GENOMIC DNA METHYLATION IN HEALTH AND DISEASE

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A hair perhaps divides False and True;
Yes, and a single Alif were the clue-
Could you but find it -to the Treasure -house,
And peradventure to THE MASTER too;

Omar Khayyam, Persian poet, 1048-1122

To my family

ABSTRACT

Cytosine methylation is one of the important epigenetic mechanisms controlling the vertebrate genome. Changes in genomic DNA methylation have been demonstrated in aging, auto-immune diseases and cancer. Aberrant DNA methylation patterns, involving both hypomethylation and hypermethylation, are associated with pathological events in cancer and other diseases.

The aim of this thesis was to shed light on DNA methylation and its control in disease. Initially, a novel assay to estimate global DNA methylation was developed which was named LUminometric Methylation Assay (LUMA). In this assay, DNA cleavage by methylation-sensitive restriction enzymes is coupled to a polymerase extension assay by Pyrosequencing™. LUMA is a quantitative, highly reproducible and easy to scale up assay which can be applied as a useful method to analyze genome-wide DNA methylation in a variety of physiological and pathological conditions including etiologic, diagnostic and prognostic aspects of cancer.

In the next step of the study, it was shown, for the first time, that global DNA hypermethylation is correlated with systemic inflammation in chronic kidney disease patients. Moreover, it was demonstrated that there is an association between global DNA hypermethylation and survival rate in end stage renal patients starting dialysis treatment. A correlation between genomic DNA hypermethylation and cardiovascular disease was revealed in this study. It was suggested that DNA methylation can be used as a strong novel prognostic marker in these renal patients.

Changes in DNA methylation by TrichostatinA (TSA) treatment was investigated in Hep3B cells as part of the study. To address this, the effect of TSA on DNA methylation was studied at gene specific and global levels. It was shown that TSA treatment results in genomic hypomethylation by affecting DNA methyltransferases both at the protein level and by changes in the DNA methyltransferases (DNMTs) mobility in the nucleus.

Finally, the impact of cytomegalovirus (CMV) infection on the host cell DNA methylation machinery was investigated. Using several experimental approaches it was shown that CMV infection alters the DNA-methylation machinery of the host cell leading to profound global inhibition of DNA methylation by intracellular relocalization of DNMT1. It was also suggested that defects in the DNMT1 function render non-permissive cells to be permissive to CMV infection. These findings may also help to understand the etiology of CMV pathogenesis and develop new therapy.

Based on the data achieved during this study, it has been suggested that cellular DNA methylation is a dynamic process which is changed based on cellular responses to physiological environment.

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II. Khan R, Schmidt-Mende J, **Karimi M**, Gogvadze V, Hassan M, Ekström TJ, Zhivotovsky B, Hellström-Lindberg E. "Hypomethylation and apoptosis in 5-azacytidine-treated myeloid cells."
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Archives Physiology Biochemistry, 2008 114(5):301-30

CONTENTS

LIST OF ABBREVIATIONS

5metC	5 methyl cytosine
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
CEA	Cytosine extension assay
CGI	CpG island
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-guanine
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
FCS	Fluorescence correlation spectroscopy
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDACI	Histone deacetylase inhibitor
LUMA	Luminometric methylation assay
NNA	Nearest neighbor analysis
PCR	Polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
SAM	S-adenosyl-l-methionine
TSA	Trichostatin A

1 INTRODUCTION

1.1 EPIGENETICS

The term epigenetics refers to heritable properties/changes in gene function that occur in the absence of alteration in DNA sequence. Gene expression control at the chromatin level is essential for all eukaryotic organisms, and is especially important in multicellular organisms to orchestrate key biological processes such as differentiation, imprinting, X-chromosome inactivation and aging. Cellular differentiation is the best example of epigenetic changes. All multi-cellular organisms, including animals originate from a single cell after fertilization of an egg by a sperm. Therefore all cells in a given organism will have the same genome, but during fetal development each cell will gain a specific set of epigenetic marks. The sum of these epigenetic marks in each cell, called "epigenome", defines the identity of each cell during differentiation. Epigenetic marks change again during aging where cellular functions are demolished overtime while the cellular genome is still intact (Fraga & Esteller, 2007). Therefore, it is important to study the epigenome to understand the biological and biomedical significance of epigenetic phenomena. However, it should be kept in mind that the epigenome is highly variable between cells and fluctuates according to local conditions, even within a single cell. Hence, it has been suggested that there is at least as many epigenomes as there are cell types.

The main epigenetic marks are covalent modifications of histone tails, DNA cytosine methylation and microRNA silencing. In most primitive eukaryotic species, epigenetic regulation is initiated and maintained only by modifications of histone tails and microRNA. Higher eukaryotes, especially vertebrates, utilise DNA cytosine methylation patterns as an extra layer of epigenetic mark which is maintained during DNA replication and is easily inherited (Bird, 2007; Suzuki & Bird, 2008).

The studies in this thesis have been focused on histone modifications and DNA methylation which are discussed in more details in the following sections.

