory domain for maintaining the differential expression of the *H19* and *IGF2* genes in humans. Loss of differential methylation at this ICR is associated with loss of imprinting in various types of cancers (Ulaner et al., 2003). In addition the overgrowth disorder Beckwith-Weidemann Syndrome (BWS) and the Silver-Russell Syndrome (SRS) (characterized by reduced growth) are strongly associated with defects in *H19/IGF2* imprinting (Gicquel et al., 2005, Ideraabdullah et al., 2008, Reik et al., 1995).

In the current model of imprinting regulation at the *H19/IGF2* locus, the ICR functions as a methylation regulated enhancer blocker (Hark et al., 2000). The CTCF proteins bind to the unmethylated maternally inherited allele of the ICR because it is a suitable binding site for CTCF. The CTCF binding creates an insulator or physical boundary on the maternal allele

between the *IGF2* promoter and the downstream enhancer, preventing the enhancers from activating transcription of IGF2 and leaving them available to activate transcription of *H19* (Figure 6). On the methylated paternally inherited allele ICR, the CTCF is unable to bind (due to presence of methylation) allowing downstream enhancers to activate transcription of *IGF2* while the *H19* is silenced (Engel et al., 2006, Lewis et al., 2004)(Engel et al., 2006; Lewis et al., et al., 2004) (Figure 6). The insulator mechanism has been demonstrated both *in vitro* and in a mouse model (Bell and Felsenfeld, 2000, Schoenherr et al., 2003). In addition CTCF binding to the unmethylated maternal ICR might be necessary to prevent *de novo* methylation of the maternal allele. Targeted mutation of the CTCF binding sites demonstrates that these sites are necessary for imprint maintenance and not for the establishment of the imprints (Szabo et al., 2004). The CTCF binding in the ICR is the major organiser of chromatin composition in the maternal allele along the entire imprinted domain (Han et al., 2008)(Han et al., 2008). CTCF recruits active histone tail modification marks to the ICR and recruits, at a distance, the Polycomb-mediated H3K27me3 repressive marks at the *Igf2* promoter (Li et al., 2008).

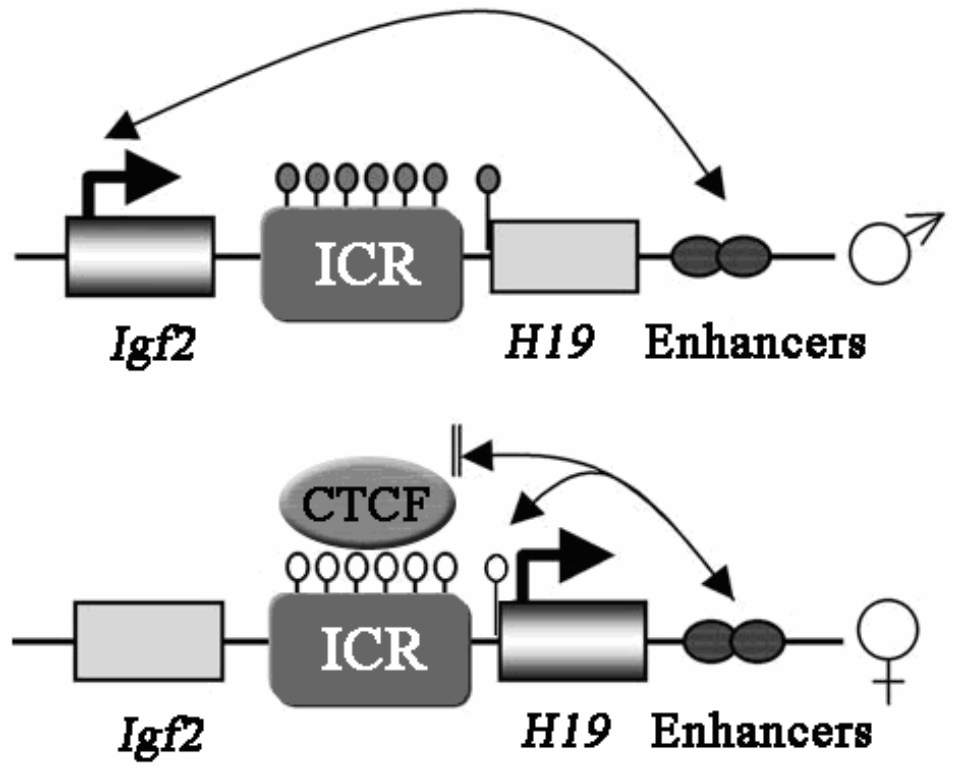


Figure 6: The *H19* ICR.

The *H19 ICR* in the context of the *H19/Igf2* imprinted domain in the mouse. Filled and unfilled lollipops represent methylated and unmethylated CpG dinucleotides, respectively. Arrows represent transcription start sites of the *Igf2* and *H19* genes; when CTCF binds to the unmethylated maternal allele it blocks access of maternal *Igf2* to enhancer sequences located downstream of *H19;* conversely the insulator function of the *H19 ICR* is abrogated by methylation on the paternal allele, blocking CTCF and allowing paternal *Igf2* access to the enhancers. Thus, paternal *Igf2* is expressed and maternal *Igf2* is silenced (Engel et al., 2006).

1.7.3.2. CDKNIC/KCNQ1OT1 imprinting cluster

The *CDKNIC/KCNQ1OT1* locus is located on chromosome 11p15.5 in humans, and contains one paternally expressed ncRNA gene, *potassium channel KQT-family member 1 OT 1* (*KCNQ1OT1*) also called *LIT1.* It also contains eight maternally expressed protein coding genes, including *potassium channel KQT-family member 1* (*KCNQ1*), *cyclic dependent kinase inhibitor 1C* (*CDKNIC*) also called $p57^{KIP2}$ and *pleckstrin homology-like domain family A member 2* (*PHILDA2/TSSC3*), *OSBPL5*, *SLC22A18*, SLC22A18AS, *KCNQ1DN* and *ASCL2* (Chiesa et al., 2012, Reik and Walter, 2001) (Figure 7). The locus is regulated by a maternally methylated ICR, *KvDMR1* (also called *ICR2*). The *KvDMR1* is located within intron 10 of the *KCNQ1* gene and is methylated on the maternal allele and unmethylated on the paternal allele. *KvDMR1* has been shown to be methylated in the oocytes but not in sperm in mice

and humans, thus indicating the *KvDMR1* carries a germline epigenetic imprint ((Geuns et al., 2007b, Yatsuki et al., 2002). *CDKNIC* encodes a cyclin-dependent kinase inhibitor (*CDKI*) that belongs to the CIP/KIP family of cell cycle regulators and is considered to be a putative tumor suppressor gene (Watanabe et al., 1998). Overexpression of *CDKNIC* can arrest cells in G1. Cells that have exited the cell cycle express this protein (Maher and Reik, 2000). In Beckwith-Wiedemann syndrome (BWS), germ-line mutations of *CDKNIC* have been identified and shown to cause loss of cell cycle inhibition (Maher and Reik, 2000). Decrease in the expression of *CDKNIC* has been shown in sporadic cancers and embryonic tumours (Higashimoto et al., 2006).Therefore CDKNIC is a critical protein in BWS and cancers.

KCNQ1OT1, mentioned above, is a non-coding antisense transcript to *KCNQ1* gene, spanning about 60 kb and 54 kb in human and mice, respectively. Its promoter resides within *KvDMR1* (Lee et al., 1999, Mitsuya et al., 1999, Smilinich et al., 1999). Hypomethylation of the promoter of *KCNQ1OT1* or *KvDMR1* on the paternal allele lead to expression of *KCNQ1OT1* and repression of adjacent protein coding imprinted genes, whereas hypermethylation *of KvDMR1* on the maternal allele leads to repression of the ncRNA and activation of the adjacent protein coding imprinted genes (Ideraabdullah et al., 2008). In mice deletion of the *KvDMR1* on the paternal allele result in repression of *Kcnq1ot1* and depression of normally paternally silent imprinted genes (Fitzpatrick et al., 2002) suggesting that transcription of *KCNQ1OT1* is essential to silencing the 8 protein coding imprinted genes in *cis*.

KvDMR1 has been shown to function as a bi-directional silencer in regulating imprinting in the *CDKNIC/KCNQ1OT1* locus (Thakur et al., 2003). *KvDMR1* uses the non-coding RNA model of imprinting to regulate imprinting, where, transcription of *KCNQ1OT1* or the transcript itself is required for bi-directional silencing of maternally expressed genes in *cis* (Mancini-Dinardo et al., 2006). It is suggested that the *KCNQ1OT1* transcript silences its neighbouring genes in a similar manner as *XIST,* the non-coding RNA which is the driving force behind the process of X chromosome inactivation. The Xist RNA coats the future inactive X and triggers the events that lead to gene silencing along the length of the X chromosome (Heard, 2004, Reik and Lewis, 2005). Therefore the *KCNQ1OT1* transcript may silence genes in cis, by coating them and recruiting heterochromatin factors.

Histone modification has been reported at this ICR: H3 Lys9 di-methylation (H3m2K9) has been shown to be abundant on the methylated maternal *KvDMR1* in both humans and mice indicating a condensed and inactive heterochromatin. While on the other hand H3Ac, H4Ac and H3Lys4 di-methylation (H3me2 K4) were observed to be abundant on the unmethylated paternal *KvDMR1* allele in both human and mice, indicating open chromatin and active transcription (Higashimoto et al., 2003). The histone modification state at the KvDMR1 was associated with DNA methylation status and the expression of *KCNQ1OT1.*

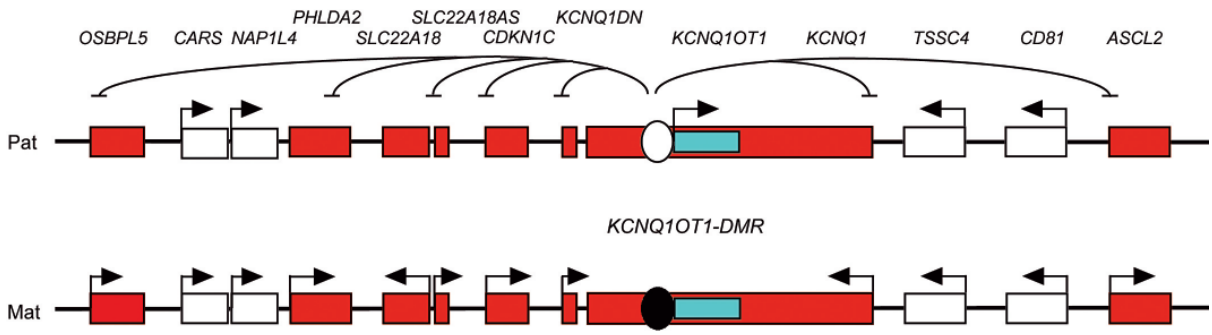


Figure 7: Human *CDKN1C / KCNQ1OT1* imprinted domain

Maternally and paternally expressed genes are indicated by red and blue boxes, respectively. DNA methylation status at *KvDMR* is shown by the white oval (unmethylated) and by the black oval (methylated). Unmethylated *KvDMR1* on the paternal allele (Pat), which works as a silencer and as a promoter for *KCNQ1OT1* RNA transcription, represses the surrounding maternally expressed genes. Methylated *KvDMR1* on the maternal allele (Mat) cannot work as a silencer, and *KCNQ1OT1* RNA cannot be transcribed. As a result, surrounding maternal expressed genes are transcribed (Higashimoto et al., 2006).

1.7.3.3. DLK1/GTL2 imprinting locus

The *DLK1/GTL2* imprinting locus is located on distal mouse chromosome 12 and on chromosome 14q32 in humans. It has been demonstrated that the organisation and imprinting of the *DLK1/GTL2* imprinting locus is highly conserved between mouse and human species (Kobayashi et al., 2000, Miyoshi et al., 2000). The *DLK1/GTL2* domain contains the paternally expressed delta- like homologue 1 (*Dlk1*) gene and maternally expressed non-coding RNA transcript, gene trap line 2 (*GTL2*). The human orthologue of *Gtl2 is called MEG3 (*maternally expressed gene 3*)* (Geuns et al., 2007a, Kobayashi et al., 2000). *DLK1* and *GTL2* (*MEG3*) are located within a 1 Mb imprinting cluster containing additional

imprinted genes including two paternally expressed protein coding genes [type III iodothyronine deiodinase (*DIO3*) gene and tetrotransposon-like gene1 (*RTL 1*)], several maternally expressed non-coding RNAs, a maternally active cluster of small nucleolar RNAs (*C/D box snoRNAs*) and numerous maternally active microRNA encoding genes (Figure 8) (Geuns et al., 2007a). Therefore this imprinting cluster deviates from other imprinting clusters exhibiting a single critical non-coding RNA as it contains several maternally expressed non-coding RNAs.

GTL2 (*MEG3*) appears to lack an open reading frame and it expresses a non-coding poly-adenylated transcript with unknown function in both humans and mice (Wylie et al., 2000). The *DLK1* gene codes for a cell-surface transmembrane glycoprotein, containing six epidermal growth factor- like (EGF-like) repeat motifs (in its extracellular domain), similar to those present in notch/delta/serrate family of signalling molecules (Kawakami et al., 2006). *DLK1* has been identified as preadipocyte factor -1 (PREF-1), a crucial negative regulator of adipocyte differentiation (Smas and Sul, 1993) and zona glomerulosa specific protein (ZOG), a gene involved in zonal differentiation of the adrenal gland (Okamoto et al., 1998). It has also been implicated in pancreatic islet cell differentiation and critically involved in regulating the cellularity of developing thymocytes (Kaneta et al., 2000). *DLK* has been shown to be overexpressed in the adrenal medulla neuroendocrine tumour, pheochromocytoma and Wilms tumour (Helman et al., 1987). All these findings indicate that *DKL1* plays an important role in normal cellular differentiation and carcinogenesis (Geuns et al., 2007a, Laborda, 2000).

Imprinting in the *DLK1/GTL2* cluster is regulated by an intergenic differentially imprinted region (termed *IG-DMR*) located between *DLK* and *GTL2* (Edwards et al., 2008). *IG-DMR* is methylated on the paternal allele and unmethylated on the maternal allele and has been shown to be methylated during spermatogenesis and remain unmethylated in the maternal germline in both mice and humans (Lin et al., 2003, Takada et al., 2002). The *IG-DMR* is therefore an ICR and regulates imprinting of all the genes in the cluster (Lin et al., 2003). Targeted deletion of the *IG-DMR* has been shown to repress all maternal-specific transcripts (*GTL2* and all the other noncoding RNA genes) while expressing paternal-specific transcripts (*Dlk1*, *Rtl1* and *Dio3*); and perinatally lethal when the deletion is inherited maternally.

However no effects are seen when the deletion is paternally inherited (Lin et al., 2003). This is unusual because the *IG-DMR* is normally unmethylated on the maternal allele and methylated on the paternal allele, therefore suggesting that it is the maternally inherited unmethylated copy that is essential for maintaining repression of protein-coding genes and activation of the non-coding RNA (da Rocha et al., 2008). On the other hand methylation of the paternal chromosome seems to be important for the expression of the protein coding-genes because failure to maintain paternal methylation results in considerable *Dlk1* repression (Schmidt et al., 2000).

The exact mechanism(s) regulating imprinting at the *DLK1/GTL2* domain is unknown, however there are several proposed models for the regulation of imprinting at the *DLK1/GTL2* domain: 1. *IG-DMR* may act as an insulator element, as described for the *IGF2/H19* locus (Figure 6); 2. *IG-DMR* may use the non-coding RNA model as described for *CDKNIC/KCNQ1OT1* locus; 3. The maternally inherited unmethylated *IG-DMR* may acts as a positive transcriptional regulator for *Gtl2* and its associated transcripts and is required for repression of the maternally silent imprinted genes. *Gtl2* transcription might prevent the ability of the enhancers to act on the protein coding genes in the region (da Rocha et al., 2008); 4. The last possibility is that interaction between the unmethylated *IG-DMR* and other long range *cis*-acting regulators confer a conformation on the maternal chromosome that places the protein coding genes in a repressive chromatin 'compartment' (da Rocha et al., 2008)

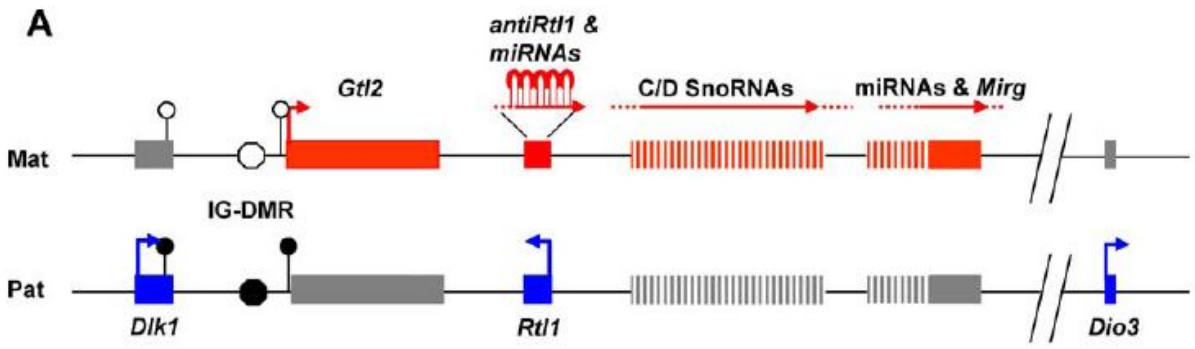


Figure 8: The *DLK-Gtl2* imprinting domain in the mouse

It shows genes expressed from the paternal chromosome (blue) and noncoding RNAs (red) expressed from the maternally inherited chromosome. The imprinting control region for the domain is the paternally methylated *IG-DMR* (circle) (Edwards et al., 2008).

1.7.3.4. PEG3 imprinting locus

Paternally expressed gene 3 (*Peg3*) is the first gene detected on the proximal imprinted domain region of mouse chromosome 7 (Kim et al., 1997, Kuroiwa et al., 1996). Five additional imprinted genes have been identified subsequently within the surrounding genomic region, and they include the paternally expressed gene *Usp29* and Zf264, and the maternally expressed genes Zim1, Zin2 and Zim3 (Kim et al., 2003). The human homologs of most of these mouse imprinted genes are located in the syntenically homologous region of the long arm of chromosome 19q13.4 (Kohda et al., 2001) and human *PEG3* and *ZIM2* are also imprinted (Murphy et al., 2001). Mouse *Peg3* encodes a Kruppel-type (C2H2) zinc finger containing protein, most of which are thought to function as transcription factors (Kim et al., 1997, Pieler and Bellefroid, 1994). Mouse *Peg3* is expressed in mesodermal tissues during embryogenesis and high level of expression has been shown in the central nervous system in adults (Kuroiwa et al., 1996). *Peg3* knockout mice have been found to show growth retardation before birth as well as impairment of maternal behaviour of the adult female that resulted in death of offspring (Li et al., 1999). In the *Peg3* mutant female mouse the number of oxytocin-positive neurons in the hypothalamus is reduced as compared to that in the wild type female. Thus it is suggested that *Peg3* plays a role in growth, differentiation and survival of neural cells (Kim et al., 1997). Both human and mouse P*EG3* are strongly expressed in the brain suggesting that it may have an important and conserved role in neuronal cells. Human *PEG3* is expressed in the placenta consistent with the notion that

most imprinted genes are involved with embryogenesis (Barlow, 1995). A decrease in human *PEG3* expression has been commonly observed in glioma cell lines (Kohda et al., 2001).

The *PEG3* locus is regulated by a *PEG3*-DMR which has been identified as a CpG island region of about 5kb surrounding the first exon of *Peg3* and *Usp29* and is conserved in mice and humans (Kim et al., 2003). The CpG island was shown to be methylated in an allele specific pattern in somatic cells (Li et al., 2000) and germ cells (Lucifero et al., 2002), therefore it is an ICR. Comparison analysis of *PEG3-DMR* sequences derived from human, mouse and cow has shown that the region contains an evolutionary conserved element, and multiple binding sites for the Gli-type transcription factor (Kim et al., 2003) . A study by Kim et al (2003) showed that YY1-binding to the motifs is methylation-sensitive and that all of the YY1 sites are differentially methylated between the two parental alleles *in vivo*. Functional assays of *PEG3-DMR* suggest that the YY1-binding region acts as a methylation-sensitive insulator that may play a role in imprinting control of *Peg 3* and neighbouring genes (Kim et al., 2003).

All the four loci mentioned above were investigated for the present study. What they have in common is that their DMRs have methylation that is established in the gametes and therefore important in controlling the imprinting of genes in their imprinting cluster. Both the maternal and paternal methylated ICRs were represented. The selected imprinted clusters are implicated in growth, embryonic development and neurogenesis. Therefore they are good candidates in terms of their biological impacts in line with FAS features like growth and brain defects.

1.8. Effect of Ethanol on DNA methylation

DNA methylation has been proposed as a possible candidate mechanism of alcohol teratogenesis resulting in the development of the features that characterise the FASD phenotype. This proposition follows early studies of a mouse model by Garro et al., (1991) where they demonstrated that acute alcohol exposure in pregnant mice resulted in global hypomethylation in mid-gestation mouse fetuses. There were also lower levels of methyltrasferase activity in fetuses of alcohol fed mice as compared to controls (Garro et al.,

1991). They also demonstrated that acetaldehyde, a byproduct of alcohol metabolism, inhibited DNA methyltransferase activities even at low concentration (Garro et al., 1991). Since DNA methylation is known to play an important role in the regulation of gene expression during embryogenesis, ethanol associated alterations in fetal DNA methylation may contribute to the developmental abnormalities seen in FAS.

Haycock and Ramsay (2009) studied a locus specific effect of alcohol, where they looked at imprinting of the *Igf2/H19* locus in mouse embryos and placentae after maternal alcohol exposure in the preimplantation stage. Severe growth retardation was observed in embryos and placentae in the alcohol exposed group compared to controls, however no epigenetic changes at *H19 ICR* were observed in the embryos but hypomethylation was observed in placentae *H19 ICR* (Haycock and Ramsay, 2009).

A study by Kaminen-Ahola (2010) tested the hypothesis that epigenetics is involved in gestational reprogramming of the adult phenotype when exposed to alcohol *in utero* or preconception. They used an epigenetically sensitive allele in mice, Agouti viable yellow (A^{vy}) (whose expression is closely linked to their epigenetic state), as a reporter to detect epigenetic alterations caused by alcohol exposure. They used two mouse models, one to study the effect of maternal gestational alcohol consumption and the other to study the effect of maternal preconception alcohol exposure on the phenotype of her offspring. They found that both maternal preconception and gestational alcohol exposure affected the expression of the A^{vy} allele in the offspring. Maternal alcohol exposure was found to induce hypermethylation at the A^{vy} locus, which was associated with increasing the probability of transcriptional silencing at the A^{vy} locus, resulting in more mice with an agouti colored coat. Postnatal growth retardation and craniofacial dysmorphologies significant in FAS affected individuals were reported following gestational alcohol exposure. Genome-wide expression studies demonstrated that several genes (those associated with growth and development of the nervous system) were significantly down regulated in alcohol exposed mice (Kaminen-Ahola et al., 2010).

Strouder et al. (2011) used a mouse model to evaluate the possible effect of low dose alcohol exposure in pregnant mice (administered from gestational day 10-18 which is a period consistent with the major event of organogenesis and fetal development), on DNA

methylation patterns of selected imprinted genes (*H19, Gtl2, Peg1 , Snrpn* and *Peg3*) in different tissues of the offspring. The tissues included were tail, liver, skeletal muscle, hippocampus and sperm DNAs from male offspring over two generations. They also looked at the transgenerational effect of alcohol exposure. No difference in methylation status was observed in the 5 imprinted genes from different tissues (tail, liver, muscle hippocampus and new born whole brain) between controls and alcohol fed offspring. The effects observed were a 3% ($p<0.05$) decrease in the number of methylated CpGs of *H19* in the F1 offspring sperm, a 4% ($p<0.05$) decrease in the number of methylated CpGs of *H19* in the F2 offspring brain and a 26% ($p<0.05$) decrease in sperm concentration. CpGs 1 and 2 of the *H19* CTCF binding site 2 exhibited significant methylation percentage losses. Their observations suggest that hypomethylation at *H19* may contribute to decreased spermatogenesis seen in the offspring (Stouder et al., 2011).

In a study by Bielawski et al. (2002) male rats were treated with alcohol before breeding; it was shown that alcohol exposure resulted in a decrease in cytosine methyltransferase 1(*DNMT1*) mRNA levels in their sperm compared with controls and a significant decrease in mean fetal weight was also observed. These finding suggest that paternal alcohol exposure before conception may be one of the mechanism causing altered genomic imprinting and thus disrupting offspring development. It has been shown that deficiency of methyltransferase activity in mutant mice resulted in hypomethylation, expression of imprinted genes like *H19* which are normally silent on the paternal allele and repression of the normally active paternal *Igf2* gene, with an increase in embryonic lethality in mice (Li et al., 1993). These suggest that appropriate levels of DNA methyltransferases are critical for maintaining proper transcriptional activation of the paternal allele, and thus normal development (Bielawski et al., 2002). Knezovich and colleagues, investigated the effect of paternal alcohol exposure on DNA methylation at *H19* and *Rasgrf1* (paternally methylated ICRs), in exposed sperm and somatic DNA of sired offspring (Knezovich and Ramsay, 2012). They reported a significant reduction in DNA methylation at the H19 ICR in offspring of ethanol-treated sires which corresponded to reduced weight at postnatal day 35 to 42.

A study by Liu et al. (2009) used a mouse model to assess the effect of alcohol exposure at early embryonic neurulation, on genome-wide DNA methylation and gene expression. Alcohol induced alterations in DNA methylation were observed particularly in genes on

chromosomes 7, 10, and X. An increase in methylation in genes known to play a role in metabolism (*Cyp4f13*) and a decrease in methylation in genes associated with development (*Nlgn2, Sox21, Elavl2* and *Sim1*), imprinting (*Igf2r*) and chromatin (*Hist1h3d*) was observed. Altered methylation was associated with significant changes in the expression of about 84 genes. This study was the first to use a mouse model for FASD that showed that alcohol exposure during early neurulation can induce aberrant changes in DNA methylation with associated changes in gene expression, which together contribute to observed abnormal fetal development (Liu et al., 2009).

A study by Ouko et al. (2009) looked at sperm DNA of male alcoholics and controls in trying to establish a link between alcohol use in men and DNA methylation at two paternally imprinted loci. They found that there was a pattern of decreased methylation correlated with alcohol consumption at two imprinted ICRs, *H19 ICR* and *IG-DMR*, with significant differences observed at the *IG-DMR* between the non-drinking group and heavy alcohol consuming group (Ouko et al., 2009).

The studies described in this section support alteration in epigenetic mechanism (e.g. DNA methylation) as a contributing factor for the development of features observed in FAS. It is suggested that the effect is mediated through the interruption of the one carbon pathway by alcohol (Fowler et al., 2012, Halsted et al., 2002, Liu et al., 2009)(Liu at al., 2009, Halsted et al., 2002, Frowler et al., 2012).

1.9. One carbon metabolism and Alcohol

One carbon Metabolism (OCM) can be described as a sequence of biochemical reactions whereby one carbon atom (e.g. methyl group) is transferred from a donor to another compound or methyl acceptor (e.g. DNA, proteins and neurotransmitters) in a series of steps. OCM encompasses folate, methionine and choline metabolism (Bailey et al., 2012). OCM is essential for the biosynthesis of universal methyl donor, S-adenosyl methionine (SAM) and also for the production of compounds that are important for DNA synthesis. SAM makes methyl groups available for epigenetic processes like DNA methylation.

Folate is a coenzyme that plays a vital role as a source of methyl group in the OCM in mammals. Humans cannot synthesise folate even though little amount can be synthesised

from the intestinal flora (Wani et al., 2012); therefore they mainly obtain folate from dietary sources.

Folate circulates in the blood in the form of 5-methyl tetrahydrofolate (5MTHF) (Friso et al., 2002) and therefore enters the OCM as such. In the presence of B_{12}, the enzyme methionine synthase (MS) transfers the methyl group from 5MTHF to homocysteine converting it to methionine (Figure 9). Homocysteine can also be remethylated to methionine by a folate independent pathway (Kohlmeier et al., 2005) that involves methyl donor betaine. Methionine is then converted to SAM in the presence of methionine adenosyl transferase (MAT) and ATP. SAM serves as a substrate for DMNT enzymes where the methyl groups are transferred from SAM to the DNA for the DNA methylation processes. Once SAM loses its methyl group it becomes S-adenosylhomocycteine (SAH) which can be hydrolysed to homocysteine and adenosine by SAH hydrolase. SAH is a strong competitive inhibitor for DNMTs since it has the ability to bind with high affinity to methyltransferases, therefore can inhibit transmethylation reactions and affect DNA methylation processes (De Cabo et al., 1995). The intracellular ratio of SAM to SAH is a critical determinant of methylation capacity of the cell and thus transmethylation reactions (Lu, 2000, Schalinske and Nieman, 2005, Yi et al., 2000). The homocysteine can either be remethylated back to form methionine or be irreversibly catabolised to cystathionine by cystathionine synthase through the transsulfuration pathway.

OCM pathway is essential in maintenance of normal development and any disturbance in the pathway will result in disorders such as birth defects, cardiovascular disorders, neurological disorders, and cancer (Schalinske and Nieman, 2005). Alcohol is one of the factors that can disturb the OCM, leading to perturbation of DNA methylation processes. There are several plausible mechanisms that have been suggested. Firstly through its metabolite acetaldehyde, which has been reported to inhibit DNMT activity (Bonsch et al., 2006, Garro et al., 1991) and also alcohol itself has been reported to reduce DNMT mRNA levels (Bielawski et al., 2002). Secondly alcohol may reduce the pool of methyl donors by inducing folate deficiency, which may be due to poor diet (common in chronic drinkers). Alcohol may also impair folate transport system in the kidneys and intestines, leading to reduction in folate intestinal absorption and increase in folate renal excretion (due to reduced renal folate re-absorption) and therefore folate deficiency (Hamid et al., 2009).

Lastly alcohol may directly reduce the activity of enzymes involved in the OCM including methionine adenosyl transferase (MAT) and methionine synthase (MS), therefore causing reduction in the SAM and its precursor methionine and also an increase in SAH. The effects of the enzymes will eventually lead to reduction in the SAM to SAH ratio and therefore the methylation capacity of the cell (Hamid et al., 2009, Stickel et al., 2000).

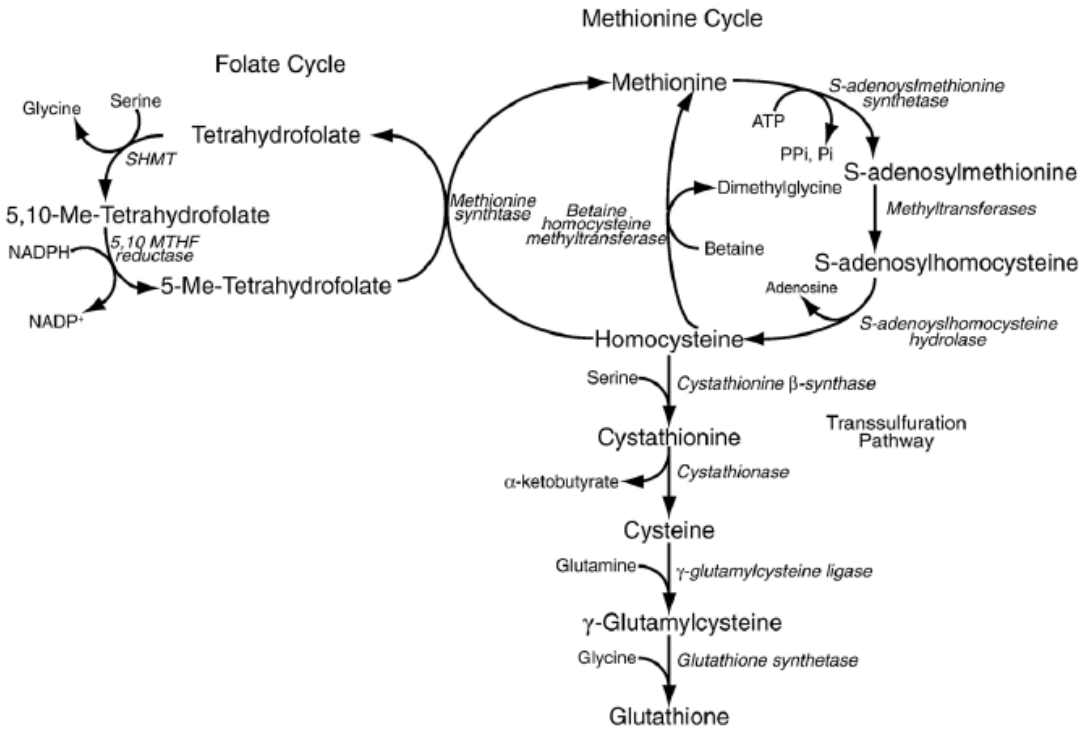


Figure 9: Schematic representation of the biochemistry of one-carbon metabolism (Hitchler and Domann, 2007).

1.10. Study Aim and objectives

Aim

The main aim of the study was to examine the effect of alcohol on DNA methylation of imprinting control regions (ICRs) of specific imprinted genes in children with FAS and unaffected individuals in a case control study.

Hypothesis

Alcohol exposure during prenatal development will result in epigenetic modifications at imprinted loci, such as a reduction or increase in DNA methylation at ICRs that could be observed in the blood derived DNA of children with FAS.

Specific Objectives

- To select specific imprinted loci to examine for epigenetic changes.
- To examine DNA methylation at ICRs of selected imprinted loci in case and control samples using the pyrosequencing method.
- To asses methylation status in different tissues i.e. blood and buccal tissues
- To asses potential DNA methylation differences at different ICRs between cases and controls (locus averaged and CpG site specific).
- To examine the effect of confounders on methylation in cases and controls.
- To determine the effect size difference in methylation between cases and controls at different imprinted loci after adjusting for confounding factors

2. Subjects and Methods

Method describing the preparations of solutions are available in Appendix B, unless otherwise stated.

2.1. Study participants

2.1.1. Case control study participants

The participants of this study (both cases and controls) are of mixed ancestry, referred to as "Coloureds" in the South African context. This population has been reported to have the highest prevalence of FASD /FAS in the world. Most of the South African Coloureds reside in the Western Cape region of South Africa and their population is estimated to be about 4 million people, which is approximately 9% of the South African population. They form a unique genetically admixed population in which the Africans (Khoisan, local Bantu-speakers and other Africans from other parts of Africa), Europeans and south Asian population groups have contributed to the admixture (Quintana-Murci et al., 2010).

The FAS cases were recruited from several areas of the Western Cape (Wellington, Philippi, Nyanga, Michell's plain and Bredasdorp) and the Northern Cape (De Aar and Upington). The FAS cases were diagnosed by a team of trained clinicians from the Division of Human Genetics, National Health Laboratory Service (NHLS), Braamfontein, Johannesburg and also by a clinical team of the Foundation for Alcohol related Research (FARR) lead by Prof Denis Viljoen. FARR is a non-governmental, non-profit organisation established in 1997 by Prof D Viljoen. Their main focus is substance abuse with FASD (including FAS) as their primary interest (FARR, 2008).

The control participants were mainly from the Northern Cape (De Aar and Upington). Western Cape control samples were available, however they were not used for this study because they were collected from a blood bank, and did not have information about age and ethnicity. The controls were randomly selected and not phenotyped (no phenotype data was collected). The cases and controls were ethnically matched and partially geographically matched (no controls from the Western Cape).

2.1.2. Case control participant recruitment and sample collection

I was not part of the participant recruitment and sample collection for the case control study. The recruitment of participants and collection of samples was done in previous studies between the years 1999 and 2005 (as part of the larger study initiated by Prof Denis Viljoen). Ethics approval for the study was obtained from University of the Witwatersrand Committee for Research on Human Subjects (Medical) and ethics protocol number M02/10/41 and M03/10/20 was issued for collection of FAS and controls participants, respectively (Appendix A). Information sheets and consent forms for FAS cases and controls are also shown in Appendix A. For the molecular analysis performed in the present study by myself, ethics approval was obtained from the University of the Witwatersrand, Human Research Ethics Committee (Medical), protocol number: M080548 (Appendix A).

The initial screening of FAS or partial FAS (PFAS) involved measurements of head circumference, body height and weight. If measurements were below the 10^{th} percentile for growth within their age group, the participants were examined by two physicians. When both physicians were in agreement on the diagnosis, a maternal interview was conducted. If a maternal history of alcohol abuse during pregnancy was identified, a neurodevelopmental examination of the child was performed. Finally a case conference was held to determine the most accurate diagnosis. Most of the FAS cases were children of school entry age or much younger and therefore their parents or guardians had to give informed consent.

The ethnic origins of the participants' parents and grandparents were recorded. Control Individuals were also asked about their geographic origins. No information was obtained on whether the control individuals were prenatally exposed to alcohol or not. The participants had to be 18 years and older for informed consent purposes (See information sheet for ethics protocol number: M03/10/20, Appendix A). The individuals were not phenotyped because they did not undergo any physical or neurological examinations.

Blood samples and buccal swabs were collected by nurses from the Division of Human Genetic, NHLS, Braamfontein, Johannesburg. Either 10ml blood (collected in ethylenediamine tetra-acetic acid (EDTA) anti-coagulant tubes) or buccal swabs were collected from the cases and controls. The blood samples and buccal swabs were stored in

cooler boxes and sent to the Molecular Genetics Laboratory at the NHLS, Braamfontein, Johannesburg.

2.1.3. Participants and sample collection for assessment of methylation status in different tissues

This part of the study was performed to establish if the blood and buccal tissue samples reflected similar imprinting profiles at the *H19 ICR*. This was done because in the case control study the participants donated either a blood or buccal tissue sample. The study was done under the ethics approval protocol number: M080548, after permission to do the study under the mentioned protocol number was requested and granted (see Appendix A for information sheet, informed consent form and approval letter).

Fifty adult volunteers (assumed to be healthy) were recruited from NHLS, Braamfontein, Johannesburg, for participation in this study. After the aim and objectives of the study were explained, the participants were asked to sign a consent form if willing to participate. Blood (5-10 ml in EDTA tubes) was drawn by a qualified nurse from the NHLS, Braamfontein, Johannesburg; the tubes were mixed gently and thoroughly. The samples were stored at 4^{o}C.

The buccal tissue swab was collected using a nylon bristle cytology brush which was supplied with the Gentra Puregene buccal cell kit (Qiagen, Valencia, CA). As advised by the kit's instruction protocol, the participants were asked to wait for at least 1 hour after eating or drinking. The inside of the mouth was scraped 10 times with buccal collection brush by participants themselves. The collection brush's head was immersed completely in a clearly labelled 1.5 ml tube containing lysis solution (provided with the kit) and the handle cut off using a sterilised scissors. The tube was closed and stored at 4^{o}C.

2.2. DNA Extraction

I performed DNA extractions for blood samples and buccal tissues for the study to assess the methylation status in different tissues. For the case and control study the DNA samples were extracted previously and were already available.

2.2.1. Blood DNA extraction

Blood DNA was extracted according to a modified protocol from Miller et al., (1988)(Miller et al., 1988). Five to ten milliliters of blood collected into EDTA vacutainer tubes was mixed gently and thoroughly, before being decanted into a clearly labeled 50 ml polypropylene tube. The blood in the tube was stored in the -20^{o}C freezer until used. Before starting with DNA extraction the frozen whole blood was thawed at room temperature. Once thawed, the 50 ml tube was filled up to the 40 ml mark with Sucrose-Triton X lysing buffer (which was kept cold during the procedure). The Sucrose-Triton X lysing buffer lyses the red blood cells. The tube was inverted several times to mix it then it was centrifuged at 1200 x *g* at 4^{o}C for 10 minutes. After centrifugation a reddish white pellet was visible at the bottom. The supernatant fluid (containing the lysed red blood cells) was discarded carefully making sure that the pellet does not dislodge. 20 ml of cold Sucrose-Triton X-lysing buffer was added to the pellet and mixed by inversion. The tube was put at -40^{o}C for 5 minutes. Afterwards the tube was centrifuged for 5 minutes at 1200 x *g* and the supernatant discarded. 3 ml of T20E5, 0.2 ml of 10% SDS and 0.5 ml of Proteinase-K mix (see Table B1, Appendix B) was added to the pellet and the tube mixed by inversion. The tube was then incubated overnight in a 42^{o}C incubator (without agitation). After the incubation 1 ml saturated NaCl was added to the lysate and it was mixed gently by inversion for 15 seconds. The tube was then placed in a -40^{o}C freezer for 5-10 minutes followed by centrifugation at 1200 x *g* at room temperature. A white pellet-containing protein should be visible at the bottom of the tube after centrifugation, if not, the tube was centrifuged again. The supernatant-containing the DNA was transferred to a new clearly labeled 50 ml tube. Two volumes of 100% absolute ethanol kept at room temperature were added to the supernatant to precipitate the DNA. The tube was agitated gently and the DNA was spooled or fished out then washed in 1 ml of ice cold 70% ethanol. The washed DNA was air dried and re-suspended in an appropriate amount of TE (TRIS-EDTA) buffer, depending on the amount of pellet. The DNA was stored at 4^{o}C until use.

2.2.2. Buccal tissue DNA

The buccal tissue DNA was extracted using the Gentra Puregene buccal cell kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. The protocol followed is for purification of genomic DNA from 1 buccal DNA brush.

As described above under sample collection, the buccal brush head was immersed in a clearly labelled 1.5 ml tube containing lysis solution (provided with the kit). The tube was then incubated at 65^{o}C for 15 – 60 minutes. 1.5 µl of Puregene proteinase K (20 mg/ml) was added to the lysate and tube was mixed with inversion 25 times, and then incubated at 55^{o}C for 1 hour (up to overnight if maximum yield required). After incubation the collection brush heads were removed from the lysis solution, scraping it on the insides of the tubes to recover as much liquid as possible. 1.5 µl of RNase A solution was added to the cell lysate and the tube was mixed by inverting 25 times before it was incubated at 37^{o}C for 15 minutes. Then it was cooled on ice for 1 minute. 100 µl of protein precipitation solution was added to the tube and then vortexed vigorously for 20 seconds at high speed, and then incubated on ice for 5 minutes. The tube was centrifuged at 15 000 x *g* for 3 minutes, to separate the tube contents into a white tight pellet (containing the protein) and a supernatant. The supernatant was carefully poured (making sure not to disturb the pellet) into a new tube containing 300 µl of Isopropanol and 0.5 µl glycogen solution. The tube was then mixed 50 times by gentle inversion and centrifuged for 5 minutes at 15 000 x *g*. After centrifugation the supernatant was carefully discarded and the tube was drained on a clean piece of paper towel, taking care that the pellet remains in the tube. 300 µl of 70% ethanol was added to wash the DNA pellet by inverting several times. The tube was centrifuged for 1 min at 15 000 x *g*, thereafter the supernatant was carefully discarded, the tube was then drained on a piece of clean paper towel making sure the pellet remain in the tube. The pellet was allowed to air dry for up to 15 min. 20 µl DNA hydration solution was added to the dried pellet and vortexed for 5 seconds to mix. The DNA was incubated for an hour at 65^{o}C to dissolve the DNA, and further incubated overnight at room temperature to make sure it is completely dissolved. After incubation the tube was briefly centrifuged and the concentration and quality of the DNA was checked before the DNA was transferred to a 1.5 ml tube. The concentration was determined and the samples were stored at -20^{o}C until use.

