University of the Witwatersrand

Division of Human Genetics

School of Pathology

Faculty of Health Sciences

METHYLATION PROFILING OF PATERNALLY IMPRINTED LOCI IN MALE GAMETES FOLLOWING ALCOHOL EXPOSURE

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine

DECLARATION

I, Punita Navnitlal Pitamber, hereby declare this dissertation to be my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been previously submitted for any degree or examination at this, or any other university.

Punita N Pitamber

20 APRIL 2012

Date

DEDICATION

To: My Parents

PRESENTATIONS AND PUBLICATIONS

The following presentations arose from this research:

Oral Presentation:

Pitamber, P., Lombard, Z. & Ramsay, M. Comparative sequence analysis of the mouse imprinted gene *Rasgrf1* and its non-imprinted human homolog *RASGRF1*

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Pitamber, P., Lombard, Z. & Ramsay, M. Comparative sequence analysis of the mouse imprinted gene *Rasgrf1* and its non-imprinted human homolog *RASGRF1*

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- Joint African Society of Human Genetics and South African Society of Human Genetics Congress, Cape Town International Convention Center, South Africa (4-9 March 2011)
- Environmental Epigenomics and Disease Susceptibility Keystone Symposium, North Carolina, USA (27 March – 1 April 2011)

Patel S., **Pitamber P.**, Lombard Z. & Ramsay M. Effect of alcohol on global and

locus-specific DNA methylation in spermatozoa: Implications for Fetal Alcohol Spectrum Disorder.

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Publication:

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ABSTRACT

Fetal Alcohol Syndrome (FAS), the most severe form of Fetal Alcohol Spectrum Disorder (FASD), has traditionally been associated with maternal alcohol consumption during pregnancy. However, a number of animal studies have shown an association between paternal preconception alcohol consumption and developmental abnormalities in the offspring that resemble the features of FAS. Dysregulation of epigenetic factors (such as DNA methylation) in the presence of alcohol may provide a plausible mechanism by which paternal alcohol consumption could result in offspring affected with features of FAS. Imprinted genes are expressed in a parent-of-origin manner due to DNA methylation at distinct differentially methylated regions (DMRs) and are essential for normal embryonic development.

There are only two known paternally methylated DMRs in humans, with an additional one described in mice - associated with *Rasgrf1*. The first aim of this study was to determine whether the human *RASGRF1* gene contains a DMR and whether this DMR is paternally methylated. In order to assess the imprint status of *RASGRF1*, a number of computational assessments were done to identify key features of imprinted loci. Pyrosequencing analysis was used to assess the methylation status of various CpG islands surrounding *RASGRF1* in peripheral blood and sperm DNA samples. The *RASGRF1*-associated CpG regions were not found to exhibit differential methylation in a parent-of-origin manner.

The second aim of the study was to examine the effect of paternal alcohol consumption on the methylation status of the IG-DMR locus in male gametes and to determine whether alcohol is correlated with methylation in a dose-dependant manner. Methylation assessment was done using the quantitative pyrosequencing technology. While an overall reduction in methylation was noted in males who consumed alcohol after adjusting for confounding variables, the amount of alcohol consumed did not correlate with overall methylation. When analyzed by individual CpG sites, alcohol consumption was found to correlate preferentially with demethylation at CpG 3 while alcohol-dosage preferentially correlated with demethylation at CpG 7. Age was significantly correlated with an increase in the overall methylation at *IG-DMR* and at individual sites within *IG-DMR*.

In conclusion, these findings support the hypothesis that paternal preconception alcohol consumption can lead to hypomethylation of normally hypermethylated DMRs of specific imprinted genes in human sperm. This in turn could have significant implications with regard to the regulation of developmentally significant genes in the zygote and fetus, resulting in developmental, behavioral and neuro-cognitive disorders.

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ABBREVIATIONS

µl	microlitre
°C	degree Celsius
5-C	5-Carbon position
AS	Angelman Syndrome
bp	base pairs
C	cytosine
CGI	CpG Island
CTCF	CTCF-binding factor
ddH_2O	de-ionised distilled water
df	degrees of freedom
dH_2O	distilled water
DALY	Disability Adjusted Life Years
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	deoxyribonucleic acid triphosphate
DTT	dithiothreitol
FASD	Fetal Alcohol Spectrum Disorder
FAS	Fetal Alcohol Syndrome
G	guanine
ICF	Immunodeficiency, centromeric instability and facial anomaly
ICR	Imprinting Control Region
IUPAC	International Union of Pure and Applied Chemistry
Kb	kilobase
MAT	methionine adenosyltransferase
MEG	maternally expressed gene
min	minutes
mir	micro-RNA
MS	methionine synthase
MTHFR	methylene tetrahydrofolate reductase
ncRNA	non-coding RNA

ng	nanogram
PCR	polymerase chain reaction
PEG	paternally expressed gene
PGC	primordial germ cell
PWS	Prader-Willi Syndrome
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
S-AdoHcyst	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine (*S*-AdoMet)
sec	seconds
SHMT	serine hydroxy methyl transferase
SNP	single nucleotide polymorphism
T	thymine
Ta °C	annealing temperature
THF	tetrahydrofolate
U	uracil
UCSC	University of California, Santa Cruz
V/cm	volts per centimetre
w/v	weight per volume

CHAPTER 1

INTRODUCTION

"It would seem that if alcohol is a vice, then virtue is unattractive, and if drinking is a malady, then good health alone is not enough to satisfy man."

Jean-Charles Sournia (1917-2000)

With the many negative consequences associated with alcohol consumption, which include harm to the unborn fetus through preconception and prenatal alcohol exposure, it is hard to fathom why many people are so easily influenced by the brief satisfaction achieved by consuming alcohol.

1.1 ALCOHOL AND THE ASSOCIATED BURDEN IN SOUTH AFRICA

For many, alcoholism is a disease of the alcoholic and the alcoholic alone. This is untrue. Alcoholism is also a disease of the relative, friend and co-worker and affects society as a whole. This is because the consequences of drinking go far beyond the individual drinker's health and well-being. They include harm to the unborn fetus, domestic violence, sexual assault and child abuse, violent crime, vandalism and reduced productivity at work.

At the Sixtieth World Health Assembly in 2007, the World Health Organization (WHO) ranked alcohol abuse as the fifth leading risk factor for premature death and disability in the world (WHO 2007). In 2002, it was estimated that global alcohol-related disease accounted for 3.7% of total deaths and 4.4% of all Disability-Adjusted Life Years (DALY) (WHO 2007). In 2007, a study was carried out by Schneider et al. to estimate the burden of disease attributable to alcohol use in South Africa in the year 2000. It was discovered that alcohol accounted for 7.1% of total deaths and 7% of all DALYs (Schneider et al. 2007).

1.1.1 THE INFAMOUS "DOP" SYSTEM

The ramifications of immense alcohol consumption, sculpted by the political history of South Africa, are widespread. During the early years of colonial settlement in the Cape Colony, indigenous pastoralists and coastal people were enticed into service on settler farms with payment taking the form of tobacco, bread and wine (Scully 1992 as cited in London 1999). This led to the birth of the infamous "dop" system, which

would over the next 300 years allow farmers to exert social control over their farm workers (Scully 1992 as cited in London 1999).

Fruit, grape and wine production dominate the Western Cape Province of South Africa. For this reason, the farmers in this region distributed alcohol daily to their workers as partial payment for labor (May et al. 2005). Although the "dop" system has been outlawed for over 40 years now, heavy, episodic alcohol consumption remains a major form of recreation in this region. Furthermore, increased availability of commercial alcoholic beverages such as beer, distilled spirits and wine has exacerbated severe drinking (London et al. 1995 and Parry, Bennets 1998 as cited in May et al. 2000).

1.1.2 FETAL ALCOHOL SYNDROME: A MAJOR HEALTH CHALLENGE IN SOUTH AFRICA

A sustained culture of alcohol intake has led to severe consequences for the population residing in the Western Cape Province and nowhere is the effect of excessive alcohol consumption more evident than on the children of this region. This agricultural and wine-producing region has the highest prevalence of Fetal Alcohol Syndrome (FAS) in the world with 68.0 – 89.2 per 1000 children of school-going age being affected (May et al. 2007). This figure has drastically increased from the two previous studies carried out by May et al. (2000) and Viljoen et al. (2005) in the Western Cape Province where the prevalence was found to be 40.5 – 46.4 per 1000 and 65.3 – 74.2 per 1000 children of school-going age, respectively (May et al. 2000, Viljoen et al. 2005). In comparison it is estimated that the rate of FAS in the United States of America ranges between 0.5 – 2.0 per 1000 births (May, Gossage 2001).

The reason for this high FAS burden is not fully understood. It is however known that a large amount of alcohol consumption during pregnancy is a primary risk factor for FAS (May et al. 2008). It has been reported that in the Western Cape, almost 34% of urban women and 46% - 51% of rural women consume alcohol during pregnancy in a binge pattern (Croxford, Viljoen 1999).

1.2 FETAL ALCOHOL SPECTRUM DISORDER

Fetal alcohol spectrum disorders (FASD) is an umbrella term used to encompass a spectrum of structural anomalies and behavioral and neuro-cognitive disabilities caused by prenatal alcohol exposure (Barr, Streissguth 2001). According to the Institute of Medicine's revised classification system, there are currently six recognized diagnoses that form part of the FASD continuum: partial FAS (PFAS) with confirmed maternal alcohol exposure; partial FAS (PFAS) without confirmed maternal alcohol exposure; alcohol-related birth defects (ARBD); alcohol-related neuro-developmental disorder (ARND); FAS without confirmed maternal alcohol exposure; and FAS with confirmed maternal alcohol exposure (Hoyme et al. 2005). The latter of these diagnoses is the most severe form of FASD.

Children born with FAS usually have distinct anomalous features. These include distinct facial dysmorphology (shortened palpebral fissures, smooth philtrum and thin vermilion border of the upper lip), pre- and post-natal growth retardation and mental, behavioral and social deficits (Jones, Smith 1973).

1.2.1 FETAL ALCOHOL SYNDROME: AN HISTORICAL OVERVIEW

An understanding of the teratogenic potential of alcohol dates back many centuries to ancient Roman and Greek mythology, where it was believed that intoxication at the moment of conception could lead to the birth of a defective child (Haggard, Jellinek 1942 as cited in Jones, Smith 1973). However, it was only during England's 'Gin Epidemic' (1720 – 1751) that the ill effects of alcohol consumption on reproduction were first observed. A group of physicians called gin "a cause of weak, feeble, and distempered children" (Warner, Rosett 1975 as cited in Sanders 2009). In a report to the House of Commons it was noted, "Unhappy mothers habituate themselves to these distilled liquors, whose children are born weak and sickly, and often look shrivel'd and old as though they had numbered many years" (George 1966 as cited in Abel 2001). A decrease in fertility and fecundity was also observed during this time (Abel 1998 as cited in Abel 2001). In spite of this, it is only in the last hundred years that concrete evidence has been found linking prenatal alcohol exposure to deleterious effects in offspring (Jones, Smith 1973, Lemoine et al. 1968 as cited in Hoyme et al. 2005, Sullivan 1899 as reviewed in Calhoun, Warren 2007).

In 1899, Dr. W.C. Sullivan undertook the first empirical study linking maternal alcohol consumption to fetal damage. He described offspring born to imprisoned alcoholic women as having a characteristic pattern of birth defects (Sullivan 1899 as cited in Hoyme et al. 2005). He also noticed an increase in the rate of infant mortality and stillbirths when he compared children of alcoholic women to those of controls (Sullivan 1899 as reviewed in Calhoun, Warren 2007). No correlation was observed between these effects and alcohol consumption of the fathers (Sullivan 1899 as cited

in Hoyme et al. 2005). From this study it was deduced that maternal drinking was the primary cause of damage to the fetus (Golden 2005 as reviewed in Calhoun, Warren 2007).

Approximately 70 years later, Lemoine et al. (1968) documented common physical, behavioral and neuro-developmental patterns in children born to mothers who drank heavily during pregnancy. Despite this insight, this study did not present any definitive diagnostic criteria for diagnosing FAS or FASD (Lemoine et al. 1968 as cited in Hoyme et al. 2005).

It was only in 1973 that two dysmorphologists, Jones and Smith, described in detail the consistent pattern of malformations among children affected by *in utero* alcohol exposure (Jones, Smith 1973). These malformations included microcephaly, prenatal and postnatal growth deficiency, developmental delay, short palpebral fissures, epicanthal folds, flattened midface, and altered palmer crease patterns. They also provided diagnostic criteria for the condition which they coined Fetal Alcohol Syndrome (Jones, Smith 1973).

1.2.2 RISK FACTORS FOR FAS

The primary risk factor for FAS is alcohol consumption during pregnancy (Abel, Hannigan 1995). In addition, there must be certain permissive and provocative factors that serve to mediate the effects of alcohol in the fetus, since not all children exposed to alcohol prenatally are diagnosed with FAS (Abel 1984, Abel, Hannigan 1995). The

estimated incidence of FAS among women who drink heavily is 4.3% of all live births (Abel 1995).

Abel and Hannigan (1995) put forth an hypothesis, integrating maternal socio-behavioral risk factors with biological conditions, to explain why only a small proportion of women who consume alcohol during pregnancy give birth to children with FAS (Figure 1.1). There are a number of permissive (socio-behavioral) factors such as excessive alcohol consumption, smoking, low socio-economic status (SES), fewer social resources such as education, income and spirituality and culture that increase fetal vulnerability to alcohol's teratogenic effects (May et al. 2005, Abel, Hannigan 1995, Warren et al. 2001). These external factors are responsible for creating certain internal biological conditions that are "provocative" for FAS such as high blood alcohol concentrations (BAC), under-nutrition or increased stress (Abel, Hannigan 1995, May et al. 2005). The severity of FAS depends upon the interplay between these permissive and provocative factors, triggering a number of cellular events responsible for alcohol-related damage to the fetus - such as the accumulation of free radicals, hypoxia and apoptosis.

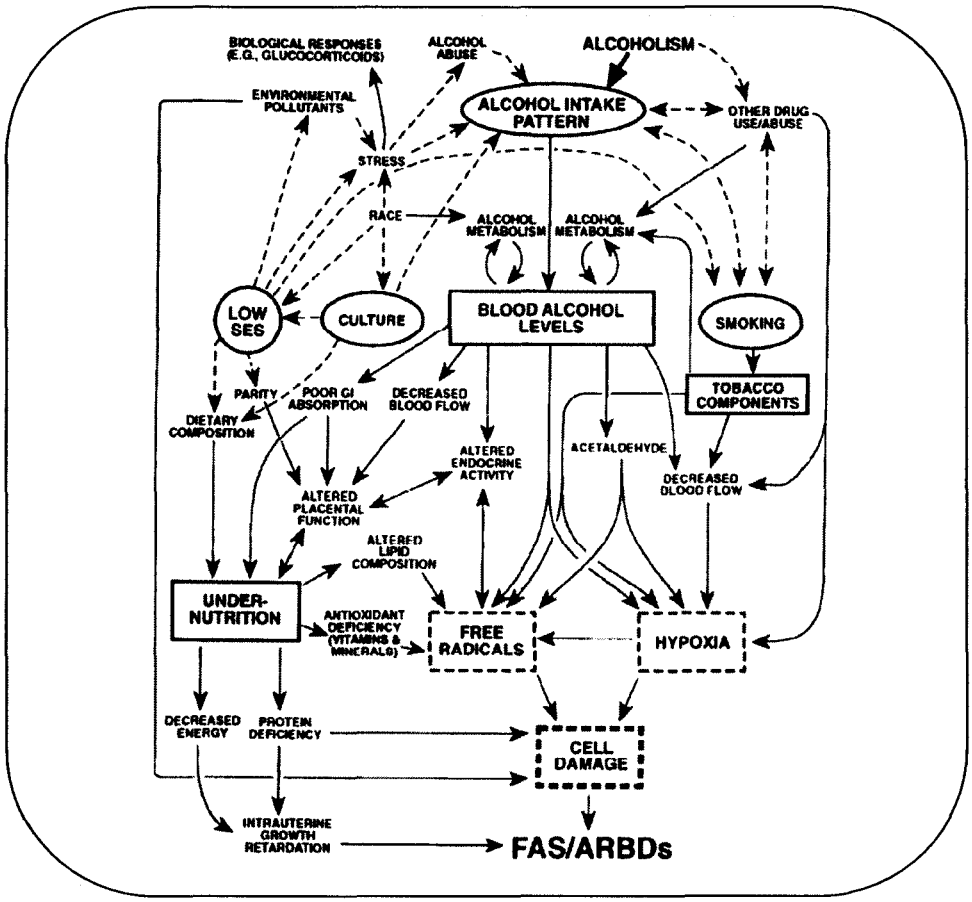

Figure 1.1: Schematic representation of the complex interplay between various permissive and provocative maternal risk factors and the mechanisms responsible for Alcohol-Related Birth Defects (ARBDs), including FAS. Permissive factors are found inside the circles; Provocative factors are found inside solid squares. These risk factors act on the maternal/placental and fetal units via several pathways, which show associations among various environmental, demographic and behavioral variables (dotted-line arrows). Biological relationships and physiological pathways are indicated by solid-line arrows. Obtained from (Abel, Hannigan 1995).

Another set of risk factors that may influence fetal vulnerability to FAS is genetic variation. Animal models, in particular mouse models, have been extensively studied in an effort to elucidate the genetic factors that govern susceptibility to the teratogenic effects of alcohol. The reason for this is that mice exposed to alcohol *in utero* manifest the same full spectrum of FAS anomalies as those observed in affected humans (Becker, Diaz-Granados & Randall 1996).

Chernoff et al. (1980) carried out one of the first animal studies focusing on genetics and prenatal alcohol risk. Diallelic crosses were performed on three mouse strains with each strain differing in their ability to metabolize ethanol. This resulted in the dams of each strain having a different peak blood ethanol concentration, which correlates to alcohol dehydrogenase (ADH) activity. Thus the severity of the teratogenic effects of ethanol was, at least in part, dependant on the genetics of the mother (Chernoff 1980). In addition, Ogawa et al. (2005) demonstrated that fetal genetics also had a part to play in developing FAS. In this study, two mouse strains were exposed *in utero* to the same amount and pattern of alcohol during the same developmental period. This resulted in each strain displaying unique vulnerability in specific organs - suggesting that this differential vulnerability is also attributed by the fetal genetic make-up (Ogawa et al. 2005). A number of other mouse studies have demonstrated similar strain-related differences depending on the frequency, quantity and timing of alcohol exposure (Boehm et al. 1997, Gilliam et al. 1997, Thomas et al. 1998).

In humans, just as in the mouse models, the teratogenic effects of alcohol show much inter-individual variability. A number of studies done in humans have related these differences to polymorphisms in alcohol-metabolizing enzymes such as alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and cytochrome P450 2E1 (CYP2E1) (McCarver 2001, Viljoen et al. 2001, Jacobson et al. 2006). Studies done on twin-pairs have shown higher rates of discordance for FAS in dizygotic twins when compared to monozygotic twins (Streissguth, Dehaene 1993).

The majority of FAS research conducted thus far has focused on maternal and fetal genetic risk factors following maternal alcohol consumption during pregnancy. However, studies have shown that preconception paternal alcohol abuse could result in offspring born with characteristic FAS symptoms. One study reported that 75% of children with FAS were fathered by heavy drinkers or alcoholics (Abel 1993). This suggests that preconception paternal alcohol abuse could result in alcohol-induced alterations in the paternal genome, which could be passed onto the offspring through the male gamete, resulting in FAS-like features.

1.3 RELATIONSHIP BETWEEN PRECONCEPTION PATERNAL ALCOHOL CONSUMPTION AND FASD

There is a growing body of evidence, both from animal and human studies, linking the adverse effects of paternal alcohol abuse to offspring development. While a number of animal studies have indicated an association between preconception paternal alcohol consumption and malformations (Mankes et al. 1982, Abel 1993, Bielawski, Abel 1997), altered organ weights (Abel 1993) and behavioral and developmental anomalies (Abel, Tan 1988, Ledig et al. 1998, Meek et al. 2007), some studies have found no effect of paternal alcohol consumption on fetal growth and development(Randall et al. 1982, Abel, Tan 1988).

A number of studies conducted on humans have investigated the effects of preconception alcohol consumption on offspring and the results are in agreement with those found in animal studies. While some studies have found no association between paternal alcohol consumption and low infant birth weight – one of the attributes

associated with FAS (Savitz et al. 1992, Windham et al. 1995, Passaro et al. 1998) – a number of studies have reported on the negative outcomes observed in children fathered by heavy drinkers. A study conducted by Little et al. (1987) found that infants fathered by regular drinkers were found to weigh significantly less then those fathered by occasional drinkers (Little, Sing 1987). Children of alcoholic fathers have also been found to be more prone to psychiatric disorders including generalized anxiety and a higher rate of substance abuse (Mathew et al. 1993, Malone, Iacono & McGue 2002). There is also some suggestive evidence indicating that children born to alcoholic fathers, rather than having adoptive alcoholic fathers, suffer more often from psychosocial adjustment (Andreas, O'Farrell 2007) and intellectual and cognitive deficit (Hegedus, Alterman & Tarter 1984, Noll et al. 1992, Weinberg 1997, Ozkaragoz, Satz & Noble 1997). This suggests that genetic factors play a crucial role in male-mediated effects on offspring.

It has been postulated that the adverse effects noted in offspring, due to paternal alcohol consumption, could be mediated through direct effects of alcohol on the male gametes (Abel, Moore 1987).

1.3.1 THE EFFECT OF ALCOHOL ON MALE GERM CELLS

Chronic alcohol consumption in men has been associated with infertility (Donnelly et al. 1999). Alcohol is a direct testicular toxin that can cause the atrophy of testes, alter testosterone production, disrupt spermatogenesis and adversely affect sperm concentration, morphology and motility in humans and animals (Van Thiel et al.

1975, Lester, Van Thiel 1977, Pajarinen et al. 1996, Muthusami, Chinnaswamy 2005, Gaur, Talekar & Pathak 2010).

Charles Stockard in 1913 provided the first empirical evidence associating preconception paternal alcohol exposure with "definite injury" to male gametes (Stockard, Papanicolaou 1916). He subjected male guinea pigs to alcohol fumes and noted that these males "beget defective offspring" after being mated with normal vigorous females. These defects, affecting the central nervous system and including gross deformities of the eyes and paralysis of limbs, got progressively worse in each subsequent generation. Stockard suggested that alcohol could potentially alter the chromatin within the male gametes and this could result in the transmission of deformities seen through three generations (Stockard, Papanicolaou 1916).

At present very little evidence exists to support the effects of the teratogenic properties of alcohol on sperm.

A study carried out by Bielawski et al. (2002) was the first to demonstrate the effect of alcohol on sperm cytosine methyltransferase messenger RNA (mRNA) levels. These methyltransferase enzymes are crucial for maintaining correct DNA methylation patterns in imprinted genes (Li, Bestor & Jaenisch 1992). The sperm of alcohol-treated male mice was found to have significantly lower levels of cytosine methyltransferase mRNA. The authors suggested that decreased mRNA levels, following chronic ethanol exposure, are associated with decreased enzyme levels that can subsequently lead to a decrease in DNA methylation levels (hypomethylation). This could result in the expression of paternal alleles that would not normally be

expressed. As a consequence, abnormally high levels of gene products could accumulate, resulting in decreased fetal weight and an increased frequency of fetal runts (Bielawski et al. 2002).

Another such study done by Ouko et al. (2009) in human sperm found chronic alcohol consumption to be associated with modestly decreased methylation levels at specific CpG sites of two normally methylated, male imprinted loci (*H19* and IG-DMR). These loci are involved in regulating the expression of genes that are important for normal embryonic growth and development (Wylie et al. 2000, Gabory, Jammes & Dandolo 2010). The study demonstrated an inverse correlation between the amount of alcohol consumed and the degree of methylation at the IG-DMR locus, with the lowest methylation levels observed in heavy drinkers and the highest methylation levels observed in non-drinkers (Ouko et al. 2009).

Both the above-mentioned studies have shown that chronic alcohol consumption has the ability to alter DNA methylation levels in male germ cells, indicating that this epigenetic modification may be a possible mechanism underlying the paternally mediated effects on FASD.

1.4 EPIGENETIC THEORY

The term epigenetics has been defined a number of times since Conrad Waddington first coined the word. Initially the term was used to explain "the interactions of genes with their environment, which bring the phenotype into being" (Waddington, 1942 as cited in Dolinoy, Weidman & Jirtle 2007). Today epigenetics can be simply defined

as referring to the heritable and transient changes in gene expression that do not entail a change in the DNA sequence but requires an interplay of epigenetic marks such as DNA methylation, histone modifications and non-coding RNAs (ncRNAs) (as reviewed in Reamon-Buettner, Borlak 2007, Hirst, Marra 2009).

The genetic make-up of almost all nucleated somatic cells of a multi-cellular organism is identical. However, during development cells undergo differentiation to generate a diversity of cell types. Each cell type has its own epigenetic signature, which together with the genotype and the environmental influences (i.e. hormone, morphogen or growth factor), gives rise to the phenotype and hence determines the function of the cell type (as reviewed in Morgan et al. 2005, Crews 2008). This process of cellular decision-making during development was metaphorically illustrated by Waddington and referred to as the "epigenetic landscape" (Figure 1.2).

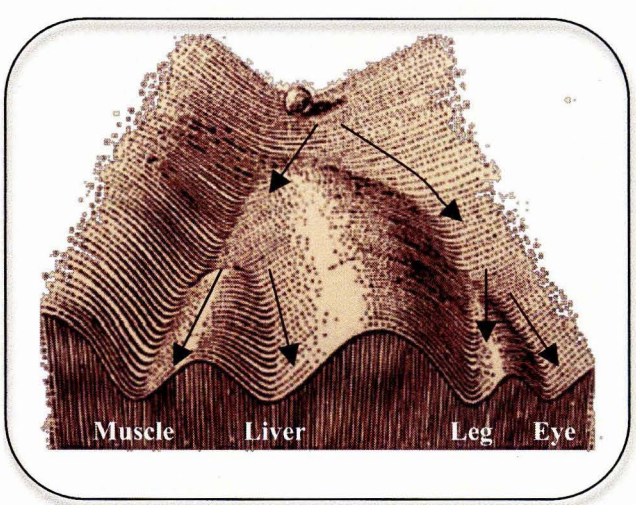

Figure 1.2: Conrad Waddington's illustration of the epigenetic landscape. During development the undifferentiated cell, at the top, travels down a landscape of ridges and valleys where multiple factors influence the cell to travel down a certain course. The fate of the cell is dependant upon the course traveled. Modified from (Goldberg, Allis & Bernstein 2007).

More recently, Waddington's classical epigenetic landscape illustration has been modified to include all known epigenetic factors responsible for regulating gene expression. This is important for generating a diversity of cell types during mammalian development (Figure 1.3).

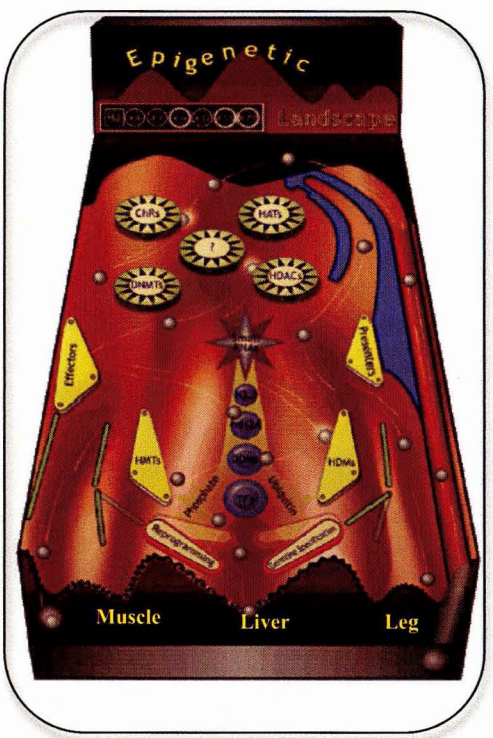

Figure 1.3: A current view of the epigenetic landscape. During cellular development and tissue differentiation, cells will be directed down a certain course by a complex interplay between various epigenetic regulatory factors such as DNA methyltransferases (DNMTs), chromatin remodelers (ChR), histone-modifying proteins (HDACs, histone deacetylases; HATs, histone acetyltransferases; HDMs, histone demethylases) and transcription factors (TFs). Modified from (Goldberg, Allis & Bernstein 2007).

Apart from playing an important role in generating a diversity of cell types during development, epigenetic regulation is also critical in maintaining the stability and integrity of the genome and in the silencing of repetitive transposable elements, thereby inhibiting their transcription (as reviewed in Reamon-Buettner, Borlak 2007, Slotkin, Martienssen 2007). Environmental, toxicological and nutritional factors can

affect the maintenance of epigenetic patterns leading to epigenetic dysregulation (Feil 2006). The latter can lead to neuro-developmental and neuro-degenerative disorders as well as several cancers and autoimmune diseases (Vaissiere et al. 2009, Feng et al. 2008, Huang, Akbarian 2007, Wang, Oelze & Schumacher 2008, Javierre et al. 2010).

There are three epigenetic regulatory mechanisms that form part of the epigenetic regulatory network: DNA methylation, histone modifications and RNA-associated pathways. Since this study focuses on DNA methylation, it will be discussed in more detail in the subsequent sections. Histone proteins are crucial for packaging DNA into nucleosomes. Histones are subject to numerous post-translational modifications of their N-terminal tails including acetylation, phosphorylation, ubiquitination and methylation, all of which can influence gene activity (Kouzarides 2007). Histone acetylation, phosphorylation and ubiquitination are associated with a transcriptionally active chromatin structure while histone deacetylation is associated with an inactive chromatin structure leading to transcriptional repression (Hebbes, Thorne & Crane-Robinson 1988, Nickel, Allis & Davie 1989, Kadosh, Struhl 1998, Nowak, Corces 2000). Histone methylation on the other hand, depending on its position, is either associated with transcriptional repression or activation (Bannister, Kouzarides 2005). Regulatory non-protein-coding RNAs (ncRNAs) are critical for transcriptional and post-transcriptional regulation and in modifying chromatin structure. There are a large number of ncRNAs, each with a different regulatory function. These ncRNAs include small interfering RNAs which are responsible for the silencing of transposable elements (as reviewed in Carthew, Sontheimer 2009), microRNAs that regulate gene-expression in a sequence-specific manner (as reviewed in He, Hannon 2004) and long RNAs which are involved in X-chromosome inactivation and DNA imprinting

(Redrup et al. 2009). Together DNA methylation, histone modifications and ncRNAs all interact to establish cell-type specific epigenomes.

1.4.1 DNA METHYLATION – A KEY EPIGENETIC MARK

DNA methylation is the most widely studied epigenetic regulatory mechanism. It is a covalent modification in which methyl groups are added to the 5-C position of cytosine residues, predominantly within CpG dinucleotides. CpG dinucleotides tend to cluster in regions referred to as CpG islands. Approximately 80% of the CpG dinucleotides in the human genome are methylated (silenced) with the exception of CpG islands (as reviewed in Jaenisch, Bird 2003). CpG islands are generally unmethylated and associated with the 5' end of housekeeping and tissue-specific genes, with the exception of CpG islands at loci that undergo genomic imprinting and X-chromosome inactivation (Shen et al. 2007).

The primary role of DNA methylation is in cellular differentiation and tissue-specific gene regulation during development. Apart from this, DNA methylation is also involved in X-chromosome inactivation, genomic imprinting and in silencing of transposable elements (as reviewed in Bird 2002, Miranda, Jones 2007). DNA methylation is associated with repressed chromatin and inhibition of promoter activity, either directly by preventing the binding of transcription factors to their target sequences or indirectly by the recruitment of methyl-binding proteins and histone deacetylases (Ng, Jeppesen & Bird 2000).

Various factors including carcinogen exposures, inflammation, stress and diet have the ability to alter DNA methylation patterns (Jirtle, Skinner 2007, Christensen et al. 2009, Murgatroyd et al. 2009). Disruptions in normal DNA methylation patterns can result in several human diseases including cancer, a number of imprinting disorders and DNA repeat-instability disorders such as Fragile X mental retardation (as reviewed in Robertson 2005).

1.4.2 DNMT ENZYMES AND DNA METHYLATION

DNA methylation is mediated by DNA methyltransferase (DNMT) enzymes that catalyze the transfer of methyl groups from *S*-adenosylmethionine (SAM), a methyl donor, to cytosine residues of CpG dinucleotides (Bestor 2000). These enzymes are crucial in maintaining the DNA methylation patterns following DNA replication thereby stabilizing the cellular identity of daughter cells. There are four known DNMT enzymes: DNMT1; DNMT3A; DNMT3B and DNMT3L but only the first three display catalytic activity. DNMT1 plays a role in the maintenance of parental methylation patterns in daughter cells following DNA replication (Leonhardt et al. 1992). DNMT3A and DNMT3B are referred to as *de novo* methyltransferases and are responsible for establishing methylation patterns during embryogenesis (Okano et al. 1999). The third member of the DNMT3 family, DNMT3L, lacks catalytic activity but is capable of establishing methylation imprints in male and female germ-lines during gametogenesis through interactions with the other DNMT3 enzymes (Bourc'his et al. 2001, Kato et al. 2007).

A number of mouse studies have been done to determine the importance of DNMT enzymes in development. *Dnmt1* null mice exhibited genome-wide hypomethylation and did not survive past embryonic development (Li, Bestor & Jaenisch 1992). In comparison, *Dnmt3a* null mice developed normally but became runted and died approximately 4 weeks after birth while *Dnmt3b* null mice were not viable due to multiple developmental defects. *Dnmt3a* and *Dnmt3b* double mutant mice displayed abnormal morphology and died shortly after gastrulation (Okano et al. 1999). Lastly, the *Dnmt3l* null mice exhibited hypomethylation of specific imprinted regions in oocytes and male sterility due to a loss of spermatogonia (Bourc'his et al. 2001, La Salle et al. 2007). In humans there is only one known genetic disease associated with a DNMT enzyme – Immunodeficiency, Centromeric instability and Facial anomalies (ICF) syndrome. The majority of the patients presenting with ICF have mutations in the *DNMT3B* gene that leads to hypomethylation of specific regions of the genome (Hansen et al. 1999, Kondo et al. 2000, Tao et al. 2002). Apart from this, these patients present with impaired cellular immunity, facial anomalies, mental retardation and numerous cytogenetic abnormalities primarily involving the juxtacentromeric heterochromatin (Jin et al. 2008).

Thus it can be seen that these enzymes are crucial for generating and maintaining epigenetic control of gene expression during embryogenesis, gametogenesis and genomic imprinting.

1.5 GENOMIC IMPRINTING

The discovery of genomic imprinting in mammals occurred in the mid-1980s following nuclear transplantation studies conducted in mice. Diploid androgenotes derived from two male pronuclei and diploid gynogenotes derived from two female pronuclei failed to develop beyond mid-gestation, thereby suggesting that diploidy alone is not sufficient for normal embryonic growth and development (Barton, Surani & Norris 1984, Surani, Barton & Norris 1984). It was proposed that the maternal and paternal genomic contributions to the embryo are functionally different with certain genes being inherited in a functional form from one parent, and a non-functional form from the other (McGrath, Solter 1984). The mono-allelic behavior of these genes, now referred to as imprinted genes, is dependent on the parental origin of the gene (as reviewed in Jirtle, Skinner 2007).

1.5.1 THE EVOLUTIONARY THEORIES OF IMPRINTING

Almost all placental mammals studied to date have retained imprinted genes in their genomes (Jirtle, Weidman 2007). Imprinted genes are effectively haploid and therefore confer a selective disadvantage to mammals, making them vulnerable to recessive mutations and epigenetic modifications. Although several hypotheses have been put forth explaining this paradox, only two of the more plausible hypotheses will be discussed here.

The ovarian time bomb hypothesis proposes that genomic imprinting may have evolved to prevent the spontaneous development of unfertilized oocytes (a process also known as parthenogenesis), which could lead to ovarian trophoblastic disease

(Weisstein, Feldman & Spencer 2002). Therefore inactivating the maternal copy of the growth-enhancing gene in the embryo reduces this risk while the addition of a single paternal copy of the growth-inhibitor to the embryo creates a balanced environment for successful embryogenesis (as reviewed in Morison, Ramsay & Spencer 2005). It is possible that the action of imprinted genes in preventing parthenogenesis has certain advantages in mammalian development.

The most widely accepted theory for the evolution of imprinting is the conflict hypothesis. This theory states that there are conflicting interests between the males and females over the allocation of maternal resources during the growth and development of offspring, especially in the event of multiple paternities (Moore, Haig 1991). Therefore if a father is to ensure that his genome is most efficiently propagated, his germ-line will have to imprint genes in a manner that will promote the growth and survival of his offspring by demanding more nutrient resources from the mother (as reviewed in Tycko, Morison 2002). Conversely, if the mother is to have her genome propagated successfully her genes need to be imprinted such that her resources are equally allocated to all her current and future offspring, regardless of paternity (Das, Hampton & Jirtle 2009). Evidence for this theory comes from mice with germ-line knockouts of *Igf2* and *Igf2r* imprinted genes. The *Igf2* gene encodes an embryonic growth factor and is expressed from the paternal chromosome only, while the *Igf2r* gene is expressed from the maternal chromosome only and encodes a receptor that binds and transports the Igf2 protein to lysosomes (DeChiara, Robertson & Efstratiadis 1991, Lau et al. 1994). A study carried out by DeChiara et al. (1991) demonstrated that a loss of function of *Igf2* resulted in growth deficient offspring (DeChiara, Robertson & Efstratiadis 1991). On the contrary, mutations of the

maternal *Igf2r* gene resulted in embryonic over-growth and perinatal lethality (Lau et al. 1994). The resulting aberrant prenatal growth seen in both knockouts demonstrates the balance required between maternal and paternal genomes for the regulation of normal embryonic growth.

