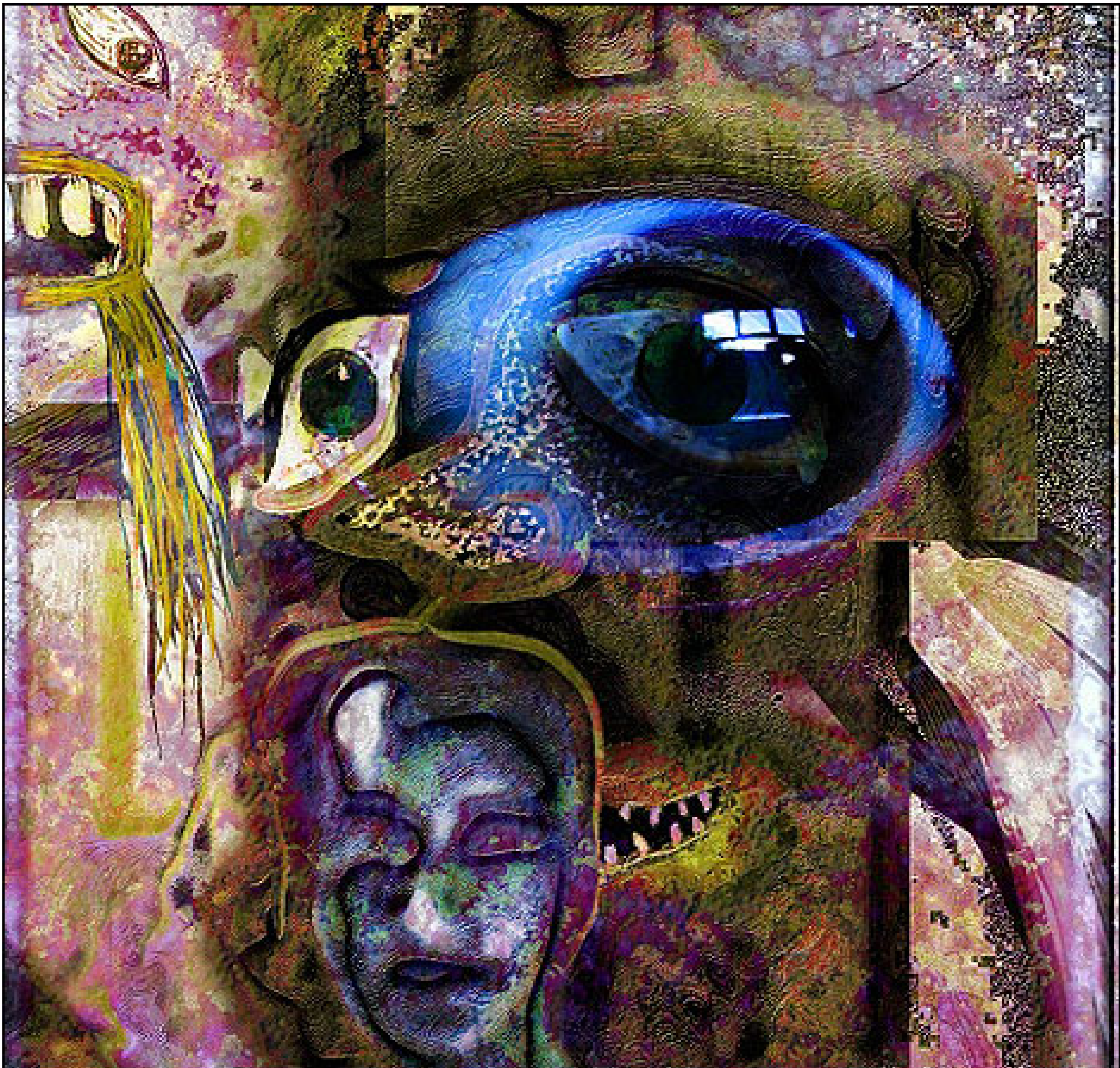
Epigenetic modifications in schizophrenia



MSc Thesis

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Contents

Part 1:
Abstract… pp3

Part 2:

Introduction…pp4-32

- Epigenetics…pp4-5
- DNA methylation…5-7
- DNA methylation and specific gene expression…pp7-9
- Hypermutability of CpG sequences…pp9-10
- Chromatin remodeling…pp10-13
- Relationship between DNA methylation and chromatin modification…pp14
- Epigenetics: Environment meets genome…pp14-17
- Transmission of epigenetic modification across genome…pp17-18
- Relevance of epigenetics to complex disease…pp18-21
- Schizophrenia: the epigenetic perspective…pp21-26
- Previous studies…pp26-33

Part 3:

Aim…pp33

Part 4:

Materials and methods…pp34-42

Part 5:

Results…pp42-50

Part 6:

Discussion…pp50-58

Conclusion…pp58-59

Part 7:

References…pp60-74

Part 1: Abstract

Epigenetic misregulation is consistent with various non-Mendelian features of schizophrenia. To date, however, few studies have investigated the role of DNA methylation in schizophrenia. In this pilot study, we used LUMA (LUminometric Methylation Assay) to assess global DNA methylation level in peripheral leukocytes in 183 schizophrenic patients and 171 blood donors. We found evidence for global hypomethylation of affected samples versus unaffected ($p < 0.0001$) and interestingly patients with early onset of the disease presented lower levels of DNA methylation compared to the other patients ($p=0.018$). Bisulfite pyrosequencing, a quantitative method to assess site-specific gene methylation, revealed hypermethylation of 5-HTT and S-COMT in SZ patients (n=76 for 5-HTT and n=52 for S-COMT) compared to controls (n=84 and 52, respectively) ($p=0.023$ for 5-HTT and $p=0.052$ for S-COMT) with a gender effect among the patients. More specifically, male SZ showed a higher degree of 5-HTT DNA methylation versus females in CpG1 and CpG2 (22.83% vs 19.62%; $r1=0.39$, $p1=0.0005$ and 21.83% vs 19.88%; $r2=0.24$, $p2=0.0341$). In addition, a positive correlation between the degree of 5-HTT methylation and age was found among SZ individuals ($p=0.033$). Regarding S-COMT, female SZ appeared to have a higher level of methylation vs males (43.43% versus 40.3%, $p=0.012$). On the other hand, MB-COMT, PRODH and DTNBP1 promoter showed no degree of DNA methylation both in patients and controls.

Part 2: Introduction

Epigenetics

The human genome is composed of three billion base pairs divided into 23 DNA molecules that make up chromosomes, and 30.000-50.000 genes encoded in only 3% of the DNA sequence (Ewing and Green, 2000). The function of 40% of the genes and the remaining non-gene encoding genome is unknown. Some portion of the genome is needed to maintain chromosome structure and regulate gene expression (Sadock and Sadock, 2000).

Potentially, all genes could be expressed in all cells. However, gene function and regulation is modulated by genetic and epigenetic factors. Epigenetic mechanisms allow short-term adaptation of genomic DNA and cells to the local environment (Hartl and Jones, 2001).

Adaptation to the environment is one of the fundamental regulatory processes in biology and is found among both simple and complex organisms. In a changing environment, simple organisms enhance species survival by high rates of spontaneous mutation achieved by several means: short maturation rates, rapid rates of reproduction, recombination through sexual reproduction, and large numbers of offspring. Then, by a process of natural selection, organisms that are adapted to their environment will survive and multiply. The process of natural selection also affects complex multicellular organisms and promotes adaptive changes. However, this simple strategy for survival becomes less effective in multicellular organisms as the ecological niche becomes more complex and the rates of maturation and fertility decrease. As a result, changes in the environment outpace the rate of genetic evolutionary change, which is limited by generation time (Colvis et al., 2005).

The term ***epigenetics*** derives from the Greek prefix “epi” that literally means “above” or “in addition to” genetics. It refers to processes that physically occur with or on genes, and

involves the physical support of genetic processes, the chromatin. Originally, long before the notion of chromatin even existed, the developmental biologist Conrad Hal Waddington (1905–1975) defined epigenetics as "… the interactions of genes with their environment which bring the phenotype into being", emphasizing that epigenetic mechanisms vary in response to a given environment. Waddington later referred to an equally important characteristic of epigenetic modifications by stating that "…it is possible that an adaptive response can be fixed without waiting for the occurrence of a mutation…".This notion of non-genetic transmission of acquired morphological and behavioural traits had already been proposed by Jean-Baptiste Lamarck (1744–1829), but met with fierce criticism, essentially due to Lamarck's inclination to place his observations in the perspective of adaptive evolution. The modern definition of epigenetics now integrates Waddington's early assumptions, but excludes most of Lamarck's views. *Epigenetics* is nowadays most commonly defined as the ensemble of alterations in gene functions that are heritable through both mitosis and meiosis, but that cannot be explained by changes in the DNA sequence itself (Bird et al., 2007; Jaenisch et al., 2003).

At the molecular level, epigenetic mechanisms are biochemical modifications i) of the DNA and ii) histone proteins, the major constituents of chromatin. Recent findings have revealed that additional mechanisms involving RNA interference and prion proteins also contribute to epigenetic regulation.

DNA methylation

DNA methylation plays a key role in gene regulation. It is a common method of gene silencing that can be inherited without changing the nucleotide sequence of the involved gene and thus is part of the epigenetic code.

In humans and other higher organisms, the vast majority of DNA methylation involves the addition of a methyl group to the cytosine ring carbon in 5 CpG3 of DNAs (*figure 1*). This reaction is catalyzed by multiple methyltransferases, including DNMT1, DNMT2, DNMT3A, and DNMT3B, which are encoded on different chromosomes (19p13.2, 10p15.1, 2p23, and 20q11.2, respectively; Bestor, 2000; Kim et al., 2002). Methyl groups are provided by *S*-adenosyl methionine. Recent data have also demonstrated triggering of DNA methylation by histone methylation (Lachner et al., 2001; Li, 2002). In the human genome, approximately 4-8% of Cs are methylated, and 5-mC constitutes only about 1% of the total residues that make up the genome (Petronis et al., 1999). Potentially, there are 100 million CpG dinucleotide methylation targets in the diploid mammalian genome. The dinucleotide CpG occurs once per 80 dinucleotides throughout 98% of the genome. However, the frequency is increased in "CpG islands" located within and around the regulatory regions of genes (Costello and Plass, 2001).

There are approximately 29,000 CpG islands, sequences rich in CpG dinucleotides, in the human genome, and 50-60% of all genes contain a CpG island (Singal and Ginder, 1999; Bird, 2002). In vertebrates, 70% of CpGs are methylated, while the promoter regions of active genes, in general, are less than 30% methylated (Kress et al., 2001). Usually CpG islands of expressed genes have low levels of methylation.

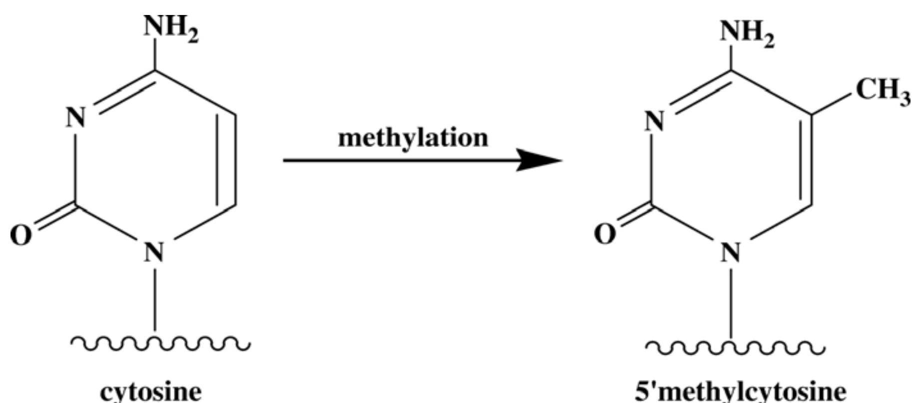


Figure 1: Methylation of cytosine. DNA methylation involves the addition of a methyl group to the 5-carbon position of cytosine residue; *Roberts et al, Australian and New Zealand Journal of Psychiatry, 2008.*

DNA methylation and specific gene expression

Four proteins - MeCP2, MBD1, MBD2, and MBD3 (encoded on chromosomes X, 18q21, 18q21, and 19, respectively) - bind specifically to methylated DNA (Hendrich et al., 1999). Concurrently, histones are deacetylated and the chromatin structure of the chromosome is modified reducing transcription. (Hendrich et al., 1999; Bird, 2002).

Dense DNA methylation is associated with irreversible silencing of gene expression, while partial methylation, a more frequent occurrence, marks genes that can be reactivated (Russo et al., 1996). In spite of evidence indicating the presence of an active demethylation process, no specific demethylating enzyme has yet been identified. However, some proteins and hormones, acting as transcription factors and binding to DNA may have such a role (Costello and Plass, 2001) and some methyl CpG-binding proteins such as MBD2 could have demethylase activity as well (Detich et al., 2002). Although demethylation is gene specific, in most instances changes are regionally rather than dinucleotide specific. However, a change in the methylation status of a few CpG dinucleotides is associated with a change in gene expression at a few loci. For example, differences in methylation at specific CpG dinucleotides in the cAMP-like response sequence element in several hormonal gene promoter regions is associated with changes in gene expression (Ngo et al., 1996).

DNA methylation is believed to be important where the timing and location of gene expression are important (Russo et al., 1996; Kress et al., 2001). Tissue-specific methylation modulation is a continuous process that allows gene expression levels to

change in specific cells at specific times, especially during critical periods of development (Kress et al., 2001).

In some cases, DNA methylation changes appear to be transmitted through meiosis (Russo et al., 1996). For example, in mice, transplantation of a nucleus from an early embryo into an egg cell led to DNA methylation increases in some genes, changes in genes expression, and a reduction of the body weight of the offspring (Roemer et al., 1997). Furthermore, these phenotypes are transmitted to the next generation of offspring. Hence, "Lamarckian" inheritance may be transmitted through DNA methylation or other epigenetic effects.

It is proposed that during early development, binding of gene-activating factors in promoter regions and recruitment of multiple transcription regulatory proteins to different sites that make up gene promoter regions may prevent methylation and lead to increased expression thereafter (Bird, 2002). Moreover, successive bindings of transcription factors to a gene's regulatory area changes nucleosome structure, which is associated with a decreased methylation rate and more capability for gene expression.

Global regulation of genomic methylation appears to be restricted to embryonic life. DNA in primordial germ-line cells is generally unmethylated. However, sperm DNA is highly methylated, whereas egg DNA appears to contain the adult methylation pattern. During early embryonic life (blastocyst stage), a period marked by large amounts of DNA replication, the paternal DNA is demethylated, but becomes methylated at implantation. During differentiation, the promoter regions of inactive genes become methylated, although, in some cases, CpG methylation is associated with active genes (Abdolmaleky et al, 2003).

The mechanism by which the genome globally demethylates soon after fertilization is not well known (Hartl and Jones, 2001). In humans, remethylation begins at about the seventh day after fertilization. In mice, demethylation starts in the myoblastic stage and

remethylation occurs after birth, indicating that both the pre- and post-natal periods are important stages for establishing adult methylation patterns (Tawa, 1990). In summary, there is a resetting of methylation patterns in early life and a subsequent dynamic demethylation, coinciding with developmental critical periods. Interference in the methylation process may result in developmental defects or even cell death (Watson and Goodman, 2002).

DNA methylation is also involved in X-chromosome inactivation. Female cells have two copies of chromosome X, while male cells have a single copy of chromosome X and one chromosome Y. Despite this, the amount of protein produced from genes encoded on chromosome X is equal in both types of cells, because one X chromosome is randomly methylated and inactivated in each female cell (Watson and Goodman, 2002) except in regions that share homology with chromosome Y. This means that females are mosaics, where some cells express the maternally inherited X chromosome and other cells express the paternally inherited X chromosome. This leads to greater variability in the phenotype of females than males and the opportunity for greater variation in chromosome X epigenetic regulation. Males inherit and express only the maternal X chromosome and the approximately 200 genes on chromosome Y.

Hypermutability of CpG sequences

The rate of mutation at 5-mCpG is 20-40 times higher than for other dinucleotides. This occurs because spontaneous deamination events convert 5-mC to thymine, and convert cytosine to uracil. Uracil is removed from DNA while the T-G mismatched base-pair is mutagenic (Hendrich et al., 1999).

Deamination accounts for the 5-20-fold lower than expected occurrence of CpGs and the higher than expected frequency of TpG dinucleotides in eukaryotic genomes (Singal and Ginder, 1999). This means that new traits may be evolved more rapidly by mutations at 5-

mC residues especially present in inactive genes or silent foreign DNA elements integrated into the genome (e.g., endogenous retroviruses). Methylated and inactive foreign DNA is prone to C-to-T mutations and the creation of new genes from reactivation sequences (Monk, 1995).