2.2.3. Quantification of genomic DNA (gDNA)

The extracted gDNA (blood and buccal) concentrations were quantified using a NanoDrop® ND-100 spectrophotometer (Thermo Fisher Scientific, MA United States of America). It is a full spectrum (220-750nm) spectrophotometer that measures absorbance of 1 µl of DNA sample with accuracy and reproducibility without dilution. The absorbance was read at

260nm for DNA and 280nm for proteins. The concentration was calculated by the NanoDrop. The ratio of 260/280 provides an estimate for purity of the gDNA. The ratio of 1.8-2.0 indicates an acceptable purity for gDNA while the ratio below 1.5 indicates protein contamination.

2.2.4. Agarose gel electrophoresis

The quality of the extracted genomic DNA was checked by electrophoresing 5 µl DNA mixed with 5 µl ficoll on a 0.8% agarose gel, at 6 V/cm. A distinct and intact bright band positioned above the largest band of the 1Kb+ DNA marker (Invitrogen, Corporation CA United States) was accepted as good quality high molecular weight DNA (see appendix C).

2.2.5. Bisulfite modification

Bisulfite treatment of DNA is a requirement for DNA methylation analysis for many epigenetics-based studies involving methylation profiling and quantification of methylation status and is currently a "gold standard'' in distinguishing between cytosine and 5-methyl cytosine (Ruga et al., 2008). During bisulfite modification, sodium bisulphite deaminates all unmethylated cytosine bases and converts them to uracil while methylated cytosines (5-methylcytosines) remain unchanged because it is resistant to the conversion. This resistance is induced by the presence of methyl at position 5 of cytosine that makes the amino acid at position 4 resistant to the bisulfite deamination (Hayatsu et al., 2007). Therefore the sequence of the treated DNA will differ from that of its original composition at unmethylated cytosine residues. During subsequent PCR reactions, the uracil bases are complemented with adenosine bases, which are in turn used as a template for thymine complementation. Thus the UpG dinucleotides of the bisulfite modified sample strand are converted to TpG (Figure 10). Primers for PCR are therefore specifically designed based on the chemically modified sequence, which can be further analysed by pyrosequencing.

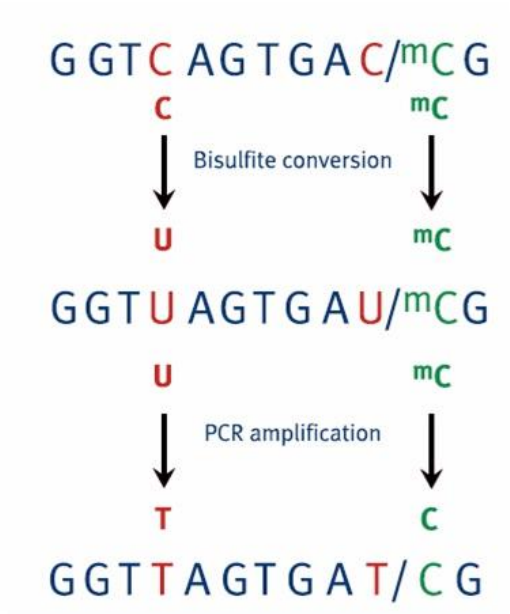


Figure 10: DNA treatment with sodium bisulfite

Methylated cytosines (^{m}C) remain as Cs while unmethylated cytosines are converted to uracil (U) and subsequently to thymine (T) during PCR (England and Pettersson, 2005).

For the present study gDNA from blood and buccal tissues were bisulfite modified using EZ-DNA Methylation Gold Kit ™ (Zymo Research, Orange, CA) according to the manufacturer's instruction (Appendix C). The protocol of the kit involves the transformation of DNA unmethylated cytosine bases, by sodium bisulfite and it combines the DNA denaturation step and sodium bisulfite step into one single step. It utelises the temperature denaturation method, instead of the chemical denaturation. The protocol is customised for better recovery of DNA and production of complete conversion of unmethylated cytosine.

The protocol recommended a DNA input of 200 – 500 ng, however 500ng of gDNA was used as starting DNA concentration for my samples. gDNA from the case control study was normalised to 50 ng/µl in 96 well plates using the TECAN FREEDOM EVO® SYSTEM (TECAN AG Trading, Switzerland) while gDNA for the study on assessment of methylation status in different tissues was manually diluted to 50 ng/µl with TE buffer.

2.3. Pyrosequencing ™ for quantitative DNA methylation analysis

Pyrosequencing™ technology is a real time sequencing method used for the analysis of short to medium length DNA sequences (Aydin et al., 2006, Ronaghi et al., 1998). It is

classified as sequencing by synthesis method that detects luminescence (proportional to the quantity of DNA and number of nucleotides) from the release of pyrophosphate (PPi) on nucleotide incorporation into the complementary strand (Tost and Gut, 2007). The released PPi is subsequently converted to light through a cascade of enzyme reactions and the generated light is seen as a peak in the Pyrogram. The signal produced is proportional to the amount of PPi produced and hence the methylation at the CpG can be detected and quantified by analysing the chemically induced C/T sequence differences (Ronaghi, 2001, Uhlmann et al., 2002). The incorporation of a cytosine is an indication of a methylated residue, whereas the incorporation of thymine indicates an original unmethylated cytosine at the CpG site. Thus the methylation status of a CpG site can be read as a C/T single nucleotide polymorphism (SNP) (Reed et al., 2010).

2.3.1. Assay design

Pyrosequencing assays were designed using PSQ Assay Design Software (Biotage). This software specifically designs an assay to amplify the region of interest that contains a number of single nucleotide polymorphisms (SNPs). In this instance the cytosine nucleotide contained within a CpG dinucleotide, that will either remain as a cytosine if methylated or converted to thymine (through bisulfite modification) if unmethylated, is actually the "polymorphism", the C/T (IUPAC code, Y). However in the case of DNA methylation, the C (methylated): T (unmethylated) ratio at a given CpG within a specific DNA sample may vary and is therefore unlike a traditional SNP where a sample is either heterozygous or homozygous. Therefore the C and T alleles are quantified and subsequently expressed as a percentage.

Once the reference sequence containing the IUPAC code Y, for each CG (YG) has been imported and the target region of approximately 100 bp selected, the assay design software generates a series of potential primer sets to amplify the region. In addition a sequencing primer is also generated for each primer set that will be used for the pyrosequencing reaction.

The technique of pyrosequencing in conjunction with pyro Q-CpG methylation offers several advantages for methylation analysis. It is highly sensitive, accurate and therefore reproducible (Huse et al., 2007) and quantifies the DNA methylation of a sample by

analysing all amplicons within a pooled PCR sample, therefore regarded as being more accurate than bisulfite sequencing PCR (BSP)(Reed et al., 2010). Reproducible quantification of consecutive CpG site is easy and fast on 96 samples in parallel. As the methylation of each site is measured in the context of the DNA sequence, the software automatically performs quality control of the raw data to make sure that the expected sites were analysed. Furthermore Cs not followed by a G are used as a quality control measure to evaluate whether the bisulfite treatment went to completion, thereby ensuring reliable data (England and Pettersson, 2005).

2.3.2. Pre-pyrosequencing PCR

For methylation analysis, pyrosequencing is done directly on the PCR product of bisulfite modified DNA (Kobayashi et al., 2006). A target region of up to 350 bp is amplified using a pair of primers complementary to the bisulfite treated DNA sequence, amplifying all strands irrespective of methylation status.

As a starting material for the pyrosequencing reaction, a PCR-amplified single stranded DNA template (with a sequencing primer hybridised to it) is required. There are several methods that can be used to generate the single stranded DNA template for pyrosequencing analysis (Ronaghi et al., 1996) and for our study streptavidin-coated Sepharose™ beads were used. When using the streptavidin-coated Sepharose™ beads method for sample preparation, one of the PCR primers has to be 5' biotin labelled (biotinylated) for immobilisation to the beads and the other unlabelled. Thus the PCR product will have one strand 5' biotin labelled and the other unlabelled. The PCR product is captured to the Sepharose beads through the biotin labelled strand, eventually a biotin labelled single stranded DNA template is generated and used as a template for pyrosequencing complementary strand.

As mentioned above, the method of pyrosequencing requires that one of the PCR primers (forward or reverse) be 5' biotinylated. In order to eliminate the need for a unique biotin-labelled primer for each primer set, a universal biotin labelled primer was used to generate labelled DNA fragments (Colella et al., 2003), therefore reducing cost. The sequence specific prepyrosequencing PCR primer that has been designated to be biotin labelled (tagged in Table 1, 2 and 4) thus requires a complementary tail or tag off which a universal primer can prime. This is achieved by the addition of a 23 bp complementary tag, 5'-

GACGGGACACCGCTGATCGTTTA-3' to the 5' end of the specified primer. Therefore for the pre-pyrosequencing PCR, 3 primers are utilised; normal, tagged and universal primer. Again the pre-pyrosequencing PCR requires more cycles (45-50) in order to make sure that all the biotinylated primers are consumed, which is important for pyrosequencing.

The PCR products were electrophoresed on a 3% agarose gel with either 100 bp or 50 bp DNA ladder to size the PCR product. A negative control was also run together with the PCR product, to check if the PCR product was not contaminated. A strong PCR product band without excess primers, primer dimmers and non- specific bands (spurious bands) were accepted as good PCR product for pyrosequencing. A PCR product gel pictures for IG-DMR and H19 ICR respectively are shown in the appendix C.

Published primer sets and designed primer sets were used to amplify a specific region within the ICR of each of the imprinted loci under investigation i.e. *IGF2/H19*; *DLK1/GTL2 (MEG3)*, *KCNQ1OT1/CDKN1C* and *PEG3* (Table 1). These ICRs are usually located between two genes within each locus. The ICR for *IGF2/H19* is called *H19 ICR*; for *DLK1/GTL2* is *IG-DMR* (intergenic region) *KCNQ1OT1/CDKN1C* is *KvDMR1* and for *PEG3* is *PEG3 DMR*.

Nested PCR was used to amplify the *H19 ICR* region (Table 1 and 2). Nested PCR is used to amplify the target sequences when the number of DNA copies is very low. It involves two consecutive rounds of amplification, where the first round uses external primers and the second uses the internal or nested primers. The first round amplifies the target region as in typical PCR while in the second round the nested primers anneal to the sequence internal to the product of the first round PCR. The first round was a normal PCR of 28 cycles using the bisulfite modified DNA as a starting template and second round PCR was a pre-pyrosequencing of 50 cycles using the first round PCR as a starting template. For the other ICRs we did not use nested PCR (Table 1).

Table 1: Locus specific information for PCR amplification and pyrosequencing

Locus (Contig)	No. CpG sites	PCR primers (5'-3')	Amplicon length (bp)	Annealing Temp (^{O}C)	Sequencing primer (5'-3')	Reference
H19 ICR (AF087017)	6	**Outer Reverse** CTTAAATCCCAAACCATAACACTA **Outer Forward** GTATATGGGTATTTTTTGGAGGT **Inner Forward** GTATATGGGTATTTTTTGGAGGT **Inner Reverse** Tag- ATATCCTATTCCCAAATAA	317 53 217	61.5 53	TGGTTGTAGTTGTGGAAT	Present study
IG-DMR (A117190)	7	**Forward** Tag- TTTATTGGGTTGGGTTTTGTTAG **Reverse** AACCAATTACAATACCACAAAATT	267	58	**Primer 1** CAATTACAATACCACAAAAT	Present study
	3				**Primer 2** CCATAAACAACTATAAACCT	Present study
KvDMR1 (U90095)	7	**Forward** TTAGTTTTTTGYGTGATGTGTTTATTA **Reverse** Tag- CCCACAAACCTCCACACC	101	55	TTGYGTGATGTGTTTATTA	(Bourque et al., 2010)
PEG3 DMR (AC006115)	7	**Reverse** Tag- CCTATAAACAACCCCACACCTATAC **Forward** TAATGAAAGTGTTTGAGATTTGTTG	272	62	GGGGGTAGTTGAGGTT	(Boissonnas et al., 2010)

* 5'-biotin-GACGGGACACCGCTGATCGTTTA-3' - universal biotin labelled tag. Note: the amplicon size excludes the 23bp 5'-biotin label.

2.3.2.1. *H19 ICR PCR*

The *H19 ICR* is the only ICR amplified using nested PCR (Table 1 and 2). The *H19 ICR* amplified region is located to contig AF087017. The *H19 ICR* contains seven CTCF binding sites, of which the sixth CTCF binding site is differentially methylated (Takai et al., 2001). Therefore the region amplified for the present study contains the sixth CTCF binding site. The sixth CTCF binding site contains 5 CpGs, but the amplified region included one extra CpG outside of the binding site, therefore the amplified region contains six CpGs (Appendix D, Figure 21). The primer set used for amplification of this region was designed using the PSQ assay design software (Biotage, AB Sweden). The PCR for this region was performed in triplicate. After a sample was bisulfite modified, it was divided into 3 aliquots of 2 µl bisulfite DNA. The aliquots were used for PCR for round 1, followed by round 2. The sequences for the primers are shown in Table 1.

Table 2: PCR conditions for *H19* ICR

PCR reagents	**Volume**	**PCR conditions**	**Cycles**
Round 1			
ddH2O 10X PCR buffer MgCl2 (25mM) dNTPs (1.25mM) Out F (10µM Out R (10µM) AmpliTaq (5U/µl) gDNA(~100ng)	29.8µl 5µl 4 µl 5 µl 2 µl 2 µl 0.2 µl 2µl	94°C - 5min	1
		94°C - 30sec 61.5°C - 30sec 72°C - 30sec	28
		72°C - 5 min	1
		4°C - ∞	
	50µl final volume		
PCR reagents	**Volume**	**PCR conditions**	**Cycles**
Round 2			
ddH2O 10X PCR buffer MgCl2 (25mM) dNTPs (1.25mM) Primer Normal (10µM) Primer tagged (1µM) Universal primer (10µM) Betaine (5M) AmpliTaq (5U/µl) DNA (from round 1)	25.1 µl 5 µl 4 µl 2.5 µl 1 µl 1 µl 1 µl 7 µl 0.4 µl 3 µl	95°oC -5min	1
		95°C - 20s 53°C - 30s 72°C - 20s	50
		72°C - 5min	1
		4°C - ∞	
	50µl final volume		

2.3.2.2. IG-DMR

The IG-*DMR* amplified region is located to contig A117190 and does not contain the CTCF binding site. It contains 15 CpGs, however only 10 CpGs in total were analysed by pyrosequencing, using two different sequencing primers named primer 1 and 2 (Table 1 and Appendix D_Figure 23). Primer 1 analysed 3 CpGs and primer 2 analysed 7 CpGs respectively from different independent PCRs. The primer set used for amplification of this region was designed using the PSQ assay design software (Biotage, AB Sweden). The PCR was run in duplicate using PCR conditions shown in Table 3.

Table 3: PCR conditions for *IG-DMR* and *KvDMR1*

***IG-DMR* PCR conditions**	**Cycles**	***KvDMR1* PCR conditions**	**Cycles**
95°C - 5min	1	95°C - 10min	1
95°C -15s 58°C - 30s 72°C – 15s	50	95°C - 40s 55°C - 40s 72°C - 40s	45
72°C – 5min	1	72°C - 7min	1
4°C - ∞		4°C - ∞	

2.3.2.3. KvDMR1

The amplified *KvDMR1* region is located to the contig U90095. The amplified region contains 7 CpGs including the differentially methylated *NotI* site that is often altered in BWS (Weksberg et al., 2001) and is used in diagnostic testing for Beckwith-Wiedemann Syndrome (BWS) (Bourque et al., 2010). Patients with BWS show loss of methylation at the *NotI* site (Bourque et al., 2011). The *NotI* restriction enzyme is methylation sensitive and its recognition sequence is 5'-GCGGCCGC-3'. It encompasses CpG 4 and 5 of our amplified *KvDMR1*, (Figure 22, and Appendix D) for *KvDMR1* sequence. This ICR does not contain a CTCF binding site. The PCR was run in duplicate. PCR and pyrosequencing primers used for amplification of the *KvDMR1* are published primers used in pyrosequencing of the region (Bourque et al., 2011) (Table 1). The forward and sequencing primer had a wobble introduced to accommodate an unavoidable CpG site in the sequence template that could either be methylated or unmethylated. PCR conditions are shown in Table 3. PCR reagent cocktails for both *KvDMR1* and *IG-DMR* were the same as round 2 PCR reagents cocktail for

H19 ICR except that 2 µl of bisulfite modified DNA was used instead of 3 µl, however, their PCR conditions were different (Table 3).

2.3.2.4. PEG3 DMR

The amplified *PEG3 DMR* region is located to contig AC006115. The amplified region contains 14 CpGs but only 7 CpGs were analysed (Figure 24, Appendix D). The ICR does not contain a CTCF binding site. The PCR was run in duplicate. PCR and pyrosequencing primers used to amplify the *PEG3 DMR* are published primers used in pyrosequencing of the region (Boissonnas et al., 2010) (Table 1). The *PEG3 DMR* PCR cocktail and conditions are shown in Table 4.

Table 4: PCR conditions for *PEG3 DMR*

PCR reagents	Volume	PCR conditions	Cycles
ddH2O 10X PCR buffer MgCl2 (25mM) dNTPs (1.25mM) Primer normal(10µM) Primer tagged (1µM) Universal primer (10µM) Betaine (5M) AmpliTaq (5U/µl) DNA	9.1µl 2.5µl 2.5µl 2 µl 0.8µl 0.8 µl 0.8µl 5µl 0.4 µl 2µl	 95°C - 15min	 1
		95°C - 30s 62°C - 30s 72°C - 30s	50
		72°C – 5min	1
		4°C - ∞	
	25 µl		

2.4. Pyrosequencing Preparation and run

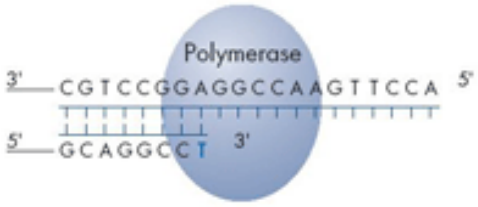
For a detailed method see Appendix C

Prior to the actual pyrosequencing run, a DNA preparation step is performed outside the PSQ 96 MA Pyrosequencer ™ (Biotage, Uppsala, Sweden). In preparation PCR amplicons are incubated with streptavidin-coated Sepharose™ beads and binding buffer. During incubation the PCR amplicons are bound to the Sepharose beads through the biotin-labelled strand. The non-biotinylated strand is eventually removed by treatment with 70% EtOH and NaOH denaturation solution and separated from the biotinylated strand that is attached to the beads. The immobilised strand is then washed with washing buffer (pH 7.6) that neutralise

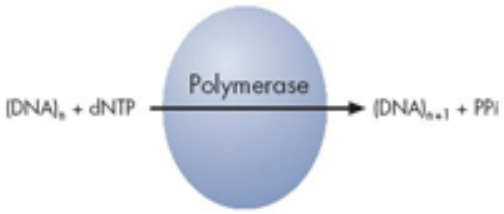
the pH. The beads with attached DNA strand are transferred to an annealing buffer containing the sequencing primer in a pyrosequencing plate.

The sequencing primer hybridises to the bead bound single stranded DNA fragment (Figure 11a) after heating at 80°C and allowed to cool. The following steps occur inside the machine; the sequencing primer hybridised to the DNA strand is incubated with four enzyme cocktail, namely DNA polymerase, ATP sulfurylase, luciferase and apyrase together with substrates adenosine 5' phosphosulfate (APS) and luciferin and dNTPs which are dispensed in a pre-defined dispensation order into the wells using a cartridge (Ronaghi et al., 1998). The first dNTP is added to the reaction and if it is complementary to the first nucleotide after the sequencing primer on the DNA template, it is incorporated into the complementary DNA strand by DNA polymerase (Figure 11a and b).

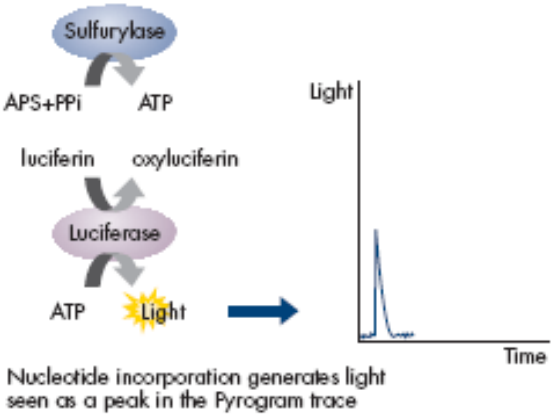
Incorporation of a dNTP results in the release of a PPi in a quantity equivalent to the amount of incorporated nucleotide (Figure 11b). The released PPi is then converted to ATP by ATP sulfurylase in the presence of APS. The resulting ATP drives the conversion of luciferin to oxyluciferin by luciferase. The oxyluciferin then generates visible light in amounts that are proportional to the amount of ATP used which is proportional to the amount of PPi released. The light is then detected by a charge coupled device (CCD) camera and recorded as a peak in a Program™ (Figure 11c). Between nucleotide incorporations, apyrase degrades all unincorporated dNTPs and unused ATP (Figure 11d), and the dNTPs incorporation continues. As the complementary strand grows a pyrogram is constructed (Figure 11e).



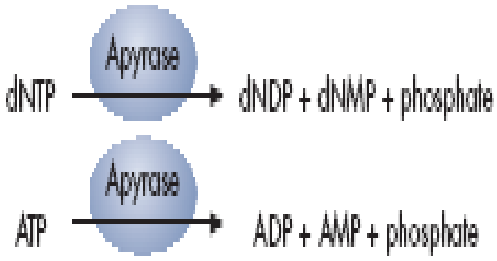
a.



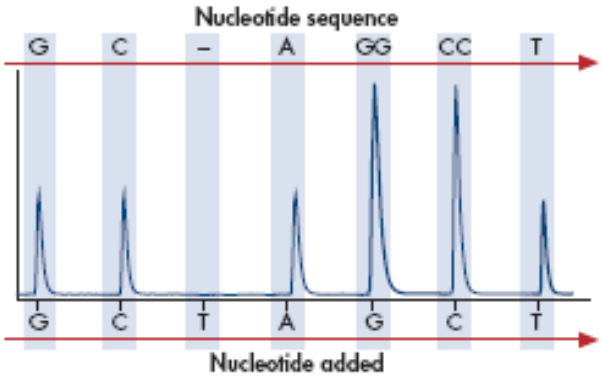
b .



c.



d.



e

Figure 11: Pyrosequencing run

The pyrosequencing run occurs in four steps the result in a pyrogram. The first step is the hybridization of the sequencing primer (a) followed by nucleotide incorporation (b), visible light production (c) and the degradation of incorporated and unused dNTPs and ATP (d) which all result in the construction of a pyrogram (e). Double peak height indicates an incorporation of two nucleotides in a row (Biotage, 2008)

2.5. Statistical Analysis

Descriptive statistics, including mean, standard deviation (SD), average and distribution, was done using SAS statistical software, version 4.22.0.9238.

Linear mixed-effects models were used to generate the results, and a biostatistician from the Medical Research Council of South Africa (MRC), Dr Lize van der Merwe assisted me with the statistical analyses. These analyses were based on joint models, where all the observations were included into a single model to simultaneously do the tests. One advantage is that it avoids some false positive results, because all the results are adjusted for each other. These models also enabled us to adjust for different kinds of random variation as random effects: those between sites, those between individuals and those within individuals (replicates). Adjusting for the variation between individuals is a different way of saying the correlation between replicates on the same individual were adjusted for. After confirming, using linear mixed-effect model that age and sex were confounders, all further models were adjusted for them, as fixed effects. All p values, effects sizes and standard errors (SE) come from interaction terms in the models. All results corresponding to p- values below 0.05 are described as significant, below 0.01 as highly significant and below 0.001 as very highly significant.

The observed methylation data are also summarised with box plots. Each box extends from the first quartile to the 3rd quartile (interquartile range), the line inside the box is at the median, and the whiskers extend to the non-outlying minimum and maximum, respectively. Outliers are shown as open circles. The freely available environment for statistical computing and graphics, R (R Core Team, 2015) and R package (Pinheiro et al., 2015) were used for these analyses.

3. Results

Part of the study results have been published in the journal Frontiers in genetics (Appendix F).

The results are presented in three sections. Firstly results on optimisation of the method are represented; the optimisation was done in order to make sure that accurate and reliable data are produced. The second results section is for testing for tissue specificity, which was done to establish if the use of two different tissues (blood and buccal tissue) in the case control study was not going to confound the results. Lastly the results on the main objective of the study, which is comparison of methylation variation/profiles at selected loci between FAS affected children and controls, are presented.

3.1. Optimising the *H19 ICR* DNA methylation assay

Methylation profiles were obtained for 50 blood DNA samples and their matching buccal tissue DNA from the same individual. Table 5 shows methylation percentages analysed at six CpG sites of *H19 ICR*. The first five CpG sites form part of the 6th CTCF binding site of *H19 ICR* while the last does not (Figure 12, Appendix D). The 6th CTCF binding site of *H19 ICR* is differentially methylated in a parent of origin manner. Only a few samples are shown in the Tables 5 and 6, a complete set of results are shown in Appendix E.

Table 5 and Figure 12 show that there are three different methylation patterns in different individuals i.e. hypomethylation (BL 002 and 008), hypermethylation (BL 011 and 027) and intermediated methylation (BL 003 and 004). In samples from somatic tissue an intermediate level methylation (35-65%) is expected at imprinted loci where one of the parental alleles is methylated and the other is not. All consecutive CpG sites showed relatively similar methylation percentages in individual samples e.g. BL002, BL008 and BL004; however in some of the individual samples, CpG site 4 behaved differently from other CpG sites of the same individual sample. CpG site 4 was completely unmethylated (mean percentage of 1, 7% and 4% respectively) in individual samples e.g. BL003, BL011 and BL0027.

Table 5: Methylation profiles of CpG sites at the 6th CTCF binding site of *H19 ICR* using primers which overlapped with sites for a known SNP

Sample ID	Methylation percentages (%)					
	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6
BL002	0	4	0	3	0	5
BL002	0	0	5	0	5	0
BL002	3	3	0	0	0	3
mean	1	2	2	1	2	3
BL008	3	5	4	2	2	4
BL008	4	5	4	2	2	4
BL008	3	5	3	3	2	7
mean	3	5	2	2	2	5
BL003	42	42	40	0	39	41
BL003	42	45	44	2	41	43
BL003	42	42	50	0	39	39
mean	42	43	45	1	40	41
BL004	33	36	35	34	34	34
BL004	31	31	31	30	32	34
BL004	30	31	31	29	29	32
mean	31	33	32	31	32	33
BL011	90	96	91	6	90	95
BL011	89	90	91	5	88	85
BL011	91	91	86	9	82	92
mean	90	92	89	7	87	91
BL027	90	92	90	3	86	88
BL027	87	97	91	6	87	87
BL027	90	96	91	3	88	88
mean	89	95	91	4	87	88

BL= blood sample

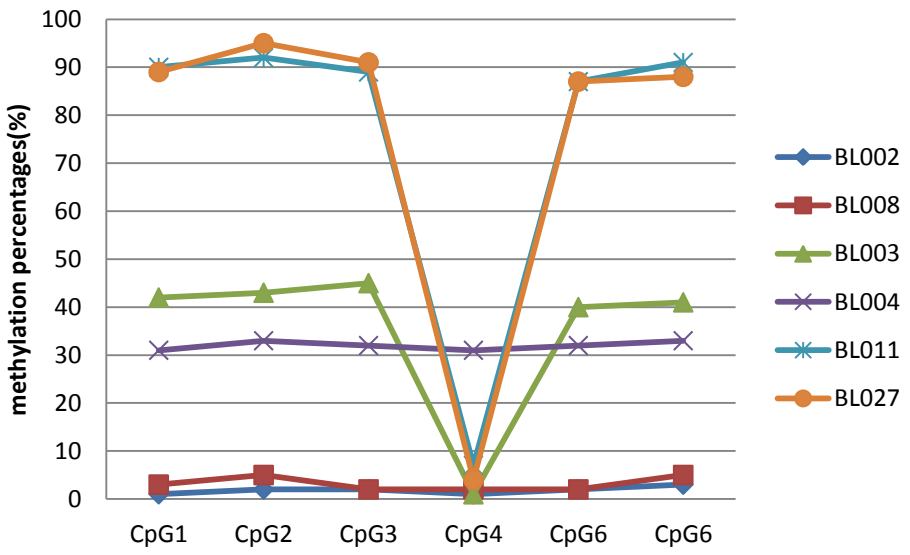


Figure 12: Methylation profiles of CpG sites at the 6th CTCF binding site of H19 ICR using primers which overlapped with sites for a known SNP.

During the course of the study, I came across a retracted article by Tost and collegues which highlighted that the variable methylation profiles shown in Table 5 may be due to biased amplification caused by the presence of SNPs in some of the binding sites of the *H19 ICR* nested primers used (Tost et al., 2007). The primers were then checked if they contained known SNPs, and indeed two of the primers were found to contain a known SNP (outer forward primer_rs11564736 and inner reverse primer_rs56125822, Appendix D). Thereafter primers were redesigned to avoid sites containing known SNPs, and the modified primers were used to amplify the same *H19 ICR* region for the same samples shown in Table 5.

Table 6: Methylation profiles of CpG sites at the 6th CTCF binding site of *H19 ICR* using modified primers that did not overlap with a known SNP site

Sample ID	Methylation percentages (%)					
	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6
BL002	48.00	48.00	50.00	49.00	52.00	49.00
BL002	49.00	49.00	48.00	49.00	51.00	49.00
BL002	48.00	48.00	49.00	49.00	52.00	49.00
Mean	48.33	48.33	49.00	49.00	51.67	49.00
BL008	55.00	57.00	53.0	51.00	53.00	53.00
BL008	56.00	56.00	52.00	51.00	54.00	52.00
BL008	55.00	57.00	53.00	51.00	53.00	53.00
Mean	55.33	56.67	52.67	51.00	53.33	52.67
BL003	56.00	57.00	54.00	0.00	53.00	53.00
BL003	55.00	57.00	53.00	1.00	52.00	52.00
BL003	56.00	57.00	54.00	0.00	53.00	53.00
Mean	55.67	57.00	53.67	0.33	52.67	52.67
BL004	52.00	55.00	50.00	52.00	49.00	53.00
BL004	50.00	56.00	52.00	52.00	50.00	52.00
BL004	52.00	55.00	50.00	52.00	49.00	53.00
Mean	51.33	55.33	50.60	52.00	49.33	52.67
BL011	50.00	54.00	51.00	2.00	51.00	52.00
BL011	49.00	55.00	51.00	1.00	53.00	55.00
BL011	50.00	54.00	51.00	2.00	51.00	52.00
Mean	49.67	54.33	51.00	1.67	51.67	53.00
BL027	56.00	61.00	56.00	2.00	55.00	56.00
BL027	55.00	60.00	56.00	0.00	52.00	54.00
BL027	56.00	61.00	56.00	2.00	55.00	56.00
Mean	55.67	60.67	56.00	1.33	54.00	55.33

BL=Blood sample

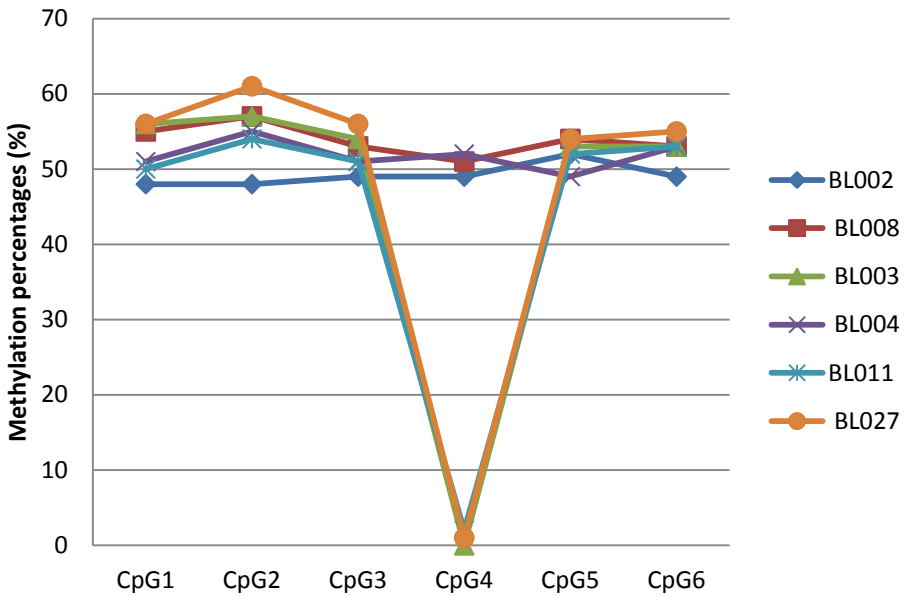


Figure 13: Methylation profiles of CpG sites at the 6th CTCF binding site of H19 ICR using modified primers that did not overlap with a known SNP site.

From the results in Table 6 and Figure 13 it is observed that the methylation pattern of all the CpG sites of *H19 ICR* in the different individual samples were about 50%, even for samples that previously reflected a hypermethylation pattern in Table 5 (BL011 and BL027). It is also observed that all the CpG sites of different individual samples reflected methylation of roughly 50% except for CpG site 4 which was still hypomethylated in some individuals the same way as in Table 5 (BL003, BL011 and BL0027). The hypomethylation at CpG site 4 is explained by the presence of a known C/T SNP (rs10732516) at this site. The T allele appeared hypomethylated at the fourth CpG site while the C allele did not.

3.2. Testing for tissue specific DNA methylation differences at *H19 ICR*

This section of the study was performed to establish if the blood and buccal tissue DNA samples reflected similar patterns of methylation at the imprinted locus, *H19 ICR*. This was done because in the case control study the participants donated either blood or buccal tissue samples. The control participants (N=58) all donated blood samples and of the 87 FAS cases, 8 donated buccal samples and the remainder donated blood (Appendix E). We wanted to verify, before performing the case control study, that the methylation profiles between the two tissues were not different, and therefore not going to confound or bias

our results. Table 7 represents methylation profiles of six CpG sites of *H19 ICR* obtained from blood and buccal tissue of the same individual.

Table 7: Methylation profiles of CpG sites at the 6th CTCF binding site of *H19 ICR* obtained from blood and buccal tissue DNA of each participant

Sample ID	Methylation percentages (%)					
	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6
BL002	0.00	4.00	0.00	3.00	0.00	5.00
BL002	0.00	0.00	5.00	0.00	5.00	0.00
BL002	3.00	3.00	0.00	0.00	0.00	3.00
Mean	1.00	2.33	1.67	1.00	1.67	2.67
BC002	4.00	7.00	3.00	4.00	4.00	5.00
BC002	4.00	4.00	3.00	3.00	3.00	6.00
BC002	0.00	4.00	3.00	0.00	0.00	4.00
Mean	2.67	5.00	3.00	2.33	2.33	5.00
BL003	42.00	46.00	40.00	0.00	39.00	41.00
BL003	42.00	45.00	44.00	2.00	41.00	43.00
BL003	42.00	45.00	50.00	3.00	42.00	39.00
Mean	42.00	45.00	44.67	1.67	40.67	41.00
BC003	41.00	49.00	41.00	3.00	41.00	45.00
BC003	43.00	48.00	42.00	4.00	44.00	45.00
BC003	41.00	47.00	42.00	3.00	43.00	44.00
Mean	41.67	48	41.67	3.33	42.67	44.67
BL011	90.00	96.00	91.00	6.00	90.00	95.00
BL011	89.00	90.00	91.00	5.00	88.00	85.00
BL011	91.00	91.00	86.00	9.00	82.00	92.00
Mean	90	92	89.00	7.00	87.00	91
BC011	91	92	89	4	87	84
BC011	87	95	88	4	83	86
BC011	82	92	83	5	82	86
Mean	86.67	93	86.67	4.33	84	85.33

BL=Blood; BC=Buccal tissue

Table 8: Comparison of methylation profiles of CpG sites at the 6th CTCF binding site of *H19 ICR* between blood and buccal DNA samples of a participant

Sample no	CpGs	Blood DNA mean methylation (%)	Buccal DNA mean methylation (%)	p-value
BL002 BC002	CpG1	1.00	2.67	0.44
	CpG2	2.33	5.00	0.16
	CpG3	1.67	3.00	0.47
	CpG4	1.00	2.33	0.44
	CpG5	1.67	2.33	0.76
	CpG6	2.67	5.00	0.21
BL003 BC003	CpG1	42.00	41.67	0.64
	CpG2	45.00	48.00	0.36
	CpG3	44.67	41.67	0.36
	CpG4	1.67	3.33	0.6
	CpG5	40.67	42.67	0.2
	CpG6	41.00	44.67	0.3
BL011 BC011	CpG1	90.00	86.67	0.28
	CpG2	92.00	93.00	0.77
	CpG3	89.00	86.67	0.35
	CpG4	7.00	4.33	0.14
	CpG5	87.00	84.00	0.4
	CpG6	91.00	85.33	0.15

BL=Blood; BC=Buccal tissue; significant: $p<0.05$

It should be noted that the methylation profiles from Table 7 and 8 come from *H19 ICR* region amplified by primers that contained SNPs in their binding region, which is why there are samples that are still hypermethylated and hypomethylated in the tables. By looking at the methylation percentages represented in Table 7 and 8, it is observed that methylation levels at all the six CpG sites analysed in blood and buccal tissues from the same individual were not different (p-values <0.005, Table 8).

3.3. Case control study of methylation variation at imprinted loci in FAS affected and control individuals

3.3.1. Study and sample description

The main objective of the study was to compare methylation profiles at four imprinted locus imprinting control regions (ICRs) between cases (FAS) and controls. In the case group two partial FAS (PFAS) DNA samples (1PFAS male and 1PFAS female) were included and were

combined with all FAS cases for all statistical analysis (Appendix F). Therefore in the discussion we only refer to the FAS group. Not all samples were typed for every locus (Appendix E). For the *H19 ICR*, some of the results are presented in triplicate and some in duplicate. The reason for this is that for *H19 ICR*, every sample was run in triplicate, but for some samples one run of the three runs would fail quality control (QC) and would therefore be excluded from the results. The other loci (*KvDMR1*, *IG-DMR* and *PEG3 DMR*) were run in duplicate for reasons of scarce sample and cost of reagents. For all four loci (Appendix F), one case sample (DNA004270) was excluded from statistical analysis because of a lack of information on age, gender and diagnosis (diagnosis=indication of whether it was a FAS or PFAS sample). Three other case samples (DNA004258, DNA004312 and DNA004313) did not have age data (Appendix F) and therefore were excluded from analysis involving age. A summary for the number of samples, gender and age distribution at the different loci in the case and control groups is shown in Table 9. For all the loci, control samples had a median age of 20 years (range 18 to 26 years) while cases had a median age of 9 years (range 1 to 16 years). The control group had a lower number of samples (50+) while the case group had a higher number of samples (70+). There is almost the same number of males and females tested at all loci in the control groups but in the case groups at *KvDMR1* and *IG-DMR* the number of males and females are almost the same, whereas at *H19 ICR* and *PEG3 DMR* there is slightly higher number of males than females. A complete data set of all loci including the replicates is shown in Appendix E and F.

Table 9: Summary table for number of samples, gender and age distribution for the different loci tested in the control and case groups

	Controls				**Cases**			
		Age	Gender			Age	Gender	
Locus	N	mean(yrs) (min:max)	Male	Female	N	mean(yrs) (min:max)	Male	Female
H19 ICR	50	21 (18:26)	27	23	73	8.7 (1:16)	41	32
KvDMR1	55	21 (18:26)	27	28	86	8.4 (1:16)	46	40
IG-DMR	56	21 (18:26)	29	27	84	8.5 (1:16)	45	39
PEG3 DMR	50	21 (18:26)	25	25	79	8.7 (1:16)	46	33

3.3.2. Locus specific methylation analysis

Results in Tables 10 up to 19 were generated by me using SAS statistical software, version 4.22.0.9238. The mean methylation of each individual at each CpG site in a locus and across a locus was calculated and analysed. The data in Tables (10-19) and box plots were not adjusted for confounders and therefore may be biased. Data for average methylation at different CpG sites and average methylation across a locus for different loci were first checked if they were normally distributed (Tables 10, 12, 14, 16 and 18), before comparison of means for sites and average methylation across a locus calculations between cases and controls were done (Tables 11, 13, 15, 17 and 19). If the data being compared were normally distributed, a t-test (a parametric test) was used to test if the means of the two groups were significantly different. If the data was not normally distributed a non-parametric test (Kruskal-Wallis test) was used. Figures 14-19 were generated by a biostatistician from the Medical Research Council of South Africa (MRC),Dr Lize van der Merwe, using a statistical joint model (R package version 3.1-102), but were put under this section because their data is unadjusted for age and gender. The adjusted analysis taking into consideration age and gender are shown in a later section.

3.3.2.1. H19 ICR

Table 10: Descriptive data for *H19 ICR* methylation percentages at different CpG sites and average methylation

Control	mean age (yrs) (min:max)	sites	Mean methylation %	Std Dev	Median	Minimum	Maximum	Normal distribution
N=50	21 (18:26)	CpG1	51.57	3.13	51.33	40.33	57.50	no
		CpG2	54.64	3.28	54.67	44.67	62.67	yes
		CpG3	52.21	3.44	52.25	40.00	58.50	no
		CpG5	51.82	3.07	51.58	42.00	59.50	yes
		CpG6	53.43	3.39	53.33	43.33	61.33	yes
		Average	52.73	3.36	53.97	42.07	59.10	yes
Cases	8.7 (1:16)	CpG1	53.37	3.53	53.58	44.67	62.50	yes
N=73		CpG2	55.64	3.77	55.5	47.00	64.50	yes
		CpG3	53.38	3.73	53.58	44.00	62.67	yes
		CpG5	53.29	3.25	53.33	45.67	61.33	yes
		CpG6	54.77	3.78	54.83	47.50	64.33	yes
		Average	54.07	2.97	52.6	46.80	61.80	yes

N=number

In the *H19 ICR* locus, six CpG sites were analysed by pyrosequencing. As already mentioned in section 3.1 all the CpG sites, except site 6, form part of the 6th CTCF binding site (which is differentially methylated) in the *H19 ICR*. All the CpG sites reflected a methylation percentage of ± 50% as is expected in a normal somatic tissue for an imprinted locus (where only one parental allele is methylated and the other is not). However CpG site 4 appeared hypomethylated (< 10%) in some samples and had methylation of ±50 percent in other samples and this is explained by the presence of a known C/T SNP (rs10732516) in this position. Therefore site 4 was excluded from the statistical analysis for *H19 ICR* (Table 10 and 11). In cases and controls methylation percentages were normally distributed in all *H19 ICR* CpG sites and in terms of methylation across the locus, except for sites 1 and 3 in controls (Table 10).

Table 11: Comparison of means at different sites and average methylation across the loci at *H19 ICR* between controls and cases

H19 ICR	sites	Control mean %	Case mean %	p-value
	CpG1	51.57	53.37	0.007*
	CpG2	54.64	55.64	0.132
	CpG3	52.21	53.38	0.081
	CpG5	51.82	53.29	0.013*
	CpG6	53.43	54.77	0.046*
	Average	52.73	54.07	0.024*

Significant:* p<0.05

Table 11 and Figure 14 represent comparisons of methylation percentage means at different CpG sites in *H19 ICR* between controls and cases. It was observed that there is a significant difference of means at CpG site 1, 5 and 6, (higher methylation in cases than controls) while no difference was observed at sites 2 and 3. Average methylation across the *H19 ICR* locus was also significantly different between cases and controls.