1.2 HISTONE MODIFICATIONS

In eukaryotic cells, genomic DNA is always associated with basic proteins called histones to form chromatin. The basic repeat element of chromatin is the nucleosome that consists of a histone octamer and 147 base pairs of DNA wrapped around it. The histone octamer consists of two copies of each of the core histones H2A, H2B, H3 and H4. Nucleosomes are connected by linker DNA (10 to 80 base pair long) to form the 10 nm chromatin fibers known as "beads on a string". These fibers are then coiled to a helical structure known as the 30 nm fiber, which in turn is condensed to form chromosomes which are visible through light microscope in dividing cells (Figure 1).
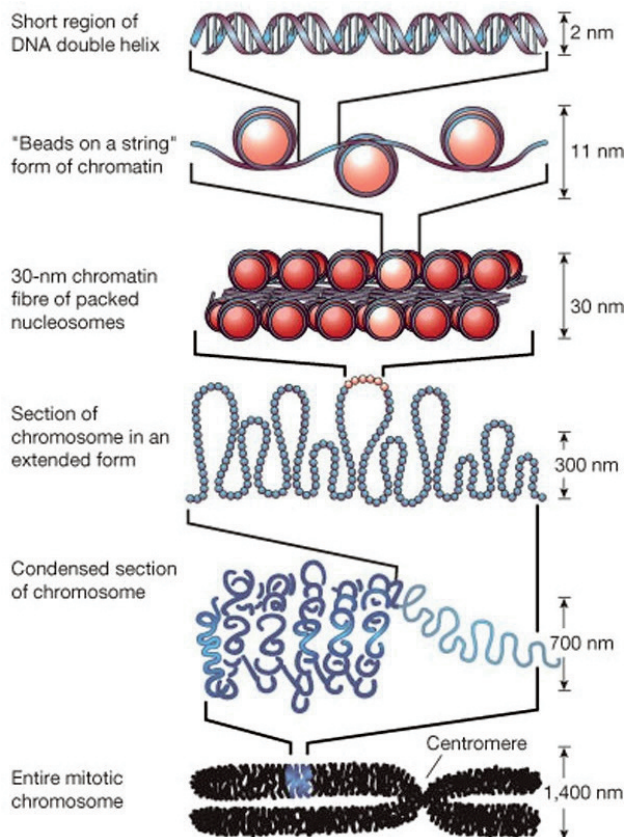
Figure 1. Different level of chromatin structure from DNA to chromosome (from Nature 2003 421:448-53 (Felsenfeld & Groudine, 2003)).

The chromatin structure is highly dynamic such that it can switch between heterochromatin (condensed) and euchromatin (relaxed) forms. The flexible structure allows chromatin to function properly in the cell to package DNA into the nucleus, to strengthen the DNA during mitosis and meiosis, to control gene expression, DNA replication and DNA repair (Felsenfeld & Groudine, 2003).

To achieve a high level of coordination in nuclear processes, cells have developed several mechanisms to spatially and temporally modulate chromatin structure and function on specific loci in the genome. These mechanisms involve chromatin remodeling by ATP-dependent remodeling enzymes, incorporation of histone variants and covalent modifications of histones. Chromatin remodeling enzymes modulate chromatin structure by sliding nucleosomes along the DNA molecule, by disrupting histone-DNA interactions and generating negative superhelical torsion in DNA (Kassabov et al., 2003). Incorporation of histone variants, of mainly H2A and H3, is another mechanism to affect chromatin structure. H2A, for example, can be replaced by
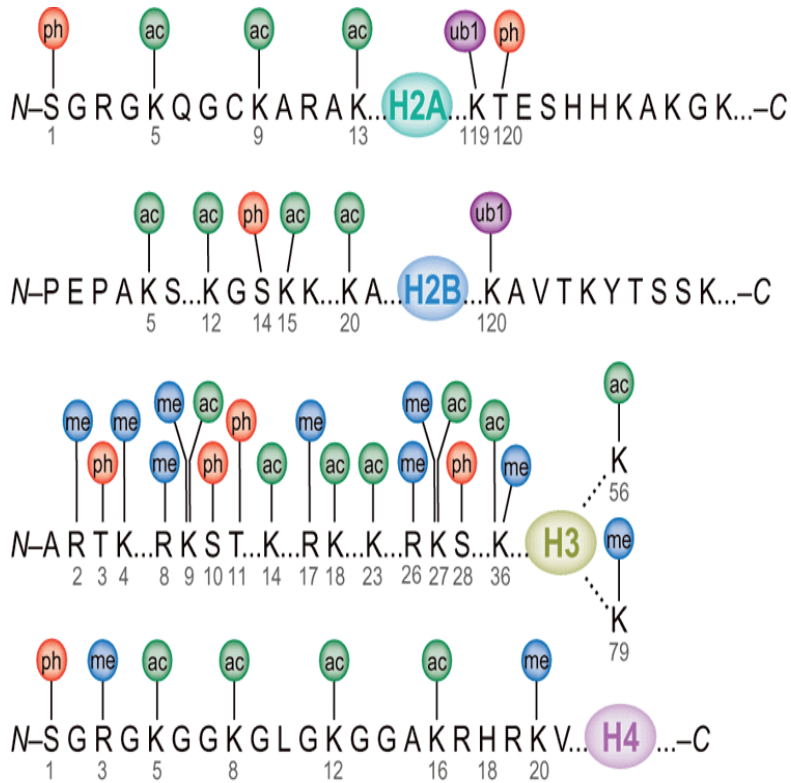
Figure 2. Histone residues which can be modified in the chromatin. Tne symbols are ac for Acetylation; me for Methylaation; ph for phosphorylation; ub1 for mono ubiquitination. (from(Bhaumik et al., 2007)

H2AX (which is associated with DNA repair), and H3 can be replaced by H3.3 (associated with gene activation) or CENPA (associated with centromeres) (Felsenfeld & Groudine, 2003). Chromatin structure and function are also controlled by covalent modifications of histones. The "histones tails" (the amino terminal ends of histones) are extended outside of the nucleosome core. They are thus accessible to enzymes for chemical modifications which in turn affect the histone DNA interaction and modulate chromatin structure. Several different types of histone modifications are known, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, deimination, ADP ribosylation and proline isomerization. There are over 60 different amino acid residues on histones which are accessible for covalent modifications (Figure 2) (Bhaumik et al., 2007). The combination of these modifications would produce over a million different possibilities for each nucleosome. This means that even in a given nucleus each single nucleosome can have its unique combination of modifications. The enormous combination of these modifications can be read out by regulatory proteins and influence gene expression. The term "histone code" is now widely used to describe the effects of histone modification patterns on the expression of individual genes, although it is still not proven if they form a true "code" or not (Turner, 2007).