Chromatin remodeling

Chromatin is the complex of DNA, histones and non-histone proteins in the cell nucleus. Remodelling of chromatin is a dynamic process that modulates gene expression. The fundamental unit of chromatin is the *nucleosome*, which consists of ~147 base pairs of DNA wrapped around a core histone octamer. Each octamer contains two copies each of the histones H2A, H2B, H3 and H4 (*fig. 2a*). The nucleosomal structure of chromatin allows DNA to be tightly packaged into the nucleus by organized folding (Felsenfeld, 2003). Intricate chromatin remodelling mechanisms ensure that DNA remains accessible to the transcriptional machinery. These epigenetic mechanisms alter gene activity by modulating DNA–protein interactions without changing the genetic code.

In simplified terms, chromatin exists in an inactivated, condensed state, *heterochromatin*, which does not allow transcription of genes, and in an activated, open state, *euchromatin*, which allows individual genes to be transcribed (*Fig.2b*). The opening of chromatin is associated with acetylation of nearby histones, although it remains unclear whether acetylation mediates or reflects chromatin decondensation. In reality, chromatin can exist in many states in between these two extremes (*Fig.2b*). Portions of chromatin are highly repressed, owing to DNA and histone methylation and the binding of repressor proteins, and might never be accessible for transcription. Other portions of chromatin are in repressed or permissive states; their basal activity is low owing to histone methylation and perhaps other modifications, but the genes are available for derepression and activation in response to transcription factors and transcriptional co-activators. Chromatin remodeling modulates

gene expression with high temporal and spatial resolution by permitting small groups of nucleosomes to become more or less open, which consequently enhances or inhibits access of the transcriptional machinery to specific promoter regions.

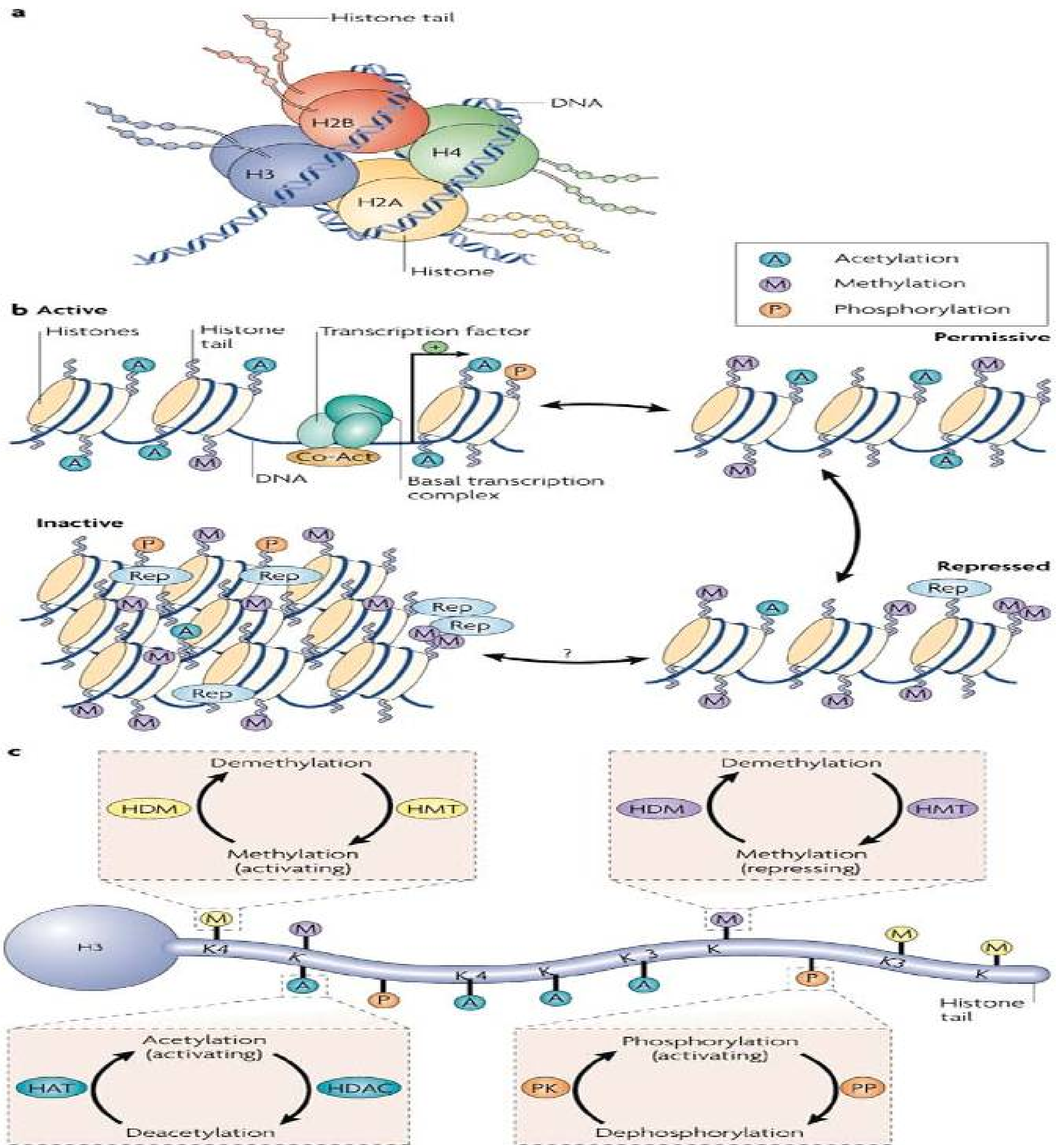
By far the best characterized chromatin remodeling mechanism in the brain is the post-translational, covalent modification of histones at distinct amino acid residues on their amino (N)-terminal tails. Such modifications include acetylation, ubiquitylation or SUMOylation at lysine (K) residues, methylation at lysine or arginine (R) residues, phosphorylation at serine (S) or threonine (T) residues, and ADP-ribosylation at glutamate (E) residues. Hyperacetylation is generally thought to promote decondensation of chromatin and an increase in gene activity, whereas hypoacetylation marks condensation and decreased activity. It has also been proposed that increased gene activity is best associated not with the level of acetylation, but with the dynamic cycling of acetylation and deacetylation. In contrast to acetylation, histone methylation can correlate with either gene activation or repression, depending on the residue undergoing methylation (Lachner et al., 2002). Phosphorylation of histones is also associated with chromatin inhibition or activation (Hake et al., 2004). The roles of histone ubiquitylation, SUMOylation and ADP ribosylation are less well understood. The diversity of histone modifications supports the 'histone code hypothesis', which posits that the sum of modifications at a particular promoter region defines a specific epigenetic state of gene activation or silencing.

The enzymes that mediate these covalent modifications are becoming increasingly understood. Many histone acetyltransferases (HATs), which catalyse acetylation, have been identified. Several transcriptional activators contain intrinsic HAT activity (Jenuwein et al., 2001; Narlikar G, 2002). Histone deacetylases (HDACs) catalyse deacetylation; they also associate with several transcriptional repressors to further repress chromatin activity. The balance between the opposing activities of HATs and HDACs maintains acetylation on core histones and is thought to be an important determinant of transcription. Methylation at

lysine or arginine residues is mediated by histone methyltransferases (HMTs). In general, histone lysine methylation is regarded as a more stable modification than other histone modifications, which seem to be more readily reversible, although the recent discovery of histone demethylases (HDMs) indicates that even methylation can be reversed (Tsankova et al., 2007).

Figure 2: ***a*** / Picture of a nucleosome showing a DNA strand wrapped around a histone octamer composed of two copies each of the histones H2A, H2B, H3 and H4. The amino (N) termini of the histones face outward from the nucleosome complex. ***b*** / Chromatin can be conceptualized as existing in two primary structural states: as active, or open, euchromatin (top left) in which histone acetylation (A) is associated with opening the nucleosome to allow binding of the basal transcriptional complex and other activators of transcription; or as inactive, or condensed, heterochromatin where all gene activity is permanently silenced (bottom left). In reality, chromatin exists in a continuum of several functional states (active; permissive (top right); repressed (bottom right); and inactive). Enrichment of histone modifications such as acetylation and methylation (M) at histone N-terminal tails and related binding of transcription factors and co-activators (Co-Act) or repressors (Rep) to chromatin modulates the transcriptional state of the nucleosome. Recent evidence suggests that inactivated chromatin may in some cases be subject to reactivation in adult nerve cells, although this remains uncertain. **c|** Summary of common covalent modifications of H3, which include acetylation, methylation and phosphorylation (P) at several amino acid residues. H3 phosphoacetylation commonly involves phosphorylation of S10 and acetylation of K14. Acetylation is catalysed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs); lysine methylation (which can be either activating or repressing) is catalysed by histone methyltransferases (HMTs) and reversed by histone demethylases (HDMs); and phosphorylation is catalysed by protein kinases (PK) and reversed by protein phosphatases (PP), which have not yet been identified with certainty. K,

lysine residue, S, serine residue; *Epigenetic regulation in psychiatric disorders, Nature Reviews Neuroscience, 2007*



Relationship between DNA methylation and chromatin modification

The mechanisms by which DNA methylation affects the regulation of gene activity are thought to be mediated in 2 ways. Firstly, methylated cytosines in transcription factor–binding sites change the affinity of DNA for the transcription factor, which in turn alters the transcriptional activity of a gene (Erlich, 1993; Tate et al., 1993). For example, DNA methylation at the promoter region of *BRCA1* exerts a suppressive effect on *BRCA1* expression by inhibiting cAMP response element-binding protein from binding to the promoter region (di Nardo et al., 2001). Secondly, methylated cytosines attract methyl-CpG–binding protein, which recruit chromatin-remodeling proteins (i.e., histone deacetylase [HDAC] complex and SWI-SNF proteins) to deacetylate the histones, resulting in transcriptional silencing (Comb M., 1990; Inamdar et al., 1991; Nan X et al., 1997; Hendrich B. et al.,1998; Ng HH et al., 1999; Fujita N et al., 1999). Aberrant epigenetic regulation (epimutations) could have the same effect as DNA mutations because an epimutation could lead to the abnormal expression of a gene by enhancing or silencing that gene. Precise timing, location, and level of gene expression are crucial for normal cell function.

Epigenetics: environment meets genome

Environmental stimuli can alter epigenetic modifications and influence gene expression

Changes in epigenetic modifications can be induced via environmental stimuli such as nutrition, maternal care/behaviour, hormones and drugs. For example: **i)** ***Nutrition*** can influence gene expression via DNA methylation. The metabolic production of methyl groups used in all biological methylation reactions is extremely dependent on diet-derived methyl donors (e.g. methionine) and critical cofactors (e.g. folic acid, vitamin B12) (Veyver,2002). In pre-implantation embryos the genome undergoes widespread demethylation resulting in

a loss of the CpG methylation pattern (Reik, W et al., 2001). The requirement to re-establish the genomic methylation pattern following implantation and the necessity to maintain this pattern during the many cycles of rapid cell proliferation in early development make the fetal and early postnatal stages of life more critically reliant on the availability of appropriate levels of dietary methyl donors and cofactors, more so than later in life (Waterland, 1999). Fang (2003) reported that polyphenoles (from green tea) can inhibit DNMT activity and reactivate methylation-silenced genes in cancer cells. In addition, a number of natural compounds found in the human diet can influence HDACs and the acetylation status of histones (Delage et al., 2008; *Table 1*). Therefore, overall availability of critical amino acids and micronutrients may alter DNA methylation and histones modifications and thus influence gene expression.

Table1: **Natural and/or dietary compounds modulating histone acetylation and/or HDAC/HAT activities**

Dietary components	**Examples of food/plant sources**
***S*-allylmercaptocysteine**	Garlic (*Allium sativum* L.)
6-methylsulfinylhexyl-isothiocyanate	Japanese horseradish (wasabi)
Allyl mercaptan	Garlic (*Allium sativum* L.)
Anacardic acid	Cashew nut
Butein	*Rhus verniciflua* (stems)
Butyrate	Dietary fiber fermentation
Copper	Ubiquitous
Curcumin	*Curcuma longa* (tumeric roots)
Diallyl disulfide (DADS)	Garlic (*Allium sativum* L.)
Dihydrocoumarin	*Melilotus officinalis* (sweet clover)

Fisetin	*Rhus toxicodendron* (leaves)
Garcinol	*Garcina indica* (fruit)
Quercetin	Apple, tea, onion, nuts, berries
Resveratrol	Red grapes, wines, eucalyptus, spruce
Sulforaphane	Broccoli, broccoli sprouts
Theophylline	Black and green tea

ii) Drugs Methamphetamine, which is known to cause schizophrenia-like phenotype with prolonged use, alters the expressional level of DNMT1 (Numachi et al., 2004). In this connection, it is interesting to note that aberrant DNMT1 expression was also observed in the GABA (gamma-aminobutyric acid)-ergic interneurons of postmortem brain tissues of schizophrenia patients (Veldic et al, 2004). A recent study showed that histone acetylation is induced in the nucleus accumbens in response to acute and chronic cocaine administration (Kumar et al., 2005). The increase in histone acetylation was mediated by decreased HDAC function (more specifically, HDAC5) (Tremolizzo et al., 2002; Chen et al, 2002). Interestingly, decreased HDAC5 function in the nucleus accumbens was also observed in chronic social defeat stress, an animal model for depression (Tremolizzo et al., 2005).

iii) *Stress* In mammals, quality of the early environment is conferred by the mother, both through nutritional and behavioral investment. In rodents, observations of mother-infant interactions during the first week postpartum indicate that there are stable natural variations in maternal behavior, particularly licking/grooming (LG), which are critical for shaping stress responsivity of adult offspring (Meaney, 2001; Champagne et al., 2003). Offspring born to mothers who exhibit high levels of LG are less anxious in a novel environment and have an attenuated corticosterone response to stress compared with offspring of low-LG mothers (Liu et al., 1997; Caldji et al., 1998). These behavioral and physiological characteristics are

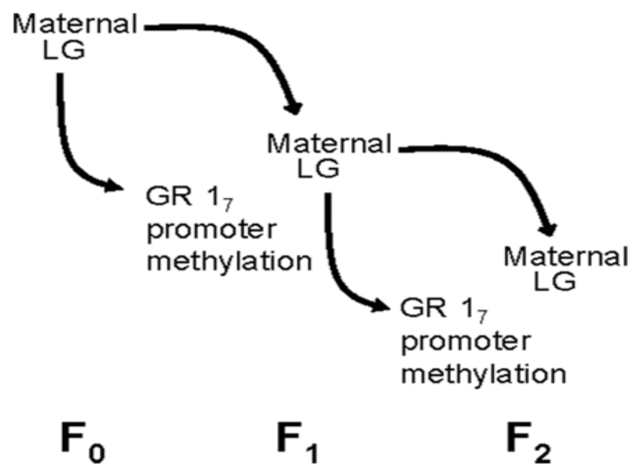
associated with expression of hippocampal glucocorticoid receptors (GRs), such that adult offspring of low-LG mothers have reduced levels of GRs compared with off-spring of high-LG mothers (Liu et al., 1997). Cross-fostering studies confirm that these phenotypes are mediated by variations in maternal care received during the postpartum period (Francis et al., 1999).

Transmission of epigenetic modification across generations

Although the transmission of traits over generations has long been the domain of genetics, there is increasing evidence in support of nongenomic and epigenetic inheritance in mammals. Natural variations in maternal care in the rat are transmitted from mother to female offspring, such that the offspring of high-LG mothers are themselves high in LG and offspring of low-LG mothers exhibit low levels of LG toward their own offspring (Francis et al., 1999; Champagne et al., 2003). Thus, generations of females inherit the maternal style exhibited by their mothers, and, hence, differential GR 1_7 promoter methylation is passed from one generation to the next via maternal behavior (*Fig.3*). This transmission is dependent on the quality of postpartum care, such that cross-fostered offspring will exhibit maternal LG that corresponds to that of their adopted mother rather than their biological mother. The importance of the mother in transmitting epigenetic modifications across generations is clear, but there is also evidence for paternal inheritance. Paternal epigenetic inheritance has also been observed in offspring born to mothers exposed to toxins. In utero exposure to estrogenic and antiandrogenic endocrine disruptors reduces fertility in male offspring, and these effects are transmitted to subsequent generations (Anway et al., 2005). Analysis of DNA methylation levels in F_2 and F_3 generation offspring confirm that the transmission of these effects involves epigenetic modifications. Thus, paternal epigenetic inheritance can be modified by environment and shape the development of subsequent offspring. These studies suggest that the persistent effects of experiences occurring early in development are mediated by epigenetic modifications that can be transmitted to multiple

generations of offspring. DNA methylation, by silencing gene expression, is critical to this process and can be targeted in later life to alter patterns of gene expression and behavior.