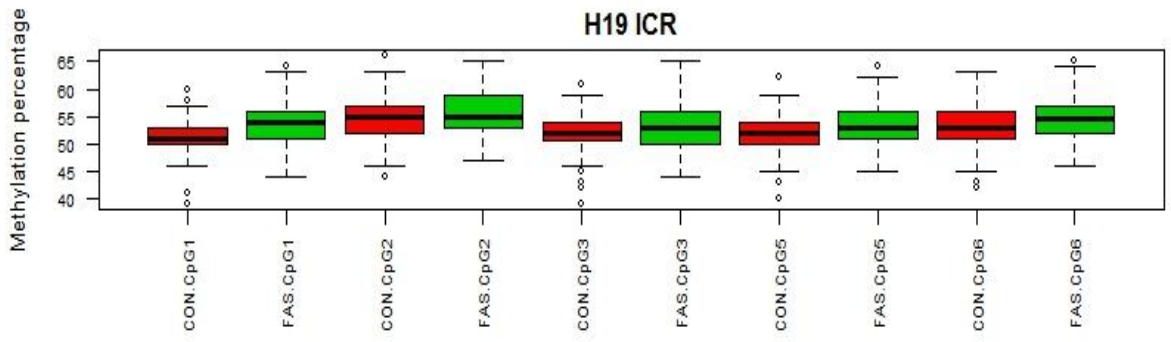


Figure 14: Boxplots summarising the observed percentage methylation at different CpG sites in H19 ICR in (CON) controls and cases (FAS), unadjusted for age and gender.

3.3.2.2. KvDMR1

Table 12: Descriptive data for *KvDMR1* methylation percentages at different CpG sites and average methylation

Control	Mean age (yrs) (min:max)	sites	Mean methy-lation %	Std Dev	Median	Minimum	Maximum	Normal distribution
N=55	21 (18:26)	CpG1	58.64	2.41	59.00	52.50	63.50	yes
		CpG2	61.06	2.82	61.50	51.50	66.50	no
		CpG3	57.16	2.16	57.50	49.50	61.50	no
		CpG4	58.42	2.06	58.50	50.50	62.00	no
		CpG5	59.23	2.47	59.50	50.50	64.50	yes
		CpG6	58.33	2.79	58.50	51.50	63.50	yes
		CpG7	60.16	2.62	60.00	54.00	67.00	yes
		Average	59.04	2.03	58.93	51.64	62.36	no
Cases	8.3 (1:16)	CpG1	59.48	2.97	59.50	52.50	70.00	no
N=86		CpG2	61.44	2.86	61.00	55.50	71.50	no
		CpG3	57.30	2.17	57.00	53.00	64.50	no
		CpG4	58.14	2.25	57.50	54.50	65.50	no
		CpG5	59.21	2.84	59.00	54.00	68.00	no
		CpG6	58.14	3.08	58.00	52.50	68.50	no
		CpG7	59.47	2.53	59.50	55.00	67.00	no
		Average	59.02	2.37	58.57	55.57	67.37	no

N=number

At *KvDMR1* seven CpG sites were analysed. All the CpG sites of the *KvDMR1* had intermediate methylation as expected of a normal imprinted locus of a somatic tissue. CpG 4 and 5 represent the two cutting sites for *NotI* restriction enzyme (Appendix D, *KvDMR1* sequence), which is methylation sensitive.

The methylation data for all the sites in the case group, including average methylation, were not normally distributed. In the control group methylation for sites 1, 5 and 6 were normally distributed while for the other sites as well as for the average methylation across the loci were not normally distributed (Table 12).

Table 13: Comparison of means at different sites and average methylation across the loci at *KvDMR1* between controls and cases

KvDMR1	**sites**	**Control mean %**	**Case mean %**	**p-value**
	CpG1	58.64	59.48	0.142
	CpG2	61.06	61.44	0.693
	CpG3	57.16	57.3	0.790
	CpG4	58.42	58.14	0.093
	CpG5	59.26	59.21	0.376
	CpG6	58.33	58.14	0.338
	CpG7	60.16	59.47	0.026*
	Average	59.04	59.02	0.215

Significant:* p<0.05

In Table 13 and Figure 15 it was observed that there is a significant difference of means only at CpG site 7, where mean methylation is lower in cases than controls. The remaining sites including sites 4 and 5 were not significantly different between the groups.

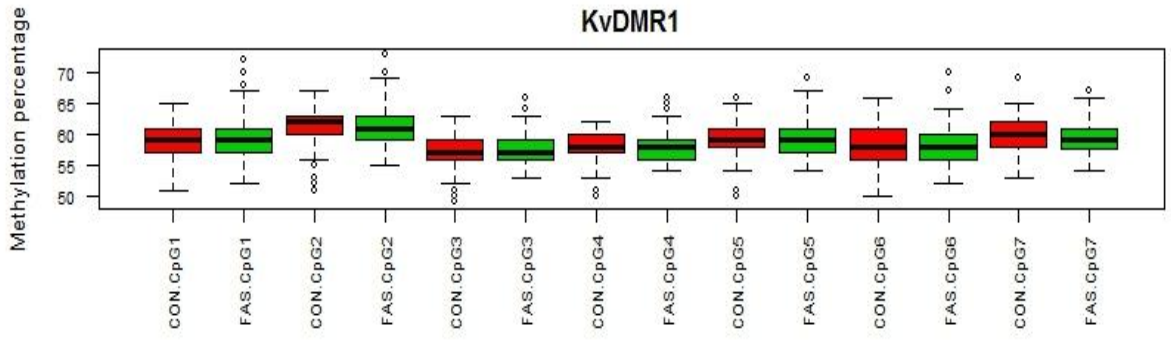


Figure 15: Boxplots summarising the observed percentage methylation at different CpG sites in KvDMR1 in (CON) controls and cases (FAS), unadjusted for age and gender.

3.3.2.3. IG-DMR (region A and B)

For the *IG-DMR*, ten CpG sites were analysed. It has been observed that *IG-DMR* has a wide variability in methylation at the different CpG sites analysed. Most of the individuals had methylation levels of above 70% at CpG 1-5 while CpG sites 6-10 have methylation of approximately 50 percent (Figure 16). The *IG-DMR* primer annealing sites were checked for the presence of known SNPs, and no known SNPs were found. Betaine solution (which is known to improve DNA amplification by reducing the secondary structure in the GC rich regions and also enhances the specificity of PCR) was used in our PCR cocktail but still the CpG site 1-5 methylation remained above 70%. The two regions appear to behave independently with regard to their methylation imprint. Since the average methylation was shown to differ significantly between CpG sites 1-5 and CpG sites 6-10 (Figure 16) they were analysed separately. The *IG-DMR* region was therefore divided into two regions, region A (CpG 1-5) and B (CpG site 6-10) and analysed independently. The average methylation was shown to differ highly significantly between the two regions; region B had 18.3 % lower methylation than region A, $p< 0.0001$(using a joint model).

In Table 14 it was observed that in the control group DNA methylation was normally distributed in all the *IG-DMR*.A region CpG sites together with the average methylation across the region, except for CpG site 5. In the case group only methylation for CpG sites 1 and 5, and average methylation across the locus, were normally distributed. CpG sites 2, 3 and 4 were not normally distributed. As mentioned above, this region does behave as expected for a normal imprinted locus.

Table 14: Descriptive data for *IG-DMR*.A methylation percentages at different CpG sites and average methylation

Control	mean age (min:max)	sites	Mean methy-lation %	Std Dev	Median	Minimum	Maximum	Normal distribution
N=56	21.2 (18:26)	CpG1	71.93	4.44	71.00	63.00	81.50	yes
		CpG2	74.83	5.14	74.00	64.00	85.50	yes
		CpG3	66.23	3.77	66.00	57.00	76.00	yes
		CpG4	72.54	3.87	73.00	65.00	82.00	yes
		CpG5	74.56	5.38	74.25	65.00	86.50	no
		Average	72.02	4.22	71.40	64.40	80.60	yes
Cases	8.5 (1:16)	CpG1	73.05	5.20	73.00	54.00	84.00	yes
N =84		CpG2	76.01	5.99	76.00	52.00	89.00	no
		CpG3	66.67	4.18	67.00	50.00	78.00	no
		CpG4	72.73	4.17	72.50	64.00	87.50	no
		CpG5	77.76	6.55	77.00	66.50	91.50	yes
		Average	73.25	4.55	73.00	63.80	84.10	yes

N=number

In Table 15 it is observed that there is no significant difference in mean methylation at almost all the CpG sites, except for site 5 whose mean methylation is significantly higher in cases than controls. Average methylation of all sites across the *IG-DMR*.A region is also not different between controls and cases.

Table 15: Comparison of means at different sites and average methylation across the loci at *IG-DMR*.A between controls and cases

IG-DMR.A	sites	Control mean %	Case mean %	p- value
	CpG1	71.93	73.05	0.188
	CpG2	74.83	76.01	0.19
	CpG3	66.23	66.69	0.492
	CpG4	72.55	72.73	0.866
	CpG5	74.56	77.76	0.007*
	Average	72.02	73.25	0.109

Significant:* $p<0.05$

Table 16: Descriptive data for *IG-DMR*.B methylation percentages at different CpG sites and average methylation

Control	mean age (min:max)	sites	Mean methy-lation %	Std Dev	Median	Minimum	Maximum	Normal distribution
N=56	21.2 (18:26)	CpG6	56.17	2.84	56.00	51.00	63.00	yes
		CpG7	56.93	2.50	56.75	52.00	65.00	no
		CpG8	52.41	2.30	52.00	49.00	59.00	no
		CpG9	51.80	2.12	51.25	49.00	58.00	yes
		CpG10	52.52	2.32	52.00	49.50	59.00	no
		Average	53.97	1.84	53.40	51.00	59.00	no
Cases	8.5 (1:16)	CpG6	57.56	3.39	57.00	50.00	66.00	yes
N=84		CpG7	57.13	2.51	57.00	52.00	64.00	yes
		CpG8	53.22	3.57	52.50	49.00	65.50	no
		CpG9	53.28	3.81	52.00	49.00	68.50	no
		CpG10	52.86	3.05	52.50	48.50	63.00	no
		Average	54.81	2.76	54.00	50.60	64.40	no

N=number

All the CpG sites for the *IG-DMR.B* reflected an intermediate methylation of about 50 percent consistent with the expected normal methylation distribution for an imprinted tissue locus (Table 16). In controls methylation of CpG 6 and 9 were normally distributed while those for the remaining sites together with the average methylation of CpG sites across the locus were not. In cases methylation for only sites 6 and 7 were normally distributed, while those of the remaining sites together with the average methylation of all CpG sites across the locus were not.

In Table 17 and Figure 16 it is observed that there is no significant difference in mean methylation at CpG site 7, 8 and 10 while at sites 6 and 9 mean methylation is significantly different between controls and cases, with cases having higher mean methylation than controls. Average methylation of all sites across the *IG-DMR*.B region is not different between controls and cases.

Table 17: Comparison of means at different sites and average methylation across the loci at *IG-DMR*.B between controls and cases

IG-DMR.B	Sites	Control mean %	Case mean %	p- value
	CpG6	56.17	57.53	0.012*
	CpG7	56.93	57.14	0.491
	CpG8	52.41	53.22	0.39
	CpG9	51.8	53.28	0.031*
	CpG10	52.52	52.87	0.866
	Average	53.97	54.81	0.084

Significant:* p<0.05

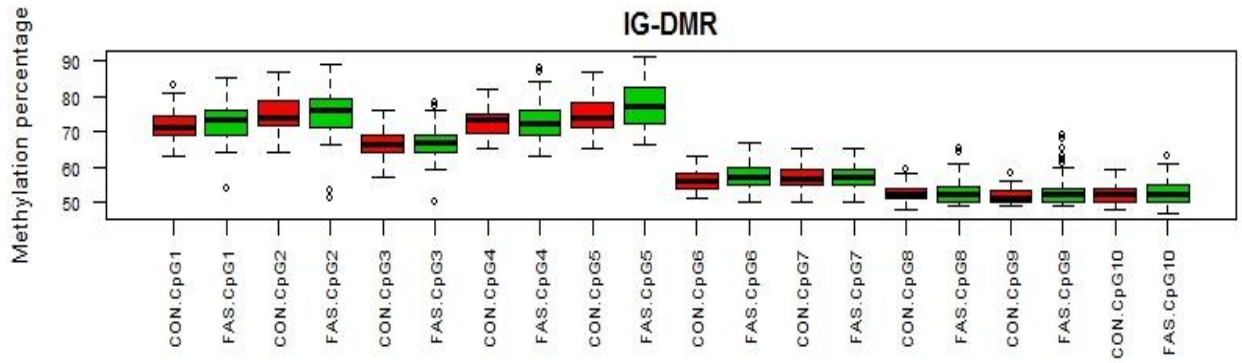


Figure 16: Boxplots summarising the observed percentage methylation at different CpG sites in IG-DMR in (CON) controls and cases (FAS), unadjusted for age and gender.

3.3.2.4. PEG3 DMR

All seven CpG sites at the *PEG3 DMR* in controls reflected an intermediate methylation percentage (30-58%), consistent with methylation levels for a normal imprinted tissue. The methylation percentage for *PEG3 DMR* sites seem to be different from the other imprinted loci (*H19 ICR*, *IG-DMR* and *KvDMR1*), their methylation is lower than 50 percent at some sites. In controls methylation of CpG sites 1-6 were normally distributed as was the average methylation of all the CpG site across the locus, but CpG 7 was not. In cases only methylation of CpG sites 1, 3, 4 and 5 as well as average methylation of CpG sites across the locus were normally distributed while the remaining sites were not.

Table 18: Descriptive data for *PEG3 DMR* methylation percentage at different CpG sites and average methylation

Control	mean age (yrs) (min:max)	sites	Mean methy-lation %	Std Dev	Median	Minimum	Maximum	Normal distribution
N=50	21.3 (18:26)	CpG1	49.97	4.94	49.75	41.00	62.00	yes
		CpG2	45.85	5.92	45.00	35.00	60.00	yes
		CpG3	45.72	5.37	45.25	34.50	57.00	yes
		CpG4	46.71	5.25	47.00	38.00	58.50	yes
		CpG5	39.12	5.15	39.00	30.00	50.50	yes
		CpG6	39.86	4.96	39.50	30.00	53.00	yes
		CpG7	40.7	5.16	40.00	32.00	50.00	no
		Average	43.99	4.81	43.61	35.07	54.79	yes
Cases	8.7 (1:16)	CpG1	44.52	4.64	44.00	33.00	53.50	yes
N=79		CpG2	40.88	4.98	41.00	29.00	58.00	no
		CpG3	39.47	4.85	39.75	26.00	51.00	yes
		CpG4	41.84	4.63	41.75	29.50	55.50	yes
		CpG5	33.46	4.45	33.50	25.00	44.00	yes
		CpG6	34.62	4.44	34.00	25.00	45.00	no
		CpG7	35.21	3.97	35.00	26.00	50.50	no
		Average	38.55	4.00	39.04	29.71	47.93	yes

N=number

In Table 19 and Figure 17 it is observed that mean methylation of all seven CpG sites is highly significantly different between controls and cases, with cases having lower mean methylation than controls. The average methylation of all sites across *PEG3 DMR* is also highly significantly different between cases and control.

Table 19: Comparison of means at different sites in *PEG3 DMR* between controls and cases

PEG3 DMR	sites	Control mean %	Case mean %	p -value
	CpG1	49.97	44.51	<0.0001*
	CpG2	45.85	40.88	<0.0001*
	CpG3	45.72	39.47	<0.0001*
	CpG4	46.71	41.84	<0.0001*
	CpG5	39.12	33.46	<0.0001*
	CpG6	39.86	34.62	<0.0001*
	CpG7	40.7	35.21	<0.0001*
	Average	43.99	38.55	<0.0001*

Significant:* p<0.05

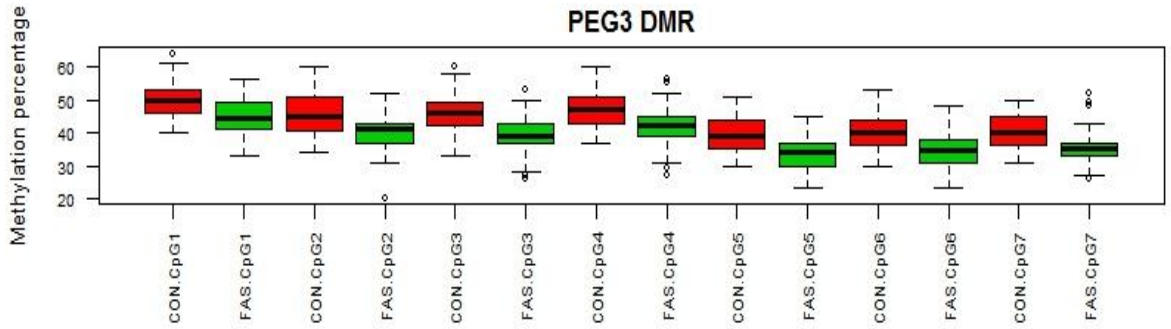


Figure 17: Boxplots summarising the observed percentage methylation at different CpG sites in PEG3 DMR in (CON) controls and cases (FAS), unadjusted for age and gender.

3.3.3. Potential confounders

Age and gender are reported confounders in DNA methylation studies; therefore it was important to examine their effect as potential confounders of DNA methylation changes in the present study. Unfortunately the study design was sub-optimal in terms of age. All cases were below 17 years of age and ranged from 1 to 16 years; all controls were above 18 years of age and ranged from 18 to 26 years. There is no overlap in age and therefore the age effect and alcohol effect on percentage methylation could not be separated. The reason for this major limitation is that ethics approval for the collection of controls stipulated that controls should be over the age of 18 in order to provide informed consent (Appendix A). In addition, when this study was initially planned there was limited knowledge about age as a confounding factor for epigenetic mechanisms. The statistical data for this section of potential confounders and effect size (from Table 20-24 and box plots from Figures 14-19) were generated by a biostatistician from MRC, Dr Lize van der Merwe, using statistical linear mixed-effects models (R package version 3.1-102). All p-values, effects and standard errors (SE) come from the linear mixed-effects models.

3.3.3.1. Age

In Tables 20 and 21 below, methylation percentages were stratified by age of the participants (FAS children and controls), in order to see if average methylation across a locus differed by age in controls and cases. The data were adjusted for gender (fixed), CpG sites, replicates and individuals (random effect). The difference is called effect. Effect is defined as

the estimated percentage difference in methylation between participants of a specific age and those one year younger, in a specific group at the specific locus.

The most significant effect is seen at both *IG-DMR* A and B in FAS cases (Table 20), where the estimated methylation percentage decreased by 0.43% and 0.38% respectively for a one year increase in age. At *KvDMR1* there is a significant estimated methylation increase by 0.19% for every year increase in age in controls however in FAS cases there is a significant decrease by 0.11. Again the highly significant effect is seen at *PEG3 DMR* in the control group, where estimated methylation percentage decrease by 0.22 % for every year increase in age. This decrease is almost half of that seen for *IG-DMR A* and *B* cases. No age effect was observed at *H19 ICR* (in both cases and controls), at both *IG-DMR* A and *B* (in controls) and at PEG3 DMR (in cases).

Table 20: The estimated effect of one year of age on percentage methylation per locus per group

Locus	Group	Effect	SE	p-value
H19 ICR	CON	-0.05	0.10	0.634
H19 ICR	FAS	0.02	0.06	0.749
IG-DMR.A	CON	0.02	0.10	0.861
IG-DMR.A	FAS	-0.43	0.06	< 0.001*
IG-DMR.B	CON	0.01	0.10	0.920
IG-DMR.B	FAS	-0.38	0.06	< 0.001*
KvDMR1	CON	0.19	0.08	0.016*
KvDMR1	FAS	-0.11	0.05	0.021*
PEG3 DMR	CON	-0.22	0.08	0.008*
PEG3 DMR	FAS	0.00	0.05	0.948

CON=controls; FAS=FAS case; SE=standard error of effect, Significant:* p<0.05

Table 21 summarises, for each CpG site, the effect of one year of age on methylation, separately for controls and FAS cases, as well as the estimated difference between cases and controls in that effect. There are five CpG sites in *IG-DMR,* one in *KvDMR1*, where the effect of age on methylation is significantly more negative in FAS cases and controls. At *IG-DMR* sites 2, 5, 6, 8, and 9, as well as at *KvDMR1* site 6, methylation decreased highly significantly with age in FAS cases but no significant effect was detected in controls. In *PEG3 DMR* site 2, the effect was significantly higher in FAS cases than controls.

Table 21: The estimated effect of one year of age on methylation percentage, at different sites of a locus, in controls and cases, separately, and the estimated difference between those effects between FAS and controls

CONTROL GROUP					FAS CASES			Estimated difference in age effect on Me between FAS and CON		
Locus	Site	Age effect	SE	P-value	Age effect	SE	P-value	FAS - CON	SE	p-value
H19 ICR	CpG1	0.02	0.16	0.89	0.03	0.1	0.758	0.01	0.19	0.962
H19 ICR	CpG2	0.04	0.16	0.811	0.06	0.1	0.577	0.02	0.19	0.924
H19 ICR	CpG3	-0.16	0.16	0.298	0.02	0.1	0.851	0.18	0.19	0.326
H19 ICR	CpG5	0.1	0.16	0.522	-0.01	0.1	0.896	-0.11	0.19	0.541
H19 ICR	CpG6	-0.09	0.16	0.546	0.08	0.1	0.448	0.17	0.19	0.36
IG-DMR	CpG1	-0.05	0.17	0.789	-0.43	0.1	< 0.001*	-0.38	0.2	0.052
IG-DMR	CpG2	-0.19	0.17	0.273	-0.72	0.1	< 0.001*	-0.53	0.2	0.007*
IG-DMR	CpG3	0.19	0.17	0.256	-0.02	0.1	0.811	-0.22	0.2	0.273
IG-DMR	CpG4	0.17	0.17	0.311	-0.17	0.1	0.089	-0.35	0.2	0.081
IG-DMR	CpG5	-0.11	0.17	0.52	-0.95	0.1	< 0.001*	-0.84	0.2	< 0.001*
IG-DMR	CpG6	-0.02	0.17	0.922	-0.45	0.1	< 0.001*	-0.43	0.2	0.03*
IG-DMR	CpG7	0.05	0.17	0.774	-0.25	0.1	0.016*	-0.3	0.2	0.134
IG-DMR	CpG8	0.03	0.17	0.878	-0.44	0.1	< 0.001*	-0.47	0.2	0.019*
IG-DMR	CpG9	-0.04	0.17	0.812	-0.52	0.1	< 0.001*	-0.48	0.2	0.016*
IG-DMR	CpG10	-0.03	0.17	0.863	-0.4	0.1	< 0.001*	-0.37	0.2	0.059
KvDMR1	CpG1	0.26	0.17	0.122	-0.11	0.1	0.255	-0.37	0.2	0.056
KvDMR1	CpG2	0.11	0.17	0.513	-0.04	0.1	0.687	-0.15	0.2	0.443
KvDMR1	CpG3	0.1	0.17	0.562	-0.14	0.1	0.148	-0.24	0.2	0.218
KvDMR1	CpG4	0.14	0.17	0.412	-0.09	0.1	0.363	-0.23	0.2	0.243
KvDMR1	CpG5	0.26	0.17	0.126	-0.03	0.1	0.769	-0.29	0.2	0.142
KvDMR1	CpG6	0.18	0.17	0.284	-0.22	0.1	0.023*	-0.4	0.2	0.038*

KvDMR1	CpG7	0.15	0.17	0.38	-0.12	0.1	0.225	-0.27	0.2	0.17
PEG3 DMR	CpG1	-0.19	0.17	0.272	0.02	0.1	0.818	0.22	0.2	0.289
PEG3 DMR	CpG2	-0.28	0.17	0.115	0.25	0.1	0.017*	0.53	0.2	0.01*
PEG3 DMR	CpG3	-0.03	0.17	0.858	0.09	0.1	0.376	0.12	0.2	0.543
PEG3 DMR	CpG4	-0.11	0.17	0.523	0.03	0.1	0.793	0.14	0.2	0.495
PEG3 DMR	CpG5	-0.22	0.17	0.201	-0.14	0.1	0.194	0.09	0.2	0.665
PEG3 DMR	CpG6	-0.19	0.17	0.284	-0.16	0.1	0.125	0.03	0.2	0.895
PEG3 DMR	CpG7	-0.37	0.17	0.033*	0.03	0.1	0.799	0.4	0.2	0.05

SE=standard error; CON=controls; FAS=FAS case; Significant:* p<0.05

3.3.3.2. Gender

Table 22 gives the difference in percentage methylation between males and females, in cases and controls. The data were adjusted for CpG sites, replicate and individuals. The data were not adjusted for age because it was done as a baseline to see if gender needed to be adjusted for. Effect is the percentage difference in estimated methylation between males and females at specific locus. The box plots summarising the percentage methylation by gender are shown in Figure 18.

The gender effect was shown to be highly significant at *PEG3 DMR* in FAS cases, where males had an estimated 1.11% more methyation than females on average. In contrast in controls at *PEG3 DMR* males had a modest but, significant 0.84% lower average methylation in males than females. However average methylation did not differ by gender in control and FAS cases at *H19 ICR*, *IG-DMR A*, *IG-DMR* B and *KvDMR1*.

In light of these differences, age and gender were adjusted for in the subsequent analyses in the following section to assess differences between FAS cases and controls.

Table 22: Comparison of average methylation within a locus between genders, in FAS and controls

Locus	Group	Effect[a]	SE	p-value
H19 ICR	CON	0.33	0.48	0.495
H19 ICR	FAS	-0.16	0.4	0.687
IG-DMR.A	CON	0.37	0.46	0.428
IG-DMR.A	FAS	0.18	0.38	0.626
IG-DMR.B	CON	-0.45	0.46	0.329
IG-DMR.B	FAS	-0.6	0.38	0.112
KvDMR1	CON	0.1	0.39	0.795
KvDMR1	FAS	-0.22	0.32	0.49
PEG3 DMR	CON	-0.84	0.41	0.042*
PEG3 DMR	FAS	1.11	0.33	0.001*

CON=controls; FAS=FAS case; SE=standard error. Significant: *p<0.05

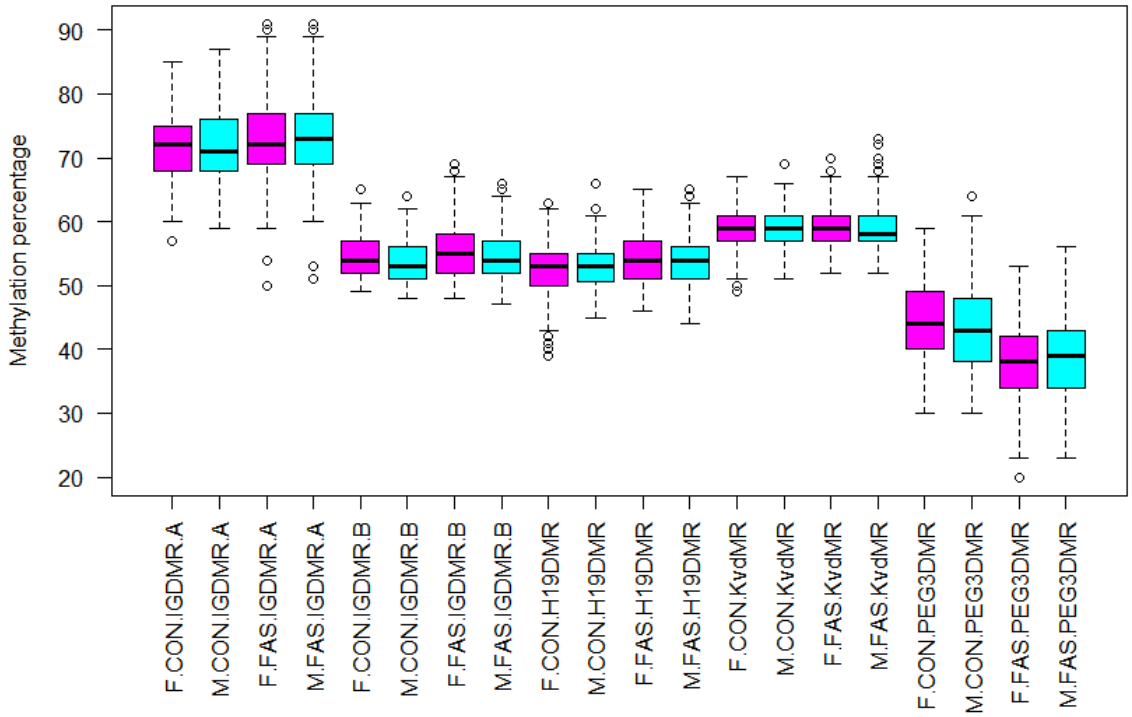


Figure 18: Boxplots summarising observed % methylation within a locus, stratified by gender, in cases (FAS) and controls (CON) and males (M) and females (F).

3.3.4. Case control comparisons - Effect size: unadjusted and adjusted statistical analysis

In this section we assessed potential differences in methylation percentages at different CpG sites and also across loci between controls and FAS cases. For simple comparison, the tables show unadjusted results on the left side and results adjusted for age and gender on the right. The random variation between sites, individuals and replicates per individual was adjusted for in all analyses. The box plots in Figures 14-19 summarise the unadjusted results in Table 23 and represent percentage methylation at different CpG sites in *H19 ICR*, *IG-DMR*, *KvDMR1* and *PEG3 DMR*.

Table 23 gives a summary of estimated difference in CpG methylation between FAS cases and controls (FAS-CON), per CpG site, unadjusted and adjusted for age and gender. Both models were adjusted for random variation between and within *KvDMR1,* were at sites 4 and 7, where methylation was significantly lower in FAS cases than controls, after adjustment for age and gender. At *PEG3 DMR*, across all CpG sites, estimated methylation

were very highly significantly lower (all p-values <0 .001) in FAS than in controls, with and without adjustment for age sex and individuals.

At *H19 ICR*, all sites and at *IG-DMR* sites 2, 5, 6 and 9, the case group had significantly higher methylation than the control group. However after adjusting for age and gender there was no longer a significant difference between controls and cases. The only significant effects detected at *KvDMR1*, were at sites 4 and 7, where methylation was significantly lower in FAS cases than controls, after adjustment for age and gender. At *PEG3 DMR*, across all CpG sites estimated methylation were very highly significantly lower (all p-values <0.001) in FAS than in controls, with and without adjustment for age and sex.

Table 23: Summary of estimated differential CpG methylation between FAS cases and controls (FAS-CON), per CpG site

		Unadjusted			Adjusted for age and gender		
Locus	**Site**	**FAS-CON**	**SE**	**p-value**	**FAS-CON**	**SE**	**p-value**
H19 ICR	CpG1	1.8	0.5	< 0.001*	0.23	0.79	0.767
H19 ICR	CpG2	1.06	0.5	0.035*	-0.49	0.79	0.537
H19 ICR	CpG3	1.17	0.5	0.019*	-0.42	0.79	0.594
H19 ICR	CpG5	1.48	0.5	0.003*	-0.05	0.79	0.95
H19 ICR	CpG6	1.35	0.5	0.007*	-0.16	0.79	0.835
IG-DMR.A	CpG1	1.02	0.53	0.054*	-0.5	0.81	0.54
IG-DMR.A	CpG2	1.07	0.53	0.043*	-0.52	0.81	0.52
IG-DMR.A	CpG3	0.48	0.53	0.364	-1.01	0.81	0.216
IG-DMR.A	CpG4	0.19	0.53	0.724	-1.27	0.81	0.117
IG-DMR.A	CpG5	3.05	0.53	< 0.001*	1.5	0.81	0.065
IG-DMR.B	CpG6	1.34	0.53	0.012*	-0.21	0.81	0.792
IG-DMR.B	CpG7	0.22	0.53	0.679	-1.31	0.81	0.106
IG-DMR.B	CpG8	0.68	0.53	0.202	-0.92	0.81	0.258
IG-DMR.B	CpG9	1.36	0.53	0.01*	-0.22	0.81	0.782
IG-DMR.B	CpG10	0.24	0.53	0.646	-1.33	0.81	0.101
KvDMR1	CpG1	0.96	0.53	0.072	-0.53	0.81	0.512
KvDMR1	CpG2	0.28	0.53	0.596	-1.21	0.81	0.138
KvDMR1	CpG3	0.28	0.53	0.595	-1.2	0.81	0.141
KvDMR1	CpG4	-0.17	0.53	0.752	-1.67	0.81	0.04*
KvDMR1	CpG5	-0.01	0.53	0.986	-1.43	0.81	0.079
KvDMR1	CpG6	-0.1	0.53	0.851	-1.55	0.81	0.057
KvDMR1	CpG7	-0.6	0.53	0.262	-2.12	0.81	0.009*
PEG3 DMR	CpG1	-5.34	0.55	< 0.001*	-6.98	0.83	< 0.001*
PEG3 DMR	CpG2	-5.08	0.55	< 0.001*	-6.69	0.83	< 0.001*

PEG3 DMR	CpG3	-6.14	0.55	< 0.001*	-7.73	0.83	< 0.001*
PEG3 DMR	CpG4	-4.74	0.55	< 0.001*	-6.43	0.83	< 0.001*
PEG3 DMR	CpG5	-5.51	0.55	< 0.001*	-7.07	0.83	< 0.001*
PEG3 DMR	CpG6	-5.11	0.55	< 0.001*	-6.67	0.83	< 0.001*
PEG3 DMR	CpG7	-5.35	0.55	< 0.001*	-6.96	0.83	< 0.001*

Effect=difference in estimated methylation between control and FAS; SE=standard error.
Significant:* p<0.05

The estimated methylation percentage difference between controls and cases across each locus is summarised in Table 24 and the observed percentage methylation in Figure 19. At the *H19 ICR* locus, cases showed a highly significant increased average methylation compared to the controls, but this was no longer significant after adjusting for age and sex. At *KvDMR1* locus showed a significant lower average methylation after age and sex were adjusted. At *IG-DMR* region A and B, in the unadjusted analysis, the average methylation was significantly higher (1.15 and 0.75% respectively) in cases than controls, however after adjusting for age and sex the direction of the effect had changed but the reduced methylation was only significant at region B. The *PEG3 DMR* also showed a highly significant difference between cases and controls and the unadjusted (p<0.001) and adjusted (p<0.001) effect sizes were similar (5.47% lower in cases before adjustment and 7.09% lower in cases after adjustment). Since the unadjusted results may be biased, only the adjusted results are emphasised in the discussion.

Table 24: Estimated differences in percentage methylation between cases and controls at each locus

	Unadjusted			**adjusted for age and gender**		
Locus	**Effect**	**SE**	**p-value**	**Effect**	**SE**	**p-value**
H19 ICR	1.36	0.31	< 0.001*	-0.17	0.41	0.674
IG-DMR.A	1.15	0.3	< 0.001*	-0.4	0.4	0.315
IG-DMR.B	0.75	0.3	0.012*	-0.84	0.4	0.035*
KvDMR1	0.01	0.25	0.967	-1.49	0.37	< 0.001*
PEG3 DMR	-5.47	0.26	< 0.001*	-7.09	0.37	< 0.001*

SE=standard error. Significant: p<0.05. Analysis were adjusted for variation between sites and variation between individuals, with and without adjustment for age and gender

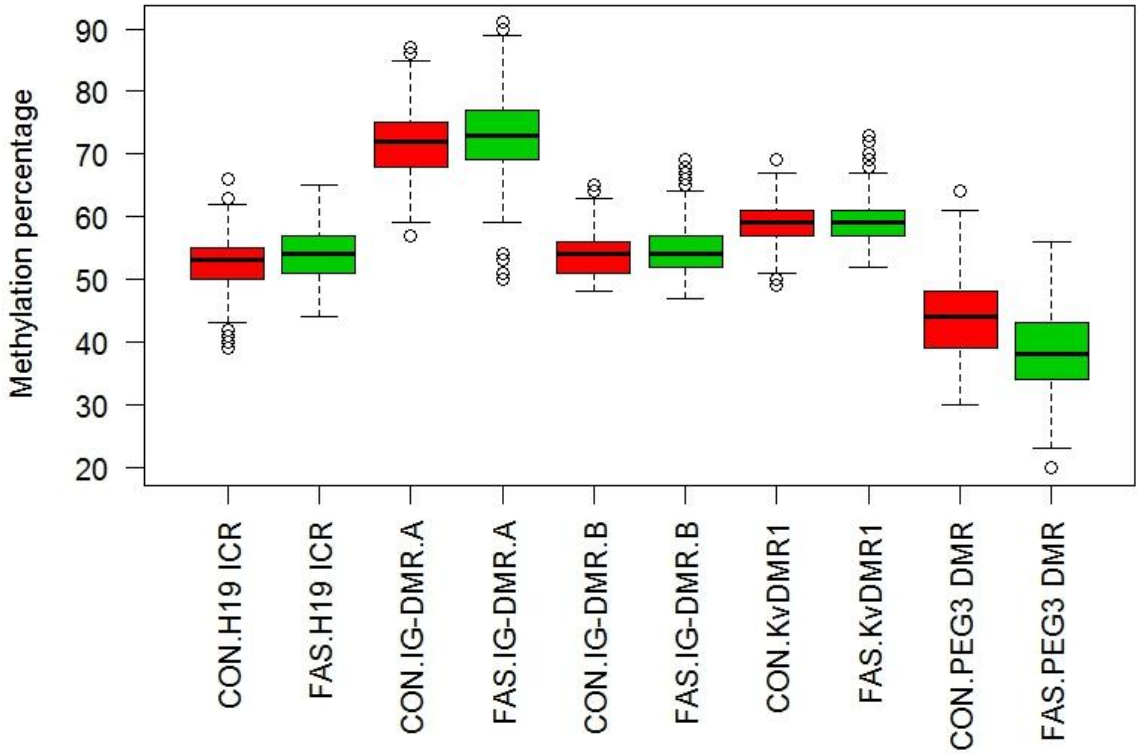


Figure 19: Box plots of observed methylation percentages across ICRs, H19 ICR, *IG-DMR*, *KvDMR1* and *PEG3 DM*R in controls and cases, unadjusted for age and gender.

4. Discussion

The hypothesis of the study is that alcohol exposure during prenatal development will result in epigenetic modifications at imprinted loci, which may be observed as a reduction or increase in DNA methylation at ICRs. The results presented in this thesis support this hypothesis, with an observed reduction in methylation at two of the four ICR loci that I investigated. The following discussion of the study will start by looking at implications of validation of the assay method, specifically with regard to *H19 ICR,* and how the findings were adopted for the other ICR assays. The assessment of tissue specific methylation at ICRs was carried out because of the fact that my study DNA samples originated from two different tissues. The effect of alcohol exposure on the methylation of four selected ICRs will be discussed in accordance with the hypothesis. However, I first start by looking at the implications of the two known confounding factors of DNA methylation, i.e. age and gender. The mechanism involved in the reduction of DNA methylation due to alcohol exposure, together with implications of a loss of imprinting at ICRs, will be explored in terms of epigenetic regulation of imprinted loci and how these may result in the clinical features of FASD.

4.1. Considerations for assay and study design optimisation

In this section I discuss the importance of optimisation of the methodology and study design prior to embarking on a study. In the first instance it is important to ensure that there is no allelic bias in PCR amplification at specific loci and that the results are reproducible. Secondly, since two different tissues were used for DNA extraction, it was important to understand whether there was tissue specific differential DNA methylation at the loci I planned to investigate. I essentially used the *H19 ICR* for this purpose, as it is the best studied and documented in the literature (Barlow and Bartolomei, 2014, Ollikainen and Craig, 2011). Therefore this section is divided into two, optimisation of the assay method for *H19 ICR* and assessment of tissue specific methylation patterns.

4.1.1. Optimising of the *H19 ICR* DNA methylation assay

In this study it was confirmed that the presence of commonly occurring SNPs in the binding region of PCR primers may lead to biased PCR amplification, which results in DNA

methylation patterns reflecting apparent hypermethylation, hypomethylation and intermediate methylation at the *H19* imprinting control region being analysed. This finding was first documented by a retraction of an *IGF2/H19* imprinting study by Tost and colleagues (Tost et al., 2007). In their study they analysed methylation profiles at the 6th CTCF binding site of *H19 ICR* in normal human tissues, and reported three methylation profiles to be present (Tost et al., 2006). However, after being made aware that the three methylation profiles could be fully explained by biased amplification caused by a SNP in the binding site of their PCR reverse primer, they then used a primer avoiding the SNP and only one methylation profile (intermediate methylation, approximately 50%) was observed in all samples. Thus they concluded that the three methylation profiles at the *H19 ICR* were due to a technical artifact (Tost et al., 2007). I went through a similar learning curve during this project and adjusted the *H19 ICR* primers for my study, such that they avoided this polymorphism, which was also present in my study group. This finding was therefore also very important for my case control study and the lesson learnt was applied to the other three ICRs (*IG-DMR*, *KvDMR1* and *PEG3 DMR*). I was careful to check the literature and annotated genome databases for the presence of SNPs in the binding sites for their PCR primers in order to eliminate biased PCR amplification.

In order to ensure reproducibility of results, my runs were done in triplicate for *H19 ICR*. This assisted me in monitoring the consistency or variation of my results, therefore ensuring quality of the data. Running samples in triplicate is advantageous over duplicate runs because if you have an outlier out of the three results you may still use the remaining two. However due to scarce sample and cost of reagents the runs for the *IG-DMR*, *KvDMR1* and *PEG3 DMR* were done in duplicate.

4.1.2. Assessment of methylation status in different tissues

It is now well established that there is differential CpG methylation between tissues (Byun et al., 2009). However when this study was started we did not know much about tissue differential methylation for ICRs and therefore it was important to do this section of the study.

One of the early objectives of the study was therefore to assess if blood and buccal tissues reflected similar methylation profiles at the 6th CTCF binding site of *H19 ICR* (db SNP

annotation database), because the case control participants donated either blood or buccal tissue for the study. All the control participants (N=58) donated blood samples, however with the FAS case participants 8 out of 87 cases donated buccal samples and the remaining 79 participants donated blood (Appendix E).

In order to assess methylation status between the two tissues, I conducted a study using independent control participant specifically recruited for this study and asked them to donate blood and buccal samples. My results showed that *H19 ICR* methylation profiles between blood and buccal tissues from the same individual were not different, and I was confident that using both tissue types in the case control study was not going to confound my results for the *H19 ICR*. However methylation status between the two tissues was not assessed at *KvDMR1*, *IG-DMR* and *PEG3 DMR* which are also included for analysis in the case control study. I made the assumption that methylation profiles at these other three ICRs are also not likely to differ between the two tissues, similar to what I observed for the *H19 ICR.* Although a limitation for my study, it was a reasonable assumption based on two studies in the literature. Bourque et al., (2010) compared average methylation profiles at *KvDMR1* between blood and saliva tissues in healthy adults and reported that their methylation patterns were similar (Bourque et al., 2010). In addition a study by Woodfine et al., (2011) examined the methylation patterns of 17 germ-line DMRs (including *H19 ICR*, *KvDMR1*, *IG-DMR* and *PEG3 DMR*) amongst several somatic tissues (including brain, breast, colon, heart, kidney and liver) and reported that the average methylation did not vary amongst the tissues, thus showing that the germ-line DMRs are stable (Woodfine et al., 2011). These studies support the assumption made in my study, that methylation profiles at the three ICRs are likely to be similar between blood and buccal tissues.