1.2.1 Histone acetylation

Acetylation is presently the most studied histone modification in the epigenome. It is associated with active chromatin structure (Jenuwein & Allis, 2001). The acetylation status of histones is determined by balanced activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze the transfer of acetyl groups from acetyl-CoA to lysine residues in the histone tails which results in neutralization of the positive charges of lysine and consequently loosening of the complex between histones and the negatively charged DNA molecule (Marmorstein & Roth, 2001). Therefore, histone acetylation is associated with a relaxed chromatin structure which is more accessible to transcription factors. HDACs on the other hand, remove acetyl groups from histone tails which results in packaging of chromatin into a condensed structure and prevents the contact of transcription factors to DNA and consequently inhibits transcription (Gray & Ekstrom, 2001). HATs and HDACs activities are recruited to specific regions of genomic DNA by interaction with particular transcription factors. HATs and HDACs are normally part of large protein complexes such as CBP/p300 (HAT) (Marmorstein & Roth, 2001) or Sin3a (HDAC) (Gray & Ekstrom, 2001) which are involved in gene activation or gene silencing, respectively.

Acetylated lysines are recognized by a special protein domain called "bromodomain", which has approximately 110 aminoacids and consists of four helix boundles with left hand twist. Until now, over one hundred bromodomain containing proteins have been identified, most of which are chromatin associated. Bromodomains are also important in protein-protein interactions between regulatory proteins (Mujtaba et al., 2007).

Dysregulation of histone acetylation has been associated with tumor development and progression, and is also implicated in some other non-cancer diseases (Egger et al., 2004). In most tumor cells, chromatin is hypoacetylated due to disrupted activity of HATs or over expression of HDACs. The hypoacetylated status of chromatin has been associated with low expression of either tumor suppressor genes or pro-apoptotic genes (Bryan et al., 2002; Mahlknecht & Hoelzer, 2000). Moreover, some HDACs are aberrantly recruited to chromatin regions by fusion proteins produced by chromosomal translocations. Two examples are PML-RARa in acute promyelocytic leukemia and AML1-ETO in acute myeloid leukemia, that can recruit HDACs to chromatin leading to specific gene repression (Amann et al., 2001; Somech et al., 2004).

1.2.2 Histone deacetylase inhibitors

Histone deacetylase inhibitors (HDACIs) constitute a relatively new class of drugs with a high potential as anti-cancer agents. Pharmacological evaluations have shown that these drugs have low toxicity for normal cells while selectively inducing apoptosis in different types of tumor cells (Armeanu et al., 2005; Marks et al., 2000). Some HDACIs have already been approved for therapy in hematological malignancies, and several others are currently undergoing phase I, phase II or phase III clinical trials (Somech et al., 2004).

HDACI treatment generally induces a rapid accumulation of acetylated histone proteins, thereby changing chromatin structure and consequently affecting gene expression. Genome-wide expression profilings have shown that HDACIs modulate genes with key functions in cell fate. Induction of the cyclin dependent kinase inhibitor p21[WAF1] is one of the most characterized effects of several HDACIs (Richon et al., 2000). HDACI treatment of multiple myeloma cells mainly affects cellular processes related to apoptosis, cell survival, drug resistance, DNA repair, and cell cycle control (Mitsiades et al., 2004; Peart et al., 2005). In addition, some studies have highlighted a role of HDACIs in counteracting angiogenesis (Kim et al., 2001; Yang et al., 2006), and tumor progression and invasiveness (Klisovic et al., 2005; Whetstine et al., 2005), which support the therapeutic potential of these drugs.

Frequently described classes of HDACIs include i) short chain fatty acids, such as butyric acid and valproic acid; ii) hydroxamic acids, such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and oxamflatin; iii) cyclic peptides, such as depsipeptide and trapoxin; and iv) benzamides as CI-994 (Emanuele et al., 2008). The hydroxamic acids class of HDACIs has been considered more by oncologists because of their significant and specific effect on cancer cells (Grant et al., 2007; Kelly & Marks, 2005). SAHA, which has already been approved for patients with hematological malignancies (Zolina®, vorinostat), is particullary interesting due to its ability to induce apoptosis in tumor cells, whereas normal cells are relatively resistant (75, 76). In addition, SAHA have exhibited synergistic antitumor effects in combination with various anticancer drugs (Drexler & Euler, 2005; Emanuele et al., 2007; Ocker et al., 2005). Other hydroxamic acids, such as scriptaid and ITF 2357, are also very promising because of their selective antitumor activity and relative low toxicity (Leoni et al., 2005; Takai et al., 2006).

The molecular mechanisms underlying selective effects of HDACIs on cancer cells have not been completely elucidated. Several groups have suggested that tumor cells are characterized by histone hypoacetylation which would result in suppression of several tumor suppressor genes (Minucci & Pelicci, 2006). Treatment of tumor cells with HDACIs would increase histone acetylation, thus inducing derepression of pro-apoptotic genes and inhibition of tumor growth (Marks et al., 2001). A typical effect of HDACIs in many tumor cells is the induction of p21[waf1] through both p53-dependent and p53-independent pathways (Archer et al., 2005). Moreover, several anti-proliferative genes including p16, p57, and p19 are upregulated by HDACIs while proliferative genes such as CDK4, CDC25, cyclins, PCNA and Ki-67 are downregulated (Mitsiades et al., 2004; Yokota et al., 2004).

HDACIs may also affect non-histone proteins which are important in other cellular processes. Examples of such proteins that control apoptosis are p53, Hsp90, NF-κB and tubulin. They are stabilized in an acetylated form after treatment with HDACIs (Lin et al., 2006). These findings suggest that HDACIs exert complex and multiple mechanisms leading to growth inhibition and induction of apoptosis is tumor cells.