Fig.3: Illustration of the behavioral transmission of epigenetic modifications across generations via maternal care, *Colvis, C. M. et al. J. Neurosci. 2005; 25:10379-10389*



Relevance of epigenetics to complex disease

There are three fundamental points that enable us to consider epigenetic factors as etiological candidates in complex disease. First, the epigenetic status of genes is more dynamic in comparison to DNA sequence and can be altered by developmental programs and the environment of the organism (Weaver et al., 2004). Furthermore, epigenetic changes may occur even in the absence of evident environmental differences, i.e., owing to stochastic reasons (Riggs et al., 1998). Second, some epigenetic signals can be transmitted along with DNA sequence across the germline generations, i.e., such signals exhibit partial meiotic stability (Richards 1996). Third, epigenetic regulation is critical for normal genomic function, such as segregation of chromosomes in mitosis, inactivation of parasitic DNA elements, and regulation of gene activity. Partial epigenetic stability, or metastability, and the primary role in controlling activities of DNA sequences can shed new light on non-Mendelian irregularities of complex diseases.

Discordance of identical monozygotic (MZ) twins has been one of the hallmarks of complex non-Mendelian disease. Concordance of MZ twins reaches only ~15% in breast cancer, 20% in ulcerative colitis, 25%–30% in multiple sclerosis, 25%–45% in diabetes, 50% in schizophrenia, and 40%–70% for Alzheimer's disease (Petronis et al., 2001). Discordance of MZ twins traditionally has been explained by the differential effect of environmental factors, which supposedly produce disease in one of the two genetically predisposed co-twins (Reiss et al.,, 1991). Identification of such factors has been very difficult, and thus far only a couple environmentally derived disease risk factors have been identified (e.g., smoking in lung cancer, diet in cardiovascular diseases). The epigenetic explanation of MZ twin discordance is that due to the partial stability of epigenetic factors, a substantial degree of disease-relevant epigenetic dissimilarity can be accumulated in such twins (Petronis et al, 2003; Fraga MF et al., 2005). Epigenetic differences in identical twins may reflect differential exposure to a wide variety of environmental factors. For example, intake of folic acid affects both the global methylation level in the genome and regulation of imprinted genes (Wolff et al, 1998; Ingrosso et al, 2003). During pregnancy, maternal dietary methyl supplements increase DNA methylation and change methylation-dependent epigenetic phenotypes in mammalian offspring (Waterland et al., 2003). There could be numerous environmental effects, including even maternal behavior, that cause some epigenetic "trace."

One of the important peculiarities of complex disease is sexual dimorphism—differential susceptibility to a disease in males and females. Multiple sclerosis, rheumatoid arthritis, Crohn's disease, panic disorder, structural heart disease, and hyperthyroidism are more common in females, whereas males are more often affected with autism, Hirschsprung's disease, ulcerative colitis, Parkinson's disease, alcoholism, allergies, and asthma (especially at a young age) (Ostrer,1999). In psychiatric diseases, such as Alzheimer's disease, schizophrenia, alcoholism, and mood and anxiety disorders, psychopathology exhibits a

number of differences between the sexes in rates of illness as well as the course of disease (Seaman, 1997). It is important to note that effects of gender in complex diseases cannot be explained by sex chromosome-linked genes. In fact, effects of gender quite often have been detected in genetic linkage and association studies on autosomal chromosomes. Although sex hormones have been the usual "culprit" in the explanation of gender effects in complex diseases, there are no specific mechanisms proposed as to how such hormones predispose to or protect from a disease (Kaminsky et al., 2006). The gender-specific effects in genetic linkage and association studies suggest that chromosomes and individual genes can be the target of sex hormones. Although such hormones cannot change DNA sequence, they can be potent modifiers of epigenetic status, which controls gene expression and various other genomic activities. It is known that hormones, including sex hormones, can control gene expression via epigenetic modifications, and therefore it can be hypothesized that differential susceptibility to complex disease in males and females is mediated by sex hormone–induced differences in epigenetic regulation of genes.

The epigenetic model of complex disease can be imagined as a chain of aberrant epigenetic events that begins with a pre-epimutation, a primary epigenetic problem that takes place during the maturation of the germline; pre-epimutation increases the risk for the disease but is not necessarily sufficient to cause the disease. The misregulation can be tolerated to some extent, and age of disease onset may depend on the effects of tissue differentiation, stochastic factors, hormones, and probably some external environmental factors (nutrition, infections, medications, addictions, etc.). It may take decades to reach a critical threshold beyond which the genome, cell, or tissue is no longer able to function normally. Only some predisposed individuals will reach the threshold of epigenetic misregulation and acquire phenotypic changes that meet the diagnostic criteria for a clinical disorder. Severity of epigenetic misregulation may fluctuate over time, which in clinical terms is called remission and relapse. In some cases, "aging" epimutations may slowly regress back to the norm. For

example, in major psychosis, this is seen as fading psychopathology or even partial recovery, which is consistent with age-dependent epigenetic changes in the genome (Petronis et al, 2008). To date, epigenetic factors in complex disease have been poorly investigated, with the exception of cancer. In addition to cancer, epigenetic studies of psychiatric diseases are now underway.

In this study, we are going to be focused on schizophrenia.

Schizophrenia: the epigenetic perspective

Schizophrenia (SCZ) from the greek roots *schizein* (σχίζειν, "to split") and *phrēn*, *phren-* (φρήν, φρεν-, "mind") is a debilitating psychiatric disorder that affects about 2% of the world`s population. Although SCZ has many neuropsychiatric manifestations, psychotic symptoms-hallucinations, delusions, and disorganized thought and behavior-have historically been the basis of diagnostic criteria. In the fourth, Revised, of the *Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV),* negative symptoms, duration of illness, and the temporal relationship to any depressive or manic syndrome are also part of the criteria but psychotic symptoms remain central (*Table 2*). Onset of symptoms typically occurs in young adulthood. The current prevailing explanatory theory is that the disorder is neurodevelopmental in origin, with the interaction between genetic factors and environment marking the beginning of brain pathophysiological processes long before the overt manifestation of clinical symptoms.

Table 2 ***Summary of the DSM-IV (Revised) Diagnostic Criteria for Schizophrenia***

1. Characteristic symptoms (at least two)

   a. Delusions
   b. Hallucinations
   c. Disorganized speech
   d. Disorganized or catatonic behavior

e. Negative symptoms (blunted affect, poverty of speech, or avolition)

2. Marked social or occupational dysfunction
3. Six months duration
4. Mood disorder: no major affective syndrome during active phase of illness, or the duration of active phase affective symptoms is brief compared to the total duration of illness.
5. Symptoms are not directly due to substance abuse or another medical illness
6. If patient has a diagnosis of autism, must have prominent delusions or hallucinations

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Over the last decades, psychiatric research has focused on the genetic predisposition with the expectation that identification of disease-specific DNA polymorphisms and mutations will revolutionize medicine and lead to new diagnostic, treatment, and prophylactic strategies.

Predisposing DNA sequence variants of some specific genes, however, are not considered to be the only cause of mental dysfunction. In complex diseases, MZ twins (who by definition carry the same DNA sequences) exhibit far from full concordance, and MZ concordance for schizophrenia is 41%–65% (Cardno, A.G. and Gottesman, I.I., 2000). Phenotypic discordance in MZ twins has traditionally been interpreted as the evidence of the role of hazardous environment. Even slightly different environments should affect the co-twins differently, and therefore such environments are called “non-shared” (Plomin and Daniels, 1987). Although psychodynamic factors are still entertained, thinking regarding environmental effects shifted from the psychological to the biological pole and now deals primarily with such factors as infectious agents and birth seasonality (Torrey et al, 1987), adverse events during the mother's pregnancy, prenatal and postnatal development, nutritional factors, and drug abuse, among others (Tsuang et al , 2001). Because it is very difficult to uncover the specific impact of a myriad of environmental events on human brain

and behavior, it has been generally accepted that it is easier to investigate several dozen genes and genomic loci than the elusive environmental factors. Hence, thus far, molecular genetic research has dominated the field of psychiatric research.

Although thus far there is no direct experimental evidence that epigenetic factors are involved in schizophrenia, shifting the emphasis from DNA sequence variation to epigenetic misregulation may provide a cohesive explanation of various nonmendelian features of this disease and a new theoretic framework for experimental approaches. From the epigenetic standpoint, schizophrenia could be imagined as the result of a chain of unfavorable epigenetic events that begins with a primary epigenetic defect, or pre-epimutation, that occurs in the germline during the error-prone epigenetic reprogramming process. Pre-epimutation increases the risk for the disease but, unlike the deterministic DNA mutations in mendelian disorders, a pre-epimutation does not necessarily indicate that the disease is inevitable. Such pre-epimutations might not cause any clinical problems for decades, although they might result in various minor cytoarchitectural changes in brain development and lead to barely detectable neuropsychological aberrations in childhood. Pre-epimutations are subject to further changes during embryogenesis, childhood, and adolescence owing to multidirectional effects of tissue differentiation, stochastic factors, hormones, and probably some external environmental factors (e.g., nutrition, medications, and addictions) (Jaenish and Bird, 2003). The peaks of susceptibility to schizophrenia seem to follow the major changes in endocrine homeostasis: late adolescence and early adulthood for both sexes, late forties in women, and the sixth decade in both sexes again, which suggests that hormonal changes might play a significant role in the further dynamics of an inherited epigenetic defect (Petronis, 2001). The phenotypic outcome depends on the overall effect of the series of pre- and postnatal impacts on pre-epimutation. Only some predisposed individuals will reach the "threshold" of epigenetic misregulation that presents with clinical schizophrenia. Severity of epigenetic misregulation might fluctuate over time, which in

clinical terms is treated as remissions and relapses. In addition to its intensity, the spectrum of psychopathology might also vary in the same patients (e.g., delusions and hallucinations might be substituted by predominantly negative symptoms). In aging patients, epimutations might start slowly regressing back to the norm, and this would be seen as fading psychopathology or even partial recovery. The advantages of the epigenetic scenario of schizophrenia, compared with the DNA sequence–based model, is that the former is consistent with long years of ostensible mental health, critical susceptibility periods, fluctuating course, and even clinical improvement after decades of the patient being affected by this debilitating disease.

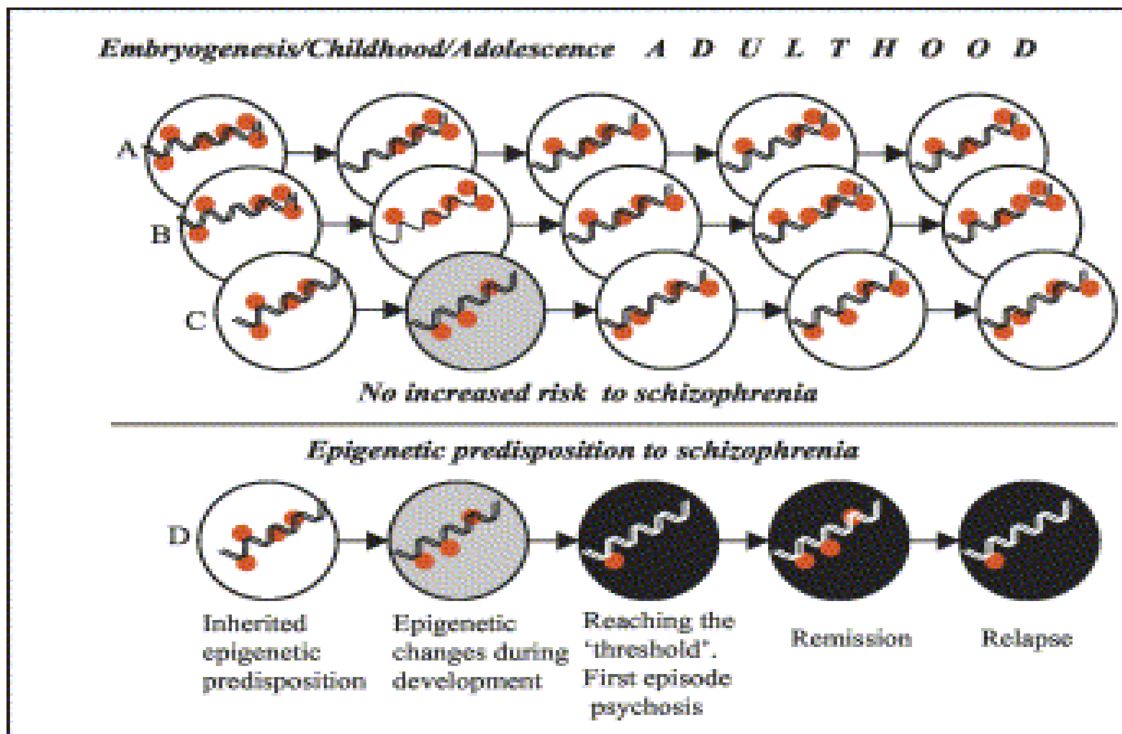


Figure 4: Epigenetic changes during development. Epigenetic status of a gene changes under the influence of various factors: cell differentiation, intra- and extracellular environment, age effects, and stochastic factors. Scenarios **A**, **B**, and **C** demonstrate normal epigenetic development of a hypothetical gene that potentially might predispose to schizophrenia (white circles: healthy individuals; gray circles: borderline psychological abnormalities). Scenario **D** illustrates how a pre-epimutation converts into a serious

epigenetic problem, which results in psychosis (black circles). Note that despite an identical starting point, epigenetic developments in **C** and **D** were very different, as were clinical outcomes; *Petronis A, Biol Psychiatry, 2004*

The epigenetic theory of schizophrenia also challenges the idea of a critical etiologic role of a hazardous environment. First, despite many decades of schizophrenia research (including the psychodynamic period), thus far nobody has been able to identify any specific exogenous factor that would unequivocally increase the risk for schizophrenia. Second, there are significant methodologic problems with the interpretation of the role of such environmental candidates. For example, if childhood head trauma is associated with a higher chance of developing schizophrenia, can it be concluded that head injury increases susceptibility to the disease? Finally, a fundamental question can be raised: does the relatively high MZ twin discordance for schizophrenia really mean that environmental differences make the twins different? There are at least several pieces of evidence arguing against the environmental effects on phenotypes of genetically identical organisms. First, adoption studies showed that the risk to a disease does not decrease if a child born to an affected parent is raised in a healthy family. Another example is the similar rate for schizophrenia among the offspring of MZ co-twins who were discordant for this disease (Gottesman, 1991).

From the epigenetic point of view, stochastic events might be a much more powerful mechanism that induces phenotypic differences in genetically identical organisms than environmental effects. The role of stochastic factors is occasionally discussed in the psychiatric literature. However, it has been generally accepted that stochasticity cannot be reliably investigated at the molecular level. It is interesting to note that stochasticity is an inseparable component of the epigenetic metastability, which presents with partial epigenetic "infidelity." There are many other examples demonstrating that the error rate of replication of epigenetic patterns is significantly higher than that observed for DNA

replication. Stochastic variations in replication of epigenetic patterns can result in phenotypic diversity in identical genetic and environmental backgrounds. Examples for this could be inbred (genetically identical) agouti and kinked tail mice that show major differences in coat color and shape of the tail, both of which are determined by differential epigenetic regulation (Rakyan et al, 2002). Similarly, MZ twins, although carrying identical (or nearly identical) DNA sequences, might exhibit many random epigenetic differences however, it is possible that only one of the two co-twins might reach the critical mass of epigenetic misregulation that results in some specific phenotype (Petronis et al, 2003). If the emphasis is shifted from environment to stochasticity, it might become clear why MZ twins reared apart are not more different from each other than MZ twins reared together. It is possible that MZ twins are different for some traits not because they are exposed to different environments but because those traits are determined by metastable epigenetic regulation on which environmental factors have minimal impact at the best. Finally, epigenetic mechanisms might provide a molecular explanation for the riddle of similar risks for the offspring of discordant MZ twins. The key element of this explanation is epigenetic tissue differences. Epigenetic misregulation might reach very different points in the brains of MZ twins, affecting only one of them, but pre-epimutations might be nearly identical in the germline of the affected and unaffected twins, resulting in the same risk to the offspring of the discordant co-twins (Petronis, 2004).