It is important to always validate an assay first before running research samples in order to avoid errors which may bias the results. With methylation studies it is important to check SNPs in the binding sites of the primers in order to avoid biased PCR amplification. Methylation profiles between blood and buccal DNA from the same individual are not different at *H19 ICR*. Even though I did not manage to physically check the methylation status for *KvDMR1*, *IG-DMR* and *PEG3 DMR* between the blood and buccal DNA in the laboratory, there is evidence from the literature (Woodfine et al., 2011) which supports that the methylation profiles between the two tissues are unlikely to differ because germ line

DMR methylation is stable. The methodology optimisation was an essential part of my study and once completed led to higher expected confidence in the results that will be presented in the following sections.

4.2. The effect of alcohol on methylation profiles of ICRs at selected imprinted loci

The main aim of the present study was to examine the effect of alcohol on DNA methylation at ICRs of specific imprinted loci in children with FAS. Children with FAS represent the outcome of alcohol exposure during fetal development. The selected imprinted loci (*IGF2/H19*, *DLK1/MEG3* (*GTL2*), *CDKNIC/ KCNQ1OT1* and *PEG3*) have been shown to be important during embryonic development and growth. I compared the DNA methylation profiles of the paternally imprinted ICRs (*H19 ICR* and *IG-DMR)* and maternally imprinted ICRs (*KvDMR1* and *PEG3 DMR)* between FAS cases and controls. Based on previous research, I proposed that prenatal alcohol exposure will result in epigenetic changes that will lead to the reduction of DNA methylation at the ICRs which will subsequently affect gene expression and contribute to developmental abnormalities seen in FAS individuals. The results of this study support the hypothesis, but only at the *KvDMR1* and *PEG3 DMR* loci, where there is a significant decrease in methylation at these two ICRs in comparison with controls. However, in this study there was no observed effect of alcohol on DNA methylation at *H19 ICR* and *IG-DMR* because there was no significant difference in DNA methylation at these loci between FAS cases and controls.

In this study I first expected a reduction in DNA methylation at selected loci in FAS cases because in an earlier study by Garro and colleagues, prenatal alcohol exposure was reported to cause global DNA hypomethylation in mice (Garro et al., 1991). In another study alcohol was shown to cause a decrease in cytosine methyltransferase mRNA in sperm of offspring of male rats which were exposed alcohol before breeding (Bielawski et al., 2002). Although a global reduction in DNA methylation may seem a reasonable expectation, further studies now suggest locus specific differences. Interestingly it is becoming clear that individual loci may be either hypermethylated or hypomethylated following alcohol exposure. A study by Kaminen-Ahola and colleagues reported that maternal alcohol exposure tended to induce hypermethylation at the A^{vy} locus (Kaminen-Ahola et al., 2010), while Haycock and Ramsay (2009) reported hypomethylation at the *H19 ICR* in mouse placenta following *in utero*

alcohol exposure (Haycock and Ramsay, 2009) and Stouder at al., (2011) also reported hypomethylation at *H19 ICR* in the brain and sperm of *in utero* exposed offspring (Stouder et al., 2011). A study by Liu et al., (2009) has demonstrated that alcohol exposure during early embryonic neurulation can induce aberrant changes in DNA methylation patterns (increased and decreased methylation) with associated changes in gene expression (Liu et al., 2009) .

It is widely suggested that the effect of alcohol on DNA methylation is mediated through the interruption of the one OCM pathway that is critical in the production of the methyl groups for the maintenance of DNA methylation (Halsted et al., 2002, Liu et al., 2009). Alcohol may interrupt the one carbon metabolic pathway by causing folate deficiency (Hamid et al., 2009), this may happen in cases of people who drink a lot in conjunction with poor diet that is lacking essential nutrients like folate. Folate (a member of B class vitamins) is one of the key molecules that makes methyl groups available (donate or transfer) for the one carbon pathway, therefore folate deficiency will interrupt the one carbon pathway and subsequently the DNA methylation (Halsted et al., 2002, Hamid et al., 2009). Secondly alcohol has been reported to reduce the intestinal absorption of folate and also increase its renal excretion, by interfering with the folate transport system (Hamid et al., 2009). Alcohol can also reduce the activity of methionine synthase which remethylates the homocysteine in the one carbon pathway, converting it to methionine which is eventually converted to S-adynosylmethionine (SAM), the universal donor for DNA methylation. Reduced activity of methionine synthase will lead to reduced levels of SAM and therefore hypomethylation (Wani et al., 2012). Lastly, as reported by Garro et al., (1991), alcohol can directly reduce the activity levels of DNA methyltransferases through its metabolite acetaldehyde. Acetaldehyde has been reported to inhibit the activity of the methyltranferases thus leading to global hypomethylation.

4.2.1. The effect of confounding factors on the levels of DNA methylation

Age has been shown to have an effect on DNA methylation (Fraga et al., 2005), while DNA methylation has been shown to differ with gender (Murphy et al., 2012). Our study participants included males and females and the ages of the control and case groups were not overlapping, cases were of a younger age and controls of older age.

4.2.1.1. Age as a confounding factor for DNA methylation

Age is reported to cause a reduction in global DNA methylation and causes dramatic changes in the distribution of 5-methylcytosine across the genome (Heyn et al., 2012, Liu et al., 2011). The promoter regions of many specific genes however, tend to switch from an unmethylated to a methylated state resulting in gene silencing in an age dependent manner. This includes the promoters of several tumor and aging related genes such as Runt related transcription factor 3(*RUNX3*) and Tazarotine-induced gene 1(*TIG1*) (Fuke et al., 2004, Liu et al., 2011, Wilson and Jones, 1983). The mechanism contributing to the age dependent changes in global methylation include a decrease in the expression of *DMNT1* (Liu et al., 2003, Lopatina et al., 2002). With respect to specific genes, methylation can either be increased or decreased depending on the specific gene investigated (Liu et al., 2003). Issa et al., (1996) reported that the *IGF2* P2-P4 promoter-associated CpG island is methylated on the silenced maternal allele in young individuals, however with age this methylation also appears on the paternal allele resulting in biallelic methylation (indicating an increase in methylation with age)(Issa et al., 1996). Longitudinal research on age effect that study the same individuals at several time points is rare (Flanagan et al., 2015, Florath et al., 2014). In two studies DNA methylation of participants was examined at two ages only, one where they were sampled 6 years apart and the other 8 years apart. It is therefore not yet clear whether age-related changes in methylation at CpG loci associated with age effect occur linearly with age (Flanagan et al., 2015, Florath et al., 2014).

In my study I examined the effect of age on the different CpG sites of the loci (*H19 ICR*, *KvDMR1*, *IG-DMR* and *PEG3 DMR*) and average methylation across each locus. In the control group, with the exception of *PEG3 DMR* CpG7 there was no CpG site age specific effect. In FAS cases however, eight out of the ten *IG-DMR* CpG sites, one *KvDMR1* site and one *PEG3 DMR* site showed a significant age effect. With a single exception, methylation in the FAS group decreased by a modest amount for every addition year of age. When examining the locus averaged-methylation and the effect of age, there was a small but significant effect for *KvDMR1 (*both cases and controls*)*, but a larger effect in the FAS cases for *IG-DMR* (for both region A and B). This effect was not observed in controls. In contrast the controls showed an age effect at the *PEG3 DMR* where estimated methylation percentage decrease by 0.22% for every year increase in age. The measure for an age effect is the difference in methylation

per additional year of age, however there was no overlap in absolute age between cases and controls in my study. From my results it would appear that age effects are more significant at younger ages (1 to 16 years) than in the older age group (18 to 26 years), in a locus-specific manner.

In this study age was shown to influence methylation at three of the four loci investigated. In alignment with my study, a study by Heijmans et al., (2008), assessed the relationship between age and *IGF2DMR* methylation in controls for the peri-conceptional famine exposure. They found that within the age group of 43 to 70 years the DNA methylation of a 10 year older group was associated with a 3.6% lower methylation (p=0.015). The magnitude of the effect (0.36% per annum) in their study was greater than that observed in my study (Heijmans et al., 2008).

4.2.1.2. Gender as a confounding factor for DNA methylation

The effect of gender on global DNA methylation and locus specific methylation has been reported. Global DNA methylation has been reported to have a tendency towards higher methylation levels in males (Fuke et al., 2004, Shimabukuro et al., 2007). The results were found to be surprising considering that in female cells, the inactivation of one X chromosome is accompanied by DNA methylation of CpG islands on the inactive chromosome (Norris et al., 1991). However, this apparent anomaly can be explained by studies that showed hypomethylation of regions of the inactive X chromosome, especially at gene poor regions, and hypermethylation of CpG islands in gene rich regions while the active X chromosome is hypermethylated in the body of genes (Hellman and Chess, 2007, Wilson et al., 2006). Another potential explanation for global methylation being higher in males than females may be due to the fact that females tend to have low circulating folate levels (Hsiung et al., 2007). Folate is required for the synthesis of the precursor of the universal methyl donor, SAM, which is essential for DNA methylation. Again folate is important for the formation of erythrocytes. Erythrocytes are regularly depleted by menstruation in females and as a result more folate would be utilised for the formation of erythrocytes leading to low blood folate and thus a decrease in DNA methylation (Terry et al., 2011).

Studies on the effect of gender on locus specific methylation have shown conflicting results. Sandovici et al., (2005) and Eckhardt et al., (2006) found no gender effect on methylation of specific Alu repeats and different loci on chromosomes 6, 20 and 22 (Eckhardt et al., 2006, Sandovici et al., 2005). Sarter et al., (2005) studied promoter regions of four autosomal genes (*MTHFR*, *CALCA* and *MGMT* and *ESR1*) and reported gender as a strong predictor of methylation at three of these autosomal genes, *MTHFR*, *CALCA* and *MGMT*, with males showing higher methylation, *ESR1* methylation levels were not gender dependent (Sarter et al., 2005). Imprinted genes in primordial germ cells, prior to meiotic division, were shown to be more highly methylated in XY cells than in XX cells (Durcova-Hills et al., 2004, Durcova-Hills et al., 2006, El-Maarri et al., 2007). A study by El-Maarrie et al., (2007) looked at the effect of gender on global methylation (Line-1 and Alu repeats) and locus specific methylation (DMRs of *H19*, *PEG3* and *NESP55*). All the DMRs of the three imprinted genes showed a small tendency towards higher methylation in males but none of them reached statistical significance.

In my study the effect of gender on methylation was shown to be significant at only one locus i.e. *PEG3 DMR*. Interestingly the effects are modest, but opposite in FAS cases and controls, with FAS males showing an increased locus-averaged methylation (1.11%) while control males had a lower locus-averaged methylation (0.84%) than females. It is not clear why gender effect on methylation is different in the two groups, but it may be due to the fact that the data were not adjusted for age when the analysis was done because it was done as a baseline comparison to decide if gender needed to be adjusted for in the main analysis. *PEG3 DMR* average methylation was shown to decrease in controls for every one year increase in age. Therefore at this locus, there may be an age gender interaction. There was no effect of gender on average methylation at *H19 ICR*, *KvDMR1* and *IG-DMR*. This shows that the effect of gender on methylation in this study is locus specific.

The study by El-Maarrie et al., (2007) mentioned above supports my results where I found no effect of gender on methylation at *H19 ICR*, *KvDMR1* and *IG-DMR,* on average. But in contrast I found a significant gender effect on *PEG3 DMR* while they did not find any. They hypothesise that the reason why imprinted genes are not significantly affected by gender may be because any factors influencing gender specific differences in methylation (if any)

act during development, or during the cell cycle at the time when methylation or epigenetic marks at an imprinted region are already established (El-Maarri et al., 2007).

Since both age and gender showed some effect on DNA methylation at one or more of the imprinted loci in this study, the discussion that follows presents age and gender adjusted analyses when comparing DNA CpG methylation between FAS cases and unaffected controls.

4.2.2. The effect of *in utero* alcohol exposure on DNA methylation at the four imprinted loci

In this study I assessed the possible effect of maternal alcohol consumption on DNA methylation at four imprinted loci (*H19 ICR*, *KvDMR1, IG-DMR* and *PEG3 DMR)* by comparing methylation levels between FAS cases and unaffected controls. After adjusting for age and gender there was no observed correlation with *in utero* alcohol exposure at the CpG site level at two of the imprinted loci, *H19 ICR* and *IG-DMR*. Interestingly, a modest effect (p=0.035) of decreased averaged methylation (0.84%) at *IG-DMR* region B was observed in FAS cases. There was no difference in average methylation across the loci and at different CpG sites at the *H19 ICR* and *KvDMR1* regions between FAS cases and controls, after adjusting for age and gender.

The *IG-DMR* is a good candidate in terms of its biological impact, in line with the features of FAS. The paternally methylated *IG-DMR* is the primary ICR at *the DLK1/GTL2 (MEG3)* imprinting domain in human chromosome 14q32, where it plays an essential role in regulating monoallelic expression of several imprinted genes including the paternally expressed *DLK1* and maternally expressed *GTL2* (Lin et al., 2003). The methylation on the paternal allele is essential in maintaining the expression of imprinted genes, because, in a mouse model, failure to maintain the paternal methylation has been shown to result in considerable *Dlk* repression while *Gtl2* expression is increased (Schmidt et al., 2000).

The *DLK1/GTL2 (MEG3)* imprinting cluster is a critical region for phenotypes associated with both maternal and paternal uniparental disomy (UPD) of human chromosome 14 (Buiting et al., 2008, Coveler et al., 2002, Kagami et al., 2005, Temple et al., 2007). Maternal uniparental disomy 14 [Upd(14)mat] and hypomethylation at paternally imprinted *IG-DMR* (Ogata et al., 2008) are characterised by pre- and postnatal growth retardation,

developmental delays, mild and moderate mental retardation, muscular hypotonia, small hands and feet, premature puberty and truncal obesity. The locus-averaged methylation of *IG-DMR* was modestly reduced, (p=0.315, region A) and moderately significant (p=0.035, region B) in FAS cases, tending towards hypomethylation which may potentially contribute to the growth and neuronal deficit in affected individuals. The magnitude of alcohol effects may be tissue specific and may play an important role in neurogenesis. These findings warrant further study and validation.

One of the key features of FAS is pre and post growth-retardation and dysregulation of imprinting at *H19 ICR* has been associated with growth disorders (Gicquel et al., 2005, Ideraabdullah et al., 2008, Reik et al., 1995). Loss of imprinting at the *H19 ICR* has been implicated in growth disorders like Russell-Silver syndrome (Azzi et al., 2009). Russell-Silver syndrome (RSS) is a developmental disorder characterized by severe intrauterine and postnatal growth retardation (<3rd percentile), atypical craniofacial features, clinodacty lV and hemihypotrophy (Binder et al., 2011, Hitchins et al., 2001). It has been shown that loss of DNA methylation (LOM) at *H19 ICR* is found in over 50% of patients with RSS (Netchine et al., 2007). Since growth retardation is one of the clinical features of FAS just like in RSS, I expected a decrease in methylation at the *H19 ICR* to be a contributing factor to the growth retardation seen in FAS children. In my study there was no effect of maternal alcohol exposure on DNA methylation at *H19 ICR* in FAS cases. In some cases of FAS there is a catch-up on their growth as they grow older (Streissguth, 2007), therefore, their growth retardation may have a different molecular aetiology to severe growth retardation seen in RSS, suggesting that loss of imprinting at the *H19 ICR* may only be observed in severe growth abnormalities. This possibility is supported by a study that analysed the methylation status at *H19 ICR* in three groups of patients with growth retardation (patients with RSS features, patients with isolated growth retardation and patients presented with clinical signs not related to RSS)(Schonherr et al., 2007). The study was done to elucidate whether epigenetic mutations at *H19 ICR* were generally involved in growth retardation. They reported *H19 ICR* hypomethylation in 20% of patients with RSS features and no cases of *H19 ICR* hypomethylation in the other two groups, thus suggesting that loss of imprinting at *H19 ICR* may be rare in growth retardation in general, but seems to be restricted to a subgroup of patients with RSS (Schonherr et al., 2007).

The findings of my study are, however, in agreement with a study done in a mouse model by Haycock and Ramsay (2009) where they reported no difference in methylation at the *H19 ICR* of mouse embryos exposed to alcohol during the preimplantation period, when compared to unexposed control embryos (Haycock and Ramsay, 2009). Interestingly *H19 ICR* hypomethylation was observed in the mouse placentas suggesting a localised effect on the extra-embryonic tissue, which could explain the effect on fetal growth. In two other related studies subtle differential DNA methylation was observed. In a study by Dawning et al., (2011), a small decrease in methylation was observed at the mouse *Igf2 DMR1* locus, with a significant decrease seen at only one CpG site, in embryos following *in utero* alcohol exposure (Downing et al., 2011). Knezovich and Ramsay (2012) reported a significant decrease at the *H19 ICR* in mice offspring following preconception paternal alcohol exposure(Knezovich and Ramsay, 2012).

One important fact to be noted about the Downing and colleagues study (2011) is that in addition to assessing DNA methylation, they also examined the gene expression of the *Igf2* gene and showed that there was an approximately 1.5 fold decrease in expression of three *Igf2* transcripts in the embryos, following alcohol exposure (Downing et al., 2011). Unfortunately the study by Haycock and Ramsay, as well as my study did not complement the DNA methylation studies with expression studies of genes controlled by the DNA methylation at ICRs that were looked at. This is a limitation for my study, although only blood and/or buccal tissue could have been examined and may not have been the most appropriate tissue to show relevant gene expression differences. Gene expression studies would have assisted me in distinguishing if expression of genes regulated by *H19 ICR* were affected or not affected by alcohol exposure in children with FAS. The importance of expression studies is demonstrated in the Downing study, because they reported no significant decrease in methylation at the mouse *Igf2 DMR1* locus in placentae following *in utero* alcohol exposure but on the other hand the expression studies showed that the expression of four *Igf2* transcripts were decreased by approximately 1.5 fold in placentae (Downing et al., 2011). This implies that alcohol can alter gene expression in the absence of changes in DNA methylation at the ICR, therefore suggesting that other epigenetic modifications that are also important in gene expression, like histone modification and micro-RNA expression, may be affected by alcohol exposure. Therefore future studies

should couple DNA methylation studies at this locus with gene expression studies to validate the effect of maternal alcohol on gene expressions at this locus.

After adjusting for age and gender, two *KvDMR1* CpG sites (4 and 7) showed significant decreased DNA methylation in FAS children which contributed to a locus-averaged decrease of 1.49% methylation in the KvDMR1. The functional impact of this difference is not clear. The biggest effect (a decrease of 7.09% methylation in FAS cases) was observed at *PEG3 DMR*, which was significantly affected by *in utero* alcohol exposure. Both loci show a decrease in methylation following alcohol exposure.

The hypomethylation at *KvDMR1* and *PEG3 DMR* is aligned to our original hypothesis suggesting that alcohol reduces DNA methylation through OCM pathway and its effect on reducing folate levels. In the next section the potential implications of hypomethylation at these loci are explored.

4.2.3. The functional impact of reduced *KvDMR1* methylation in FAS cases is unclear

KvDMR1 CpG site-specific and average locus-wide hypomethylation in response to *in utero* alcohol exposure would suggest a loss of methylation on the maternally methylated ICR which regulates the monoallelic expression of several imprinted genes located in the *CDKN1C/KCNQ1OT1* imprinting domain cluster. This imprinting domain harbours the paternally expressed non-coding antisense transcript to *KCNQ1* called *KCNQ1OT1*, and other maternally expressed protein coding genes including *KCNQ1* and *CDKN1C*. Loss of imprinting or hypomethylation at the *KvDMR1* has been widely implicated in Beckwith Wiedemann syndrome (BWS) (Azzi et al., 2009, Diaz-Meyer et al., 2003, Gaston et al., 2001). BWS is a congenital disorder characterized by pre- and postnatal overgrowth, organomegaly and a high risk of childhood tumours (Weksberg et al., 2010). Hypomethylation at this locus has also been observed in cases of BWS arising after intracytoplasmic sperm injection, *in vitro* fertilization and embryo transfer (Chen et al., 2014). The *KvDMR1* sequence that I analysed included the differentially methylated *NotI* site, which is represented by CpG sites 4 and 5. The *NotI* site at the *KvDMR1* is often altered in BWS and is used in the diagnostic testing of the BWS (Smilinich et al., 1999). Patients with BWS show loss of methylation at the *NotI* site (Bourque et al., 2010). Paradoxically, the FAS cases showed significant

hypomethylation at CpG sites 4 and 7 (1.67 and 2.1%, respectively) and also locus-averaged methylation, yet FAS affected individuals are growth restricted. It is unclear whether hypomethylation of only two of the seven CpG sites in this ICR will affect the level of expression of the imprinted genes in the cluster and what the functional effect may be.

Hypomethylation at *KvDMR1* is expected to result in a degree of biallelic expression of paternally expressed *KCNQ1OT1*, with reciprocal repression of maternally expressed imprinted genes like cyclic-dependent kinase inhibitor 1C (*CDKN1C*). *CDKN1C* encodes for cyclic-dependent kinase inhibitor (*CDKI*) that belongs to the CIP/KIP family of cell cycle regulators and is considered to be a putative tumour suppressor gene (Besson et al., 2008, Watanabe et al., 1998). Decreased expression of *CDKN1C* has been observed in sporadic cancers and embryonic tumours (Higashimoto et al., 2006). There is no evidence to suggest that cancers are more common in individuals with FAS.

To gain further insight into the gene regulation at this locus will require both gene expression and DNA methylation studies to more fully understand the impact of altered methylation at the *KvDMR1*. This is the first study to show the effect of alcohol on the methylation status at *KvDMR1* and the findings are contrary to expectation given that hypomethylation is associated with an overgrowth phenotype (BWS), in contrast to small stature in FAS case.

4.2.4. The role of alcohol induced hypomethylation at the *PEG3* imprinted gene cluster in the pathogenesis of FAS

The *PEG3* imprinting cluster is located on human chromosome 19q13.4 and is regulated by a maternally methylated ICR, the *PEG3 DMR*. The cluster includes several imprinted genes including the paternally expressed gene 3 (*PEG3)*, the imprinted zinc-finger gene 2 (*ZIM2*) and the ubiquitin-specific processing protease 29 (*USP29*), all of which are paternally expressed. Although these loci are syntenic in mouse and human, there are some interesting differences regarding their regulation, their tissue specific expression and their exon structure and genomic arrangement (Kim et al., 2004, Kim et al., 2000b, Murphy et al., 2001). *USP29*, a likely de-ubiquinating enzyme which may be involved in the turnover of many proteins, is highly expressed in the mouse brain (Kim et al., 2000a). *USP29* does not however show significant expression in the human brain. It is highly expressed in mouse and

human testis (Kim et al., 2000a). *PEG3*, on the other hand, is expressed in mouse and human brain, but most highly in human ovary but not mouse ovary (Kim et al., 1997). The *PEG3* gene is also expressed in embryonic tissues including the hypothalamus and brain. *PEG3* encodes a DNA binding protein based on its multiple zinc finger motifs (Iuchi, 2001, Relaix et al., 1996) and it is an imprinted transcription factor that has multiple target genes (Thiaville et al., 2013). It has a proposed tumour suppressive function (Nye et al., 2013) and has been shown to induce *p53*-mediated apoptosis in multiple cell types (Yamaguchi et al., 2002). A mouse knockout model targeting the *Peg3* gene has shown that it is responsible for a variety of phenotypic outcomes including altered maternal offspring rearing behaviour, low birth weight, alteration in fat tissue storage and synthesis, and lower metabolic activity (Curley et al., 2004, Li et al., 1999).

In this study I observed that maternal alcohol consumption is correlated with a significant reduction of approximately 7% methylation at the *PEG3 DMR* in FAS cases as compared to controls. The highly significant ($p<0.001$) decrease in methylation was observed for all the CpG sites analysed for this locus and also for the average methylation across this locus. It is possible that this change in methylation at the *PEG3 DMR* may affect multiple imprinted genes in the region. *PEG3* is expressed from the paternal allele (because of a lack of methylation on the paternal allele of *PEG3 DMR*) and is reciprocally repressed on the maternal allele (because of the presence of methylation on the *PEG3 DMR* maternal allele); suggesting that alcohol induced demethylation likely affects the maternal allele thus leading to derepression of the *PEG3* gene on the maternal allele, and therefore biallelic expression of PEG3. This would lead to an overall increase in PEG3 expression. Most studies have focused on the effects of reduced Peg3 (Champagne et al., 2009, Curley et al., 2004, Kim et al., 2012, Li et al., 1999), but none have yet explored the phenotypic outcome of over expression of PEG3.

Since the mouse knockout model targeting *Peg3* resulted in growth retardation due to non-functional Peg3 or reduced expression of *Peg 3* (Curley et al., 2004, Li et al., 1999), it is curious that the increased expression of *PEG3* appears to have the same outcome in humans. There is good evidence that gene regulation in this *PEG3 DMR* regulated cluster is different between species and the effect of hypomethylation in humans has not yet been

explored. The role of increased expression of *PEG3* on growth retardation, if any, remains unclear.

In a study on *Peg3* target genes by Thiaville and colleagues, it was illustrated that the Peg3 protein is able to bind to specific regions near its target genes, for example those genes that regulate mitochondrial function, tissue developmental genes and imprinted genes like Growth factor receptor bound 10 (*Grb10)* (Thiaville et al., 2013). They further analysed the expression levels of the *Peg3* bound genes in a mutant mouse model (with 75% lower levels of *Peg3* expression relative to the wild type), in order to determine the response of target genes to reduced expression of *Peg3*; and reported either a reduction or increase in several target genes. *Grb10* was reported to have reduced expression due to low expression levels of *Peg3* in the mouse (Thiaville et al., 2013). *Grb10* is an imprinted gene involved in regulating growth and development, and has been implicated as a potent growth inhibitor (Lim et al., 2004). It is maternally expressed in mice, but in humans the expression is both isoform and tissue specific. This further emphasises the differences between the species in terms of the function of the *Peg3* imprinted gene cluster. *Grb10* has been suggested as a strong candidate gene associated with the aetiology of RSS (characterised by growth retardation) because about 10% of RSS patients have been reported to have maternal UPD of chromosome 7 (that encompasses *IGFBP1*, *IGFBP2* and *Grb10*). Since *IGFBP1* and *IGFBP2* are not imprinted, *Grb10* is mooted as the gene to contribute to the pre- and post-natal growth retardation seen in RSS by inhibiting the growth promoting effect of insulin growth factor 1 (*IGF-1*) (Lim et al., 2004). Since low expression of Peg3 has been associated with reduced expression of *Grb10* (Thiaville et al., 2013), it can be speculated that high expression of *PEG3* (as expected in the case of hypomethylation at *PEG3 DMR* observed in my study), may lead to overexpression of *Grb10* and therefore growth retardation. If such regulation were equivalent in humans (which we do not know) it may suggest a mechanism by which *PEG3* may indirectly contribute to growth related features of FAS through its interaction with some of its target genes.

Gene expression studies, without correlation to their imprinting status have demonstrated upregulation of the *PEG3* gene (as well as several other genes) in intrauterine growth restriction (IUGR) placentas (Ishida and Moore, 2013). Since IUGR is a cause of reduced fetal

growth, this study supports our findings that the proposed increase in *PEG3* expression is associated with a growth restriction phenotypes.

At present it is not possible to predict with certainty what the mechanism is through which the significantly reduced methylation at *PEG3 DMR* acts to exert a phenotypic effect in FAS cases. Its role could affect both fetal growth and neuronal development and may involve dysregulation of *PEG3* target genes. The role of the *PEG3 DMR* in regulating the imprinted gene cluster in humans requires further investigations.

4.3. Limitations of the study

This study has several limitations, most of which relate to study design and the challenges working with children. There are also limitations in the technical aspects of the study and the availability of funding to do state of the art NGS approaches to genome wide methylation.

One major limitation of the present study is that the controls and cases were not age matched. The cases were of younger age (17 years and below) while the controls were of older age (18 and above). This means that age is so strongly confounded that it is not easy to tell whether differences between cases and controls are caused by age differences or by the variable under investigation. In an attempt to ameliorate the effect of age, statistical adjustment to the data was done for sex and age in order to ensure that the differences in DNA methylation between the cases and control groups were minimised by these two confounders. The assumption was made that methylation differences are linear with age, meaning that the percentage difference is the same for every year a person is older, within the case and control groups. This is not ideal, as we do not know if the relationship is linear, but it was the best option, given this challenge.

Sample size is another limitation of the study. Although the present study is large in terms of a human FAS cohort, it is still a relatively small sample size for an epigenetic association study. Increasing the sample size would increase the statistical power of the study and therefore the ability to detect small but significant differences. Performance of our study with a larger sample may increase the statistical power to detect subtle alcohol effects on

DNA methylation that could not be detected between the FAS affected offspring and unaffected controls in this study.

5-hydroxymethyl cytosine (5hmC) is the endproduct of the oxidation of 5-methyl cytosine (5mC) during the process of active DNA demethylation and TET enzymes are responsible for the oxidative process (Ito et al., 2011). Increased levels of 5hmC have been observed in brain tissues and embryonic stem cells where it is enriched in promoter regions, gene bodies and intergenic areas near genes; and it is associated positively with gene expression (Xu et al., 2011). Therefore the production of 5hmC appears to have a functional role in promoting gene expression during active DNA demethylation. Alcohol metabolism has been speculated to cause oxidation of 5mC to 5hmC (Jenner et al., 1998, Wright et al., 1999). The method that is the gold standard for the detection of 5mC is bisulfite modification; however, it is unable to differentiate between the 5mC and 5hmC and will detect both. The reason being that when 5hmC is treated with bisulfite, a stable methyl-sulfonate adduct is produced which is also read as a cytosine when sequenced (Huang et al., 2010). The bisulfite modification method therefore would present the concentration 5mC in combination with 5hmC instead of only 5mC alone (Booth et al., 2013). In my study, I was unable to use an alternative method that could differentiate between the 5mC and 5hmC. Given the importance of the implications of 5hmC to understanding gene expression and regulation, such studies should be done in future as it it would have interpretative implications.

The most profound effects of alcohol exposure are on neuronal development and the brain represents the affected tissue as it is the site where the major deficits of the disorder primarily manifest. The present study examined the effect of alcohol only at four loci, which were well chosen in line with their potential role in affecting features of FAS phenotype, however the loci could not be examined in affected tissue, such as the brain. The study reports on the epigenetic effect observed in blood and buccal tissue, which may not directly reflect tissue-specific alterations in the developing brain. For the purpose of human epigenetic studies, blood tissue is easily accessible and buccal tissue is readily available without discomfort or pain, unlike brain tissue which is impractical to obtain in living individuals. There are limited human studies that correlate the epigenetic variations between the brain tissue and blood tissue from the same individuals, however the Genotype Tissue Expression (GTEx) project may address this dilemma. Through their

compilation of information on gene expression from multiple tissues taken from a large number of deceased humans, the GTEx project will make available valuable information to researchers on which tissues /cell types are relevant to a study in relation to the diseases or disorders under investigation (GTEx Portal, 2015). However there are still limitations, for example, all these studies were done post-mortem. A recent study looked at the correlation of DNA methylation between blood and brain tissue collected during neurosurgical treatment from epileptic patient (Walton et al., 2015). Their results suggested that a subset of peripheral blood data may represent methylation status of the brain tissue.

Lastly the present study did not include gene expression studies in parallel with the DNA methylation studies at selected ICRs. Therefore the study was unable to validate if maternal alcohol exposure had an effect on the expression of genes regulated by DNA methylation at specific ICRs.

4.4. Conclusion

This is the first human study to examine epigenetic changes in children with FAS. Most similar studies on epigenetics as a mechanism for *in utero* alcohol effects reported so far are on animal models. The human FAS model is extremely complex to decipher because the time, amount of alcohol and manner of exposure is usually unclear and is at best based on the verbal recollection of drinking behaviour of a mother, often years after the pregnancy. The aim of the study was to examine the effect of maternal prenatal alcohol exposure on DNA methylation profiles of specific ICRs (*H19 ICR*, *IG-DMR*, *KvDMR1* and *PEG3 DMR*) in children with FAS. Despite some of the shortcomings indicated under limitations, the present study suggests that prenatal alcohol exposure is correlated with a reduction in DNA methylation in a locus-specific manner. The study shows significant reduction in DNA methylation at two maternally methylated ICRs, *KvDMR1* and *IG-DMR,* in children with FAS when compared to unaffected controls. The observed hypomethylation at the two ICRs supports the original hypothesis that suggests that alcohol causes a reduction in DNA methylation, through the one carbon metabolism pathway and the effect of alcohol on reducing folate levels.

The observed hypomethylation at the *KvDMR1* however has an uncertain impact on gene expression and the FAS phenotype. The largest epigenetic effect among the loci

investigated, was observed at *PEG3 DMR* where a locus-averaged 7% reduction in methylation was observed across all its 7 CpG sites. This ICR orchestrates a complex pattern of gene expression across the region with reported differences in a mouse model compared to humans. It is proposed that hypomethylation of the *PEG3 DMR* would result in an increase of the paternally expressed *PEG3* gene. *PEG3* has a DNA binding motif and is considered an imprinted transcription factor, and therefore its function is most likely mediated by altered expression of its targets. Although there is some spatiotemporal congruence of gene expression in line with the developmental origin of aspects of the FAS related phenotype, the effect and mechanism of altered gene expression of other imprinted genes controlled by *PEG3 DMR* remains unclear. Despite the uncertainty of the functional biological mechanism of the locus-specific hypomethylation of important ICRs in the blood of FAS cases, these findings support the role of epigenetic mechanism in the development of FAS.

4.5. Future Studies

As emphasised in the study limitations above, future studies to validate the results will have to employ a larger sample size and age matched cases and controls. In addition to DNA methylation at specific ICRs, inclusion of gene expression studies of genes regulated by respective ICRs will be of great importance as they will provide insight into understanding the impact of altered methylation on gene expression at specific ICRs. Gene expression studies could also answer the question of whether alcohol could alter gene expression of respective genes without observed altered DNA methylation at the ICRs that regulate their expression. Secondary DMRs are also of significant importance in regulating associated gene expression in an imprinted cluster, therefore future studies should consider expanding the repertoire of imprinted loci to include secondary DMRs such as *IGF2 DMR* and *MEG3 DMR*. Whole blood is a mixture of different blood cells and DNA methylation has been reported to vary among the different blood cell types (Reinius et al., 2012, Wu et al., 2011); therefore blood cellular heterogeneity should be considered as a confounding factor for methylation in future studies using whole blood.

Since there is evidence from human and animal model studies on the contribution of preconception paternal alcohol exposure on the development of FAS, data collection of

information on the drinking pattern of fathers of the FAS offspring may be of importance as it can be correlated with the severity of the FAS phenotype and epigenetic changes. In addition, inclusion of additional maternal information such as maternal age, maternal nutrition, and patterns of maternal alcohol consumption in the data would be valuable as it could also be correlated with epigenetic changes in the FAS offspring.

DNA methylation is known to interact with histone modifications and ncRNAs in order to maintain the stability and integrity of the genome and alcohol has also been shown to affect these other epigenetic mechanisms, therefore in future studies assessment of the effect of maternal alcohol on histone modification and RNA species in addition to DNA methylation, coupled with gene expression of associated regions, would be of great value.

Measurement of global DNA methylation gives an estimate of the overall DNA methylation level across the genome, and it can be done directly or by using surrogate markers of global DNA methylation e.g. LINE1 and Alu repeats. Therefore inclusion of measurements of global DNA methylation in addition to locus-specific DNA methylation in future studies will be useful as it will give a more general and complete picture of the effect of alcohol on DNA methylation across the genome.

Future studies may assess genome-wide methylation levels and identify novel sites of importance by employing the high throughput techniques like Illumina HumanMethylation 450 Bead chip array. This would involve DNA bisulfite modification followed by whole genome sequencing for methylation using next-generation sequencing. For gene expression (transcriptome) studies, next- generation sequence based transcriptome methodologies (RNA-seq) may be used. This includes messenger RNA (mRNA) extraction with subsequent conversion to cDNA, making a library and then doing next-generation RNA sequencing using, for example, the Illumina platform.

Since blood and buccal tissues do not represent FAS affected tissues, and there are very few human studies that have correlated the epigenetic changes between blood/buccal and brain tissue in an individual; epigenetic results obtained from blood or buccal tissue should be interpreted with caution. The use of postmortem brain tissue in future studies may be useful in solving the problem of getting access to the affected tissue, and would more accurately reflect the alcohol effect on epigenetic variation. The use of post-mortem brain

tissues has been reported in epigenetic related studies of psychiatric disorders like psychosis and depression (Pidsley and Mill, 2011, Sabunciyan et al., 2012). A study that involves post-mortem tissues will be a retrospective study design, for obvious reasons, and it would take a long time to reach a required sample size of FAS cases and controls. The National Institutes of Health Epigenomic Roadmap (Epigenomic Roadmap Project, 2010) and GTEx studies (GTEx Portal, 2015) which aim to index profiles of epigenetic difference across different cell and tissue types may shed some light on the current dearth of knowledge of specific gene expressions in different tissues, and data should be examined carefully to understand the behaviour of primary DMRs at different stages of development.

The aim of the study was to examine the effect of maternal prenatal alcohol exposure on DNA methylation profiles of specific ICRs in children with FAS. The effect of alcohol on DNA methylation was observed to be locus specific, and caused hypomethylation at two loci of the four loci examined. The study suggests alcohol's contribution to FAS phenotype through alteration of epigenetic modifications, specifically DNA methylation. Since epigenetic changes are potentially modifiable, this may present an opportunity for therapeutic intervention in FAS individuals. This is supported by animal model studies which have shown that administration of choline (a methyl donor) following prenatal alcohol exposure has beneficial effects. Supplementation of choline during postnatal development (up to 30 days) and (days 40-60; adolescent/young adult stage in rats) were shown to reduce the severity of alcohol related working memory deficit and behavioural outcome (Ryan et al., 2008, Thomas et al., 2004). If these results can be replicated in human studies it will present opportunity for mitigation of long lasting alcohol effects on the brain in FAS individuals. A plausible mechanism may be that since choline is a methyl donor, it will target DNA methylation, probably by making methyl available for methyl transferases and therefore increase DNA methylation with resultant reduction of alcohol related effects in FAS individuals.

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6. Appendices

Appendix A: Ethics

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Masemola/Ramsay

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080548**
PROJECT	Epigenetic modification at imprinted loci following alcohol exposure during prenatal development
INVESTIGATORS	Ms/Prof Masemola/Ramsay
DEPARTMENT	Human Genetics
DATE CONSIDERED	08.05.30
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.06.11 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof M Ramsay

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Masemola 19/06/08

M. Ramsay 19/6/08

A1. Information Sheet for M080548

January 2009

EPIGENETIC MODIFICATION AT IMPRINTED LOCI FOLLOWING ALCOHOL EXPOSURE DURING PRENATAL DEVELOPMENT.

INFORMATION SHEET

Dear Colleague

We would like to invite you to participate in a study that intends to investigate the epigenetic contribution to fetal alcohol syndrome (FAS). Participation is voluntary. If you choose to participate in this study, your samples will be used as controls for validating the laboratory methods to be used in the above project and again for comparison of methylation status of H19 ICR between different tissues i.e. blood and buccal swabs.

The aim of our study is to examine the effect of alcohol on the methylation of imprinting control regions (ICR) of specific imprinted genes in FAS and non FAS offspring. FAS is a devastating developmental disorder that result from alcohol exposure during fetal development, and a serious public health problem in South Africa. FAS is induced by environmental trigger, excessive alcohol exposure during foetal development, but twin concordance studies and animal models suggest that there are genetic and epigenetic susceptibility factors for developing FAS.

We are going to require blood and buccal swabs. A qualified nurse will take approximately two table spoon (2 tubes) of blood. There will only be a little discomfort. Again you will be requested to scrape the inside of the mouth 10 times with a buccal collection Brush in order to collect buccal cells. For confidentiality all samples will be deidentified. The identity of participants would not be used if the data is published in a scientific journal. There are no costs involved in participating in this study. Participation is completely voluntary. Non-participation has no consequences. If you are willing to participate please complete and sign the attached consent form.

Should you require any further information please, please do not hesitate to contact:

Ms. Matshane Lydia Masemola

Tel: 083 404 8971

Prof. Michele Ramsay

Tel: 011 489 9214

Department of Human Genetics, School of Pathology

National health laboratory Services/ WITS University

P.O. Box 1038, Johannesburg, 2000

A2. Informed Consent for M080548

CASE CODE: _____

EPIGENETIC MODIFICATION AT IMPRINTED LOCI FOLLOWING ALCOHOL EXPOSURE DURING PRENATAL DEVELOPMENT.

INFORMED CONSENT FORM

To be completed by the participant:

I,_____, have read and fully understand the information sheet and agree to participate in this study.

Date of Birth: _____

Signature: _____ Date: _____

To be completed by the researcher:

I have fully explained the procedure and purpose of the study to the participants. I have answered all the participant's questions to the best of my ability.

Signature: _____ Date: _____

A3. Ethics clearance, M02-10-41

q
UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Viljoen/Ramsay

DIVISION OF THE DEPUTY REGISTRAR (RESEARCH)
RECEIVED 2002 -12- 12
FILE:
ACTION:

CLEARANCE CERTIFICATE PROTOCOL NUMBER M02-10-41

PROJECT A Search for Gentic Factors Predisposing to Fetal alcohol Syndrome in South African Populations

INVESTIGATORS Prof's D/M Viljoen/Ramsay

DEPARTMENT School of Pathology, NHLS

DATE CONSIDERED 02-10-25

DECISION OF THE COMMITTEE Approved unconditionally

Unles otherwise specified the ethical clearance is valid for 5 years but may be renewed upon application
This ethical clearance will expire on 30 July 2007.

DATE 02-12-02 CHAIRMAN .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof D Viljoen
Dept of School of Pathology, NHLS
Works2\lain0015\HumEth97.wdb\M 02-10-41
==========
DECLARATION OF INVESTIGATOR(S)

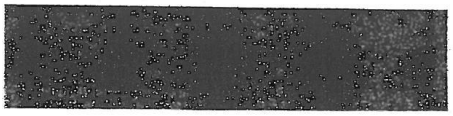
To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress form. I/we agree to inform the Committee once the study is completed.

DATE 17/1/03 SIGNATURE M. Ramsay / Viljoen

PLEASE QUOTE THE PROTOCOL NO IN ALL QUERIES .: M 02-10-41

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES



A4. Information sheet for M02-10-41

Information sheet and consent form
September 2002

Identification of Genetic Risk Factors for Fetal Alcohol Syndrome (FAS)

We would like to invite you to participate in a study aimed at finding some of the genetic causes of FAS. In our previous studies we have diagnosed one of your family members as having FAS. Participation is entirely voluntary and you will not be disadvantaged in any way if you choose not to participate.

The aim of our study is to identify genetic contributing factors to fetal alcohol syndrome (FAS) in South African populations. Although FAS is caused by alcohol abuse during pregnancy, many studies suggest that there are genetic risk factors for developing FAS. FAS is a common birth defect in certain communities in South Africa and worldwide. Although it is a preventable disorder, it is important to understand how and why FAS occurs and to identify the genetic predisposing factors for individuals at high risk of having children with FAS.

To do these studies we need to obtain blood samples from individuals with FAS and their family members, both unaffected and affected. We will also need blood samples from control subjects who are unrelated to these families, but who live in the same area and have a similar ethnic background. A doctor or a nurse will take the blood samples. There will be a little discomfort. We will take the equivalent of about two or three tablespoons of blood from each individual. All samples will be coded and any information obtained from this study will be completely confidential. Some of the samples will be kept in the laboratory for future studies. The samples may be used for further alcohol related studies in the future. You will not be disadvantaged in any way if you decide not to participate. No information will be reported back as this study is likely to take many years. The test will not involve any costs for those taking part and participation is completely voluntary.