In addition to anticancer therapy, HDACIs are also used in other diseases including urea cycle disorder (Brusilow et al., 1984), sickle cell anemia (Dover et al., 1994),

adrenoleukodystrophy (Kemp et al., 1998), cystic fibrosis (Rubenstein & Zeitlin, 1998) and fragile X syndrome (Chiurazzi et al., 1999). Valproic acid is well known in the field of neurology as an anti-epileptic drug (van Breemen et al., 2007).

1.3   DNA METHYLATION

DNA methylation refers to the addition of a methyl group to one of the four bases that constitute DNA (Figure 3). In eukaryotes, DNA methylation occur only on the 5th position of the cytosine bases in the genome. DNA methylation is a stable epigenetic mark that regulates gene expression and chromatin structure and is involved in important processes such as embryogenesis, gametogenesis imprinting, X-chromosome inactivation and silencing of repetitive DNA elements. (Li, 2002). Methyl cytosine is found in the genomic DNA of many eukaryotic organisms, ranging from fungi and plants to invertebrate and vertebrate animals. But Yeast and C.elegans, however, do not have methylation in their genomes (Colot & Rossignol, 1999).

Methylated cytosine was first reported in 1948 by using paper chromatography (Hotchkiss, 1948). Subsequently DNA methylation was proposed to be important in gene expression. It was also suggested that this modification can serve as a heritable epigenetic modification for cellular memory (Holliday & Pugh, 1975; Riggs, 1975). This theory was proposed based on the observation that methyl cytosines reside predominantly in CpG and CpNpG sequences. Since CpG and CpNpG sites are diagonally symmetric, they can provide a molecular mechanism for semi-conservative inheritance of DNA methylation patterns during DNA replication. DNA methylation was later reported to be important particularly in cancer (Feinberg & Vogelstein, 1983a). An essential role of DNA methylation for mammalian embryonic development was evidenced by early lethality in mice lacking DNA methyltransferases (DNMTs) (Li et al., 1992; Okano et al., 1999). Chromosome instability and imprinting abnormalities have also been reported in DNMTs knock out models (Chen & Li, 2004). These findings, and numerous other studies have led to the generalization that DNA methylation functions to silence promoter activity and is associated with repressed chromatin structure (Bird & Wolffe, 1999). The mammalian genome is globally methylated, meaning that all categories of DNA sequences (genes, transposons and intergenic DNA) are methylated on CpG sites (Eckhardt et al., 2006). This ubiquitous DNA methylation makes it difficult to determine whether methylation is targeted to specific genomic regions or is a default state for the whole genome.

1.3.1   CpG Islands

Extensive studies of the methylation patterns in mammalian genomes have revealed that certain CpG rich regions are not methylated under normal conditions
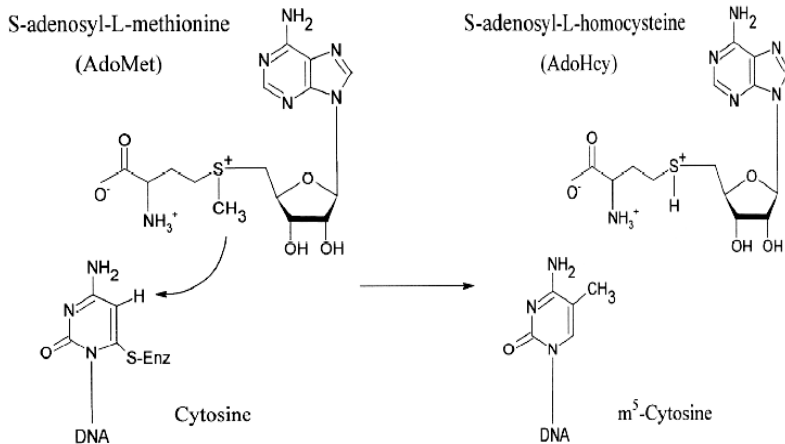
Fig 1-3. The methylation mechanism of cytosine (C) to 5-methylcytosine (m5C) by DNA methyltransferases. Methyl group is transferred from S-adenosyl-L-methionine to the C within CpG dinucleotides to form 5-methyl cytosine and S-adenosyl-L-homocysteine is released. (From (Turek-Plewa & Jagodzinski, 2005)).

These regions are called CpG islands (CGIs) (Bird, 1986). CGIs are defined as short regions of DNA with an average length of 1,000 bp (500-4,000 bp) which are relatively GC-rich (65% GC content) compared to the whole genome (40% GC content) and which have a high frequency of CpGs (4 times more frequent than the average of the genome). Genome-wide analyses of CGIs by computational prediction have suggested that approximately 56% of human gene promoters are associated with CGIs (Antequera & Bird, 1993). Interestingly about half of the predicted CGIs are located down-stream of the genes or in intergenic regions. Functional significance of intergenic CGIs remains unclear, but it is possible that at least some of them are associated to the promoters of non-coding RNAs (Suzuki & Bird, 2008).

Although most CGI are believed to remain unmethylated through-out development regardless of expression level, some CGIs become methylated during development which correlates with silencing of the associated genes (Bird, 2002). The best example is X chromosome inactivation during which several CGIs on the inactivated X chromosome are heavily methylated along with silencing of the associated genes (Mohandas et al., 1981). CGI methylation also occurs in imprinted genes and at genes which have specific expression in the germ-line (Sutcliffe et al., 1994).

1.3.2 DNA methyltransferases

DNA methyltransferases (DNMTs) are enzymes which catalyze cytosine methylation. There three DNMTs in mammals (DNMT1, DNMT3a and DNMT3b) are encoded by separate genes: (Chen & Li, 2006). All DNMTs are essential for normal development, as it was shown that mice lacking any of DNMTs will die at fetal developmental stage or early after birth (Okano et al., 1999). The DNMT3 family

members are primarily responsible for establishing the initial CpG methylation patterns *de novo*, whereas Dnmt1 maintains this pattern during DNA replication and repair (Chen & Li, 2006; Mortusewicz et al., 2005). As expected the maintenance DNMT, i.e. Dnmt1, has a 30- to 40-fold preference for hemimethylated sites (Jeltsch, 2006). However, this division of duties is not completely clear, as Dnmt1 activity is also involved in *de novo* methylation at non-CpG cytosines (Chen & Li, 2006; Grandjean et al., 2007; Jeltsch, 2006; Mortusewicz et al., 2005; Okano et al., 1999), and perhaps to some extent in CpG islands (Jair et al., 2006).