Petronis (2004) suggested that when we refer to etiologic factors of non-Mendelian disorders, such as schizophrenia, we should include DNA sequences, epigenetics, and stochasticity.

Previous studies…

Reelin and GAD67: Cognitive impairments due to a dysfunction of working memory and alterations in executive brain function are emerging as some of cardinal clinical features of

SZ disorder morbidity. From postmortem brain studies it has become clear that a downregulation of GABAeregic neurons along with a reduced level of the mRNA encoding GAD67 and reelin (*figure 4*) are the critical neuropathologal alterations that underlie important clinical features of SZ (Benes et al, 2001; Guidotti et al, 2005; Guidotti et al, 2000; Lewis et al., 2005). Reelin and GAD67 down-regulation in telencephalic GABAergic neurons of SZ patients are responsible for the downregulation of dendritic spine expression and its associated neuropil hypoplasticity (Liui et al., 2001; Selemon et al, 2003). In GABAergic neurons, DNMT1 expression is increased in cortex and basal ganglia and is layer specific. This increase likely brings about DNA hypermethylation and downregulates reelin and GAD67 promoter function (Dong et al., 2007).

The reelin promoter contains a large CpG island. Chen (2002) demonstrated that when the human reelin promoter is hypermethylated reelin expression is repressed but when the reelin promoter is hypomethylated, reelin expression can increase by as much as 80-fold above normal values. In a recent study it was shown that repeated methionine administration causes hypermethylation of the promoter and results in downregulation of reelin transcription in the heterozygous $Reln^{+/-}$ mouse model of schizophrenia. Methionine treatment also induced MeCP2 binding to the reelin promoter. In contrast, treatment with the methylation inhibitor 5-aza-2'-deoxycytidine and valproic acid upregulated reelin expression in vitro (Noh et al., 2005). Interestingly, a role for methylation in the pathogenesis of schizophrenia was suggested decades ago by clinical studies, in which treatment with the methylating agent SAM elicited psychotic episodes in some patients with schizophrenia (Antun et al., 1971).

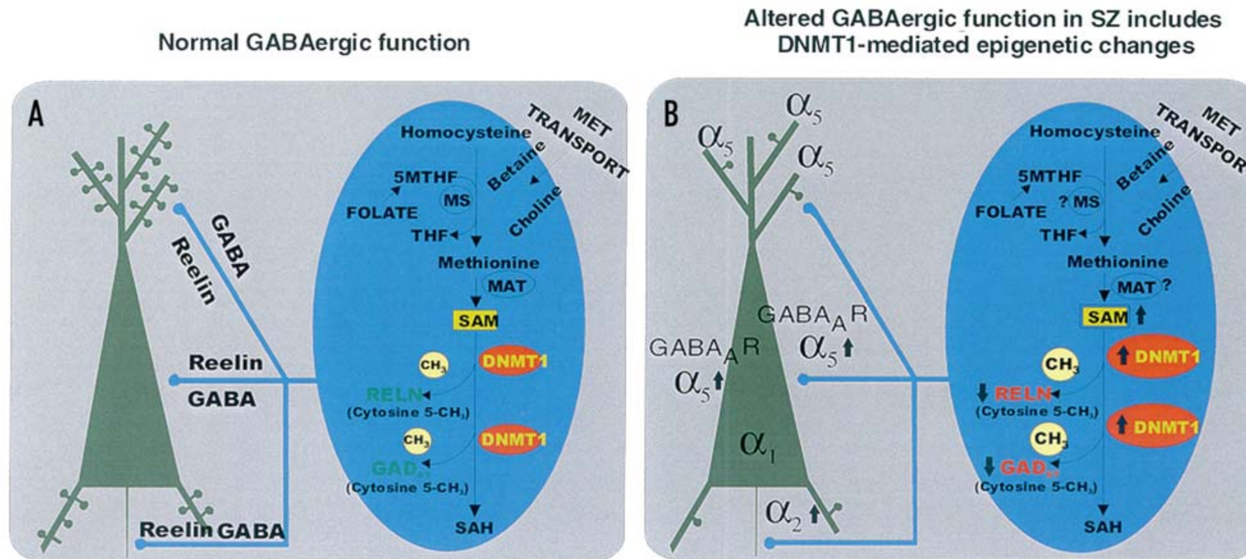


Figure 4: Cortical GABAergic function is altered in schizophrenia. The diagram depicts the cortical GABAergic interneurons in blue and the pyramidal neurons in brown. In (A) GABAergic interneurons provide inhibitory (GABA) and trophic (Reelin) inputs to pyramidal neuron apical dendrites, somata or initial axon segments. In (B) DNMT1 and SAM are overexpressed (↑) and GAD67 and reelin are downregulated (↓) in GABAergic neurons of SZ patients. The reduction of GABAergic inhibition and trophic input to the pyramidal neurons is presumably the cause of the reduction of the number of dendritic spines observed in PFC of SZ patients (11-13, 20, 21, 22, 28, 29, 33, 37, 38, 42, 57-60, 64) DNMT1 = DNA methyltransferase 1; MET = methionine; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; MTHF = 5 methyl-tetrahydrofolate; THF = tetrahydrofolate; MAT = methionineadenosyltransferase; MS = methionine synthetase; *Costa et al., Epigenetics, 2007*

SOX10: Downregulation of oligodendrocyte-related genes, referred to as oligodendrocyte dysfunction, in schizophrenia has been revealed by DNA microarray studies. Because oligodendrocyte-specific transcription factors regulate the differentiation of oligodendrocytes, genes encoding them are prime candidates for oligodendrocyte dysfunction in schizophrenia. Iwamoto (2005) first revealed that the cytosine-guanine dinucleotide (CpG) island of sex determimg region Y-box containing gene 10 (SOX10), an

oligodendrocyte transcription factor, tended to be highly methylated in brains of patients with schizophrenia, correlated with reduced expression of *SOX10*.

DRD2: Petronis (2003) investigated the DNA epigenetic modifications in the 5`regulatory region of the dopamine receptor gene in two pairs of monozygotic twins, one concordant and one discorcodant with schizophrenia. It was detected that the affected twin from the pair discordant for schizophrenia was epigenetically closer to the affected concordant twins than to his unaffected MZ co-twin. This finding was not replicated by Zhang (2006). They found that the degree of aberrant methylation of DRD2 did not reflect the disease process of schizophrenia and also no association between age and cytosines methylation of DRD2 in the regulatory region.

COMT: Catechol-O-methyltransferase (*COMT*), one of the most intensively investigated genes in psychiatric illnesses, regulates the homeostatic levels of neurotransmitter dopamine in the synapses. This gene is located in chromosome 22q11.21, a region linked by several studies to schizophrenia and bipolar disorder (Takahashi et al., 2003). COMT has two known isoforms: membrane-bound COMT (*MB-COMT*) and soluble COMT (S-*COMT*), each with its own promoter (Tenhunen et al., 1994). *MB-COMT* is the predominant form involved in the degradation of synaptic dopamine in the human brain. *MB-COMT* exhibits a functional polymorphism at codon 158 where an adenine (A) substitution for guanine (G) in the gene sequence produces a methionine (Met) substitution for valine (Val) in the COMT protein. The Val-coding allele of the Val158Met polymorphism codes for an enzyme with approximately three times higher physiological activity (Lachman et al., 1996). Several studies showed that increased dopamine degradations arising from COMT hyperactivity are associated with disturbances in attention, executive cognition and working memory performance in normal populations and schizophrenic patients and that these effects may even be progressive over time (Egan et al., 2001; Blasi et al., 2005; Bruder et al., 2005). In

contrast, the hypoactive allele (Met) of the gene is associated with less variability in reaction time and greater stability in performance (Stefanis et al., 2005).

Abdolmaleky (2006) analyzed 115 post-mortem brain samples from the frontal lobe. His study was the first to reveal that the *MB-COMT* promoter DNA is frequently hypomethylated in schizophrenia and bipolar disorder patients, compared with the controls, particularly in the left frontal lobes. Quantitative gene-expression analyses showed a corresponding increase in transcript levels of *MB-COMT* in schizophrenia and bipolar disorder patients compared with the control with an accompanying inverse correlation between *MB-COMT* and *DRD1* expression. Furthermore, there was a tendency for the enrichment of the Val allele of the *COMT* Val158Met polymorphism with *MB-COMT* hypomethylation in the patients. These findings suggest that *MB-COMT* over-expression due to promoter hypomethylation and/or hyperactive allele of *COMT* may increase dopamine degradation in the frontal lobe providing a molecular basis for the shared symptoms of schizophrenia and bipolar disorder.

Intriguing data come also from Murphy (2005), who examined the cytosine DNA methylation profile of the human S-*COMT* promoter regions, which partially overlaps with the MB-COMT coding region and covers a total of 56 cytosines. Their analysis of brain regions and individual blood samples suggests that the cytosine methylation in this region is restricted to the CpG dinucleotides only. Also, the methylation pattern is nearly identical in the brain and blood with few exceptions.

A recent study in MZ twins showed that the degree of methylation at the two CpG sites in the COMT gene was highly correlated, but there was considerable variation in the concordance of methylation levels between MZ twin-pairs. Some MZ twin-pairs showed a high degree of methylation concordance, whereas others differed markedly in their methylation profiles. Such epigenetic variation between genetically identical individuals may

play a key role in the etiology of psychopathology, and explain the incomplete phenotypic concordance observed in MZ twins (Mill et al, 2006).

In addition, Mill and colleagues (2008) performed a microarray-based epigenomic scan using CpG-island microarrays. In contrast to previous studies, they failed to replicate COMT methylation differences between SZ and BP patients vs controls. On the other hand, they found psychosis-associated brain-DNA-methylation differences in numerous loci, including many genes that have been functionally linked to disease etiology. Consistent with increasing evidence for altered glutamatergic and GABAergic neurotransmission in the pathogenesis of major psychosis, they identified epigenetic changes in loci associated with both of these neurotransmitter pathways.

5-HT2A: Interest in serotonin system genes arose several decades ago when it was detected that serotonin is widely distributed throughout the central nervous system and that the hallucinogenic drug LSD demonstrates high affinity for serotonin receptors (reviewed in Lewis et al. 1999). To our knowledge, association of 5-HTR2A with SCZ was first detected in a Japanese sample when a higher frequency of C allele of the 102T/C polymorphism was documented (Inayama et al., 1996). Although a number of studies with relatively small sample sizes were negative, a large combined study consisting of over a thousand SCZ patients and ethnically matched controls detected a significant overall association between SCZ and the 102C allele. The mechanism of C →T mutation may be of epigenetic origin since methylated cytosine (^{met}C) in the CpG dinucleotide is known to be a "hot" spot for DNA and it is likely that the C102T polymorphism resulted from the conversion of ^{met}C to uracil and eventually to thymine (Petronis, 2000).

5-HTT: In addition to its accepted role as a neurotransmitter, serotonin (5–HT) has been shown to regulate the development of the central nervous system (CNS).The serotonin transporter protein, which plays a crucial role in regulating the intensity and duration of

serotonergic signaling at synapses, has long been suggested as a promising candidate gene for various psychiatric disorders including schizophrenia (Fan et al., 2005). However, to date there is no report for 5-HTT DNA methylation in schizophrenia.

DTNBP1: Dysbindin, a coiled-coil-containing protein encoded by *DTNBP1*, was initially found to interact with α- and β -dystrobrevin (DTNA and DTNB) in the muscle and brain of mice. DTNA and DTNB are members of the dystrophin-associated protein complex (DPC), which links the cytoskeleton to the extracellular matrix and serves as a scaffold for signaling proteins (Benson et al., 2001). So far, DTNBP1 has been one of the most prominent schizophrenia susceptibility genes (Li et al., 2007). Mill (2008) showed evidence of hypermethylation in PFC brain tissue of SZ versus control subjects.

PRODH: A candidate gene for schizophrenia that has received extensive investigation, with conflicting results, is PRODH, encoding proline oxidase (POX). This enzyme, among other functions, is rate limiting in the conversion of proline to glutamate in mitochondria. PRODH along with its pseudogene are located at 22q11 is implicated in schizophrenia through a hemideletionsyndrome at this locale, 22q11 deletion syndrome. Several recent studies have found genetic associations of schizophrenia with tag and functional single nucleotide polymorphisms (SNPs) in PRODH (Kempf et al, 2008). So far, no methylation studies have been carried out for this gene.

Global methylation and schizophrenia

There are not many studies regarding schizophrenia and global methylation. Shimabukuro and colleagues (2007) reported a global hypomethylation of peripheral leukocyte DNA in male patients with schizophrenia. This effect was more marked in individuals of young ages, but there was no evidence of the above observation in females.

In addition Shimabukuro (2006) found that haloperidol treatment decreased leukocyte global DNA methylation in male rats, but unexpectedly, increased it in females. In brain, haloperidol treatment resulted in a decrease in global DNA methylation in female, but not male rats. These effects may reflect the involvement of estrogen in DNA methylation.

As mentioned before, the methyl donor for DNA methylation is *S*-adenosylmethionine (SAM), produced from the dietary amino acid methionine. After the donation of the methyl group SAM is converted to *S*-adenosylhomocysteine (SAH) which is hydrolyzed to homocysteine. This reaction is reversible; high intracellular homocysteine levels shift the equilibrium between SAH and homocysteine back to SAH production. SAH inhibits the activity of most SAM-dependent methyltransferases, including DNA methyltransferase, therefore elevated homocysteine and SAH concentrations may cause DNA hypomethylation. Such negative correlation between plasma SAH or homocysteine levels and global DNA methylation was reported in several studies.

In fact a recent meta-analysis suggested that a 5 μM increase in homocysteine is associated with a 70% higher risk for schizophrenia. Blomberg (2008) failed to find an association between global leukocyte DNA methylation and homocysteine levels in schizophrenia patients.

Aim: *The goals of our pilot study were to examine i) whether differences in global DNA methylation exist between subjects with schizophrenia and healthy individuals, ii) gene-specific methylation differences for 5-HTT, S-COMT, MB-COMT, DTNBP1, PRODH, between SZ and control subjects, and iii) at the same time explore the possibility of using blood as a surrogate for brain tissue.*

Part 4: Materials and methods

Samples:

This pilot study included 183 blood samples representing schizophrenic patients from Danderyds Hospital, Stockholm Sweden, and 171 blood donors. Patients were assessed from psychiatrists using DSM-IV criteria. DNA was extracted by a standard procedure involving phenol–chloroform purification and quantified by using a NanoDrop Spectrophotometer (ND-1000).

We used LuMA assay and bisulfite pyrosequencing for global methylation and gene-specific methylation, respectively. Before further proceeding, we are going to describe the basic principles of the above methods.

Luma: LUminometric Methylation Assay

Principles of Luma

The method is based on DNA cleavage by methylation-sensitive or -insensitive restriction enzymes followed by a bioluminometric polymerase extension assay to quantify the extent of restriction cleavage. For our experiments, we used the CpG methylation-sensitive restriction enzyme *Hpa*II and its methylation-insensitive isoschizomer *Msp*I in parallel reactions. *Eco*RI is included in all reactions as an internal reference. *Msp*I and *Hpa*II both leave 5′-CG overhangs after DNA cleavage, whereas *Eco*RI produces 5′-AATT overhangs, which are then filled in a polymerase extension assay with stepwise addition of dNTPs. Following successful extension of a dNTP, inorganic pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase and adenosine-5′-phosphosulfate. Luciferin is subsequently converted to oxyluciferen by luciferase and ATP to produce a proportional amount of visible light which is detected by a charge coupled device (CCD) camera. In this assay, dNTPs are added in four sequential steps (Step 1: dATPαS, Step 2: dGTP + dCTP,

Step 3: dTTP and Step 4: dGTP + dCTP). Peaks corresponding to dATPαS (Step 1) and dTTP (Step 3) additions both represent *Eco*RI cleavage and therefore are expected to be equal to one another. Therefore, the dTTP-peak serves as a control for the dATP-peak. In Step 2, dCTP and dGTP are added together, and the corresponding peak represents *Hpa*II or *Msp*I cleavage. In Step 4, dCTP and dGTP are added again as an internal control for the completion of Step 2. The corresponding peak is expected to be zero or close to zero. *Figure 5* illustrates a typical example of the assay outcome. The *Hpa*II/*Msp*I ratio was defined as (*Hpa*II/*Eco*RI)/(*Msp*I/*Eco*RI) (*Fig.6*).