Should you require further information, please do not hesitate to contact
Professor Denis Viljoen or Professor Michele Ramsay
Department of Human Genetics
South African Institute for Medical Research
School of Pathology, PO Box 1038, Johannesburg, 2000
Tel. (011) 489 9210 and (011) 489 9214

I, ..., have read and understood this information sheet and agree to participate in the study.

Signature:..... Date:.....
I have fully explained the procedure and the purpose of the study and answered all questions to the best of my ability.

Signature:..... Date:.....

FAS Child's Name:
FAS Child's date of birth:
Relationship to FAS child:

A5. Ethics clearance M03-10-20, Information sheet and Consent form

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Lombard

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M03-10-20

PROJECT	Fetal Alchol Syndrome in the Northern Cape Province, Investigation of Population Structure and-Admixture in the Coloured Community and Collection of Control Subjects
INVESTIGATORS	Z Lombard
DEPARTMENT	School of Pathology, NHLS
DATE CONSIDERED	03-10-31

DECISION OF THE COMMITTEE Approved unconditionally

Unless otherwise specified the ethical clearance is valid for 5 years but may be renewed upon application
This ethical clearance will expire on 1 January 2008.

DATE 03-12-07 CHAIRMAN .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof M Ramsay
Dept of School of Pathology, NHLS
Works2\Iain0015\HumEth97.wdb\M 03-10-20

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress form. I/we agree to inform the Committee once the study is completed.

DATE 9/6/2004 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NO IN ALL QUERIES .: M 03-10-20
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

FETAL ALCOHOL SYNDROME IN THE NORTHERN CAPE PROVINCE: INVESTIGATION OF THE POPULATION HISTORY OF THE COLOURED COMMUNITY AND COLLECTION OF CONTROL SUBJECTS.

INFORMATION SHEET

We are researchers from WITS University, who are working together with the fetal alcohol syndrome (FAS) prevention group from WITS University and FARR. We would like to invite you to participate in a genetic study that intends to identify the genetic causes of FAS. If you choose to participate in this study, you will form part of the control group, which will be helpful in understanding:

1. The genetic heritage of the Coloured community of De Aar and Upington, in order to determine this population groups' genetic history.
2. To determine the genetics factors that may contribute to FAS.

FAS is a common birth defect that results from alcohol consumption by the mother during pregnancy. Newborn babies' facial features, growth and intellect are affected by this disorder. It has been seen before that not all mothers who drink during pregnancy have babies with FAS, suggesting that genetics factors may play a role in the development of FAS. We aim to determine which genetic factors may influence the development of FAS, and therefore need blood samples from a group of individuals with FAS, as well as a group of healthy individuals for the control group.

A good candidate for the control group should be:

1. 18 years or older
2. Male or female
3. Have four coloured grandparents
4. Live in the de Aar or Upington area.

A qualified nurse or doctor will take approximately 2-3 tablespoons of blood (2-3 tubes) from each person. There will only be a little discomfort. All information obtained from this study will be kept completely confidential; therefore blood samples will be coded as soon as they reach the laboratory. Participation is entirely voluntary and you will not be disadvantaged in any way if you choose not to participate. Furthermore, should you feel uncomfortable at any time with your participation in this study, you are free to withdraw from the study, without any negative consequences. Some of these samples will be kept in the laboratory for future studies on the coloured community of the Northern Cape, related to their population structure and health. Unfortunately a study like this takes many years to complete and we will be unable to report back to you personally on the outcome of this study. There are no costs involved in participating in this study. If you are willing to participate, please complete and sign the attached consent form. Thank you for volunteering to be part of this study.

Should you require any further information, please do not hesitate to contact:

Prof. Michele Ramsay
Tel: 011 489 9214

Ms. Zané Lombard
Tel: 011 489 9222

Department of Human Genetics, School of Pathology
National health laboratory Service/WITS University
PO Box 1038, Johannesburg, 2000

CASE CODE: _____

FETAL ALCOHOL SYNDROME IN THE NORTHERN CAPE PROVINCE: INVESTIGATION OF THE POPULATION HISTORY OF THE COLOURED COMMUNITY AND COLLECTION OF CONTROL SUBJECTS.

INFORMED CONSENT FORM

To be completed by the participant:

I, _____, have read and fully understand the information sheet and agree to participate in this study.

Date of Birth: _____

Signature: _____ Date: _____

To be completed by the researcher/nurse/doctor collecting the sample:

I have fully explained the procedure and the purpose of the study to the participant. I have answered all the participant's questions to the best of my ability.

I have confirmed that the participant (*please tick*):

Is 18 years or older ☐

Has four coloured grandparents ☐

Signature: _____ Date: _____

A6. Ethics clearance for additional samples

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

21 September 2011

Ms ML Masemola
Division of Human Genetics
National Health Laboratory Services
University

Dear Masemola

RE: **Protocol M080548: Epigenetic Modification at Imprinted Loci following Alcohol exposure during Prenatal Development**
Request for additional samples

This letter serves to confirm that the Co-Chair of the Human Research Ethics Committee (Medical) has reviewed and approved your to "collect 40 samples (blood and buccal swabs) from members in your Division" as detailed in your letter dated 17/07/2011.

Thank you for keeping us informed and updated

Please accept my apology for the delay in sending this to you.

Yours sincerely,

Anisa Keshav
Secretary
Human Research Ethics Committee (Medical)

Appendix B: Solution Preparations

Blood DNA Extraction Solutions

1 M Tris – HCL pH 8
121.1 g Tris
Make up to 1 L with dH2O
Adjust pH
Autoclave

0.5 M EDTA
93.06 g EDTA
Make up to 500 ml with dH2O
pH to 8.0 with NaOH
NB: EDTA will only dissolve once correct pH is reached

1 M $MgCL_2$
101.66 g $MgCl_2$
Make up to 500 ml with dH_2O
Autoclave

Sucrose-Triton-X Lysing buffer
10 ml 1M Tris-HCL pH8
5 ml 1M $MgCL_2$
10 ml Triton-X 100
Make up to 1L with dH_20
Autoclave
Keep solution chilled at 4^oC
Add 105.9 g sucrose just before use.
(Do not keep longer than 1 day)

20 mM Tris 5 mM EDTA (T20E5)
20 ml 1 M Tris-HCL(pH8)
10 ml 0.5 M EDTA (pH8)
Make up to 1L with dH_20
Autoclave

1X Tris EDTA (TE) Buffer
10 ml 1M Tris-HCL (pH8)
2 ml 0.5 EDTA
Make up to 1 L with dH_20
Autoclave

Saturated NaCl

Autoclave 100 ml dH_2O

Slowly add 40 g NaCl until absolutely saturated i.e. some NaCl will precipitate out.

NB: Before use agitate and let NaCl settle. Use clear supernatant

10% SDS

Add 10 g SDS to 100 ml autoclaved dH_2O

10 mg/ml Proteinase K

Add 100 mg to 10 ml autoclaved dH_2O

Make aliquots of 1ml into 1.5 ml eppendorf tubes and store at -20^{o}C until use

The proteinase K stock is available from Roche

Table B1: Proteinase K mix preparation according to number of samples

No of samples	10% SDS	0.5 M EDTA	10 mg/ml Proteinase-K	H2O	Total
1	50 µl	2 µl	100 µl	348 µl	500 µl
2	100 µl	4 µl	200 µl	696 µl	1 ml
3	150 µl	6 µl	300 µl	1044 µl	1.5 ml
4	200 µl	8 µl	400 µl	1392 µl	2 ml
5	250 µl	10 µl	500 µl	1740 µl	2.5 ml
6	300 µl	12 µl	600 µl	2088 µl	3 ml
7	350 µl	14 µl	700 µl	2436 µl	3.5 ml
8	400 µl	16 µl	800 µl	2784 µl	4 ml
9	450 µl	18 µl	900 µl	3132 µl	4.5 ml
10	500 µl	20 µl	1000 µl	3480 µl	5 ml
11	550 µl	22 µl	1100 µl	3828 µl	5.5 ml
12	600 µl	24 µl	1200 µl	4176 µl	6 ml
13	650 µl	26 µl	1300 µl	4524 µl	6.5 ml
14	700 µl	28 µl	1400 µl	4872 µl	7 ml
15	750 µl	30 µl	1500 µl	5220 µl	7.5 ml
16	800 µl	32 µl	1600 µl	5568 µl	8 ml
17	850 µl	34 µl	1700 µl	5916 µl	8.5 ml
18	900 µl	36 µl	1800 µl	6264 µl	9 ml

Proteinase- K Mix

For 16 extractions

800 µl 10% SDS

32 µl 0.5M EDTA

5568 µl autoclaved dH_2O

Add 1600 µl proteinase- K (10 mg/ml stock), just before use

Pyrosequencing Solutions

Binding buffer (pH 7.6)
10 mM Tris-HCL (1.21 g Tris- HCL)
2 M NaCl (117 g NaCl)
1 mM EDTA (0.292 ml EDTA))
0.1% Tween 20 (1 ml Tween 20)
Dissolve in 900 ml ddH_2O, adjust pH with 1 M HCL, Add 1 ml Tween 20, Make up to 1000 ml with ddH2O

Annealing buffer (pH 7.6)
20 mM Tris (2.42 g Tris)
2 mM Mg-Acetate tetra hydrate (0.43 g)
Dissolve in 900 ml ddH_2O, once completely dissolved, make up to 1000 ml with ddH_2O
Denaturation solution
0.2 M NaOH (8 g NaOH)
Dissolve in 900ml ddH_2O, once completely dissolved, make up to 1000ml with ddH_2O
Store at room temperature

Washing buffer (pH 7.6)
10 mM Tris (1.21 g Tris)
Dissolve in 900ml ddH2O, Adjust pH with 4 M Acetic acid, and make up to 1000 ml with ddH_2O

70% Ethanol
700 ml absolute ETOH
30 ml ddH_2O

Other Solutions

Ficoll-Bromophenol Blue Loading dye (Ficoll)
50% sucrose crystal (50 g)
0.5 M EDTA_ pH7.0 (0.1 ml)
0.1% bromophenol blue dye (0.1 g)
10% Ficoll powder (10 g)
Dissolve in 100 ml of ddH_2O
Store at 4^oC

100bp molecular weight marker
1 µl Ficoll (prepared above)
1 µl 100 bp DNA molecular marker (1µg/µl)
Make up to 10 µl by adding 8 µl of ddH_2O
Store at 4^oC

50 bp molecular weight marker
1 µl Ficoll (prepared above)
1 µl 50bp DNA molecular marker (1 µg/µl)
Make up to 10 µl by adding 8 µl of ddH_2O
Store at 4^oC
The 100 bp and 50 bp are all available commercially from Invitrogen

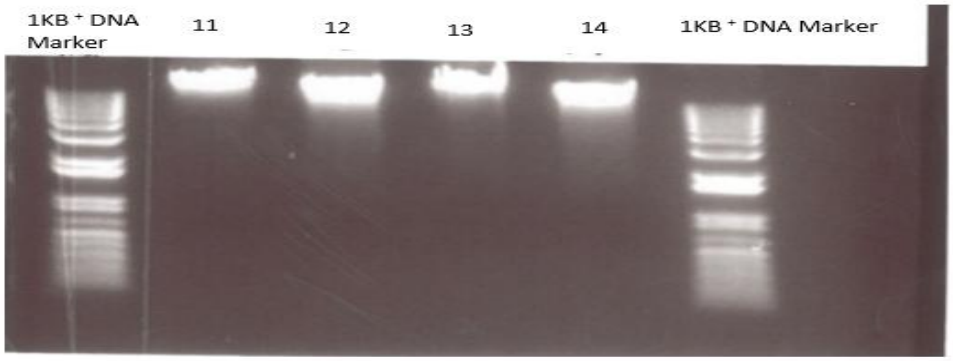
1.25 mM dNTP mix
12.5 µl dATP (100 mM)
12.5 µl dTTP (100 mM)
12.5 µl dCTP (100 mM)
12.5 µl dGTP (100 mM)
950 µl ddH2O (autoclaved)
Make aliquots and store at -20^oC

0.8% Agarose gel
3.2 g Agarose powder
400 ml 1X TBE buffer
12 µl EtBr (10mg/ml)
EtBr (Ethidium Bromide): 3µl EtBr /100 ml of 1X TBE buffer

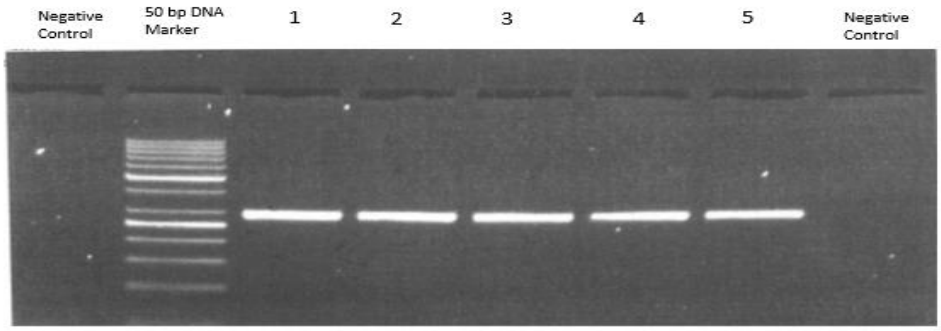
3% Agarose gel
12 g Agarose
400 ml 1X TBE buffer
12 µl EtBr (10 mg/ml)
EtBr (Ethidium Bromide): 3 µl EtBr /100ml of 1X TBE buffer
EtBr solution (Sigma Aldrich)

10 X TBE buffer
216 g Tris
110 g Boric acid
14.88 g EDTA
Make up to 2L with ddH_2O.
Autoclave and store at room temperature

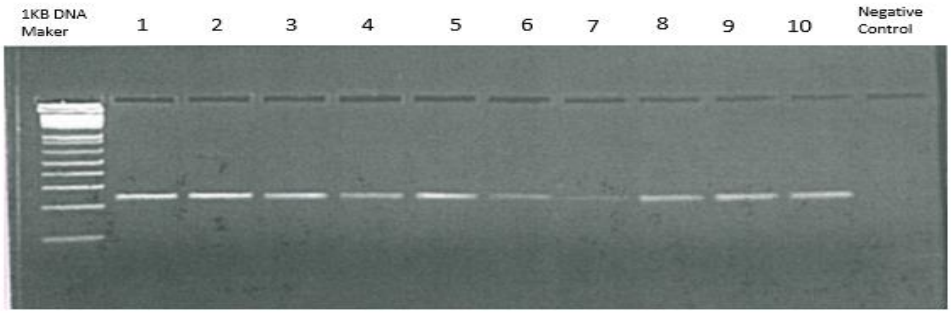
Appendix C: Gels



Whole gDNA gel



IG-DMR PCR gel (267bp amplicon length)



H19 ICR PCR gel (217bp amplicon length)

Appendix D: Protocols

EZ DNA Methylation-Gold kit protocol

Preparation of CT Conversion Reagent

Add 900 µl ddH2O, 300 µl M-Dilution and 50 µl M-Dissolving to the CT Conversation tube. Mix the tube with frequent vortexing and shaking for 10 minutes to dissolve the tube contents.

- Add 130 µl of the prepared CT conversion reagent solution to 20 µl of DNA sample in a PCR tube. Mix the sample by flicking then centrifuged briefly to collect the liquid to the bottom of the tube.
  Place the sample tubes in a thermal cycler and perform the following steps:

  98^{o}C for 10 minutes

  64^{o}C for 2.5 hours

  4^{o}C storage for up to 20 hours

- Add 600 µl of M-Binding buffer into a Zymo-Spin TM Spin IC Column and place the column into a provided collection tubes.
- Load the samples from the thermal cycler onto the column containing M-Binding buffer. Close the cap and mix inverted the column several times.
- Centrifuged the tubes at full speed (≥10 000 x *g*) for 30 seconds, discard the flow- through.
- Add 100 µl of M-Washing buffer to the column and centrifuged at full speed for 30 seconds.
- Add 200 µl M-Desulphonation of buffer to the column and let stand at room temperature (20-30^{o}C) for 15-20 minutes. After incubation centrifuge at full speed for 30 seconds.
- Add 200 µl of M-Washing buffer to the column. Spin at full speed for 30 seconds. Repeat the washing step once.
- Place the column in a sterile 1.5 ml microcentrifuge tube. Add 10µl of M-Elution buffer directly to the column matrix then centrifuge at full speed for 30 seconds to elude the DNA.
- The DNA is ready for immediate use or can be stored at -20^{o}C until later use.

Pyrosequencing preparation protocol

Pyrosequencing was performed using the PSQ 96MA system (Biotage).

The following protocol was taken from Biotage's sample preparation protocol guidelines: Sample preparation guidelines for PSQ ^{TM} 96 and PSQMA systems.

- For all the samples; electrophorese 5 ul of the prepyrosequencing PCR product mixed with 5ul of ficoll on a 3% gel to check if the PCR was successful.
- Set up your run on the PSQ96MA software programme as directed in PSQ 'Getting Started Guide' manual.

1. Cleaning of the vacuum prep needles and Checking if needles sucks properly

- Fill the 96 PCR plate with 80 µl of ddH_2O. Switch on the vacuum pump and apply the vacuum by turning the switch 'on', lower the vacuum prep tool onto the plate for 20 secs or more so

that all ddH_2O is taken up by the vacuum prep tool. (All wells should be empty after this). If there is still ddH2O it means the needles are non functional, therefore the wells should not be used for the run. Place the vacuum prep on the 'parking position'.

2. Immobilisation of PCR products to the beads

- 40-45 µl PCR DNA was added to a clean/sterile 96 well MicroAmp PCR plate
- Shake the bottle of Streptavidin – coated Sepharose™ High performance beads gently until a homogenous solution is obtained.
- Make up a master mix of sepharose beads and binding buffer by adding 6 ul of sepharose beads and 40 µl of binding buffer for each sample.
- Add 46 µl of Sepharose-binding buffer mix to samples previously added to the 96 well MicroAmp PCR plate.
- Cover the MicroAmp PCR plate and place on the orbital shaker set at 300 r.p.m for 10 minutes.
- Prepare the PSQ 96 plate Low well with the sequencing primer and annealing buffer. For each sample add 38.4 µl annealing buffer and 1.6 µl of 10 µM sequencing primer. Make a master mix depending on how many samples you have and then aliquot 40µl into each well corresponding to the samples wells in the MicroAmp PCR plate.

3. Strand separation of products

- Place the 4 troughs on the Vacuum Prep station in order shown in the diagram in the manual page 5.
- Fill each trough with the following :
  180 ml of 70% EtOH (trough 1)
  120 ml of Denaturation buffer (trough 2)
  180 ml of Washing buffer pH 7.6 (trough 3)
  180 ml of ddH2O (trough 4)
- After the agitation of the 96 MicroAmp PCR plate was complete, the samples were resuspended using a pipette before vacuum prep tool was applied to the samples.
- Turn the vacuum pump on and apply the vacuum. Capture the beads-containing the immobilised template on the filter probes by lowering the vacuum prep tool into the 96 MicroAmp PCR plate.
- Make sure all the Sepharose beads have been captured onto the filter probes. Then move the Vacuum Prep Tool sequentially to a trough containing 70% alcohol for 20secs, Denaturation solution for 20 secs, Washing buffer for 20 secs and lastly ddH2O for 20secs. After the last trough allow the liquid to drain completely from the probes by holding the prep tool up at 90^{o}Cfor a few seconds and return to horizontal position.
- Turn off the Vacuum off to release the vacuum.
- Place the PSQ 96 well Low plate (containing the sequencing primer and annealing buffer mix on the work station. Release the beads from the filter probes onto the plate by shaking the vacuum prep tool while allowing the filter probes to rest on the bottom of the wells.

4. Primer Annealing

- Heat the PSQ plate containing the beads, sequencing primer and annealing primer on a heating block at 80^{o}C for 3 minutes.
- Allow to cool at room temperature.

5. Cartridge preparation (this should be done preferable while waiting for the PSQ plate to cool at room temperature)

- Put the cartridge on the bench with the label facing you. Add the specific amount of enzyme, substrate and dNTPs into their respective wells. The amounts are specified from the pyrosequencing software after entering the necessary information for the run and are calculated according to how many samples are going to be analysed at a time.
- Place the PSQ plate and cartridge in their respective positions in the pyrosequencer, and start the run.

Appendix E: DNA sequences

Bisulfite modified DNA sequences for different ICRs analysed

GAAATATTTTAGGTTATTTAAGT**CG**GG**CG**TTATAGGGTTTATAGGGGT**CG**TGAGGTATAGGATATTTA

TGGGAGTTATAT**CG**GGTTA**CG**TGTTTGATTTATTTTAGGGTGTATTGTTGAAGGTTGGGGAGATGGGA

GGAGATATTAGGGGAATAATGAGGTGTTTTAGTTTTATGGATGATGGGGATTT**CG**GTTTTAGTGTGAA

ATTTTTTT**CG**TAGGGTTTTTGGTAGGTATAGAGTTTGGGGGTTTTTGTATAGTATATGGGTATTTTTG

GAGGTTTTTTTTT**CG**GTTTTAT**CG**TTTGGATGGTACGGAATTGGTTGTAGTTGTGGAAT**CG**GAAGTGG

TCGCGCGGCGGTAGTGTAGGTTTATATATTATAGTT**CG**AGTT**CG**TTTTAATTGGGGTT**CG**TT**CG**TGGA

AA**CG**TTT**CG**GGTTATTTAAGTTA**CGCG**T**CG**TAGGGTTTA**CG**GGGGTTATTTGGGAATAGGATATTTAT

GGGAGT**CG**TATTAGATTTTTAGGT**CG**GGTATTATTTATAGTTT**CG**TGGTTT**CG**GGTTATATTT**CG**AGG

GTTTTAGTGTTATGGTTTGGGATTTAAGTTA**CG**TTTATTTATGTGATGATTATAGT

Figure 20: Bisulfied modified *H19* ICR sequence with primers binding in regions containing known SNPs. The nested PCR primers were used innitially for amplification of H19ICR in the pilot study.

Outer foward primer: 5'-GAGTT**CG**GGGGTTTTTGTATAGTAT-3',(G=rs11564736)

Outer reverse primer: 5'-CTTAAATCCCAAACCATAACACTA-3'(5'-TAGTGTTATGGTTTGGGATTTAAG-3')

Inner foward primer: 5'-GTATATGGGTATTTTTGGAGGT-3'

Inner reverse primer: 5'-ATATCCTATTCCCAAATAAC-3' (5'-GTTATTTGGGAATAGGATAT-3')
G=rs56125822

Sequencing primer: 5'-TGGTTGTAGTTGTGGAAT-3'

The underlined yellow highlighted sequence is the 6th CTCF binding site and the last CG (part of the underlined but not highlighted with yellow) is not part of the CTCF binding site but was included in the pyrosequencing analysis. The C in CTCF binding sequence represents the known SNP (C/T), rs10732516. The SNP caused variability of methylation at this CpG site.

GAAATATTTTAGGTTATTTAAGT**CG**GG**CG**TTATAGGGTTTATAGGGGT**CG**TGAGGTATAGGATATTTA

TGGGAGTTATAT**CG**GGTTA**CG**TGTTTGATTTATTTTAGGGTGTATTGTTGAAGGTTGGGGAGATGGGA

GGAGATATTAGGGGAATAATGAGGTGTTTTAGTTTTATGGATGATGGGGATTT**CG**GTTTTAGTGTGAA

ATTTTTTT**CG**TAGGGTTTTTGGTAGGTATAGAGTT**CG**GGGGTTTTTGTATAGTATATGGGTATTTTTG

GAGGTTTTTTTTT**CG**GTTTTAT**CG**TTTGGATGGTACGGAATTGGTTGTAGTTGTGGAAT**CG**GAAGTGG

TCGCGCGGCGGTAGTGTAGGTTTATATATTATAGTT**CG**AGTT**CG**TTTTAATTGGGGTT**CG**TT**CG**TGGA

AA**CG**TTT**CG**GGTTATTTAAGTTA**CGCG**T**CG**TAGGGTTTA**CG**GGGGTTATTTGGGAATAGGATATTTAT

GGGAGT**CG**TATTAGATTTTTAGGT**CG**GGTATTATTTATAGTTT**CG**TGGTTT**CG**GGTTATATTT**CG**AGG

GTTTTAGTGTTATGGTTTGGGATTTAAGTTA**CG**TTTATTTATGTGATGATTATAGT

Figure 21: Bisulfite modified *H19* ICR sequence with primers that did not contain SNPs in their binding sites. The primers were used for the case control study.

Outer reverse primer (purple): CTTAAATCCCAAACCATAACACTA (TAGTGTTATGGTTTGGGATTTAAG)

Inner foward primer (blue): 5′-GTATATGGGTATTTTTGGAGGT-3′ (also used as Outer foward primer)

Inner reverse primer (blue): 5′-ATATCCTATTCCCAAATAA-3′ (5′-TTATTTGGGAATAGGATAT-3′)

Sequencing primer (red): 5′-TGGTTGTAGTTGTGGAAT-3′

GG**CGCG**AGT**CG**T**CG**TTTTTTTTGTTTTTATT**CG**T**CG**TTTTTGAGTATTAT**CG**GGGGT**CG**GGGTTAG**CG**
TTAGTTTTAG**CG**TTGGTAT**CG**T**CG**GGGTGAGTTGGAGATA**CG**GGTTAGTTTTTTGCGTGATGTGTTTA
TTATTT**CG**GGGTGAT**CGCG**TGAGGATA**GCGGTCGT**ATTT**CG**ATATTGTTGTGGGTTTT**CG**GTGTGGAG
GTTTGTGGG**CG**TTTAGGTTA**CG**TT**CG**AGATTAGTTTTTT**CG**T**CG**G**CG**T**CG**TTGTAG**CG**ATTTT**CG**AAT
T**CG**GGTAAGGTT

Figure 22: Bisulfite modified *KvDMR1* Sequence

Forward primer (blue and underlined part of red): 5′-TTAGTTTTTTGYGTGATGTGTTTATTA-3′

Reverse labeled primer (purple): 5′-CCCACAAACCTCCACACC-3′ (5′-GGTGTGGAGGTTTGTGGG-3′)

Sequencing primer (red): 5′-TTGYGTGATGTGTTTATTA-3′

Not1 restriction site (highlighted in green): G**CG**GT**CG**T and the recognition site is

5′...GCGGCCGC...3′
3′...CGCCGGCG...5′

The highlighted yellow region is the sequence of the CpG sites analysed by pyro.

TTTAGGTTGGAATTGTTAAGAGTTTGTGGATTTGTGAGAAATGATTTCGTTTATTGGGTTGGGTTTTG
TTAGTTGTTTGTGGTTTATTAGTTGTTCGCGGTTTATTAGTTGTTCGCGATTTATTAG TGTTTGCGG
TTTATTAGTTGTTTGTGGTTTATTAGTTGTTCGTGGTTTATTAGTTGTTCGTGGTTTATAGTTGTTCG
AGGTTTATAGTTGTTTATGGTTTGTTAATTGTTAGCGATTTGTTAATTGCGAGTGGTTCGTTAGTTGT
TCGCGGTTCGTTAAATTCGTAATTTTGTGGTATTGTAATTGGTTATAATGGATT

Figure 23: Bisulfite modified *IG-DMR* sequence region.

Forward primer (Bio), blue: 5'- TTTATTGGGTTGGGTTTTGTTAG – 3'

Reverse primer: 5' red – AACCAATTACAATACCACAAAATT – 3' (5'-AATTTTGTGGTATTGTAATTGGTT-3')

Sequencing primer (reverse) region1, underlined red: 5' – CAATTACAATACCACAAAAT – 3' (5'-ATTTTGTGGTATTGTAATTG-3')

Sequencing primer (reverse) region2; underlined dark red: 5' – CCATAAACAACTATAAACCT – 3' (5'-AGGTTTATAGTTGTTTATGG-3')

Region 1: highlighted yellow sequence (7 CpG sites)

Region 2: highlighted dark yellow sequence (3 CpG sites)

GTTGGAGTTTGTTGCGTAGGTGTTGTTTTGGTTGGTTGGTGGTAGATGGGGCGGGGTAAGGTTGAAGT
GATAGGGTGGTATTGGATTTAGTGGTTTGTTTAAGTTTTATTTATTTGGGCGTTATTTTTAATGAAAG
TGTTTGAGATTTGTTGCGTAGGCGTTGTTTTAGTTGGTTGGGCGAGATAAGGTTCGTTCGTTTGGGCG
TTATTTTTGATGGGGGTAGTTGAGGTTTGTCGCGTAGGCGTTGTTCGGATTGGTTGGCGGTAGATGCG
GCGGGGTAAGGTTGAAGTGGTTGTAGGTGGTATGGGCGGGACGGTTTGTTTAAGTTTCGTTTATTTGG
GTGTTATTTTTGATCGGGGCGGTTGCGGTTTGTCGTATAGGTGTGGGGTTGTTTATAGGGAGGGGCGG
GGTTACGGTTGTTGTGTTTGTTTGTTTTGTGGTGATGGAAAGTTGTGGAGTGTCGCGTTTTTTGGGTT
GTGTGTGTTGGTCGTTAGGTTGTT

Figure 24: Bisulfite modified *PEG3* DMR sequence

PEG3 Forward primer (blue): 5'-TAATGAAAGTGTTTGAGATTTGTTG-3'

PEG3 Reverse primer (Bio) (purple): 5'-CCTATAAACAACCCCACACCTATAC-3' (5'-GTATAGGTGTGGGGTTGTTTATAGG-3')

PEG3 Sequencing Primer 1(red): 5'-GGGGGTAGTTGAGGTT-3'

Appendix F: Complete data

Table 25: Methylation profiles at 6 CpG sites of *H19 ICR* obtained from blood and buccal tissue DNA.

	% Methylation					
Sample ID	**CpG 1**	**CpG 2**	**CpG 3**	**CpG 4**	**CpG 5**	**CpG 6**
BL001	36	35	34	33	35	35
BL001	34	35	34	34	36	37
BL001	37	37	34	34	34	36
Mean	**36**	**36**	**34**	**34**	**35**	**36**
BS001	33	35	36	32	31	37
BS001	40	40	34	38	36	40
BS001	35	37	34	35	33	37
Mean	**36**	**37**	**35**	**35**	**33**	**38**
BL002	0	4	0	3	0	5
BL002	0	0	5	0	5	0
BL002	3	3	0	0	0	3
Mean	**1**	**2**	**2**	**1**	**2**	**3**
BS002	4	7	3	4	4	5
BS002	4	4	3	3	3	6
BS002	0	4	3	0	0	4
Mean	**3**	**5**	**3**	**2**	**2**	**5**
BL003	42	42	40	0	39	41
BL003	42	45	44	2	41	43
BL003	42	42	50	0	39	39
Mean	**42**	**43**	**45**	**1**	**40**	**41**
BS003	41	49	41	3	41	45
B0003	43	48	42	4	44	45
BS003	41	47	42	3	43	44
Mean	**42**	**48**	**42**	**3**	**43**	**45**
BL004	33	36	35	34	34	34
BL004	31	31	31	30	32	34
B0004	30	31	31	29	29	32
Mean	**31**	**33**	**32**	**31**	**32**	**33**
BS004	39	42	40	39	38	41
BS004	36	41	40	39	37	43
BS004	33	34	32	30	31	33
Mean	**36**	**39**	**37**	**36**	**35**	**39**
BL005	34	31	33	27	33	34
BL005	31	34	33	29	31	33
BL005	31	35	33	31	31	32
Mean	**32**	**33**	**33**	**29**	**32**	**33**
BS005	42	48	42	43	44	45
BS005	41	46	39	42	41	41

BS005	41	44	44	41	42	44
Mean	**41**	**46**	**42**	**42**	**42**	**43**
BL 06	37	34	35	34	36	35
BL 06	39	39	39	37	39	39
BL 06	36	37	37	36	37	40
Mean	37	37	37	36	37	38
BS006	36	39	37	37	39	42
BS006	39	45	40	41	40	44
BS006	39	44	39	40	38	41
Mean	**38**	**43**	**39**	**39**	**39**	**42**
BL008	3	5	4	2	2	4
BL008	4	5	3	2	2	7
BL008	3	5	2	3	2	5
Mean	**3**	**5**	**3**	**2**	**2**	**5**
BS 08	2	5	2	3	3	6
BS 08	2	4	2	2	2	5
BS 08	3	8	0	5	4	6
Mean	**2**	**6**	**1**	**3**	**3**	**6**
BL009	29	32	31	29	31	31
BL009	32	32	30	28	31	34
BL009	30	31	31	31	30	31
Mean	**30**	**32**	**31**	**29**	**31**	**32**
BS009	41	47	40	42	35	34
BS009	32	36	35	36	35	35
BS009	35	43	37	36	39	33
Mean	**36**	**42**	**37**	**38**	**36**	**34**
BL010	31	34	30	31	30	32
BL010	34	33	35	31	33	34
BL010	34	32	33	31	33	35
Mean	**33**	**33**	**33**	**31**	**32**	**34**
BS010	32	34	33	31	33	34
BS010	38	39	40	38	40	42
BS010	29	32	30	32	31	31
Mean	**33**	**35**	**34**	**34**	**35**	**36**
BL011	90	96	91	6	90	95
BL011	89	90	91	5	88	85
BL011	91	91	86	9	82	92
Mean	**90**	**92**	**89**	**7**	**87**	**91**
BS011	91	92	89	4	87	84
BS011	87	95	88	4	83	86
BS011	82	92	83	5	82	86
Mean	**87**	**93**	**87**	**4**	**84**	**85**
BL012	35	38	35	35	34	34
BL012	32	34	30	32	32	35

BL012	29	31	30	31	29	30
Mean	**32**	**34**	**32**	**33**	**32**	**33**
BS012	40	42	40	40	40	43
BS012	34	37	35	32	36	38
BS012	35	42	35	37	38	39
Mean	**36**	**40**	**37**	**36**	**38**	**40**
BL013	36	38	37	34	35	36
BL013	37	38	36	35	34	37
BL013	35	38	37	35	36	34
Mean	**36**	**38**	**37**	**35**	**35**	**36**
BS013	38	42	36	38	39	39
BS013	39	44	38	39	39	42
BS013	38	43	39	41	41	39
Mean	**38**	**43**	**38**	**39**	**40**	**40**
BL014	37	39	38	36	36	36
BL014	37	38	34	36	37	36
BL014	34	38	36	34	32	38
Mean	**36**	**38**	**36**	**35**	**35**	**37**
BS 14	38	39	40	39	40	41
BS 14	31	34	33	33	32	33
BS 14	36	34	35	40	38	35
Mean	**35**	**36**	**36**	**37**	**37**	**36**
BL015	37	37	37	2	36	37
BL015	38	40	40	4	38	42
BL015	42	42	42	0	40	41
Mean	**39**	**40**	**40**	**2**	**38**	**40**
BS015	49	50	46	0	45	45
BS015	39	36	35	5	36	39
BS015	40	43	37	4	38	46
Mean	**43**	**43**	**39**	**3**	**40**	**43**
BL016	36	38	36	32	35	34
BL016	33	33	33	33	30	34
BL016	32	35	32	32	32	34
Mean	34	35	34	32	32	34
BS016	33	31	34	32	34	35
BS016	34	37	36	32	36	38
BS016	26	28	27	29	29	33
Mean	**31**	**32**	**32**	**31**	**33**	**35**
BL017	32	34	30	31	32	33
BL017	35	37	34	33	34	36
BL017	33	36	32	32	33	34
Mean	**33**	**36**	**32**	**32**	**33**	**34**
BS017	35	38	36	38	37	36
BS017	35	40	35	37	36	39

BS017	35	36	32	33	36	35
Mean	**35**	**38**	**34**	**36**	**36**	**37**
BL018	30	32	31	30	32	32
BL018	33	35	34	31	35	35
BL018	32	36	29	31	20	36
Mean	**32**	**34**	**31**	**31**	**29**	**34**
BS018	24	28	27	24	26	29
BS018	28	30	29	29	29	31
BS018	35	35	32	29	31	31
Mean	**29**	**31**	**29**	**27**	**29**	**30**
BL019	28	30	25	25	24	26
BL019	30	34	28	29	29	34
BL019	29	32	28	29	28	31
Mean	**29**	**32**	**27**	**28**	**27**	**30**
BS019	29	28	28	27	29	29
BS019	28	30	34	30	30	36
BS019	30	30	30	30	29	32
Mean	29	29	31	29	29	32
BL020	29	29	27	26	28	29
BL020	30	33	30	31	29	34
BL020	33	34	30	30	32	34
Mean	**31**	**32**	**29**	**29**	**30**	**32**
BS020	27	32	26	29	27	30
BS020	34	31	32	30	31	35
BS020	29	30	31	29	29	34
Mean	**30**	**31**	**30**	**29**	**29**	**33**
BL021	3	5	2	2	0	4
BL021	4	5	4	4	4	7
BL021	3	5	0	3	3	0
Mean	**3**	**5**	**2**	**3**	**2**	**4**
BS021	6	7	5	5	6	5
BS021	0	6	0	0	8	8
BS021	0	4	0	0	2	4
Mean	**2**	**6**	**2**	**2**	**5**	**6**
BL022	28	31	28	28	29	32
BL022	32	35	29	30	29	33
BL022	32	35	28	31	30	30
Mean	**31**	**34**	**28**	**30**	**29**	**32**
BS022	32	35	26	27	30	33
BS022	29	34	30	29	31	32
BS022	32	33	30	30	28	30
	31	34	29	29	30	32
BL023	33	35	32	30	31	34
BL023	32	31	29	28	31	32

BL023	33	37	32	34	31	35
Mean	**33**	**34**	**31**	**31**	**31**	**34**
BS023	34	32	36	33	34	34
BS023	28	29	32	27	30	33
BS023	34	38	36	34	36	37
Mean	**32**	**33**	**35**	**31**	**33**	**35**
BL024	34	37	34	36	34	37
BL024	30	35	28	31	30	33
BL024	35	36	34	33	33	36
Mean	**33**	**36**	**32**	**33**	**32**	**35**
BS024	32	34	32	33	34	35
BS024	35	37	36	36	33	37
BS024	32	33	31	31	32	31
Mean	**33**	**35**	**33**	**33**	**33**	**34**
BL025	34	36	33	33	33	35
BL025	34	35	33	33	32	36
BL025	33	33	32	32	32	37
Mean	34	35	33	33	32	36
BS025	36	35	36	32	35	34
BS025	30	32	29	33	32	32
BS025	36	36	40	34	39	37
Mean	**34**	**34**	**35**	**33**	**35**	**34**
BL026	39	40	39	2	36	38
BL026	38	39	38	2	36	39
BL026	39	40	38	1	36	39
Mean	**39**	**40**	**38**	**2**	**36**	**39**
BS026	36	41	37	2	36	39
BS026	37	40	36	2	35	38
BS026	38	44	37	0	37	40
Mean	**37**	**42**	**37**	**1**	**36**	**39**
BL027	90	92	90	3	86	88
BL027	87	97	91	6	87	87
BL027	90	96	91	3	88	88
Mean	**89**	**95**	**91**	**4**	**87**	**88**
BS027	88	95	87	3	86	89
BS027	88	94	87	4	85	88
BS027	89	91	88	4	86	85
Mean	**88**	**93**	**87**	**4**	**86**	**87**
BL028	3	4	3	2	2	4
BL028	2	4	0	3	0	5
BL028	2	3	3	2	1	6
Mean	**2**	**4**	**2**	**2**	**1**	**5**
BS028	2	4	2	3	2	5
BS028	3	4	3	2	1	7

BS028	2	4	0	3	2	5
Mean	**2**	**4**	**2**	**2**	**2**	**6**
BL029	36	39	37	2	36	37
BL029	36	39	38	4	34	39
BL029	33	36	35	2	32	35
Mean	**35**	**38**	**37**	**3**	**34**	**37**
BS029	33	36	30	0	28	31
BS029	33	35	33	3	32	34
BS029	35	37	34	3	35	38
Mean	**34**	**36**	**32**	**2**	**32**	**34**
BL030	87	89	84	86	85	88
BL030	85	88	87	91	70	90
BL030	86	87	86	87	88	86
Mean	**86**	**88**	**86**	**88**	**81**	**88**
BS030	88	88	85	86	83	87
BS030	84	87	86	90	70	90
BS0030	87	87	86	88	87	87
Mean	**86**	**87**	**86**	**88**	**80**	**88**
BL031	28	30	29	29	29	31
BL031	33	33	30	30	27	34
BL031	35	36	33	34	33	36
Mean	**32**	**33**	**31**	**31**	**30**	**34**
BS031	33	38	33	31	32	32
BS031	37	42	37	35	34	37
BS031	34	36	33	33	33	38
Mean	**35**	**39**	**34**	**33**	**33**	**36**
BL032	33	34	35	32	32	36
BL032	34	35	37	32	34	36
BL032	36	38	35	36	34	36
Mean	**34**	**36**	**36**	**33**	**33**	**36**
BS032	35	35	33	33	33	34
BS032	29	32	31	31	30	33
BS032	32	34	32	32	32	34
Mean	**32**	**34**	**32**	**32**	**32**	**34**
BL033	33	34	34	33	32	37
BL033	33	37	35	36	33	39
BL033	32	34	33	32	32	35
Mean	**33**	**35**	**34**	**34**	**32**	**37**
BS033	37	39	34	36	35	37
BS033	39	41	37	35	36	39
BS033	35	36	36	34	35	37
Mean	**37**	**39**	**36**	**35**	**35**	**38**
BL034	36	34	35	0	35	35
BL034	34	36	34	2	33	33

BL034	38	39	35	1	34	36
Mean	**36**	**36**	**35**	**1**	**34**	**35**
BS034	38	40	37	3	36	38
BS034	35	39	38	3	40	38
BS034	33	39	32	4	33	36
Mean	**35**	**39**	**36**	**3**	**36**	**37**
BL035	37	39	35	37	35	38
BL035	36	36	34	35	32	35
BL035	37	37	38	36	37	38
Mean	**37**	**37**	**36**	**36**	**35**	**37**
BS035	41	42	38	40	39	41
BS035	39	40	40	37	37	42
BS035	41	44	41	43	41	44
Mean	**40**	**42**	**40**	**40**	**39**	**42**
BL036	39	31	36	36	37	39
BL036	34	38	34	34	34	37
BL036	35	37	36	36	35	37
Mean	**36**	**35**	**35**	**35**	**35**	**38**
BS036	36	39	36	36	36	37
BS036	37	43	40	40	38	42
BS036	41	44	38	40	39	41
Mean	**38**	**42**	**38**	**39**	**38**	**40**
BL037	3	4	4	0	4	6
BL037	3	4	2	2	3	4
BL037	2	3	2	2	2	4
Mean	**3**	**4**	**3**	**1**	**3**	**5**
BS037	0	5	6	0	0	7
BS037	3	4	3	3	3	6
BS037	4	0	0	0	0	0
Mean	**2**	**3**	**3**	**1**	**1**	**4**
BL038	36	36	34	32	32	37
BL038	38	41	39	37	38	41
BL038	37	44	33	34	32	41
Mean	**37**	**40**	**35**	**34**	**34**	**40**
BS038	39	35	36	32	36	32
BS038	39	39	42	39	38	40
BS038	39	37	38	36	37	36
Mean	**39**	**37**	**39**	**36**	**37**	**36**
BL039	35	36	34	0	35	37
BL039	30	31	29	3	27	30
BL039	34	40	33	0	35	36
Mean	**33**	**36**	**32**	**1**	**32**	**34**
BS039	34	37	34	3	33	39
BS039	33	37	34	0	33	38