In addition to DNMT3a and DNMT3b, the Dnmt3 family includes one regulatory factor, Dnmt3-Like protein (Dnmt3L) (Bestor, 2000). Dnmt3a and Dnmt3b have similar domain structure: both contain a variable region at the N terminus, followed by a PWWP domain that may be involved in nonspecific DNA binding (Lukasik et al., 2006), a cystein rich Zn-binding domain (comprising six CXXC motifs), and a C-terminal catalytic domain. Dnmt3L is very similar to that of Dnmt3a and Dnmt3b in the cystein rich Zn-binding domain, but it lacks the conserved C-terminal catalytic domain (Bestor, 2000).

It is still not clear how DNA methylation patterns are established in the genome and how DNMTs activity is modulated. Structural analysis has shown that large conformational changes are involved in Dnmt1's transition to the active state. These changes involve interactions between the amino-proximal and catalytic domains (Fatemi et al., 2001) and/or phosphorylation of Ser515 (Goyal et al., 2007).

DNMT3L is involved in targeting DNMT3a and DNMT3b to genomic regions. It also stimulates their activity (Kareta et al., 2006). Dnmt3L appears to stabilize the conformation of the active-site loop of Dnmt3a, which contains the catalytic nucleophile residue (Cys706), via interactions with the C-terminal portion of that loop (G718-L719-Y720) (Jia et al., 2007). Interestingly, point mutations of the codons corresponding to G718–L719 in human Dnmt3b are associated with the disease ICF (immunodeficiency, centromere instability, and facial anomalies) (Xu et al., 1999).

Although great efforts have been invested to understand the mechanisms for DNA methylation in mammals, understanding the basis for establishing, maintaining, and disturbing DNA-methylation patterns will require a much more research on the interaction between form and function in the DNMT proteins (Cheng & Blumenthal, 2008).

1.3.3 DNA methylation in different diseases

DNA methylation is considered to be quite stable in an individual cell. However, DNA methylation patterns are influenced by environmental factors such as diet, inflammation and aging (Cropley et al., 2006; Yung & Julius, 2008). Significant changes in DNA methylation has been observed in aging studies. The overall level of DNA methylation in many tissues of diverse organisms, ranging from salmon, to humans, has been reported to decline with age (Golbus et al., 1990; Wilson et al., 1987). One potential mechanism can be the decline in DNMT1 activity with age

(Lopatina et al., 2002). However, this declination in DNA methylation is not a general phenomenon. Depending on the specific strain of the animal and the tissue examined, methylation levels can be either increased, decreased, or remain unchanged. For example, comparison of DNA methylation levels in 12 and 24 months old male albino rats revealed age-related decrease in 5-deoxy-methylcytosine content in the brain and heart, increased levels in the kidney, and no change in the spleen, lung and liver DNA (Vanyushin et al., 1973).

Additionally, certain methylation sensitive genes are differentially affected in aging. For example, the murine c-Myc gene undergoes demethylation in the spleen but shows increased methylation in the liver during aging (Ono et al., 1989). On the other hand, c-fos is hyper-methylated in the liver but not in the brain or spleen in aging (Liu et al., 2003). Wilson et al. examined the methyl cytosine content of liver, brain, and small intestinal mucosa from two rodent species (C57BL/6 and the longer living white-footed mice), and found that the rate of decline in DNA methylation content was inversely correlated to life span (Wilson et al., 1987). They also reported that the genomic DNA methylation density of human bronchial epithelial cells decline with age, and suggested that the maximum life span may be potentially related to the stability of the DNA methylation patterns (Wilson et al., 1987).

In a very interesting international study on human identical twins, Fraga *et al.* reported that young twin pairs had almost identical global and gene-specific DNA methylation patterns. By contrast, the older twin pairs in their 50s and 60s had very little similarity in their DNA methylation patterns (Fraga et al., 2005). Based on these findings, it can be speculated that cumulative errors in copying epigenetic information during cell division over one's life span is part of the aging process. Methylation changes that occur in aging have also been associated significantly with cancer development (Liu et al., 2003).

The role of DNA methylation in cancer development was initially proposed from observational studies of epigenetic patterns in normal and neoplastic tissues. Early studies using global measurement of 5-methylcytosine content suggested that decreased DNA methylation levels is a common feature of cancer (Lapeyre & Becker, 1979). Subsequently, this loss of DNA methylation was also shown for individual genes and was suggested as a mechanism to activate gene expression (Feinberg & Vogelstein, 1983b). Marked loss of 5-methylcytosine was also shown to be associated with chromosomal instability (Ehrlich, 2002) and to lead to an increased cancer incidence in certain animal models (Gaudet et al., 2003).

Global hypomethylation is just one characteristic of cancer. At the same time, many genes were shown to have increased DNA methylation in their promoters which results in silencing of gene expression and loss of protein function (Baylin et al., 1998)(Herman & Baylin, 2003). Genomic screening of 98 different primary human tumors has revealed that each single tumor has approximately 600 aberrantly methylated CpG islands. Some of these methylated CpG islands are present in genomic regions containing unknown genes which may play an important role in tumorigenesis (Costello et al., 2002). Many of these genes also play important physiological roles in

the creation or perpetuation of the neoplastic phenotype. Finally, reducing methylation in mouse models was also shown to prevent cancer formation under specific circumstances (Belinsky et al., 2003; Laird et al., 1995), providing further evidence for a pathogenic role of increased DNA methylation in cancer.