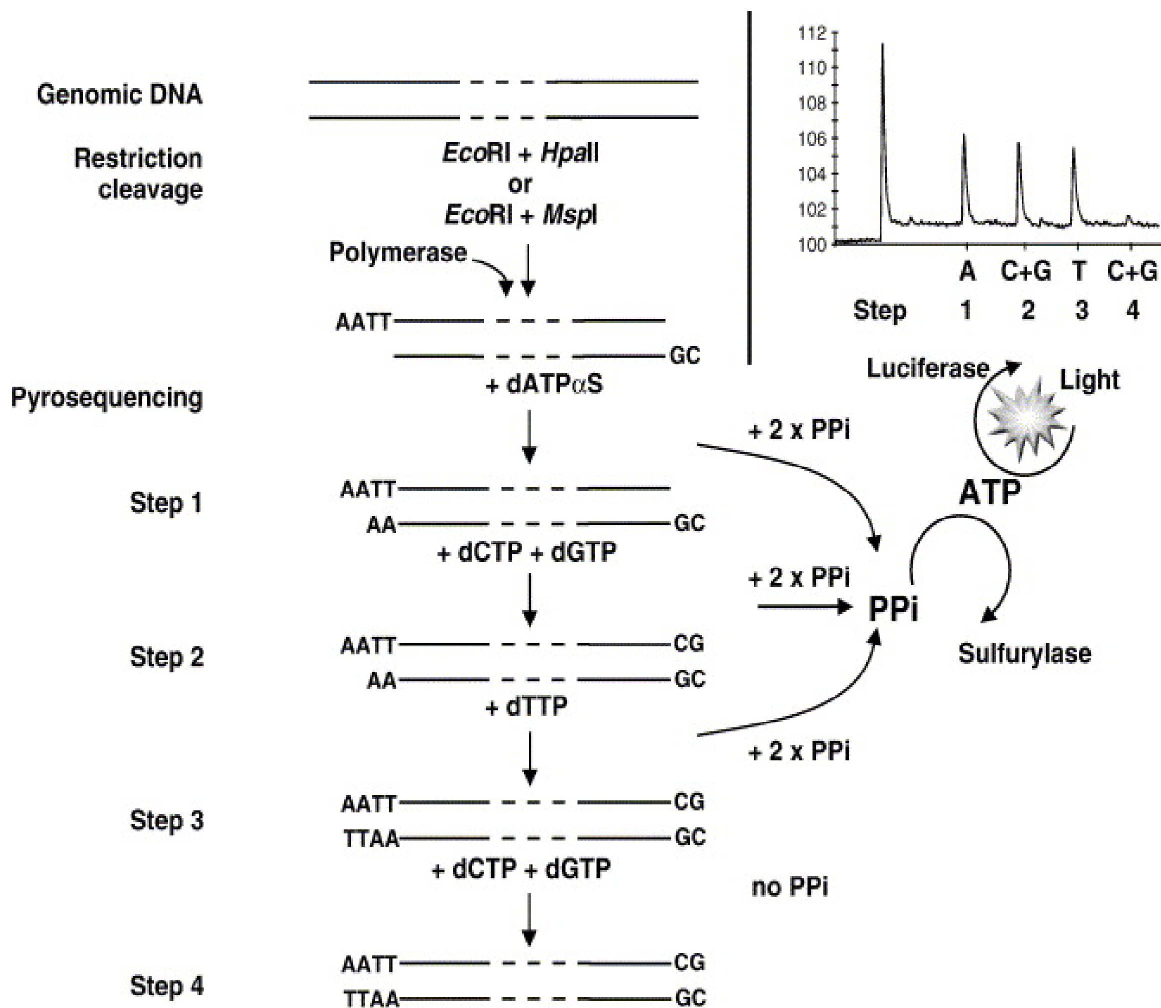


Fig. 5: General strategy for analysis of global DNA methylation with the LUMA assay. Genomic DNA of the test sample is cleaved with two combinations of restriction enzymes,

either HpaII + EcoRI or MspI + EcoRI. The extent of cleavage is then determined by a polymerase extension assay based on a four-step pyrosequencing reaction. After each nucleotide dispensation, inorganic pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase. Luciferin is converted by Luciferase and ATP to produce a proportional amount of visible light that is detected by a CCD camera. The amount of light is directly proportional to the number of overhangs produced by the respective restriction enzymes. The A and T peaks correspond to pyrosequencing Step 1 and Step 3, reflecting the EcoRI cleavage and should be equal. The C + G peak resulting from pyrosequencing Step 2 illustrates HpaII or MspI cleavage. The second C + G peak originating from Step 4 is an internal control that should be close to zero; *Karimi et al., Experimental Cell Research, 2006*

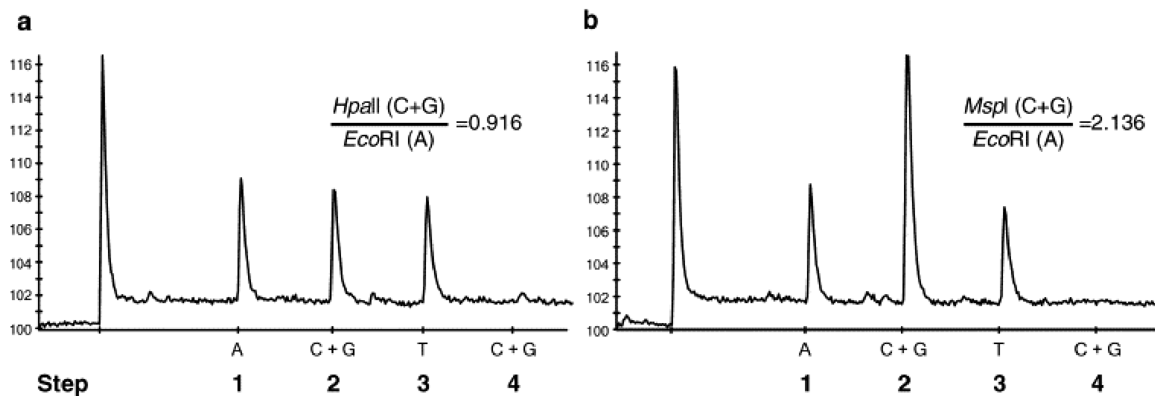


***Fig**. 6:* Typical pyrograms for LUMA assays of genomic DNA from normal peripheral blood leukocytes (PBL) cleaved with (a) HpaII + EcoRI and (b) MspI + EcoRI. S peak = substrate peak; A peak = dATPαS dispensation; First C + G peak = first dCTP + dGTP dispensation; T peak = dTTP dispensation; second C + G peak = second dCTP + dGTP dispensation. In this example, the HpaII/MspI ratio [(HpaII/EcoRI)/ (MspI/EcoRI)] is determined as 0.429 (i.e., 0.916/2.136); *Karimi et al, Experimental Cell Research, 2006*

Bisulfite treatment principle

This method takes advantage of a chemical reaction using sodium bisulfite, which can selectively deaminate cytosine but not 5-methylcytosine to uracil. This leads to a primary sequence change in the DNA that will allow distinguishing cytosine from 5-methylcytosine. Once this conversion has taken place the sequence differences between a methylated and unmethylated cytosine can be exploited by either direct sequencing, restriction digestion (COBRA), nucleotide extension assays (MS-SnuPE), primer-specific PCR (MSP) or pyrosequencing (*fig.7*).

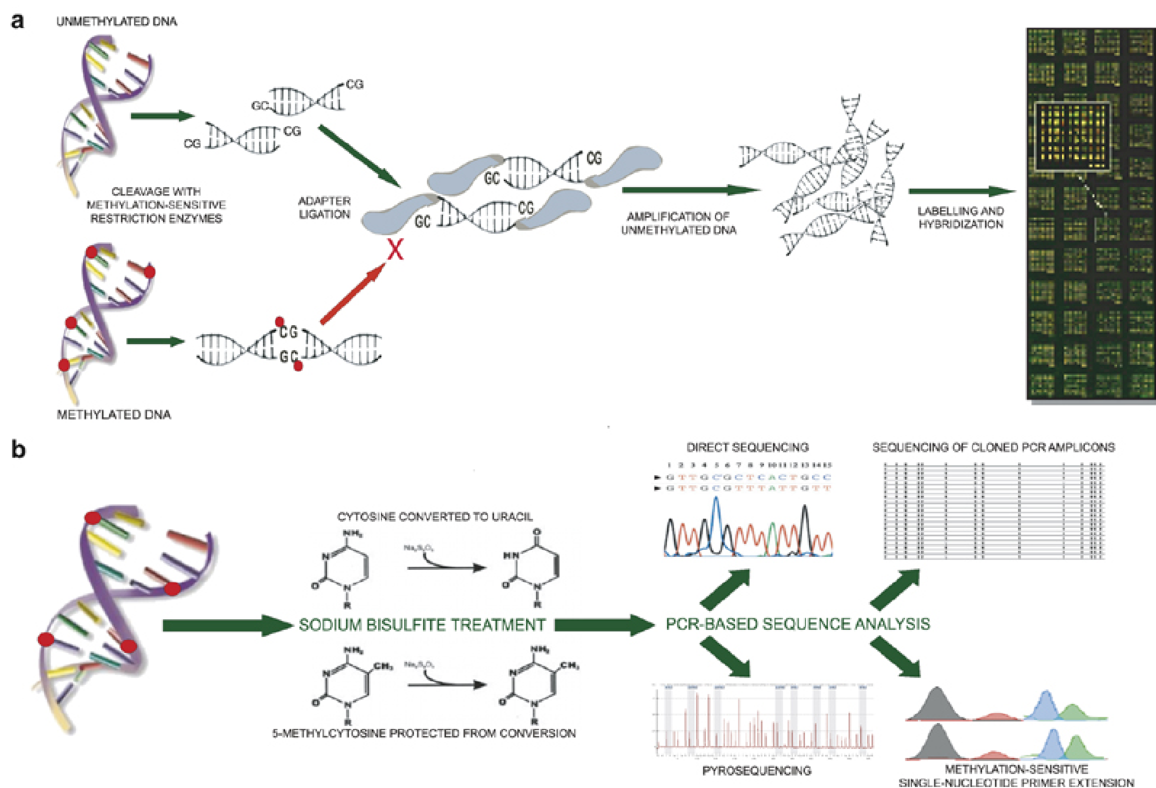


Fig.7: Experimental approaches to investigate DNA methylation. (**a**) Microarray-based methylation profiling using microarrays can investigate DNA methylation changes at a genome-wide level. In this example the unmethylated fraction of genomic DNA is enriched using methylation-sensitive restriction enzymes and adaptor-ligation PCR and subsequently

hybridized on microarrays (**b**) locus-specific methylation analysis using sodium bisulfite treatment and PCR-based sequence analysis; *Mill et al, Molecular Psychiatry, 2007*

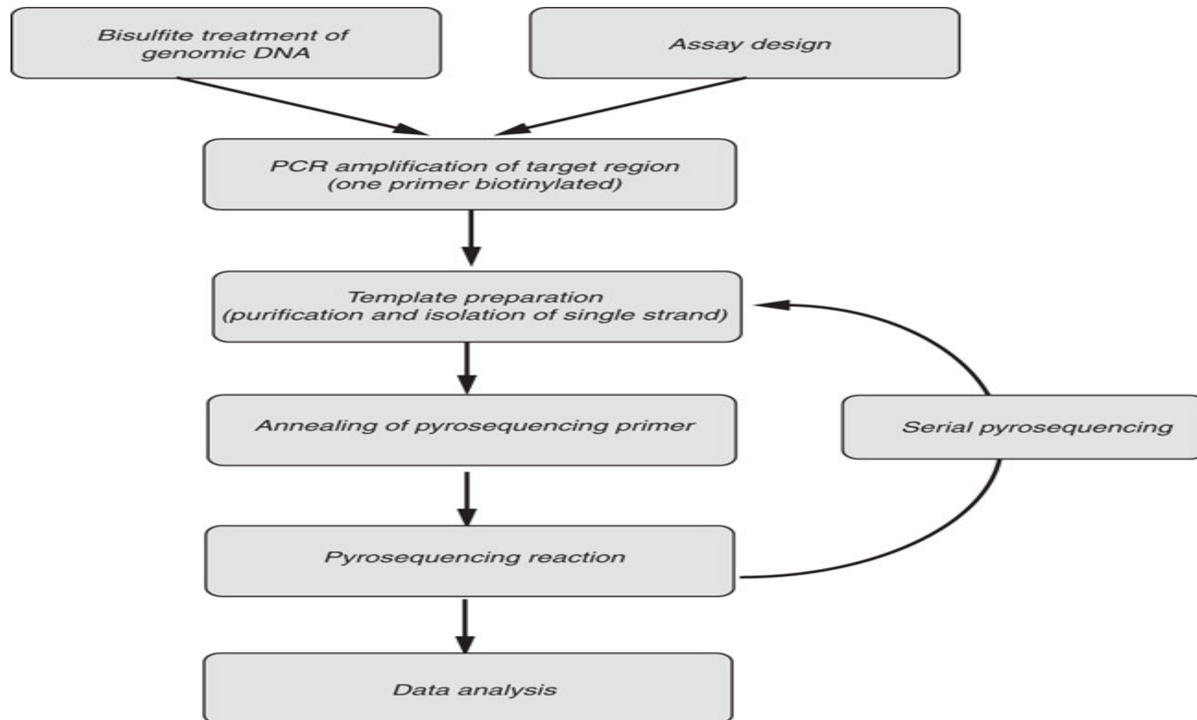
In this study we used the bisulfite pyrosequencing approach.

Bisulfite pyrosequencing

Pyrosequencing is a sequencing-by-synthesis method that relies on the luminometric detection of pyrophosphate release upon nucleotide incorporation via an enzymatic cascade. Because there is a direct correlation between sequence data and the amount of nucleotides incorporated during the reaction, it provides an accurate method for detecting the precise methylation level at any CpG site giving truly quantitative data that are not achievable using standard bisulfite-sequencing protocols.

Pyrosequencing-based methylation analysis investigates quantitatively the degree of methylation at CpG positions in close proximity after bisulfite treatment of genomic DNA. The procedure for a pyrosequencing assay can be divided into several steps, as shown in *Figure 8*. The preparative steps include the design of the assay and treatment of the samples of interest and references with sodium bisulfite. A target region of up to 350 bp is then amplified by PCR using a pair of primers complementary to the bisulfite-treated DNA sequence, amplifying all states irrespective of methylation status. One of these two amplification primers carries a biotin label at its 5'-terminus. The incorporated biotinylated primer is subsequently immobilized on streptavidin-coated beads used to purify and render the PCR product single-stranded (as only one strand is biotinylated). A pyrosequencing primer complementary to the single-stranded template is then hybridized to the template, and the pyrosequencing reaction is performed by the sequential addition of single nucleotides in a predefined order. After pyrosequencing, the template can be repurified to analyze a second part of the template with a new sequencing primer (serial pyrosequencing).

Fig 8: Outline of the procedure for the pyrosequencing assay for DNA methylation analysis; *TosT J, Nature Protocols, 2007*



LuMA:

Restriction enzymes (HpaII, MspI and EcoRI), were purchased from New England Biolabs (Beverly, MA, USA). PSQ^{TM} 96 SNP reagents for pyrosequencing were purchased from Biotage AB (Uppsala, Sweden). Luminometric Methylation Assay was run as described above. Briefly, genomic DNA (200–500 ng) was cleaved with HpaII + EcoRI or MspI + EcoRI in two separate reactions and was run in a 96-well format. The digestion reactions were run in a PSQ96^{TM} MA system (Biotage AB). Peak heights were calculated using the PSQ96^{TM} MA software. The HpaII/EcoRI and MspI/EcoRI ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions. The HpaII/MspI ratio was defined as (HpaII/EcoRI)/(MspI/EcoRI).

Bisulfite treatment:

From each sample, 500 ng genomic DNA were bisulfite treated with EZ DNA Gold-Methylation Kit (Zymo Research, Orange, CA, USA) and used for Pyrosequencing.

Bisulfite Primer Design and PCR Amplification:

Primers were designed with Pyrosequencing Assay Design Software v1.0.6 (Biotage, Uppsala, Sweden). The identification of the promoter sequences for MB-COMT, S-COMT, 5-HTT and DTNBP1 were based on the papers of *Abdolmaleky et al (2006), Murphy et al (2005), Philibert et al (2007*) and *Liao et al* (2004), respectively. For Prodh putative promoter region, ProScan analysis program was used. A full list of primer sequences and annealing temperatures for each PCR reaction can be found in *Table* 3. PCR amplifications were performed with a standard hot-start PCR protocol (initial activation step: 95° 15min, denaturation: 95° 20 sec, annealing temperature 20sec, extension: 72° for 20 sec, number of cycles 38 and final extension at 72° for 10 min) in 50 µl volume reactions containing 1.5 µl of sodium-bisulfite-treated DNA, 10µM primers, 10mM dNTPs and a master mix containing hot-start *Taq* polymerase (QIAGEN). All PCR reactions were checked on a 2.0% agarose gel to ensure successful amplification and specificity before proceeding with pyrosequencing.