BS039	35	38	34	0	32	36
Mean	**34**	**37**	**34**	**1**	**33**	**37**
BL040	34	37	36	34	39	38
BL040	34	34	31	32	32	33
BL040	35	38	31	36	33	34
Mean	**34**	**36**	**33**	**34**	**35**	**35**
BS040	31	37	35	35	35	37
BS040	35	34	34	32	34	33
BS040	30	38	30	36	36	34
Mean	**32**	**36**	**33**	**34**	**35**	**35**
BL041	24	27	24	24	22	26
BL041	31	32	33	31	31	32
BL041	28	30	30	28	28	29
Mean	**28**	**30**	**29**	**28**	**27**	**29**
BS041	41	41	41	41	38	43
BS041	30	36	29	33	30	36
BS041	38	39	39	40	38	39
Mean	**36**	**39**	**36**	**38**	**35**	**39**
BL042	36	42	41	0	37	42
BL042	39	38	38	0	38	40
BL042	34	34	30	3	30	35
Mean	**36**	**38**	**36**	**1**	**35**	**39**
BS042	38	34	34	0	32	38
BS042	37	40	35	0	34	39
BS042	34	33	34	0	32	35
Mean	**36**	**36**	**34**	**0**	**33**	**37**
BL043	37	37	37	37	34	36
B0 43	35	38	34	35	34	38
BL043	34	37	34	33	34	36
Mean	**35**	**37**	**35**	**35**	**34**	**37**
BS043	34	37	34	33	34	36
BS043	33	36	33	33	28	38
BS043	37	37	37	37	34	36
Mean	**35**	**37**	**35**	**34**	**32**	**37**
BL044	36	41	36	36	36	39
BL044	36	38	38	40	35	36
BL044	36	37	36	34	36	39
Mean	**36**	**39**	**37**	**37**	**36**	**38**
BS044	35	35	37	29	31	36
BS044	35	37	36	31	34	36
BS044	36	38	35	33	36	36
Mean	**35**	**37**	**36**	**31**	**34**	**36**
BL045	36	43	42	0	35	42
BL045	43	45	40	2	39	41

BL045	32	37	32	1	32	34
Mean	**37**	**42**	**38**	**1**	**35**	**39**
BS045	32	36	42	5	32	32
BS045	39	41	39	4	38	42
BS045	37	38	37	0	37	39
Mean	**36**	**38**	**39**	**3**	**36**	**38**
BL046	29	31	30	2	29	32
BL046	30	32	31	0	31	32
BL046	30	31	30	0	30	31
Mean	**30**	**31**	**30**	**1**	**30**	**32**
BS046	30	32	31	0	31	32
BS046	30	31	30	0	30	31
BS046	29	31	30	2	29	32
Mean	**30**	**31**	**30**	**1**	**30**	**32**
BL047	37	35	34	6	34	36
BL047	32	32	34	3	33	33
BL047	38	37	38	0	37	37
Mean	**36**	**35**	**35**	**3**	**35**	**35**
BS047	37	36	36	5	35	39
BS047	36	41	33	5	33	37
BS047	39	40	37	5	36	41
Mean	**37**	**39**	**35**	**5**	**35**	**39**
BL048	30	30	33	30	31	32
BL048	34	35	34	36	33	33
BL048	33	35	34	35	33	32
Mean	**32**	**33**	**34**	**34**	**32**	**32**
BS048	40	43	38	39	40	40
BS048	37	40	37	39	36	37
BS048	39	42	38	40	38	38
Mean	**39**	**42**	**38**	**39**	**38**	**38**
BL049	30	28	29	28	28	33
B0 49	31	31	31	30	29	33
BL049	29	29	31	27	29	30
Mean	**30**	**29**	**30**	**28**	**29**	**32**
BS049	31	33	32	31	32	33
BS049	28	31	25	28	25	26
BS049	34	36	30	31	31	29
Mean	**31**	**33**	**29**	**30**	**29**	**29**
BL050	31	30	32	29	31	31
BL050	32	34	36	33	36	33
BL050	30	30	30	29	29	33
Mean	**31**	**31**	**33**	**30**	**32**	**32**
BS050	30	30	30	29	29	33
BS050	32	34	36	33	36	33

BS050	31	30	32	29	31	31
Mean	**31**	**31**	**33**	**30**	**32**	**32**
BL051	33	27	26	25	26	26
BL051	31	34	33	32	32	34
BL051	31	28	36	29	34	34
	28	**30**	**32**	**29**	**31**	**31**
BS051	30	30	23	24	27	30
BS051	29	29	30	29	29	31
BS051	32	37	29	32	32	34
	30	**32**	**27**	**28**	**29**	**32**
BL052	28	26	29	28	27	29
BL052	25	27	26	26	25	30
BL052	28	31	32	26	31	31
	27	**28**	**29**	**27**	**28**	**30**
BS052	31	33	27	29	26	28
BS052	32	32	27	28	31	33
BS052	32	35	32	31	32	33
Mean	**32**	**33**	**29**	**29**	**30**	**31**

Table 26: Typed control samples for different ICRs

Controls	***H19ICR***	***KvDMR1***	***IG-DMR***	***PEG3DMR***
DNA004392	Yes	Yes	Yes	Yes
DNA004393	Yes	Yes	Yes	Yes
DNA004394	Yes	Yes	Yes	Yes
DNA004395	Yes	Yes	Yes	Yes
DNA004396	Yes	Yes	Yes	Yes
DNA004398	Yes	Yes	Yes	Yes
DNA004399	No	Yes	Yes	Yes
DNA004401	Yes	Yes	Yes	Yes
DNA004402	Yes	Yes	Yes	No
DNA004404	No	Yes	Yes	Yes
DNA004406	Yes	Yes	Yes	Yes
DNA004407	Yes	Yes	Yes	Yes
DNA004408	Yes	Yes	Yes	Yes
DNA004410	Yes	Yes	Yes	No
DNA004411	Yes	Yes	Yes	Yes
DNA004412	Yes	Yes	Yes	Yes
DNA004413	Yes	Yes	Yes	Yes
DNA004414	Yes	Yes	Yes	Yes
DNA004415	Yes	Yes	Yes	Yes
DNA004418	Yes	Yes	Yes	Yes
DNA004420	Yes	Yes	Yes	Yes

DNA004421	Yes	Yes	Yes	Yes
DNA004422	No	Yes	Yes	Yes
DNA004425	Yes	Yes	Yes	Yes
DNA004426	Yes	Yes	Yes	Yes
DNA004428	Yes	Yes	Yes	Yes
DNA004429	Yes	Yes	Yes	Yes
DNA004430	No	Yes	No	No
DNA004431	Yes	Yes	Yes	Yes
DNA004434	Yes	No	Yes	Yes
DNA004437	No	Yes	Yes	Yes
DNA004440	Yes	Yes	Yes	Yes
DNA004441	Yes	Yes	Yes	Yes
DNA004442	Yes	Yes	Yes	Yes
DNA004448	Yes	Yes	Yes	Yes
DNA004450	Yes	Yes	Yes	Yes
DNA004456	Yes	Yes	Yes	No
DNA004460	Yes	Yes	Yes	No
DNA004461	Yes	Yes	Yes	No
DNA004463	No	Yes	Yes	Yes
DNA004465	No	Yes	Yes	Yes
DNA004466	Yes	Yes	Yes	Yes
DNA004476	Yes	Yes	Yes	Yes
DNA004477	Yes	Yes	Yes	Yes
DNA004481	No	Yes	Yes	Yes
DNA004482	Yes	Yes	no	Yes
DNA004483	Yes	Yes	Yes	Yes
DNA004487	Yes	Yes	Yes	Yes
DNA004492	Yes	Yes	Yes	Yes
DNA004494	Yes	Yes	Yes	No
DNA004496	Yes	Yes	Yes	No
DNA004497	Yes	Yes	Yes	Yes
DNA004498	Yes	Yes	Yes	Yes
DNA004500	Yes	Yes	Yes	Yes
DNA004501	Yes	Yes	Yes	Yes
DNA004664	Yes	No	Yes	Yes
DNA004668	Yes	No	Yes	Yes
DNA004690	Yes	Yes	Yes	Yes

All samples are blood DNA samples

Table 27: Typed case samples for different ICRs

Cases	*H19 ICR*	*KvDMR1*	*IG-DMR*	*PEG3 DMR*
DNA003985	Yes	Yes	Yes	Yes
DNA003989	Yes	Yes	Yes	Yes
DNA003991	Yes	Yes	Yes	Yes
DNA004010	Yes	Yes	Yes	Yes
DNA004012	Yes	Yes	Yes	Yes
DNA004013	Yes	Yes	Yes	Yes
DNA004017	Yes	Yes	Yes	No
DNA004021	Yes	Yes	Yes	Yes
DNA004024	Yes	Yes	Yes	Yes
DNA004029	Yes	Yes	Yes	Yes
DNA004032	Yes	Yes	Yes	Yes
DNA004035	Yes	Yes	Yes	Yes
DNA004042	No	Yes	Yes	Yes
DNA004052	Yes	Yes	Yes	Yes
DNA004053	Yes	Yes	Yes	Yes
DNA004055	Yes	Yes	Yes	Yes
DNA004059	Yes	Yes	Yes	Yes
DNA004061	Yes	Yes	Yes	Yes
DNA004063	Yes	Yes	Yes	Yes
DNA004064	Yes	Yes	Yes	Yes
DNA004065	No	Yes	Yes	Yes
DNA004068	Yes	Yes	Yes	Yes
DNA004069	Yes	Yes	Yes	Yes
DNA004072	Yes	Yes	Yes	Yes
DNA004074	Yes	Yes	Yes	Yes
DNA004076	Yes	Yes	Yes	Yes
DNA004082	Yes	Yes	Yes	Yes
DNA004094	Yes	Yes	Yes	Yes
DNA004126	Yes	Yes	Yes	Yes
DNA004141	Yes	Yes	Yes	No
DNA004146	No	Yes	No	Yes
DNA004157	No	Yes	Yes	No
DNA004161	No	Yes	Yes	No
DNA004166	No	Yes	No	Yes
DNA004168	Yes	Yes	Yes	Yes
DNA004172	Yes	Yes	Yes	Yes
DNA004191	Yes	Yes	Yes	Yes
DNA004193	Yes	Yes	Yes	Yes
DNA004198	Yes	Yes	Yes	Yes
DNA004200	Yes	Yes	Yes	Yes

DNA004204	Yes	Yes	Yes	Yes
DNA004225	Yes	Yes	Yes	Yes
DNA004227	Yes	Yes	Yes	Yes
DNA004231	Yes	Yes	Yes	Yes
DNA004233	Yes	Yes	Yes	Yes
DNA004241	Yes	Yes	Yes	Yes
DNA004246	Yes	Yes	Yes	Yes
DNA004249	Yes	Yes	Yes	Yes
DNA004253	Yes	Yes	Yes	Yes
DNA004258	Yes	Yes	Yes	Yes
DNA004267	Yes	Yes	Yes	Yes
DNA004270	Yes	Yes	Yes	Yes
DNA004280	Yes	Yes	Yes	Yes
DNA004290	Yes	Yes	Yes	Yes
DNA004292	Yes	Yes	Yes	Yes
DNA004296	Yes	Yes	Yes	Yes
DNA004300	Yes	Yes	Yes	Yes
DNA004301	Yes	Yes	Yes	Yes
DNA004304	Yes	Yes	Yes	Yes
DNA004305	Yes	Yes	Yes	Yes
DNA004308	Yes	Yes	Yes	No
DNA004312	Yes	Yes	Yes	Yes
DNA004313	Yes	Yes	Yes	Yes
DNA004317	Yes	Yes	Yes	Yes
DNA004325	Yes	Yes	Yes	Yes
DNA004327	Yes	Yes	Yes	Yes
DNA004331	Yes	Yes	Yes	Yes
DNA004335	Yes	Yes	Yes	Yes
DNA004337	Yes	Yes	Yes	Yes
DNA004343	Yes	Yes	Yes	Yes
DNA004348	No	Yes	Yes	Yes
DNA004350	No	Yes	Yes	Yes
DNA004354	Yes	Yes	Yes	No
DNA004356	Yes	Yes	Yes	Yes
DNA004360	Yes	Yes	Yes	Yes
DNA004365	Yes	Yes	Yes	Yes
DNA004370	No	Yes	Yes	Yes
DNA004373	Yes	Yes	Yes	Yes
DNA004375	no	Yes	Yes	Yes
DNA004378	Yes	Yes	Yes	Yes
DNA004380	No	Yes	Yes	Yes
DNA004382	Yes	Yes	Yes	Yes
DNA004384	No	Yes	Yes	Yes

DNA004386	Yes	Yes	Yes	Yes
DNA004388	No	Yes	Yes	Yes
DNA004390	Yes	Yes	Yes	No
DNA004391	Yes	Yes	Yes	Yes

Highlighted grey=buccal DNA samples; unhighlighted =blood DNA samples

Table 28: Complete data for *H19 ICR* control samples and runs.

H19 ICR								
Sample ID	**Gender**	**Age(yrs)**	**Run**	**% Methylations**				
				CpG1	CpG2	CpG3	CpG5	CpG6
DNA004392	F	25	1	51	52	54	52	54
			2	56	57	56	56	57
			3	54	58	56	55	58
DNA004393	M	19	1	52	54	54	54	54
			2	51	55	53	54	53
			3	57	58	58	57	59
DNA004394	M	20	1	51	50	51	51	54
			2	53	55	52	52	56
			3	50	54	50	52	51
DNA004395	F	26	1	53	55	53	52	53
			2	58	61	59	59	58
			3	55	58	56	54	53
DNA004396	F	25	1	50	50	39	49	49
			2	54	53	43	56	54
			3	53	53	45	55	54
DNA004398	M	19	1	51	52	53	52	56
			2	46	49	47	47	51
			3	49	46	48	48	52
DNA004401	F	22	1	51	53	51	49	53
			2	48	50	52	51	54
			3	52	56	53	53	53
DNA004402	F	21	1	52	53	54	51	53
			2	48	51	49	48	48
			3	54	56	53	51	52
DNA004406	F	22	1	47	50	46	45	48
			2	48	52	48	49	50
			3	47	50	46	45	48
DNA004407	F	24	1	41	46	39	43	45
			2	39	44	39	40	43
			3	41	44	42	43	42
DNA004408	M	24	1	54	60	52	54	53
			2	56	62	58	57	58
			3	58	66	58	58	59
DNA004410	F	20	1	54	58	54	56	56

			2	56	60	56	57	59
			3	52	56	52	54	53
DNA004411	M	19	1	51	53	51	51	52
			2	53	56	53	53	55
			3	49	49	48	48	49
DNA004412	M	23	1	51	59	52	53	55
			2	50	58	57	54	54
			3	50	57	54	55	57
DNA004413	F	19	1	54	57	53	53	55
			2	51	52	50	49	51
			3	49	56	50	51	54
DNA004414	M	23	1	56	62	58	53	54
			2	55	58	55	54	54
			3	56	60	57	54	54
DNA004415	F	21	1	49	54	51	50	50
			2	50	55	50	50	51
			3	47	53	51	50	49
DNA004418	F	19	1	50	52	50	50	50
			2	55	57	55	54	55
			3	56	58	56	53	55
DNA004420	M	19	1	48	52	48	48	51
			2	47	50	48	47	49
			3	48	51	48	48	50
DNA004421	M	18	1	56	56	55	55	60
			2	57	57	57	55	61
DNA004425	F	22	1	58	58	59	56	62
			2	56	54	55	55	59
			3	57	57	55	56	63
DNA004426	F	24	1	47	56	48	49	51
			2	52	53	52	51	54
DNA004428	F	20	1	46	52	51	48	52
			2	50	51	50	51	51
			3	46	52	51	48	54
DNA004429	F	24	1	55	58	56	57	57
			2	60	63	61	62	62
DNA004431	M	25	1	50	50	52	51	54
			2	53	56	54	53	56
			3	50	50	52	51	54
DNA004434	M	24	1	51	55	51	51	53
			2	50	54	52	51	53
			3	52	54	52	51	53
DNA004440	M	21	1	52	57	50	52	54
			2	54	59	53	54	58
			3	54	60	52	52	53
DNA004441	M	19	1	52	58	54	54	56

			2	53	59	54	52	56
			3	52	58	54	54	56
DNA004442	M	20	1	53	56	54	53	53
			2	53	59	53	55	54
			3	58	59	54	55	55
DNA004448	M	18	1	50	55	49	52	50
			2	53	57	54	53	56
			3	50	53	52	50	55
DNA004450	F	19	1	50	53	53	51	51
			2	51	57	54	53	54
DNA004456	M	20	1	47	53	50	48	50
			2	47	53	50	48	50
			3	47	50	49	47	53
DNA004460	M	18	1	56	59	57	58	59
			2	53	55	52	53	56
			3	55	58	56	57	58
DNA004461	F	24	1	53	56	55	54	52
			2	47	52	52	49	51
			3	53	53	55	51	56
DNA004466	F	18	1	51	52	53	50	53
			2	51	55	53	51	53
			3	53	57	55	52	59
DNA004476	M	19	1	52	55	53	51	53
			2	53	56	51	52	50
DNA004477	F	24	1	52	55	51	52	52
			2	52	53	51	52	54
			3	53	56	52	53	53
DNA004482	F	19	1	52	61	54	59	56
			2	50	58	52	57	54
DNA004483	F	19	1	52	55	51	49	51
			2	52	58	55	53	58
DNA004487	M	20	1	55	57	56	53	57
			2	55	57	56	53	57
			3	53	56	54	48	53
DNA004492	F	21	1	51	53	51	51	49
			2	51	54	51	51	48
DNA004494	M	20	1	50	52	53	48	52
			2	52	55	53	51	52
			3	49	51	52	47	51
DNA004496	M	18	1	49	51	49	48	51
			2	48	49	48	48	48
			3	48	54	50	51	50
DNA004497	M	19	1	51	52	53	51	50
			2	51	52	53	51	50
			3	53	54	55	53	52

DNA004498	F	24	1	49	56	53	53	51
			2	55	59	53	55	54
			3	50	53	49	50	50
DNA004500	M	18	1	49	50	53	48	49
			2	53	52	52	49	52
DNA004501	M	18	1	53	56	57	53	58
			2	55	58	59	55	60
DNA004664	M	22	1	50	52	51	51	51
			2	51	53	53	52	52
			3	49	51	51	51	50
DNA004668	M	22	1	50	52	51	51	58
			2	51	53	52	51	59
DNA004690	M	24	1	47	50	47	48	47
			2	46	49	46	47	45
			3	48	51	48	50	48

Table 29: Complete data for *H19 ICR* case samples and runs

***H19* ICR cases**									
Sample ID	**Gender**	**Age (yrs)**	**Phenotype**	**Run**	**% Methylations**				
					CpG1	CpG2	CpG3	CpG5	CpG6
DNA003985	F	9	FAS	1	49	51	49	48	53
				2	50	53	51	49	48
				3	52	54	50	50	53
DNA003989	F	9	FAS	1	52	64	54	53	61
				2	52	64	54	53	61
				3	55	64	56	61	62
DNA003991	F	12	FAS	1	54	55	51	52	54
				2	55	56	54	54	57
				3	56	60	56	56	58
DNA004010	M	9	FAS	1	56	59	53	54	57
				2	57	64	58	57	60
				3	55	62	60	60	63
DNA004012	F	9	FAS	1	56	59	57	59	59
				2	56	63	58	58	62
				3	56	59	57	59	59
DNA004013	M	9	FAS	1	56	55	52	53	56
				2	56	55	52	53	56
				3	56	55	56	55	55
DNA004017	F	9	FAS	1	48	54	49	49	52
				2	50	55	48	50	54
				3	50	58	53	52	55
DNA004021	M	9	FAS	1	45	50	44	46	50
				2	44	49	44	45	50

				3	45	50	44	46	50
DNA004024	F	7	FAS	1	53	58	52	52	57
				2	54	57	53	52	59
				3	49	54	50	48	54
DNA004029	F	9	FAS	1	52	54	52	54	55
				2	54	56	53	53	53
				3	53	58	54	54	58
DNA004032	M	10	FAS	1	49	52	52	51	52
				2	48	51	51	50	51
				3	48	49	46	47	51
DNA004035	M	9	FAS	1	50	54	52	51	54
				2	56	60	57	56	59
DNA004052	M	10	FAS	1	54	57	56	55	65
				2	54	57	56	55	65
				3	55	58	57	56	63
DNA004053	F	11	FAS	1	50	54	51	52	56
				2	52	53	52	53	57
				3	48	55	50	51	54
DNA004055	M	11	FAS	1	51	56	49	53	49
				2	51	57	49	53	54
				3	53	57	52	51	54
DNA004059	F	10	FAS	1	53	55	50	53	54
				2	55	55	56	57	54
				3	55	57	56	57	55
DNA004061	F	10	FAS	1	51	54	51	51	51
				2	51	59	55	53	57
				3	55	57	56	56	57
DNA004063	M	10	FAS	1	52	54	54	54	56
				2	51	55	54	54	55
				3	51	55	57	52	55
DNA004064	F	10	FAS	1	52	53	52	55	54
				2	46	51	48	50	49
				3	48	51	50	48	49
DNA004068	M	16	FAS	1	59	59	60	58	59
				2	63	64	65	64	64
				3	61	64	63	62	62
DNA004069	F	10	FAS	1	53	58	55	57	56
				2	57	61	55	56	56
				3	53	56	53	53	53
DNA004072	F	6	FAS	1	56	57	50	55	61
				2	54	62	57	58	58
DNA004074	F	10	FAS	1	51	55	50	53	50
				2	49	53	50	50	52
DNA004076	F	10	FAS	1	52	60	53	55	55
				2	57	61	56	57	56

DNA004082	M	2	FAS	1	56	58	57	56	55
				2	58	60	59	57	56
				3	53	54	55	54	53
DNA004094	F	11	FAS	1	56	64	55	57	60
				2	59	65	61	62	62
DNA004126	M	2	FAS	1	48	55	49	51	49
				2	49	52	50	52	50
				3	49	52	50	52	50
DNA004141	F	2	FAS	1	59	61	57	58	59
				2	56	61	55	54	58
				3	59	61	56	58	59
DNA004168	M	3	FAS	1	54	59	59	55	56
				2	60	53	59	57	52
DNA004172	F	16	FAS	1	49	53	50	51	51
				2	49	50	49	46	48
DNA004191	F	10	FAS	1	54	53	53	54	51
				2	55	58	55	52	51
DNA004193	M	10	FAS	1	51	53	50	51	52
				2	53	55	52	53	54
DNA004198	M	10	FAS	1	53	61	54	54	57
				2	53	60	52	52	52
				3	50	59	50	52	50
DNA004200	F	10	FAS	1	53	51	56	55	51
				2	54	54	54	54	52
				3	54	57	53	53	52
DNA004204	M	10	FAS	1	56	59	56	53	57
				2	52	54	52	52	53
				3	54	57	54	53	55
DNA004225	M	10	FAS	1	56	56	55	54	55
				2	52	52	52	52	51
				3	57	56	55	56	52
DNA004227	F	11	FAS	1	51	51	50	49	53
				2	57	55	54	53	58
DNA004231	M	11	FAS	1	58	59	58	56	58
				2	55	57	56	53	53
				3	58	59	58	56	58
DNA004233	F	10	FAS	1	57	57	56	55	57
				2	59	60	59	58	61
				3	59	60	58	57	61
DNA004241	F	10	FAS	1	59	59	55	54	62
				2	58	59	59	58	60
DNA004246	M	11	FAS	1	54	56	54	53	54
				2	55	55	56	54	55
DNA004249	M	11	FAS	1	57	56	56	54	55
				2	62	59	60	57	57

				3	59	57	58	55	56
DNA004253	M	11	FAS	1	64	64	63	60	63
				2	61	63	60	57	59
DNA004258	M	?	PFAS	1	58	56	56	53	63
				2	55	60	59	59	61
DNA004267	F	1	PFAS	1	57	58	57	56	57
				2	59	59	63	58	58
DNA004270	?	?	?	1	54	56	51	52	55
				2	50	54	54	53	54
				3	56	54	50	52	56
DNA004280	F	11	FAS	1	56	60	56	56	58
				2	57	58	59	58	57
DNA004290	M	14	FAS	1	60	58	58	56	60
				2	55	57	59	56	55
DNA004292	F	12	FAS	1	53	52	52	52	55
				2	53	51	54	52	54
				3	54	51	55	51	54
DNA004296	M	7	FAS	1	49	50	48	52	53
				2	48	49	47	50	52
				3	50	52	49	53	53
DNA004300	F	7	FAS	1	49	47	49	46	47
				2	49	47	49	47	48
DNA004301	M	6	FAS	1	61	62	63	60	63
				2	57	60	57	55	58
DNA004304	M	16	FAS	1	50	51	48	50	48
				2	52	53	50	52	50
DNA004305	M	4	FAS	1	50	51	51	50	51
				2	56	55	55	54	53
				3	54	55	53	54	55
DNA004308	F	5	FAS	1	51	52	49	49	51
				2	49	48	49	49	46
DNA004312	M	?	FAS	1	54	53	49	53	47
				2	53	56	53	53	51
DNA004313	M	?	FAS	1	52	54	55	49	50
				2	51	55	57	50	51
DNA004317	M	13	FAS	1	47	48	47	47	48
				2	46	47	46	46	47
DNA004325	M	2	FAS	1	56	57	57	56	54
				2	54	56	53	55	54
				3	57	57	58	56	56
DNA004327	M	3	FAS	1	54	55	55	55	53
				2	55	56	54	56	54
DNA004331	M	8	FAS	1	56	52	56	56	56
				2	55	54	54	54	51
				3	54	54	53	54	51

DNA004335	M	2	FAS	1	50	53	50	49	53
				2	51	56	54	56	52
				3	55	56	55	55	54
DNA004337	F	2	FAS	1	51	52	51	51	55
				2	52	53	52	52	56
DNA004343	F	10	FAS	1	54	55	53	54	54
				2	54	56	53	54	55
				3	55	56	54	55	55
DNA004354	M	8	FAS	1	54	55	50	54	56
				2	54	55	51	54	56
				3	54	54	50	52	54
DNA004356	M	9	FAS	1	51	51	51	51	51
				2	52	57	55	52	56
				3	49	50	48	47	51
DNA004360	M	10	FAS	1	47	54	48	50	51
				2	49	48	49	48	49
				3	49	54	49	50	51
DNA004365	M	10	FAS	1	58	59	58	58	60
				2	58	63	58	57	60
				3	56	60	56	55	57
DNA004373	F	7	FAS	1	53	50	51	50	53
				2	49	52	50	50	52
DNA004378	M	8	FAS	1	51	57	52	53	55
				2	49	54	49	50	54
				3	49	51	50	51	54
DNA004382	M	9	FAS	1	58	59	57	56	59
				2	54	58	53	54	56
				3	54	59	50	53	55
DNA004386	M	8	FAS	1	45	49	46	48	51
				2	47	49	46	47	47
				3	49	55	49	51	53
DNA004390	F	7	FAS	1	51	53	48	54	58
				2	51	53	49	49	52
DNA004391	M	7	FAS	1	48	50	49	46	49
				2	50	50	48	47	50

? = no information

Table 30: Complete data for *IG-DMR* control samples and runs

Complete data for *IG-DMR* control samples and runs													
Controls	**Gender**	**Age(yrs)**	**Run**	**Reg 1**							**Reg2**		
				CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10
DNA004392	F	25	1	63	64	65	67	66	52	55	56	55	56
			2	63	64	65	67	66	52	55	50	51	52
DNA004393	M	19	1	79	83	70	78	85	61	60	54	54	57
			2	80	84	73	78	87	59	61	56	51	53
DNA004394	M	20	1	67	69	62	67	67	53	56	51	51	52
			2	69	71	63	69	68	52	57	50	49	48
DNA004395	F	26	1	70	72	65	71	72	55	58	54	52	54
			2	70	72	65	76	75	57	59	54	53	52
DNA004396	F	25	1	78	80	73	79	79	60	61	54	52	53
			2	78	80	73	78	78	59	61	58	52	57
DNA004398	M	19	1	72	75	62	71	71	56	54	55	53	56
			2	72	75	62	71	71	56	54	55	53	56
DNA004399	F	21	1	71	72	64	72	72	55	55	51	50	50
			2	74	79	69	78	79	59	59	51	50	50
DNA004401	F	22	1	71	73	65	73	74	56	55	51	51	52
			2	71	73	65	73	74	56	55	51	51	52
DNA004402	F	21	1	81	85	72	80	85	63	65	52	51	54
			2	81	85	72	80	85	63	65	52	51	54
DNA004404	F	25	1	76	77	70	78	79	54	55	53	49	50
			2	76	77	70	78	79	54	55	53	49	50
DNA004406	F	22	1	74	78	70	74	75	58	60	52	51	52
			2	74	72	72	73	74	55	59	52	49	52
DNA004407	F	24	1	74	75	66	73	74	57	58	50	50	50
			2	75	76	67	74	75	57	58	50	50	50
DNA004408	M	24	1	74	73	68	73	76	58	56	57	56	56
			2	69	73	64	72	74	57	56	57	56	56

DNA004410	F	20	1	67	71	57	68	69	53	55	49	49	49
			2	67	71	57	68	69	53	55	49	49	50
DNA004411	M	19	1	73	75	67	73	74	55	58	49	49	49
			2	74	77	71	75	77	58	60	50	49	51
DNA004412	M	23	1	80	84	72	78	85	62	61	54	51	51
			2	83	87	72	78	85	61	61	52	51	50
DNA004413	F	19	1	70	73	65	72	72	55	54	57	55	56
			2	70	73	65	70	70	57	56	57	55	56
DNA004414	M	23	1	81	85	76	82	77	61	61	55	54	53
			2	81	85	76	82	76	60	60	54	54	53
DNA004415	F	21	1	74	79	74	74	77	58	60	58	53	58
			2	74	79	74	74	77	58	60	58	53	58
DNA004418	F	19	1	71	74	68	76	75	56	58	50	50	50
			2	71	74	68	76	75	56	58	50	50	50
DNA004420	M	19	1	75	79	69	78	80	57	55	54	54	55
			2	75	79	69	75	80	58	55	54	51	55
DNA004421	M	18	1	78	86	69	78	87	58	58	51	51	51
			2	80	82	71	77	86	60	60	52	53	57
DNA004422	F	20	1	70	73	63	70	73	54	56	51	49	50
			2	70	73	63	69	73	55	56	54	52	52
DNA004425	F	22	1	73	77	70	78	79	58	58	53	54	50
			2	74	78	67	76	78	60	60	51	53	52
DNA004426	F	24	1	73	75	66	73	77	58	59	51	53	55
			2	73	75	66	73	77	58	59	51	53	55
DNA004428	F	20	1	74	77	72	75	75	58	57	59	58	59
			2	77	81	67	78	78	61	62	59	58	59
DNA004429	F	24	1	73	79	66	73	78	57	60	52	53	53
			2	73	79	66	73	78	57	60	52	53	53
DNA004431	M	25	1	70	72	68	73	72	55	56	48	49	50
			2	70	72	68	73	72	55	56	51	50	49
DNA004434	M	24	1	70	70	65	71	72	54	56	51	51	51

			2	70	70	65	71	72	54	56	52	52	52
DNA004437	M	23	1	81	85	69	76	84	60	57	53	56	52
			2	79	85	69	76	85	62	59	54	56	51
DNA004440	M	21	1	78	84	69	76	85	62	59	52	50	54
			2	76	83	70	76	85	62	59	57	55	57
DNA004441	M	19	1	65	65	61	65	66	52	55	51	51	49
			2	65	65	61	65	66	52	55	51	51	52
DNA004442	M	20	1	69	71	65	72	70	54	57	52	50	51
			2	69	71	65	72	70	54	57	52	52	49
DNA004448	M	18	1	72	74	66	68	71	57	56	52	51	51
			2	71	74	67	68	73	58	61	52	51	51
DNA004450	F	19	1	71	74	66	73	73	55	57	55	55	55
			2	71	74	66	73	73	55	57	55	55	55
DNA004456	M	20	1	69	73	64	68	72	55	50	54	54	54
			2	70	73	67	70	70	53	54	54	50	54
DNA004460	M	18	1	67	70	63	68	70	53	55	51	51	50
			2	67	71	64	69	71	54	64	51	51	50
DNA004461	F	24	1	66	67	61	67	66	54	54	52	51	52
			2	66	67	61	67	66	54	54	50	50	50
DNA004463	M	25	1	70	73	67	72	73	56	58	53	52	55
			2	70	73	67	72	73	56	58	53	52	55
DNA004465	F	20	1	71	74	65	72	75	60	56	50	51	50
			2	71	74	65	72	75	60	56	50	51	50
DNA004466	F	18	1	68	70	62	67	69	55	54	50	52	52
			2	69	70	62	68	70	53	55	50	52	52
DNA004476	M	19	1	76	79	65	74	79	56	55	51	50	50
			2	76	79	65	74	79	56	55	51	50	50
DNA004477	F	24	1	65	68	61	65	68	52	54	49	52	52
			2	66	67	60	66	66	52	52	49	52	52
DNA004481	F	20	1	72	75	68	73	76	57	57	53	53	55
			2	67	70	65	68	72	53	57	49	50	51

DNA004483	F	19	1	75	79	64	67	81	59	58	56	58	55
			2	79	80	66	70	81	57	57	56	58	55
DNA004487	M	20	1	70	74	63	69	71	56	56	52	50	53
			2	70	74	63	69	71	56	56	52	50	53
DNA004492	F	21	1	71	76	65	74	75	57	57	54	55	54
			2	71	74	68	75	74	56	55	55	51	53
DNA004494	M	20	1	71	70	70	70	71	54	55	53	53	51
			2	67	72	69	68	71	54	55	53	53	51
DNA004496	M	18	1	70	72	64	71	73	52	54	50	50	53
			2	69	69	64	70	69	52	55	50	50	53
DNA004497	M	19	1	76	78	66	75	81	59	57	50	49	49
			2	76	78	66	75	81	59	57	50	49	53
DNA004498	F	24	1	70	72	60	70	74	55	54	50	49	53
			2	70	72	60	70	74	55	54	52	49	50
DNA004500	M	18	1	73	76	62	75	76	54	54	52	54	54
			2	73	76	70	77	75	57	59	51	54	54
DNA004501	M	18	1	66	74	59	71	70	57	53	53	52	51
			2	66	70	61	68	69	52	55	50	50	50
DNA004664	M	22	1	71	76	67	75	75	55	58	52	51	53
			2	71	76	67	75	75	55	58	52	51	53
DNA004668	M	22	1	67	71	65	71	69	51	54	51	50	52
			2	67	71	65	71	69	51	54	51	50	52
DNA004690	M	24	1	64	66	62	67	65	51	53	51	50	50
			2	64	66	62	67	65	51	53	51	50	50

Table 31: Complete data for case samples for *IG-DMR* and runs:

Complete data for case samples for *IG-DMR* and runs														
Sample ID	**Gender**	**Age(yrs)**	**Phenotype**	**Run**	**Reg 1**							**Reg2**		
					CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10
DNA003985	F	9	FAS	1	71	76	67	70	75	56	54	52	54	53
				2	75	80	67	75	78	60	57	52	54	53
DNA003989	F	9	FAS	1	74	77	70	76	78	60	60	50	52	52
				2	74	77	70	76	78	60	60	50	52	52
DNA003991	F	12	FAS	1	74	76	67	76	79	60	58	57	60	56
				2	74	76	67	76	79	60	58	57	60	56
DNA004010	M	9	FAS	1	74	78	69	75	78	57	57	53	53	54
				2	74	78	69	75	78	57	57	53	53	54
DNA004012	F	9	FAS	1	75	78	69	74	80	57	58	52	51	49
				2	75	78	69	74	80	57	58	52	51	49
DNA004013	M	9	FAS	1	75	78	74	79	79	62	58	53	52	53
				2	75	78	74	79	79	62	58	53	52	53
DNA004017	F	9	FAS	1	71	73	66	70	74	55	56	52	50	51
				2	71	77	65	72	75	56	56	52	50	51
DNA004021	M	9	FAS	1	74	77	69	75	80	59	59	54	51	53
				2	74	77	69	75	80	59	59	54	51	53
DNA004024	F	7	FAS	1	75	77	69	75	78	57	59	50	50	50
				2	75	77	69	75	78	57	59	50	50	50
DNA004029	F	9	FAS	1	81	84	78	79	88	61	61	55	54	57
				2	81	84	78	79	88	61	61	55	54	57
DNA004032	M	10	FAS	1	68	72	65	69	69	53	57	50	50	52
				2	67	70	64	69	69	53	57	50	50	52
DNA004035	M	9	FAS	1	73	76	66	73	77	55	57	50	50	51
				2	74	76	71	74	77	60	58	50	50	51
DNA004042	F	9	FAS	1	71	74	67	74	75	56	56	51	52	51
				2	72	76	67	73	77	59	57	49	51	50

DNA004052	M	10	FAS	1	71	75	64	71	77	57	54	55	54	53
				2	73	73	68	77	76	58	56	53	54	54
DNA004053	F	11	FAS	1	69	72	64	69	72	55	54	50	49	49
				2	67	67	59	66	69	51	54	49	49	50
DNA004055	M	11	FAS	1	74	76	69	74	74	56	60	50	49	50
				2	73	77	69	77	76	58	57	50	49	50
DNA004059	F	10	FAS	1	67	68	60	67	67	55	55	50	50	50
				2	66	66	62	67	66	52	50	50	50	50
DNA004061	F	10	FAS	1	69	74	63	70	72	56	57	53	54	53
				2	64	70	62	69	70	54	53	53	54	53
DNA004063	M	10	FAS	1	68	71	62	68	72	55	58	51	51	49
				4	69	72	66	68	71	55	55	51	51	50
DNA004064	F	10	FAS	1	66	72	64	68	72	55	58	55	55	53
				2	69	70	64	71	72	51	56	55	55	53
DNA004065	M	8	FAS	1	77	83	68	75	87	58	58	51	53	52
				2	77	83	68	75	87	58	58	55	54	54
DNA004068	M	16	FAS	1	68	69	61	67	67	53	55	53	52	53
				2	68	69	61	67	67	53	55	53	52	53
DNA004069	F	10	FAS	1	66	69	66	69	69	52	52	50	50	50
				2	66	69	64	68	68	52	52	50	50	50
DNA004072	F	6	FAS	1	70	69	65	71	73	56	57	52	53	52
				2	70	69	65	71	73	55	55	52	53	52
DNA004074	F	10	FAS	1	77	79	64	76	79	56	57	51	51	51
				2	73	75	68	74	77	57	58	50	50	48
DNA004076	F	10	FAS	1	75	78	67	73	80	59	56	54	56	56
				2	72	75	69	73	79	59	57	54	56	56
DNA004082	M	2	FAS	1	74	79	69	76	82	62	60	54	54	55
				2	74	79	69	76	82	62	60	54	54	55
DNA004094	F	11	FAS	1	78	81	66	73	85	66	61	58	63	60
				2	78	81	66	76	82	62	60	58	60	56
DNA004126	M	2	FAS	1	77	86	64	71	90	62	58	64	65	58

				2	79	84	62	76	89	66	59	59	63	58
DNA004141	F	2	FAS	1	75	75	61	69	84	60	60	61	60	58
				2	78	81	67	70	85	65	58	64	62	55
DNA004157	F	1	FAS	1	54	74	50	66	75	50	54	53	54	56
				2	54	74	50	66	75	50	54	53	54	56
DNA004161	F	5	FAS	1	76	77	68	69	69	62	64	60	60	60
				2	76	77	68	69	69	62	64	60	60	60
DNA004168	M	3	FAS	1	79	81	64	74	85	60	60	57	55	54
				2	75	77	67	68	83	60	65	57	55	54
DNA004172	F	16	FAS	1	67	67	63	68	68	53	55	50	50	50
				2	68	69	65	71	70	56	59	50	50	50
DNA004191	F	10	FAS	1	77	80	71	79	82	55	59	50	52	53
				2	76	77	70	77	85	56	55	53	53	54
DNA004193	M	10	FAS	1	76	79	70	75	78	60	55	50	50	51
				2	76	79	70	75	78	60	55	50	50	51
DNA004198	M	10	FAS	1	73	73	67	71	71	50	50	51	50	50
				2	73	74	66	73	74	53	56	51	50	50
DNA004200	F	10	FAS	1	85	87	67	73	86	59	58	59	60	57
				2	78	82	71	72	86	60	59	61	61	59
DNA004204	M	10	FAS	1	65	68	62	67	68	52	53	49	50	49
				2	65	68	62	67	68	52	53	49	50	49
DNA004225	M	10	FAS	1	74	79	71	76	78	59	59	57	56	55
				2	73	76	68	73	82	59	63	53	53	53
DNA004227	F	11	FAS	1	72	77	67	72	77	57	60	50	49	51
				2	72	76	68	72	76	57	57	52	51	51
DNA004231	M	11	FAS	1	72	77	68	72	77	54	57	50	50	50
				2	72	75	67	74	76	52	53	50	50	50
DNA004233	F	10	FAS	1	67	71	65	68	70	55	55	55	55	52
				2	65	68	63	68	71	56	54	55	54	53
DNA004241	F	10	FAS	1	79	83	73	75	85	58	57	58	52	53
				2	79	83	76	78	86	57	58	55	50	52

DNA004246	M	11	FAS	1	67	69	61	68	69	53	56	54	54	53
				2	67	70	64	69	69	54	55	52	53	52
DNA004249	M	11	FAS	1	71	73	69	71	74	57	58	52	51	52
				2	71	73	65	71	73	56	56	51	53	53
DNA004253	M	11	FAS	1	68	70	64	69	71	53	56	51	50	50
				2	65	70	63	69	70	53	56	51	49	52
DNA004258	M	?	PFAS	1	76	80	61	63	81	59	57	65	62	60
				2	78	83	65	68	85	58	60	61	60	56
DNA004267	F	1	PFAS	1	80	89	69	76	90	65	61	65	68	63
				2	85	88	66	74	91	67	60	65	69	61
DNA004270	?	?	?	1	84	85	63	73	91	63	56	65	62	63
				2	81	88	69	75	92	64	59	66	67	63
DNA004280	F	11	FAS	1	79	82	76	79	86	60	61	52	50	53
				2	78	81	77	81	84	61	61	54	51	52
DNA004290	M	14	FAS	1	69	69	60	67	70	55	55	52	54	52
				2	69	69	60	67	70	55	55	52	54	52
DNA004292	F	12	FAS	1	67	71	64	69	71	55	55	51	51	51
				2	70	72	67	71	73	56	57	51	51	50
DNA004296	M	7	FAS	1	69	69	63	68	73	57	57	49	51	50
				2	71	74	64	71	74	56	56	49	51	50
DNA004300	F	7	FAS	1	71	77	65	72	79	55	57	51	51	50
				2	71	75	67	73	77	58	59	52	49	50
DNA004301	M	6	FAS	1	81	87	71	77	88	63	59	54	50	55
				2	80	86	70	77	91	65	60	54	54	55
DNA004304	M	16	FAS	1	80	82	70	76	85	59	59	50	52	52
				2	78	82	70	78	85	59	58	50	52	52
DNA004305	M	4	FAS	1	74	76	69	74	80	56	57	52	57	55
				2	75	75	67	76	79	60	57	52	55	55
DNA004308	F	5	FAS	1	80	77	75	80	85	61	60	50	51	52
				2	78	82	71	78	86	66	63	51	53	53
DNA004312	M	?	FAS	1	69	74	63	72	74	57	55	50	49	52