1.5.2 IMPRINTED GENES

To date approximately 100 imprinted genes have been identified in mice (http://www.har.mrc.ac.uk/research/genomic_imprinting/) and only 80 identified in humans (Cooper, Constancia 2010). While the majority of the human imprinted genes have mouse orthologs and share a conserved imprint status, some imprinted genes in the mouse have human orthologs that fail to exhibit an imprint status (as reviewed in Morison, Ramsay & Spencer 2005). Many of these genes play a vital role in embryonic growth and regulation of cellular proliferation (http://www.har.mrc.ac.uk/research/genomic_imprinting/), while others influence behavior and neuro-developmental processes (as reviewed in Wilkinson, Davies & Isles 2007).

Imprinted genes are regulated by DNA methylation (Li, Beard & Jaenisch 1993). Allele-specific DNA methylation is confined to differentially methylated regions (DMRs) and these are found in close proximity to imprinted genes. There are two classes of DMRs: primary DMRs (also referred to as Imprinting Control Regions [ICRs]), which acquire gamete-specific methylation during spermatogenesis or oogenesis and secondary DMRs, which acquire differential methylation patterns following fertilization (Kobayashi et al. 2006). To date only 18 known maternally

methylated primary DMRs have been identified in mice and only four known paternally methylated primary DMRs (Smith et al. 2003, Kobayashi et al. 2006, Wood et al. 2007, Hiura et al. 2010). Of the four paternally methylated DMRs found in mice, only three are known to share an imprint status with human orthologs: the *H19/IGF2* DMR, the *DLK1/GTL2* locus DMR (known as IG-DMR) and the *GPR1/ZDBF2* locus DMR (Davis et al. 1999, Geuns et al. 2007, Kobayashi et al. 2009b). These DMRs are all intergenic. The additional mouse locus displaying a paternal-specific DMR, *Rasgrf1*, has a human ortholog but the imprint status thereof is unknown (de la Puente et al. 2002).

Poorly established DNA methylation patterns of imprinted genes have been the cause of a number of behavioral and developmental disorders such as Prader-Willi syndrome (PWS), Angelman syndrome (AS) and Beckwith-Weidemann syndrome (Buiting et al. 1995, Brown et al. 1996).

1.5.3 EPIGENETIC REPROGRAMMING OF IMPRINTED GENES

There are two critical periods in mammalian development when DNA methylation undergoes genome-wide reprogramming (Figure 1.4). This reprogramming is not only crucial in erasing and re-establishing the correct epigenetic marks during embryonic development but also in restoring the totipotency of the fertilized egg (as reviewed in Reik, Dean & Walter 2001).

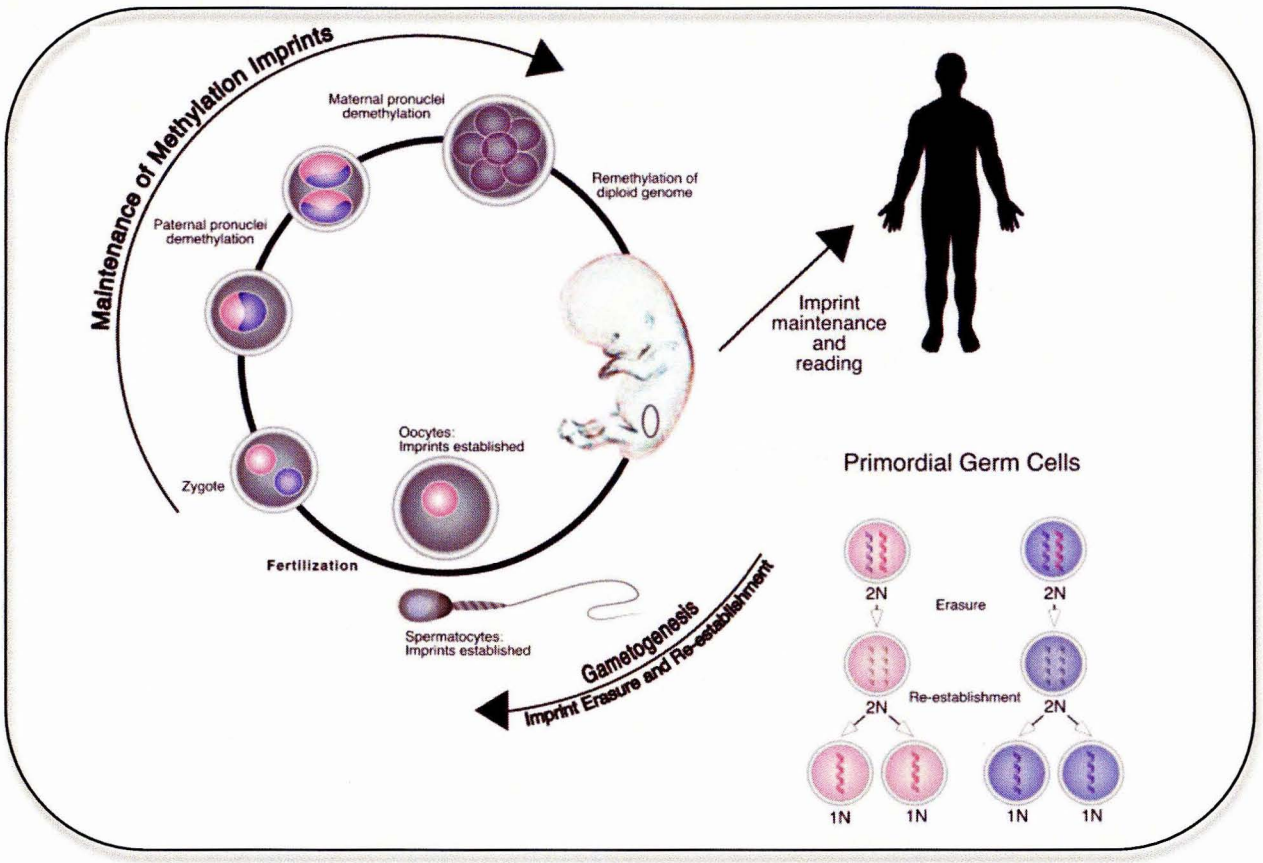

Figure 1.4: Epigenetic reprogramming in mammalian development Obtained from (Murphy, Jirtle 2003).

The first wave of demethylation occurs in early embryogenesis when the maternal and paternal genomes come together to form a zygote. Upon fertilization, the paternal genome undergoes chromatin remodeling whereby protamines are replaced by histones, this is followed by rapid and active demethylation that occurs prior to DNA replication. During this time the maternal genome completes meiosis and undergoes passive demethylation in a replication-dependant manner (as reviewed in Morgan et al. 2005). The methylation marks on imprinted genes resist this wave of global demethylation and maintain their parent-of-origin specific methylation patterns throughout development (as reviewed in Guibert, Forné & Weber 2009). Around the time of implantation, the embryo undergoes a wave of *de novo* methylation in a

lineage-specific manner (Kafri et al. 1992). These methylation patterns are then faithfully maintained throughout the lifespan of the individual.

The second wave of demethylation occurs during gametogenesis in the primordial germ cells (PGCs) of the embryo. This coincides with the migration of PGCs into the genital ridge (Hajkova et al. 2002). This particular wave of global demethylation includes the erasure of imprinting marks. Upon sex determination, methylation patterns are re-established both globally and at imprinted loci where the methylation of the imprints is dependant on the sex of the offspring (as reviewed in Murphy, Jirtle 2003). This germ-line epigenetic reprogramming is not only important for resetting of parental imprints for the next generation but is also equally important in preventing epimutations from being passed onto the next generation.

The gonadal sex determination stage in embryonic development is most sensitive to environmental insults that could alter the epigenome of the gamete. This together with incomplete erasure and re-establishment of epigenetic marks in the germ-line could allow for the transmission of incorrect epigenetic signatures to the subsequent generation (as reviewed in Skinner, Guerrero-Bosagna 2009).

1.6 THE EFFECT OF ETHANOL ON ONE-CARBON METABOLISM

Folate, a member of the B-complex vitamins, is a cofactor in one carbon transfer. It not only plays a key role in nucleic acid biosynthesis but is also responsible for maintaining DNA methylation in conjunction with another cofactor, *S*-adenosylmethionine (SAM) (as reviewed in Appling 1991, Hitchler, Domann 2007).

SAM plays a role in DNA and histone methylation reactions by acting as a methyl group donor (Hitchler, Domann 2007). Both these co-factors are biochemically linked via the folate and methionine cycles (Figure 1.5).

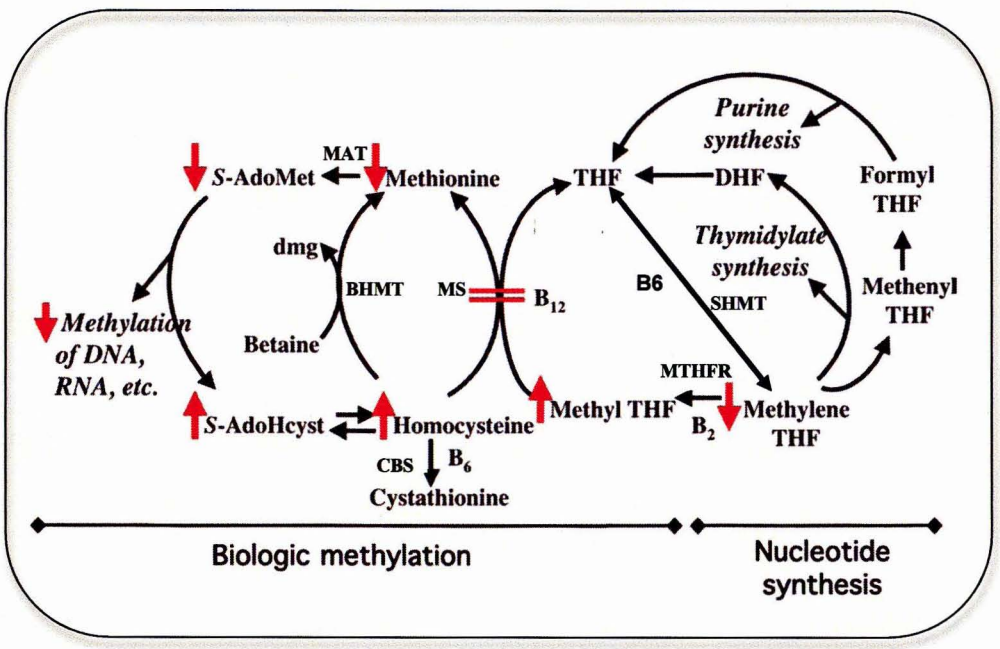

Figure 1.5: Schematic representation of the biochemical pathway of one-carbon metabolism. Shown here are the intricate interactions between the folate and methionine cycles. Two key enzymes, MS and MTHFR, link these two cycles. Modified from (Mason, Choi 2005). The red arrows indicate the effects that ethanol consumption can have on each substrate. DHF = Dihydrofolate; dmg = dimethylglycine; *S*-AdoHcyst = *S*-adenosylhomocysteine; *S*-AdoMet (SAM) = *S*-adenosylmethionine; THF = tetrahydrofolate; MS = methionine synthase; MAT = methionine adenosyltransferase; BHMT = betaine:homocysteine methyltransferase; MTHFR = methylenetetrahydrofolate reductase; SHMT = serine hydroxymethyltransferase; CBS = cystathionine β-synthase.

Intracellularly, folate as 5-methyl THF functions as a methyl donor for homocysteine remethylation. The resulting THF is converted to methylene THF by SHMT, which donates a methyl group to uracil converting it to thymidine. This is used for DNA synthesis and repair (as reviewed in Hamid, Wani & Kaur 2009). The MTHFR enzyme has the ability to reduce methylene THF to 5-methyl THF donating a methyl

group to homocysteine to produce methionine. The B12-dependant MS enzyme catalyzes this conversion of homocysteine to methionine. Hereafter, MAT catalyses the addition of ATP to methionine, generating *S*-AdoMet. *S*-AdoMet then serves as a universal donor of methyl groups in various methylation reactions (Schalinske, Nieman 2005).

Mammals cannot synthesize folate and thus must obtain the vitamin from exogenous nutritional sources via intestinal absorption (Balamurugan, Said 2006). Chronic ethanol consumption has a multifaceted impact on the bioavailability of dietary folate and subsequent metabolism of folate and methionine (Mason, Choi 2005). Excessive ethanol consumption has been reported to interfere with the absorption of dietary folate (Halsted, Robles & Mezey 1973, Halsted et al. 2002). This can have an indirect impact on folate and methionine metabolism. However, ethanol can also have a direct impact on one-carbon metabolism (Figure 1.5). It has the ability to inhibit the reaction catalyzed by MS. This will lead to the subsequent decrease of downstream products such as methionine and *S*-AdoMet and an increase of some of the precursors of the reaction such as homocysteine and *S*-AdoH-cyst. *S*-AdoH-cyst is a potent inhibitor of methyltransferase enzymes – enzymes that are crucial for maintaining the correct DNA methylation patterns. An increase in *S*-AdoH-cyst can result in a considerable degree of genomic hypomethylation (Lu et al. 2000, Castro et al. 2005). In addition, inhibition of MS by ethanol results in an increase of 5-methyl THF, which cannot be catalyzed into THF resulting in a loss of methylene-, methenyl- and formyl THF. This loss leads to defective DNA synthesis and repair (Mason, Choi 2005).

Disruption in the folate and methionine metabolism can lead to several human diseases including several types of cancers, neural tube defects and neuro-degenerative diseases (van Engeland et al. 2003, Pitkin 2007, Flatley et al. 2009, Naj et al. 2010).

1.7 A PLAUSIBLE EPIGENETIC MECHANISM FOR FAS – FROM A PATERNAL PERSPECTIVE

The first paper to demonstrate the link between the long-term effects of preconception and prenatal alcohol exposure and epigenetic changes established in the fetus was only recently published (Kaminen-Ahola et al. 2010). In this study the researchers used a mouse model demonstrating that maternal ethanol consumption, prior to or following fertilization, has the ability to alter the expression of A^{vy}, an epigenetically sensitive allele, in the offspring. The resulting phenotypes were comparable to the FASD phenotypes, which included a small skull size, differences in the shape of the skull and postnatal growth restriction (Kaminen-Ahola et al. 2010). These results display the direct relationship between maternal alcohol consumption before and during pregnancy and changes in DNA methylation in the offspring. It is known that DNA methylation plays a crucial role in regulating the expression of imprinted genes during embryogenesis and that environmental insults (such as alcohol) during this time can promote developmental abnormalities as seen in offspring affected with FAS (Skinner, Manikkam & Guerrero-Bosagna 2010).

However, paternal preconception alcohol consumption can also have an impact on the development of the offspring – indirectly through the male gametes. It is known that

alcohol consumption has a negative impact on sperm cytosine methyltransferase mRNA levels possibly through the effect of alcohol on one-carbon metabolism (Bielawski et al. 2002). This leads to a decrease in DNA methylation that could result in the expression of paternal alleles that would not normally be expressed (Bielawski et al. 2002, Ouko et al. 2009). Could this alteration in gene expression be transmitted to the offspring and result in early life origins of disease? A study done by Knezovich and Ramsay (awaiting publication) on preconception paternal alcohol exposure on three paternally imprinted loci (*H19, Rasgrf1* and Ig-DMR) did not find any of these loci to be significantly demethylated in the sperm samples of the alcohol-exposed males. However, the offspring sired by alcohol treated males had significantly lower weights during the weaning period when compared to the controls and this was accompanied by significant reductions in DNA methylation at the *H19* ICR in the former. The lack of demethylation in sperm samples due to alcohol exposure and the significant reduction in methylation at the same loci in the offspring suggests that alcohol could have potentially induced the oxidation of 5-methylcytosine (5-mc) to form 5-hyroxymethylation (5-hmc) – an intermediary modification found at the stage between demethylation in gametes and remethylation of differentiated cells in embryos. This 5-hmc is found at high levels in the male pronucleus, Purkinje cells and embryonic stem cells (Wossidlo et al. 2011). This intermediary in the male sperm could then later manifest as demethylated DNA in the offspring. Another study looking at the impact of diet on offspring development, done by Ng et al. (2010), demonstrated that a father's high-fat diet resulted in a diabetes-like condition only in his daughters. The paternal high-fat diet resulted in the altered expression of numerous genes belonging to regulatory pathways involved in insulin and glucose metabolism in the offspring. This study suggested that diet could have an impact on

spermatogenesis and on the epigenetic reprogramming of the germ-line (Ng et al. 2010). These two studies demonstrate the link between a paternal environmental exposure and a negative outcome in the offspring through altered DNA methylation levels.

1.8 STUDY HYPOTHESIS

Hypothesis: Alcohol consumption induces hypomethylation of normally hypermethylated DMRs, associated with paternally imprinted genes, in the sperm of men who consume alcohol. This alteration in methylation occurs in a dose-dependant manner such that men with higher drinking frequencies display a higher level of hypomethylation compared to men with lower drinking frequencies.

Rationale: Alcohol is a direct testicular toxin and is known to disrupt spermatogenesis. Alcohol is also known to affect one-carbon metabolism by decreasing *S*-AdoMet, the methyl donor and increasing *S*-AdoH-cyst, a potent inhibitor of methyltransferase enzymes. Thus alcohol could have a negative impact on spermatogenesis resulting in the hypomethylation of DMRs and subsequent expression of paternally imprinted loci in the sperm. This could have significant implications with regard to the regulation of developmentally significant genes in the zygote and fetus resulting in developmental, behavioral and neuro-cognitive disorders.

1.9 AIM OF THE STUDY

The current study had two aims.

The first aim of the study was to determine whether the human *RASGRF1* gene contains a DMR and whether this DMR is paternally methylated.

The second aim of the study was to examine the effect of paternal alcohol consumption on the methylation status of the *RASGRF1* DMR (if one was identified) and the IG-DMR loci in male gametes and to determine whether alcohol affects methylation in a dose-dependant manner. While *RASGRF1* is a developmentally significant gene, IG-DMR is a key regulator of the *DLK1/GTL2* imprinted gene cluster. Therefore if a link between alcohol consumption in men and hypomethylation of normally hypermethylated loci is found, this will allow for a better understanding of the possible epigenetic mechanism underlying the paternally mediated effects on FASD.

1.9.1 OBJECTIVES OF THE STUDY

Objective 1: To determine the methylation status of *RASGRF1* in human sperm and peripheral blood samples.

Objective 2: To quantitatively assess the overall methylation status of the *RASGRF1* DMR (if one was identified) and IG-DMR and to assess the methylation status of

each individual CpG site within the respective DMRs, following alcohol consumption.

Objective 3: To quantitatively assess the effect of drinking frequency on the overall methylation status of the *RASGRF1* DMR (if one was identified) and IG-DMR and to assess the methylation status of each individual CpG site within the respective DMRs.

The objectives will be discussed separately with Chapter 2 dedicated to *RASGRF1* and Chapter 3 dedicated to IG-DMR.

CHAPTER 2

IN SEARCH OF A PATERNALLY METHYLATED DMR FOR HUMAN *RASGRF1*

2.1 INTRODUCTION

2.1.1 COMMON FEATURES OF IMPRINTED GENES

The differential marking of imprinted genes at the primary DMRs (also known as ICRs) in a parent-of-origin manner is a key feature of all imprinted genes. Most ICRs overlap with CpG islands and the imprints at these regions are established during gametogenesis and are maintained throughout development (Hutter, Helms & Paulsen 2006, Lim, Ferguson-Smith 2010). Differential marking of imprinted genes can also occur at secondary DMRs following fertilization. The establishment of the secondary DMRs is thought to be set up by the ICRs (Lopes et al. 2003). Although DNA methylation at the ICR is key for the reciprocal expression of these genes, differences in histone modifications at the ICR of the two parental chromosomes are also found to contribute to the reciprocal expression profiles (as reviewed in Kacem, Feil 2009). Histone H3 di- and tri-methylation at lysine 9 (H3K9me2/3) and histone H3 di- and tri-methylation at lysine 27 (H3K27me2/3) are found to be preferentially enriched on the methylated allele, while histone H3 acetylation, H4 acetylation and histone H3 di- and tri-methylation at lysine 4 (H3K4me2/3) are found to be enriched on the unmethylated allele (Fournier et al. 2002, Umlauf et al. 2004, Barski et al. 2007). However, it is important to note that DNA methylation is the most consistent hallmark of imprinting and is crucial for establishing and maintaining imprints (Delaval, Feil 2004).

The frequent presence of tandem repeats, simple repeats and long interspersed nuclear elements (LINEs) in the vicinity of imprinted regions is another common feature of imprinted genes (Allen et al. 2003, Hutter, Helms & Paulsen 2006). Tandem repeat

elements tend to occur more frequently within or adjacent to CpG islands associated with imprinted genes than they do with CpG islands of biallelically expressed genes (Hutter, Helms & Paulsen 2006). It is thought that these repeat elements play an essential role in regulating the establishment of methylation of the adjacent ICR, in addition they are involved in various epigenetic silencing and heterochromatin formation processes (Volpe et al. 2002, Reinhart, Paoloni-Giacobino & Chaillet 2006, Brideau et al. 2010).

Transcription factors and chromatin insulators, Yin-Yang 1 (YY1) and CTCF, present within the ICR have prominent roles in the epigenetic modifications and transcriptional control of associated imprinted genes (Filippova et al. 1996, Kim et al. 2006). However, it should be noted that these factors are not exclusively located within ICRs. YY1 binding sites are found in many heterochromatic regions throughout the genome while CTCF-binding sites are ubiquitous throughout the genome (Kim T.H. et al. 2007, Kim 2008).

Having listed all the common features of imprinted genes, it is the ICR that is the key feature of imprinted genes due to its regulatory function. It is the methylation status of the ICR that has direct influence on the expression of genes at the locus (Yoon et al. 2002).

2.1.2 THE IMPORTANCE OF IMPRINTING CONTROL REGIONS IN NORMAL DEVELOPMENT

A number of studies have emphasized the role of the ICR in establishing and maintaining imprints. A mouse study done by Williamson et al. (2006) focused on the

targeted deletion of the *Nespas* ICR at the complex *Gnas* cluster. Upon inheriting the deletion on the paternal allele, offspring did not survive past postnatal day two. This was ascribed to the altered expression of all transcripts in the *Gnas* cluster and altered methylation of the *Nesp* DMR and the *Exon1A* DMR (Williamson et al. 2006). Another such study demonstrated the effect of deleting the ICR that regulated the *H19/Igf2* locus in mice. Under normal circumstance, *H19* is hypermethylated on the paternal allele while *Igf2* is hypermethylated on the maternal allele. Targeted deletion of the ICR in mice has shown that when the ICR deletion is paternally inherited *H19* is activated and *Igf2* is silenced. The opposite is true when the deletion is maternally inherited where the *H19* locus is silenced and *Igf2* is expressed (Thorvaldsen, Duran & Bartolomei 1998). In humans, there exists a large imprinted domain on chromosome 15q11-13. This region houses a number of maternally and paternally expressed genes and ncRNAs that are regulated by a bipartite ICR (Prader-Willi syndrome [PWS]-ICR and Angelman syndrome [AS]-ICR). Microdeletions on the paternally inherited PWS-ICR result in the silencing of the paternally expressed genes resulting in PWS syndrome while microdeletions on the maternally inherited AS-ICR results in AS (Buiting et al. 1995, as reviewed in Edwards, Ferguson-Smith 2007).

One would expect these important regulatory elements, that establish and maintain gene imprints, to be conserved between various species. However, studies have shown that some genes display discordant imprinting between the mouse and human genomes (Li et al. 2005, Monk et al. 2006).

2.1.3 FEATURES OF THE PATERNALLY IMPRINTED *RASGRF1* IN MICE

Rasgrf1 (*Ras protein-specific guanine nucleotide-releasing factor 1)* forms part of the MAPK signaling pathway and has been found to play a role in long-term memory consolidation and growth control (Brambilla et al. 1997, Itier et al. 1998). *Rasgrf1,* found on mouse chromosome 9 [NCBI Gene ID: 19417], contains a paternally imprinted DMR and is exclusively expressed from the paternal allele in the neonatal brain and liver (Dockery et al. 2009). A binary switch located 30 kb upstream of the *Rasgrf1* promoter is responsible for controlling the imprint status of the gene in mice (Figure 2.1). The first component of this binary switch is a repeat unit consisting of forty copies of a 41 bp repeat element, which is responsible for establishing methylation of the second component of the switch, the DMR (Holmes, Chang & Soloway 2006). This DMR serves as a CCCTC-binding factor (CTCF) binding site on the unmethylated maternal allele. Binding of a CTCF protein blocks enhancer-to-promoter interactions resulting in the silencing of the maternal allele of *Rasgrf1*. On the paternal allele, the CTCF-binding factor is unable to bind to the methylated DMR resulting in expression of *Rasgrf1* from the paternal allele (Yoon et al. 2005).

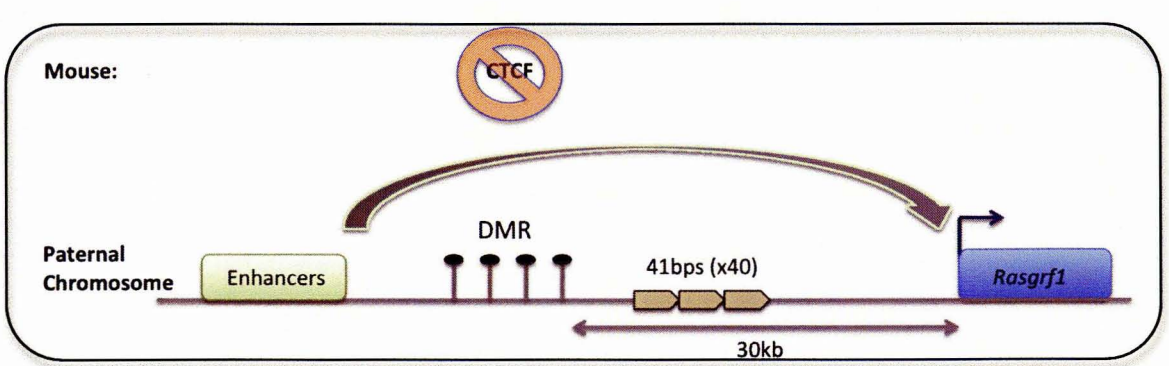

Figure 2.1: A schematic representation of a binary switch located upstream of *Rasgrf1*. Shown here is the paternal allele with a methylated DMR (indicated by the filled in lollipops), which prevents the CTCF binding factor from binding to the DMR thereby allowing promoter-to-enhancer interactions.

The mouse DMR is a primary DMR established in the male germ-line (Yoon et al. 2002). It is located between *Rasgrf1* and a brain-specific micro-RNA (*Mir*-184) that is imprinted and expressed from the paternal allele in the brain (Figure 2.2) (Nomura et al. 2008). Both these genes have human homologs and they are transcribed in opposite directions. The mouse and human genomes display conserved synteny of *Rasgrf1* and *Mir*-184.

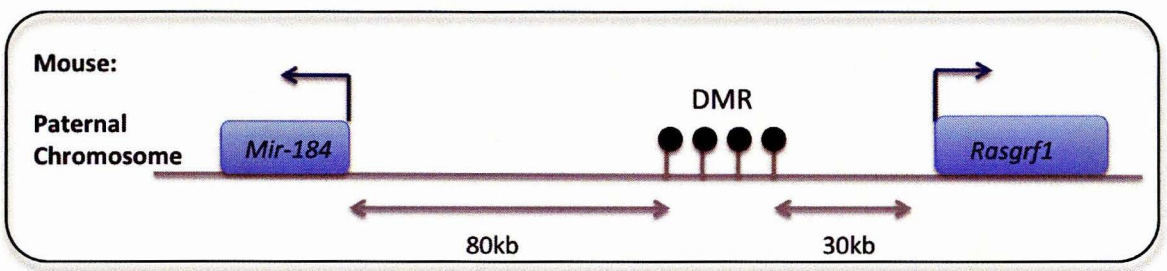

Figure 2.2: A schematic representation of the position of the mouse DMR relative to *Rasgrf1* and the *Mir*-184.

The human ortholog of *Rasgrf1* is found on chromosome 15 [NCBI Gene ID: 5923] but its imprint status is unknown. The reason for focusing on this gene is that studies conducted by Brambilla et al. (1997) have shown that mice lacking the Rasgrf1 protein demonstrated severely impaired memory consolidation (Brambilla et al. 1997). Another study done in mice by Itier et al. (1998) observed that mice lacking the *Rasgrf1* gene suffered from postnatal growth retardation, which persisted into adulthood (Itier et al. 1998). The phenotypes observed in both these studies are similar to those seen in offspring affected by FASD. Therefore if this gene is found to be paternally imprinted in humans and if a link between alcohol consumption in men and hypomethylation of the normally hypermethylated allele is found, this will allow

for a better understanding of the possible epigenetic mechanism underlying the paternally mediated effects on FASD.

Therefore the aim of this part of the study was to determine whether the human *RASGRF1* gene contains a DMR and whether this DMR is paternally methylated. In order to assess the imprint status of *RASGRF1*, a number of computational assessments were done to identify key imprinting features, while pyrosequencing analysis was used to assess the methylation status of various CpG islands surrounding *RASGRF1*.

2.2 SUBJECTS AND METHODOLOGY

2.2.1 COMPUTATIONAL ASSESSMENT

2.2.1.1 SEARCHING FOR SEQUENCE SIMILARITY

The BLAST-like Alignment Tool (BLAT) was used to identify sequence similarity using the mouse DMR [NCBI Reference Sequence: NT_039476.7] and the 40 copies of the 41 bp repeat element unit as query sequences and comparing them against the human genome on Ensembl. The Ensembl database can be found at http://www.ensembl.org/index.html.

2.2.1.2 IDENTIFICATION OF CTCF BINDING SITES

Knowing that the mouse *Rasgrf1* DMR is regulated by a CTCF-binding protein (Yoon et al. 2005), a starting point for assessment was to identify all CTCF binding sites

within human *RASGRF1* and between *RASGRF1* and the *MIR*-184. The *in silico* CTCF Binding Site Database (CTCFBSDB) (http://insulatordb.uthsc.edu/storm.php) contains both experimentally identified and computationally predicted CTCF binding sites. The query sequence was uploaded in the CTCFBS prediction tool. This tool searches the query sequence for CTCFBS core motifs, which are represented by position weight matrices (PWM). A match between a query sequence and a PWM is represented by a score that corresponds to the log-odds of the observed sequence being generated by the motif versus being generated by the background. A large positive score is indicative of a good match for a potential CTCFBS.

2.2.1.3 IDENTIFICATION OF EVOLUTIONARY CONSERVED REGIONS AMONG VARIOUS SPECIES

The mouse DMR is located between *Rasgrf1* and a brain-specific micro-RNA (*Mir-184*). The mouse and human genomes display conserved synteny of *Rasgrf1* and *Mir*-184. Identifying regions of conservation between these two genes in multiple species could suggest the presence of regulatory elements. The start co-ordinate of *Rasgrf1* (mouse chr9: 89,804,613 [NCBI37/mm9]; human chr15: 77,041,341 [NCBI36/hg18]) and the start co-ordinate of *Mir*-184 (mouse chr9: 89,697,098 [NCBI37/mm9]; human chr15: 77,289,185 [NCBI36/hg18]), in mice and in humans, was obtained from the UCSC Genome Browser and used in the Evolutionary Conserved Region (ECR) browser to visualize conservation-profiles (Ovcharenko et al. 2004). These co-ordinates gave a defined region in which the mouse DMR lies. Hence, the mouse was used as a base genome first, followed by using the human (hg18) as a base genome. The ECRs were identified using a minimum identity of 70% per 100 bp sequence.

The ECR browser can be found at http://ecrbrowser.dcode.org/ whilst the UCSC Genome browser is accessible at http://genome.ucsc.edu/index.html.

2.2.1.4 SEARCHING FOR CPG ISLANDS AS POTENTIAL SITES FOR DIFFERENTIAL METHYLATION

It is known that DMRs in mice have an average G+C content of 54%, suggesting that these regions are generally CpG rich (Kobayashi et al. 2006). Identifying CpG rich regions between the human *RASGRF1* and *MIR*-184 would allow for identification of possible DMRs that would need to be validated in the laboratory.

A CpG Island (CGI) Prediction tool, part of UCSC's plethora of bioinformatic tools, was used to identify CGIs between *RASGRF1* and *MIR-184*. All CpG islands on this webpage are scored quantitatively based on their CGI strength. High CpG island strength is indicated by:

1. An absence of DNA methylation
2. Presence of promoter activity
3. Open chromatin structure.

The scores allow *bona fide* CGIs – which have high CGI strength – to be distinguished from CpG rich regions devoid of *bona fide* CGI characteristics (Bock et al. 2007). The best indicator of CGI strength and most predictive of *bona fide* CGI is the "combined epigenetic score", which can assume one of three scores:

- 0.67 – high confidence for at least two of the three high CGI strengths
- 0.50 – equal chance of the CGI to be a *bona fide* island or a false positive
- 0.33 – high confidence for at least one of the three high CGI strengths (Bock et al. 2007)

The CGI Prediction tool can be found under "Custom Annotation Tracks" on the UCSC Genome Browser website.

2.2.1.5 SEARCH FOR TANDEM REPEATS WITHIN AND ADJACENT TO PREDICTED CPG ISLANDS

The presence of tandem repeats within or adjacent to the CGIs predicted using the CGI Prediction Tool would allow for the identification of CGIs that could serve as potential ICRs.

The sequences of the CGIs, together with 1000 bp up- and downstream of the CGI regions, were used in the Tandem Repeat Finder (TRF) program (version 4.03) to identify tandem repeats. The reason behind using 1000 bp up- and downstream was that tandem repeats associated with ICRs are either found within or adjacent to the ICRs (Thorvaldsen, Duran & Bartolomei 1998, Okamura et al. 2000, Takada et al. 2002). The repeats associated with *Rasgrf1* in mice are found within the ICR (Brideau et al. 2010).

This program utilizes an algorithm to detect tandem repeats and does not require the need to specify the repeat pattern or repeat size. The repeats are modeled based on the percentage identity and frequency of indels between adjacent repeat copies in the sequence (Benson 1999). Default parameters were used for this study. The TRF program can be found at http://tandem.bu.edu/trf/trf.html.

2.2.2 MOLECULAR ASSESSMENT

All reagent and equipment suppliers are listed in Appendix C.

2.2.2.1 STUDY PARTICIPANTS

The methylation status of the putative DMRs was assessed using peripheral human blood (n = 10) and human sperm (n = 8) samples from unrelated participants, after obtaining written informed consent. The random blood DNA samples that were used in this study were collected over a number of years from members of the laboratory. The male volunteers were recruited through the Cryobank, a private andrology laboratory and sperm bank in Parktown, Johannesburg, South Africa. Detailed information regarding the collection of samples from the male volunteers is given in Chapter 3, section 3.2.2. Eight random sperm samples were selected and used for this part of the project.

Ethics approval for sample collection was obtained from the University of the Witwatersrand, Committee for Research on Human Subjects – Medical (M050706 and M090555; *shown in Appendix A.1 and A.2, respectively).*

2.2.2.2 DNA EXTRACTION

Following routine semen analysis, samples collected were stored at -40°C upon arrival at the laboratory. Batches of samples were defrosted and DNA extracted using the QIAamp® DNA Micro Kit. The supplementary protocol "Purification of DNA from

epithelial cells mixed with sperm cells" was used *(full protocol shown in Appendix F)*. This protocol utilizes a differential extraction method (Gill, Jeffreys & Werrett 1985).

On average 1 ml of sperm sample was added to a 1.5 ml microcentrifuge tube, containing buffer ATL and Proteinase K. This step allowed for the preferential lysis of epithelial cells by Proteinase K. These cells were removed from the cell mixture following four wash and centrifugation steps at 14000 rpm for 5 min. The remaining pellet containing sperm cells was subjected to Proteinase K and dithiothreitol (DTT) treatment. The latter is responsible for disrupting the sulfur bonds in the outer membrane of the sperm cell, releasing the DNA into solution. The sperm cell pellet together with the Proteinase K and DTT solution was incubated overnight, at 56°C, to ensure complete digestion of the sperm heads (Voorhees, Ferrance & Landers 2006). Following incubation, buffer AL and carrier RNA were added to all samples, with the latter enhancing binding of DNA to the QIAamp MinElute column membrane. The addition of carrier RNA is important especially when relatively few target molecules are present as is the case with sperm DNA. The buffer AL, carrier RNA and sperm cell DNA mixture was incubated at 70°C for 10 min, where after two wash and centrifugation steps at 14000 rpm for 1 min ensured the removal of all excess buffers and other solutions.

Sperm DNA was eluted in 35 µl of buffer AE and stored at -20°C. The DNA was quantified using the Nanodrop® ND-1000 Spectrophotometer. DNA yields ranged from 13 ng/µl to 338 ng/µl. The DNA concentration together with the purity of the samples is shown in Appendix F.

The diagnostic staff members in the laboratory were responsible for extracting DNA from the random blood samples. The salting out procedure was used (Miller, Dykes & Polesky 1988). The DNA samples were resuspended in TE buffer and stored at 4°C. DNA yields ranged from 100 ng/µl to 398 ng/µl.

2.2.2.3 BISULFITE MODIFICATION

Bisulfite modification is used to detect and quantify the level of DNA methylation at specific CpG sites within the genome. The principle of bisulfite modification lies in the fact that it is a selective chemical conversion method (Hayatsu, Wataya & Kazushige 1970). Treatment of DNA with bisulfite converts all unmethylated cytosines to uracil bases by a process of deamination, while all methylated cytosines (referred to as 5-methylcytosine) remain unchanged. During subsequent PCR reactions, uracil bases are complemented with adenosine bases, which are in turn used as a template for thymine complementation. Thus, the UpG dinucleotides are converted to TpG dinucleotides during PCR. The methylated cytosines are resistant to bisulfite treatment and will therefore remain as cytosines following PCR (Figure 2.3).