Site-Specific DNA-Methylation Analysis with Pyrosequencing:

For pyrosequencing analysis, bisulfite-PCR products were processed according to the manufacturer's standard protocol (Biotage). In brief, 2µl of streptavidin-sepharose beads (Streptabidin Sepharose High Performance, GE, Healthcare), 40 µl of binding buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl) and 18 µl MilliQ water were mixed with 40 µl of PCR product for 15 min at room temperature. After application of the vacuum, the beads were treated with a denaturation solution (0.2 N NaOH) for 8 sec and washed with washing buffer (10 mM Tris-acetate at pH 7.6). The beads were then suspended with 25 µl of annealing

buffer (20 mM Tris-acetate, 2 mM Mg-acetate at pH 7.6) containing 0.3µM of sequencing primer. The template-sequencing primer mixture was transferred onto a PyroMark Q24 plate (Biotage), heated to 90°C for 2 min, and finally cooled to room temperature. The percentage methylation at each CpG site was calculated from the raw data by use of PyroMark Q24 Software (Biotage, Uppsala, Sweden).

Genotyping:

For 5HTTLPR genotyping, primers (Thermo Scientific, Germany) flanking the 5-HTTLPR of the 5-HTT gene (forward: 5'-TGAATGCCAGCACCTAACCCCTAA-3' and reverse: 5'-GAATACTGGTAGGGTGCAAGGAGA-3') were used. Each 25 µl PCR reaction contained 10x reaction buffer (10 mM Tris-HCL, 50 mM KCL, 1.5mM MgCl2, pH 8.3, 0.5 U polymerase; New England, Biolabs), 10mM dNTPs, 10 µM primer, 2 µl DNA template. The PCR product was analyzed in 2.5% agarose gel.

Table 3: Primer sequences and annealing temperatures of PCR reactions

Primer name	Oligo sequence	Annealing Temperature
Prodh_forw	5-GTGTTAGGAGGTGGGT-3	53.5
Prodh_rev	5- CAACACCAACAAACTCC-3 biotin	
Prodh_seq	5- GTGTTAGGAGGTGGGT-3	
Dtnbp1_for	5- GAGGTTTGAGGAGAGGAGAT-3	53.5
Dtnpbp1_rev	5- ACCCCCTCAAATCCCTTT-3 biotin	
Dtnbp1_seq	5- GAGGTTTGAGGAGAGGA-3	

5-HTT_forw	5- AGTTTTAGTTTTGGTTTTTGTTGA-3	57.6
5-HTT_rev	5-TTTCTTTCCAAACCTTCTTCC-3 biotin	
5-HTT_seq	5-AGTTTTGGTTTTTGTTGA-3	
S-COMT_forw	5-GATGGTGGTATTTTAAGTAAAGGG-3	57.6
S-COMT_rev	5-CTCCTTAATATCACCCATAAACAA-3 biotin	
S-COMT_seq	5-GGTGGTATTTTAAGTAAAGG-3	
MB-COMT_forw	5- GGGGGTTATTTGTGGTTAGAAGTA-3	57.6
MB-COMT_rev	5-ACAACCCTAACTACCCCAAAAAC-3 biotin	
MB-COMT_seq	5- TTATTTGTGGTTAGAAGTAG-3	

Data analysis:

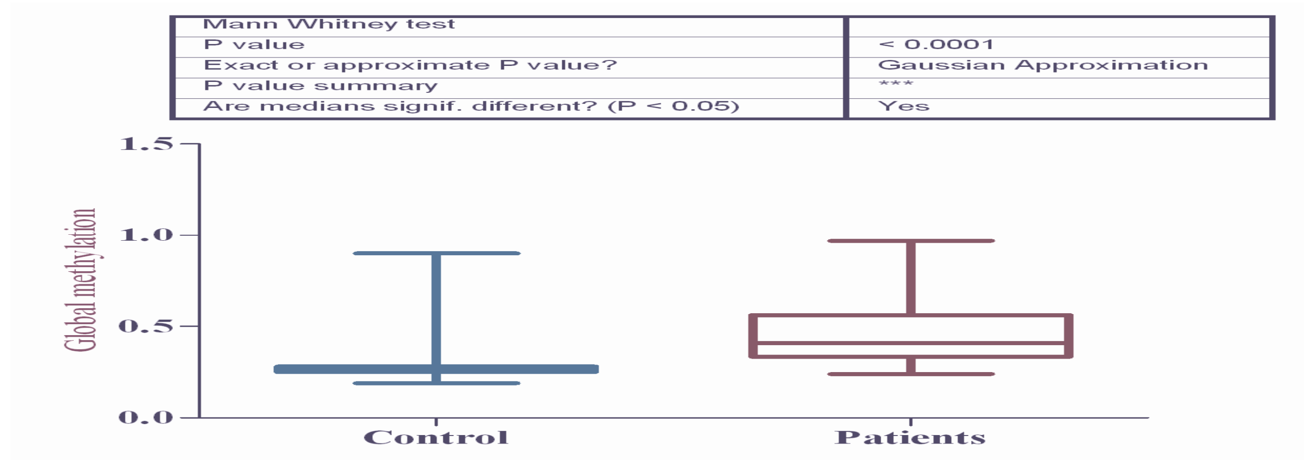
Results are presented as means and standard deviation (SD). Gene-specific methylation is given as percentage of methylation of CpG islands. Gene-methylation was not normally distributed according to the Kolmogorov–Smirnov test. Therefore, we used non-parametric tests like Mann–Whitney U for statistical analysis and Spearman correlation. We applied a significance level of $p < 0.05$. Data were analyzed employing SPSSTM for Windows15.0 (SPSS Inc., Chicago, IL).

Part 5: Results

Global methylation analysis:

Schizophrenic patients (n=183) had a significantly higher ratio of HpaII/MspI (0.449± 0.145 versus 0.273±0.062; p< 0.0001) compared with 171 controls, which indicates DNA hypomethylation in their peripheral blood cells (*Fig.9a*). In addition patients of early onset of the disease (n=18) showed global hypomethylation compared with the other patients (0.515±0.121 vs 0.44±0.14; r=-0.174, p=0.018) (*fig.9b*). No other correlations were observed when other variables were taken into consideration.

9a.



Mann Whitney test	
P value	< 0.0001
Exact or approximate P value?	Gaussian Approximation
P value summary	***
Are medians signif. different? (P < 0.05)	Yes

9b.

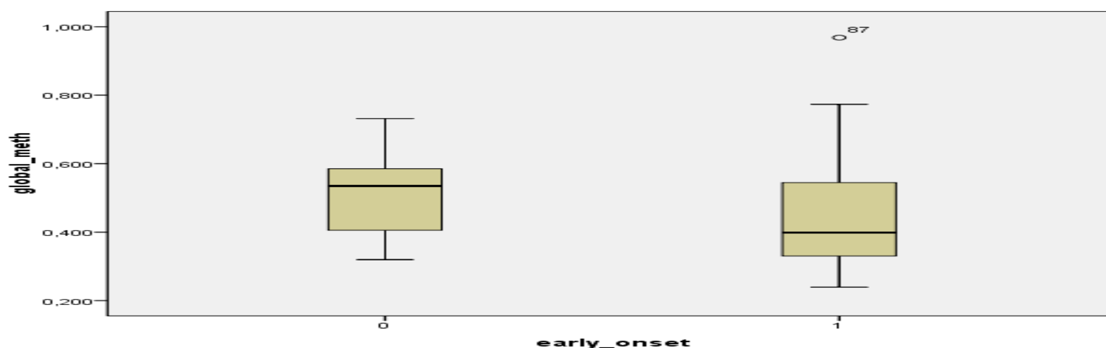


fig. 9: Global methylation analysis: **a)** Patients showed global hypomethylation compared with the blood donors. **b)** Patients of early onset of the disease had a higher ration of HpaII/MspI compared with the patients without early onset of the disease; **0**: patients with early onset of the disease, **1**: patients without early onset of the disease

Specific-gene methylation:

5-HTT:

Schizophrenic patients (n1=76) showed a higher degree of 5-HTT average methylation compared to the 84 blood donors (19,297% versus 18,356%, p=0,023) (*fig. 10a and b*). Since no demographic data were available for the blood donors, we conducted a statistical analysis between patients. We found a positive correlation between the degree of methylation and age (r=0.249; p=0.033). When other variables were taken into consideration such as 5HTTLPR genotyping, gender, early onset of the disease, smoking and drinking, no further effect on the degree of methylation was found.

Fig.10a: SZ patients had a higher degree of 5-HTT methylation compared to controls

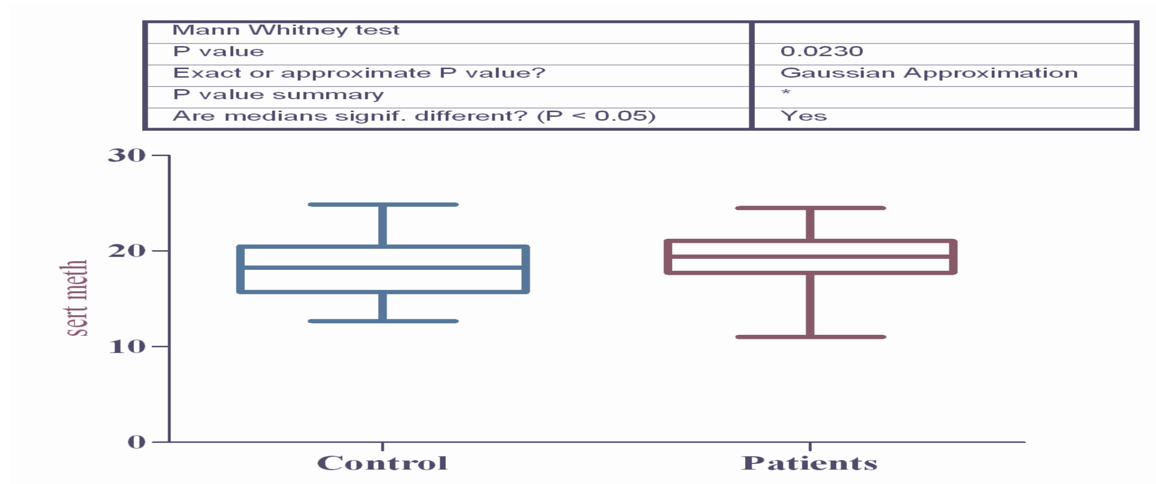
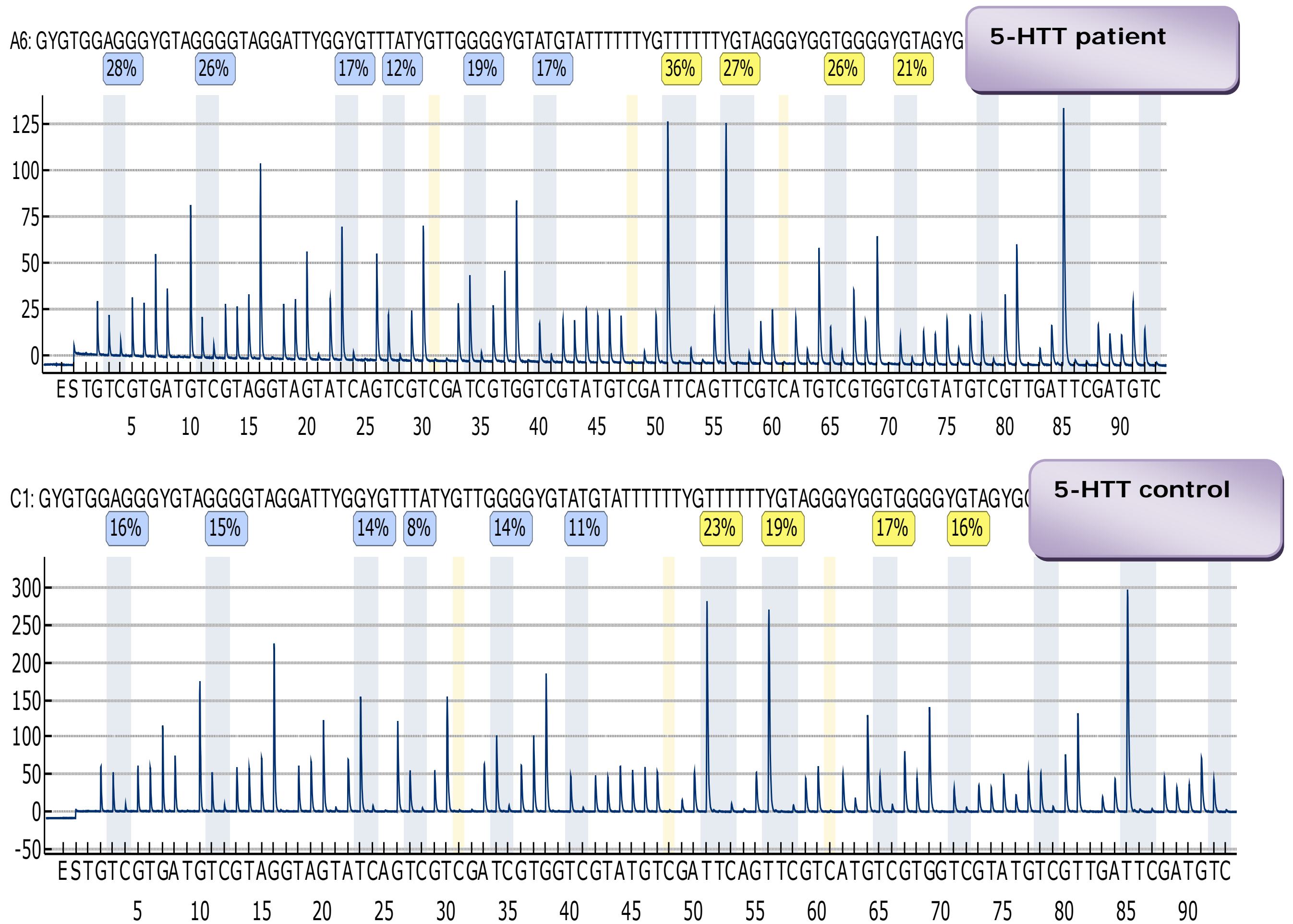


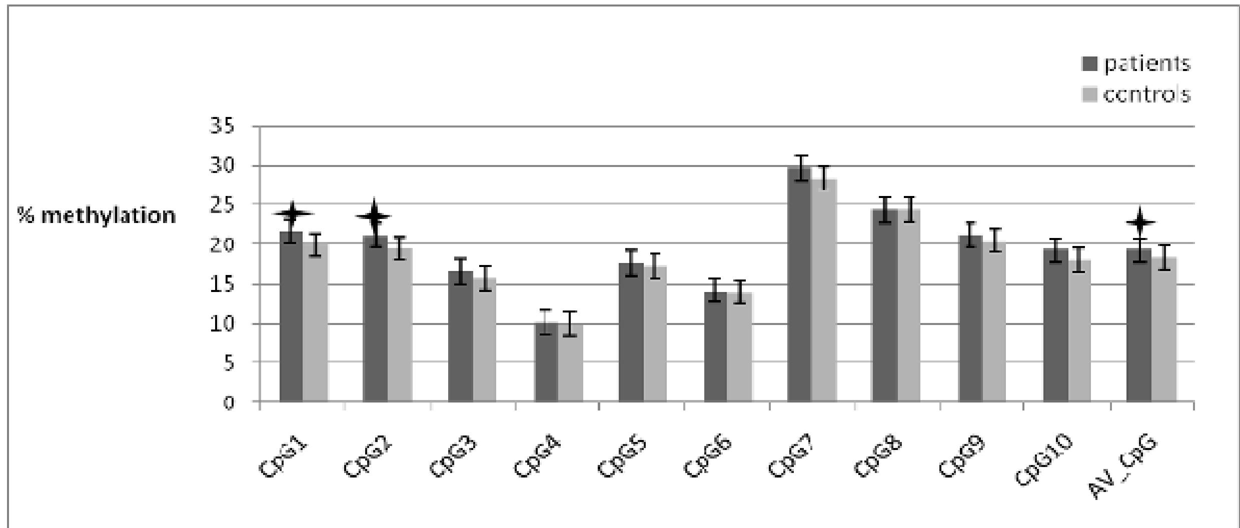
Fig.10b: Example pyrograms demonstrating hypermethylation of a schizophrenic patient compared to a control subject