1.3.4 DNA methylation inhibitors

Aberrant DNA methylation in cancer and other diseases has led to a renewed interest in DNA methylation inhibitors as potential therapeutic drugs. It was originally believed that such drugs would inhibit DNA methylation in a non-specific way, and may be too toxic to be used in patients. Nevertheless, a therapeutic ratio for hypomethylation therapy exists and is related to the fact that tumors are much more dependent on gene silencing (e.g., of tumor suppressor genes) for their phenotype than normal adult cells. The effects of hypomethylation therapy are then the sum of multiple effects on cellular physiology, and it is likely that the net effect is favorable therapeutically. Indeed, this non-specificity can be viewed as advantageous (multiple defects are corrected simultaneously) while realizing its potential problems (risk of toxicity, cancer induction, etc.) (Issa, 2007).

Being a postsynthetic event in proliferating cells, DNA methylation is critically dependent on continued activity of DNMTs. Inhibition of the activity of these proteins will therefore result in progressive reduction of DNA methylation in newly dividing cells (Egger et al., 2004). DNA methylation inhibitors generally fall into three classes: (a) nucleoside inhibitors; (b) non-nucleoside weak inhibitors, often discovered by chance; and (c) rationally designed inhibitors.

The two most studied DNA methylation inhibitors are 5-Azacytidine and 5-aza-2-deoxycytidine. Both are cytosine analogues that trap DNMTs and target them for degradation (Egger et al., 2004). At low doses which do not inhibit proliferation, these drugs are effective hypomethylating agents and have shown clinical activity as anticancer agents. Other nucleoside inhibitors include zebularine, which seems to be promising but is not currently clinically approved (Cheng et al., 2003), and 5-fluoro-2-deoxycytidine (Gowher & Jeltsch, 2004), which is now applied in clinical trials.

One limitation of nucleoside analogues is the requirement for active DNA synthesis for their action, which limits the activity of the drugs in slow proliferating cells (including potentially cancer stem cells). Therefore, there is an interest in developing different classes of inhibitors for DNMTs, and this is an area of active investigation. Among the new drugs are orally available weak inhibitors such as procainamide and hydralazine (Cornacchia et al., 1988). The mechanism of action of these drugs is not understood yet and their clinical applications are limited since they induce low level of hypomethylation (Chuang et al., 2005). However, they could be useful models for the design of new non-nucleoside DNA methylation inhibitors.

Rationally designed DNMT inhibitors is a promising approach to find new drugs (Siedlecki et al., 2006). One limitation to this approach is the fact that there are three separate DNMT genes encoding for 3 different enzymes with DNA methyltransferase

activity. DNMT1 is the most abundant DNA methyltransferase in cancer cells, but it is doubtful whether inhibition of this enzyme alone is sufficient to induce hypomethylation and a therapeutic effect in cancer cells (Rhee et al., 2000; Robert et al., 2003). It has been reported that there is cooperation between different DNA methyltransferases (Rhee et al., 2002). Therefore, drug design strategies need to take into account the inhibition of several DNA methyltransferases simultaneously to be effective. In addition, there is considerable interest to induce gene specific hypomethylation and some theoretical approaches have been reported using unmethylated oligonucleotides (Yao et al., 2003) or engineered transcription factors (Jouvenot et al., 2003), but none of these is of practical clinical utility at the present time.

1.4   CYTOMEGALOVIRUS INFECTION

Human cytomegalovirus (HCMV), a member of the β-herpesvirus group, is a one of the most common viral infections in the world. Depending on socio-economic class and geographic location, HCMV infection is found in 60 - 90% of the world's population. Seroprevalence is age dependent ranging from 58.9% of 6 years old individuals up to 90.8% in individuals aged 80 or more (Staras et al., 2008). Like other herpesviruses, primary infection with HCMV in immunocompetent individuals is followed by persistence of latent infection with minimal or no clinical manifestations. In contrast, primary infection or reactivation of virus is life-threatening in immunocompromised individuals, such as immunosupressed solid organ and allogenic bone marrow recipients and HIV-infected patients, who are at great risk of developing severe diseases such as pneumonia, gastrointestinal disease and retinitis (Griffiths & Walter, 2005). HCMV is also the most common cause of congenital viral infection in humans. Approximately 5 - 10% of congenitally infected infants have symptomatic disease at birth, associated with microcephaly, intracranial calcifications, hearing loss and mental defects. However, the majority of the infected newborns appears to be normal at birth but may further develop HCMV associated diseases (Dollard et al., 2007).

The CMV genome is a large, double stranded DNA of 220-240 kb. Molecular characterization of CMV strains suggests that the viral genome has 252 open reading frames, but only 45 to 57 of these genes are essential for viral DNA replication. Hence, the vast majority of viral proteins are involved in modulation of virus- host interactions (Dunn et al., 2003). Upon infection of permissive cells, three classes of viral genes are expressed, immediate early (IE), early (E), and late (LA). Expression of IE genes is the first step in the viral infection cycle, which is followed by expression of early genes and subsequent viral DNA replication. Expression of late proteins are required for production of structural proteins and entry into virion assembly step (Landolfo et al., 2003).

The replication of the HCMV genome is a very complex process, which requires the activity of several viral proteins. These proteins are essential to permit the initiation

of DNA replication, the extension of the nascent DNA strands and also the termination of the whole process. In a transient transfection-replication assay, it has been shown that 11 loci are required to complement HCMV DNA replication (Pari & Anders, 1993; Pari et al., 1993). Based on sequence and biochemical similarities to homologues of HSV-1 replication proteins, it was suggested that six of the identified loci encode replication fork proteins. The six encoded proteins include the DNA polymerase catalytic subunit (UL54), the single-stranded DNA-binding protein (UL57), the polymerase accessory protein (UL44) and the three subunits of the helicase-primase complex (UL70, UL102, and UL105). The five additional loci needed to complement HCMV DNA replication have no obvious homologues in alpha- and gamma-herpesviruses. Three required loci encode known IE regulatory proteins [UL36-38, IRS1/TRS1 and the major immediateearly (MIE) region spanning (UL122-123)]; the remaining two loci (UL84 and UL112-113) encode early, nucleus-localised proteins (Mercorelli et al., 2008). Recently, UL84 has been proposed to be a key factor in the initiation of HCMV DNA replication and thus to represent the viral origin-binding protein (Colletti et al., 2007).