				2	69	78	66	74	77	57	55	52	51	51
DNA004313	M	?	FAS	1	70	74	67	73	75	58	56	49	52	51
				2	71	74	68	72	74	56	57	49	51	51
DNA004317	M	13	FAS	1	66	70	60	66	71	52	52	50	51	49
				2	66	70	60	66	71	52	52	50	51	49
DNA004325	M	2	FAS	1	73	79	66	70	84	56	54	52	52	52
				2	72	80	67	70	83	57	55	54	54	53
DNA004327	M	3	FAS	1	79	83	67	75	86	56	54	58	57	55
				2	76	83	70	77	86	61	60	53	52	52
DNA004331	M	8	FAS	1	80	53	75	84	87	64	61	55	55	55
				2	78	51	74	81	88	64	59	53	55	55
DNA004335	M	2	FAS	1	74	79	62	69	82	59	55	50	54	51
				2	74	79	62	69	80	59	55	50	54	51
DNA004337	F	2	FAS	1	83	86	69	75	89	63	60	55	58	58
				2	84	89	73	79	90	60	60	55	58	58
DNA004343	F	10	FAS	1	71	72	65	72	75	57	56	51	52	51
				2	68	71	64	71	71	60	55	50	49	50
DNA004348	F	10	FAS	1	72	75	64	72	74	56	56	52	49	54
				2	72	75	64	73	74	59	58	53	52	51
DNA004350	F	8	FAS	1	69	71	62	72	73	56	59	56	55	57
				1	71	73	63	72	73	56	57	55	54	56
DNA004354	M	8	FAS	1	75	81	71	79	81	59	58	53	54	50
				2	75	81	71	79	81	59	58	52	52	55
DNA004356	M	9	FAS	1	72	76	67	71	75	55	52	54	51	49
				2	73	75	68	73	72	56	52	55	54	52
DNA004360	M	10	FAS	1	73	76	69	73	83	58	57	52	54	50
				2	78	74	73	76	80	60	59	51	50	50
DNA004365	M	10	FAS	1	72	76	69	71	73	57	56	50	53	50
				2	72	76	69	72	76	59	58	51	52	50
DNA004370	F	11	FAS	1	68	71	65	69	71	56	55	50	51	50
				2	68	71	65	69	71	56	55	50	51	50

DNA004373	F	7	FAS	1	67	70	63	71	70	55	53	55	51	50
				2	68	71	66	68	69	53	53	53	53	49
DNA004375	M	8	FAS	1	74	74	68	72	76	57	58	52	50	50
				2	72	74	68	75	76	57	57	54	54	47
DNA004378	M	8	FAS	1	76	78	68	76	79	55	60	50	49	52
				2	76	78	68	76	79	55	60	56	54	56
DNA004380	M	8	FAS	1	66	71	62	71	72	57	55	61	60	59
				2	67	70	62	68	72	55	55	56	57	55
DNA004382	M	9	FAS	1	70	71	63	70	71	56	54	54	50	53
				2	66	68	60	68	68	55	52	54	50	53
DNA004384	F	6	FAS	1	71	79	64	71	78	55	57	55	53	53
				2	71	79	64	71	78	55	57	50	53	53
DNA004386	M	8	FAS	1	83	86	74	87	87	61	60	53	49	49
				2	85	87	76	88	88	64	59	53	50	51
DNA004388	M	6	FAS	1	71	69	66	64	74	59	60	53	51	53
				2	71	69	66	64	74	60	57	52	55	50
DNA004390	F	7	FAS	1	81	83	67	78	87	61	58	60	57	59
				2	83	83	67	78	88	61	59	52	55	56
DNA004391	M	7	FAS	1	81	89	72	79	88	62	62	52	57	56
				2	81	89	72	80	88	59	61	52	52	53

? = no information

Table 32: Complete data for *KvDMR1* control samples and runs:

Complete data for *KvDMR1* control samples and runs										
Controls	**Gender**	**Age(yrs)**	**run**	**Methylation %**						
				CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7
DNA004392	F	25	1	62	62	60	60	60	58	62
			2	57	63	60	59	63	61	64
DNA004393	M	19	1	63	63	61	59	60	60	63
			2	60	62	59	62	60	61	65
DNA004394	M	20	1	55	62	58	58	61	58	69
			2	58	62	58	60	61	60	65
DNA004395	F	26	1	63	60	61	61	64	60	63
			2	59	62	62	61	65	61	63
DNA004396	F	25	1	61	62	63	62	65	61	64
			2	62	65	60	61	63	62	62
DNA004398	M	19	1	57	63	59	60	57	56	63
			2	59	63	58	60	63	60	60
DNA004399	F	21	1	57	62	58	60	60	58	63
			2	59	63	58	59	60	61	63
DNA004401	F	22	1	52	52	49	50	50	52	53
			2	54	51	50	51	51	53	55
DNA004402	F	21	1	59	61	56	58	59	59	61
			2	56	57	59	57	57	57	60
DNA004404	F	25	1	62	61	58	59	59	61	61
			2	65	59	57	60	59	62	63
DNA004406	F	22	1	56	62	57	58	60	59	61
			2	57	61	53	60	59	58	59
DNA004407	F	24	1	62	62	58	59	61	61	57
			2	58	59	59	60	58	61	63
DNA004408	M	24	1	59	59	58	59	63	60	64
			2	59	60	53	58	58	57	58

DNA004410	F	20	1	62	61	55	55	58	58	57
			2	62	61	55	56	59	56	55
DNA004411	M	19	1	61	57	58	57	55	58	55
			2	61	57	57	57	56	55	55
DNA004412	M	23	1	61	63	60	60	62	60	58
			2	60	64	60	60	62	60	58
DNA004413	F	19	1	58	59	55	57	56	50	58
			2	57	57	56	56	56	55	58
DNA004414	M	23	1	64	61	59	62	60	62	63
			2	61	66	59	62	64	63	64
DNA004415	F	21	1	65	63	57	57	60	58	59
			2	62	62	59	61	60	62	62
DNA004418	F	19	1	55	58	58	57	59	56	60
			2	58	60	55	58	57	61	62
DNA004420	M	19	1	55	62	56	56	61	56	55
			2	59	61	56	56	59	57	55
DNA004421	M	18	1	59	60	62	62	63	59	64
			2	60	64	61	60	63	60	62
DNA004422	F	20	1	61	57	57	57	58	56	59
			2	58	59	59	59	58	59	59
DNA004425	F	22	1	59	60	54	56	58	53	57
			2	59	60	55	56	59	55	57
DNA004426	F	24	1	58	60	56	58	61	54	59
			2	59	57	59	58	59	56	58
DNA004428	F	20	1	58	62	61	62	66	60	63
			2	57	66	57	58	63	61	60
DNA004429	F	24	1	56	59	57	56	59	55	59
			2	58	63	57	58	59	56	58
DNA004430	M	18	1	55	60	57	58	60	56	59
			2	57	58	56	58	58	57	61
DNA004431	M	25	1	61	62	57	59	59	58	60

			2	56	63	56	58	61	57	60
DNA004437	M	23	1	61	58	57	58	58	55	58
			2	57	64	57	57	60	56	58
DNA004440	M	21	1	59	66	58	60	65	57	60
			2	59	61	58	59	62	57	59
DNA004441	M	19	1	62	62	56	57	60	57	57
			2	59	60	56	58	58	58	59
DNA004442	M	20	1	56	62	59	59	56	58	61
			2	60	63	58	58	56	57	61
DNA004448	M	18	1	61	64	55	56	60	59	58
			2	60	64	57	58	59	59	61
DNA004450	F	19	1	55	64	57	59	62	56	59
			2	58	59	58	59	59	57	61
DNA004456	M	20	1	59	64	54	56	60	54	57
			2	54	62	57	59	62	55	60
DNA004460	M	18	1	60	66	59	60	61	62	64
			2	58	61	59	61	57	61	63
DNA004461	F	24	1	60	67	59	61	62	61	64
			2	59	62	57	60	57	59	61
DNA004463	M	25	1	59	66	54	58	62	62	62
			2	57	62	55	57	58	60	60
DNA004465	F	20	1	57	61	57	58	60	57	59
			2	60	62	55	59	61	57	61
DNA004466	F	18	1	60	67	60	62	62	59	64
			2	60	66	59	62	59	64	64
DNA004476	M	19	1	59	66	58	60	61	61	60
			2	64	63	59	60	60	66	64
DNA004477	F	24	1	62	64	56	58	59	60	61
			2	63	64	58	59	58	64	59
DNA004481	F	20	1	60	60	55	57	57	57	58
			2	55	62	57	57	61	56	62

DNA004482	F	19	1	54	60	57	60	57	60	63
			2	52	60	56	56	58	57	59
DNA004483	F	19	1	56	59	56	58	57	59	59
			2	59	58	56	57	57	58	60
DNA004487	M	20	1	59	61	58	60	58	64	62
			2	61	63	57	59	59	63	60
DNA004492	F	21	1	59	62	58	60	58	64	61
			2	56	66	58	61	63	61	62
DNA004494	M	20	1	62	63	60	62	58	63	62
			2	57	65	57	59	60	60	59
DNA004496	M	18	1	56	55	56	56	55	56	58
			2	57	57	57	58	58	58	59
DNA004497	M	19	1	57	63	56	57	60	57	57
			2	53	59	52	53	55	52	54
DNA004498	F	24	1	55	60	56	59	59	54	59
			2	58	62	55	58	57	57	61
DNA004500	M	18	1	51	53	51	53	56	51	54
			2	54	56	53	54	54	52	55
DNA004501	M	18	1	57	59	56	58	54	56	61
			2	61	60	56	57	58	59	59
DNA004690	M	24	1	61	64	57	58	61	60	63
			2	59	59	57	60	57	58	62

Table 33: Complete data for *KvDMR1* case samples and runs:

Complete data for *KvDMR1* case samples and runs											
Cases	**Gender**	**Age(yrs)**	**Phenotype**		**Methylation %**						
				run	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7
DNA003985	F	9	FAS	1	57	59	57	58	57	60	59
				2	62	59	58	59	57	59	62
DNA003989	F	9	FAS	1	62	63	58	59	59	63	60
				2	59	60	62	63	61	63	62
DNA003991	F	12	FAS	1	55	60	58	57	63	57	58
				2	55	62	56	57	59	55	59
DNA004010	M	9	FAS	1	59	62	56	58	59	58	58
				2	63	63	58	60	59	63	60
DNA004012	F	9	FAS	1	57	59	57	57	61	56	59
				2	62	64	57	58	62	57	57
DNA004013	M	9	FAS	1	63	62	61	61	60	64	61
				2	62	62	61	61	59	63	60
DNA004017	F	9	FAS	1	61	63	59	58	62	60	58
				2	59	63	58	60	59	60	61
DNA004021	M	9	FAS	1	57	59	58	60	57	61	62
				2	62	62	57	59	58	58	57
DNA004024	F	7	FAS	1	59	60	59	60	58	61	63
				2	63	64	58	59	61	60	58
DNA004029	F	9	FAS	1	58	60	57	58	58	59	58
				2	54	60	55	55	58	54	56
DNA004032	M	10	FAS	1	57	58	57	57	58	56	60
				2	54	60	58	58	60	56	60
DNA004035	M	9	FAS	1	58	62	56	57	60	56	61
				2	57	65	55	56	58	60	59
DNA004042	F	9	FAS	1	60	61	59	60	60	58	60
				2	61	62	59	60	59	60	61
DNA004052	M	10	FAS	1	62	63	60	59	61	62	57
				2	58	59	58	58	58	56	57
DNA004053	F	11	FAS	1	58	58	55	58	59	56	58
				2	58	60	56	58	58	57	61

DNA004055	M	11	FAS	1	59	64	58	59	61	59	60
				2	59	61	59	60	60	58	60
DNA004059	F	10	FAS	1	58	60	58	58	58	57	60
				2	60	62	54	55	58	58	60
DNA004061	F	10	FAS	1	61	64	58	59	62	59	61
				2	59	58	58	59	57	59	61
DNA004063	M	10	FAS	1	58	60	58	59	60	59	61
				2	63	65	59	59	59	58	61
DNA004064	F	10	FAS	1	61	64	55	57	60	57	60
				2	59	63	58	59	61	58	58
DNA004065	M	8	FAS	1	63	67	58	60	64	61	60
				2	57	63	59	59	59	61	61
DNA004068	M	16	FAS	1	61	62	55	57	60	57	57
				2	59	63	55	58	59	58	60
DNA004069	F	10	FAS	1	55	61	56	56	61	55	60
				2	56	62	54	56	60	54	57
DNA004072	F	6	FAS	1	62	64	60	60	65	60	64
				2	67	70	60	61	65	62	63
DNA004074	F	10	FAS	1	56	61	57	58	60	59	60
				2	57	64	58	60	60	57	60
DNA004076	F	10	FAS	1	61	63	59	59	59	61	62
				2	61	65	59	60	62	58	59
DNA004082	M	2	FAS	1	58	63	55	55	59	56	56
				2	57	59	57	58	58	57	58
DNA004094	F	11	FAS	1	60	60	60	60	59	62	60
				2	59	62	56	60	60	57	56
DNA004126	M	2	FAS	1	57	59	57	59	57	61	60
				2	61	63	58	59	57	61	57
DNA004141	F	2	FAS	1	61	63	59	59	58	61	62
				2	60	63	62	62	61	61	62
DNA004146	F	2	FAS	1	61	62	61	58	61	64	64
				2	65	63	59	61	62	63	64
DNA004157	F	1	FAS	1	65	63	60	62	61	62	62
				2	61	63	55	58	60	58	59

DNA004161	F	5	FAS	1	64	68	64	64	61	67	64
				2	60	65	62	64	65	62	64
DNA004166	M	2	FAS	1	72	73	64	65	69	70	65
				2	68	70	62	65	67	67	66
DNA004168	M	3	FAS	1	64	70	62	63	63	67	67
				2	66	68	64	65	66	67	67
DNA004172	F	16	FAS	1	61	64	56	58	62	58	59
				2	59	59	60	60	58	58	61
DNA004191	F	10	FAS	1	61	65	61	61	63	61	65
				2	65	70	60	62	66	64	64
DNA004193	M	10	FAS	1	59	64	59	60	61	60	63
				2	57	61	57	58	62	56	60
DNA004198	M	10	FAS	1	61	61	59	61	59	60	64
				2	58	61	60	60	61	58	62
DNA004200	F	10	FAS	1	61	61	60	59	61	61	60
				2	65	67	57	58	64	60	60
DNA004204	M	10	FAS	1	60	63	60	60	62	63	62
				2	58	64	60	61	65	60	63
DNA004225	M	10	FAS	1	65	69	60	62	65	61	61
				2	60	66	59	62	67	59	65
DNA004227	F	11	FAS	1	58	62	58	57	58	56	61
				2	62	62	57	58	58	59	62
DNA004231	M	11	FAS	1	62	65	56	58	62	59	59
				2	68	66	60	62	62	64	66
DNA004233	F	10	FAS	1	59	65	60	60	65	59	64
				2	62	65	58	60	61	64	63
DNA004241	F	10	FAS	1	62	62	56	58	59	57	59
				2	61	64	56	58	59	57	61
DNA004246	M	11	FAS	1	58	59	56	59	57	56	60
				2	61	59	56	59	58	59	61
DNA004249	M	11	FAS	1	58	59	57	59	57	58	59
				2	56	62	56	58	60	58	60
DNA004253	M	11	FAS	1	57	58	55	58	56	53	60
				2	57	57	56	55	59	53	56

DNA004258	M	?	PFAS	1	56	60	56	59	57	56	59
				2	60	59	57	59	56	56	58
DNA004267	F	1	PFAS	1	58	59	58	57	56	59	59
				2	56	62	54	57	56	59	60
DNA004270	?	?	?	1	56	62	53	56	59	54	59
				2	58	62	53	57	55	57	59
DNA004280	F	11	FAS	1	53	60	58	58	59	54	61
				2	52	60	57	57	60	55	58
DNA004290	M	14	FAS	1	58	59	56	58	56	56	58
				2	57	61	54	56	57	56	57
DNA004292	F	12	FAS	1	61	61	56	57	59	56	59
				2	61	59	57	59	57	54	55
DNA004296	M	7	FAS	1	60	63	56	57	60	56	58
				2	60	58	57	57	57	56	58
DNA004300	F	7	FAS	1	55	62	56	56	60	55	58
				2	57	62	56	57	60	55	59
DNA004301	M	6	FAS	1	63	59	57	57	57	54	59
				2	61	61	55	56	59	56	59
DNA004304	M	16	FAS	1	61	62	57	57	57	59	57
				2	61	62	57	58	61	58	59
DNA004305	M	4	FAS	1	62	61	54	56	58	57	57
				2	57	59	58	58	55	59	58
DNA004308	F	5	FAS	1	59	60	57	59	57	59	59
				2	56	61	57	56	55	58	58
DNA004312	M	?	FAS	1	60	60	55	55	54	54	58
				2	60	60	55	57	54	54	58
DNA004313	M	?	FAS	1	54	62	54	56	56	57	61
				2	57	57	55	55	56	55	58
DNA004317	M	13	FAS	1	59	61	56	56	56	57	57
				2	58	61	54	57	56	59	57
DNA004325	M	2	FAS	1	54	58	56	57	57	56	59
				2	56	58	55	55	56	55	56
DNA004327	M	3	FAS	1	55	56	56	55	58	56	55
				2	56	57	57	57	57	56	59

DNA004331	M	8	FAS	1	62	55	57	56	57	55	58
				2	62	56	56	55	56	55	55
DNA004335	M	2	FAS	1	58	59	60	59	58	59	61
				2	56	57	56	56	58	55	58
DNA004337	F	2	FAS	1	57	58	56	58	55	56	58
				2	57	56	56	55	57	54	58
DNA004343	F	10	FAS	1	63	62	56	58	57	58	61
				2	60	60	54	56	56	56	56
DNA004348	F	10	FAS	1	58	63	55	55	57	57	57
				2	58	57	56	56	54	55	55
DNA004350	F	8	FAS	1	58	58	58	58	57	56	60
				2	60	60	57	57	58	56	59
DNA004354	M	8	FAS	1	59	57	54	55	56	54	56
				2	59	59	54	55	55	54	56
DNA004356	M	9	FAS	1	60	57	56	56	56	53	57
				2	60	57	56	54	54	53	55
DNA004360	M	10	FAS	1	58	59	54	54	57	57	55
				2	57	61	56	55	57	57	56
DNA004365	M	10	FAS	1	59	63	54	56	57	54	57
				2	58	58	55	56	57	55	56
DNA004370	F	11	FAS	1	59	60	54	55	58	54	57
				2	56	59	55	55	57	55	54
DNA004373	F	7	FAS	1	61	61	56	58	57	59	58
				2	56	57	56	56	55	56	57
DNA004375	M	8	FAS	1	54	61	54	55	59	58	55
				2	54	61	54	55	59	58	55
DNA004378	M	8	FAS	1	55	59	58	58	60	57	59
				2	59	59	57	58	58	56	57
DNA004380	M	8	FAS	1	67	65	59	57	67	63	65
				2	66	61	61	60	64	59	64
DNA004382	M	9	FAS	1	54	57	56	56	56	52	57
				2	59	57	56	54	55	53	56
DNA004384	F	6	FAS	1	61	59	53	55	56	55	56
				2	61	58	54	54	55	56	56

DNA004386	M	8	FAS	1	56	61	56	56	57	56	58
				2	58	61	59	59	61	56	59
DNA004388	M	6	FAS	1	67	70	66	65	69	63	67
				2	70	69	63	66	67	64	65
DNA004390	F	7	FAS	1	62	63	56	57	61	56	58
				2	60	61	57	58	59	55	57
DNA004391	M	7	FAS	1	55	58	58	57	60	62	58
				2	60	61	58	56	60	60	57

? = no information

Table 34: Complete data for *PEG3 DMR* control samples:

Complete data for *PEG3 DMR* control samples										
Sample ID	**Gender**	**Age(yrs)**	**Run**	**Methylation %**						
				CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7
DNA004392	F	25	1	43	38	38	38	30	34	33
			2	45	37	35	43	30	30	33
DNA004393	M	19	1	50	43	42	43	36	38	37
			2	49	39	41	46	32	35	37
DNA004394	M	20	1	44	40	46	42	37	36	39
			2	45	40	46	38	35	38	41
DNA004395	F	26	1	47	40	40	45	32	33	37
			2	52	39	43	41	34	35	33
DNA004396	F	25	1	48	49	44	39	35	37	37
			2	51	43	45	43	34	37	39
DNA004398	M	19	1	49	45	43	43	36	34	36
			2	47	46	40	46	42	39	39
DNA004399	F	21	1	57	55	54	54	44	45	46
			2	56	58	58	53	47	44	50
DNA004401	F	22	1	53	47	47	50	42	44	43
			2	53	52	47	54	43	50	42
DNA004404	F	25	1	55	51	50	55	40	41	39
			2	49	51	48	49	38	41	40
DNA004406	F	22	1	50	45	44	49	36	35	35
			2	46	44	40	47	33	35	38
DNA004407	F	24	1	45	37	42	37	39	39	36
			2	50	42	42	39	39	39	36
DNA004408	M	24	1	48	43	47	45	41	53	40
			2	48	43	47	45	41	53	40
DNA004411	M	19	1	53	44	48	51	41	45	47
			2	53	45	48	51	41	43	47

DNA004412	M	23	1	51	43	40	40	32	33	35
			2	51	39	43	45	32	35	34
DNA004413	F	19	1	45	38	36	39	34	35	36
			2	46	37	42	43	37	39	35
DNA004414	M	23	1	61	54	55	57	48	44	48
			2	58	54	55	54	47	48	48
DNA004415	F	21	1	53	51	53	48	43	40	48
			2	50	51	53	49	43	40	48
DNA004418	F	19	1	50	37	39	43	38	39	39
			2	50	39	41	43	38	39	39
DNA004420	M	19	1	54	47	45	52	41	44	41
			2	54	46	46	52	42	44	43
DNA004421	M	18	1	59	60	54	55	45	46	49
			2	59	60	54	55	45	46	49
DNA004422	F	20	1	47	51	47	47	45	46	49
			2	48	52	48	46	45	46	49
DNA004425	F	22	1	45	40	40	45	37	37	38
			2	43	44	44	46	39	37	41
DNA004426	F	24	1	53	49	48	48	44	41	44
			2	53	49	48	48	44	41	44
DNA004428	F	20	1	52	47	45	47	44	40	42
			2	54	43	45	48	38	38	38
DNA004429	F	24	1	50	48	53	52	46	48	45
			2	54	49	53	52	46	48	45
DNA004431	M	25	1	55	46	52	48	45	42	44
			2	54	45	52	48	45	42	44
DNA004434	M	24	1	49	45	43	46	35	30	36
			2	48	45	43	45	35	30	35
DNA004437	M	23	1	44	40	39	40	38	32	33
			2	41	39	37	39	33	32	32

DNA004440	M	21	1	50	45	46	47	39	38	38
			2	48	44	43	46	35	35	38
DNA004441	M	19	1	49	42	45	40	36	38	37
			2	44	38	39	40	31	34	34
DNA004442	M	20	1	48	47	46	40	37	40	40
			2	46	47	44	40	39	42	39
DNA004448	M	18	1	43	39	43	40	33	37	33
			2	41	38	36	40	30	32	32
DNA004450	F	19	1	43	38	40	40	32	37	34
			2	41	36	38	40	32	37	34
DNA004463	M	25	1	49	45	46	50	39	41	43
			2	51	45	48	49	38	42	39
DNA004465	F	20	1	48	53	46	53	44	44	42
			2	48	50	45	51	44	44	41
DNA004466	F	18	1	57	53	50	49	48	44	49
			2	54	52	49	48	48	42	47
DNA004476	M	19	1	53	46	47	51	40	37	44
			2	53	48	47	47	41	37	42
DNA004477	F	24	1	45	44	43	45	40	36	40
			2	44	40	40	43	35	35	40
DNA004481	F	20	1	55	53	50	48	38	42	49
			2	56	51	50	46	40	42	45
DNA004482	F	19	1	59	57	57	53	47	49	42
			2	56	54	51	50	46	44	45
DNA004483	F	19	1	50	46	47	45	39	42	42
			2	50	44	42	41	33	38	41
DNA004487	M	20	1	59	51	52	57	43	46	48
			2	54	49	47	54	38	43	43
DNA004492	F	21	1	52	47	47	47	41	40	40
			2	48	48	48	50	39	44	46

DNA004497	M	19	1	64	60	60	60	51	48	50
			2	60	55	54	57	50	48	50
DNA004498	F	24	1	51	52	54	51	44	42	46
			2	56	52	52	56	44	45	47
DNA004500	M	18	1	51	51	49	51	44	40	46
			2	52	54	52	52	47	41	50
DNA004501	M	18	1	42	36	36	40	30	32	33
			2	44	34	33	37	30	33	31
DNA004664	M	22	1	48	47	44	48	37	40	41
			2	50	42	44	50	34	35	36
DNA004668	M	22	1	43	44	44	47	34	38	36
			2	43	44	44	47	34	38	36
DNA004690	M	24	1	42	41	38	38	33	35	37
			2	40	39	38	41	33	34	33

Table 35: Complete data for *PEG3 DMR* case samples and runs:

Complete data for *PEG3 DMR* case samples and runs											
Sample ID	**Gender**	**Age(yrs)**	**Phenotype**	**Run**	**Methylation %**						
					CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7
DNA003985	F	9	FAS	1	47	48	40	45	38	40	39
				2	53	49	46	49	38	42	37
DNA003989	F	9	FAS	1	44	40	28	29	35	23	34
				2	44	39	30	34	30	27	31
DNA003991	F	12	FAS	2	44	40	29	32	34	30	33
				2	39	39	31	27	32	25	32
DNA004010	M	9	FAS	1	50	47	46	47	40	41	40
				2	49	43	46	42	39	37	34
DNA004012	F	9	FAS	1	49	42	39	40	34	34	36
				2	50	44	39	42	34	35	36
DNA004013	M	9	FAS	1	40	34	33	35	30	31	33
				2	40	34	33	36	29	31	33
DNA004021	M	9	FAS	1	50	51	49	47	40	42	41
				2	47	47	49	47	42	45	41
DNA004024	F	7	FAS	1	44	40	38	41	37	36	36
				2	48	41	38	42	36	39	36
DNA004029	F	9	FAS	1	40	40	39	39	34	35	36
				2	42	37	38	40	32	33	33
DNA004032	M	10	FAS	1	40	40	34	42	30	32	32
				2	40	36	33	40	26	27	27
DNA004035	M	9	FAS	1	50	41	39	45	30	32	36
				2	47	37	39	45	29	37	30
DNA004042	F	9	FAS	1	40	37	34	42	28	32	29
				2	45	41	39	46	33	36	33
DNA004052	M	10	FAS	1	50	43	44	45	33	36	36
				2	47	42	45	42	34	34	35

DNA004053	F	11	FAS	1	37	34	34	35	25	29	35
				2	37	34	34	35	25	29	35
DNA004055	M	11	FAS	1	39	41	37	39	28	30	33
				2	42	39	38	37	24	28	28
DNA004059	F	10	FAS	1	41	42	38	43	31	33	34
				2	41	42	38	43	31	33	34
DNA004061	F	10	FAS	1	47	43	40	41	34	36	36
				2	48	42	44	41	37	36	37
DNA004063	M	10	FAS	1	52	48	47	50	37	40	39
				2	50	45	42	47	35	38	39
DNA004064	F	10	FAS	1	47	46	43	46	35	39	37
				2	50	43	46	47	40	39	39
DNA004065	M	8	FAS	1	44	43	40	41	40	35	35
				2	47	41	43	40	37	37	37
DNA004068	M	16	FAS	1	40	43	38	40	36	32	33
				2	40	43	38	40	36	32	33
DNA004069	F	10	FAS	1	45	44	40	46	38	36	35
				2	47	48	44	50	43	40	41
DNA004072	F	6	FAS	1	44	44	42	42	40	45	38
				2	44	44	42	42	40	45	38
DNA004074	F	10	FAS	1	41	42	39	40	38	37	35
				2	44	45	44	45	40	38	35
DNA004076	F	10	FAS	1	46	44	40	42	36	33	37
				2	44	38	37	41	34	32	33
DNA004082	M	2	FAS	1	46	45	43	43	39	41	37
				2	41	42	38	38	34	35	35
DNA004094	F	11	FAS	1	42	37	38	41	34	35	36
				2	37	41	33	40	33	29	33
DNA004126	M	2	FAS	1	33	31	26	35	27	30	26
				2	33	31	26	35	27	30	26
DNA004146	F	2	FAS	1	47	43	42	46	40	39	40

				2	42	38	38	41	35	35	35
DNA004166	M	2	FAS	1	44	36	27	44	30	30	32
				2	42	40	30	39	29	30	34
DNA004168	M	3	FAS	1	51	43	41	44	35	38	37
				2	50	44	45	47	38	38	37
DNA004172	F	16	FAS	1	40	37	34	40	27	29	30
				2	40	36	35	41	26	28	30
DNA004191	F	10	FAS	1	52	48	44	44	33	34	36
				2	49	49	42	49	35	38	39
DNA004193	M	10	FAS	1	47	43	40	42	34	31	37
				2	41	38	39	40	31	36	36
DNA004198	M	10	FAS	1	54	51	47	52	37	40	32
				2	53	49	50	51	39	38	38
DNA004200	F	10	FAS	1	40	33	33	35	31	27	27
				2	39	34	34	36	29	31	30
DNA004204	M	10	FAS	1	42	43	41	39	37	37	36
				2	45	42	42	44	35	36	37
DNA004225	M	10	FAS	1	49	43	41	43	32	34	38
				2	46	45	38	45	36	29	37
DNA004227	F	11	FAS	1	43	36	34	38	28	29	31
				2	44	42	39	44	31	35	36
DNA004231	M	11	FAS	1	45	44	42	44	29	34	32
				2	51	44	44	44	30	36	36
DNA004233	F	10	FAS	1	49	42	41	43	32	33	34
				2	46	36	39	40	26	31	30
DNA004241	F	10	FAS	1	47	44	39	45	35	37	37
				2	51	45	45	50	39	42	40
DNA004246	M	11	FAS	1	47	40	43	45	33	36	37
				2	44	41	38	42	30	31	33
DNA004249	M	11	FAS	1	36	33	33	34	25	26	28
				2	36	34	33	34	25	27	30

DNA004253	M	11	FAS	1	42	45	40	50	32	32	35
				2	45	41	38	47	29	32	30
DNA004258	M	?	PFAS	1	49	43	45	49	37	38	37
				2	49	43	45	49	37	38	37
DNA004267	F	1	PFAS	1	41	34	34	38	28	30	28
				2	41	34	34	38	28	30	28
DNA004270	?	?	?	1	49	42	44	42	34	37	36
				2	44	41	40	43	33	35	35
DNA004280	F	11	FAS	1	38	38	38	43	29	32	33
				2	39	38	39	43	30	32	33
DNA004290	M	14	FAS	1	50	43	41	40	32	33	33
				2	49	43	42	40	32	33	33
DNA004292	F	12	FAS	1	46	37	39	43	31	34	35
				2	46	37	39	43	31	34	35
DNA004296	M	7	FAS	1	43	36	34	41	31	32	33
				2	45	41	38	43	37	36	36
DNA004300	F	7	FAS	1	47	40	44	43	33	37	36
				2	48	45	43	48	37	38	37
DNA004301	M	6	FAS	1	40	38	37	38	28	32	32
				2	35	33	32	34	23	27	26
DNA004304	M	16	FAS	1	46	43	38	43	35	39	40
				2	47	41	44	43	34	42	39
DNA004305	M	4	FAS	1	50	35	49	56	44	48	48
				2	56	41	53	55	42	42	42
DNA004312	M	??	FAS	1	41	36	35	37	27	30	33
				2	45	39	39	40	31	29	34
DNA004313	M	??	FAS	1	49	49	41	50	36	38	41
				2	46	43	38	47	33	35	38
DNA004317	M	13	FAS	1	43	40	42	40	35	30	35
				2	45	45	43	45	36	36	39
DNA004325	M	2	FAS	1	42	39	40	38	31	32	32

				2	42	34	34	39	32	32	32
DNA004327	M	3	FAS	1	50	43	47	45	42	40	39
				2	50	43	44	40	38	36	35
DNA004331	M	8	FAS	1	39	37	33	40	30	31	33
				2	41	39	39	39	34	35	33
DNA004335	M	2	FAS	1	47	42	41	44	37	38	39
				2	45	41	44	46	33	36	38
DNA004337	F	2	FAS	1	41	20	32	39	29	29	29
				2	36	38	32	33	26	28	26
DNA004343	F	10	FAS	1	42	40	42	44	39	40	42
				2	46	39	40	43	39	40	42
DNA004348	M	9	FAS	1	41	39	42	37	31	38	37
				2	42	40	42	37	36	40	39
DNA004350	F	8	FAS	1	46	37	39	39	28	30	31
				2	41	32	37	35	34	31	32
DNA004356	M	9	FAS	1	51	41	41	46	35	36	37
				2	51	41	41	46	35	36	37
DNA004360	M	10	FAS	1	43	42	40	43	33	38	40
				2	43	43	42	37	34	34	39
DNA004365	M	10	FAS	1	42	33	35	39	28	32	34
				2	43	34	36	40	29	33	35
DNA004370	F	11	FAS	1	42	42	43	42	38	37	38
				2	45	38	38	45	36	35	37
DNA004373	F	7	FAS	1	36	36	34	34	28	26	29
				2	40	39	36	37	32	31	35
DNA004375	M	8	FAS	1	42	37	40	37	34	35	36
				2	36	34	37	32	31	29	32
DNA004378	M	8	FAS	1	36	33	32	39	27	33	32
				2	36	33	32	39	27	33	32
DNA004380	M	8	FAS	1	34	32	35	31	30	29	31
				2	34	32	37	41	25	30	32

DNA004382	M	9	FAS	1	51	49	46	42	34	34	35
				2	51	49	46	42	34	34	35
DNA004384	F	6	FAS	1	48	41	44	39	34	35	37
				2	44	44	44	38	33	34	34
DNA004386	M	8	FAS	1	49	45	46	46	38	42	42
				2	55	45	45	50	39	42	43
DNA004388	M	6	FAS	2	51	49	45	45	44	44	42
				2	51	49	45	45	44	44	42
DNA004391	M	7	FAS	1	50	49	46	47	40	43	49
				2	52	52	50	52	45	44	52

? = no information

Appendix F: Published article related to the present study

frontiers in GENETICS

ORIGINAL RESEARCH ARTICLE
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Reduced DNA methylation at the *PEG3 DMR* and *KvDMR1* loci in children exposed to alcohol *in utero*: a South African Fetal Alcohol Syndrome cohort study

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Fetal alcohol syndrome (FAS) is a devastating developmental disorder resulting from alcohol exposure during fetal development. It is a considerable public health problem worldwide and is characterized by central nervous system abnormalities, dysmorphic facial features, and growth retardation. Imprinted genes are known to play an important role in growth and development and therefore four imprinting control regions (ICRs), *H19 ICR*, *IG-DMR*, *KvDMR1* and *PEG3 DMR* were examined. It is proposed that DNA methylation changes may contribute to developmental abnormalities seen in FAS and which persist into adulthood. The participants included FAS children and controls from the Western and Northern Cape Provinces. DNA samples extracted from blood and buccal cells were bisulfite modified, the ICRs were amplified by PCR and pyrosequencing was used to derive a quantitative estimate of methylation at selected CpG dinucleotides: *H19 ICR* (six CpG sites; 50 controls and 73 cases); *KvDMR1* (7, 55, and 86); *IG-DMR* (10, 56, and 84); and *PEG3 DMR* (7, 50, and 79). The most profound effects of alcohol exposure are on neuronal development. In this study we report on epigenetic effects observed in blood which may not directly reflect tissue-specific alterations in the developing brain. After adjusting for age and sex (known confounders for DNA methylation), there was a significant difference at *KvDMR1* and *PEG3 DMR*, but not the *H19 ICR*, with only a small effect (0.84% lower in cases; $p = 0.035$) at *IG-DMR*. The two maternally imprinted loci, *KvDMR1* and *PEG3 DMR*, showed lower average locus-wide methylation in the FAS cases (1.49%; $p < 0.001$ and 7.09%; $p < 0.001$, respectively). The largest effect was at the *PEG3 DMR* though the functional impact is uncertain. This study supports the role of epigenetic modulation as a mechanism for the teratogenic effects of alcohol by altering the methylation profiles of imprinted loci in a locus-specific manner.

Keywords: fetal alcohol syndrome, imprinted genes, epigenetics, *PEG3*, *KvDMR1*, *H19 ICR*, *IG-DMR*

INTRODUCTION

Alcohol is a potent teratogen with devastating effects on the developing fetus. The most profound effects of prenatal alcohol exposure are on neuronal development, resulting in adverse cognitive and behavioral outcomes with lifelong implications, distinct dysmorphic features (shortened palpebral fissures, smooth philtrum, and thin vermilion border to the upper lip), and pre- and postnatal growth retardation (Stratton et al., 1996; Riley and McGee, 2005; Floyd et al., 2006). The outcomes are collectively referred to as fetal alcohol spectrum disorders (FASD) and range in severity with fetal alcohol syndrome (FAS) at the most severe end of the spectrum (Sokol et al., 2003). FAS is the leading cause of preventable mental retardation and developmental disability in the world. It is an international problem that shows no racial boundaries (Clarren and Smith, 1978; Masotti et al., 2006) and the consequences of prenatal alcohol exposure represent a major public health problem worldwide (May and Gossage, 2001; Sokol et al., 2003; Riley et al., 2011).

The worldwide average prevalence of FAS is estimated at 0.97 per 1000 live births, yet in some communities it is much higher (Abel and Hannigan, 1995; May and Gossage, 2001; McKinstry, 2005). Notably, the prevalence of FAS in South Africa is one of the highest reported in the world, at 40.5–46.4 per 1000 children of school going age in the Western Cape Province (May et al., 2000),

confirmed in two follow up studies from the same area reporting an increasing prevalence of 65.2–74.2 (Viljoen et al., 2005) and 68–89.2 per 1000 (May et al., 2007). In addition a study in the Northern Cape Province reported a similar prevalence of 67.2 per 1000 (Urban et al., 2008).

Fetal alcohol syndrome is a complex multifactorial condition and although prenatal alcohol exposure is the primary trigger, twin concordance studies and animal models suggest a significant genetic susceptibility for the development of FAS (Streissguth and Dehaene, 1993; Becker et al., 1996). Recent studies have proposed an epigenetic etiology and supporting evidence for such a mechanism is accumulating (Garro et al., 1991; Haycock, 2009; Ungerer et al., 2013). Gene expression disturbances can be caused by changes in DNA methylation, molecular modification of histones and through RNA interference. These mechanisms work together to produce a unique, and reversible epigenetic signature that regulates gene expression through chromatin remodeling. DNA methylation has been investigated extensively as a mechanism of alcohol teratogenesis.

Genomic imprinting is an epigenetic phenomenon resulting in mono-allelic gene expression according to the parent of origin in a locus-specific manner. It is mediated by differential DNA methylation and imprinted loci play an important role during normal development (Jirtle et al., 2000; Rodenhiser and Mann, 2006). The DNA methylation status can be influenced by the environment leading to a functional impact mediated by changes in the epigenome (Jirtle and Skinner, 2007). Imprinted genes are therefore suitable candidates for investigating the effects of teratogens on disease etiology. Almost all imprinted genes contain differentially methylated regions (DMRs) which serve as a mark that differentiates the paternal allele from the maternal allele. Some DMRs which regulate the methylation patterns of a duster of imprinted genes are referred to as primary DMRs or imprinting control regions (ICRs). The CpG methylation at ICRs is established in the gametes and maintained in somatic tissues of offspring throughout development (Smallwood and Kelsey, 2012). Despite this trend, they may still be subject to tissue-specific effects and extrapolation from the tissue under investigation should be done with care. On the other hand, the imprinting of secondary DMRs is established after fertilization (Geuns et al., 2007). Individual loci may be hyper- or hypomethylated following alcohol exposure. A study by Kaminen-Ahola et al. (2010) reported that maternal alcohol exposure tended to induce hypermethylation at the A^{vy} locus, while Haycock and Ramsay (2009) reported hypomethylation at the *H19 ICR* in mouse placenta following *in utero* alcohol exposure and Stouder et al. (2011) also showed hypomethylation at the *H19 ICR* in the brain and sperm of *in utero* exposed offspring (Stouder et al., 2011). A study by Liu et al. (2009) has demonstrated that alcohol exposure during early neurulation can induce aberrant changes in DNA methylation with associated changes in gene expression in mice. These studies support an epigenetic mechanism as a contributing factor for the development of features observed in FASD. It is widely suggested that the effect is mediated through the interruption of the one carbon pathway that is critical in production of the methyl groups in the maintenance of DNA methylation (Halsted et al., 2002; Liu et al., 2009). Alcohol exposure is correlated with reduced DNA methylation through several plausible mechanisms. Firstly acetaldehyde, a metabolite of alcohol metabolism, inhibits methyltransferase activity, and secondly, folate deficiency as a result of alcohol consumption and poor nutrition, reduces the pool of methyl donors.

In this study we examined quantitative changes in DNA methylation in blood and buccal cells from individuals with FAS, compared to unaffected controls, at four ICRs that regulate gene expression at loci that are important during fetal growth and development: *H19 ICR*, *KvDMR1*, *IG-DMR*, and *PEG3 DMR*.

MATERIALS AND METHODS

STUDY PARTICIPANTS AND SAMPLE COLLECTION

The study participants included 87 individuals with a diagnosis of FAS and 58 controls. All participants were of mixed ancestry, referred to as "Coloreds" in the South African context, and were resident in the Western Cape and Northern Cape provinces of South Africa. The FAS cases were recruited from Wellington in the Western Cape and De Aar and Upington in the Northern Cape. They were diagnosed by a team of trained clinicians from the Division of Human Genetics, NHLS, Braamfontein, Johannesburg, and also the Foundation for Alcohol Related Research (FARR; http://www.farr-sa.co.za), led by Denis Viljoen. The control participants were recruited from the Northern Cape and no phenotype data were collected. The cases and controls were not age matched. The FAS cases has a median age of 9 years (range 1–16 years) and the control participants were 17–26 years of age (median age 20 years). Adult participants provided informed consent and the parents or guardians of minor participants provided informed consent on their behalf. Ethics approval for the study was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Medical) (Protocol numbers M02/10/41, M03/10/20 and M080548). Venous blood samples were collected into EDTA by qualified phlebotomists and buccal swabs were collected by the research staff.

DNA EXTRACTION FROM BLOOD AND BUCCAL TISSUES

DNA was extracted from whole blood using a manual salting out method according to a modified protocol from Miller et al. (1988). The buccal tissue DNA was extracted using the Gentra Puregene buccal cell kit (Qiagen, Valencia, CA, USA).

DNA BISULFITE MODIFICATION AND PCR AMPLIFICATION

Genomic DNA was bisulfite modified using the EZ-DNA Methylation Gold Kit ™ (Zymo Research, Orange, CA, USA). Published primer sets and custom designed primer sets were used to amplify specific regions within the ICRs of four imprinted loci: *H19 ICR*; *IG-DMR*; *KvDMR1*; and *PEG3 DMR*. Each locus is described briefly and the details of the PCR and sequencing primers are shown in **Table 1**.