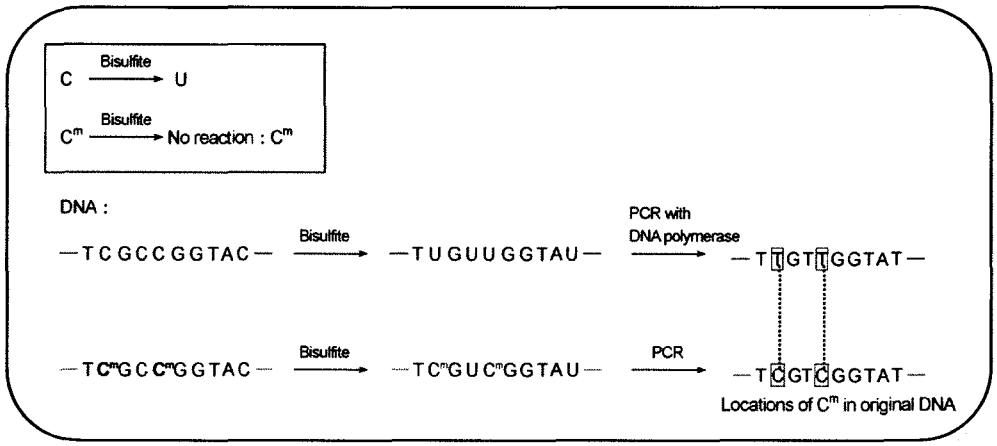

Figure 2.3: Principle of bisulfite modification. All unmethylated cytosines (C) are first converted to uracil (U) and then to thymine (T) during PCR (top strand), while all methylated cytosines (C^m) remain as Cs (bottom strand). Obtained from (Hayatsu 2008)

Following DNA extraction, 400 ng of DNA was subjected to bisulfite modification using the EZ DNA Methylation Gold™ Kit. The procedure was carried out according to the manufacturer's specifications *(full protocol shown in Appendix G)*.

2.2.2.4 PYROSEQUENCING TECHNOLOGY FOR QUANTITATIVE METHYLATION ANALYSIS

Pyrosequencing is a quantitative real-time sequencing-by-synthesis technique that requires bisulfite modified single-stranded DNA templates to synthesize complementary strands. This method utilizes a cascade of enzymatic reactions that generates light in a quantitative manner, which is directly proportional to the number of nucleotides incorporated into the complementary strand (Figure 2.4) (Ronaghi et al. 1996). The light generated is sequentially displayed as peaks in a pyrogram.

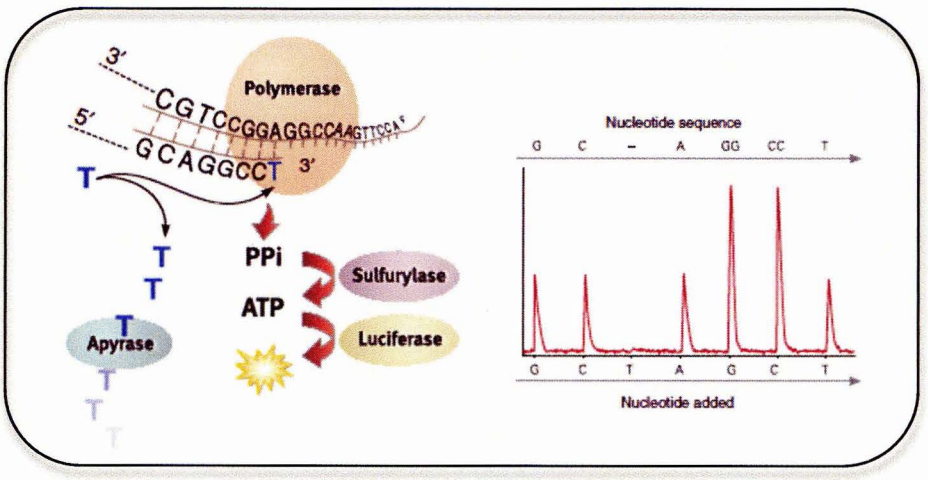

Figure 2.4: The principle of pyrosequencing. This technique relies on the release of pyrophosphate (PPi) following the successful incorporation of a nucleotide, which is complementary to the nucleotide in the single-strand template. The PPi is used in a sulfurylase reaction, releasing ATP. A luciferase enzyme uses the released ATP to convert luciferin to oxyluciferin. This reaction produces a light signal proportional to the quantity of PPi released. The light is captured by a CCD camera and recorded as peaks in a pyrogram Obtained from (England, Pettersson 2005).

This technique was initially developed for the analysis of single nucleotide polymorphism (SNP) variation. The resulting pyrogram would indicate the genotype of the sample – whether the sample is heterozygous or homozygous at the specific SNP position.

It is only recently that pyrosequencing has been adapted to study DNA methylation. The technique requires PCR amplification of the target region, using bisulfite treated genomic DNA. During PCR amplification the cytosine nucleotide, if methylated, will remain cytosine (C) while the unmethylated cytosine will be converted to thymine (T). This in essence is reflected as a C/T SNP. However, the difference with DNA methylation is that unlike traditional SNP analysis, the C (methylated):T (unmethylated) ratio at a specific CpG may vary between samples. In this way, the

pyrophosphate released following the successful incorporation of the C and T alleles is quantified and expressed as a percentage on the pyrogram.

Pyrosequencing is capable of analyzing all amplicons within a pooled PCR sample thereby accurately quantifying multiple CpG sites with high resolution and reproducibility. Furthermore, the pyrosequencing assays contain several internal controls for bisulfite treatment to evaluate the completion of the bisulfite conversion step thereby ensuring reliable data (England, Pettersson 2005).

2.2.2.4.1 PSQ ASSAY DESIGN SOFTWARE

Pyrosequencing assays were designed using the PSQ Assay Design software version 1.0.6 (Biotage, Uppsala, Sweden). The sequences of interest were imported into the design software with the CG sites denoted by the IUPAC codes as YG (for the forward strand) and CR (for the reverse strand). With this information, the software automatically generates a series of suitable primer sets for PCR and pyrosequencing.

For each CGI predicted using the CGI Prediction tool, three primers were designed, a forward, reverse and a sequencing primer. To reduce the cost per assay, a biotin-labeled universal primer was used to generate labeled PCR products (Colella et al. 2003). A 23 bp complementary tag (5' – GACGGGACACCGCTGATCGTTTA – 3') off which the universal primer will prime was added to the 5' end of the primer designated to be biotin-labeled (Biotin; Table 2.1).

Since this study focused on assessing the methylation status of loci in sperm samples, it was imperative to control for potential contamination with somatic cell DNA.

Therefore a DMR associated with *SNRPN*, a *small nuclear ribonucleoprotein polypeptide N* gene, was used as a control for somatic cell contamination in the sperm samples. This DMR is located 5'of the gene and is found to be completely methylated in oocytes, completely unmethylated in human sperm and shows an average of 53% methylation in embryos suggesting the imprints that were established during gametogenesis are stably maintained throughout development (Geuns et al. 2003). Therefore in this study, if the DMR is found to be methylated in the sperm samples this would indicate somatic cell contamination while an absence of methylation would indicate a lack of somatic cell contamination.

2.2.2.4.2 PCR AMPLIFICATION FOR PYROSEQUENCING REACTIONS

Amplicons, for all the CGI loci and the *SNRPN* locus, were generated in a 50 µl reaction containing 2 µl of bisulfite-treated DNA, 0.2 µM each of the forward and reverse PCR primers (see list of primers Table 2.1) and 0.02 µM of the universal primer, 0.125 mM deoxynucleoside triphosphates (Bioline, London, United Kingdom), 2.0 mM $MgCl_2$, 1 x Buffer (Applied Biosystems, NJ, United States), 1 M Betaine (Sigma-Aldrich, Seelze, Germany) and 1 U of AmpliTaq Gold (Applied Biosystems, NJ, United States).

Table 2.1: PCR primers for pyrosequencing PCR reactions

Locus	Primer Sequence (5' – 3')	†Amplicon Length	Annealing Temperature
CGI_1973a			
Forward	GTGCGGAAAGATGGTGTTTT	269 bp	60°C
Reverse (Biotin)	*CTCTCATTCGCCTACACAATTAC		
CGI_1973b			
Forward	GAGGGAGGGTAGGGTTTGAGTG	292 bp	63°C
Reverse (Biotin)	*AACCCCAATACCCGAAAAATAC		
CGI_1974			
Forward	GGTTTGTTTGGGTTTAGTAGAGAA	235 bp	60°C
Reverse (Biotin)	*ACAACCTACCTTCAAAATCATCTC		
CGI_1975a			
Forward	AGGAGAGAAGATGGAATTTGATT	186 bp	59°C
Reverse (Biotin)	*AATTTCCCCACACTCCTAAAATAA		
CGI_1975b			
Forward (Biotin)	*TTAGGAGTGTGGGGAAATTATTG	110 bp	59°C
Reverse	TCTCTCCCTTCAAAACACAAAAT		
§***SNRPN***			
Forward	AGGGAGTTGGGATTTTTGTATT	261 bp	58°C
Reverse (Biotin)	*CCCCAAACTATCTCTTAAAAAAAAC		

† Amplicon length includes the 23 bp complementary tag sequence

*A 23 bp complementary tag (5'– GACGGGACACCGCTGATCGTTTA – 3') was added to the 5' end of all biotin-labelled primers (Colella et al. 2003)

§ Published primer set (White et al. 2006)

The PCR for each CGI locus was performed in a GeneAmp® 2720 thermal cycler (Applied Biosystems, NJ, United States) with the following conditions for all reactions; 95°C for 5 min, followed by 50 cycles of 95°C for 15 sec, Ta°C for 30 sec (see Table 2.1 for Ta°C), and 72°C for 15 sec; with a final extension step of 72 °C for 5 min; and a final hold at 15°C. The PCR amplifications for each sperm sample and each blood sample were performed in duplicate and triplicate, respectively.

The PCR for the *SNRPN* locus was performed in a GeneAmp® 2720 thermal cycler (Applied Biosystems, NJ, United States) with the following conditions for all reactions; 94°C for 7 min, followed by 50 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec; with a final extension step of 72 °C for 7 min; and a final hold at 15°C. The PCR amplifications for each sperm sample were done in triplicate.

Standard electrophoretic techniques were used to resolve the PCR amplified products. A 3% (w/v) agarose gel containing 1 mg/L ethidium bromide was used to visualize the amplified products at 6 V/cm in 1 x TBE buffer. A 50 bp DNA molecular size marker was used as a size standard.

2.2.2.4.3 PYROSEQUENCING REACTIONS AND DATA ANALYSIS

A mix consisting of 6 µl of Streptavidin Sepharose™ HP and 40 µl of binding buffer was added to each well of a 96-well PCR plate containing bisulfite modified PCR products (45 µl). The plate was left on a shaker for 10 min at 300 rpm. This was done to facilitate binding of the PCR products to the sepharose beads via the biotin-labeled universal primer. The vacuum prep tool was used to capture the sepharose beads containing the PCR products. In order to prepare single-stranded PCR products, the vacuum prep tool was placed in four different troughs containing 70% ethanol, denaturation solution, washing buffer and ddH_20 for 60 sec each. The sepharose beads with the single-stranded PCR products were released into a PSQ 96 Plate Low™ containing a mix of 45 µl annealing buffer and 1.6 µl of a 10 µM corresponding sequencing primer (Table 2.2). The PSQ 96 Plate Low™ was heated to 80°C for 3

min to facilitate binding of the sequencing primer to the single-stranded PCR products.

A reagent cartridge was prepared with the required amounts of enzyme, substrate and dNTPs using the PyroMark® Gold Q96 Kit (Qiagen, Valencia, CA, United States). The PSQ 96 Plate Low™ was placed into the PSQ 96MA instrument along with the cartridge, prior to starting the run. Pyrosequencing data was analysed using the Pyro Q-CpG Software (Biotage, Uppsala, Sweden). The full pyrosequencing protocol is shown *Appendix H.*

Table 2.2: Primers for pyrosequencing assays

Locus	Sequencing Primer Sequence (5' – 3')	Number of CpG Sites Analyzed	§Sequence to Analyze
***CGI_1973*a**	**F:** GGTGTTTTTTTTTATAGGG	13	TTG**Y**G**Y**GGGTATTTATTTT**Y**GTTGTAAAG**Y**GTT**Y**GGGTTAGG**Y**G**Y**GGTTTGTTT**Y**GG GT**Y**GGGG**Y**GAGTT**Y**GTTGTTTTTT**Y**G**Y**GGTAGATAG**Y**GATG
***CGI_1973*b**	**F:** GGAGGTATTAAGTTGTA	8	GTTTG**Y**G**Y**G**Y**GGTTTTG**Y**GTTT**Y**GG**Y**GGGGTTG**Y**GTG**Y**GTGTGTTTTTGTT
***CGI_1974*a**	**F:** GATTAGTTTTTTATAGTGTT	3	**Y**GGTTTTGTAGTTTGAAGGTTTGAGAATAT**Y**GG**Y**GGGGAGTGGGTG
***CGI_1974*b**	**F:** GTGGGTGTATGGGAAG	4	GA**Y**GATGGTTTTA**Y**GGTTTTGT**Y**GTTAA**Y**GGTGGTTTTTAGAGATAATGTG
***CGI_1975*a**	**F:** AGAGTTTTTTTTGAAGGTAG	4	**Y**GAGAGGGG**Y**GTTTA**Y**GTGGT**Y**GATTTATAGAGTT
***CGI_1975*b**	**R:** CCCTTCAAAACACAAAAT	3	TC**R**CATAAAAC**R**TAAAACTAAACCCTCCCC**R**TATTTTTAAA
SNRPN	**F:** GTAGAGGTAGGTTGG	4	**Y**G**Y**GTATGTTTAGG**Y**GGGGATGTGTG**Y**GAAG

§The sequence to analyze is immediately 3' to the sequencing primer binding site on the biotinylated strand

The positions of the CpG sites analyzed are shown by their IUPAC codes in bold font.

2.3 RESULTS

2.3.1 COMPUTATIONAL ASSESSMENT

2.3.1.1 SEARCHING FOR SEQUENCE SIMILARITY

The BLAST algorithm was used to identify regions in the human genome that had sequence similarity to the mouse DMR and the 40 copies of the 41 bp repeat element. No sequence similarity was identified.

2.3.1.2 IDENTIFICATION OF CTCF BINDING SITES

REN_20, MIT_LM2, MIT_LM7 and MIT_LM23 are PWM motif sequences that correspond to CTCF binding sites (Kim T.H. et al. 2007, Xie et al. 2007). The *in silico* CTCFBS prediction tool used these four motifs to search for putative CTCF binding sites in a region spanning 249 841 bp (Ensembl co-ordinates chr15: 79252289-79502130 [release 61, GRCh37.p2]). This region included *RASGRF1* and 118 915 bp upstream of the gene to the first base of *MIR*-184. Four putative CTCF binding sites were identified each with a PWN score greater than 3.0 (Table 2.3). A score greater than 3.0 indicates that the putative CTCF binding site matches more closely to the PWM motif compared with the random background sequence (Pan, Phan 2008).

Table 2.3: *In silico* predicted CTCF binding site motif sequences within *RASGRF1* and between *RASGRF1* and *MIR*-184

Motif PWN*	Putative CTCF Binding Site Sequence	Motif Start Location§	Motif Length	Motif Orientation	PWN Score
REN_20	TGAGCCACCAGAGGGCGGAG	182100	20	+	22.2079
MIT_LM2	ATGCCACCAGGTGGCAGTT	1433	19	+	20.6626
MIT_LM7	TAAGCACCAGAGGGCGCTGT	205840	20	+	21.708
MIT_LM23	TCACCACCAGGTGGCGCTTG	25632	20	+	25.8977

* The *in silico* CTCFBS prediction tool used four PWM motifs to search for putative CTCF binding sites between *RASGRF1* and *MIR*-184.

§ The motif start location is based on the 249 841 bp (Ensembl co-ordinates chr15: 79252289-79502130 [release 61, GRCh37.p2]) query sequence and gives the exact location of the putative CTCF binding site within this sequence. In this table the motif start location uses Ensembl co-ordinate 79502130 as point 0 and all motif start locations are relative to this point.

The putative CTCF binding site sequences corresponding to REN_20 and MIT_LM7 were found within *RASGRF1*. MIT_LM2 and MIT_LM23 were both found in the intergenic region between *MIR*-184 and *RASGRF1*.

2.3.1.3 IDENTIFICATION OF EVOLUTIONARY CONSERVED REGIONS AMONG VARIOUS SPECIES

The mouse genome was selected as the base genome and the intergenic region (indicated by the red peaks) between *Rasgrf1* and the *Mir*-184, within which the mouse DMR lies, was visualised for conservation-profiles. The mouse genome was compared to eight vertebrate species including *Homo sapiens* (humans), *Pan troglodytes* (chimp), *Macaca mulatta* (rhesus macaque), *Xenopus tropicalis* (frog), *Gallus gallus* (chicken), *Fugu rubripes* (pufferfish) and the *Monodelphis domestica*

(opossum). A number of conserved intergenic regions were identified (Figure 2.5). The mouse DMR co-ordinates (chr9: 89774524-89774827) from UCSC were used to search for conservation between species in the ECR browser. This DMR was not conserved in any of the eight vertebrate species.

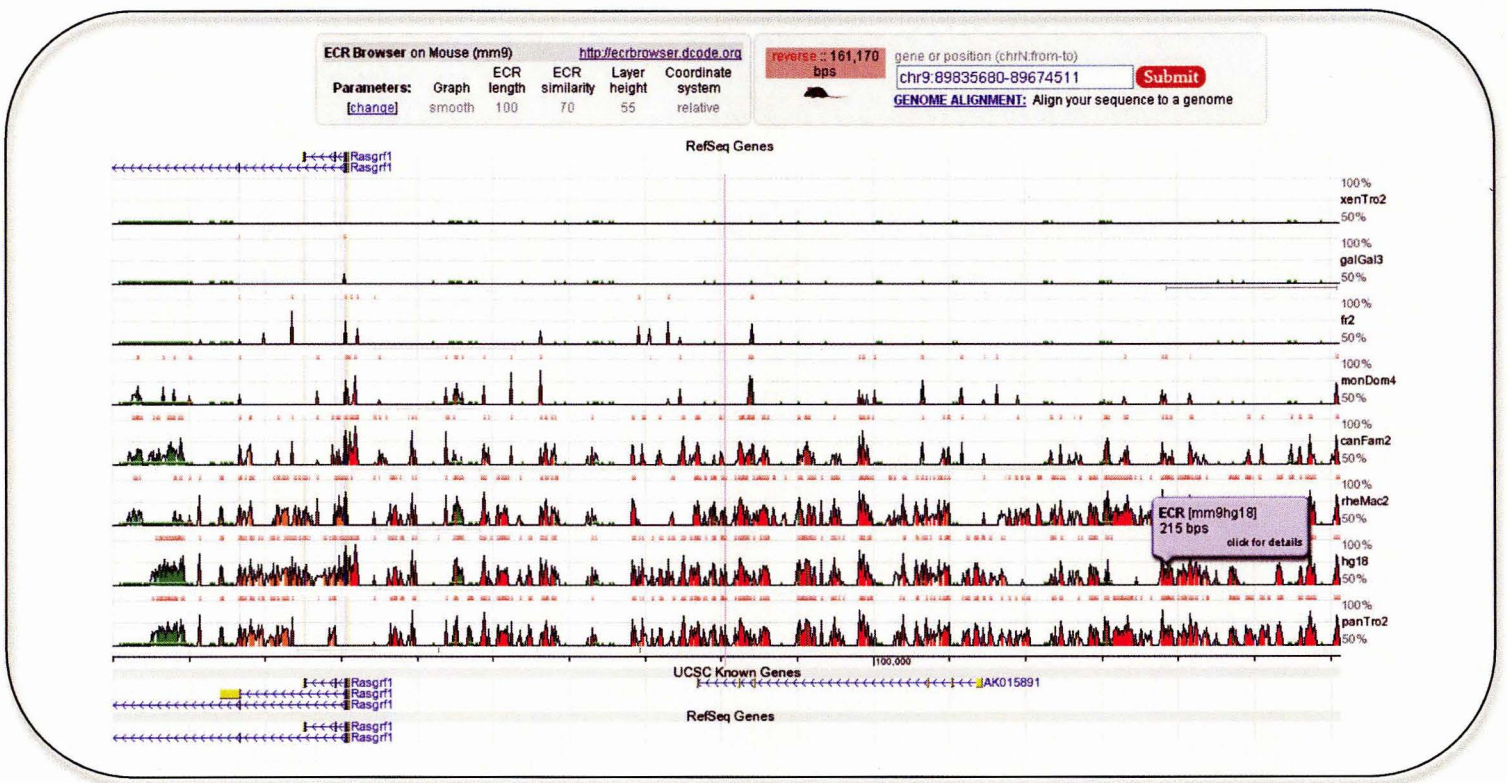

Figure 2.5: Conservation profiles of various vertebrate species between the *Rasgrf1* and *Mir*-184 loci, using the mouse genome as the base genome. In order to identify regions of conservation the genomes of eight vertebrate species were compared relative to the mouse genome. The red peak represents intergenic regions while the pink bars above the peak indicate conservation. The height of the peak indicates degree of conservation. The blue bubble to the right of *AK015891* represents the *MIR*-184 in the human genome. The figure was generated using http://ecrbrowser.dcode.org/. PanTro2 (*Pan troglodytes*), hg18 (*Homo sapiens*), rheMac2 (*Macaca mulatta*), canFam2 (*Canis familiaris*), monDom4 (*Monodelphis domestica*), fr2 (*Fugu rubripes*), galGal3 (*Gallus* gallus), xenTro2 (*Xenopus tropicalis*).

The human genome (hg18) was then used as a base genome and the region between *RASGRF1* and *MIR*-184 was assessed to identify evolutionary conserved intergenic

regions. The intergenic region at the promoter of *RASGRF1* was conserved in five of eight species while the first coding exon (indicated by the blue peak) was conserved in all eight vertebrate species. The *MIR*-184 was conserved in five of the eight species (Figure 2.6).

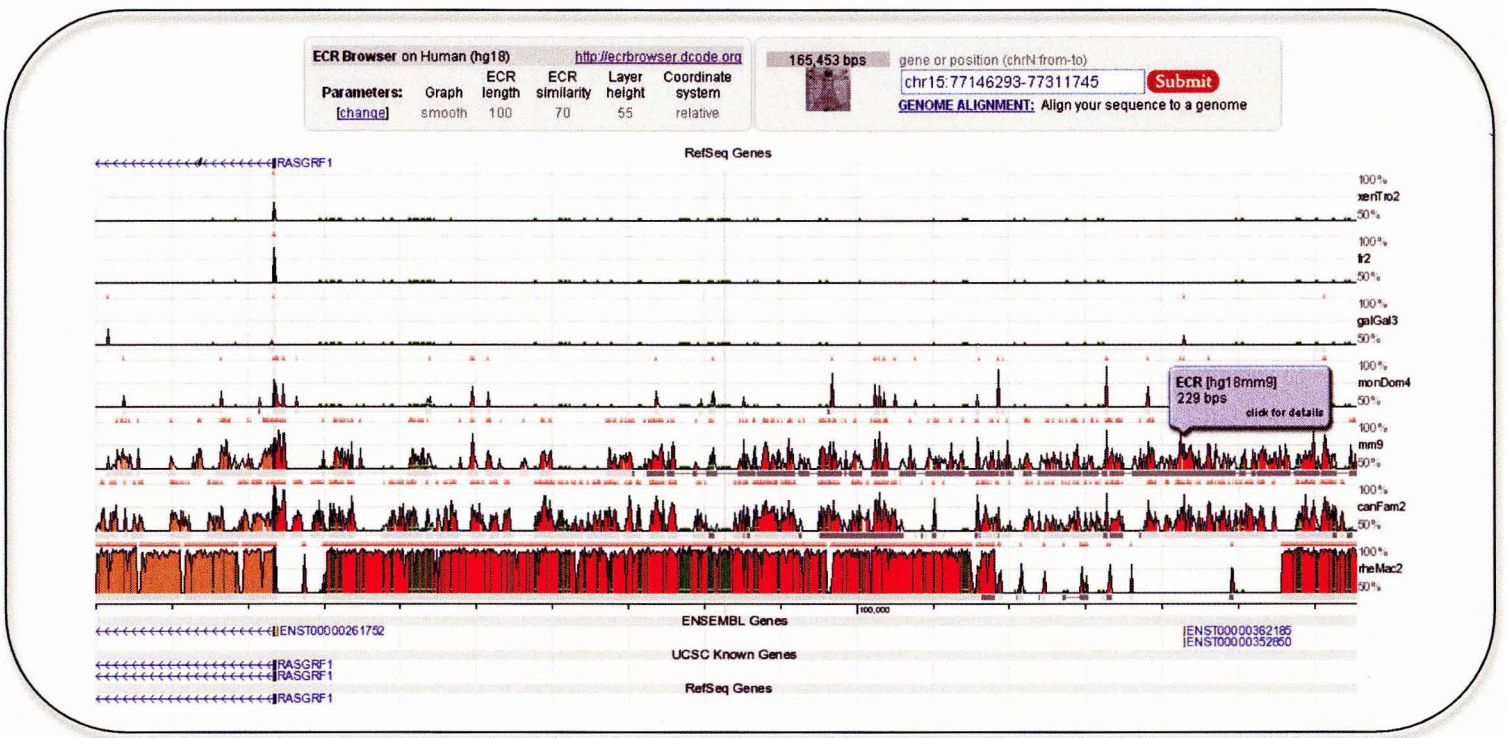

Figure 2.6: **Conservation profiles of various vertebrate species between the *RASGRF1* and *MIR*-184 loci, using the human genome as the base genome.** In order to identify regions of conservation the genomes of eight vertebrate species were compared relative to the human genome. The red peaks represent intergenic regions while the pink bars above the peaks indicate conservation. The height of the peak indicates degree of conservation. The blue bubble to the right represents the *Mir*-184 in the mouse genome. The figure was generated using http://ecrbrowser.dcode.org/. PanTro2 (*Pan troglodytes*), hg18 (*Homo sapiens*), rheMac2 (*Macaca mulatta*), canFam2 (*Canis familiaris*), monDom4 (*Monodelphis domestica*), fr2 (*Fugu rubripes*), galGal3 (*Gallus gallus*), xenTro2 (*Xenopus tropicalis*).

2.3.1.4 SEARCHING FOR CPG ISLANDS AS POTENTIAL SITES FOR DIFFERENTIAL METHYLATION

All the CGI tracks were viewed simultaneously on UCSC's CGI prediction tool. The panel containing the combined epigenetic scores was used to select the appropriate CGI. The reason behind choosing this specific score is that it provides a precise estimate of CGI strength. The CGI strength indicates the CGI's inherent tendency to exhibit an open and transcriptionally competent chromatin structure (Bock et al. 2007). Three CGIs were identified between the human *RASGRF1* and *MIR*-184: CGI_1973 (chr15: 77168729-77171326) at the promoter of *RASGRF1*; CGI_1974 (chr15: 77258299-77258552) and CGI_1975 (chr15: 77269392-77269597) [NCBI36/hg18].

2.3.1.5 IDENTIFICATION OF TANDEM REPEATS WITHIN OR ADJACENT TO CPG ISLANDS

The TRF program only detects repeats which occur in a head-to-tail manner (Hutter, Helms & Paulsen 2006). Only one out of the three CGIs identified, using the CGI Prediction tool, had direct tandem repeats associated with it. There were a total of eight repeat elements found within and directly adjacent to CGI_1973 (Table 2.4). However, the TRF program identifies overlapping repeat elements therefore two elements were regarded as one if the smaller one overlapped with the larger one. The smaller repeat motif was chosen as a consensus sequence (Hutter, Helms & Paulsen 2006).

Table 2.4: Tandem repeats detected within and adjacent to CGI_1973

Indices*	Period Size	Copy Number	Percent Matches	Percent Indels	Consensus Sequence
2608--2638	12	2.6	89	0	CACACACACACG
3537--4321	7	110.4	71	20	ACACACC
3537--4315	9	86.9	72	20	ACACACACC
3537--4329	14	56.4	69	17	ACACACACCACACA
3537--4329	16	49.4	70	17	ACACACACACCACACA
3537--4321	2	424.5	71	17	AC
4356--4433	2	39.5	74	12	CA
4356--4406	23	2.3	86	3	CACACAACACACGCAGCACACCA

*Indices represent the position of the beginning of the repeat relative to the detected pattern.
All rows highlighted in grey indicate overlapping repeat elements with the smallest repeat AC overlapping with all the other repeats detected. Rows highlighted in pink represent overlapping repeat elements, which can be represented by one repeat element with consensus sequence CA.

2.3.1 MOLECULAR ASSESSMENT OF THE PUTATIVE CPG ISLANDS WHICH COULD SERVE AS SITES OF DIFFERENTIAL METHYLATION

Bisulfite-treated PCR products for each of the three CGIs and *SNRPN* (used as a control for somatic cell contamination) were quantitatively assayed using pyrosequencing technology. Pyrosequencing was performed on PCR products from sperm (in duplicate on n=8) and blood (in triplicate on n=10) DNA samples, respectively. The average methylation percentage for each CpG site was calculated by averaging the duplicate and triplicate values for each CpG site per sperm and blood sample, respectively (*shown in Appendix I – RASGRF1 and SNRPN Data*). The C (methylated) and the T (unmethylated) alleles were quantified and subsequently expressed as a percentage. When examining the duplicate data set for each sperm sample and the triplicate data set for each blood sample, a greater than 6%

methylation difference per CpG site was used as a cut-off value for unsuccessful replication. As a result, certain replicates on the sperm and blood samples were excluded due to more than one of the CpG site differing by more than 6% when comparing across the duplicate and triplicate data sets, respectively. It should be noted that the triplicate data set obtained for each blood sample was not as reproducible as the duplicate data set obtained for each sperm sample. Technical variation could be one possible reason for the degree of variability observed within the triplicate data sets. The blood samples were done first before much experience was built up. In addition, blood samples contain double the chromosome complement of sperm samples. This could add to the variability observed within triplicate data sets.

Figure 2.7 illustrates the methylation percentages in each tissue type for each region evaluated.

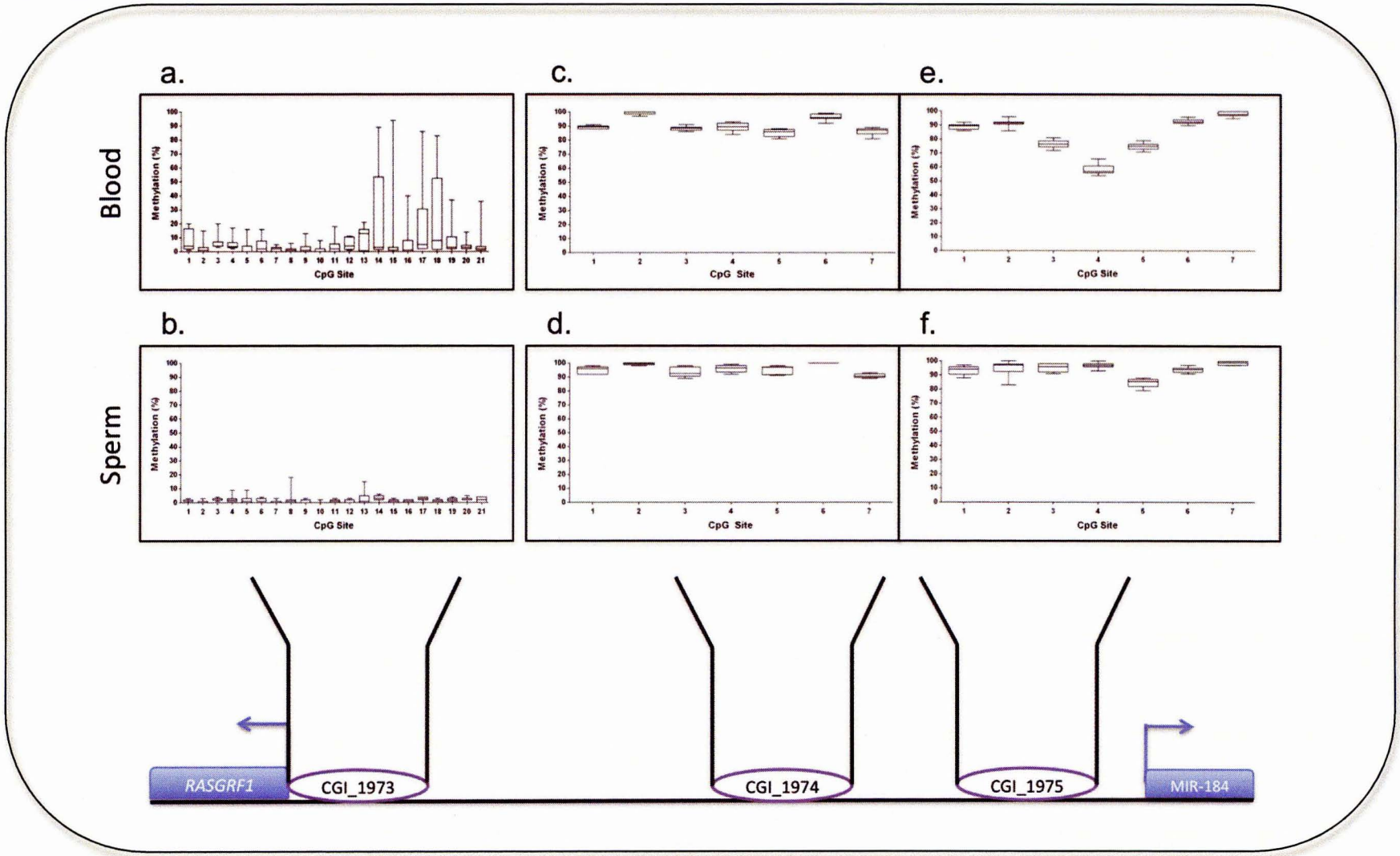

Figure 2.7: A schematic representation of *RASGRF1* and *MIR*-184 and the positions of the putative CpG islands relative to the two genes. The box and whisker plots above (a – f) represent the methylation percentages based on the average of the duplicate and triplicate results obtained for individual CpG sites for the sperm (n=8) and blood (n=10) samples, respectively.

Due to the size of CGI_1973, found at the promoter of *RASGRF1*, a smaller region found to be rich in CG dinucleotides within this CGI was selected for further analysis. This smaller region was then split into two sub-regions CGI_1973a and CGI_1973b (bordering exon1 of *RASGRF1*) and each was assayed separately. CGI_1974 and 1975 were difficult regions not easily amenable to pyrosequencing and therefore CGI_1974 was covered using two sequencing primers while CGI_1975 was amplified in two separate PCR reactions using two sets of PCR primers. It should be noted that the data from sub-region CGI_1973a and CGI_1973b were analyzed collectively as one region. This also applies to the data obtained from sub-region CGI_1974a and CGI_1974b and CGI_1975a and CGI_1975b.

The entire CGI_1973 region was 2598 bp in length with a G+C content of 64%. However, only a region spanning 515 bp with a G+C content of 70% was investigated. CpG sites 1 to 13 were found to be hypomethylated in blood and sperm DNA. The methylation percentages for blood samples were very variable and displayed considerable inter-site and inter-individual variation at CpG sites 14 to 21 (Figure 2.7a). It should be kept in mind that these CpG sites border exon1 of the *RASGRF1* gene. In comparison the methylation percentages of the sperm samples showed relative hypomethylation of this region with little or no inter-site and inter-individual variation (Figure 2.7b).

CGI_1974 was 212 bp in length with a G+C content of 56%. The methylation percentages of the blood and sperm samples showed relative hypermethylation (Figure 2.7c and 2.7d).

The CGI_1975 region was 231 bp in length with a G+C content of 51%. The methylation percentages of the blood and sperm samples showed relative hypermethylation. The blood samples showed a methylation percentage of between 50% and 60% at CpG site 4 and the two sites (CpG 3 and 5) on either side displayed a methylation level of between 70% and 90% (Figure 2.7e). The methylation percentages of the sperm samples at these sites ranged between 90 and 100% except for site 5 where the methylation percentage was between 70% and 90% (Figure 2.7f).

The methylation status of the *SNRPN* DMR (found to be hypermethylated on the maternal allele) was assessed to detect the presence of somatic cell DNA contamination in sperm samples. Only 22 (19.5%) of the 113 sperm samples collected were used for the somatic cell contamination assessment. The mean methylation of this DMR was found to be 2.43% with a standard deviation of 1.35. The theoretical methylation value for DMRs in somatic tissue is 50% (Cooper, Constancia 2010). However studies have shown that somatic DMRs display methylation levels between 35% and 65% (Woodfine, Huddleston & Murrell 2011). Therefore the low level of methylation at the *SNRPN* DMR within the sperm samples may suggest that the paternal allele may not be completely devoid of methylation but rather displays basal levels of methylation or that there could have been low levels of contamination with somatic cells. However, it was concluded that the samples were not significantly contaminated with somatic cells.

2.4 DISCUSSION

The primary aim of this part of the study was to determine whether the human *RASGRF1* gene has a DMR that is paternally methylated. Methylation analysis of three putative DMRs in the vicinity of this gene in sperm and blood samples indicate that *RASGRF1*-associated CpG rich regions do not exhibit differential methylation in a parent-of-origin manner, as is seen in its mouse counter-part.

The results presented in this study demonstrate that the human genome does not contain any homologous sequences to the mouse DMR and to the 40 copies of the 41 nucleotide repeat element. Two of the most common features of any imprinted region are the presence of a DMR and adjacent to this, tandem repeats. It is often expected that DMRs among various mammalian species have a conserved DNA sequence because of their key regulatory function in imprinted regions. However this is not universally true. Instead, the presence of tandem repeats is a common feature of functionally orthologous DMRs (Hutter et al. 2010). There are only a handful of repeats that are highly conserved between the mouse and human. In all other cases, the number of repeat elements and their arrangement within the DMRs tend to be highly variable (Paulsen et al. 2005).

The mouse *Rasgrf1* and the human ortholog *RASGRF1* display both organisational similarities and differences. The mouse gene contains 26 exons while the human gene possesses two extra exons. The sizes of exons and intron-exon junction sequences are highly conserved. The 5' sequence upstream of exon 1 is not only highly conserved between the mouse and the human but is also highly conserved in other vertebrates.

This is consistent with the presence of important regulatory elements in this region (de la Puente et al. 2002). However it should be noted that organizational similarity in the genomes of species does not necessarily relate to the conservation of imprinting between species (Okamura et al. 2000). As an example, the mouse and human *IGF2R* genes share conserved intronic CpG islands with large direct repeats that are maternally expressed in the mouse but are biallelically expressed in humans (Kim K.P. et al. 2007).