The genomes of herpes viruses, including HCMV, exist as episomes during infection. The episomes are associated with nucleosomes in the infected host cells, but when encapsidated into virions, the viral DNA lacks histones (Murphy et al., 2002). It was recently reported that the host cell nucleosome deposition machinery, targets CMV DNA upon infection, resulting in a stepwise and dynamic viral-chromatin assembly (Nitzsche et al., 2008). Based on these finding it was suggested that epigenetic events are involved in all viral DNA based processes during CMV infection including genome replication, DNA damage response, and the temporal cascade of viral gene transcription (Nitzsche et al., 2008). In addition, an *in vitro* model has been defined for CMV latency and reactivation by chromatin remodeling in dendritic cells (Reeves et al., 2005). Indeed, a number of studies have provided evidence suggesting that the CMV gene expression is controlled by epigenetic mechanisms such as histone modifications and DNA methylation (Hummel et al., 2007; Ioudinkova et al., 2006).

Antiviral drugs have been developed and employed for either prophylactic or pre-emptive therapies and for direct treatment for HCMV disease (Singh, 2006). Five compounds have been currently licensed to treat established HCMV infections—ganciclovir, its oral prodrug valganciclovir, foscarnet, cidofovir and fomivirsen. High dose acyclovir, and more recently, valaciclovir have also been used as a prophylactic agent against HCMV especially in bone marrow and renal transplant recipients, albeit they lack sufficient potency to be used for treatment of active HCMV disease (Mercorelli et al., 2008). With the exception of fomivirsen, an antisense oligonucleotide which is targeted to the HCMV immediate-early (IE) gene locus and has been licensed for intra-ocular use only, all of the other compounds target, either directly or indirectly, the viral DNA polymerase (Biron, 2006). The currently available anti-HCMV drugs have several drawbacks that limit their clinical utility. Some of the compounds have limited oral bioavailability, and thus must be administered intravenously. In addition, most of the anti-HCMV drugs exhibit significant toxicity; for example, nephrotoxicity is a dose-limiting factor for cidofovir and foscarnet, whereas bone marrow suppression that results in granulocytopoenia and thrombocytopenia is the most common toxic side

effect seen with ganciclovir. The emergence of drug-resistant viral strains also poses an increasing problem for disease management. Since most of the approved anti-HCMV compounds share a similar mechanism of action, targeting the viral DNA polymerase, mutant viruses resistant to one drug are commonly resistant to others (Villarreal, 2003). Therefore, finding new targets for anti-viral therapy is essential in the HCMV research.

HCMV infection has been reported to be associated with several human malignancies, including brain, prostate, and colon cancer, suggesting a potential role for HCMV in oncogenesis (Harkins et al., 2002). Active HCMV infection was demonstrated in 90% of human malignant gliomas while the non-malignant adjacent tissue was free of HCMV infection (Cobbs et al., 2002). Accumulating evidence indicate that HCMV viral gene products can modify signaling pathways underlying cellular processes such as apoptosis, proliferation, migration and transformation (Cinatl et al., 2004). For example, transcriptional activation of cellular oncogenes, including c-FOS, c-MYC, and c-JUN is induced by HCMV infection (Boldogh et al., 1990). During evolution, DNA viruses have acquired the capacity to modulate cellular signaling pathways to activate cell cycle and inhibit apoptosis. This is done by activation of PI3-K/AKT axis or interference with p53 and Rb check points (O'Shea, 2005). In HCMV, IE-72 and IE-86 have been reported to disrupt cellular processes by direct interaction with retinoblastoma protein p107 and p53. Such interactions would inhibit p53 pro-apoptotic functions and activation of E2-F controlled genes (Poma et al., 1996; Speir et al., 1994). Recently, it was shown that HCMV infection caused rapid activation of the PI3-K /AKT pathway and led to phosphorylation of phospholipase C in infected cells. It was suggested that HCMV infection promotes glioma cell invasiveness trough increased extracellular matrix dependent migration (Cobbs et al., 2007).

1.5   CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) is a worldwide public health problem. In the United States, there has been a rising incidence and prevalence of kidney failure, with poor outcome. The incidence and prevalence of end-stage renal disease (ESRD) have doubled in the past 10 years and is continuing to rise steadily in the future. Nearly, 8 million people (4% of the adult U.S. population) have moderate or severe CKD, and another 450,000 have end-stage renal disease (ESRD) (Coresh et al., 2003). In 2003, total Medicare and private insurers' costs for ESRD treatment in US were $27.0 billion and accounted for more than 15% of Medicare expenditures (Weiner, 2007). The mean number of comorbid conditions in dialysis patients is approximately 4 per patient, the mean number of hospital days per year is approximately 15, and self-reported quality of life is far lower than the general population (Bakris et al., 2000).

Despite advances in dialysis and transplantation, there is still poor prognosis for kidney failure patients. More than 63,000 deaths of patients with ESRD were reported in 1998, and annual mortality rate of dialysis patients is more than 20%. Expected lifetimes of patients treated by dialysis were quite short, varying (depending on gender

and race) from 7.1 to 11.5 years for 40 – 44 years old patients, and from 2.7 to 3.9 years for patients aged 60 to 64 years. Morbidity of kidney failure is also very high in these patients (Jaar et al., 2005).