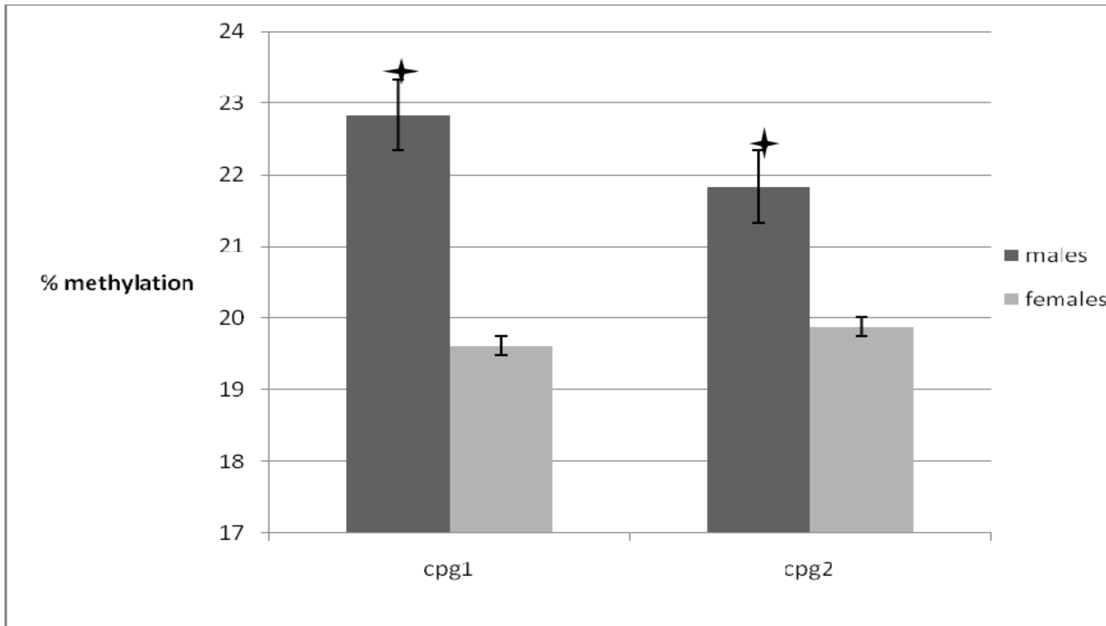
When we performed the statistical analysis per CpG, there was a strong correlation between the degree of methylation at the 10 CpGs (p<0.0001). In addition, the difference on the percentage of methylation between patients and controls was increased in CpG1 (21.66% versus 19.93%; Z=2.9, p=0.003) and CpG2 (21.13% versus 19.5%; Z=2.9, p=0.003) (*Fig.11a*). There was also a strong correlation in patients between the degree of methylation and age, especially in CpGs 6 and 8 (r6=0.285, p6=0.0144; r8=0.46, p8<0.0001, respectively). Interestingly, in CpG1 and CpG2 a gender effect appeared, where male patients were hypermethylated compared to the females (22.83% vs 19.62%; r1=0.39, p1=0.0005 and 21.83% vs 19.88%; r2=0.24, p2=0.0341) (*fig.11b*). CpGs 2 and 6 are putative binding sites for the transcription factor MTF-1, which has been implicated in DNA methylation (Ratke et al, 1996) (*fig.12*)

Fig.11: a) DNA methylation at the ten CpGs; Bars denote standard error, ***b)*** Bars denoting difference in degree of methylation between male and female patients in CpG1 and CpG2; asterisks show statistical significance.

a)



b)



1 2 6

GCGTGGAGGGCGCAGGGGCAGGATCCGGCGCCCACCGCTGGGGCGCATGCACCTCCTCGC

8

CTCCTCGCAGGGCGGTGGGGCGCAGCGGTTCTATCCCCCTCCCCGAGGCGG

Fig.12:Predicted transcription binding sites in the region of 5-HTT analyzed by pyrosequencing. Boxes indicate CpG sites with the largest DNA methylation differences in affected individuals. Green marked sequence: putative binding sites for MTF-1

S-COMT:

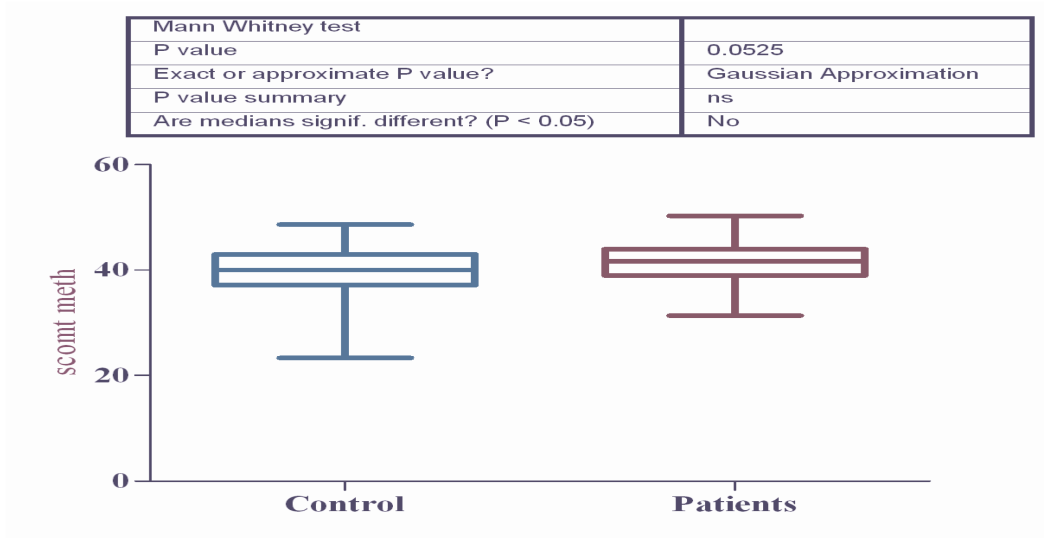
In S-COMT average methylation there was a trend for significance between the two groups (p=0.052), where patients (n=52) presented a higher degree of methylation compared with the 52 controls (41.55% versus 39.24%) (*Fig.13a and b).* In controversy with 5-HTT, female affected individuals had a higher percentage of methylation compared with males (43.43% versus 40.3%, r=-0.352, p=0.012) *(Fig.13c).* No other significant correlation was found between the percentage of average methylation and the other variables.

When we conducted the analysis per CpG, a strong correlation in the degree of methylation of the 3 CpGs appeared (p<0.0001). Patients showed a statistically significant higher degree in methylation in CpG2 and CpG3 vs the control subjects (50.96% versus 47.56% p2=0.005, Z2=2.78, 49.67% versus 47.21% p3=0.045, Z3=2, respectively) (*fig.13d*). Moreover the correlation between gender and methylation became significant in CpG2 and CpG3 (52.667% vs 49.806%, r2=0.277, p2=0.0462; 51.38% vs48.52%, r3=0.326, p3=0.0180) (*fig.13c*). Interestingly, CpG3 is putative binding site for GATA-1, GATA-2 and C-ETS elements, which have been implicated in DNA methylation (Murphy et al., 2005).

Fig.13: a) Patients had a higher degree of methylation compared to controls, ***b)*** Example pyrograms demonstrating S-COMT hypermethylation of a schizophrenic patient compared to a control subject, ***c)*** Bars denoting difference in degree of methylation between male and

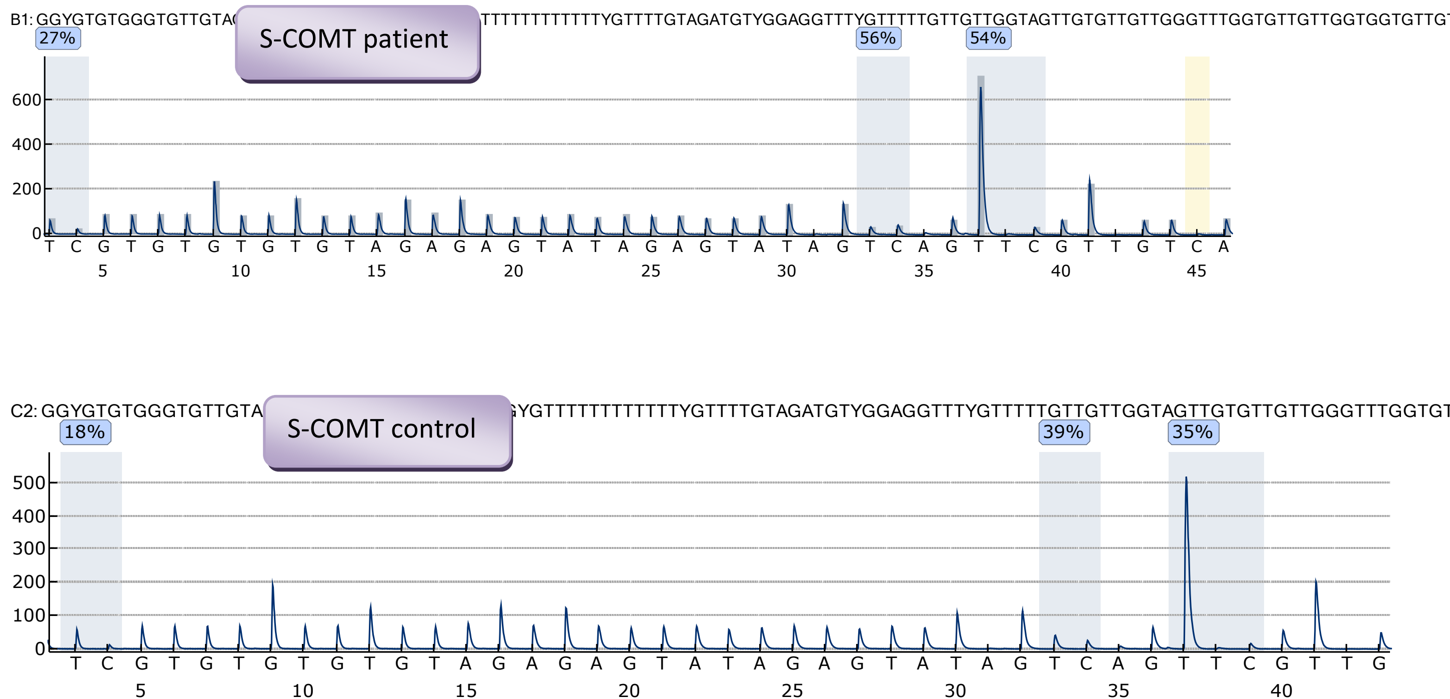
female patients in CpG2 and CpG3, ***d***) DNA methylation at the three CpGs; asterisks show statistical significance.

13a)

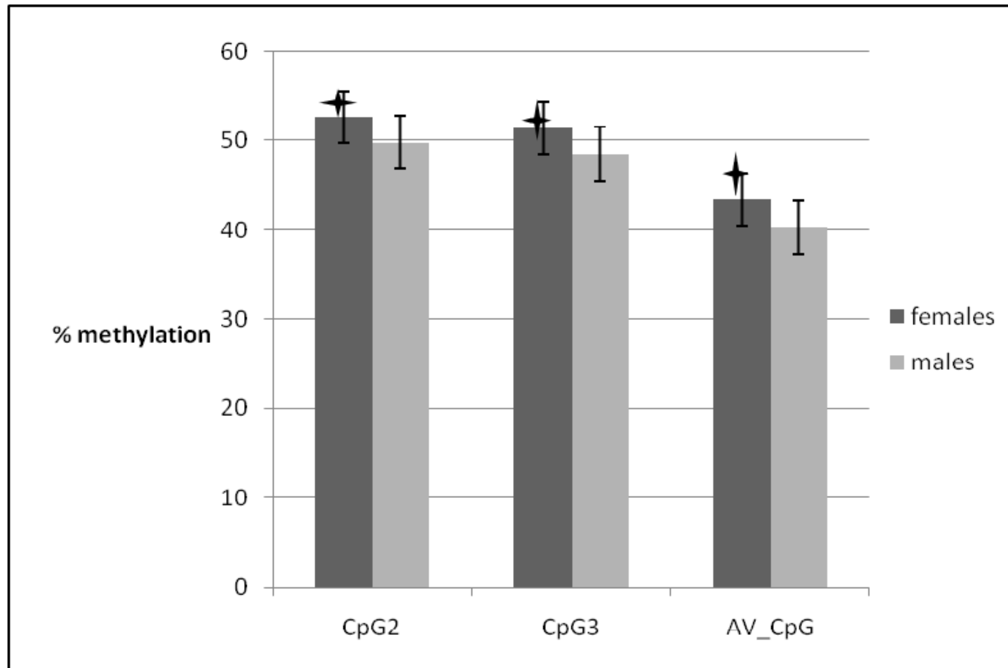


Mann Whitney test	
P value	0.0525
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No

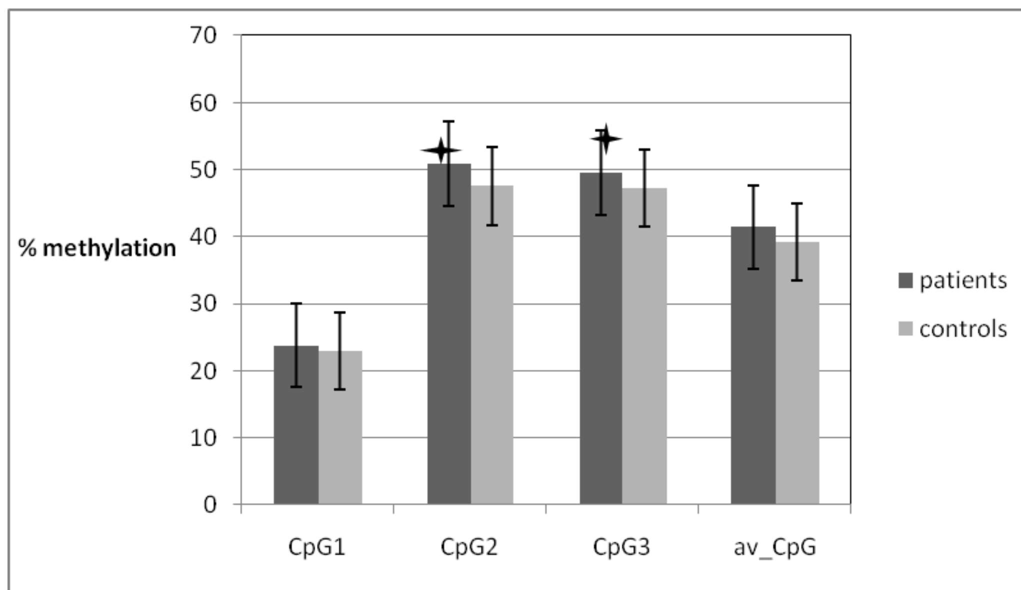
13b)



13c)



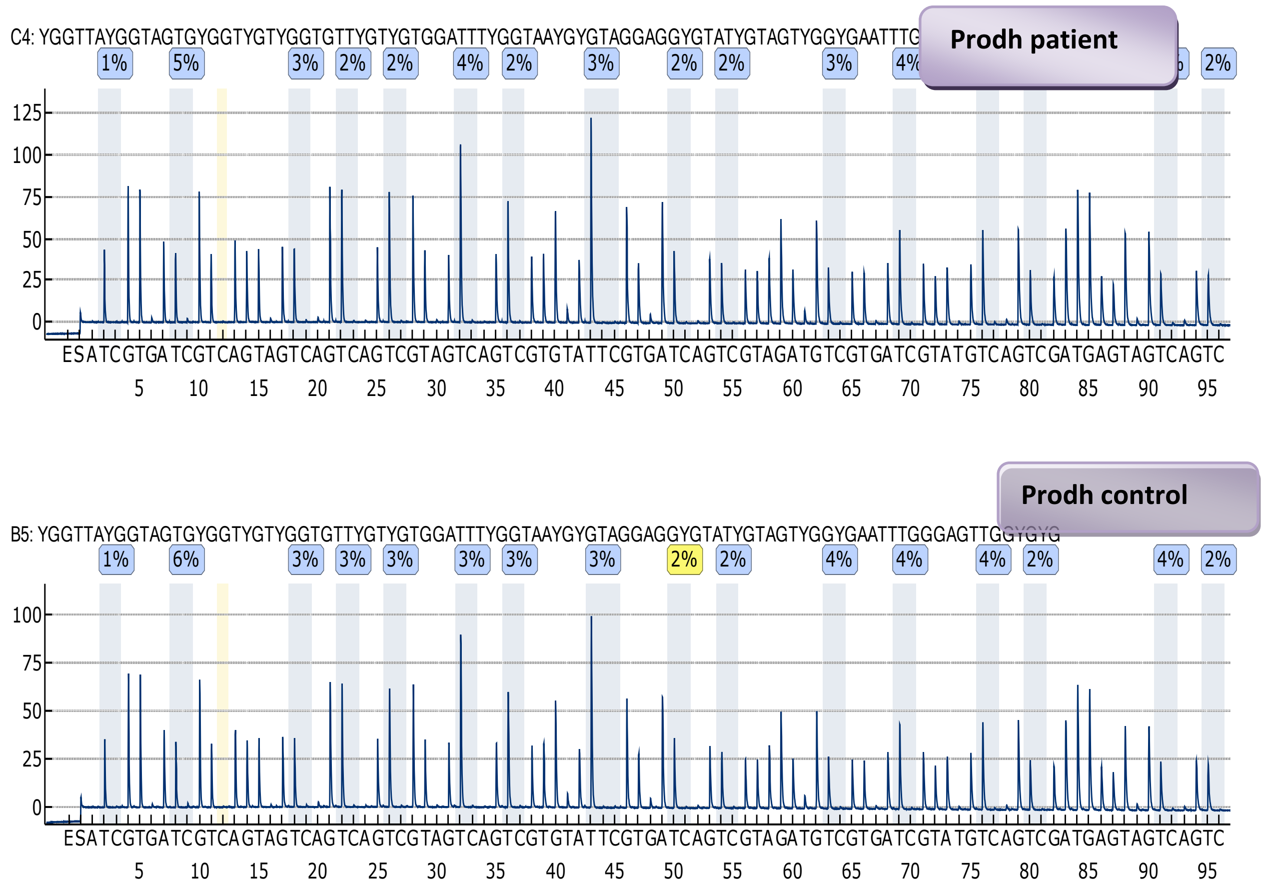
13d)



MB-COMT*, *PRODH*, *DTNBP1

In the above genes there was no methylation, either in patients or controls. The average percentage of methylation was approximately 5% (*fig.15*)

Fig.15: Example pyrograms denoting the degree of Prodh methylation for a SZ and a healthy individual.