The CTCF binding protein is a ubiquitously expressed 11 zinc finger protein that plays a key role in chromatin insulation and is the only protein known to exhibit enhancer-blocking activity (Bao, Zhou & Cui 2008, Williams, Flavell 2008). In this study two of the four putative CTCF binding sites corresponding to MIT_LM2 and MIT_LM2 were located in the intergenic region between *MIR*-184 and *RASGRF1*. The position of these binding sites is consistent with a potential role of CTCF as an insulator protein (Kim T.H. et al. 2007). The other two putative binding sites corresponding to REN_20 and MIT_LM7 were found within *RASGRF1*. It is known that almost half of the CTCF binding sites within the human genome are located within genes and many of these segregate alternative promoters of a single gene (Kim T.H. et al. 2007). The protocadherin gamma locus, the T-cell receptor locus and the immunoglobulin heavy chain locus all have intragenic CTCF binding sites that segregate transcriptional start sites that display differential activities across tissues (Kim T.H. et al. 2007). *RASGRF1* is known to have four transcripts and therefore the identification of two putative CTCF binding sites within this gene may have a role in segregating the transcriptional start sites based on their activities across various

tissues. Functional studies would need to be done to determine whether these putative CTCF binding sites actually bind the CTCF protein.

In some instances CTCF binding sites are found within DMRs. An example of such a CTCF binding site is that which is present at the mouse *Rasgrf1* DMR (Yoon et al. 2005). Another example is the CTCF binding site present at the *H19/Igf2* ICR, which serves as a primary DMR established in the germ-line (Szabo et al. 2004). The hypomethylated maternal ICR serves as a binding site for the CTCF protein. Once the protein binds to this region it acts as an insulator preventing the interactions of the enhancers with the promoter of Igf2 (Szabo et al. 2004). In this study the putative CTCF binding sites were not found to occur in the predicted CpG islands. This suggests that *RASGRF1*, if imprinted, would be unlikely to be regulated by a CTCF binding protein as is its mouse ortholog. However, if *RASGRF1* is imprinted in humans there are other factors, besides the CTCF binding protein, which could mediate imprinting. These factors could be cis- or trans-acting and could contain multiple elements that work in synergy to establish and maintain the imprint status (Ogawa et al. 2006).

The mouse and human genomes display conserved synteny of *Rasgrf1* and *Mir*-184. In mice the *Rasgrf1* DMR is found in the intergenic region. The ECR browser results demonstrate that the majority of the intergenic regions are conserved in the *Pan troglodytes, Macaca mulatta, Canis familiaris, Mus musculus* and *Monodelphis domestica.* The conservation of these regions across multiple vertebrate species suggests that one or more of these regions could contain possible *cis*-regulatory

elements such as enhancers or repressors crucial for gene regulation (Visel, Bristow & Pennacchio 2007, Nakagawa et al. 2008).

The majority of the CGIs in the mouse and human genomes are hypomethylated and a small proportion of the hypermethylated CGIs are located at imprinted loci in the germ-line (Henckel, Arnaud 2010). The hypermethylated CGIs of potential imprinted loci are expected to display monoallelic methylation (50% methylation) in somatic tissues (Strichman-Almashanu et al. 2002). For a region to be regarded as a DMR, it is proposed that the following criteria need to be fulfilled: the sequence should be rich in CpG dinucleotides with an average G+C content of 54% (Kobayashi et al. 2006) or above and display methylation levels between 35% and 65% in somatic tissue (Woodfine, Huddleston & Murrell 2011) over four consecutive CpG sites (Kobayashi et al. 2006).

CGI_1973, a putative CGI identified using the CGI Prediction tool, is located in the 5' sequence upstream of exon 1, a region that harbors the promoter sequence for *RASGRF1* and has been found to share high homology with the mouse sequence (de la Puente et al. 2002). The methylation assessment of this region in blood samples displayed a deviation from the expected 50% methylation for imprinted loci in somatic tissue, indicating that the selected CGI is not differentially methylated. This is due to the fact that CGIs associated with promoters of tissue-specific genes are generally unmethylated in both expressing and non-expressing tissues although exceptions are being identified (Bird 2002, Song et al. 2005, Weber et al. 2007). It is however interesting to note that part of this CGI (CpG sites 14 to 21) displays great inter-individual and inter-site variation in blood samples only. The difference in

methylation patterns between individuals in peripheral blood samples is unlikely to be attributed to technical errors. To test the reproducibility of the PCR and pyrosequencing results, we performed each PCR and pyrosequencing reaction in duplicate for sperm samples and in triplicate for blood samples. The similar methylation profiles observed between individuals in the sperm samples, for this region and other regions, demonstrates that our techniques were reproducible. The possible reason behind the high inter-individual variation observed in peripheral blood could be that CpG sites 14 to 21 in this specific region are not tightly regulated, making this region more responsive to the effects of various environmental factors including carcinogen exposures, inflammation, stress and diet which all have the ability to alter DNA methylation patterns (Jirtle, Skinner 2007, Christensen et al. 2009, Murgatroyd et al. 2009).

The other two putative CGIs identified in this study, CGI_1974 and CGI_1975, lie in the intergenic region between *RASGRF1* and the *MIR*-184. Both sample types were found to be hypermethylated in these regions and showed a lack of inter-individual variation. This suggests that this region is under tight regulation and may harbor transposable elements that are normally hypermethylated in the genome (Geiman, Robertson 2002, Slotkin, Martienssen 2007). The methylation profiles of the sperm samples for regions CGI_1974 and CGI_1975 displayed tight methylation patterns. These results are contrary to that found by Flanagan et al. (2006) who demonstrated the presence of significant inter-individual DNA methylation variation in human male germ cells. However, studies in mice and other organisms have shown that certain loci display partial epigenetic stability during meiosis, which may be a possible explanation for the results obtained in this study (Flanagan et al. 2006). In somatic

tissues DMRs are expected to exhibit 50% average methylation over several consecutive CpG sites (Cooper, Constancia 2010). However the paternal allele, as shown by the sperm samples, is found to be almost completely hypermethylated over seven CpG sites of the CGI_1975 region, with a methylation level between 90% and 100%, suggesting a possible paternal parent-of-origin effect. In the blood samples only one CpG site, CpG 4, displayed a methylation percentage between 50% and 60%. The surrounding CpG sites had significantly higher methylation, indicating that this is not a DMR for *RASGRF1*.

Tandem repeats are found to occur frequently within the human genome, however they tend to occur more frequently within or adjacent to CpG islands associated with imprinted genes (Hutter, Helms & Paulsen 2006). The tandem repeats identified using the TRF tool were found to be associated with only one of the CGIs identified, CGI_1973. This CGI is located at the promoter region of *RASGRF1* and was found to be hypomethylated both in the peripheral blood and in the sperm samples. Owing to the fact that the CGI_1973 was unmethylated in both tissue types, it can be deduced that the tandem repeats associated with this promoter CGI is not involved in establishing methylation at this locus but rather plays a role in regulating gene expression by binding transcription factors and influencing splicing efficiency, RNA stability and RNA-to-protein interactions (Martin et al. 2005, Shah, Hile & Eckert 2010). The presence of repeats but the absence of differential methylation at CGI_1973 in peripheral blood suggests that this CGI does not serve as a DMR for *RASGRF1*.

2.5 LIMITATIONS OF THE STUDY

One of the limitations of this part of the study is that no expression data was available to confirm the imprint status of *RASGRF1*. *Rasgrf1* is expressed in the neonatal brain and liver, in mice. Therefore, in order to assess allele-specific expression in humans large numbers of neonatal human brain and liver samples would be needed to identify an individual heterozygous for a SNP in the coding region of the gene. This sample would then be subjected to allele-specific real-time PCR to quantitate the amount of paternally and maternally derived *RASGRF1* RNA present in the brain and liver. This would indicate the imprint status of this gene in both tissue types. Ethics approval and sample collection would be required and due to the sensitive ethical issues this was not done.

Tissues are composed of multiple cell types, with each cell type displaying a unique epigenetic signature (Morgan et al. 2005). The use of peripheral blood in this study was not ideal as blood is made up of various cells types, including monocytes, basophils and lymphocytes. As a result, DNA from peripheral blood constitutes a mixture of epigenomes from a diversity of cells. Therefore, differences between individuals in terms of blood cell populations may itself contribute to or mask epigenetic differences.

In this study DNA methylation of the putative DMRs was only analyzed using sperm and peripheral blood samples. The methylation profiles of the DMRs in various other tissues including ovarian teratomas, brain tissue and liver tissue would be useful in the event that *RASGRF1* displays tissue-specific differential methylation.

2.6 CONCLUSION

The CGIs evaluated in this study did not fit the criteria of being a DMR. The human *RASGRF1*-associated CpG rich regions do not exhibit differential methylation in a parent-of-origin manner. However, gene expression studies would need to be performed to confirm the imprint status of this gene. Due to the absence of a DMR for *RASGRF1,* no further work relating to alcohol exposure was carried out on the methylation status at this locus.

CHAPTER 3

THE EFFECT OF ALCOHOL CONSUMPTION ON IG-DMR IN MALE GAMETES

3.1 INTRODUCTION

3.1.1 THE ROLE OF THE SPERM EPIGENOME IN OFFSPRING DEVELOPMENT

There are two critical periods during the spermatogenic process where various environmental and lifestyle factors can have a direct or indirect impact on the male gametes (Figure 3.1).

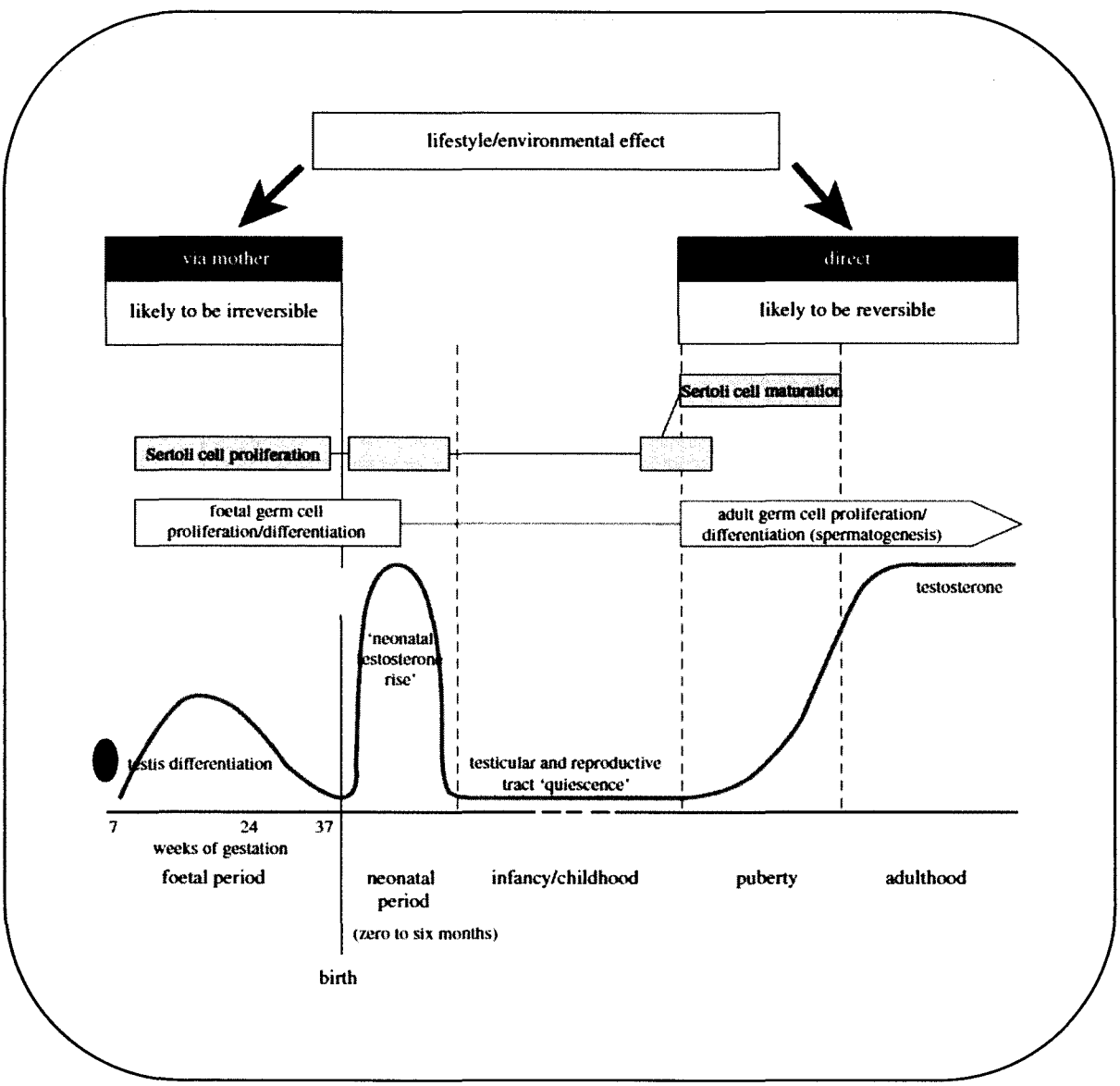

Figure 3.1: A schematic representation of two critical time points at which environmental factors can have a negative impact on spermatogenesis. Obtained from (Sharpe 2010).

The first critical period is during gonadal sex determination when the foundations for spermatogenesis are laid down and primordial germ cell development occurs. During this period a wave of demethylation is responsible for erasing the imprint marks and re-establishing methylation patterns. This occurs both globally and at imprinted loci where the methylation of the imprints occur in a sex-dependant manner (as reviewed in Murphy, Jirtle 2003). This germ-line epigenetic reprogramming is not only important for resetting of parental imprints for the next generation but also in preventing epimutations from being passed onto the next generation (Godmann, Lambrot & Kimmins 2009).

A classic study carried out by Anway et al. (2005) looked at the effect of exposing a gestating rat to vinclozolin, an endocrine disruptor, during the fetal gonadal sex determination stage. This resulted not only in the F1 male offspring having an increase in spermatogenic cell apoptosis and a decrease in sperm number and motility but this indirect exposure also had a negative impact on the health of these offspring in later life. The negative effects were a result of altered DNA methylation of the male germ line that occurred due to maternal vinclozolin exposure and did not only affect the F1 male generation but all males in many subsequent generations (F2-F4) as well (Anway et al. 2005). Another more recent study done in mice focused on the effect of prenatal alcohol exposure on DNA methylation levels of five imprinted genes in male offspring. The F1 male offspring displayed hypomethylation of the normally hypermethylated *H19*-ICR in sperm only. These males were then mated to normal females and the resulting offspring (F2) displayed considerable hypomethylation of *H19*-ICR in whole brain regions. This study demonstrated that

the altered *H19*-ICR in the sperm of the F1 males might have been passed onto the F2 generation (Stouder, Somm & Paoloni-Giacobino 2011).

The second period during which environmental and lifestyle factors can impact on spermatogenesis extends from the onset of puberty through to adulthood. It is during this period that the spermatogenic process is directly vulnerable to the adverse effects of toxic agents associated with the general environment and lifestyle of the individual (Sharpe 2010).

In 2005, Barton et al. carried out a study looking at the effect of chronic paternal exposure of a widely used anticancer agent, cyclophosphamide, on rat offspring development. Chronic exposure of male sperm to cyclophosphamide did not affect the viability of the sperm but affected the epigenetic reprogramming of the zygotes. This led to disrupted embryonic development and resulted in an increase in neurological deficits and malformations (Auroux et al. 1990, Hales, Crosman & Robaire 1992, Barton, Robaire & Hales 2005). These abnormalities were found to be transmitted to subsequent generations (Auroux et al. 1990, Hales, Crosman & Robaire 1992). A similar study focused on the consequences of long-term paternal cocaine exposure in mice. Male mice were trained to self-administer cocaine in multi-hour inhalation sessions prior to coitus. Following mating with females not exposed to cocaine, the offspring were found to have impaired visuo-spatial attention and spatial working memory. This negative effect was thought to be a consequence of altered DNMT1 levels in the male gametes, which resulted in the alteration of normal parental imprint patterns found in the sperm (He, Lidow & Lidow 2006). Another such study looking at male rats exposed to tamoxifen, a selective estrogen receptor modulator, found a

significant decrease in DNA methylation at the *H19/Igf2* ICR in the spermatozoa. Following mating, an increase in post-implantation embryo loss was noted and this was found to be significantly associated with hypomethylation of the *H19/Igf2* ICR, a key regulator of growth control. This suggests that the hypomethylated *H19/Igf2* ICR in the spermatozoa was inherited by the subsequent generation (Pathak et al. 2009). There has been one study done in humans that looked at the effect of alcohol consumption on two paternally imprinted ICRs, the *H19 ICR* and IG-DMR in male gametes. These ICRs are associated with the imprinted *H19/IGF2* and *DLK1/GTL2* loci, respectively. While a general trend towards reduced overall methylation was noted at the *H19* locus, a modestly significant difference was noted in the methylation levels of the non-drinkers and heavy-drinkers at the IG-DMR locus (Ouko et al. 2009).

All of the studies mentioned above demonstrate the ability of environmental insults (e.g. irradiation, therapeutic drugs, pesticides, endocrine disruptors, smoking and alcohol) to alter the epigenome of the gametes either at the point where the foundation for spermatogenesis is laid down or when spermatogenesis is initiated during the pubertal stage. This consequently affects the fertility and the health of future offspring.

3.1.2 METHYLATION DEFECTS ASSOCIATED WITH DISRUPTIVE SPERMATOGENESIS

Male infertility accounts for roughly 40% of the cases of infertility (Gaur, Talekar & Pathak 2010). A number of lifestyle, environmental, genetic and epigenetic factors contribute towards this etiology.

Alcohol and cigarette smoke are two major lifestyle factors that have a negative impact on male fertility. Alcohol has been shown to not only adversely affect the normal morphological development and maturation of spermatozoa resulting in teratozoospermia but also decrease spermatozoa production known as oligozoospermia (as reviewed in Emanuele, Emanuele 1998, Gaur, Talekar & Pathak 2010, Sermondade et al. 2010). The toxins produced by cigarette smoke have been found to have a negative effect on the motility of the sperm resulting in asthenozoospermia (Kunzle et al. 2003, Gaur, Talekar & Pathak 2010).

Several recent studies have focused on imprinting defects in sperm from men suffering from severely reduced sperm counts or oligozoospermia (Marques et al. 2004, Kobayashi et al. 2007, Marques et al. 2008). The altered imprint patterns in these infertile men can have serious implications for children conceived by assisted reproductive techniques (ART). This is due to the fact that imprinted genes resist the wave of global demethylation that occurs following fertilization. This would prevent the erasure of any epimutations present in imprinted genes in the sperm at the time of fertilization.

Two independent studies focused on the methylation defects at the *H19/IGF2* paternally methylated ICR and *MEST* a maternally imprinted locus in sperm from infertile men suffering from oligozoospermia. Global DNA methylation levels of non-imprinted repetitive elements (*Alu* and *LINE-1*) were found to be normal in these men. However, both studies found the *H19/IFG2* ICR to be hypomethylated and *MEST* to be hypermethylated, suggesting that altered methylation patterns are specific and affect imprinted loci in different ways (Kobayashi et al. 2007, Marques et al.

2008). These aberrant methylation patterns may contribute significantly to Silver-Russell syndrome in children born through ART (Bliek et al. 2006, Kagami et al. 2007). Another study done by Kobayashi et al. (2009a) showed methylation defects at the *H19* and *GTL2* imprinted loci in 17 out of 78 ART embryos. Altered methylation patterns at these loci were also found in the sperm of the infertile fathers suggesting that the abnormal hypomethylation at these loci were transmitted to the offspring via the sperm and not as a consequence of ART (Kobayashi et al. 2009a). These findings suggest an association between the epigenetic regulation of imprinted genes and defects in spermatogenesis, raising concerns about the effect that these aberrant imprints in male gametes could have on the subsequent generation (as reviewed in Filipponi, Feil 2009).

Several growth and developmental disorders such as Beckwith-Weidemann syndrome, Angelman syndrome and Prader-Willi syndrome are a result of aberrations in the methylation patterns of ICRs (Buiting et al. 1995, Brown et al. 1996, Gicquel et al. 2005).

3.1.3 IG-DMR – A KEY REGULATOR OF THE *DLK1-DIO3* IMPRINTED DOMAIN

The majority of imprinted genes tend to occur in discrete clusters throughout the genome with their expression being coordinately regulated by the differentially methylated ICRs (Edwards, Ferguson-Smith 2007). One such region of the human genome that harbors an imprinted gene cluster is 14q32.2. This 1 Mb imprinted domain carries a number of paternally expressed genes (PEGs) such as *DLK1, DIO3* and *RTL1* and non-coding maternally expressed genes (MEGs) such as *GTL2, RTL1as*

and *MEG8* (Irving et al. 2010). This region is regulated by a germ-line derived primary intergenic DMR, IG-DMR, and a post-fertilization derived secondary *MEG3*-DMR. IG-DMR acts in a hierarchical manner to regulate the methylation of *MEG3*-DMR in somatic tissue (Kagami et al. 2010). For the purpose of this study only IG-DMR will be discussed further.

The IG-DMR acquires its methylation mark in the male germ-line while the oocytes carry the unmethylated maternally inherited copy (Takada et al. 2002). It is only one of three known paternally methylated primary DMRs in humans (Cooper, Constancia 2010). This DMR lies in the intergenic region between two reciprocally expressed genes, the paternally expressed *DLK1* gene and the maternally expressed *GTL2* gene and is required for maintaining the correct imprint status of these genes (Figure 3.2). The human, mouse and sheep genomes display conserved synteny of this region (Paulsen et al. 2001).

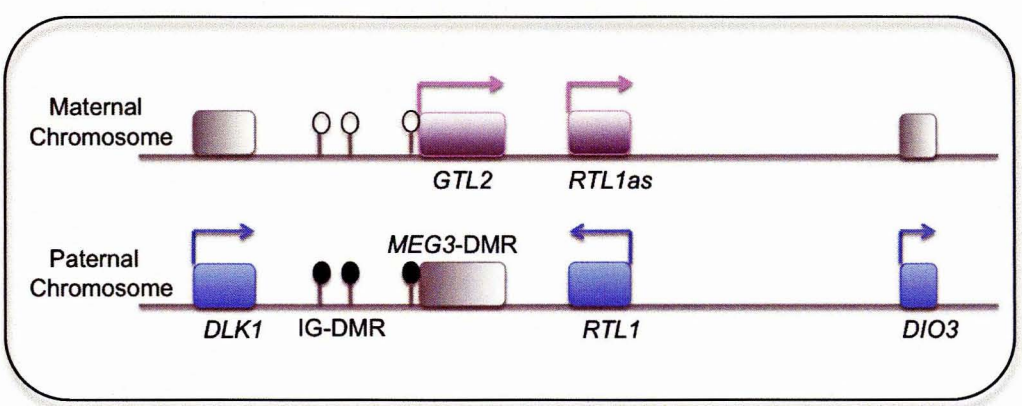

Figure 3.2: Schematic representation of the 1 Mb imprinted domain found on human chromosome 14q32.2. The IG-DMR lies between *DLK1* and *GTL2* and is found to be hypermethylated on the paternal allele and hypomethylated on the maternal allele. The *MEG3*-DMR is a secondary DMR and is found at the promoter of *GTL2* (also known as *MEG3*).

The *DLK1 (Drosophila homologue-like1)* gene forms part of the Notch-Delta gene family, encoding transmembrane proteins containing epidermal growth factor-like repeats (Wylie et al. 2000). It is involved in cellular signaling and differentiation, postnatal growth and tumorigenesis (Laborda 2000, Moon et al. 2002, Yin et al. 2006). The maternally expressed *GTL2 (Gene Trap Locus 2)* gene produces a non-coding RNA transcript with an unknown function (Schuster-Gossler et al. 1998).

Mouse, human and sheep studies, focusing on imprinting anomalies, have provided evidence for the role of the intergenic DMR (IG-DMR), found between the *DLK1* and *GTL2* genes, in growth regulation. A number of case studies have reported that loss of methylation at IG-DMR on the paternal allele leads to a clinical presentation of maternal uniparental disomy [UPD(14)mat] of chromosome 14 with biparental inheritance of chromosome 14 (Temple et al. 2007, Buiting et al. 2008). Patients with UPD(14)mat present with pre- and post-natal growth retardation, developmental delay and short stature (Buiting et al. 2008). Paternal UPD of chromosome 14 [UPD(14)pat] is less common but a few case studies have shown that hypermethylation at IG-DMR on the maternal allele can result in paternalization of this region (Kagami et al. 2008). The characteristics associated with UPD(14)pat are usually severe and include distinct facial dysmorphology including short palpebral fissures, severe developmental delay, growth retardation and skeletal anomalies (Murphy et al. 2003, Stevenson et al. 2004). Epimutations at the IG-DMR result in the aberrant expression of imprinted genes within the 1 Mb domain with an increased expression of PEGs and absent expression of MEGs in UPD(14)pat and increased expression of MEGs and absent expression of PEGs in UPD(14)mat. The phenotypes associated with UPD(14)pat and UPD(14)mat have similarities to those phenotypes seen in offspring affected by FAS.

The primary aim of this study was to examine the effect of paternal alcohol consumption on the methylation status of IG-DMR in sperm DNA and to determine whether alcohol is correlated with methylation in a dose-dependant manner. This assessment was conducted on sperm samples donated by men who consume alcohol and those who do not drink. The IG-DMR is found between two paternally imprinted genes that are critical for embryonic development. Therefore if a link between alcohol consumption in men and hypomethylation of the normally hypermethylated DMR is found, this would contribute to a better understanding of the possible epigenetic mechanism underlying the paternally mediated effects on FASD.

3.2 SUBJECTS AND METHODOLOGY

Reagent and equipment suppliers are listed in Appendix C.

3.2.1 STUDY PARTICIPANTS

The male volunteers were recruited through the Cryobank, a private andrology laboratory and sperm bank in Parktown, Johannesburg, South Africa. The main reason for these men attending the sperm bank was for routine semen analysis; an initial evaluation to determine whether the couples' infertility is attributed to male infertility (Lamb 2010).

Ethics approval for sample collection was obtained from the University of the Witwatersrand, Committee for Research on Human Subjects – Medical (M090555; *shown in Appendix A.2*).

3.2.1.1 SAMPLE COLLECTION

The male volunteers were invited to participate and if they agreed, were asked to fill out an informed consent form and were given a detailed information sheet together with a brief verbal information session *(Consent form and Information sheet are shown in Appendix B.1 and B.2, respectively)*. A questionnaire was administered to these volunteers to assess their drinking patterns and recreational drug and cigarette use. A small section of the questionnaire incorporated questions from the short Michigan Alcoholism Screening Test (sMAST) *(Participant questionnaire is shown in Appendix B.3)*. This was used to assess the psychological aspects of alcohol consumption (Selzer, Vinokur & van Rooijen 1975).

The volunteers were requested to abstain from sexual intercourse and/or ejaculation 48 hours prior to semen sample collection. This was followed by routine semen analysis, done at the Cryobank. The analysis was done to assess sperm concentration, count, morphology and motility. The World Health Organization (WHO) has defined the following as lower reference limits for the parameters assessed during semen analysis:

- Semen Volume: 1.5ml
- Total sperm number: 39 million per ejaculate
- Sperm concentration: 15 million per ml
- Total (progressive and non-progressive) motility: 40%
- Morphologically normal forms: 4% (Cooper et al. 2010)

A total of 149 sperm samples were collected from the Cyrobank, of which only 113 were used in this study. Of the 36 samples that were not used, 7 were azoospermic and did not contain any sperm cells while the remaining 29 samples were disregarded on the basis of insufficient information. A summary of the participant information and semen parameters is shown in *Table E.1, Appendix E.*

3.2.1.2 SAMPLE CLASSIFICATION

A dichotomous classification approach was used for this part of the study, based on questionnaire information (*shown in Appendix B.3*). Individuals were either classified as non-drinkers (controls) or as drinkers based on their reported alcohol consumption. The non-drinkers were individuals who were teetotalers or those individuals who had not consumed alcohol in the past two years. Men consuming as little as one drink per month were placed into the alcohol-consuming group. The amount of alcohol consumed by the men in this group was calculated as a product of the number of drinks consumed per session and the number of sessions per month. This was referred to as drinking frequency and gave an indication of the dosage of alcohol consumed by the men on a monthly basis.

The breakdown of the ethnicities within each treatment group is shown in *Table E.2, Appendix E.*

3.2.3 METHODOLOGY

Molecular methods mentioned here are described in detail in Chapter 2, section 2.2.2.

Once the samples were collected, DNA was extracted and stored at -20°C. Batches of samples were subjected to bisulfite modification followed by PCR.

3.2.3.1 PCR AMPLIFICATION FOR PYROSEQUENCING REACTIONS

A nested PCR was performed for this locus. This involved two consecutive rounds of PCR, where the first round made use of outer primers to amplify the target region while the second round used inner or nested primers to amplify a smaller region within the target region amplified during the first round of PCR (see list of primers Table 3.1). This technique is useful in maximizing amplification when dealing with relatively low quantities of DNA, as is the case with sperm samples. The outer primer set was previously published (Kagami et al. 2010) while the inner primer set was designed using the PSQ Assay Design Software version 1.0.6 (Biotage, Uppsala, Sweden), mentioned in Chapter 2.

Table 3.1: PCR primers for pyrosequencing PCR reactions

	IG-DMR Locus	Primer Sequence (5' – 3')	†Amplicon Length	Annealing Temperature
§**Outer Primers**	Forward	*GTTAAGAGTTTGTGGATTTGTGAGAAATG	452 bp	57°C
	Reverse	CTAAAAATCACCAAAACCCATAAAATCAC		
Inner Primers	Forward	*TTTATTGGGTTGGGTTTTGTTAG	290 bp	58°C
	Reverse	AACCAATTACAATACCACAAAATT		

§ Published primer set (Kagami et al. 2010)

† Amplicon length includes the 23 bp complementary tag sequence

*A 23 bp complementary tag (5'– GACGGGACACCGCTGATCGTTTA – 3') was added to the 5' end of all biotin-labelled primers (Colella et al. 2003)

For round 1 PCR, amplicons were generated in a 50 µl reaction containing 2 µl of bisulfite-treated DNA, 0.4 µM each of the outer forward and reverse PCR primers, 0.125 mM deoxyribonucleotide triphosphates (Bioline, London, United Kingdom), 2.0 mM $MgCl_2$, 1 x Buffer (Applied Biosystems, NJ, United States), 1x GC-rich solution (Roche, Mannheim, Germany) and 1 U of AmpliTaq Gold (Applied Biosystems, NJ, United States). Round 2 PCR amplicons were generated in a 50 µl reaction containing 2 µl of round 1 PCR product, 0.02 µM of the inner forward and 0.2 µM of the reverse PCR primers and 0.2 µM of the universal primer, 0.125 mM deoxyribonucleotide triphosphates (Bioline, London, United Kingdom), 2.0 mM $MgCl_2$, 1 x Buffer (Applied Biosystems, NJ, United States), 1 M Betaine (Sigma-Aldrich, Seelze, Germany) and 1 U of AmpliTaq Gold (Applied Biosystems, NJ, United States).

PCR was performed in a GeneAmp® 2720 thermal cycler (Applied Biosystems, NJ, United States) with the following conditions for both PCR reactions; 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, Ta°C for 30 sec (see Table 3.1 for Ta°C),

and 72°C for 15 sec; with a final extension step of 72°C for 5 min; and a final hold at 15°C. The PCR amplifications for each sperm sample were performed in triplicate.

The maternally imprinted *SNPRN* DMR, mentioned in Chapter 2, was also amplified during this part of the study to assess potential somatic cell contamination. The PCR reaction and thermal cycling condition for this DMR region was described in Chapter 2.

Standard electrophoretic techniques were used to resolve the PCR amplified products. A 3% (w/v) agarose gel containing 1 mg/L ethidium bromide was used to visualize the amplified products at 6 V/cm in 1 x TBE buffer. A 50 bp DNA molecular size marker was used as a size standard.

3.2.3.2 PYROSEQUENCING REACTIONS AND DATA ANALYSIS

Due to the length of the IG-DMR PCR product (290 bp), two sequencing primers were used to cover the region in order to assess the quantitative methylation of the all the CpGs within IG-DMR using pyrosequencing technology (Table 3.2).

Table 3.2: Primers for pyrosequencing assays

Locus	Sequencing Primer Sequence (5' – 3')	Number of CpG Sites Analyzed	§Sequence to Analyze
IG-DMR_reg1	**R:** CAATTACAATACCACAAAAT	7	TAC**R**AATTTAAC**R**AACC**R**C**R**AACAACTAAC**R**AACCACTC**R**CAATTAA CAA ATC**R**CTAACAATTAACAAACCATAAACAA
IG-DMR_reg2	**R:** CCATAAACAACTATAAACCT	3	C**R**AACAACTATAAACCAC**R**AACAACTAATAAACCAC**R**AACAACTAA TAAACCACAAACAACT

§The sequence to analyze is immediately 3' to the sequencing primer binding site on the biotinylated strand

The positions of the CpG sites analyzed are shown by their IUPAC codes in bold font.

The assays have several built-in bisulfite conversion quality control checks. These checks are designed to analyze cytosines that do not form part of CpG dinucleotides and are therefore analyzed as C to T sequence variation. All cytosines not part of CpG dinucleotides are expected to be unmethylated and therefore should undergo complete bisulfite conversion.

The pyrosequencing data were analyzed using the Pyro Q-CpG software (Biotage, Uppsala, Sweden). The data generated can either be scored as passed, check or failed. This gives the user confidence in the quality of the assay and the resulting data generated. Pyrosequencing was performed in triplicate for each sample. When examining the triplicate data set for each sample, a greater than 6% methylation difference per CpG site was used as a cut-off value for unsuccessful replication. As a result, one sample was excluded from this part of the study due to the replicates displaying a greater than 6% methylation difference at more than one CpG site.

3.2.3.3 STATISTICAL ANALYSES

All statistical analyses were performed using Statistica (StatSoft, Inc., version 8.0) unless specified otherwise.

The methylation status of each individual CpG site within the IG-DMR was assessed. In addition, the total methylation of all the CpG sites making up the IG-DMR was also assessed.

Descriptive statistics was done to summarize the data in a quantitative manner. The data were described in terms of central tendency (the means and medians of the data) and dispersion (the standard deviation and range of the data). The data obtained from this part of the study were continuous in nature. Normality testing was done using the Shapiro-Wilks test to determine whether the data was normally distributed (normal distribution if $p > 0.20$). Due to the non-normal distribution of DNA methylation across certain CpG sites, non-parametric analyses were performed. These analyses use the median values rather than mean values.

3.2.3.3.1 HYPOTHESIS TESTING

In this study it was hypothesized that alcohol consumption results in the hypomethylation of the paternally methylated IG-DMR in men who consume alcohol compared to the non-drinkers or controls. It was also hypothesized that alcohol could affect methylation in a dose-dependant manner such that men with higher drinking frequencies tend to display a higher level of hypomethylation compared to men with lower drinking frequencies.

To determine whether DNA methylation differences exist between the control and the alcohol-consuming groups, the non-parametric Mann-Whitney U test was done. Both the methylation of individual CpG sites and the total methylation across all CpG sites were assessed. Spearman's correlations were performed, on the combined group, to determine whether methylation at individual CpG sites or across all CpG sites is influenced by alcohol in a dose-dependent manner.

Significant inter-individual epigenetic variation has been reported to occur in human germ cells (Flanagan et al. 2006). To visualize this variation within and between the two treatment groups the DNA methylation percentage of each individual CpG site for each sperm sample and the total DNA methylation percentage across all CpG sites for each sample were represented in variability plots.

3.2.3.3.2 CONFOUNDING VARIABLES

A number of potential confounding variables were identified in this study due to the nature of the study. These included:

- Age,
- Smoking,
- Drug use,
- Sperm concentration (million/ml),
- Sperm morphology (percentage of normal sperm) and
- Sperm motility (percentage progressive and non-progressive).

These variables may influence DNA methylation.

Spearman's correlations were performed for the continuous variables (age, sperm concentration, sperm morphology and sperm motility) while the Mann-Whitney U test was performed for binary variables (smoking and drugs). These analyses were done on the combined group, to determine the relationship between each variable and DNA methylation at the level of individual CpG sites and across all CpG sites within IG-DMR. Once significant associations were identified with particular CpG sites, the responsible confounding variables were used to build a multiple regression model.

These variables were entered into the model such that the effect of each independent variable on the single dependant variable (in this case methylation) could be estimated when in the presence of other variables. In situations where two independent variables were found to highly correlated, a forward stepwise method was used for multiple regression analysis. This advanced method enters independent variables into the model, one at a time in sequence. If the variable contributes to the model, it is retained but all other variables in the model would be re-tested to see whether they still contribute to the success of the model. If a variable is not found to significantly contribute to the model, it will be excluded.

Multiple regression analysis was done twice for each CpG site, first including treatment (no alcohol consumption vs. alcohol consumption) followed by including drinking frequency into the analysis. These two independent variables were most important in terms of testing the hypothesis. The resulting model equation has been written in the form of:

$$Y = B_0 + B_1X_1$$

Methylation (%) = Intercept + Regression coefficient (Independent variable)

where Y is the dependent variable that was to be predicted; X_1 represents the independent variable that was used to predict Y; B_1 is the regression coefficient that describes the size of the effect that the independent variable X_1 has on the dependent variable Y, and B_0 is the value Y is predicted to have when all the independent variables are equal to zero.

3.2.3.3.3 ABNORMAL SPERM PARAMETERS, METHYLATION AND ALCOHOL

Literature has shown that altered imprint patterns can be associated with sperm parameter abnormalities. The WHO reference values for human sperm parameters (Cooper et al. 2010) were used to classify individuals into various sperm abnormality groups. Individuals with 3 or less abnormal parameters were included in this study. It was important to ascertain whether methylation differences exist between the group of individuals with normal sperm parameters and those with abnormal sperm parameters. Due to the effect of alcohol on the overall DNA methylation status, only the control samples (non-drinkers) were categorized according to sperm abnormality parameters.

Alcohol is known to adversely affect the production, development and maturation of sperm. The Mann-Whitney U test was performed to assess whether sperm parameters differ between the control and alcohol-consuming group. The effect of alcohol dosage on the sperm parameters was also assessed using Spearman's correlations. Finally, the Fisher's exact test was used to determine whether the number of individuals with abnormal parameters within the alcohol-consuming group was significantly different to the number of individuals with abnormal parameters within the control group.