The pre-pyrosequencing PCR step requires that one of the primers is 5′ biotin labeled. In this study we used a universal biotin labeled primer (5′-biotin-GACGGGACACCGCTGATCGTTTA-3′) which was included in the PCR cocktail to generate labeled DNA fragments (Colella et al., 2003). The sequences of primers

Table 1 | Locus specific information for PCR amplification and pyrosequencing.

Locus (contig)	No. CpG sites	PCR primers (5′–3′)	Amplicon length (bp)	Annealing Temp (°C)	Sequencing primer (5′–3′)	Reference
H19 ICR (AF087017)	6	Outer reverse CTTAAATCCCAAACCATAACACTA Outer forward GTATATGGGTATTTTTTGGAGGT Inner forward GTATATGGGTATTTTTTGGAGGT Inner reverse Tag-ATATCCTATTCCCAAATAA	217	61.5 53	TGGTTGTAGTTGTGGAAT	Present study
IG-DMR (A117190)	7	Forward Tag-TTTATTGGGTTGGGTTTTGTTAG	267	58	Primer 1 CAATTACAATACCACAAAAT	Present study
	3	Reverse AACCAATTACAATACCACAAAATT			Primer 2 CCATAAACAACTATAAACCT	Present study
KvDMR1 (U90095)	7	Forward TTAGTTTTTTGYGTGATGTGTTTATTA Reverse Tag-CCCACAAACCTCCACACC	101	55	TTGYGTGATGTGTTTATTA	Bourque et al. (2010)
PEG3 DMR (AC006115)	7	Reverse Tag-CCTATAAACAACCCCACACCTATAC Forward TAATGAAAGTGTTTGAGATTTGTTG	272	62	GGGGGTAGTTGAGGTT	Boissonnas et al. (2010)

Tag-5′-biotin-GACGGGACACCGCTGATCGTTTA-3′—universal biotin labeled tag.

that were designed to be biotin labeled therefore had a 23 bp complementary tag sequence added to their 5′ ends for the priming of the universal biotin labeled primer. These primers are shown in **Table 1** as "tag" primers. Unless specified to the contrary, primer sets were designed using the PSQ assay design software (Biotage, Uppsala country, Sweden).

The *H19 ICR* contains seven CTCF binding sites, of which the sixth is differentially methylated. The sixth CTCF binding site was the target region in this study and contains five CpGs, but the amplified region included one extra CpG which was also included in the analysis. For the *H19 ICR* amplification, nested PCR was used with an outer and an inner PCR primer sets. The PCR reactions for this region were performed in triplicate.

The amplified *IG-DMR* region contains 15 CpGs, but only 10 CpGs were analyzed using two different sequencing primers (1 and 2), where one analyzed three and the other analyzed seven CpG sites. PCR primers used for amplification of the *KvDMR1* are published primers and the amplicon contains seven CpGs, including a differentially methylated *NotI* site (Bourque et al., 2010). The PCR forward primer and pyrosequencing sequencing primer had a wobble introduced to accommodate an unavoidable CpG site in the sequence template that could either be methylated or unmethylated. The *PEG3 DMR* amplified region contains 14 CpGs but only seven CpGs were analyzed. The PCR assays for *IG-DMR*, *KvDMR1*, and *PEG3 DMR* were run in duplicate.

PYROSEQUENCING FOR QUANTIFICATION OF DNA METHYLATION ANALYSIS

DNA methylation of the different amplified ICRs was quantified by pyrosequencing using the PSQ 96MA system with the PyroGold SQA reagent kit (Biotage, Uppsala, Uppsala country, Sweden). Pyrosequencing assays and sequencing primers (**Table 1**) were designed using PSQ Assay Design Software and the sequencing was done in triplicate (*H19 ICR*) or duplicate (*IG-DMR*, *KvDMR1*, and *PEG3 DMR*). The percentage methylation for each of the CpG sites within the target region was calculated using Pyro Q-CpG software (Biotage, Uppsala, Uppsala country, Sweden). Two non-CpG cytosine bases were included in all the pyrosequencing assays as internal controls to assess successful bisulfite conversion. Samples containing >5% unsuccessfully converted non-CpG cytosines were discarded.

STATISTICAL ANALYSIS

We analyzed methylation data for 145 individuals, 87 FAS cases, 58 controls. Not every individual provided complete data. There was no age overlap due to the cases being of primary school age (younger than 17 years old, mean age 9 years) and the controls being 17 years or older. This means that the age effect (difference between young and older) cannot be distinguished from the fetal alcohol (case-control) effect in this study. However, the effect per additional year of age could be estimated within each group. Both groups had similar gender distributions, as summarized in **Table 2**.

Linear mixed-effects models were used to generate all the results reported here. These analyses are based on joint models, where all the original methylation observations (individual replicates) are put into a single model to simultaneously do the tests. One advantage is that it avoids some false positive results, because all the results are adjusted for each other. These models also enabled us to adjust for different kinds of random variation as random effects: that between sites, and that between individuals, and that within individuals (between replicates). Adjusting for

Table 2 | Summary table for number of samples, sex and age distribution for the different loci tested in the control and case groups.

	Controls				Cases			
		Age	Sex			Age	Sex	
Locus	*N*	Mean (years) (min:max)	Male	Female	*N*	Mean (years) (min:max)	Male	Female
H19 ICR	50	21 (17:26)	27	23	73	8.7 (1:16)	41	32
KvDMR1	55	21 (17:26)	27	28	86	8.4 (1:16)	46	40
IG-DMR	56	21 (17:26)	29	27	84	8.5 (1:16)	45	39
PEG3 DMR	50	21 (17:26)	25	25	79	8.7 (1:16)	46	33

the variation between individuals is a different way of saying we adjusted for the correlation between replicates on the same individual. After confirming, using linear mixed-effects models, that age and sex were confounders, all further models were adjusted for them, as fixed effects. All *p*-values, effects sizes and standard errors (SE) come from interaction terms in the models. All results corresponding to *p*-values below 0.05 are described as significant, below 0.01 as highly significant and below 0.001 as very highly significant.

The observed methylation data are summarized with boxplots. Each box extends from the first quartile to the third quartile (interquartile range), the line inside the box is at the median, and the whiskers extend to the non-outlying minimum and maximum, respectively. Outliers are shown as open circles. The freely available environment for statistical computing and graphics, R (R Core Team, 2014), and R package (Pinheiro et al., 2015), were used for these analyses.

RESULTS

The 87 FAS cases were recruited from several areas of the Western Cape and the Northern Cape, whereas the 58 control participants were mainly recruited from the Northern Cape. There are differences in the numbers of individuals tested per locus, due to failure to amplify in specific samples for specific loci. Similar percentages per sex were tested, 30 (52%) males and 28 (48%) females in the controls and 47 (54%) males and 40 (46%) females in the cases. The control participants ($N = 58$) all donated blood samples and of the 87 FAS cases, eight donated buccal samples and the remainder donated blood. A summary for the number of samples, sex and age distribution at the different loci in the case and control groups is shown in **Table 2**.

To address tissue specificity of DNA methylation at an imprinted locus, we showed that there was no significant difference in percentage methylation at the *H19 ICR* locus CpG sites between buccal and blood samples from 50 random participants from another study (data not shown). Methylation status between the two tissues was not assessed at *KvDMR1*, *IG-DMR*, and *PEG3 DMR*. Based on two previous studies, we concluded that methylation profiles at these ICRs are unlikely to differ between the two tissues. Bourque et al. (2010) compared average methylation profiles at *KvDMR1* between blood and saliva tissues in healthy adults and reported that their methylation patterns were similar. In addition Woodfine et al. (2011) examined the methylation patterns of 17 germline DMRs (including *H19 ICR*, *KvDMR1*, *IG-DMR*, and *PEG3 DMR*) amongst several somatic tissues (including brain, breast, colon, heart, kidney, and liver) and reported that the average methylation did not vary amongst the tissues. It is therefore unlikely that the origin of the tissue for the DNA methylation studies is a significant confounder in this study.

Figure 1 contains box plots summarizing the observed percentage methylation at individual CpG sites at all loci (*H19 ICR*, *KvDMR1*, *IG-DMR*, and *PEG3 DMR*), in controls (CON) and cases (FAS). **Figure 2** contains box plots summarizing the observed percentage average methylation at each locus: *H19 ICR*, *KvDMR1*, *IG DMR*, and *PEG3 DMR*, in controls (CON) and cases (FAS). It is not possible to visualize the data after correction for age and sex.

AGE AND SEX AS POTENTIAL CONFOUNDERS IN DNA METHYLATION STUDIES ON IMPRINTED LOCI

Age and sex are reported confounders in DNA methylation studies and their effects were investigated in the present study. The results for sex are summarized in **Table 3** and for age in **Table 4**. The sex effect was highly significant at *PEG3 DMR* in FAS cases, where males had an estimated 1.11% more methylation than females. In contrast, in controls at *PEG3 DMR*, males had a significant estimated 0.84% lower methylation compared to that in females. However estimated methylation did not differ by sex in control nor in FAS cases at any of *H19 ICR*, *IG-DMR.A*, *IG-DMR.B*, and *KvDMR1*. Since there was a significant difference at one locus, sex was adjusted for in downstream analyses. It was observed that *IG-DMR* has a wide variability in methylation at the different CpG sites analyzed. Most of the individuals had methylation of above 70% at CpG 1–5 while CpG site 6–10 have methylation of about 50%. Therefore *IG-DMR* was split into two regions for this analysis: sites 1–5 called *IG-DMR.A* and sites 6–10 called *IG-DMR.B*.

Due to ethical considerations in the selection of control participants, the study design was sub-optimal in terms of age. All cases were below 17 years of age and all controls were 17 years and above, where the latter were able to give individual informed consent, but the parents or guardians consented to the participation of the cases. This means that age is strongly confounded and that it is not possible to tell whether any differences between cases and controls are caused by the age difference or not. However, the effect of age inside each of the groups could and was investigated.

Table 4 shows estimates of the difference in methylation percentage over 1 year of age, together with its SE and *p*-values in cases and controls.

The largest effects are seen at *IG-DMR.A* and *IG-DMR.B* in FAS cases, where the estimated methylation percentage decreased

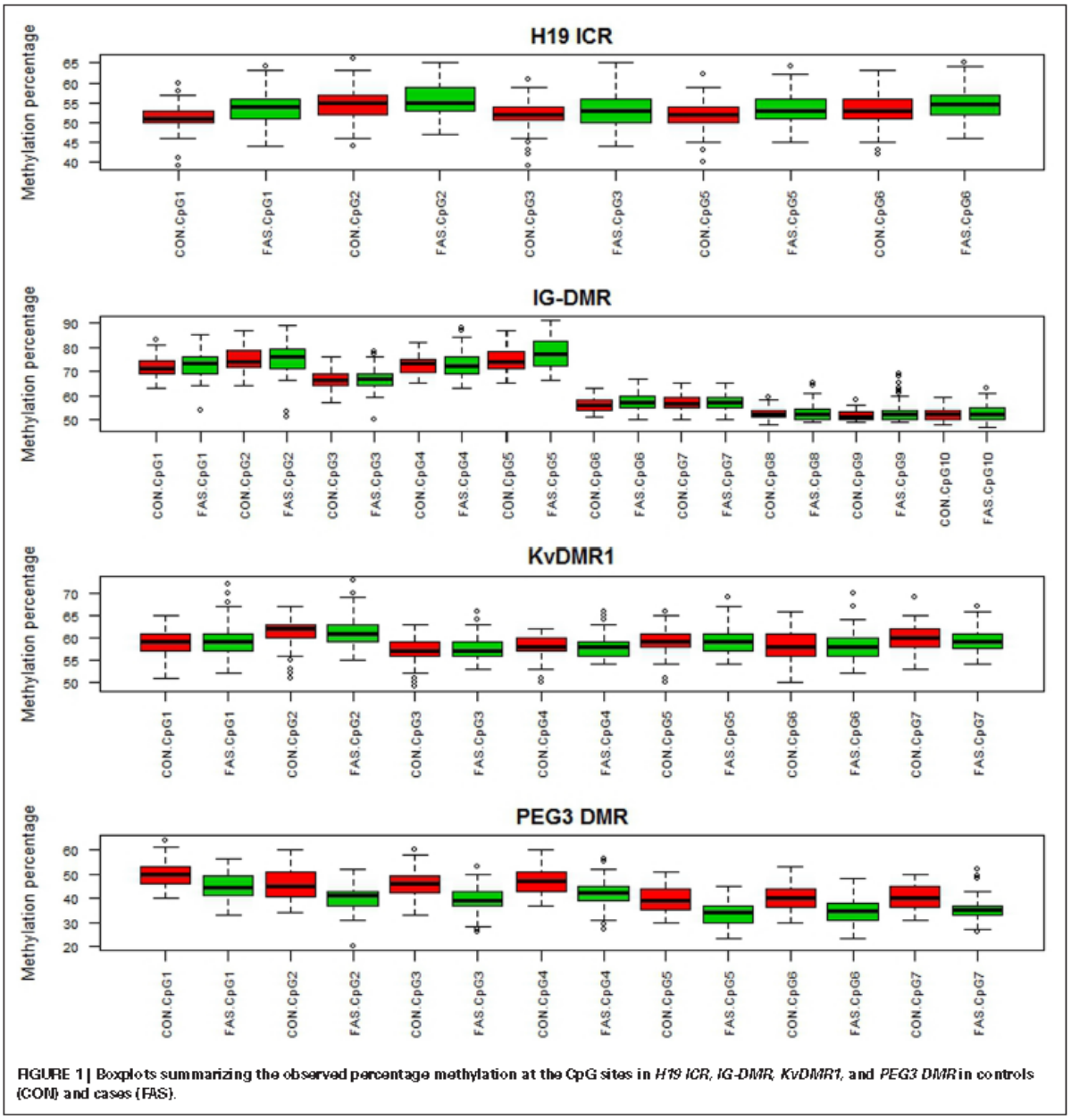

FIGURE 1 | Boxplots summarizing the observed percentage methylation at the CpG sites in *H19 ICR*, *IG-DMR*, *KvDMR1*, and *PEG3 DMR* in controls (CON) and cases (FAS).

by 0.43 and 0.38% respectively, for a 1 year increase in age. At *KvDMR1*, for every year increase in age, there is a significant estimated methylation increase of 0.19% in controls but in FAS cases there is a significant decrease by 0.11%. Again the highly significant effect is seen at *PEG3 DMR* in the control group, where estimated methylation percentage decreases by 0.22% for every year increase in age. No age effect was observed at *H19 ICR* (either in cases or controls), nor at *IG-DMR.A* and *IG-DMR.B* (in controls) nor at *PEG3 DMR* (in cases). **Table 5** summarizes, for each CpG site, the effect of 1 year of age on methylation, separately for controls and FAS cases, as well as the estimated difference between cases and controls in that effect. There are five CpG sites in *IG-DMR*, one in *KvDMR1*, where the effect of age on methylation is significantly lower in FAS cases and controls. At *IG-DMR* sites 2, 5, 6, 8, and 9, as well as at *KvDMR1* site 6, methylation decreased highly significantly with age in FAS cases but no significant effect was detected in controls. In *PEG3 DMR* site 2, the effect was significantly higher in FAS cases than controls.

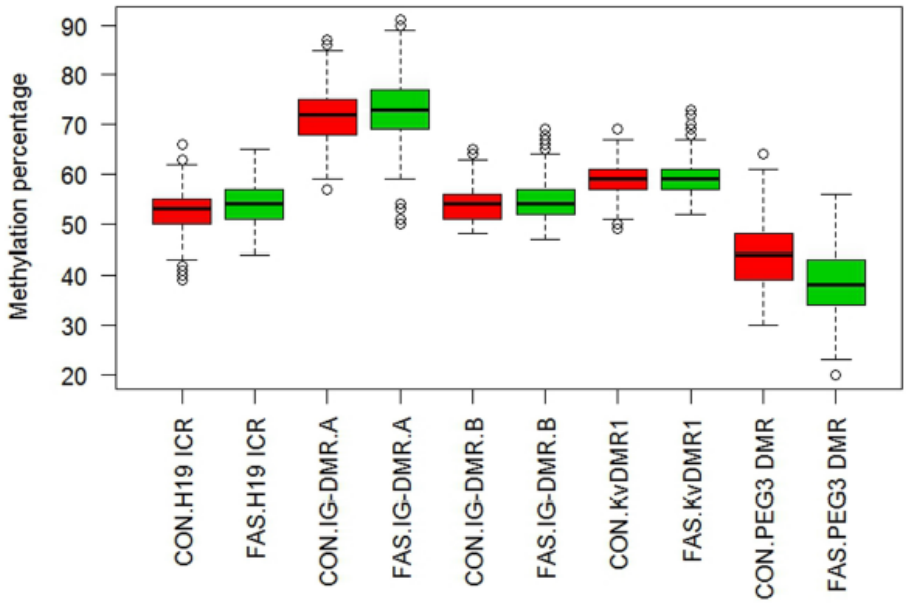

FIGURE 2 | Boxplot of observed percentage methylation per locus, *H19 ICR*, *IG-DMR*, *KvDMR1*, and *PEG3 DMR*, in controls (CON) and cases (FAS). A significant difference was detected for *H19 ICR* between cases and controls ($p = 0.024$), but after adjustment for sex and age this was no longer significant. The estimated methylation percentage difference between cases and controls at *PEG3 DMR* was highly significant ($p < 0.001$) and remained so after adjustment for age and sex.

Table 3 | Comparison of methylation within a locus between sexes, separately in FAS and controls.

Locus	Group	Effect	SE	*P*-value
H19 ICR	CON	0.33	0.48	0.495
H19 ICR	FAS	−0.16	0.40	0.687
IG-DMR1.A	CON	0.37	0.46	0.428
IG-DMR1.A	FAS	0.18	0.38	0.626
IG-DMR1.B	CON	−0.45	0.46	0.329
IG-DMR1.B	FAS	−0.60	0.38	0.112
KvDMR1	CON	0.10	0.39	0.795
KvDMR1	FAS	−0.22	0.32	0.490
PEG3 DMR	CON	−0.84	0.41	0.042
PEG3 DMR	FAS	1.11	0.33	0.001

CON, controls; FAS, FAS case; Effect, the estimated percentage difference in methylation between males and females in the specific group at the specific locus, using linear mixed-effects models, as described in methods section; SE, standard error of the effect estimate. Significant: $p < 0.05$. Analyses are adjusted for variation between sites and also for variation between individuals and within individuals as random effects.

Table 4 | The estimated effect of 1 year of age on % methylation per locus per group.

Locus	Group	Effect	SE	*P*-value
H19 ICR	CON	−0.05	0.10	0.634
H19 ICR	FAS	0.02	0.06	0.749
IG-DMR.A	CON	0.02	0.10	0.861
IG-DMR.A	FAS	−0.43	0.06	<0.001
IG-DMR.B	CON	0.01	0.10	0.920
IG-DMR.B	FAS	−0.38	0.06	<0.001
KvDMR1	CON	0.19	0.08	0.016
KvDMR1	FAS	−0.11	0.05	0.021
PEG3 DMR	CON	−0.22	0.08	0.008
PEG3 DMR	FAS	0.00	0.05	0.948

CON, controls; FAS, FAS case; Effect, estimated percentage difference in methylation between patients of a specific age and those 1 year younger, in the specific group at the specific locus, using linear mixed-effects models, as described in methods section; SE, standard error of the effect. Significant: $p < 0.05$. Analysis is adjusted for sex (fixed), CpG sites, individuals and replicates (random effects).

In light of these differences, sex and age were adjusted for in the subsequent analyses to assess differences between FAS cases and controls.

THE EFFECT OF ALCOHOL ON DNA METHYLATION AT DIFFERENT LOCI (FAS CASES COMPARED TO UNAFFECTED CONTROLS)

Unadjusted and adjusted results are presented to assess potential differences in methylation percentages at different CpG sites and also across loci, between controls and FAS cases. The random variation between sites, individuals and replicates per individual was adjusted for in all analyses.

Table 6 gives a summary of the estimated differences in CpG methylation between FAS cases and controls (FAS-CON), per CpG site, unadjusted and adjusted for age and sex. Both models were adjusted for random variation between and within individuals.

Table 5 | The estimated effect of 1 year of age on % methylation per CpG site per locus.

		Control group			FAS cases			Estimated difference in age effect on methylation between FAS and CON		
Locus	**Site**	**Age effect**	**SE**	***P*-value**	**Age effect**	**SE**	***P*-value**	**FAS-CON**	**SE**	***P*-value**
H19 ICR	CpG1	0.02	0.16	0.890	0.03	0.10	0.758	0.01	0.19	0.962
H19 ICR	CpG2	0.04	0.16	0.811	0.06	0.10	0.577	0.02	0.19	0.924
H19 ICR	CpG3	−0.16	0.16	0.298	0.02	0.10	0.851	0.18	0.19	0.326
H19 ICR	CpG5	0.10	0.16	0.522	−0.01	0.10	0.896	−0.11	0.19	0.541
H19 ICR	CpG6	−0.09	0.16	0.546	0.08	0.10	0.448	0.17	0.19	0.360
IG-DMR	CpG1	−0.05	0.17	0.789	−0.43	0.10	<0.001	−0.38	0.20	0.052
IG-DMR	CpG2	−0.19	0.17	0.273	−0.72	0.10	<0.001	−0.53	0.20	0.007
IG-DMR	CpG3	0.19	0.17	0.256	−0.02	0.10	0.811	−0.22	0.20	0.273
IG-DMR	CpG4	0.17	0.17	0.311	−0.17	0.10	0.089	−0.35	0.20	0.081
IG-DMR	CpG5	−0.11	0.17	0.520	−0.95	0.10	<0.001	−0.84	0.20	<0.001
IG-DMR	CpG6	−0.02	0.17	0.922	−0.45	0.10	<0.001	−0.43	0.20	0.030
IG-DMR	CpG7	0.05	0.17	0.774	−0.25	0.10	0.016	−0.30	0.20	0.134
IG-DMR	CpG8	0.03	0.17	0.878	−0.44	0.10	<0.001	−0.47	0.20	0.019
IG-DMR	CpG9	−0.04	0.17	0.812	−0.52	0.10	<0.001	−0.48	0.20	0.016
IG-DMR	CpG10	−0.03	0.17	0.863	−0.40	0.10	<0.001	−0.37	0.20	0.059
KvdMR	CpG1	0.26	0.17	0.122	−0.11	0.10	0.255	−0.37	0.20	0.056
KvdMR	CpG2	0.11	0.17	0.513	−0.04	0.10	0.687	−0.15	0.20	0.443
KvdMR	CpG3	0.10	0.17	0.562	−0.14	0.10	0.148	−0.24	0.20	0.218
KvdMR	CpG4	0.14	0.17	0.412	−0.09	0.10	0.363	−0.23	0.20	0.243
KvdMR	CpG5	0.26	0.17	0.126	−0.03	0.10	0.769	−0.29	0.20	0.142
KvdMR	CpG6	0.18	0.17	0.284	−0.22	0.10	0.023	−0.40	0.20	0.038
KvdMR	CpG7	0.15	0.17	0.380	−0.12	0.10	0.225	−0.27	0.20	0.170
PEG3 DMR	CpG1	−0.19	0.17	0.272	0.02	0.10	0.818	0.22	0.20	0.289
PEG3 DMR	CpG2	−0.28	0.17	0.115	0.25	0.10	0.017	0.53	0.20	0.010
PEG3 DMR	CpG3	−0.03	0.17	0.858	0.09	0.10	0.376	0.12	0.20	0.543
PEG3 DMR	CpG4	−0.11	0.17	0.523	0.03	0.10	0.793	0.14	0.20	0.495
PEG3 DMR	CpG5	−0.22	0.17	0.201	−0.14	0.10	0.194	0.09	0.20	0.665
PEG3 DMR	CpG6	−0.19	0.17	0.284	−0.16	0.10	0.125	0.03	0.20	0.895
PEG3 DMR	CpG7	−0.37	0.17	0.033	0.03	0.10	0.799	0.40	0.20	0.050

Effect is the estimated percentage difference in methylation between individuals of a specific age and those 1 year younger, in the specific group at the specific locus, using linear mixed-effects models, as described in methods section.

At *H19 ICR*, all six sites, and at *IG-DMR* sites 2, 5, 6, and 9, the case group had significantly higher methylation than the control group. However after adjusting for age and sex there was no longer a significant difference between controls and cases. The only significant effects detected at *KvDMR1*, were at sites 4 and 7, where methylation was significantly lower in FAS cases than controls, after adjustment for age and sex. At *PEG3 DMR*, across all CpG sites, estimated methylation was very highly significantly lower (all p-values < 0.001) in FAS than in controls, with and without adjustment for age and sex.

The estimated methylation percentage difference between controls and cases across each locus is summarized in **Table 7** and the observed percentage methylation is shown in **Figure 2**. At the *H19 ICR* locus, cases showed a highly significant increased average methylation compared to the controls, but this was no longer significant after adjusting for age and sex. At *KvDMR1* locus showed a significant lower average methylation after age and sex were adjusted. In the unadjusted analysis, the average methylation was significantly higher (1.15 and 0.75% respectively) in cases than controls, however after adjusting for age and sex the direction of the effect had changed but the reduced methylation was only significant at region B. The *PEG3 DMR* also showed a highly significant difference between cases and controls and the unadjusted ($p < 0.001$) and adjusted ($p < 0.001$) effect sizes were similar (5.47% lower in cases before adjustment and 7.09% lower in cases after adjustment).

DISCUSSION

Epigenetic modulation is increasingly studied as an important mechanism to explain fetal outcome based on environmental exposures during *in utero* development, with some effects lasting into adulthood. This includes maternal diet and exposure to teratogens, like alcohol, but may also include factors like stress. Since imprinted loci play an important role in fetal development, cellular differentiation and growth, we decided to investigate the levels of CpG methylation at four primary DMRs in children with FAS compared to methylation in unaffected controls. Our understanding of the relationship between DNA methylation with regard to sex, age and cell type remains incomplete, but in addition to inter-individual variation, it is clear that there are locus-specific effects. It is therefore expected that teratogens would also display locus-specific effects explaining their impact on fetal outcome. In addition, tissue-specific DNA methylation and tissue-specific epigenetic responses to prenatal alcohol exposure could potentially confound the interpretation of our study as we examined blood and buccal

Table 6 | Summary of estimated differential CpG methylation between FAS cases and controls (FAS-CON), per CpG site.

		Unadjusted			Adjusted for age and sex		
Locus	Site	FAS-CON	SE	*P*-value	FAS-CON	SE	*P*-value
H19 ICR	CpG1	1.80	0.5	<0.001	0.23	0.79	0.767
H19 ICR	CpG2	1.06	0.5	0.035	−0.49	0.79	0.537
H19 ICR	CpG3	1.17	0.5	0.019	−0.42	0.79	0.594
H19 ICR	CpG5	1.48	0.5	0.003	−0.05	0.79	0.950
H19 ICR	CpG6	1.35	0.5	0.007	−0.16	0.79	0.835
IG-DMR.A	CpG1	1.02	0.53	0.054	−0.50	0.81	0.540
IG-DMR.A	CpG2	1.07	0.53	0.043	−0.52	0.81	0.520
IG-DMR.A	CpG3	0.48	0.53	0.364	−1.01	0.81	0.216
IG-DMR.A	CpG4	0.19	0.53	0.724	−1.27	0.81	0.117
IG-DMR.A	CpG5	3.05	0.53	<0.001	1.50	0.81	0.065
IG-DMR.B	CpG6	1.34	0.53	0.012	−0.21	0.81	0.792
IG-DMR.B	CpG7	0.22	0.53	0.679	−1.31	0.81	0.106
IG-DMR.B	CpG8	0.68	0.53	0.202	−0.92	0.81	0.258
IG-DMR.B	CpG9	1.36	0.53	0.010	−0.22	0.81	0.782
IG-DMR.B	CpG10	0.24	0.53	0.646	−1.33	0.81	0.101
KvDMR1	CpG1	0.96	0.53	0.072	−0.53	0.81	0.512
KvDMR1	CpG2	0.28	0.53	0.596	−1.21	0.81	0.138
KvDMR1	CpG3	0.28	0.53	0.595	−1.20	0.81	0.141
KvDMR1	CpG4	−0.17	0.53	0.752	−1.67	0.81	0.040
KvDMR1	CpG5	−0.01	0.53	0.986	−1.43	0.81	0.079
KvDMR1	CpG6	−0.10	0.53	0.851	−1.55	0.81	0.057
KvDMR1	CpG7	−0.60	0.53	0.262	−2.12	0.81	0.009
PEG3 DMR	CpG1	−5.34	0.55	<0.001	−6.98	0.83	<0.001
PEG3 DMR	CpG2	−5.06	0.55	<0.001	−6.69	0.83	<0.001
PEG3 DMR	CpG3	−6.14	0.55	<0.001	−7.73	0.83	<0.001
PEG3 DMR	CpG4	−4.74	0.55	<0.001	−6.43	0.83	<0.001
PEG3 DMR	CpG5	−5.51	0.55	<0.001	−7.07	0.83	<0.001
PEG3 DMR	CpG6	−5.11	0.55	<0.001	−6.67	0.83	<0.001
PEG3 DMR	CpG7	−5.35	0.55	<0.001	−6.96	0.83	<0.001

The analysis was unadjusted and adjusted for age and sex. Both models were adjusted for random variation between and within individuals.

Table 7 | Estimated differences in percentage methylation between cases and controls at each locus.

	Unadjusted			Adjusted for age and sex		
Locus	Effect	SE	*P*-value	Effect	SE	*P*-value
H19 ICR	1.36	0.31	<0.001	−0.17	0.41	0.674
IG-DMR1.A	1.15	0.30	<0.001	−0.40	0.40	0.315
IG-DMR1.B	0.75	0.30	0.012	−0.84	0.40	0.035
KvDMR1	0.01	0.25	0.967	−1.49	0.37	<0.001
PEG3 DMR	−5.47	0.26	<0.001	−7.09	0.37	<0.001

SE, standard error. Significant: $p < 0.05$. Analyses were adjusted for variation between sites and variation between individuals, with and without adjustment for age and sex.

DNA from the participants, rather than neuronal tissue derived DNA.

SEX AND AGE DEMONSTRATE LOCUS SPECIFIC METHYLATION EFFECTS ON SELECTED ICRs

The effect of sex on global DNA methylation and locus-specific methylation has been reported. Global DNA methylation has a tendency toward higher methylation levels in males (Fuke et al., 2004; Shimabukuro et al., 2007). Studies on the effect of sex on locus-specific methylation have shown both increases and decreases in DNA methylation (Sandovici et al., 2005; Sarter et al., 2005; Eckhardt et al., 2006; El-Maarri et al., 2007).

In this study, the effect of sex on methylation was shown to be significant at only one locus, *PEG3 DMR*. Interestingly the effects are modest, but opposite in FAS cases and controls, with the former showing increased methylation (1.11%) in males and the latter a decrease of 0.84% in males. It is not clear why the sex effect on methylation is different in the two groups, but it may be due to the fact that the data were not adjusted for age when the analysis was done because it was done as a baseline comparison to decide if sex needed to be adjusted for in the main analysis. *PEG3 DMR* average methylation was shown to decrease in controls for every 1 year increase in age, suggesting that there may be an age sex interaction at this locus. There was no effect of sex on average methylation at *H19 ICR*, *KvDMR1*, and *IG-DMR*.

Age is reported to cause a reduction in global DNA methylation and causes dramatic changes in the distribution of 5-methylcytosine across the genome (Liu et al., 2011). With respect to specific genes, methylation can either be increased or decreased depending on the gene investigated (as reviewed by Liu et al., 2003). Issa et al. (1996) reported that the *IGF2 P2-P4* promoter-associated CpG island is methylated on the silenced maternal allele in young individuals, however with age this methylation also appears on the paternal allele resulting in biallelic methylation (indicating an overall increase in methylation with age). The promoter regions of many genes tend to switch from an unmethylated to a methylated state resulting in gene silencing

in an age dependent manner. This includes the promoters of several tumor and aging related genes (Wilson and Jones, 1983; Fuke et al., 2004; Liu et al., 2011). The mechanism contributing to the age dependent changes in global methylation includes a decrease in the expression of *DMNT1* (Lopatina et al., 2002; Liu et al., 2003). Longitudinal research on age effects that study the same individuals at several time points is rare (Florath et al., 2014; Flanagan et al., 2015). In two studies DNA methylation of participants was examined at two ages only, one where they were sampled 6 years apart and the other 8 years apart. It is therefore not yet clear whether age-related changes in methylation at CpG loci associated with age effects occur linearly with age.

We examined the effect of age on the different CpG sites and average methylation across each locus, separately in FAS cases and controls. In the control group, with the exception of *PEG3 DMR* CpG7, there was no CpG site specific age effect. In the FAS cases however, eight out of the 10 *IG-DMR* CpG sites, one *KvDMR1* site and one *PEG3 DMR* site showed a significant age effect. With a single exception, methylation in the FAS group decreased by a modest amount for every additional year of age. When examining the locus-averaged methylation and the effect of age, there was a small but significant effect for *KvDMR1*, but a larger effect in the FAS cases for *IG-DMR* (for both region A and B). This effect was not observed in controls. In contrast, the controls showed an age effect at the *PEG3 DMR*. The measure for an age effect is "difference in methylation per additional year of age"; however there was no overlap in absolute age between cases and controls. From our results, it would appear that age effects are more significant at younger ages (1–16 years) than in older age groups (17–26), in a locus-specific manner.

In this study age was shown to influence methylation at three of the four loci investigated. In alignment with our findings, a study on periconceptional famine exposure (Heijmans et al., 2008) found that within the age group of 43–70 years, the DNA methylation at the *IGF2 DMR* of a 10 year older group was associated with a 3.6% lower methylation ($p = 0.015$) in controls. The magnitude (0.36% per annum) of the effect in their study was greater than that observed in our study.

Since both sex and age showed some effect on DNA methylation at one or more of the imprinted loci in this study, we present sex and age adjusted analyses when comparing DNA CpG methylation between FAS cases and unaffected controls.

THE EFFECT OF *IN UTERO* ALCOHOL EXPOSURE ON DNA METHYLATION AT FOUR IMPRINTED LOCI

We assessed the possible effect of maternal alcohol consumption on DNA methylation at *H19 ICR*, *KvDMR1*, *IG-DMR*, and *PEG3 DMR*, by comparing methylation levels between FAS cases and unaffected controls. After adjustment for sex and age there was no observed correlation with *in utero* alcohol exposure at the CpG site level at two of the imprinted loci, *H19 ICR* and *IG-DMR*. Interestingly, a modest effect ($p = 0.035$) of decreased methylation (0.84%) for *IG-DMR* Region B was observed in FAS cases. The *IG-DMR* Region B shows roughly 50% methylation, in line with a parent of origin allelic effect whereas Region A had an overall higher methylation percentage.

The *IG-DMR* is a good candidate in terms of its potential biological impact, in line with the features of FAS. The paternally methylated *IG-DMR* is the primary ICR at the *DLK1/GTL2* (*MEG3*) imprinting domain on human chromosome 14q32, where it plays an essential role in regulating the monoallelic expression of several imprinted genes including the paternally expressed *DLK1* and maternally expressed *GTL2* genes (Lin et al., 2003). The methylation on the paternal allele is essential in maintaining the expression of imprinted genes, because failure to maintain the paternal methylation has been shown to result in considerable *Dlk* repression while *Gtl2* expression is increased (Schmidt et al., 2000).

The *DLK1/GTL2* (*MEG3*) imprinting cluster is a critical region for the phenotypes associated with both maternal and paternal uniparental disomy (UPD) of chromosome 14 (Coveler et al., 2002; Kagami et al., 2005; Temple et al., 2007; Buiting et al., 2008). Maternal uniparental disomy 14 [Upd(14)mat] and hypomethylation at the paternally imprinted *IG-DMR* (Ogata et al., 2008) are characterized by pre- and postnatal growth retardation, developmental delays, mild to moderate mental retardation, muscular hypotonia, small hands and feet, premature puberty and truncal obesity. The locus-averaged methylation of the *IG-DMR* was modestly reduced in FAS cases, tending toward hypomethylation and which may potentially contribute to the growth and neuronal deficits in affected individuals. The magnitude of alcohol effects may be tissue specific and may play a more important role in neurogenesis. These findings merit further study and validation.

After adjustment of sex and age, two *KvDMR1* CpG sites (4 and 7) showed significantly decreased DNA methylation in FAS cases which contributed to a locus-averaged decrease of 1.49% methylation in the *KvDMR1*. The functional impact of this difference is not clear. The biggest effect (a decrease of 7.09% methylation in FAS cases) was observed at the *PEG3 DMR*. Interestingly, it is the two maternally imprinted loci, *KvDMR1* and *PEG3 DMR*, which are significantly affected by *in utero* alcohol exposure and both show a decrease in methylation following alcohol exposure.

One of the key features of FAS is pre- and post-natal growth retardation and dysregulation of imprinting at *H19 ICR* has been associated with growth disorders (Reik et al., 1995; Gicquel et al., 2005; Ideraabdullah et al., 2008). The findings of our study are, however, in agreement with a study done in a mouse model by Haycock and Ramsay (2009) where they reported no difference in methylation at the *H19 ICR* of mouse embryos exposed to alcohol during the preimplantation period, when compared to unexposed control embryos. Interestingly *H19 ICR* hypomethylation was observed in the mouse placentas suggesting a localized effect on the extra-embryonic tissue, which could explain the effect on fetal growth. In two other related studies subtle differential DNA methylation was observed. Knezovich and Ramsay (2012) reported a significant decrease in methylation at the *H19 ICR* in mouse offspring following preconception paternal alcohol exposure and Downing et al. (2011) reported a subtle decrease in methylation at the mouse *Igf2 DMR1* locus in embryos following *in utero* alcohol exposure.

The hypomethylation at *KvDMR1* and *PEG3 DMR* is aligned to our original hypothesis suggesting that alcohol reduces DNA

methylation through the one carbon metabolism pathway and its effect on reducing folate levels. In the next sections the potential implications of hypomethylation at these loci are explored.

THE FUNCTIONAL IMPACT OF REDUCED *KvDMR1* METHYLATION IN FAS CASES IS UNCLEAR

KvDMR1 CpG site-specific and average locus-wide hypomethylation in response to *in utero* alcohol exposure would suggest a loss of methylation on the maternally methylated ICR which regulates the monoallelic expression of several imprinted genes located in the *CDKN1C/KCNQ1OT1* imprinting domain cluster. This imprinting domain harbors the paternally expressed non-coding antisense transcript to *KCNQ1* called *KCNQ1OT1*, and other maternally expressed protein coding genes including *KCNQ1* and *CDKN1C1*. Loss of imprinting, or hypomethylation, at the *KvDMR1* has been widely implicated in the Beckwith–Wiedemann syndrome (BWS; Gaston et al., 2001; Diaz-Meyer et al., 2003; Azzi et al., 2009), a congenital disorder characterized by pre- and postnatal overgrowth, organomegaly, and a high risk of childhood tumors (Weksberg et al., 2010). Paradoxically, the FAS cases showed significant hypomethylation at CpG sites 4 and 7 (1.67 and 2.1%, respectively), yet FAS affected individuals are growth restricted. It is unclear whether hypomethylation of only two of the seven CpG sites in this ICR will affect the levels of expression of the imprinted genes in the cluster and what the functional effect may be.

To gain further insight into gene regulation at this locus will require both gene expression and DNA methylation studies to more fully understand the impact of altered methylation at the *KvDMR1*. This is the first study to show the effect of alcohol on methylation status at *KvDMR1* and the findings are counter intuitive given that hypomethylation is associated with an overgrowth phenotype (BWS).

UNDERSTANDING THE ROLE OF ALCOHOL INDUCED HYPOMETHYLATION AT THE *PEG3* IMPRINTED GENE CLUSTER IN THE PATHOGENESIS OF FAS REQUIRES FURTHER KNOWLEDGE OF THE ICR CONTROLLED GENE EXPRESSION IN THIS REGION

The *PEG3* imprinting cluster is located on human chromosome 19q13.4 and is regulated by a maternally methylated ICR, the *PEG3 DMR*. The cluster includes several imprinted genes including the paternally expressed 3 gene (*PEG3*), the imprinted zinc-finger gene 2 (*ZIM2*) gene and the *USP29* gene, all of which are paternally expressed. Although these loci are syntenic in mouse and human, there are some interesting differences regarding their regulation, their tissue specific expression, and their exon structure and genomic arrangement. The *PEG3* gene is expressed in embryonic tissues, including the hypothalamus and brain, and in adult mouse and human brain, but most highly in human ovary, but not mouse ovary. *PEG3* encodes a DNA binding protein based on its multiple zing finger motifs (Relaix et al., 1996; Iuchi, 2001) and is an imprinted transcription factor that has multiple target genes (Thiaville et al., 2013). It has a proposed tumor suppressive function (Nye et al., 2013) and has been shown to induce *p53*-mediated apoptosis in multiple cell types (Yamaguchi et al., 2002). A mouse knockout model targeting the *Peg3* gene has shown that it is responsible for a variety of phenotypic outcomes including altered maternal offspring rearing behavior, low birth weight, alteration in fat tissue storage and synthesis, and lower metabolic activity (Li et al., 1999; Curley et al., 2004).

We observed that maternal alcohol consumption is correlated with a significant reduction of ~7% methylation at the *PEG3 DMR* in FAS cases. The highly significant decrease in methylation was observed for all the CpG sites analyzed for this locus and also for the average methylation across this locus. It is possible that this change in the *PEG3 ICR* may affect multiple imprinted genes in the region. *PEG3* is expressed from the paternal allele and is reciprocally repressed on the maternal allele, suggesting that alcohol induced demethylation likely affects the maternal allele thus leading to derepression of the *PEG3* gene on the maternal allele, and therefore biallelic expression of *PEG3*. This would lead to an overall increase in *PEG3* expression. Several studies have focussed on the effects of reduced *Peg3*, but none has explored the phenotypic outcome of over expression of *PEG3*.

Gene expression studies, without correlation to their imprinting status, have demonstrated upregulation of *PEG3* (as well as several other genes) in intrauterine growth restriction (IUGR) placentas (reviewed in Ishida and Moore, 2013). Since IUGR is a cause of reduced fetal growth, this study supports our finding that the proposed increase in *PEG3* expression could be associated with a growth restriction phenotype. The role of the *PEG3 DMR* in regulating the imprinted gene cluster in humans requires further investigation.

CONCLUSION

Despite limitations in the study design, including the lack of age matching between cases and controls, the relatively small sample size, and the inaccessibility of neuronal tissue, significant differences in DNA methylation were observed at two primary DMRs when comparing FAS cases with unaffected controls. The observed hypomethylation at the *KvDMR1* has uncertain functional impact on gene expression and the FAS phenotype. The largest epigenetic effect among the loci investigated, was a locus-averaged 7% reduction in DNA methylation at the *PEG3 DMR* which was observed across all seven CpG sites. This ICR orchestrates a complex pattern of gene expression across the region with reported differences in mouse models compared to humans. It is proposed that hypomethylation of the *PEG3 DMR* would result in an increase in the paternally expressed *PEG3* gene. *PEG3* has a DNA binding motif and is considered an imprinted transcription factor, and therefore its function is most likely mediated by altered expression of its targets. Although there is some spatiotemporal congruence of gene expression in line with the developmental origins of the FAS related phenotype, the effect and mechanism of altered expression of *PEG3* and the other imprinted genes controlled by the *PEG3 DMR* remains unclear. Despite the uncertainty of the functional biological mechanism of the locus-specific hypomethylation of important ICRs in the blood of FAS cases, these findings support the role of an epigenetic mechanism in the development of FAS.

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