Part 6: Discussion

Global Methylation

In the present study we investigated the potential link between epigenetics and schizophrenia in peripheral leukocytes. A main finding of this study is the global hypomethylation in schizophrenic patients compared to the blood donors and that the effect is more marked in patients with early onset of the disease vs the other patients.

We have yet only begun to scratch the surface of the roles and interactions of abnormal DNA methylation in schizophrenia, since there is no sufficient literature available. Our results confirm previous findings (Shimabukuro et al., 2006), although in our study there was no association between gender and methylation.

In 1952, Osmond proposed a 'transmethylation' hypothesis on schizophrenia, based on the idea of excessive and misdirected transmethylation resulting in the production of psychotoxic methylated metabolites. The hypothesis was later modified to rather suggest an insufficient transmethylation process in the patients and was referred to as the 'one-carbon cycle' hypothesis (Regland et al., 2005).The observed hypomethylation in schizophrenic patients in the current study supports the above model in which a suboptimal or altered epigenetic state alters susceptibility to schizophrenia. Kelsoe (1982) observed significantly lower kinetic parameters (KM and Vmax) for methionine adenosyltransferase activity in erythrocytes obtained from early onset schizophrenics when compared to samples from normal subjects. Moreover other studies (Smythies et al., 1986; Regland et al., 1994) report lower levels methylenetetrahydrofolate reductase in SZ patients. These are enzymes of one-carbon metabolism involved in the synthesis of the methyl donor *S*-adenosylmethionine.

The global hypomethylation reported in early schizophrenics compared with the other patients was also a new finding. In our data, there was no correlation of early onset of the disease with age and gender. Several lines of research are highly suggestive of a severe form of the disorder with possibly poorer prognosis and more neurodevelopmental insults presenting earlier in life (Kyriakopoulos et al., 2007). Thus the observed result might be a secondary effect of antipsychotic medication, since this subpopulation of patients usually begins pharmacotherapy earlier in life and receives multiple drugs and higher doses.

To our knowledge this is one of the first reports about early onset of the disease and hypomethylation. Our results also suggested that there are differences in the methylation of leukocyte DNA in schizophrenics and blood donors. In the light of the fact that most methylated cytosines are derived from repetitive sequences such as satellite DNA, it is clear that differences in methylation at the global level may be meaningful. However, the results of the current will need to be developed further.

Specific gene methylation

Alterations in the brain monoamines dopamine (DA) and serotonin (5-HT) have been implicated in the etiology and/or pharmacotherapy of multiple mental disorders including schizophrenia. Basic science research has shown that 5-HT receptors modulate dopaminergic function in the three DA pathways: mesolimbic, mesocortical, nigrostriatal, which are responsible for the positive, negative and extrapyramidical symptoms (from antipsychotic medication) of SZ respectively. In general terms, raphe stimulation inhibits the activity of dopaminergic neurons (Abi-Dargham, 2007). On closer examination though, serotonergic receptors have different and apparently opposing effects on dopamine. Moreover, their mechanism of action may differ as a function of multiple factors, including receptor subtype (Lucas and Spampinato, 2000), region (Arborelius et al.,1993), dose (in the case of pharmacological probes) (Goldstein et al., 1989), route/duration of administration (Minabe et al., 2001) and concomitant receptor activity (Seeman et al., 1976; Goldstein et al., 1989; Andersson et al., 1995; Liegeois et al., 2002).

Thus, 5-HTT, the major regulator of serotonin concentration in the synaptic cleft and S-COMT, one of the most important enzymes in the catabolism of catecholamines, are good candidates in investigating the DA-5HT interaction in SZ.

The results of this study demonstrate 5-HTT hypermethylation of the schizophrenics compared with the blood donors, which is more evident in CpG1 and CpG2, regions where male patients had a higher degree in methylation compared to the females.

To our knowledge, this is the first report on 5-HTT methylation in schizophrenia, since previous studies were conducted in depressed and alcoholic patients (Philibert et al., 2007). The direct evidence for gene-environment interplay in the aetiology of depression was given by Caspi et al (2003), who found that 5HTTLPR polymorphism regulates the effect of

stressful life events on susceptibility to depression, where individuals carrying at least one 'short' allele were more vulnerable to stressful life events.

Although the role of 5-HTT polymorphisms in depression is well established, when it comes to schizophrenia, the scenario is different. Numerous studies have been carried out on schizophrenia and 5-HTT polymorphisms. A recent meta-analysis (Fan et al, 2005) showed no statistically significant association between the short allele and SZ. However the 5HTTLPR polymorphism may play a role in other personality traits or even as a modifying locus on the presentation of schizophrenia as suggested by previous studies (Kaiser et al., 2001; Malhotra et al, 1998; Anguelova et al, 2003; Munafo et al, 2003). In contrast, it is demonstrated a highly significant association between Stin2.12 allele of the Stin2 polymorphism. In other studies, they observed significant differences between patients and controls only after 5-HTT haplotype analysis (Zaboli et al, 2007).

In our study, there was no significant evidence for an association between 5HTTLPR and schizophrenia. In addition we did not observe any correlation of the level of the methylation and 5HTTLPR polymorphism. Of course we cannot exclude the possibility that there is gene-environment interaction for 5-HTT. In future we could perform Stin2.12 or a 5HTTLPR-STIN2.12 genotyping analysis and investigate if such interplay exists.

As we mentioned in the introduction part, hypermethylation of a gene usually results in its down-regulation in mRNA level. Previous studies have demonstrated region-specific differences in SERT mRNA expression in brains from schizophrenic patients compared to controls. Expression of SERT mRNA in the frontal cortex was significantly increased, whereas expression in the temporal cortex was significantly decreased (Hermandez and Sokolov, 1997). Hranilovic (2004) investigated the potential modulation of 5-HTT transcription by 5-HTTLPR and VNTR-2 in lymphoblastoid cell lines from schizophrenic patients. The results indicated weak individual influence, but possible combined effect, of 5-

HTTLPR and VNTR-2 polymorphisms on 5-HTT gene expression. Since expression analysis in our pilot study was not possible, our results are inconclusive.

A trend of S-COMT hypermethylation of the patients compared with the controls was observed, which became statistically significant in CpG2 and CpG3. In the same region a gender effect appeared where female patients had higher S-COMT degree of methylation compared to the males.

We investigated the same region as previously done by Murphy (2005). Using bisulfite-genomic sequencing, they found that four of the sites were totally methylated in all individuals assessed, but the two adjacent CpG sites showed evidence for partial methylation. In the present study, it was not possible to quantify all of the six CpGs, described above, but only the first three. In CpG1 and CpG2, we showed partial methylation in contrast to the study. The difference in the results may be due to the methods used. In our study we used Pyrosequencing, which is an accurate and quantitative method to determine the degree of methylation.

As we mentioned in the introduction, previous studies in COMT DNA methylation are controversial. Some studies found no evidence for DNA methylation changes in SCZ (Mill et al., 2008; Demster et al., 2006), whereas others demonstrate hypomethylation of MB-COMT promoter (Abdolmaleky et al., 2006). One possible explanation might be the lack of specificity of the *COMT* probe used to any one form of *COMT* in the studies of Mill and Demster. When it comes to expression analysis, the scenario is not clear again. Data from several studies imply a great variability from no difference to decrease and even increase between SZ patients and controls (Tunbridge et al., 2004; Bray et al., 2003; Abdolmaleky et al., 2006). Regarding the gene-environment interplay, no statistically significant correlation between COMT DNA methylation and genotyping was shown, whereas there was an association between COMT genotyping and mRNA levels (Mill et al., 2008; Demster et

al., 2006). In our pilot study, we did not perform genotyping and expression analysis, so we can just make speculations about the interpretation of our results. One of our ongoing goals is to further proceed to the above, in order to have more conclusive and robust data.

In both genes, there was a gender effect in the degree of DNA methylation. There is a considerable clinical, epidemiological and neurochemical evidence to support a role for sex specific factors in the etiology of SZ (Mill et al., 2008). A protective effect of estrogen had previously been suggested by Seeman (1996). In his study, it was suggested that low estrogen phases correlate with more severe symptoms, and high estrogen phases correlate with less severe symptoms. A decade of experimental estrogen studies has shown that the sex hormone has potent neuromodulatory effects. Sumner and Fink (1995), Fink et al. (1998), Shughrue et al. (1997), McEwen et al. (1981) and Woolley and McEwen (1994) showed that oestrogen acts with similar functional effects not only on D2-receptors, but also on 5-HT_2, glutamate (NMDA) and GABA receptors on both the protein and the genomic level. In animal experiments, for example, estrogen stimulates the serotonin transporter gene. The functional effect seems to be analogous to the protective, antipsychotic effects of a reduced D2-receptor sensitivity (Fink, 1995).

It can be hypothesized that sex-specific differences represent underlying differences in etiology that can be mediated by epigenetic processes. For example, although sex hormones cannot change DNA sequence, it is known they can be potent modifiers of epigenetic status and gene expression. There are several reports of the female sex hormone estrogen, for example, altering the chromatin configuration and DNA-methylation profile of specific loci in the genome, potentially controlling gene expression in a sex-specific manner. Given the sexual dimorphism of SZ-including differences in rate of occurrence, symptoms and time course- the differential methylation changes observed in male and female patients should not come as a surprise.

We also observed a positive correlation between 5-HTT methylation and age. This finding is in agreement with earlier studies. Though epigenetic traits are generally stably inherited through numerous cell divisions, over time, epigenetic errors do occur in somatic cells. The frequency appears to increase with age and thought to be more frequent than mutations. In mice, epigenetic loss of repression of a gene was reported in older mice compared with younger mice (Benett-Baker et al., 2003). In the same study, it was suggested that it is likely that epigenetic error is a common feature of normal aging cells. In humans, a study observed that young monozygotic twins were virtually identical on measures of 5-methylcytosine DNA and histone acetylation, whereas in older twins, the differences in these measures were often fairly striking (Fraga et al., 2005). These differences in older twins might have occurred independently of or in concert with exogenous exposures.

Regarding MB-COMT, PRODH and DTNBP, the unmethylated status of their promoters that was found was due to use of the wrong material. As we mentioned above, previous studies have demonstrated hypomethylation of MB-COMT promoter (Abdolmaleky et al., 2006) as well as hypermethylation of DTNBP1 in affected females (Mill et al., 2008).

In our pilot study, brain tissue was not available, so we used blood as a surrogate for brain. Several published studies suggested that schizophrenia is accompanied by alterations in the function of the immune system and of periphreral blood lymphocytes (PBL) (Nikkila et al., 1995). A significantly increased number of activated lymphocytes have also been detected in the CSF of schizophrenic patients (Nikkila et al., 2001). In addition it has been well established a close relationship between the central nervous system and the immunological functions of PBL (Gladkevich et al., 2004). PBL expresses several receptors including cytokines, catecholamine (CA), dopamine, and acetylcholine, which act as communicators between the nervous and immune systems. Though different methylation patterns between striatum and lymphocytes have been reported in schizophrenia patients (Popendikyte et al., 1999 and Abdolmaleky et al., 2005), the analysis of DNA methylation in PBLs from living

subjects offers several advantages compared with evidence from postmortem brain tissue, such as the possibility of completely matching subject characteristics including age, gender and similar genetic background (Middleton et al., 2005). Since the use of brain biopsies of living schizophrenic patients for the purposes of biochemical investigation is unrealistic, the application of PBL presents a feasible substitute in schizophrenia research. Furthermore, it will be essential to find biomarkers in PBL for both the diagnosis and prognosis of schizophrenia.

Of course, this is only one point of view. We found no degree of methylation in three of our genes. The inconsistency of our results compared with previous findings was due to our material. So the use of brain tissue in DNA methylation studies remains a critical issue. It is noteworthy that absolute methylation changes between SZ and control samples were relatively small. Therefore, DNA methylation changes appear to be more subtle compared to those observed in brain neoplasia. These features emphasize that DNA in SZ is best approached by quantitative methodology for which bisulfite sequencing remains the gold standard. Given this lack of dramatic methylation changes in SZ, it comes as no surprise that earlier studies reporting positive findings were not consistently replicated. Some of these inconsistencies may be due to differences in methodology, the specific CpG nucleotides assayed and clinical populations. Furthermore it must be noted that maybe for the genes we examined, the degree of methylation may not necessarily correlate with functional consequences (i.e., decrease in mRNA transcript levels); rather the specific location of the CpG nucleotide-for example within a transcription binding site-may be more critical for mediating transcriptional repression.

Connor (2008) suggested that even when brain tissue is used, the interpretation of DNA methylation studies is complicated. To date, most methylation studies in brain have utilized DNA extracted from whole tissue homogenates. However, specific subpopulations of cells within specific brain regions are thought to be affected in SZ and psychiatric disorders in

general. Thus, if alterations in DNA methylation within specific cell populations do indeed play a role in the pathoetiology of SZ, some methylation changes may be *diluted*- or even undetectable- due to the averaging of methylation signals from a heterogenous pool of cells.

Conclusion

In summary, our preliminary data reveal global hypomethylation in SZ patients compared to blood donors and this effect is more evident to patients with early onset of the disease compared with the others. In specific-gene methylation, (i) 5-HTT: SZ patients were significantly hypemethylated compared to controls and the degree of methylation became greater in CpG1 and CpG2, region where male patients had a higher level of methylation compared to females. In addition there was a positive correlation between age and the degree of methylation. (ii) S-COMT: We demonstrate a trend for significance, where SZ individuals were hypermethylated compared to controls, which became statistically significant in CpG2 and CpG3, where female patients showed a greater level of methylation. (iii) PRODH, MB-COMT, DTNBP1: There was no methylation both in affected and unaffected samples, due to utilization of the wrong material.

Of course, we should note the limitations of our pilot study, (i.e., Blood as a surrogate of brain; no demographic information of blood donors), which make our data inconclusive. We suggest that further investigation of mRNA levels, as well as genotyping analysis should be performed. In addition our findings should be confirmed in post-mortem brain tissue.

Nevertheless, one should keep in mind that our understanding of the epigenetic role in mental disorders is just in its infancy. The ultimate significance remains to be determined. But it is now clear that environmental stimuli may influence disease susceptibility by changing epigenetic modifications of relevant genes. Furthermore, interventions designed to produce epigenetic modifications may offer new approaches to the treatments of schizophrenia and mental disorders in general.

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