For all statistical tests performed, a p-value < 0.05 was considered to be significant.

3.3 RESULTS

All samples were assayed in triplicate on the Pyrosequencer. The average methylation percentage for each CpG site was calculated by averaging the triplicate values for each CpG site per sample. The average methylation percentage per CpG site was then averaged across all CpG sites analyzed to give an average methylation precentage per sample (*shown in Appendix I – IG-DMR Data and SNRPN Data*). This was then used to calculate the average methylation percentage per group.

3.3.1 DETECTION OF SOMATIC DNA CONTAMINATION IN SPERM SAMPLES

As discussed in Chapter 2, a DMR associated with *SNRPN*, a maternally imprinted locus was used to control for somatic cell DNA contamination in the sperm samples. A total of 22 (19.5%) samples out of the 113 samples collected were assayed for this locus (*shown in Appendix I – SNRPN Data*). The mean methylation of this DMR was found to be 2.43% with a standard deviation of 1.35. The theoretical methylation value for DMRs in somatic tissue is 50% (Cooper, Constancia 2010). However studies have shown that somatic DMRs display methylation levels between 35% and 65% (Woodfine, Huddleston & Murrell 2011). Therefore the low level of methylation at the *SNRPN* DMR within the sperm samples indicates that the paternal allele may not be completely devoid of methylation but rather displays basal levels of methylation. It was concluded that the samples were not significantly contaminated with somatic cells. The sole purpose of incorporating this locus into the study was as a proxy for somatic cell contamination and therefore the data for *SNRPN* was not included in subsequent analyses.

3.3.2 THE EFFECT OF ALCOHOL CONSUMPTION ON DNA METHYLATION

3.3.2.1 CHARACTERISTICS OF THE DATASET

Complete results were obtained for 112 (99%) of the 113 samples. One sample was removed from this study due to inconsistent triplicate results. Of the 16 CpG sites present within the IG-DMR CpG island, only ten were analyzed using pyrosequencing. Consistent results could not be obtained for the remaining six CpG sites due to the number of repeats in the region (Geuns et al. 2007).

3.3.2.2 DNA METHYLATION ANALYSIS OF INDIVIDUAL CPG SITES IN SPERM SAMPLES

The methylation levels of the individual CpG sites are given in Table 3.3.

Table 3.3: Per group and individual CpG methylation levels for the IG-DMR locus within the two treatment groups (N = 112)

	Controls		Alcohol Consumers		[§]Mann-Whitney U Test	
Sample size (N)	34		78			
	[†]**Mean (SD)**	**Median (Range)**	**Mean (SD)**	**Median (Range)**	**P-Values**	
Average Methylation (%) per CpG site:						
CpG 1	93.25 (1.15)	93.33 (90.67-96.33)	92.82 (1.93)	93.00 (83.67-96.00)	0.527	
CpG 2	93.99 (0.92)	94.00 (91.67-96.00)	92.57 (6.81)	93.67 (48.00-95.67)	0.133	
CpG 3	97.54 (1.18)	97.67 (94.67-100.00)	96.96 (1.34)	97.33 (89.67-99.33)	0.032	*
CpG 4	67.33 (4.63)	67.00 (59.33-78.67)	66.15 (5.89)	66.50 (50.67-78.67)	0.569	
CpG 5	77.52 (2.76)	78.50 (70.00-82.00)	77.38 (3.38)	78.33 (66.00-82.67)	0.914	
CpG 6	96.46 (1.65)	97.00 (91.33-98.67)	96.42 (2.01)	97.00 (90.33-99.33)	0.709	
CpG 7	98.05 (0.96)	98.00 (96.00-100.00)	97.39 (1.73)	98.00 (88.33-99.67)	0.070	
CpG 8	88.03 (1.49)	88.33 (85.00-90.33)	87.50 (1.86)	87.67 (80.67-90.67)	0.179	
CpG 9	97.42 (0.91)	97.33 (95.00-99.33)	96.39 (5.74)	97.33 (51.00-99.33)	0.314	
CpG 10	97.86 (0.70)	98.00 (96.67-99.33)	97.71 (0.77)	98.00 (95.00-99.33)	0.595	
Average Methylation (%) per group	90.76 (0.90)	90.83 (89.10-92.47)	90.13 (1.43)	90.50 (86.23-93.23)	0.052	
Drinking Frequency (drinks/month)	N/A	N/A	35.94 (51.45)	15.00 (1.00-360.00)	N/A	

[§] The Mann-Whitney U test evaluates the differences in the medians of the two groups based on assigning a rank to each data value

[†] The mean values of the individual CpG sites were used for constructing the bar graph

* Significant at $p<0.05$ level

The mean methylation for the individual CpG sites and per group was marginally higher in the controls when compared to the alcohol consumers. The overall methylation and individual CpG methylation between individuals within the control group ranged between 89.10% and 92.47% while the methylation between individuals within the alcohol-consuming group was between 86.23% and 93.23%. The standard deviation, which gives an indication of the spread of the data from the mean, was found to be lower in the controls compared to the alcohol-consuming group. The data of the alcohol-consuming group were spread over a larger range of values when compared to the controls. The variability of the methylation within and between groups as well as within and between individual CpG sites is shown in Figure 3.3 and Figure 3.4, respectively.

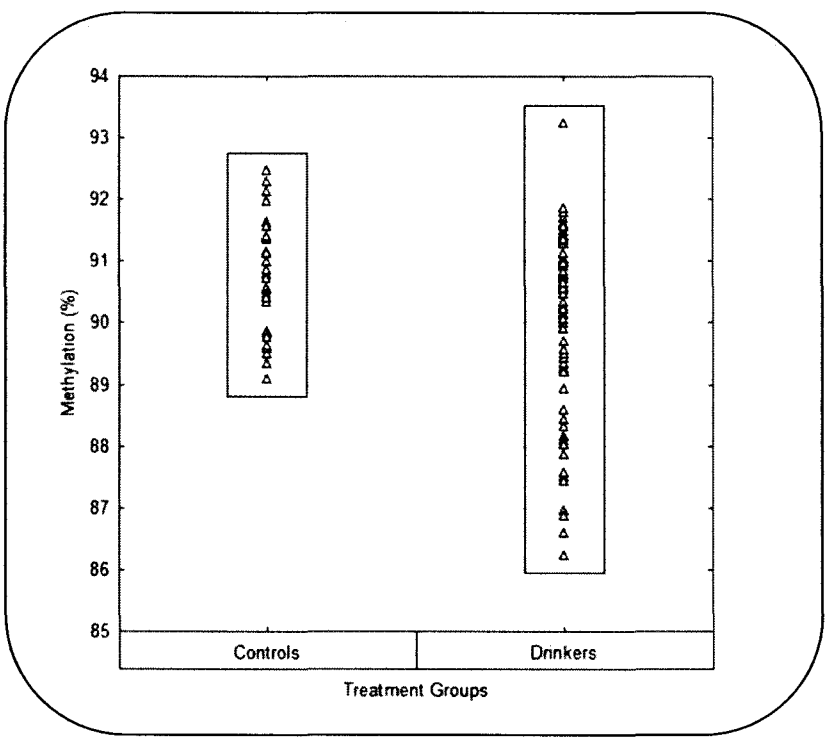

Figure 3.3: Variability plot illustrating the spread in average methylation percentage at IG-DMR, within and between the two treatment groups.

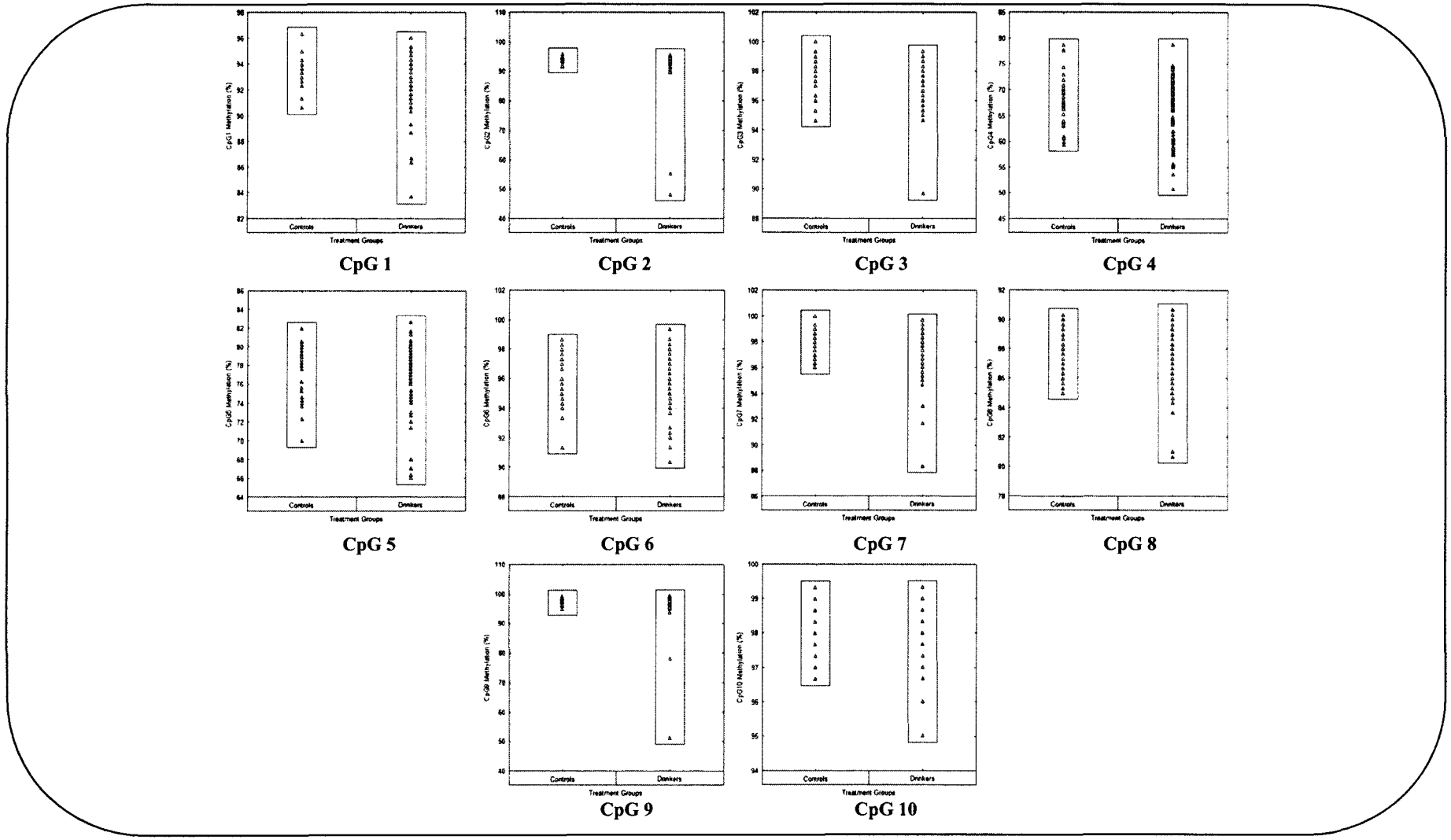

Figure 3.4: Variability plots illustrating the spread in methylation percentage at individual CpG sites within and between the two treatment groups.

The non-parametric Mann-Whitney U test was performed to assess whether a significant difference exists between the medians of the two groups at each CpG site, following alcohol consumption (Mann-Whitney p-values are indicated in Table 3.3). Only one of the ten CpG sites was found to be significantly different between the two groups (Figure 3.5). The methylation percentage at CpG 3 was significantly different (p=0.032) between the two groups with the alcohol-consuming group showing a reduction in methylation at this site. CpG 7 was the only other site that showed a trend towards being significantly different (p=0.070) between the two groups, with the alcohol-consuming group showing a modest reduction in methylation at this site.

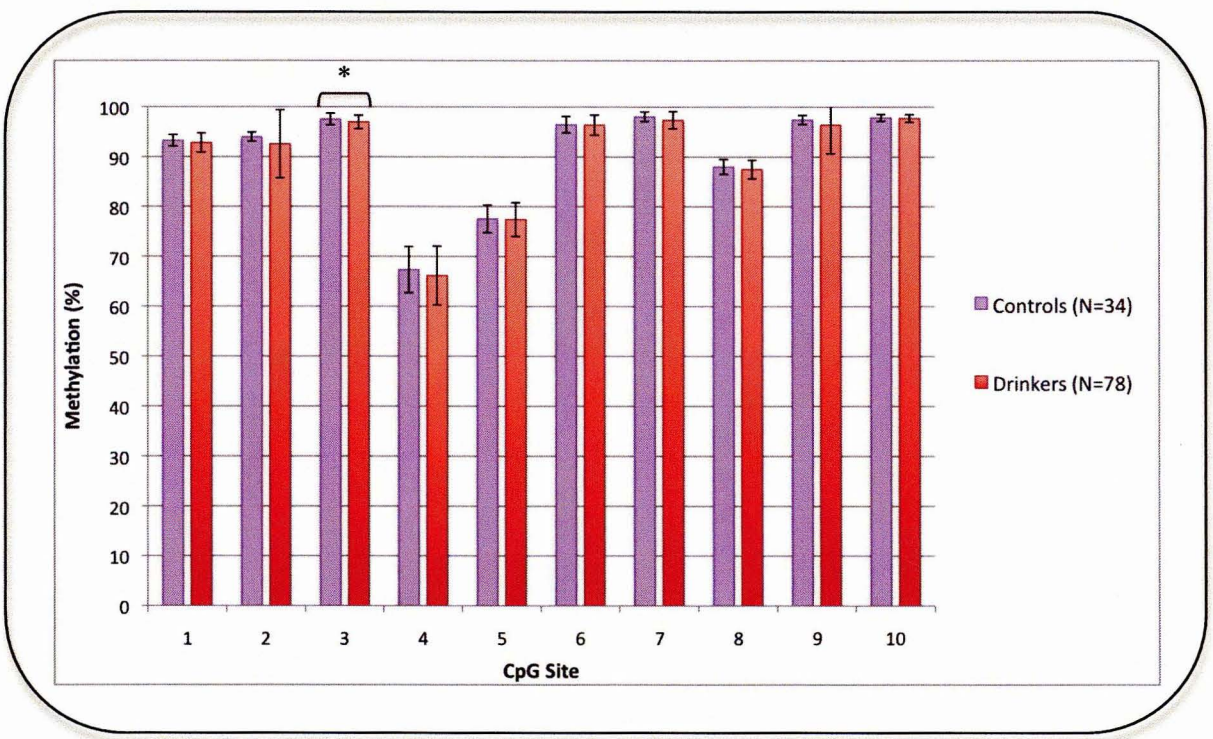

Figure 3.5: Average DNA methylation levels for individual CpG sites between the two treatment groups. Note: The error bars represent the standard deviation around the mean.

Spearman's correlations were performed to determine the presence of relationships, if any, between drinking frequency and DNA methylation at individual CpG sites.

These correlations were done on the combined (controls and drinkers) group. A notable trend towards a reduced methylation level at CpG 3 was observed with increasing drinking frequency (Spearman's rho=-0.179, p=0.059). A similar but significant relationship was observed between the DNA methylation level at CpG 7 and drinking frequency. DNA methylation significantly decreased at this site as drinking frequency increased (Spearman's rho=-0.197, p=0.037), however, the effect size is very small (Figure 3.6).

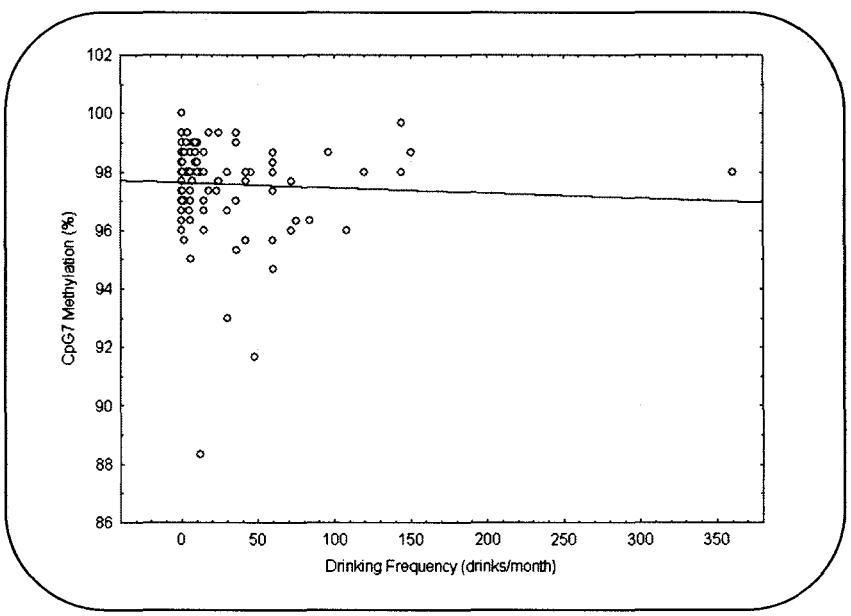

Figure 3.6: Correlation plot displaying the relationship between the methylation levels at CpG site 7 and drinking frequency.

3.3.2.3 DNA METHYLATION ANALYSIS PER GROUP

The methylation levels per group are shown in Table 3.3. Alcohol consumption was correlated with a borderline significant decrease in methylation (p=0.052) in the alcohol-consuming group without taking any confounders into account (Figure 3.7).

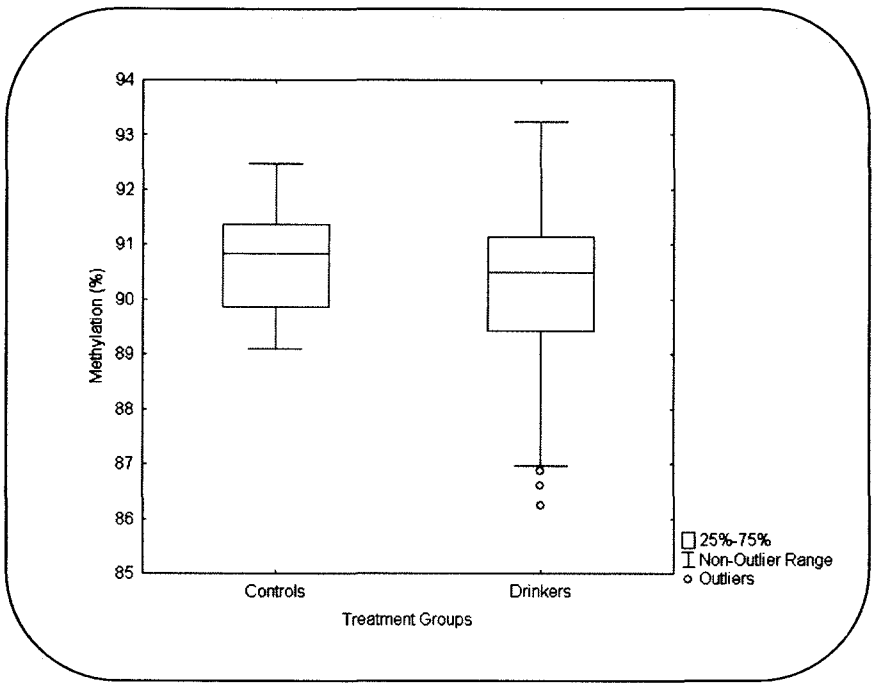

Figure 3.7: Box plot illustrating the difference in average methylation levels between the control and alcohol-consuming groups.

No association was noted when correlating drinking frequency with average methylation (Spearman's rho=0.009; p=0.920).

3.3.2.4 THE EFFECT OF CONFOUNDING VARIABLES ON DNA METHYLATION

The quantitative statistics of the potential confounding variables are shown in Table 3.4.

No significant differences were noted between the two groups for five of the six variables. However, a trend towards increased cigarette use was observed within the alcohol-consuming group.

Table 3.4: Potential confounding variables within the two treatment groups

	Controls		Alcohol Consumers		[§]Mann-Whitney U Test
Sample size (N)	34		78		
Variable	**Mean (SD)**	**Median (Range)**	**Mean (SD)**	**Median (Range)**	**P-Value**
Age (years)	34.32 (5.87)	35.00 (22.00-50.00)	33.86 (5.45)	34.00 (20.00-49.00)	0.697
Sperm Concentration (million/ml)	51.96 (35.32)	43.00 (11.00-167.00)	58.39 (58.43)	41.50 (4.20-310.00)	0.626
Sperm Morphology (% normal)	11.00 (5.64)	10.50 (1.00-21.00)	11.21 (6.38)	12.00 (1.00-25.00)	0.859
Sperm Motility (%)	54.41 (10.57)	52.50 (30.00-70.00)	55.64 (11.18)	60.00 (20.00-75.00)	0.440
	Count (Frequency)		**Count (Frequency)**		**χ^2 Test**
Cigarette Use	6 (17.65%)		31 (39.74%)		$\chi^2 = 3.500$, df=1, p=0.061
Drug Use	1 (2.94%)		5 (6.41%)		$\chi^2 = 0.532$, df=1, p=0.466

[§] The Mann-Whitney U test evaluates the differences in the medians of the two groups based on assigning a rank to each data value

The effect of the six confounding variables on methylation was assessed at individual CpG sites and across all CpG sites. The effects were assessed on the combined group.

3.3.2.4.1 THE EFFECT OF CONFOUNDERS ON METHYLATION LEVELS OF INDIVIDUAL CPG SITES

Only those confounders that had a significant relationship with DNA methylation at individual CpG sites and those that had a relationship that tended towards significance are represented in Table 3.5.

Table 3.5: Spearman's correlation analysis between the confounding variables and DNA methylation at specific CpG sites

Confounding Variable	CpG Site	Spearman's rho	P-value	
Age	CpG 3	0.188	0.048	*
	CpG 4	0.411	0.00001	*
	CpG 5	0.189	0.046	*
	CpG 6	0.183	0.054	
	CpG 7	0.169	0.074	
Sperm Concentration	CpG 8	0.195	0.039	*
Sperm Morphology	CpG 8	0.292	0.002	*
Sperm Motility	CpG 4	-0.184	0.052	

*Significant at $p<0.05$ level

It should be noted that the relationship between the confounders and methylation was site-specific.

Age and DNA methylation displayed a significantly positive relationship at three of the ten CpG sites analyzed. The methylation levels at CpG sites 3, 4 and 5 significantly increased as age increased. In addition, methylation levels at CpG sites 6 and 7 showed a trend towards higher methylation as age increased.

The methylation level at CpG 8 displayed a significant positive relationship with sperm concentration ($p=0.039$) and sperm morphology ($p=0.002$). Therefore methylation levels increased as sperm concentration increased and as the percentage of morphologically normal sperm increased.

A negative correlation was noted between the methylation level at CpG 4 and sperm motility, with a trend towards increased methylation with a decrease in sperm motility ($p=0.052$).

The Mann-Whitney U test was done to assess the effect of drugs and smoking on DNA methylation. DNA methylation levels at only one out of the ten CpG sites analyzed was affected by drugs and smoking. The methylation level at CpG 3 was significantly reduced in the presence of drug usage ($p=0.048$). In addition the methylation level at CpG 3 showed a modest trend towards lower methylation in the presence of smoking ($p=0.085$).

Multiple regression analyses were performed for select CpG sites where the methylation at these sites was significantly influenced by more than one independent variable (Table 3.6). The analysis was done twice for each CpG site. While treatment

was incorporated into the first model, the second model incorporated drinking frequency.

Table 3.6: Multiple regression analysis for specific CpG sites

		Methylation			
	Variable	**%**	**SE**	**P-value**	
Alcohol Consumption					
CpG 3	Intercept	97.08	1.03	0.000	
	Treatment	-0.52	0.26	0.046	*
	Age	0.05	0.02	0.015	*
	Drug usage	-0.81	0.53	0.128	
	Adjusted $R^2 = 0.09$				
CpG 4	Intercept	52.26	3.41	0.000	
	Treatment	-0.97	1.01	0.338	
	Age	0.47	0.08	0.000	*
	Adjusted $R^2 = 0.22$				
CpG 8	Intercept	87.81	0.68	0.000	
	Treatment	-0.55	0.35	0.123	
	Sperm Morphology	0.06	0.03	0.010	*
	Adjusted $R^2 = 0.06$				
Drinking Frequency					
CpG 3	Intercept	96.20	0.96	0.000	
	Drinking Frequency	0.0003	0.003	0.899	
	Age	0.05	0.02	0.014	*
	Drug usage	-0.89	0.54	0.102	
	Adjusted $R^2 = 0.05$				
CpG 4	Intercept	50.26	2.93	0.000	
	Drinking Frequency	0.006	0.01	0.563	
	Age	0.47	0.08	0.000	*
	Adjusted $R^2 = 0.21$				
CpG 5	Intercept	73.48	1.87	0.000	
	Drinking Frequency	0.009	0.006	0.168	
	Age	0.11	0.05	0.044	*

	Adjusted R^2=0.03				
CpG 8	Intercept	86.89	0.34	0.000	
	Sperm morphology	0.06	0.03	0.011	*
	Adjusted R^2=0.05				

*Significant at p<0.05 level

While treatment displayed a negative regression coefficient for each of the CpG sites in Table 3.6, it was only found to significantly predict a decrease in methylation (%) at CpG 3 when in the presence of other variables. Contrary to this, drinking frequency displayed a positive coefficient for each of the CpG sites. However, these coefficients were so close to zero that drinking frequency was not considered to be a predictor of methylation at any of the sites when in the presence of other variables.

Age was a significant predictor of methylation (%) at CpG 3 and 4 in the presence of alcohol consumption. For every year increase in age, methylation (%) significantly increased. In contrast, age was not found to be a significant predictor of methylation (%) at CpG 5 *(shown in Table J.1, Appendix J)*. When including drinking frequency into the model, in place of treatment, age was still found to significantly predict methylation (%) at CpG 3 and 4.

Sperm concentration and sperm morphology were both significantly correlated with methylation at CpG 8 (Table 3.5). However, multicollinearity was observed between these two predictor variables (Spearman's rho=0.493, p=0.000). Due to this strong positive correlation, a forward stepwise method was used for multiple regression analysis. Sperm morphology was found to significantly predict methylation at CpG 8 when incorporating treatment into the model. For every percentage increase in normal

sperm morphology, methylation would increase by 0.06%. When the stepwise method was used incorporating drinking frequency, sperm concentration and sperm morphology, sperm morphology was found to be the only predictor variable that contributed significantly to the success of the model.

The variance in methylation (%), explained by the adjusted R^2 value in each of the models, at each CpG site was minimal. This suggests that other variables could be responsible for the remaining variance that is unaccounted for.

3.3.2.4.2 THE EFFECT OF CONFOUNDERS ON METHYLATION LEVELS ACROSS ALL CPG SITES

Age was the only confounder significantly associated with average methylation across all CpG sites. A strong positive correlation was noted (Spearman's rho=0.280; p=0.003). As age increased so did the average methylation across all CpG sites. While no correlation was observed between sperm concentration and DNA methylation (Spearman's rho=-0.002, p=0.984), a slight positive correlation was observed between sperm morphology and DNA methylation (Spearman's rho=0.050, p=0.603). On the other hand, sperm motility displayed a negative but non-significant association with methylation (Spearman's rho=-0.126, p=0.187).

The Mann-Whitney U test found no influence of smoking and drugs on methylation across all CpG sites (p=0.627 and p=0.220, respectively).

Two multiple regression analyses were performed, with both analyses incorporating age as the independent variable. In addition one model incorporated treatment as the

second variable and the other model incorporated drinking frequency as the second variable.

Model 1:

Average Methylation (%) = 89.72 – 0.61(Treatment) + 0.05(Age)

The average methylation (%) across all CpG sites was predicted to significantly decrease by 0.61% in the presence of alcohol consumption (p=0.021) and increase by 0.05% for every year increase in age (p=0.029). In the event that the regression coefficients for the two independent variables were zero, average methylation was predicted to be 89.72%. This model accounted for 7.31% of variation in average methylation across all CpG sites.

Model 2:

Average Methylation (%) = 88.50 + 0.002(Drinking frequency) + 0.05(Age)

Drinking frequency was not considered to be a predictor of average methylation (%) due to the regression coefficient being so close to zero (p=0.285). However, age was found to be a significant predictor of average methyaltion with methylation significantly increasing by 0.05% for every year increase in age (p=0.022). Average methylation was predicted to be 88.50% in the event that the regression coefficients of the two independent variables were zero. These two predictor variables only accounted for 3.70% of the variation in average methylation across all CpG sites.

Therefore modeling both treatment and age together revealed highly significant, independent associations with DNA methylation. Alcohol dosage did not seem to play an important role in predicting methylation.

3.3.3 THE ASSOCIATION BETWEEN ABNORMAL SPERM PARAMETERS AND DNA METHYLATION

Three sperm parameters were assessed in this study: sperm concentration, sperm morphology and sperm motility. Individuals with sperm concentration levels less than 15 million per ml were classified as being oligozoospermic, while individuals with a sperm morphology percentage less than four percent were classified as being teratozoospermic and individuals with total sperm motility percentage less than 40% were classified as being asthenozoospermic. It should be noted that some samples had more than one sperm abnormality. The control samples were categorized into the various sperm abnormality groups *(shown in Table K.1, Appendix K)*. However, due to the small sample size of each category, no statistical analysis could be performed to determine whether abnormal sperm parameters are correlated with alterations in methylation patterns.

3.3.4 THE EFFECT OF ALCOHOL ON ABNORMAL SPERM PARAMETERS

No significant differences were observed in the sperm parameters between the control and alcohol-consuming groups when performing the Mann-Whitney U test (Table 3.4).

When correlating alcohol dosage to the sperm parameters, no relationship was observed between drinking frequency and sperm morphology (Spearman's rho=-0.035, p=0.715) while drinking frequency and sperm motility displayed a slight positive but non-significant correlation (Spearman's rho=0.068, p=0.477). There was only one slight trend with drinking frequency, which was towards decreasing sperm concentration (Spearman's rho=-0.161, p=0.091).

The numbers of individuals with abnormal sperm parameters in the control group were lower than the numbers of individuals with abnormal sperm parameters in the alcohol-consuming group *(shown in Table L.1, Appendix L)*. No significant differences were observed between the numbers of individuals with abnormal parameters in the alcohol-consuming group and the numbers of individuals with abnormal parameters in the control group.

In summary the results of this study show no alteration in methylation levels between the normal and abnormal sperm parameter groups. The sperm parameters were not statistically influenced by alcohol consumption nor were they influenced by the amount of alcohol consumed.

3.4 DISCUSSION

The primary aim of this part of the study was to determine whether paternal alcohol consumption is correlated with an altered methylation status of IG-DMR and to determine whether there is a dose-dependant correlation. The results presented here demonstrate that paternal alcohol consumption is correlated with a modest decrease in

the overall methylation at the IG-DMR locus regardless of the amount of alcohol consumed thus supporting the hypothesis put forth in Chapter 1. These results are in agreement with those published by Ouko et al. (2009) who also focused on the IG-DMR locus in human sperm (Ouko et al. 2009).

3.4.1 EFFECT OF ALCOHOL ON METHYLATION AT IG-DMR

Alcohol is known to be correlated with methylation changes in a locus-specific manner, with increased methylation at certain loci and decreased methylation at other loci (Liu et al. 2009). A recent study conducted to determine the effect of alcohol on global methylation in human sperm found a trend towards decreased methylation (personal communication) suggesting that more loci could be hypomethylated following alcohol exposure. A modest decrease in the overall methylation at the IG-DMR locus was observed in this study. Alcohol consumption is known to interrupt the critical one-carbon metabolism pathway by inhibiting the reaction catalyzed by methionine synthase. This subsequently leads to an increase of potent inhibitors of the DNMT enzymes that are critical for establishing and maintaining methylation patterns (Halsted, Villanueva & Devlin 2002, Liu et al. 2009). This serves as a possible explanation for the overall decrease in methylation observed at IG-DMR.

In addition to the overall reduction in methylation following alcohol consumption, CpG 3 showed significantly reduced DNA methylation levels in the alcohol-consuming group while another site, CpG 7, showed a trend towards reduced methylation in the same group. It is not uncommon to observe reduced methylation levels at specific CpG sites following alcohol exposure and these findings agree with

previous findings (Haycock, Ramsay 2009, Ouko et al. 2009, Qiang et al. 2010 Stouder, Somm & Paoloni-Giacobino 2011). The mechanism and reason behind why certain CpG sites are preferentially demethylated remains unclear. However, a study done by Oka et al. (2006) found certain CpG sites within the *Fgf-1* gene to be preferentially methylated by Dnmt3a (Oka et al. 2006). Therefore the single CpG site found to be preferentially demethylated in this study might fall within a DNMT3L recognition sequence. This DNMT enzyme is responsible for establishing methylation imprints in the male and female germ-lines (Kato et al. 2007). Alcohol consumption is known to inhibit DNMT enzymes through the one-carbon metabolism pathway (Halsted, Villanueva & Devlin 2002) therefore this recognition site would unusually be hypomethylated due to the inhibition of DNMT enzymes and this could trigger a wave of demethylation across adjacent CpG sites. The mechanism underlying this preferential demethylation of select CpG sites remain undetermined. However, a possible reason for the preferential demethylation of specific CpG sites may lie in the conformation of the chromatin. Under normal circumstances the chromatin is highly condensed and hypermethylated at this region. Specific CpG sites that serve as DNMT recognition sites may be preferentially exposed for easy accessibility. Following alcohol consumption, these specific CpG sites become preferentially demethylated due to the inhibition of the DNMT enzymes via the one-carbon metabolism pathway. The other CpG sites that are less accessible due to the conformation of the chromatin remain methylated until a wave of demethylation spreads from the preferentially demethylated CpG site. It is important to note that IG-DMR does not bind a CTCF-binding protein nor is it known to bind transcription factors (Carr et al. 2007), therefore the reduced methylation at CpG 3 may not affect the binding of any such factors.

While drinking frequency was not correlated with an overall alteration in DNA methylation, site-specific methylation alterations were noted. Drinking frequency was correlated with a decrease in methylation at CpG 7 while a trend towards reduced methylation was noted at CpG 3. These findings corroborate the results obtained by Biermann et al. (2009), where alcohol-drinking patterns negatively correlated with methylation at five CpG sites found within the NR2B promoter (Biermann et al. 2009). A possible reason for this preferential demethylation could again be attributed to the conformational structure of the chromatin in this region.

3.4.2 EFFECT OF CONFOUNDERS ON DNA METHYLATION

Besides age, none of the other confounders were found to influence the overall DNA methylation at IG-DMR. It is often thought that older age is associated with decreased methylation. However, studies have shown that methylation tends to increase with age at several loci, including the telomere maintenance loci (Siegmund et al. 2007, Christensen et al. 2009). IG-DMR is associated with a CpG island (Takada et al. 2002). The age-related increase in methylation at this locus is consistent with literature that has demonstrated that age-related increases in methylation tend to be associated with loci found within CpG islands (Kwabi-Addo et al. 2007).

An interesting observation was made regarding the proportion of men who participate in co-morbid smoking and drinking behavior. The proportion noted in this study strongly parallels what has been reported in the United States (Grant et al. 2004). In terms of DNA methylation, no alterations in methylation patterns were observed following smoking and illegal drug usage. This is contrary to other studies that have

shown smoking and drug use to be correlated with altered DNA methylation patterns (Launay et al. 2009, Nielsen et al. 2009, Breitling et al. 2011). A possible reason for the absence of a correlation between smoking and DNA methylation and between illegal drug use and DNA methylation in this study may be that methylation alterations were present in the sperm samples of smokers and drug users but that the alterations were present in only a minor pool of the spermatozoa, with the vast majority of the spermatozoa having normal methylation patterns and this could have masked the altered imprint patterns.

3.4.3 INTER-INDIVIDUAL EPIGENETIC VARIATION

Visual inspection of the overall methylation and individual CpG methylation between individuals in the same treatment group and between treatment groups showed high levels of inter-individual variation within and between the two treatment groups. This epigenetic variability in human germ cells has been noted previously (Flanagan et al. 2006). The methylation levels of the control group were more tightly clustered but still showed a considerable level of inter-individual variation compared to the alcohol-consuming group, which showed a greater spread of methylation levels.

Age is known to influence DNA methylation at specific loci (Boks et al. 2009, Christensen et al. 2009), however the spread in methylation levels observed in the alcohol-consuming group could not be attributed to age since the age range of the control and alcohol-consuming group was comparable. The fact that the control individuals display epigenetic variation, albeit to a limited extent, suggests that this locus displays some epigenetic plasticity in the germ cells. In the alcohol-consuming

group, this plasticity may render this locus more susceptible to environmental influences such as alcohol and long-term exposure to alcohol could further increase the epigenetic variability observed. The observations reported here are similar to those reported by Li et al. (2011) who exposed identical mice to long-term methyl donor dietary supplements. The mice exposed to the methyl donors had higher levels of epigenetic variation compared to the controls and the variation was found to increase with an increase in the duration of exposure (Li et al. 2011).

3.4.2 LACK OF ASSOCIATION BETWEEN ABNORMAL SPERM PARAMETERS AND DNA METHYLATION

The main focus of this study was not to examine whether methylation alterations are present and could be responsible for abnormal sperm parameters. However, a number of the sperm samples used in this study were outside the normal range for at least one of the parameters. It was thus important to determine whether the abnormal parameters were correlated with altered methylation patterns and if the abnormal parameters may be correlated with the overall reduction in methylation observed following alcohol exposure. However, this question could not be assessed in this study due to the small sample size of the various sperm abnormality groups. It would be crucial to assess this question as other studies have shown that men with abnormal sperm parameters display altered methylation patterns at certain imprinted loci (Kobayashi et al. 2007, Marques et al. 2008, Marques et al. 2009, Poplinski et al. 2010). These studies suggest that the defects in spermatogenesis could be a result of epigenetic dysregulation of imprinting in the male germ-line (as reviewed in Filipponi, Feil 2009).

3.4.5 ABNORMAL SPERM PARAMETERS AND ALCOHOL – NO RELATIONSHIP

The negative impact of alcohol on the male reproductive system has been known for decades. Not only is alcohol linked to male infertility but it has also been shown to alter testosterone production, disrupt spermatogenesis and adversely affect sperm concentration, morphology and motility in humans and animals (Pajarinen et al. 1996, Muthusami, Chinnaswamy 2005, Sermondade et al. 2010). The aim of this study was not to determine the cytotoxic effects of alcohol on sperm quality, however it was interesting to note that the alcohol-consuming group had a slightly higher number of individuals with abnormal sperm parameters than the control group, although this was not significant. This suggests that alcohol may not responsible for the disruption in spermatogenesis. A number of other environmental exposures and toxins such as therapeutic drugs, pesticides and irradiation (as reviewed in Delbes, Hales & Robaire 2010) could be responsible for the abnormal parameters seen in this group of individuals. Alternatively, a lack of significance could be due to the relatively small sample sizes of the various sperm abnormality categories.

3.4.6 THE EFFECT OF PATERNAL ALCOHOL CONSUMPTION ON OFFSPRING DEVELOPMENT

The alterations in methylation patterns observed at the IG-DMR locus in sperm following paternal alcohol consumption could have negative consequences for the development of the offspring. It is plausible that sperm with subtle differences in DNA methylation may still have the ability to fertilize oocytes and give rise to embryos. However, these embryos would inherit the altered imprint from the paternal allele. While the paternal and maternal genomes undergo a wave of demethylation

during embryogenesis, the imprinted genes resist this wave of demethylation. Therefore the altered methylation pattern at IG-DMR would likely be stably maintained in the embryo, affecting the regulation of the *DLK1/GTL2* locus. This may affect the post-natal development of the offspring.

A study done by Knezovich and Ramsay (awaiting publication) focused on the effects of preconception paternal alcohol consumption on imprinted loci in mouse sperm and their offspring. The results were contrary to those found in this study, as no significant demethylation was observed at the Ig-DMR locus between the sucrose-treated and alcohol-exposed mouse sperm. However, significant decreases in methylation were observed at the imprinted loci in the offspring of the alcohol-treated males. These offspring were found to weigh significantly less during the weaning period, compared to the offspring fathered by the sucrose-fed males. The researchers suggested that alcohol could have potentially induced the oxidation of 5-methylcytosine (5-mc) to form 5-hyroxymethylation (5-hmc) in the sperm. This intermediary modification would then later manifest as demethylated DNA in the offspring. Alternatively alcohol could have affected other epigenetic mechanisms and these alterations could have been inherited by the offspring.

Besides DNA methylation, sperm cells are also capable of transmitting other epigenetic signals, such as ncRNAs and histone modifications, to the oocyte upon fertilization. Various cytoplasmic RNAs are transmitted by the sperm to the oocyte and are crucial for normal embryonic development including establishing of imprints, early embryo patterning and morphometric patterning (Ostermeier et al. 2004, as reviewed in Boerke, Dieleman & Gadella 2007). Studies done in mice have

demonstrated that RNAs transmitted by the sperm have the ability to epigenetically alter the phenotype of the offspring. In one study a microRNA, miRNA-124, important for the development of the nervous system, was injected into the male pronucleus upon fertilization. This resulted in a giant phenotype in the progeny, which was observed from the blastocyst stage through to adulthood (Grandjean et al. 2009). Another study injected miRNA-1, important for cardiac growth, into the male pronucleus upon fertilization and found the progeny to suffer from cardiac hypertrophy (Wagner et al. 2008). Both these studies provide evidence that RNA species serve as an alternative mechanism of epigenetic inheritance.

The other alternative mechanism of epigenetic inheritance is through histone modifications. Upon maturation the haploid genome in the sperm head undergoes compaction, which is achieved through the replacement of majority of the histones with protamines (Braun 2001). However, 5% to 15% of the genome is still bound to histones and these regions are significantly enriched at loci important for embryonic development, including promoters of miRNAs, imprinted genes and at genes of key transcription and signaling factors (Tanphaichitr et al. 1978, Hammoud et al. 2009). A study done by Gardiner-Garden et al. (1998) demonstrated the association of human sperm-derived histones with two globin genes actively transcribed in the embryonic yolk sac, while protamines were found to be associated with two globin genes that were silent in the embryonic sac (Gardiner-Garden et al. 1998). Another study done in zebrafish found a strong correlation between the genes bearing active chromatin marks in sperm and the expression of these genes in the early embryo. Most of these genes were involved in driving the cell cycle and promoting metabolism (Wu, Zhang & Cairns 2011).

Alcohol exposure is known to not only affect DNA methylation, but has also been shown to affect ncRNAs and histone modifications. The effect of alcohol on miRNAs and its potential role in teratology is gaining much interest. One study noted that the exposure of mouse fetal cerebral-cortex neurons to ethanol was associated with the suppression of a collection of miRNAs and a subsequent increase in the levels of mRNA associated with genes targeted by the respective miRNAs. This resulted in the premature maturation of the cortical stem and progenitor cells (Sathyan, Golden & Miranda 2007). Similarly, bioinformatics analysis of microarray data from a fetus affected with FAS identified an up regulation of the 3' untranslated regions of various mRNAs. miRNAs are known to bind to the 3' untranslated regions of target mRNAs to induce cleavage and prevent translation. Therefore the increased 3' untranslated regions were indicative of decreased capabilities of miRNAs to degrade the target mRNAs following alcohol consumption (Wang et al. 2008). This could be responsible for mediating alcohol teratology during development.

The effects of alcohol on histone modifications have also drawn much attention. In a more recent study done in mice, perinatal ethanol exposure was found to decrease the expression and function of a histone acetyl transferase, CBP. This subsequently resulted in a decrease in the lysine H3 and H4 acetylation in the cerebellum resulting in neuro-developmental deficits (Guo et al. 2011). Studies done in rats have revealed that while alcohol leads to the acetylation of H3K9, it decreases methylation at the same residue resulting in the down regulation of various genes. Concurrently methylation at H3K4 is increased, resulting in the up regulation of various genes (Kim, Shukla 2006, Pal-Bhadra et al. 2007). An increase in acetylation following

alcohol exposure has been related to transcriptional activation (Park, Lim & Shukla 2005).

In summary, if alcohol has a negative impact on the regulation of miRNAs and significantly alters histone modifications and both these epigenetic regulatory mechanisms, together with DNA methylation, are transmitted to the oocyte during fertilization then it could be argued that preconception paternal alcohol consumption could have a detrimental impact on the life long health of the child by altering these three epigenetic processes, which are crucial for normal embryonic development and in maintaining the stability and integrity of the genome. The paternally mediated effects on FASD may be more complicated than originally thought.

3.5 LIMITATIONS OF THE STUDY

The small sample size was the main weakness of this study. As a result of this, there was an uneven spread of the number of drinks consumed per month by individuals within the alcohol-consuming group. While the majority of the alcohol-consuming individuals drank less than 20 drinks per month, only one individual drank 360 drinks per month. Therefore, increasing the sample size would enable for a more accurate classification of the individuals into three distinct and mutually exclusive catergories: light, moderate and heavy drinkers.

The current study only assessed the methylation status of one paternally methylated locus, IG-DMR. Alcohol is known to increase and decrease methylation in a locus-specific manner. Therefore maternally imprinted loci and non-imprinted Alu

elements, both found to be hypomethylated in the male germ-line under normal circumstances, would have added valuable information if included in the study.

Another drawback of this study is that the DNA methylation work presented here was not coupled with expression studies. Expression studies focusing on IG-DMR expression would provide valuable information on whether a reduction in DNA methylation is coupled with a reduction in gene expression. Alcohol consumption is thought to reduce the mRNA levels of the DNMT enzymes in sperm (Bielawski et al. 2002). This decrease in mRNA levels would indicate a reduction in DNMT enzymes, which are crucial for maintaining the correct DNA methylation levels. Therefore expression studies on the DNMT enzymes in the sperm could explain whether the decrease in methylation at the IG-DMR locus is indeed due to a decrease in the DNMT enzymes levels or whether another mechanism exists to regulate DNA methylation patterns following alcohol exposure.

3.6 CONCLUSION

The findings of this study suggest that alcohol consumption, regardless of the amount consumed, is correlated with a modest, but significant, reduction in overall DNA methylation at a paternally imprinted locus, IG-DMR, in male sperm. These findings support the hypothesis that alcohol consumption can lead to hypomethylation of normally hypermethylated DMRs of specific imprinted genes in the human sperm and this in turn could have significant implications with regard to the regulation of developmentally significant genes in the zygote and fetus resulting in developmental, behavioral and neuro-cognitive disorders.

Only one out of the ten CpG sites was significantly demethylated in the alcohol-consuming group with another CpG site showing a trend towards reduced methylation. Drinking frequency significantly reduced the methylation at this latter CpG and showed a trend towards decreasing methylation former CpG site.

It is proposed that alcohol consumption could potentially negatively influence the other epigenetic regulatory mechanisms in the sperm, which are also transmitted to the oocyte during fertilization. This in conjunction with altered DNA methylation patterns at imprinted genes could have significant implications for the health of the offspring born to men who consume alcohol during the preconception period. However, the amount of alcohol and the duration of exposure that may be harmful are not known. In addition, it is likely that there are inter-individual differences with regard to susceptibility to do harm.

FAS is a major health burden in South Africa and the attention has always been on maternal alcohol consumption during pregnancy. However, the results from this study and similar studies focusing on the effects of alcohol on epigenetic modifications in sperm could be used to develop more effective prevention strategies not only directed towards women but equally directed towards men.

In conclusion, these findings contribute to understanding the role of epigenetic modifications as possible mechanisms for paternal alcohol related effects on a fetus.

CHAPTER 4

CONCLUDING REMARKS

4.1 RATIONALE FOR THE STUDY

The majority of FAS research conducted thus far has focused on maternal and fetal genetic risk factors following maternal alcohol consumption during pregnancy. However, studies have shown that preconception paternal alcohol abuse could result in offspring born with characteristic FASD symptoms. The mechanism by which this occurs is poorly understood and a number of recent studies have proposed that epigenetic mechanisms may have a part to play. This body of work therefore focused on exploring whether DNA methylation could serve as a possible epigenetic mechanism responsible for the paternal contribution to FASD, a major health burden in South Africa.

4.2 SUMMARY OF THE FINDINGS

Two loci (*RASGRF1* and IG-DMR) were selected based on their functional role in normal embryonic development, growth regulation and memory. Abnormalities at these loci, in rodent models, result in phenotypes similar to those observed in offspring affected with FASD.

The imprint status of *RASGRF1* in humans was unknown at the start of this project. *RASGRF1* was chosen for investigation because it is paternally imprinted in rats and mice. A number of computational assessments were done to identify key imprinting features, while molecular techniques assessed the methylation status of various CGIs surrounding *RASGRF1*. The methylation levels at these CGIs suggested that *RASGRF1* is not differentially methylated in a parent-of-origin manner. Therefore the

effect alcohol on DNA methylation was only examined at the IG-DMR locus in human sperm samples. An overall reduction in methylation was observed in the alcohol-consuming group with certain CpG sites more demethylated than others. Age was independently and significantly correlated with an increase in overall DNA methylation at this locus.

4.3 IMPLICATIONS OF THE FINDINGS

This body of research has demonstrated that alcohol consumption has the potential to alter methylation patterns in human sperm DNA. However, could this modest effect have a physiological or phenotypic consequence for the offspring born to men who consume alcohol? It is known that alcohol can also disrupt alternative mechanisms of epigenetic inheritance, such as RNA interference and histone modifications. The outcome of these two mechanisms, together with DNA methylation, are transmitted through the sperm to the oocyte during fertilization and could therefore have serious implications for the normal development and lifelong health of a child born to a father who consumes alcohol in the preconception period. This has important public health implications, highlighting the need for urgent research focusing on the epigenetic mechanisms underlying the possible paternally mediated effects on FASD.

4.4 FUTURE DIRECTIONS

As outlined in the study limitations in Chapter 3, future studies of this nature will require a larger sample size such that individuals can be classified into three distinct categories based on drinking frequency: light drinkers, moderate drinkers and heavy

drinkers. These categories will allow for more meaningful results when evaluating whether alcohol is correlated with methylation in a dose-dependant manner.

In addition to increasing the sample size, future studies are required to explore the effects of alcohol on a repertoire of imprinted loci that include both paternally and maternally imprinted loci and transposable elements, such as the Alu elements, in sperm samples. It would be of great value to correlate the methylation data obtained from the imprinted loci with gene expression data of the associated genes. This could provide insight into the role of imprinting as a possible mechanism responsible for paternally mediated effects on FASD.

DNA methylation is known to act in concert with ncRNAs and histones to maintain the stability and integrity of the genome and alcohol is known to disrupt all three epigenetic modifications in the sperm. Therefore in conjunction with the DNA methylation analysis, future studies should assess the effect of alcohol on RNA species and histone modifications. This could give a clearer picture of the paternally mediated effects on FASD.

Lastly, an invaluable study would be to start a cohort, where samples would be collected from men who consume alcohol, wives or partners who do not consume alcohol and their biological children. The children would be followed preferably from birth throughout childhood into adulthood. Microarray analysis would be done in the sperm samples to determine which genes are up and down regulated. In addition, alterations in the various epigenetic modifications would be characterized. Various tissue types would be collected from the children and microarray analysis would be

done to determine whether gene expression is influenced by the changes identified in the sperm. The various epigenetic modifications in the different tissue types would need to be characterised and compared. The reason for using different tissue types is that epigenetic modifications are tissue-specific and are influenced by stochastic and temporal events (Schneider et al. 2010). This cohort study could assist in confirming whether the epigenetic alterations in sperm samples, following alcohol consumption, do indeed affect the normal development of the child. In addition, this study could assist in answering questions related to alcohol dosage effects and the effects of inter-individual DNA methylation variation in sperm and the resulting consequences in the children. If alterations in DNA methylation in human sperm are transmitted to the oocyte, could the epigenetic variation lead to variable penetrance of diseases and disorders in offspring?

One key question arising from this body of research remains to be answered:

- How many sperm cycles should pass following abstinence from alcohol to ensure that the effects of alcohol are not passed onto the subsequent generation?

APPENDICES

APPENDIX A

ETHICS CLEARANCE CERTIFICATES

A.1 Ethics clearance certificate for random control samples (blood)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ramsay/Soodyal et al

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M050706**
PROJECT	Genetic Disease Related Studies in Southern African Population: Control Samples
INVESTIGATORS	Professors M/H Ramsay/Soodyal et al
DEPARTMENT	School of Pathology/Human Genetics
DATE CONSIDERED	05.07.29
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 05.08.01 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof A Krause

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10005, 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

M. Ramsay 29/8/05
H Soodyal 29/8/05
29/8/05

A.2 Ethics clearance certificate for current study

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Misses P Pitamber/S Patel

CLEARANCE CERTIFICATE	**M090555**
PROJECT	P: methylation Profiling of Two Paternally Imprinted loci, RASGRF and IG-DMR, in Male Gametes following Alcohol Exposure/S: Profiling the Epigenetic Signatures at the H19 DMR in Sperm of Alcoholic Males-Implications for Fetal...
INVESTIGATORS	Misses P Pitamber/S Patel.
DEPARTMENT	School of Pathology/Division of Human Genetics
DATE CONSIDERED	09.05.29
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 2009/09/14 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof M Ramsay

UNIVERSITY OF THE WITWATERSRAND
PROF AJ WOODIWISS
Co-Chair
2009 -09- 14
HREC (Medical)
JOHANNESBURG

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...

APPENDIX B

CONSENT FORM, INFORMATION SHEET AND QUESTIONNAIRE

B.1 Consent form

Study Titles:

1) Methylation Profiling of Two Paternally Imprinted loci, *RASGRF* and *IG*-DMR, in Male Gametes following Alcohol Exposure

2) Profiling the Epigenetic Signatures at the *H19* DMR in Sperm of Alcoholic Males-Implications for Fetal Alcohol Spectrum Disorders (FASD)

Investigators: Miss Punita Navnitlal Pitamber and Miss Sanam Harishkumar Patel

Site of Investigation: .....
Date: .....
Code: .....

Consent Form

The information that you divulge in the questionnaire will be kept confidential

Do you understand the information related to this study?	Yes	No
Do you have any questions about the research?	Yes	No
Do we have your permission to administer the questionnaire?	Yes	No
Are you willing to contribute a semen sample?	Yes	No
Do we have your permission to conduct the described research on your semen sample?	Yes	No
Do you give permission for storage of your sample, to be used for future research on the effect of alcohol on sperm?	Yes	No

Full Name of Participant
(Participation in these studies can be anonymous):
_____
Signature: _____

Date: _____

I have answered all the questions from the participant to the best of my ability.

Name of Study Co-ordinator: _____

Signature: _____

B.2 Participant information sheet

The National Health Laboratory Service

University of the Witwatersrand, School Of Pathology
Division Human Genetics

Hospital Street, Johannesburg 2001
Telephone: +27-11-489-9224/9223/9211

PO Box 1038, Johannesburg 2000
Telefax: +27-11-489-9226 or +27-11-489-9209

Prof A Christianson 489-9239
Prof H Soodyall 489-9208

Prof A Krause 489-9219
Dr T Lane 489-9221

Prof M Ramsay 489-9214

Information Sheet

STUDY TITLES:

1) Methylation Profiling of Two Paternally Imprinted loci, RASGRF and IG-DMR, in Male Gametes following Alcohol Exposure

2) Profiling the Epigenetic Signatures at the H19 DMR in Sperm of Alcoholic Males-Implications for Fetal Alcohol Spectrum Disorders (FASD)

INVESTIGATORS: Miss Punita Navnitlal Pitamber and Miss Sanam Harishkumar Patel
(MSc [Med] Research Students)

Good Day,

We, Punita Pitamber and Sanam Patel, master's students from the Division of Human Genetics at the NHLS would like to invite you to participate in our research studies. Both studies focus on the effect of alcohol consumption on sperm DNA and your participation in this study is entirely voluntary. You can withdraw from the studies at any point without consequence to you by contacting the project investigators. Before agreeing to participate, it is important that you read the following document to understand the purpose of the studies. If you have any questions about the studies or the terms used, please do not hesitate to ask one of the study co-ordinators.

We know that children born to mothers who drink during pregnancy can develop a range of disorders known as Fetal Alcohol Spectrum Disorders (FASD). Children with any of these disorders can show symptoms such as brain damage, growth problems and facial morphologies. However, recent research in mice has shown that some symptoms of FASD such as growth retardation can develop in babies born to fathers who drank before conception and mothers who drank no alcohol before or during pregnancy. One way that this could happen is through alcohol changing the sperm at the level of the DNA. Specific patterns in the father's sperm DNA are unique and different from the patterns on the mother's DNA. These patterns are very important for normal development of children during pregnancy. Sperm DNA contains all the information that is passed from father to child. If alcohol changes the patterns on the DNA, in sperm, then the child could develop symptoms of FASD. The aim of our studies is to understand how alcohol can change the patterns contained in the DNA of sperm and in doing so affect the normal development of children.

B.2 Participant information sheet (cont'd)

The National Health Laboratory Service

University of the Witwatersrand, School Of Pathology
Division Human Genetics

Hospital Street, Johannesburg 2001
Telephone: +27-11-489-9224/9223/9211

PO Box 1038, Johannesburg 2000
Telefax: +27-11-489-9226 or +27-11-489-9209

Prof A Christianson 489-9239
Prof H Soodyall 489-9208
Prof A Krause 489-9219
Dr T Lane 489-9221
Prof M Ramsay 489-9214

If you volunteer to participate in these studies we would request that you complete a questionnaire, which will take about 30-45minutes to complete. You will be asked to answer questions about your family and ethnic history, sexual health, drinking, smoking and drug use patterns. A few questions will be asked about your parents' drinking habits. Some of the questions may be sensitive and if you feel you cannot answer them, you may leave them unanswered. You may participate in this study without giving your name. If you reveal information that may have serious legal implications we may be obligated to disclose it if compelled by a court of law.

After completing the questionnaire, we will ask you to provide us with a semen sample. A private room will be made available for this purpose. Semen should only be given if you have not ejaculated in the past two days. In the laboratory, specific tests will be performed on the sperm DNA to determine if any changes have occurred in the DNA patterns. We shall not test for any general health conditions (e.g. sexually transmitted diseases). If you permit, on completion of these studies your samples will be stored in the lab for future research. This future research will be carried out subject to approval by the Human Research Ethics Committee (Medical). If not, your samples will be destroyed on completion of these studies. The samples will be given a unique identification code to ensure anonymity. The results from the test are for this research only and will not be returned to you. However, should you wish to discuss further any issues related to or raised in the course of this research, you could approach the investigators with regard to appropriate referral for counselling.

Thank you for your time.

Sincerely,

_____
Punita Pitamber

_____
Sanam Patel
Tel: (011) 489 9225

B.3 Participant questionnaire

Study Titles:

1) Methylation Profiling of Two Paternally Imprinted loci, *RASGRF* and *IG*-DMR, in Male Gametes following Alcohol Exposure

2) Profiling the Epigenetic Signatures at the *H19* DMR in Sperm of Alcoholic Males-Implications for Fetal Alcohol Spectrum Disorders (FASD)

Investigators: Miss Punita Navnitlal Pitamber and Miss Sanam Harishkumar Patel

Site of Investigation: .....
Date: .....
Code: .....

Questionnaire

Please answer the following questions:

A. Personal History

1. Age: __
2. Your first language: _____
3. Your place of birth: _____
4. Mother's first language: _____
5. Mother's place of birth: _____
6. Father's first language: _____
7. Father's place of birth: _____
8. Have you ever conceived a child? Yes No
9. Have your genitals ever been seriously injured? Yes No
10. Have you ever contracted any sexually transmitted diseases? Yes No
11. Have you ejaculated in the past 2 days? Yes No

B. sMAST Questions

Please circle the appropriate answer

1. Do you feel you are a normal drinker of alcohol? Yes No
2. Do you ever feel bad about drinking? Yes No
3. Do friends or relatives think you are a normal drinker? Yes No
4. Do your spouse/partner or parents worry or complain about your drinking? Yes No

B.3 Participant questionnaire (cont'd)

5. Are you always able to stop drinking when you want to? Yes No
6. Have you ever attended a meeting of Alcoholics Anonymous or any similar group? Yes No
7. Has drinking ever created problems between you and your spouse? Yes No
8. Have you ever gotten into trouble at work because of drinking? Yes No
9. Have you ever neglected your obligations, your family, or your work for 2 days or more days in a row because you were drinking? Yes No
10. Have you ever gone to anyone for help about your drinking? Yes No
11. Have you ever been in hospital because of drinking? Yes No
12. Have you ever been arrested, even for a few hours, because of drinking? Yes No
13. Have you ever been arrested for drunk driving or driving after drinking? Yes No

C. Drinking Frequency

1. How old were you when you started drinking regularly? ____
2. How often do you drink? (Tick the one that fits you best)

i.	Never	vi.	1-2 days a week
ii.	Once a year at most	vii.	Almost every day
iii.	Several times a year	viii.	Every day
iv.	Once a month	xi.	Not sure
v.	2-3 times a month		

3. Have you stopped drinking? Yes No
   If *yes* at what age? _____
4. What is your usual beverage or beverages? _____
5. On days that you drink, how many drinks do you have? _____

D. Family Drinking History

	Mother	**Father**
1. Is he / she alive?	Yes No	Yes No
2. Cause of death?	_____	_____
3. Current age of parent or age at death?	_____	_____
4. Does he / she drink alcohol regularly?	Yes No	Yes No
5. If not currently a drinker did they drink when you were growing up?	Yes No	Yes No

B.3. Participant questionnaire (cont'd)

E. Smoking

1. When did you last use tobacco (smoke or chew) or snuff?
   - Never
   - More than a year ago
   - In the past year
   - In the past 30 days
   - Don't recall
2. How old were you when you first smoked or used tobacco? _____
3. Are you still smoking or using tobacco? Yes No
4. How old where you when you stopped using tobacco? _____
5. How many cigarettes a day do/did you smoke? _____

F. Other Drug Use

1. Have you ever used any drugs regularly? Yes No
2. What drugs have you used? _____

   (e.g. dagga, glue, ecstasy (XTC, E, Adam), marijuana, cocaine, heroin, sugars, mandrax,

   amphetamines (bennies, dexies), Ice (meth, crystal), CAT)
3. How old were you when you started using drugs regularly? _____
4. How often do/did you use drugs?

   i. Never
   ii. Once a year at most
   iii. Several times a year
   iv. Once a month
   v. 2-3 times a month
   vi. 1-2 days a week
   vii. Almost every day
   viii. Every day
   ix. Not sure
5. When did you last use drugs?
   - Never
   - More than a year ago
   - In the past year
   - 1-6 months ago
   - In the past 30 days
   - Don't recall

Thank you very much for your co-operation ☺

APPENDIX C

REAGENT AND EQUIPMENT SUPPLIERS

Table C.1 List of reagent suppliers

Reagent	Supplier
QIAamp® DNA Micro Kit	Qiagen, Valencia, CA, United States of America
EZ DNA methylation kit	Zymo Research, Orange, CA, United States of America
dNTPs	Bioline, London, United Kingdom
Primers	IDT, Iowa, United States of America
AmpliTaq Gold Taq polymerase, 10x Buffer & $MgCl_2$	Applied Biosystems, NJ, United States
Agarose (Molecular Grade)	Whitehead Scientific, Brackenfell, South Africa
Ethidium Bromide aqueous solution	Sigma Aldrich, MO, United States of America
50bp ladder	New England Biolabs, MA, United States of America
Sepharose Beads HP	GE Health, Uppsala, Sweden

Table C.2 List of equipment suppliers

Equipment	Supplier
Microfuge® 18 Microcentrifuge	Beckman Coulter, Brea, CA, United States of America
Nanodrop® ND-1000 Spectrophotometer	Thermo Fisher Scientific, MA United States of America
PSQ 96 MA Pyrosequencer™	Biotage, Uppsala, Sweden
GeneAmp 2720 thermal cycler	Applied Biosystems, NJ, United States of America

APPENDIX D

PREPARATION OF SOLUTIONS

1x Tris EDTA (TE) buffer

10ml 1M Tris-HCl (pH 8.0)

2ml 0.5M EDTA

Make up to 1L with dH_20

1M Tris-HCl (pH 8.0)

121.1g Tris

Dissolve in 800ml with dH_20, adjust pH to 8.0 with HCl. Make up to 1L with dH_20.

0.5M EDTA

93.06g EDTA

Dissolve in 400ml with dH_20, adjust pH to 8.0 with NaOH. EDTA will only dissolve if the pH is correct. Make up to 500ml with dH_20.

10M NaOH

4g Sodium Hydroxide

Make up to 10ml with dH_20

3% (w/v) Agarose gel

3g Agarose (Molecular Grade) per 100ml dH_20

3µl Ethidium bromide (10mg/ml) per 100ml 1x TBE

10x Tris boric EDTA

216g Tris

110g Boric Acid

18.6g EDTA

Make up to 2L with dH_20

dNTPs

12.5 µl dATP (10mM)

12.5 µl dTTP (10mM)

12.5 µl dCTP (10mM)

12.5 µl dGTP (10mM)

Make up to 1ml using ddH_20

Binding buffer (pH 7.6)

1.21g Tris

117g Sodium Chloride

0.292g EDTA

1ml Tween20

Make up to 900ml with ddH_20, adjust pH to 7.6 with HCl. Add 1 ml Tween20 and make up to 1L with ddH_20.

Annealing buffer (pH 7.6)

2.42g Tris

0.43g Magnesium Acetate Tetrahydrate

Make up to 900ml with ddH_20, adjust pH to 7.6 with acetic acid. Make up to 1L with ddH_20.

70% Ethanol

700ml Absolute Ethanol

Make up to 1L with ddH_20

Denaturation solution (0.2M Sodium Hydroxide)

8g Sodium Hydroxide

Make up to 1L with ddH_20

Washing buffer (pH 7.6)

1.21g Tris

Make up to 900ml with ddH_20, adjust pH to 7.6 with acetic acid. Make up to 1L with ddH_20.

APPENDIX E

PARTICIPANT INFORMATION

Table E.1 Participant information

Sample ID	*Ethnicity	Age	# of Years Drinking	Drinking Frequency (units/month)	Smoking	Drug Use	Total Sperm Concentration (10^6/ml)	Normal Sperm Morphology (%)	Sperm Motility 0.5 hrs (%)
HSS 019	B	25	0	0	No	No	110	15	60
HSS 025	B	38	0	0	No	No	44	5	60
HSS 027	I	28	0	0	No	No	54	20	60
HSS 031	B	32	0	0	No	No	30	7	45
HSS 032	W	29		Stopped for the last 4 years	No	No	**13**	11	50
HSS 033	I	36		Stopped for the last 6 years	Yes	No	85	15	50
HSS 035	B	29	0	0	No	No	18	10	40
HSS 037	B	33		Stopped for the last 4 years	No	No	137	7	60
HSS 038	B	37	0	0	No	No	23	8	60
HSS 042	B	40		Stopped for the last 9 years	No	No	18	12	50
HSS 046	B	33	0	0	No	No	77	5	50
HSS 050	I	50		Stopped for the last 20 years	No	No	42	4	40
HSS 055	C	29		Stopped for the past 2 years	No	No	22	6	60
HSS 056	B	30	0	0	Yes	No	21	11	50
HSS 070	B	34	0	0	No	No	73	14	70
HSS 074	B	30	0	0	No	No	72	6	40
HSS 075	B	40	0	0	No	No	36	16	70
HSS 077	I	28	0	0	No	No	39	5	50
HSS 079	B	39	0	0	No	No	167	20	50
HSS 082	I	22	0	0	No	No	56	15	65
HSS 085	B	38	0	0	No	No	57	10	50
HSS 088	I	38	0	0	Yes	No	28	13	65
HSS 089	B	38	0	0	No	No	36	15	70
HSS 094	I	28	0	0	No	No	83	16	65
HSS 096	B	36	0	0	No	No	23	10	40
HSS 097	B	36	0	0	No	No	**11**	**1**	40
HSS 107	I	43	0	0	Yes	No	68	16	55
HSS 116	B	45	0	0	No	No	52	19	55
HSS 121	I	28	0	0	No	No	53	18	70

HSS 131	C	33	0	0	No	No	70	10	50
HSS 136	B	36	0	0	No	No	37	21	50
HSS 141	W	37	0	0	Yes	No	32	**1**	**30**
HSS 142	C	36		Stopped for the past 6 years	Yes	No	65.5	**3**	70
HSS 149	B	33	0	0	No	Yes	**14**	9	60

Sample ID	*Ethnicity	Age	# Years Drinking	Drinking Frequency (units/month)	Smoking	Drug Use	Total Sperm Concentration (10^6/ml)	Normal Sperm Morphology (%)	Sperm Motility 0.5 hrs (%)
HSS 001	B	23	6	30	Yes	No	76	15	60
HSS 003	W	31	12	15	Yes	No	102	18	50
HSS 004	W	27	9	72	Yes	No	96	14	60
HSS 005	W	28	4	6	No	No	70	19	65
HSS 006	W	38	20	3	No	No	**10**	13	60
HSS 008	W	22	6	60	Yes	No	24	15	60
HSS 010	B	35	10	6	No	No	16	9	60
HSS 011	B	20	2	75	No	No	40	20	60
HSS 016	W	26	7	6	No	No	62	12	60
HSS 017	B	31	21	15	Yes	No	110	11	60
HSS 018	B	34	4	15	No	No	28	14	60
HSS 020	B	34	16	120	No	No	18	18	60
HSS 021	W	34	16	72	Yes	No	**8.8**	**2**	60
HSS 022	W	29	11	30	No	No	**4.4**	10	50
HSS 023	W	33	12	6	Yes	Yes	280	17	40
HSS 024	I	33	15	1	No	No	128	12	60
HSS 028	C	36	18	1	No	No	81	19	60
HSS 036	B	40	22	36	No	No	44	5	45
HSS 040	B	38	22	18	No	Yes	**14**	10	50
HSS 043	I	29	10	9	No	No	86	16	50
HSS 044	B	34	13	2	Yes	No	**10**	**1**	45
HSS 048	B	37	17	60	No	No	**11**	**1**	40
HSS 051	I	35	15	4	Yes	No	110	9	45
HSS 052	B	39	21	60	No	No	**10**	5	60
HSS 053	C	33	15	84	No	Yes	70	16	70
HSS 054	I	30	7	14	Yes	No	80	5	65

HSS 057	W	40	19	150	Yes	No	27	4	50
HSS 058	W	34	17	36	No	No	99	12	60
HSS 061	W	35	19	8	No	No	76	15	70
HSS 062	W	29	12	2	Yes	No	162	20	75
HSS 064	C	35	17	72	No	No	**6.3**	4	**20**
HSS 067	B	36	18	1	No	No	45	20	45
HSS 068	W	39	22	30	Yes	No	38	14	55
HSS 069	W	35	16	12	No	No	20	15	50
HSS 071	B	34	7	24	Yes	No	**7**	12	40
HSS 072	B	35	15	6	Yes	No	25	15	70
HSS 073	B	46	28	6	No	No	24	10	40
HSS 076	B	28	8	36	Yes	No	176	20	65
HSS 078	W	45	15	15	Yes	No	43	17	40
HSS 081	B	34	13	30	No	No	123	18	65
HSS 083	B	39	14	5	No	No	54	19	70
HSS 084	B	37	18	24	No	No	29	16	60
HSS 086	W	39	21	6	No	No	83	20	60
HSS 090	W	25	9	45	Yes	No	28	9	60
HSS 092	W	27	12	108	Yes	No	58	8	60
HSS 095	B	28	5	144	No	No	**4.2**	5	60
HSS 098	B	35	20	48	No	No	48	19	70
HSS 102	B	41	13	60	No	No	**11**	**2**	**30**
HSS 103	W	31	15	2	No	Yes	151.2	**2**	70
HSS 105	W	36	18	1	No	No	310	14	60
HSS 110	W	35	16	9	Yes	No	16	**1**	40
HSS 111	W	33	12	23	Yes	No	28	6	60
HSS 112	B	42	22	42	No	No	**6**	8	45
HSS 114	B	49	24	60	Yes	No	**9**	**2**	50
HSS 117	W	31	15	7	Yes	No	**5.9**	6	50
HSS 118	B	48	13	5	No	No	**8**	**1**	**20**
HSS 119	W	30	11	4	Yes	No	28	15	50
HSS 120	B	35	18	15	No	No	49	17	**30**
HSS 122	W	31	13	6	No	No	155	25	65
HSS 123	B	36	16	144	No	No	62	20	60
HSS 124	W	29	10	42	No	No	36	11	60
HSS 125	W	34	16	15	Yes	No	17	**1**	60

HSS 126	I	29	8	3	No	No	63	4	70
HSS 127	B	30	5	36	No	No	56	10	70
HSS 128	W	29	11	6	No	No	**12**	**1**	50
HSS 129	B	41	16	36	Yes	No	66	16	60
HSS 130	B	32	17	360	Yes	No	58	7	60
HSS 132	B	39	18	42	No	No	95	15	60
HSS 133	B	33	18	96	No	No	**5.3**	**3**	60
HSS 134	B	33	15	12	Yes	No	47	15	65
HSS 135	B	33	9	10	No	No	55	8	60
HSS 137	W	34	16	1	No	No	23	4	50
HSS 140	W	32	14	9	Yes	No	101	16	70
HSS 143	C	33	16	10	Yes	No	18	**2**	50
HSS 144	B	31	6	15	No	No	37	10	50
HSS 145	W	38	17	30	Yes	No	27	4	60
HSS 146	I	34	14	60	No	Yes	124	15	60
HSS 147	B	26	10	18	No	No	28	17	60
HSS 148	B	39	18	10	No	No	162	**3**	60

*Ethnicity Key – B : Black
W: White
C : Coloured
I : Indian

Sperm parameters in bold represent values lower than the WHO lower reference limits (Cooper et al. 2010).

Table E.2 Ethnicity breakdown within two treatment groups

		Controls	Alcohol Consumers
Sample size (N)		34	78
		Count (Frequency)	**Count (Frequency)**
Ethnicity:			
	White	2 (5.77%)	32 (41.03%)
	Black	20 (58.82%)	37 (47.44%)
	Colored	3 (8.82%)	4 (5.13%)
	Indian	9 (26.47%)	5 (6.41%)

APPENDIX F

SPERM DNA EXTRACTION PROTOCOL, DNA CONCENTRATION AND PURITY OF SAMPLES

The following protocol "Purification of DNA from epithelial cells mixed with sperm cells using the QIAamp® DNA Micro Kit" is the supplementary protocol to the QIAamp® DNA Micro Kit. This protocol is designed for purification of total (genomic and mitochondrial) DNA from fabrics or swabs containing epithelial cells mixed with sperm cells using the QIAamp DNA Micro Kit.

Protocol:

1. Place the swab or a piece of fabrics (≤0.5cm2) in a 2 ml microcentrifuge tube (not provided).Separate the cotton or DACRON® swab from its shaft by hand or using scissors. *In our case approximately 900 µl of sperm sample was added to the microcentrifuge tube.*
2. Add 20 µl proteinase K and 500µl Buffer ATL to the sample. Close the cap and mix by pulse-vortexing for 10 s.
3. Place the 2 ml tube in a thermomixer or heated orbital incubator, and incubate at 56°C with shaking at 900 rpm for at least 1 h.
4. Briefly centrifuge the 2 ml tube to remove drops from the inside of the lid.
5. Remove the solid material from the tube.
6. Centrifuge the tube for 5 min at full speed. Carefully transfer all but 30 µl of the supernatant to a new tube without disturbing the pellet.

Note: For isolation of DNA from epithelial cells, transfer 300 µl of the supernatant into a 2 ml microcentrifuge tube and continue with step 12.

7. Resuspend the pellet in 500 µl Buffer ATL. Close the lid and mix by pulse-vortexing for 10 s. Centrifuge the tube for 5 min at full speed. Carefully aspirate and discard all but 30 µl of the supernatant without disturbing the pellet.
8. Repeat step 7 at least three times.

Note: The ratio of epithelial cells to sperm cells influences the number of repeats needed for purification of sperm nuclei.

9. Add 300 µl Buffer ATL, 10 µl proteinase K, and 10 µl 1 M DTT to the pellet. Close the

lid and mix by pulse-vortexing for 10 s.

10. Place the 2 ml tube in a thermomixer or heated orbital incubator, and incubate at 56°C with shaking at 900 rpm for at least 1 hour.
11. Briefly centrifuge the tube to remove drops from the inside of the lid.
12. Add 300 µl Buffer AL, close the lid, and mix by pulse-vortexing for 10 s.

Note: To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution. A white precipitate may form when Buffer AL is added to Buffer ATL. The precipitate does not interfere with the QIAamp procedure and will dissolve during incubation in step 13.

Note: If carrier RNA is required (see the QIAamp DNA Micro Handbook), add 1 µg dissolved carrier RNA to 300 µl Buffer AL. Note that carrier RNA does not dissolve in Buffer AL. It must first be dissolved in Buffer AE and then added to Buffer AL.

13. Place the tube in the thermomixer or heated orbital incubator, and incubate at 70°C with shaking at 900 rpm for 10 min.
14. Centrifuge the tube at full speed (20,000 x **g**; 14,000 rpm) for 1 min.
15. Carefully transfer the supernatant from step 14 to the QIAamp MinElute® column without wetting the rim.
16. Close the lid, and centrifuge at 6000 x **g** (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

Note: If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute Column is empty.

17. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x **g** (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
18. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW2 without

wetting the rim. Close the lid and centrifuge at 6000 x **g** (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

19. Centrifuge at full speed (20,000 x **g**; 14,000 rpm) for 3 min to dry the membrane completely.

Note: This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

20. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 20–50 µl Buffer AE or distilled water to the center of the membrane.

Important: Ensure that Buffer AE or distilled water is equilibrated to room temperature (15–25°C). Dispense Buffer AE or distilled water onto the center of the membrane to ensure complete elution of bound DNA.

21. Close the lid and incubate at room temperature (15–25°C) for 1 min. Centrifuge at full speed (20,000 x **g**; 14,000 rpm) for 1 min. Incubating the QIAamp MinElute column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

Table F.1. DNA concentration and purity for all sperm samples used in the study

Sample	DNA Concentration (ng/µl)	Purity Ratio (260:280)
HSS 001	53.2	2.00
HSS003	85.56	1.85
HSS004	49.19	2.10
HSS005	66.81	1.93
HSS006	76.85	2.07
HSS008	57.94	2.06
HSS010	59.74	2.06
HSS011	44.85	2.00
HSS016	64.63	2.06
HSS017	55.42	2.01
HSS018	65	2.05
HSS019	58.83	1.98
HSS020	57.06	1.94
HSS021	72.56	2.04
HSS022	79.12	2.04
HSS023	94.39	1.97
HSS024	94.36	2.03
HSS025	66.69	1.96
HSS027	86.4	2.03
HSS028	92.3	2.03
HSS031	60.3	1.88
HSS 032	39.8	1.99
HSS033	101.5	1.97
HSS035	68.4	1.95
HSS036	86.8	2.04
HSS037	188.8	1.88
HSS038	226.9	1.94
HSS040	31.2	2.00
HSS042	82.1	1.91
HSS043	51.1	2.09
HSS044	46.1	1.87
HSS046	47	2.02
HSS048	49.1	1.73
HSS050	67.6	2.01
HSS051	42.7	2.06
HSS052	76.4	2.06
HSS053	106.1	1.98
HSS054	237.3	1.46
HSS055	43.1	2.03
HSS056	39.7	1.97
HSS057	70.7	2.07
HSS058	39.8	2.02
HSS061	60.8	1.95
HSS062	60	2.06
HSS 064	62.2	1.99
HSS 067	44.2	1.93
HSS 068	47.2	2.03
HSS 069	69.2	1.99

HSS 070	98.4	2.06
HSS071	28.56	2.07
HSS 072	32.2	2.05
HSS 073	72	1.99
HSS 074	43.3	2.09
HSS 075	70.4	1.91
HSS 076	89.1	1.99
HSS 077	17	1.40
HSS 078	99.7	2.06
HSS 079	48.3	2.06
HSS 081	37.5	2.13
HSS 082	64.6	1.95
HSS 083	39.2	1.70
HSS 084	30.2	1.47
HSS 085	80.2	2.06
HSS 086	78.8	1.92
HSS 088	38.4	2.02
HSS 089	48.4	2.02
HSS 090	67	2.03
HSS 092	52.9	2.04
HSS 094	96.5	2.10
HSS 095	59.7	1.43
HSS 096	33.7	2.09
HSS097	34.47	1.94
HSS 098	29.9	2.05
HSS102	55.98	1.83
HSS 103	89.3	2.03
HSS 105	74.1	2.08
HSS 107	61.6	2.00
HSS 110	26.5	2.10
HSS 111	119	1.68
HSS112	77.27	1.87
HSS 114	50.3	2.10
HSS 116	72	1.87
HSS 117	51.5	2.09
HSS118	75.22	1.89
HSS 119	17.6	2.03
HSS 120	40.3	2.14
HSS 121	31.7	2.06
HSS 122	86.7	1.94
HSS 123	73.2	1.93
HSS 124	28.8	2.07
HSS 125	31.9	1.2
HSS 126	61.5	1.84
HSS 127	80.3	2.08
HSS 128	41.5	2.00
HSS 129	67	1.75
HSS 130	80.1	1.96
HSS 131	111.7	2.06
HSS 132	134	2.06
HSS 133	79.6	2.01
HSS 134	69	2.11

HSS 135	108.3	1.86
HSS 136	34.9	2.08
HSS 137	65.9	2.04
HSS 140	80.2	1.97
HSS 141	59.2	2.19
HSS 142	35.4	1.99
HSS 143	55.7	2.13
HSS 144	48.8	2.03
HSS 145	28.5	1.89
HSS 146	66.8	1.96
HSS 147	52.5	2.00
HSS 148	39.6	2.07
HSS 149	45.8	1.88

APPENDIX G

BISULFITE MODIFICATION PROTOCOL

The following protocol was taken from the Zymo Research's EZ DNA Methylation-Gold™ Kit instruction manual.

Reagent preparation:

- Preparation of CT Conversion Reagent

The CT Conversion Reagent supplied within this kit is a solid mixture and must be prepared prior to first use. Prepare as follows:

1. Add 900 µl water, 300 µl of M-Dilution Buffer, and 50 µl M-Dissolving Buffer to a tube of CT Conversion Reagent.
2. Mix at room temperature with frequent vortexing or shaking for 10 minutes.

- Preparation of M-Wash Buffer

Add 96 ml of 100% ethanol to the 24 ml M-Wash Buffer concentrate before use.

Protocol for bisulfite modification:

1. Add 130 µl of the CT Conversion Reagent to 20 µl of your DNA sample in a PCR tube. If the volume of the DNA sample is less than 20 µl, make up the difference with water. Mix the sample by flicking the tube or pipetting the sample up and down, then centrifuge the liquid to the bottom of the tube.
2. Place the sample tube in a thermal cycler and perform the following steps:
    - 98°C for 10 minutes
    - 64°C for 2.5 hours
    - 4°C storage up to 20 hours.
3. Add 600 µl of M-Binding Buffer to a Zymo-Spin™ IC Column and place the column into a provided collection tube.
4. Load the sample (from Step 2) into the Zymo-Spin™ IC column containing the M-Binding buffer. Close the cap and mix by inverting the column several times.

5. Centrifuge at full speed (>10,000 x **g**) for 30 seconds. Discard the flow-through.
6. Add 100 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.
7. Add 200 µl of M-Desulphonation Buffer to the column and let stand at room temperature (20°C – 30°C) for 15 - 20 minutes. After the incubation, centrifuge at full speed for 30 seconds.
8. Add 200 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 µl of M-Wash Buffer and centrifuge for an additional 30 seconds.
9. Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl of M-Elution Buffer directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

The elution volume can be > 10 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

APPENDIX H

PYROSEQUENCING PROTOCOL

Once the PCR is complete, the products should be run on a 3% agarose gel to ensure PCR was successful.

Setting up PSQ run

1. Clean the needles of the vacuum prep:
   - Aliquot 80 µl of ddH_20 into all wells of a 96 well plate. Switch on the vacuum pump and lower the vacuum prep tool onto the plate for 20 sec so that all the ddH_2O is taken up my the vacuum prep tool (all wells should be empty after this).

2. Immobilization of PCR products to beads:
   - Use all remaining PCR products for this.
   - Shake the bottle of streptavidin sepharose beads until a homogenous solution is obtained.
   - Make up a master mix of sepharose and binding buffer so that there is 6 µl of sepharose and 40 µl of binding buffer for each sample.
   - Aliquot 43 µl of the sepharose/binding buffer mix to each sample.
   - Over the 96 well plate with a plastic seal and place on a shaker for 10 min at 300 rpm.
   - Prepare the PSQ 96 well plate with the sequencing primer and annealing buffer. For each sample add 1.6 µl of 10 µM sequencing primer to 38.4 µl of annealing buffer.
   - Aliquot 40 µl into each well of the PSQ plate.

3. Strand separation of PCR products:
   - Place four troughs on the Vacuum Prep work station in the following order and fill each

     trough with the following:

     i. 70% Ethanol
     ii. Denaturation buffer
     iii. Washing Buffer
     iv. ddH_20

   - Turn the vacuum pup on and apply the vacuum to the 96 well plate containing the samples

     sepharose/binding buffer mix. The beads with the immobilized templates will be captured

     on the filter probes of the vacuum prep tool.

- Make sure all the liquid has been captured onto the filter probes. Move the vacuum prep
  tool to the 70% ethanol trough for 60 sec, then to the denaturation buffer trough for 60 sec,
  then to the washing buffer trough for 60 sec and lastly to the ddH_2O trough for 60 sec.
- Hold the vacuum prep tool up at 90° to all for all the liquid to completely drain from it for
  a few seconds and return to a horizontal position.
- Turn the vacuum off to release the vacuum.
- Place the PSQ 96 well plate on the workstation and 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 with the sequencing primer and annealing buffer on a heating block set
     at 80°C for 3 min.
   - Allow to cool to room temperature.

5. Cartridge preparation
   - Add the required amount of enzyme, substrate and dNTPs into the cartridge, as specified
     by the software one all the necessary information has been entered.
   - Place the PSQ plate and cartridge into the pyrosequencer and start the run.

Analysis

Click on the run once it is complete and press analyze to analyze all the samples.

APPENDIX I

PYROSEQUENCING DATA

RASGRF1 DATA

- **CGI_1973**

NOTE: Boxes encompassing replicate data sets at individual CpG sites indicate the exclusion of that specific CpG site from the analysis. This is due the a greater than 6% methylation difference between the replicates.

Sample ID		CpG Sites Analyzed																					Average Methylation (%) per Sample
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	
Blood Samples																							
HBS 001	PCR1	5	0	5	0	0	0	0	0	0	6	0	0	0	4	0	0	3	42	4	13	4	
	PCR2	0	0	3	5	0	0	0	0	2	0	0	3	4	0	0	0	0	48	7	14	0	
	PCR3 (excluded)	2	0	4	6	0	0	0	0	0	0	0	3	3	2	0	0	38	4	37	5	4	
	Average Methylation (%)	**3**	**0**	**4**	**3**	**0**	**0**	**0**	**0**	**1**	**3**	**0**	**2**	**2**	**2**	**0**	**0**	**2**	**45**	**6**	**14**	**2**	**4**
HBS 002	PCR1	4	2	4	3	0	3	0	3	5	0	3	3	4	2	0	0	2	1	1	2	2	
	PCR2	3	0	2	2	0	3	0	2	2	0	0	0	0	2	2	0	3	0	0	3	2	
	PCR3	4	3	6	18	0	4	0	0	0	3	6	3	0	0	0	0	2	0	0	2	0	
	Average Methylation (%)	**4**	**2**	**4**	**8**	**0**	**3**	**0**	**2**	**2**	**1**	**3**	**2**	**1**	**1**	**1**	**0**	**2**	**0**	**0**	**2**	**1**	**2**
HBS 003	PCR1	7	4	7	5	0	0	0	0	0	0	4	10	13	3	2	0	37	3	37	4	3	
	PCR2	7	4	6	5	0	0	0	0	0	0	2	10	13	4	2	0	39	5	36	0	3	
	PCR3	7	0	6	0	0	0	5	0	0	0	0	10	16	2	0	0	40	3	39	5	3	
	Average Methylation (%)	**7**	**3**	**6**	**3**	**0**	**0**	**2**	**0**	**0**	**0**	**2**	**10**	**14**	**3**	**1**	**0**	**39**	**4**	**37**	**3**	**3**	**7**
HBS 004	PCR1	3	0	3	2	2	15	0	3	0	0	2	2	0	90	95	39	88	59	6	2	37	
	PCR2	3	0	0	5	0	16	0	0	0	0	0	0	0	90	95	41	86	59	7	4	36	
	PCR3	5	3	5	4	3	4	3	3	0	3	4	0	0	88	92	41	84	63	8	6	35	
	Average Methylation (%)	**4**	**1**	**3**	**4**	**2**	**12**	**1**	**2**	**0**	**1**	**2**	**1**	**0**	**89**	**94**	**40**	**86**	**60**	**7**	**4**	**36**	**22**
HBS 005	PCR1	16	15	6	5	16	0	3	2	5	0	4	4	13	24	0	0	0	11	0	0	0	
	PCR2	16	15	6	5	15	2	4	2	4	0	2	4	12	21	4	5	5	11	4	6	0	

	PCR3 (excuded)	5	5	6	6	12	6	0	20	7	0	8	0	16	20	14	9	10	13	11	6	0	
	Average Methylation (%)	**16**	**15**	**6**	**5**	**16**	**1**	**4**	**2**	**5**	**0**	**3**	**4**	**13**	**23**	**2**	**3**	**3**	**11**	**2**	**3**	**0**	**6**
HBS 006	PCR1	2	0	3	2	0	0	0	0	0	0	0	2	0	18	0	0	8	8	13	0	0	
	PCR2	0	0	4	4	0	0	4	0	0	0	0	0	0	16	2	1	9	7	14	3	2	
	PCR3 (excluded)	7	7	4	5	0	8	4	5	0	0	0	0	6	16	2	2	9	7	14	3	2	
	Average Methylation (%)	**1**	**0**	**4**	**3**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**1**	**0**	**17**	**1**	**1**	**9**	**8**	**14**	**2**	**1**	**3**
HBS 007	PCR1	1	0	20	16	0	16	0	2	13	7	18	5	13	0	2	12	22	82	2	1	2	
	PCR2	0	0	20	17	0	15	0	0	12	8	17	6	15	0	0	14	21	83	4	5	5	
	PCR3 (excluded)	4	0	6	5	0	4	0	0	5	0	0	4	0	4	3	13	21	88	3	2	3	
	Average Methylation (%)	**1**	**0**	**20**	**17**	**0**	**16**	**0**	**1**	**13**	**8**	**18**	**6**	**14**	**0**	**1**	**13**	**22**	**83**	**3**	**3**	**4**	**11**
HBS 008	PCR1	16	0	4	0	0	4	5	0	0	0	0	10	18	3	3	0	2	0	3	0	0	
	PCR2	17	0	4	4	0	3	5	0	0	0	0	11	17	2	0	0	2	0	0	0	3	
	PCR3 (excluded)	4	5	6	5	0	9	0	0	0	0	0	0	0	2	1	1	2	2	0	2	2	
	Average Methylation (%)	**17**	**0**	**4**	**2**	**0**	**4**	**5**	**0**	**0**	**0**	**0**	**11**	**18**	**3**	**2**	**0**	**2**	**0**	**2**	**0**	**2**	**3**
HBS 009	PCR1	0	0	3	3	3	2	2	0	0	0	4	5	46	0	10	0	0	0	7	8	9	
(excluded)	PCR2	7	6	7	11	0	5	5	0	0	0	0	5	8	4	81	3	2	0	1	1	2	
	PCR3	2	0	2	3	4	2	2	0	0	0	48	4	49	8	68	11	9	0	0	11	0	
	Average Methylation (%)	**3**	**2**	**4**	**6**	**2**	**3**	**3**	**0**	**0**	**0**	**17**	**5**	**34**	**4**	**53**	**5**	**4**	**0**	**3**	**7**	**4**	**8**
HBS 010	PCR1	21	0	8	5	5	8	4	5	4	0	8	11	20	84	6	4	4	3	0	4	0	
	PCR2 (excluded)	33	0	26	0	20	15	0	0	0	0	30	0	23	3	1	0	4	0	2	3	4	
	PCR3	19	5	7	8	6	9	0	6	0	0	8	11	21	83	1	2	5	2	4	6	5	
	Average Methylation (%)	**20**	**3**	**8**	**7**	**6**	**9**	**2**	**6**	**2**	**0**	**8**	**11**	**21**	**84**	**4**	**3**	**5**	**3**	**2**	**5**	**3**	**10**
Sperm Samples																							
HSS 027	PCR1	4	0	7	5	6	4	6	4	4	0	7	4	7	5	4	3	4	3	4	5	4	
(excluded)	PCR2	7	9	12	9	0	9	0	8	0	0	24	8	0	2	0	0	2	2	2	2	2	
	Average Methylation (%)	**6**	**5**	**10**	**7**	**3**	**7**	**3**	**6**	**2**	**0**	**16**	**6**	**4**	**4**	**2**	**2**	**3**	**3**	**3**	**4**	**3**	**4**
HSS 033	PCR1	0	0	2	2	0	4	0	0	0	0	2	0	0	2	1	1	2	0	0	2	0	

	PCR2	0	0	2	0	0	4	0	0	3	0	4	0	3	3	0	0	4	0	0	4	0	
	Average Methylation (%)	**0**	**0**	**2**	**1**	**0**	**4**	**0**	**0**	**2**	**0**	**3**	**0**	**2**	**3**	**1**	**1**	**3**	**0**	**0**	**3**	**0**	**1**
HSS 035	PCR1	0	0	2	0	0	2	0	0	0	0	0	0	0	6	0	0	4	0	0	0	0	
	PCR2	2	0	2	2	0	0	0	0	0	0	0	0	0	5	2	1	3	2	2	3	2	
	Average Methylation (%)	**1**	**0**	**2**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**6**	**1**	**1**	**4**	**1**	**1**	**2**	**1**	**1**
HSS 038	PCR1	2	0	3	2	0	3	0	0	0	0	0	0	0	5	0	0	3	0	2	4	3	
	PCR2	2	0	3	3	0	3	1	0	0	0	0	1	1	5	4	3	4	2	3	5	4	
	Average Methylation (%)	**2**	**0**	**3**	**3**	**0**	**3**	**1**	**0**	**0**	**0**	**0**	**1**	**1**	**5**	**2**	**2**	**4**	**1**	**3**	**5**	**4**	**2**
HSS 048	PCR1	1	1	4	2	2	1	1	1	0	0	2	2	2	3	5	0	3	4	2	3	3	
	PCR2	2	0	3	2	0	0	0	0	0	0	0	4	0	3	0	0	3	2	2	2	3	
	Average Methylation (%)	**2**	**1**	**4**	**2**	**1**	**1**	**1**	**1**	**0**	**0**	**1**	**3**	**1**	**3**	**3**	**0**	**3**	**3**	**2**	**3**	**3**	**2**
HSS 061	PCR1	2	0	2	0	2	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	4	
	PCR2	0	0	0	6	0	0	0	0	0	0	0	0	0	3	0	3	3	3	3	4	4	
	Average Methylation (%)	**1**	**0**	**1**	**3**	**1**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**2**	**2**	**2**	**2**	**2**	**4**	**1**
HSS 077	PCR1	2	0	2	14	3	0	2	0	0	0	0	1	4	0	0	0	0	0	5	0	0	
	PCR2	3	6	4	3	3	6	3	4	6	3	4	3	5	0	0	0	0	0	0	0	0	
	Average Methylation (%)	**3**	**3**	**3**	**9**	**3**	**3**	**3**	**2**	**3**	**2**	**2**	**2**	**5**	**0**	**0**	**0**	**0**	**0**	**3**	**0**	**0**	**2**
HSS 078	PCR1	2	0	2	0	9	0	0	18	0	0	0	0	14	3	0	3	4	0	3	2	3	
	PCR2	2	0	3	2	9	2	0	17	2	0	3	0	16	4	0	1	3	1	5	2	0	
	Average Methylation (%)	**2**	**0**	**3**	**1**	**9**	**1**	**0**	**18**	**1**	**0**	**2**	**0**	**15**	**4**	**0**	**2**	**4**	**1**	**4**	**2**	**2**	**3**

- **CGI_1974**

Sample ID		CpG Sites Analyzed							Average Methylation (%) per Sample
		1	2	3	4	5	6	7	
Blood Samples									
HBS 001	PCR1	87	100	90	90	85	97	85	
	PCR2	91	99	91	92	90	95	89	
	PCR3	92	96	92	92	84	95	89	
	Average Methylation (%)	**90**	**98**	**91**	**91**	**86**	**96**	**88**	**91**
HBS 002	PCR1	93	100	91	92	88	99	86	
	PCR2	88	100	90	91	89	99	87	
	PCR3	88	100	85	92	85	100	87	
	Average Methylation (%)	**90**	**100**	**89**	**92**	**87**	**99**	**87**	**92**
HBS 003	PCR1	86	98	84	92	88	94	87	
	PCR2	94	100	87	94	89	96	91	
	PCR3	82	98	88	90	86	100	89	
	Average Methylation (%)	**87**	**99**	**86**	**92**	**88**	**97**	**89**	**92**
HBS 004 (excluded)	PCR1	86	100	81	89	84	94	84	
	PCR2	81	98	88	91	85	99	89	
	PCR3	87	100	90	92	73	92	84	
	Average Methylation (%)	**85**	**99**	**86**	**91**	**81**	**95**	**86**	**89**
HBS 005	PCR1	89	100	85	84	79	95	82	
	PCR2	89	100	89	89	83	97	88	
	PCR3 (excluded)	90	100	87	91	87	100	84	
	Average Methylation (%)	**89**	**100**	**87**	**87**	**81**	**96**	**85**	**89**
HBS 006	PCR1	88	100	88	86	90	94	85	
	PCR2	87	100	89	88	84	100	90	
	PCR3	90	100	89	96	87	100	90	
	Average Methylation (%)	**88**	**100**	**89**	**90**	**87**	**98**	**88**	**92**
HBS 007	PCR1	88	100	82	89	87	94	84	
	PCR2	90	100	90	86	82	94	86	
	PCR3	94	100	91	85	84	100	88	
	Average Methylation (%)	**91**	**100**	**88**	**87**	**84**	**96**	**86**	**91**
HBS 008	PCR1	90	100	84	85	84	97	80	
	PCR2	86	94	89	91	85	100	88	
	PCR3 (excluded)	92	100	91	85	78	93	86	
	Average Methylation (%)	**88**	**97**	**87**	**88**	**85**	**99**	**84**	**90**
HBS 009	PCR1	91	100	90	92	88	95	88	
	PCR2	88	100	84	93	87	96	86	
	PCR3	85	98	90	94	89	94	89	
	Average Methylation (%)	**88**	**99**	**88**	**93**	**88**	**95**	**88**	**91**

HBS 010	PCR1 (excluded)	86	100	81	91	79	100	83	
	PCR2	89	100	87	86	84	93	81	
	PCR3	90	100	90	82	78	91	81	
	Average Methylation (%)	**90**	**100**	**89**	**84**	**81**	**92**	**81**	**88**
Sperm Samples									
HSS 027	PCR1	96	100	93	88	83	97	87	
(excluded)	PCR2	94	100	92	96	90	100	93	
	Average Methylation (%)	**95**	**100**	**93**	**92**	**87**	**99**	**90**	**94**
HSS 033	PCR1	96	100	94	100	91	100	89	
	PCR2	100	96	100	95	90	100	89	
	Average Methylation (%)	**98**	**98**	**97**	**98**	**91**	**100**	**89**	**96**
HSS 035	PCR1	92	98	92	95	100	100	92	
	PCR2	91	99	86	94	94	100	89	
	Average Methylation (%)	**92**	**99**	**89**	**95**	**97**	**100**	**91**	**94**
HSS 038	PCR1	94	100	90	100	100	100	89	
	PCR2	95	100	92	96	94	100	94	
	Average Methylation (%)	**95**	**100**	**91**	**98**	**97**	**100**	**92**	**96**
HSS 048	PCR1	100	100	93	100	100	99	93	
	PCR2	94	100	90	97	94	100	93	
	Average Methylation (%)	**97**	**100**	**92**	**99**	**97**	**100**	**93**	**97**
HSS 061	PCR1	100	100	100	95	96	100	93	
	PCR2	94	97	95	89	100	100	87	
	Average Methylation (%)	**97**	**99**	**98**	**92**	**98**	**100**	**90**	**96**
HSS 077	PCR1	84	100	89	89	85	100	87	
(excluded)	PCR2	96	100	92	97	93	100	92	
	Average Methylation (%)	**90**	**100**	**91**	**93**	**89**	**100**	**90**	**93**
HSS 078	PCR1	90	100	91	95	93	100	91	
	PCR2	93	100	94	93	90	100	88	
	Average Methylation (%)	**92**	**100**	**93**	**94**	**92**	**100**	**90**	**94**

- **CGI_1975**

Sample ID		**CpG Sites Analysed**							**Average Methylation (%) per Sample**
		1	**2**	**3**	**4**	**5**	**6**	**7**	
Blood Samples									
HBS 001	PCR1	84	93	78	57	73	87	100	
	PCR2	89	88	84	57	72	92	97	

	PCR3 (excluded)	80	81	85	48	76	94	100	
	Average methylation (%)	**87**	**91**	**81**	**57**	**73**	**90**	**99**	**82**
HBS 002	PCR1	91	94	77	57	78	91	93	
	PCR2 (excluded)	81	80	74	51	81	85	86	
	PCR3	88	89	73	54	79	92	96	
	Average methylation (%)	**90**	**92**	**75**	**56**	**79**	**92**	**95**	**82**
HBS 003	PCR1	92	93	76	67	77	93	100	
	PCR2	90	94	77	67	75	94	95	
	PCR3	88	88	75	63	76	100	100	
	Average methylation (%)	**90**	**92**	**76**	**66**	**76**	**96**	**98**	**85**
HBS 004	PCR1	90	92	80	55	74	91	100	
	PCR2 (excluded)	81	84	73	63	76	93	98	
	PCR3	89	90	82	58	76	94	100	
	Average methylation (%)	**90**	**91**	**81**	**57**	**75**	**93**	**100**	**84**
HBS 005	PCR1	90	89	73	57	75	90	94	
	PCR2	87	90	71	55	74	92	95	
	PCR3 (excluded)	83	91	66	55	75	93	100	
	Average methylation (%)	**89**	**90**	**72**	**56**	**75**	**91**	**95**	**81**
HBS 006	PCR1	90	93	78	60	73	94	100	
	PCR2 (excluded)	87	88	80	60	97	94	75	
	PCR3	89	92	77	61	78	94	100	
	Average methylation (%)	**90**	**93**	**78**	**61**	**76**	**94**	**100**	**84**
HBS 007	PCR1	86	86	76	55	72	93	100	
	PCR2	88	89	77	51	68	90	95	
	PCR3	86	84	78	55	74	94	100	
	Average methylation (%)	**87**	**86**	**77**	**54**	**71**	**92**	**98**	**81**
HBS 008	PCR1	90	94	75	59	74	91	100	
	PCR2	89	100	74	56	74	93	100	
	PCR3	87	94	71	62	72	92	95	
	Average methylation (%)	**89**	**96**	**73**	**59**	**73**	**92**	**98**	**83**
HBS 009	PCR1	93	91	79	61	76	93	95	
	PCR2 (excluded)	82	92	81	58	71	100	92	
	PCR3	91	91	77	61	79	95	100	
	Average methylation (%)	**92**	**91**	**78**	**61**	**78**	**94**	**98**	**84**
HBS 010	PCR1	89	93	78	59	71	94	100	
	PCR2	84	92	79	57	74	91	100	
	PCR3	86	91	72	55	74	94	100	
	Average methylation (%)	**86**	**92**	**76**	**57**	**73**	**93**	**100**	**83**
Sperm Samples									
HSS 027	PCR1	88	83	96	93	78	90	94	
	PCR2	87	82	100	92	83	92	100	
	Average methylation (%)	**88**	**83**	**98**	**93**	**81**	**91**	**97**	**90**

HSS 033	PCR1	97	96	95	98	76	95	94	
	PCR2	93	91	93	96	81	95	100	
	Average methylation (%)	**95**	**94**	**94**	**97**	**79**	**95**	**97**	**93**
HSS 035	PCR1	100	100	100	95	86	93	100	
	PCR2	94	94	96	99	87	94	97	
	Average methylation (%)	**97**	**97**	**98**	**97**	**87**	**94**	**99**	**95**
HSS 038	PCR1	95	89	89	97	87	95	97	
	PCR2	96	95	94	98	85	94	100	
	Average methylation (%)	**96**	**92**	**92**	**98**	**86**	**95**	**99**	**94**
HSS 048	PCR1	87	100	100	98	83	91	100	
	PCR2	92	94	96	97	87	94	100	
	Average methylation (%)	**90**	**97**	**98**	**98**	**85**	**93**	**100**	**94**
HSS 061	PCR1	92	94	92	100	83	90	100	
	PCR2	91	100	90	100	86	94	100	
	Average methylation (%)	**92**	**97**	**91**	**100**	**85**	**92**	**100**	**94**
HSS 077	PCR1	90	100	100	93	85	94	100	
	PCR2	95	100	96	99	88	100	100	
	Average methylation (%)	**93**	**100**	**98**	**96**	**87**	**97**	**100**	**96**
HSS 078	PCR1	95	100	93	96	86	94	100	
	PCR2	95	96	92	95	89	94	96	
	Average methylation (%)	**95**	**98**	**93**	**96**	**88**	**94**	**98**	**94**

SNRPN DATA

Sample ID		CpG Sites Analyzed				Average Methylation (%) per Sample
		1	**2**	**3**	**4**	
Controls						
HSS 032	PCR1	4	4	4	5	
	PCR2	4	0	4	4	
	PCR3	0	0	0	0	
	Average Methylation (%)	**3**	**1**	**3**	**3**	**2**
HSS 037	PCR1	0	3	0	4	
	PCR2	5	6	5	6	
	PCR3	0	0	0	7	
	Average Methylation (%)	**2**	**3**	**2**	**6**	**3**
HSS 038	PCR1	6	0	0	5	
	PCR2	0	0	0	0	

	PCR3	5	5	4	5	
	Average Methylation (%)	**4**	**2**	**1**	**3**	**3**
HSS 085	PCR1	0	0	0	3	
	PCR2	0	5	4	4	
	PCR3	0	0	0	5	
	Average Methylation (%)	**0**	**2**	**1**	**4**	**2**
HSS 088	PCR1	0	0	0	0	
	PCR2	5	0	0	0	
	PCR3	5	0	4	0	
	Average Methylation (%)	**3**	**0**	**1**	**0**	**1**
Alcohol-Consuming Group						
HSS 005	PCR1	4	0	0	0	
	PCR2	0	0	0	0	
	PCR3	0	0	0	0	
	Average Methylation (%)	**1**	**0**	**0**	**0**	**0**
HSS 006	PCR1	6	5	6	6	
	PCR2	0	0	0	8	
	PCR3	4	5	5	7	
	Average Methylation (%)	**3**	**3**	**4**	**7**	**4**
HSS 017	PCR1	0	5	0	7	
	PCR2	4	4	0	7	
	PCR3	0	0	2	4	
	Average Methylation (%)	**1**	**3**	**1**	**6**	**3**
HSS 018	PCR1	0	0	0	0	
	PCR2	0	0	0	0	
	PCR3	0	4	5	5	
	Average Methylation (%)	**0**	**1**	**2**	**2**	**1**
HSS 020	PCR1	0	0	0	5	
	PCR2	0	6	5	7	
	PCR3	4	5	5	6	
	Average Methylation (%)	**1**	**4**	**3**	**6**	**4**
HSS 022	PCR1	0	0	0	0	
	PCR2	0	0	0	0	
	PCR3	0	0	0	0	
	Average Methylation (%)	**0**	**0**	**0**	**0**	**0**
HSS 023	PCR1	6	6	4	4	
	PCR2	3	3	3	4	
	PCR3	6	7	6	7	
	Average Methylation (%)	**5**	**5**	**4**	**5**	**5**
HSS 024	PCR1	3	0	4	4	
	PCR2	0	0	0	9	
	PCR3	4	4	5	6	

	Average Methylation (%)	**2**	**1**	**3**	**6**	**3**
HSS 043	PCR1	0	0	0	0	
	PCR2	4	0	0	0	
	PCR3	5	3	4	5	
	Average Methylation (%)	**3**	**1**	**1**	**2**	**2**
HSS 048	PCR1	4	5	5	5	
	PCR2	0	6	0	9	
	PCR3	5	4	0	5	
	Average Methylation (%)	**3**	**5**	**2**	**6**	**4**
HSS 054	PCR1	3	0	0	5	
	PCR2	4	0	0	5	
	PCR3	6	5	4	7	
	Average Methylation (%)	**4**	**2**	**1**	**6**	**3**
HSS 067	PCR1	6	5	0	7	
	PCR2	6	3	4	4	
	PCR3	3	5	0	5	
	Average Methylation (%)	**5**	**4**	**1**	**5**	**4**
HSS 073	PCR1	4	4	4	4	
	PCR2	0	0	0	0	
	PCR3	3	0	3	4	
	Average Methylation (%)	**2**	**1**	**2**	**3**	**2**
HSS 081	PCR1	0	0	0	0	
	PCR2	0	4	0	6	
	PCR3	0	0	0	0	
	Average Methylation (%)	**0**	**1**	**0**	**2**	**1**
HSS 122	PCR1	0	0	0	4	
	PCR2	0	0	0	0	
	PCR3	5	0	0	0	
	Average Methylation (%)	**2**	**0**	**0**	**1**	**1**
HSS 128	PCR1	0	0	0	0	
	PCR2	4	3	5	4	
	PCR3	0	0	5	6	
	Average Methylation (%)	**1**	**1**	**3**	**3**	**2**
HSS 129	PCR1	5	0	6	7	
	PCR2	0	0	0	6	
	PCR3	0	4	5	6	
	Average Methylation (%)	**2**	**1**	**4**	**6**	**3**

IG-DMR Data

Sample ID		CpG Sites Analyzed										Average Methylation (%) per Sample
		1	2	3	4	5	6	7	8	9	10	
Controls												
HSS 019	PCR1	91	93	98	69	80	98	98	92	98	99	
	PCR2	93	93	98	69	82	95	98	90	99	98	
	PCR3	93	95	98	68	79	98	92	89	99	99	
	Average Methylation (%)	**92**	**94**	**98**	**69**	**80**	**97**	**96**	**90**	**99**	**99**	**91**
HSS 025	PCR1	95	95	99	63	80	98	98	85	99	97	
	PCR2	93	94	98	62	78	98	98	86	99	97	
	PCR3	94	92	95	64	79	97	98	85	98	96	
	Average Methylation (%)	**94**	**94**	**97**	**63**	**79**	**98**	**98**	**85**	**99**	**97**	**90**
HSS 027	PCR1	92	94	97	66	74	94	99	90	97	99	
	PCR2	95	96	97	69	77	97	99	89	97	99	
	PCR3	95	94	95	68	75	96	99	89	97	99	
	Average Methylation (%)	**94**	**95**	**96**	**68**	**75**	**96**	**99**	**89**	**97**	**99**	**91**
HSS 031	PCR1	97	94	100	71	80	97	99	86	99	98	
	PCR2	96	95	100	70	80	97	99	89	98	98	
	PCR3	96	95	100	69	81	97	99	88	98	98	
	Average Methylation (%)	**96**	**95**	**100**	**70**	**80**	**97**	**99**	**88**	**98**	**98**	**92**
HSS 032	PCR1	94	93	97	66	77	94	98	89	98	98	
	PCR2	92	93	97	67	79	96	98	88	97	98	
	PCR3	92	94	97	67	78	95	98	90	98	98	
	Average Methylation (%)	**93**	**93**	**97**	**67**	**78**	**95**	**98**	**89**	**98**	**98**	**91**
HSS 033	PCR1	94	92	95	71	81	97	98	89	98	98	
	PCR2	94	93	94	71	80	97	98	89	98	98	
	PCR3	95	93	95	69	77	93	95	84	96	95	
	Average Methylation (%)	**94**	**93**	**95**	**70**	**79**	**96**	**97**	**87**	**97**	**97**	**91**
HSS 035	PCR1	93	95	98	66	70	95	98	84	96	98	
	PCR2	93	94	98	69	71	95	98	87	96	98	
	PCR3	93	95	98	68	69	94	98	87	96	98	
	Average Methylation (%)	**93**	**95**	**98**	**68**	**70**	**95**	**98**	**86**	**96**	**98**	**90**
HSS 037	PCR1	93	94	97	66	73	90	98	89	98	98	
	PCR2	93	94	97	68	75	93	98	89	98	98	
	PCR3	92	94	97	67	73	91	98	89	98	98	
	Average Methylation (%)	**93**	**94**	**97**	**67**	**74**	**91**	**98**	**89**	**98**	**98**	**90**
HSS 038	PCR1	94	95	99	66	75	95	97	84	97	98	
	PCR2	94	95	99	63	72	94	98	86	98	98	
	PCR3	92	94	99	61	70	94	98	85	97	98	
	Average Methylation (%)	**93**	**95**	**99**	**63**	**72**	**94**	**98**	**85**	**97**	**98**	**90**

HSS 042	PCR1	91	92	100	70	78	99	100	89	97	100	
	PCR2	94	94	100	68	78	98	99	89	97	99	
	PCR3	94	94	98	70	78	98	99	90	97	99	
	Average Methylation (%)	**93**	**93**	**99**	**69**	**78**	**98**	**99**	**89**	**97**	**99**	**92**
HSS 046	PCR1	94	95	97	69	78	97	96	89	98	97	
	PCR1	94	96	97	71	79	97	97	90	99	97	
	PCR3	93	94	99	69	80	98	97	88	99	97	
	Average Methylation (%)	**94**	**95**	**98**	**70**	**79**	**97**	**97**	**89**	**99**	**97**	**91**
HSS 050	PCR1	93	94	100	81	78	97	96	83	97	97	
	PCR2	92	93	100	76	80	98	100	88	98	98	
	PCR3	92	94	100	76	79	98	97	88	98	98	
	Average Methylation (%)	**92**	**94**	**100**	**78**	**79**	**98**	**98**	**86**	**98**	**98**	**92**
HSS 055	PCR1	93	95	97	61	75	96	98	87	98	98	
	PCR2	92	93	97	62	77	97	98	88	99	98	
	PCR3	93	95	97	57	72	94	95	84	97	97	
	Average Methylation (%)	**93**	**94**	**97**	**60**	**75**	**96**	**97**	**86**	**98**	**98**	**89**
HSS 056	PCR1	93	94	97	65	71	92	99	89	91	98	
	PCR2	93	94	97	69	75	94	98	86	97	97	
	PCR3	91	92	98	67	75	94	99	88	97	98	
	Average Methylation (%)	**92**	**93**	**97**	**67**	**74**	**93**	**99**	**88**	**95**	**98**	**90**
HSS 070	PCR1	92	94	99	65	78	98	98	89	98	98	
	PCR2	93	95	98	67	79	98	99	88	98	98	
	PCR3	94	92	95	64	79	98	98	88	98	98	
	Average Methylation (%)	**93**	**94**	**97**	**65**	**79**	**98**	**98**	**88**	**98**	**98**	**91**
HSS 074	PCR1	94	94	98	68	79	97	98	90	98	97	
	PCR2	94	94	98	68	79	98	99	88	98	97	
	PCR3	93	93	98	66	79	98	99	88	98	97	
	Average Methylation (%)	**94**	**94**	**98**	**67**	**79**	**98**	**99**	**89**	**98**	**97**	**91**
HSS 075	PCR1	93	95	97	71	82	98	99	91	98	99	
	PCR2	91	95	97	70	78	98	98	89	97	99	
	PCR3	94	95	97	72	82	98	98	91	98	99	
	Average Methylation (%)	**93**	**95**	**97**	**71**	**81**	**98**	**98**	**90**	**98**	**99**	**92**
HSS 077	PCR1	92	90	96	67	79	98	98	87	97	98	
	PCR2	94	93	97	68	81	97	98	89	98	98	
	PCR3	94	92	100	69	80	98	100	89	98	99	
	Average Methylation (%)	**93**	**92**	**98**	**68**	**80**	**98**	**99**	**88**	**98**	**98**	**91**
HSS 079	PCR1	90	91	96	77	81	97	100	89	100	97	
	PCR2	91	91	95	81	83	98	100	91	99	99	
	PCR3	93	94	98	78	82	96	100	89	99	99	
	Average Methylation (%)	**91**	**92**	**96**	**79**	**82**	**97**	**100**	**90**	**99**	**98**	**92**
HSS 082	PCR1	90	92	98	60	78	99	100	90	99	99	
	PCR2	95	95	97	62	79	98	100	91	99	99	

	PCR3	94	94	98	58	76	96	100	89	98	99	
	Average Methylation (%)	**93**	**94**	**98**	**60**	**78**	**98**	**100**	**90**	**99**	**99**	**91**
HSS 085	PCR1	94	94	98	61	78	97	99	89	98	98	
	PCR2	95	95	98	60	78	97	98	88	97	98	
	PCR3	94	94	98	62	79	97	98	88	97	98	
	Average Methylation (%)	**94**	**94**	**98**	**61**	**78**	**97**	**98**	**88**	**97**	**98**	
HSS 088	PCR1	95	95	98	68	80	99	99	90	97	97	
	PCR2	94	97	97	65	79	98	99	90	97	97	
	PCR3	96	93	97	67	81	98	99	91	97	97	
	Average Methylation (%)	**95**	**95**	**97**	**67**	**80**	**98**	**99**	**90**	**97**	**97**	**92**
HSS 089	PCR1	89	92	97	59	72	93	97	85	96	97	
	PCR2	93	96	100	60	73	93	97	87	96	98	
	PCR3	92	96	97	63	77	96	98	89	97	98	
	Average Methylation (%)	**91**	**95**	**98**	**61**	**74**	**94**	**97**	**87**	**96**	**98**	**89**
HSS 094	PCR1	94	95	98	66	80	97	98	88	97	97	
	PCR2	94	95	100	66	79	97	98	89	97	97	
	PCR3	93	93	98	67	79	97	98	88	98	97	
	Average Methylation (%)	**94**	**94**	**99**	**66**	**79**	**97**	**98**	**88**	**97**	**97**	**91**
HSS 096	PCR1	94	95	100	74	78	96	98	89	97	99	
	PCR2	96	97	98	75	78	97	98	90	97	99	
	PCR3	95	96	97	74	80	97	98	90	98	99	
	Average Methylation (%)	**95**	**96**	**98**	**74**	**79**	**97**	**98**	**90**	**97**	**99**	**92**
HSS 097	PCR1	94	95	98	67	75	98	93	86	97	98	
	PCR2	93	93	98	66	77	98	98	88	96	98	
	PCR3	94	94	98	66	75	97	98	88	96	98	
	Average Methylation (%)	**94**	**94**	**98**	**66**	**76**	**98**	**96**	**87**	**96**	**98**	**90**
HSS 107	PCR1	94	95	98	72	78	96	98	89	98	97	
	PCR2	94	95	98	72	78	96	98	88	97	97	
	PCR3	95	95	98	72	77	96	98	89	97	97	
	Average Methylation (%)	**94**	**95**	**98**	**72**	**78**	**96**	**98**	**89**	**97**	**97**	**91**
HSS 116	PCR1	89	92	98	66	76	94	96	86	96	97	
	PCR2	94	95	98	65	74	94	95	84	96	96	
	PCR3	91	93	98	70	79	98	100	87	99	99	
	Average Methylation (%)	**91**	**93**	**98**	**67**	**76**	**95**	**97**	**86**	**97**	**97**	**90**
HSS 121	PCR1	94	96	96	63	74	94	98	88	97	98	
	PCR2	94	95	96	65	76	96	98	88	97	98	
	PCR3	92	94	96	63	73	94	97	88	97	98	
	Average Methylation (%)	**93**	**95**	**96**	**64**	**74**	**95**	**98**	**88**	**97**	**98**	**90**
HSS 131	PCR1	94	94	97	59	76	97	99	88	96	97	
	PCR2	95	95	96	59	75	95	99	89	96	97	
	PCR3	94	95	98	60	75	95	98	88	96	97	
	Average Methylation (%)	**94**	**95**	**97**	**59**	**75**	**96**	**99**	**88**	**96**	**97**	**90**

HSS 136	PCR1	94	94	95	70	79	97	98	89	97	98	
	PCR2	92	94	96	69	79	97	98	87	97	98	
	PCR3	94	95	95	70	80	98	99	89	98	98	
	Average Methylation (%)	**93**	**94**	**95**	**70**	**79**	**97**	**98**	**88**	**97**	**98**	**91**
HSS 141	PCR1	91	93	97	68	81	97	99	86	97	98	
	PCR2	90	93	100	68	81	97	99	87	97	98	
	PCR3	91	92	97	65	78	97	99	85	97	96	
	Average Methylation (%)	**91**	**93**	**98**	**67**	**80**	**97**	**99**	**86**	**97**	**97**	**90**
HSS 142	PCR1	94	94	98	64	80	98	97	86	97	98	
	PCR2	93	93	98	65	80	98	97	88	96	98	
	PCR3	93	94	97	63	79	97	97	86	96	98	
	Average Methylation (%)	**93**	**94**	**98**	**64**	**80**	**98**	**97**	**87**	**96**	**98**	**90**
HSS 149	PCR1	93	93	95	72	76	98	97	87	98	98	
	PCR2	94	94	96	74	77	99	97	88	98	98	
	PCR3	93	93	95	73	76	99	97	87	98	97	
	Average Methylation (%)	**93**	**93**	**95**	**73**	**76**	**99**	**97**	**87**	**98**	**98**	**91**
Alcohol-Consuming Group												
HSS 001	PCR1	88	92	98	57	73	90	94	85	94	97	
	PCR2	92	93	97	61	73	93	97	88	98	98	
	PCR3	88	95	98	59	77	95	99	92	97	99	
	Average Methylation (%)	**89**	**93**	**98**	**59**	**74**	**93**	**97**	**88**	**96**	**98**	**89**
HSS 003	PCR1	93	95	98	57	76	93	97	89	97	97	
	PCR2	94	94	96	60	77	95	97	89	97	97	
	PCR3	94	95	98	58	75	94	96	86	97	97	
	Average Methylation (%)	**94**	**95**	**97**	**58**	**76**	**94**	**97**	**88**	**97**	**97**	**89**
HSS 004	PCR1	95	94	96	64	75	87	96	91	99	95	
	PCR2	93	94	96	67	78	92	96	90	99	95	
	PCR3	92	94	96	69	80	92	96	91	100	95	
	Average Methylation (%)	**93**	**94**	**96**	**67**	**78**	**90**	**96**	**91**	**99**	**95**	**90**
HSS 005	PCR1	92	93	95	58	66	95	98	86	97	96	
	PCR2	93	94	98	58	67	94	98	85	97	96	
	PCR3	94	95	98	60	68	96	98	87	97	96	
	Average Methylation (%)	**93**	**94**	**97**	**59**	**67**	**95**	**98**	**86**	**97**	**96**	**88**
HSS 006	PCR1	93	94	99	62	74	92	98	89	97	98	
	PCR2	93	94	99	64	77	95	98	88	97	98	
	PCR3	93	95	99	66	77	95	98	88	97	98	
	Average Methylation (%)	**93**	**94**	**99**	**64**	**76**	**94**	**98**	**88**	**97**	**98**	**90**
HSS 008	PCR1	92	94	97	54	74	94	95	83	95	97	
	PCR2	94	96	97	55	71	92	96	86	96	97	
	PCR3	92	93	100	58	77	95	96	86	95	96	
	Average Methylation (%)	**93**	**94**	**98**	**56**	**74**	**94**	**96**	**85**	**95**	**97**	**88**
HSS 010	PCR1	90	90	95	61	72	89	97	90	89	98	

	PCR2	94	95	96	66	77	95	97	89	98	98	
	PCR3	94	95	97	64	75	93	97	90	98	98	
	Average Methylation (%)	**93**	**93**	**96**	**64**	**75**	**92**	**97**	**90**	**95**	**98**	**89**
HSS 011	PCR1	94	95	94	55	77	96	94	84	98	98	
	PCR2	94	95	95	54	75	98	95	83	98	98	
	PCR3	94	96	96	57	77	97	100	84	99	98	
	Average Methylation (%)	**94**	**95**	**95**	**55**	**76**	**97**	**96**	**84**	**98**	**98**	**89**
HSS 016	PCR1	92	93	89	52	73	95	95	84	97	98	
	PCR2	92	93	91	52	73	96	95	84	97	98	
	PCR3	92	93	89	48	70	92	95	85	97	98	
	Average Methylation (%)	**92**	**93**	**90**	**51**	**72**	**94**	**95**	**84**	**97**	**98**	**87**
HSS 017	PCR1	92	92	97	64	71	95	96	79	95	98	
	PCR2	93	94	97	58	70	94	96	81	96	97	
	PCR3	93	94	97	59	73	93	96	83	96	97	
	Average Methylation (%)	**93**	**93**	**97**	**60**	**71**	**94**	**96**	**81**	**96**	**97**	**88**
HSS 018	PCR1	90	91	98	57	67	89	97	87	95	98	
	PCR2	92	94	97	56	66	93	97	87	96	96	
	PCR3	92	94	98	59	71	92	97	87	95	97	
	Average Methylation (%)	**91**	**93**	**98**	**57**	**68**	**91**	**97**	**87**	**95**	**97**	**88**
HSS 020	PCR1	92	96	98	71	80	97	98	89	98	98	
	PCR2	93	92	98	72	79	97	98	89	98	98	
	PCR3	92	96	98	70	80	97	98	90	98	98	
	Average Methylation (%)	**92**	**95**	**98**	**71**	**80**	**97**	**98**	**89**	**98**	**98**	**92**
HSS 021	PCR1	91	90	95	52	76	91	96	87	97	98	
	PCR2	93	91	95	54	78	95	96	84	98	98	
	PCR3	93	91	95	55	79	95	96	86	98	98	
	Average Methylation (%)	**92**	**91**	**95**	**54**	**78**	**94**	**96**	**86**	**98**	**98**	**88**
HSS 022	PCR1	94	95	98	68	81	98	98	88	97	98	
	PCR2	93	95	98	70	81	98	98	90	97	98	
	PCR3	93	95	98	67	80	98	98	89	98	98	
	Average Methylation (%)	**93**	**95**	**98**	**68**	**81**	**98**	**98**	**89**	**97**	**98**	**92**
HSS 023	PCR1	93	95	98	65	76	93	97	89	98	97	
	PCR2	93	95	98	69	79	96	98	89	98	98	
	PCR3	93	94	98	69	79	96	97	88	98	98	
	Average Methylation (%)	**93**	**95**	**98**	**68**	**78**	**95**	**97**	**89**	**98**	**98**	**91**
HSS 024	PCR1	94	92	98	63	78	97	98	88	97	97	
	PCR2	94	93	97	62	77	97	97	86	97	97	
	PCR3	94	94	98	66	82	97	97	89	97	97	
	Average Methylation (%)	**94**	**93**	**98**	**64**	**79**	**97**	**97**	**88**	**97**	**97**	**90**
HSS 028	PCR1	89	93	98	55	73	92	98	85	97	98	
	PCR2	86	89	98	55	72	92	98	86	97	98	
	PCR3	91	93	97	55	73	92	98	85	97	97	
	Average Methylation (%)	**89**	**92**	**98**	**55**	**73**	**92**	**98**	**85**	**97**	**98**	**88**

HSS 036	PCR1	97	95	100	65	75	96	100	86	97	97	
	PCR2	95	95	100	68	77	96	99	88	97	97	
	PCR3	96	95	98	66	78	96	99	86	97	97	
	Average Methylation (%)	**96**	**95**	**99**	**66**	**77**	**96**	**99**	**87**	**97**	**97**	**91**
HSS 040	PCR1	95	94	96	66	79	98	99	87	98	97	
	PCR2	96	96	96	67	80	98	99	88	98	97	
	PCR3	94	95	96	65	79	98	100	86	98	96	
	Average Methylation (%)	**95**	**95**	**96**	**66**	**79**	**98**	**99**	**87**	**98**	**97**	**91**
HSS 043	PCR1	94	95	98	61	76	98	99	88	97	97	
	PCR2	94	95	97	66	78	98	98	90	98	98	
	PCR3	94	95	98	63	78	97	98	89	97	98	
	Average Methylation (%)	**94**	**95**	**98**	**63**	**77**	**98**	**98**	**89**	**97**	**98**	**91**
HSS 044	PCR1	91	93	97	58	74	94	97	86	97	98	
	PCR2	92	94	94	59	75	95	97	86	97	98	
	PCR3	91	93	95	57	74	94	97	85	97	98	
	Average Methylation (%)	**91**	**93**	**95**	**58**	**74**	**94**	**97**	**86**	**97**	**98**	**88**
HSS 048	PCR1	94	93	96	59	67	91	95	85	97	98	
	PCR2	93	94	98	58	66	90	95	85	98	98	
	PCR3	94	95	98	59	65	90	94	84	96	98	
	Average Methylation (%)	**94**	**94**	**97**	**59**	**66**	**90**	**95**	**85**	**97**	**98**	**87**
HSS 051	PCR1	92	90	96	70	77	97	98	79	96	98	
	PCR2	92	90	96	69	75	96	98	81	95	98	
	PCR3	92	90	97	70	77	97	98	82	95	99	
	Average Methylation (%)	**92**	**90**	**96**	**70**	**76**	**97**	**98**	**81**	**95**	**98**	**89**
HSS 052	PCR1	94	95	98	74	79	96	96	87	97	97	
	PCR2	94	96	97	71	78	98	100	84	97	96	
	PCR3	94	96	98	73	80	98	96	88	98	97	
	Average Methylation (%)	**94**	**96**	**98**	**73**	**79**	**97**	**97**	**86**	**97**	**97**	**91**
HSS 053	PCR1	94	94	97	70	77	96	93	86	98	99	
	PCR2	94	94	97	69	76	96	98	87	97	98	
	PCR3	95	94	97	70	77	96	98	87	98	98	
	Average Methylation (%)	**94**	**94**	**97**	**70**	**77**	**96**	**96**	**87**	**98**	**98**	**91**
HSS 054	**Unable to Obtain Data**											
HSS 057	PCR1	92	93	98	71	82	97	98	86	96	97	
	PCR2	94	92	98	71	84	96	100	90	97	97	
	PCR3	92	93	98	71	82	96	98	88	96	97	
	Average Methylation (%)	**93**	**93**	**98**	**71**	**83**	**96**	**99**	**88**	**96**	**97**	**91**
HSS 058	PCR1	91	93	97	73	74	98	98	85	99	99	
	PCR2	95	95	97	72	75	98	98	85	99	99	
	PCR3	92	93	96	74	76	97	90	87	99	99	
	Average Methylation (%)	**93**	**94**	**97**	**73**	**75**	**98**	**95**	**86**	**99**	**99**	**91**

HSS 061	PCR1	94	94	99	62	73	90	99	87	98	99	
	PCR2	94	92	96	63	72	94	100	86	97	98	
	PCR3	91	89	96	65	74	92	98	87	98	99	
	Average Methylation (%)	**93**	**92**	**97**	**63**	**73**	**92**	**99**	**87**	**98**	**99**	**89**
HSS 062	PCR1	93	94	96	66	80	98	96	88	98	98	
	PCR2	93	94	97	67	79	97	96	89	98	98	
	PCR3	96	94	96	66	79	98	95	88	97	99	
	Average Methylation (%)	**94**	**94**	**96**	**66**	**79**	**98**	**96**	**88**	**98**	**98**	**91**
HSS 064	PCR1	93	90	95	68	78	98	98	87	97	98	
	PCR2	93	90	96	71	79	98	97	88	96	98	
	PCR3	93	89	96	69	78	98	98	87	96	98	
	Average Methylation (%)	**93**	**90**	**96**	**69**	**78**	**98**	**98**	**87**	**96**	**98**	**90**
HSS 067	PCR1	87	55	99	71	80	98	97	87	97	97	
	PCR2	87	55	99	73	80	98	97	89	97	97	
	PCR3	85	56	99	72	80	98	97	89	96	97	
	Average Methylation (%)	**86**	**55**	**99**	**72**	**80**	**98**	**97**	**88**	**97**	**97**	**87**
HSS 068	PCR1	94	94	95	65	76	99	98	89	99	98	
	PCR2	90	94	97	64	77	99	98	87	99	98	
	PCR3	95	94	97	65	76	98	98	87	99	97	
	Average Methylation (%)	**93**	**94**	**96**	**65**	**76**	**99**	**98**	**88**	**99**	**98**	**91**
HSS 069	PCR1	95	95	98	73	81	100	89	88	95	96	
	PCR2	94	95	97	76	80	99	88	91	95	96	
	PCR3	94	95	97	74	80	99	88	90	95	98	
	Average Methylation (%)	**94**	**95**	**97**	**74**	**80**	**99**	**88**	**90**	**95**	**97**	**91**
HSS 071	PCR1	92	93	96	61	78	97	98	87	98	98	
	PCR2	92	92	97	62	78	97	97	86	98	98	
	PCR3	95	94	95	63	79	97	98	88	98	98	
	Average Methylation (%)	**93**	**93**	**96**	**62**	**78**	**97**	**98**	**87**	**98**	**98**	**90**
HSS 072	PCR1	92	91	95	65	79	96	97	87	96	97	
	PCR2	95	96	96	63	79	95	100	87	96	97	
	PCR3	92	93	93	64	78	95	97	88	96	96	
	Average Methylation (%)	**93**	**93**	**95**	**64**	**79**	**95**	**98**	**87**	**96**	**97**	**90**
HSS 073	PCR1	95	95	98	69	74	97	98	88	96	98	
	PCR2	95	94	98	72	76	98	98	87	97	98	
	PCR3	94	94	98	70	76	98	100	88	97	98	
	Average Methylation (%)	**95**	**94**	**98**	**70**	**75**	**98**	**99**	**88**	**97**	**98**	**91**
HSS 076	PCR1	91	94	98	65	77	98	99	88	98	99	
	PCR2	92	95	98	64	76	97	100	88	97	98	
	PCR3	88	91	98	65	77	97	98	87	97	98	
	Average Methylation (%)	**90**	**93**	**98**	**65**	**77**	**97**	**99**	**88**	**97**	**98**	**90**
HSS 078	PCR1	94	94	98	76	81	97	98	88	97	98	
	PCR2	93	92	96	72	79	97	98	87	97	98	
	PCR3	94	89	96	75	81	97	98	89	96	98	

	Average Methylation (%)	**94**	**92**	**97**	**74**	**80**	**97**	**98**	**88**	**97**	**98**	**91**
HSS 081	PCR1	95	94	98	71	66	98	93	90	100	100	
	PCR2	94	94	99	73	66	98	93	91	99	99	
	PCR3	94	94	99	74	67	98	93	91	99	99	
	Average Methylation (%)	**94**	**94**	**99**	**73**	**66**	**98**	**93**	**91**	**99**	**99**	**91**
HSS 083	PCR1	91	91	96	64	80	99	98	87	94	99	
	PCR2	94	96	97	65	80	98	98	87	93	99	
	PCR3	92	93	93	64	80	98	98	88	94	99	
	Average Methylation (%)	**92**	**93**	**95**	**64**	**80**	**98**	**98**	**87**	**94**	**99**	**90**
HSS 084	PCR1	94	95	97	68	79	98	99	89	98	98	
	PCR2	95	96	98	68	79	98	100	89	100	99	
	PCR3	95	95	97	68	78	98	99	87	99	98	
	Average Methylation (%)	**95**	**95**	**97**	**68**	**79**	**98**	**99**	**88**	**99**	**98**	**92**
HSS 086	PCR1	93	95	98	68	80	97	98	90	98	97	
	PCR2	95	95	97	70	82	97	98	90	98	97	
	PCR3	94	94	97	69	79	97	98	89	98	97	
	Average Methylation (%)	**94**	**95**	**97**	**69**	**80**	**97**	**98**	**90**	**98**	**97**	**92**
HSS 090	PCR1	96	96	93	67	78	97	98	87	97	98	
	PCR2	95	95	98	67	78	97	98	87	97	98	
	PCR3	95	95	98	68	80	97	98	87	97	98	
	Average Methylation (%)	**95**	**95**	**96**	**67**	**79**	**97**	**98**	**87**	**97**	**98**	**91**
HSS 092	PCR1	94	95	98	71	79	97	96	88	97	97	
	PCR2	94	95	97	70	78	97	95	88	98	97	
	PCR3	93	95	98	73	81	98	97	89	98	98	
	Average Methylation (%)	**94**	**95**	**98**	**71**	**79**	**97**	**96**	**88**	**98**	**97**	**91**
HSS 095	PCR1	93	93	98	63	76	97	98	85	97	98	
	PCR2	92	93	96	64	75	97	98	87	97	99	
	PCR3	93	92	97	64	77	97	98	87	98	98	
	Average Methylation (%)	**93**	**93**	**97**	**64**	**76**	**97**	**98**	**86**	**97**	**98**	**90**
HSS 098	PCR1	83	95	97	69	78	96	92	89	96	96	
	PCR2	84	95	97	70	79	95	91	90	96	96	
	PCR3	84	96	97	71	81	97	92	91	96	96	
	Average Methylation (%)	**84**	**95**	**97**	**70**	**79**	**96**	**92**	**90**	**96**	**96**	**90**
HSS 102	PCR1	91	93	96	71	78	98	99	88	98	99	
	PCR2	91	95	97	70	78	97	98	88	97	98	
	PCR3	92	92	94	71	79	98	99	88	98	98	
	Average Methylation (%)	**91**	**93**	**96**	**71**	**78**	**98**	**99**	**88**	**98**	**98**	**91**
HSS 103	PCR1	93	96	95	68	77	97	99	89	98	99	
	PCR2	92	95	96	69	77	96	98	86	97	99	
	PCR3	92	95	96	69	77	97	99	88	97	99	
	Average Methylation (%)	**92**	**95**	**96**	**69**	**77**	**97**	**99**	**88**	**97**	**99**	**91**
HSS 105	PCR1	93	93	98	72	79	97	98	90	98	97	

	PCR2	93	94	98	70	77	96	98	88	98	97	
	PCR3	92	93	98	72	80	97	98	90	98	97	
	Average Methylation (%)	**93**	**93**	**98**	**71**	**79**	**97**	**98**	**89**	**98**	**97**	**91**
HSS 110	PCR1	94	95	98	71	78	98	98	86	98	98	
	PCR2	93	94	98	71	78	98	100	86	99	98	
	PCR3	93	95	98	73	79	98	98	88	98	98	
	Average Methylation (%)	**93**	**95**	**98**	**72**	**78**	**98**	**99**	**87**	**98**	**98**	**92**
HSS 111	PCR1	94	93	96	64	80	97	97	85	96	97	
	PCR2	92	92	95	69	82	97	98	86	97	97	
	PCR3	94	94	97	66	79	97	97	86	98	98	
	Average Methylation (%)	**93**	**93**	**96**	**66**	**80**	**97**	**97**	**86**	**97**	**97**	**90**
HSS 112	PCR1	94	95	97	72	80	98	98	88	98	98	
	PCR2	94	94	98	72	81	98	98	90	97	98	
	PCR3	93	93	97	71	80	98	98	90	98	98	
	Average Methylation (%)	**94**	**94**	**97**	**72**	**80**	**98**	**98**	**89**	**98**	**98**	**92**
HSS 114	PCR1	96	93	96	78	80	97	98	88	98	97	
	PCR2	94	95	98	73	78	96	98	89	98	98	
	PCR3	94	95	98	73	79	97	98	89	98	97	
	Average Methylation (%)	**95**	**94**	**97**	**75**	**79**	**97**	**98**	**89**	**98**	**97**	**92**
HSS 117	PCR1	95	92	97	57	78	97	98	84	97	97	
	PCR2	93	94	98	58	77	97	98	86	97	97	
	PCR3	94	95	97	58	78	96	97	87	97	97	
	Average Methylation (%)	**94**	**94**	**97**	**58**	**78**	**97**	**98**	**86**	**97**	**97**	**89**
HSS118	PCR1	94	95	97	73	76	97	96	86	52	98	
	PCR2	95	95	98	74	78	97	97	87	51	98	
	PCR3	94	94	98	75	80	98	97	88	50	98	
	Average Methylation (%)	**94**	**95**	**98**	**74**	**78**	**97**	**97**	**87**	**51**	**98**	**87**
HSS 119	PCR1	91	92	96	68	79	97	100	89	98	98	
	PCR2	95	95	96	71	80	97	99	88	98	99	
	PCR3	93	96	99	69	79	98	99	86	98	98	
	Average Methylation (%)	**93**	**94**	**97**	**69**	**79**	**97**	**99**	**88**	**98**	**98**	**91**
HSS 120	PCR1	93	94	96	61	77	98	100	88	98	97	
	PCR2	94	93	97	60	77	98	98	89	98	98	
	PCR3	93	93	96	61	77	99	98	89	98	97	
	Average Methylation (%)	**93**	**93**	**96**	**61**	**77**	**98**	**99**	**89**	**98**	**97**	**90**
HSS 122	PCR1	92	92	99	62	74	93	97	91	96	98	
	PCR2	97	96	98	64	75	95	97	91	96	98	
	PCR3	92	94	98	66	79	97	95	86	98	98	
	Average Methylation (%)	**94**	**94**	**98**	**64**	**76**	**95**	**96**	**89**	**97**	**98**	**90**
HSS 123	PCR1	94	94	97	76	80	97	100	89	99	97	
	PCR2	96	95	100	79	82	97	99	90	98	99	
	PCR3	95	94	99	81	82	99	100	91	99	99	
	Average Methylation (%)	**95**	**94**	**99**	**79**	**81**	**98**	**100**	**90**	**99**	**98**	**93**

HSS 124	PCR1	92	93	95	60	74	94	95	85	96	98	
	PCR2	88	91	95	60	73	94	95	84	96	97	
	PCR3	92	93	97	60	78	96	97	87	97	98	
	Average Methylation (%)	**91**	**92**	**96**	**60**	**75**	**95**	**96**	**85**	**96**	**98**	**88**
HSS 125	PCR1	93	91	97	64	80	98	97	86	99	98	
	PCR2	92	91	96	63	79	98	97	89	99	98	
	PCR3	91	92	96	66	80	98	97	88	99	98	
	Average Methylation (%)	**92**	**91**	**96**	**64**	**80**	**98**	**97**	**88**	**99**	**98**	**90**
HSS 126	PCR1	95	95	97	64	83	98	99	91	78	99	
	PCR2	93	93	98	64	81	99	99	89	78	99	
	PCR3	93	94	97	64	81	97	99	91	78	99	
	Average Methylation (%)	**94**	**94**	**97**	**64**	**82**	**98**	**99**	**90**	**78**	**99**	**90**
HSS 127	PCR1	95	94	98	72	74	98	97	87	96	98	
	PCR2	93	94	98	73	75	98	97	88	95	98	
	PCR3	93	93	98	72	74	99	97	87	96	98	
	Average Methylation (%)	**94**	**94**	**98**	**72**	**74**	**98**	**97**	**87**	**96**	**98**	**91**
HSS 128	PCR1	94	89	96	58	80	98	98	88	98	98	
	PCR2	92	92	97	58	79	98	98	86	98	98	
	PCR3	92	94	96	56	78	98	98	86	98	98	
	Average Methylation (%)	**93**	**92**	**96**	**57**	**79**	**98**	**98**	**87**	**98**	**98**	**90**
HSS 129	PCR1	94	93	100	71	78	97	97	89	97	97	
	PCR2	92	92	98	71	79	97	97	89	97	98	
	PCR3	92	93	98	70	80	97	97	89	97	98	
	Average Methylation (%)	**93**	**93**	**99**	**71**	**79**	**97**	**97**	**89**	**97**	**98**	**91**
HSS 130	PCR1	94	93	99	58	79	98	98	87	98	97	
	PCR2	94	94	96	61	80	98	98	87	98	97	
	PCR3	94	93	98	60	79	98	98	88	98	97	
	Average Methylation (%)	**94**	**93**	**98**	**60**	**79**	**98**	**98**	**87**	**98**	**97**	**90**
HSS 132	PCR1	93	94	97	75	79	98	98	90	97	98	
	PCR2	93	95	97	75	78	98	97	87	98	99	
	PCR3	93	96	98	74	78	97	98	90	98	98	
	Average Methylation (%)	**93**	**95**	**97**	**75**	**78**	**98**	**98**	**89**	**98**	**98**	**92**
HSS 133	PCR1	91	93	96	72	81	98	100	90	97	99	
	PCR2	92	93	96	72	81	99	98	90	97	99	
	PCR3	92	94	95	70	80	98	98	90	97	99	
	Average Methylation (%)	**92**	**93**	**96**	**71**	**81**	**98**	**99**	**90**	**97**	**99**	**92**
HSS 134	PCR1	95	93	97	64	79	97	98	86	97	98	
	PCR2	93	94	96	61	78	97	98	86	97	99	
	PCR3	95	95	95	59	78	96	98	87	97	99	
	Average Methylation (%)	**94**	**94**	**96**	**61**	**78**	**97**	**98**	**86**	**97**	**99**	**90**
HSS 135	PCR1	92	94	99	66	79	98	99	86	98	96	
	PCR2	90	93	99	68	79	97	99	88	98	97	

	PCR3	91	94	98	65	80	98	99	87	98	97	
	Average Methylation (%)	**91**	**94**	**99**	**66**	**79**	**98**	**99**	**87**	**98**	**97**	**91**
HSS 137	PCR1	92	92	97	64	79	97	98	88	96	97	
	PCR2	94	94	96	66	80	97	98	90	96	97	
	PCR3	92	92	100	64	79	97	99	89	96	98	
	Average Methylation (%)	**93**	**93**	**98**	**65**	**79**	**97**	**98**	**89**	**96**	**97**	**90**
HSS 140	PCR1	93	94	96	72	80	94	99	89	97	98	
	PCR2	93	95	96	73	79	97	99	89	97	98	
	PCR3	93	94	97	71	78	97	99	91	97	98	
	Average Methylation (%)	**93**	**94**	**96**	**72**	**79**	**96**	**99**	**90**	**97**	**98**	**91**
HSS 143	PCR1	94	93	96	64	80	98	100	86	98	98	
	PCR2	92	93	95	66	81	98	97	88	97	97	
	PCR3	93	94	95	64	80	99	97	89	98	98	
	Average Methylation (%)	**93**	**93**	**95**	**65**	**80**	**98**	**98**	**88**	**98**	**98**	**91**
HSS 144	PCR1	95	93	97	66	77	96	98	86	98	97	
	PCR2	94	94	97	67	80	94	98	89	97	97	
	PCR3	94	94	97	68	78	97	98	87	97	97	
	Average Methylation (%)	**94**	**94**	**97**	**67**	**78**	**96**	**98**	**87**	**97**	**97**	**91**
HSS 145	PCR1	94	93	98	70	78	97	98	87	97	97	
	PCR2	93	94	97	69	79	97	98	89	98	98	
	PCR3	93	93	97	69	80	98	98	89	98	98	
	Average Methylation (%)	**93**	**93**	**97**	**69**	**79**	**97**	**98**	**88**	**98**	**98**	**91**
HSS 146	PCR1	93	93	95	70	81	98	98	86	98	98	
	PCR2	93	93	95	70	82	99	98	88	97	99	
	PCR3	94	94	97	70	81	99	99	87	98	98	
	Average Methylation (%)	**93**	**93**	**96**	**70**	**81**	**99**	**98**	**87**	**98**	**98**	**91**
HSS 147	PCR1	94	93	97	68	82	98	98	88	98	97	
	PCR2	93	93	97	69	80	97	97	87	97	98	
	PCR3	93	93	97	67	79	97	97	86	97	98	
	Average Methylation (%)	**93**	**93**	**97**	**68**	**80**	**97**	**97**	**87**	**97**	**98**	**91**
HSS 148	PCR1	86	48	98	74	79	97	100	87	98	98	
	PCR2	87	48	98	72	79	97	98	88	98	97	
	PCR3	87	48	98	74	78	97	97	87	97	97	
	Average Methylation (%)	**87**	**48**	**98**	**73**	**79**	**97**	**98**	**87**	**98**	**97**	**86**

APPENDIX J

MULTIPLE REGRESSION ANALYSIS

Table J.1: Multiple regression analysis for CpG5

		Methylation		
	Variable	**%**	**SE**	**P-value**
Alcohol Consumption				
CpG 5	Intercept	74.02	2.20	0.000
	Treatment	-0.09	0.65	0.895
	Age	0.10	0.05	0.056
	Adjusted $R^2 = 0.02$			

When examining the effect of potential confounders on the methylation levels of individual CpG sites, the methylation levels at CpG 5 was found to increase significantly as age increased (Spearman's rho=0.189, p=0.046). However, when age was incorporated into the mutliple regression model together with treatment (no alcohol consumption vs. alcohol consumption), age was not found to be a significant predictor of methylation in the presence of alcohol consumption at this site.

APPENDIX K

ABNORMAL SPERM PARAMETERS AND DNA METHYLATION

Table K.1: Number of individuals with normal and abnormal sperm parameters within the control group

Sperm Abnormality Categories		Sample Size (N)
Sperm Concentration	Normozoospermia	29
	§Oligozoospermia	2
Sperm Morphology	Normozoospermia	29
	‡Teratozoospermia	1
Sperm Concentration & Sperm Morphology	Normozoospermia	29
	Oligozoospermia & Teratozoospermia	1
Sperm Morphology & Sperm Motility	Normozoospermia	29
	Teratozoospermia & ✧Asthenozoospermia	1

§ Oligozoospermic – Individuals with sperm concentration levels less than 15 million per ml.

‡ Teratozoospermic – Individuals with a sperm morphology percentage less than four percent.

✧Asthenozoospermic – Individuals with a total sperm motility percentage less than 40%.

APPENDIX L

ABNORMAL SPERM PARAMETERS AND ALCOHOL CONSUMPTION

Table L.1: Fisher's exact test comparing the number of individuals with abnormal sperm parameters in the two treatment groups

Sperm Abnormality Categories		Controls (N=34)	Alcohol Consumers (N=78)	§Fisher's Exact
		Count (Frequency)		P-value
Sperm Concentration	Normozoospermia	29 (25.89%)	55 (49.11%)	0.295
	Oligozoospermia	2 (1.79%)	8 (7.14%)	
Sperm Morphology	Normozoospermia	29 (25.89%)	55 (49.11%)	0.344
	Teratozoospermia	1 (0.89%)	5(4.46%)	
Sperm Motility	Normozoospermia	29 (25.89%)	55 (49.11%)	0.659
	Asthenozoospermia	0 (0.00%)	1 (0.89%)	
Sperm Concentration and Sperm Morphology	Normozoospermia	29 (25.89%)	55 (49.11%)	0.260
	Oligozoospermia and Teratozoospermia	1 (0.89%)	6 (5.36%)	
Sperm Concentration and Sperm Motility	Normozoospermia	29 (25.89%)	55 (49.11%)	0.659
	Oligozoospermia and Asthenozoospermia	0 (0.00%)	1 (0.89%)	
Sperm Morphology and Sperm Motility	Normozoospermia	29 (25.89%)	55 (49.11%)	0.353
	Teratozoospermia and Asthenozoospermia	1 (0.89%)	0 (0.00%)	
Sperm Concentration, Sperm Morphology and Sperm Motility	Normozoospermia	29 (25.89%)	55 (49.11%)	0.437
	Oligozoospermia, Teratozoospermia and Asthenozoospermia	0 (0.00%)	2 (1.79%)	

§One-tailed test

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INTERNET RESOURCES

The following online resources were used:

CTCF Binding Site Database (CTCFBSDB)	http://insulatordb.uthsc.edu/storm.php [Accessed April 2010 – February 2011]
Ensembl	http://www.ensembl.org/index.html [Accessed June 2009 – July 2011]
Evolutionary Conserved Region browser (ECR browser)	http://ecrbrowser.dcode.org/ [Accessed April 2010 – June 2010]
MRC Harwell: An international centre for mouse genetics	http://www.har.mrc.ac.uk/research/genomic_imprinting/ [Accessed February 2010]
Tandem Repeat Finder program	http://tandem.bu.edu/trf/trf.html [Accessed June 2011]
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