Chronic kidney disease is defined as progressive loss of renal function. The main risk factors for CKD are diabetes and high blood pressure which would cause damages to kidneys. The symptoms of the disease vary a lot in different patients, depending on the stage of the disease and risk factors in each individual patient. CKD is normally identified by a blood test for creatinine. A high level of creatinine indicates problems in glomerular filtration rates resulting in decreased capacity of the kidneys to filter wastes from blood. Detailed investigation of kidney damage includes different imaging of kidneys, blood tests and renal biopsy (Vassalotti et al., 2007). The severity of kidney damage in CKD is defined in five stages, with stage 1 being the mildest and usually with few symptoms and stage 5 being severe illness with no kidney function. Stage 5 is also referred as end stage renal disease (ESRD) or chronic renal failure.

There are several risk factors associated with CKD including: age, race, diabetes, hypertension, inflammatory diseases, systemic infection, urinary stones, family history of CKD, low birth weight, life style and exposure to certain chemicals. These factors should be considered carefully in patient management and treatment (Keane & Eknoyan, 1999).

Cardiovascular disease (CVD) is the leading cause of mortality in patients with ESRD. Currently, atherosclerotic heart disease accounts for approximately 55% of all deaths in these patients. A combination of factors are involved, including higher prevalence of CVD in this population compared with the general population, an increased risk of adverse outcomes after coronary interventions, and complications in traditional primary and secondary prevention strategies in patients with ESRD. Increased cardiovascular disease extends to other CKD patients who do not have ESRD (Manjunath et al., 2003). Because of high prevalence of CVD among patients with renal failure, there is considerable attempt to develop strategies to decrease or delay the progression and complications of atherosclerosis in these patients (Baber et al., 2007).

One aspect of the CKD pathogenesis is uremia (presence of urine in blood) meaning that the blood wastes are not removed from the blood. Increased level of urine substances in the blood would mean that physiological environment is changed. Since some of this factors such as homocysteine, are involved in epigenetic processes, it is essential to study epigenetic markers as part of CKD pathogenesis (Stenvinkel & Ekstrom, 2008). It has already been reported that several features of uremia such as hyperhomocysteinemia (Ingrosso et al., 2003), inflammation (Stenvinkel et al., 2007), dyslipidemia (Lund et al., 2004), and oxidative stress (Valinluck et al., 2004), all may be associated with aberrant DNA methylation and changes in chromatin organization. In patients with vascular disease, increased homocysteine and S-adenosylhomocysteine (SAH) concentrations is associated with DNA hypomethylation (Castro et al., 2003). Thus, elevation of plasma homocysteine seems to have a suppressive effect on methylation reactions, mediated by an increase in intracellular SAH. However, the effect of homocysteine on DNA methylation seems to be complex and could be

context-sensitive (Jiang et al., 2007). Folic acid, which is an intrinsic component of methyl donor metabolism, is another factor that affects global DNA-methylation status in the genome, and can modulate the effects of high homocysteine levels on DNA methylation (Yi-Deng et al., 2007). Thus, vitamin status and nutrition should be taken into account when analyzing the interactions between unbalanced DNA methylation, cardiovascular disease and outcome in CKD patients. Such studies may thus lead to a better understanding how the uremic milieu contributes to cardiovascular disease and also lead to a better treatment for the patients.

2 AIMS OF THE STUDY

The overall aim of this study has been to increase our knowledge about global DNA methylation levels and the cellular mechanisms behind it´s control, as well as to elucidate alterations in the DNA methylation machinery under different pathological and physiological conditions. Specifically the aims were:

I.   To develop a high throughput assay for quantification of global DNA methylation. LUMA was developed as a cheap and easy to scale up assay which could be used as a fishing tool to analyze genome-wide DNA methylation in a variety of physiological and pathological conditions.

II.   To evaluate the association between aberrant DNA methylation and inflammation, renal function, and homocystein level in peripheral blood leucocytes of end stage renal disease patients. To investigate the independent impact of DNA methylation on survival rate in patients starting renal replacement therapy.

III. To characterize global and gene-specific DNA methylation changes following treatment with HDAC inhibitor of *in vitro* cell models. To measure DNMTs interactions with nuclear components by fluorescence correlation

IV.   To asses DNA methylation changes upon cytomegalovirus infection and to evaluate the impact of host cell DNA methylation machinery on permissiveness to cytomegalovirus infection.

3 METHODS

Several molecular techniques were applied during this study which are presented in the respective individual papers. Two methods, however, were especially important and are therefore discussed in more details. Luminometric methylation analysis (LUMA) was developed early in this study and subsequently applied in quantification of global methylation levels. Fluorescence correlation spectroscopy (FCS) was used to monitor the interaction between DNMTs and nuclear components.

3.1 LUMINOMETRIC METHYLATION ASSAY (LUMA)

When this study was started, measurement of global DNA methylation was a challenge in the field. Although several assays had been reported previously (Fraga & Esteller, 2002) they were either labor intensive, required large amounts of genomic DNA, or involved radioactive isotopes. One of the earliest methylation assays relies on methylation sensitive and insensitive restriction endonucleases (Cedar et al., 1979). A pair of isoschizomer endonucleases is used where one enzyme is able to cut only unmethylated DNA, whereas the other cuts both methylated and unmethylated DNA. *Hpa*II and *Msp*I is the most commonly used isoschizomer pair in DNA methylation studies. The target sequence for both enzymes is CCGG. However, *Hpa*II is not able to cut if the internal cytosine is methylated (CmCGG) (Cedar et al., 1979). Several methods based on *Hpa*II and *Msp*I have been described to determine genome-wide CpG methylation such as: self-primed in situ labeling (SPRINS) (Andersen et al., 1998), methylation-sensitive arbitrarily primed polymerase chain reaction (AP-PCR) (Liang et al., 2002), non-methylated genomic sites coincidence cloning (NGSCC) (Azhikina et al., 2004), differential methylation hybridization (DMH) (Yan et al., 2002), and methylation target array (MTA) (Chen et al., 2003).

In an interesting paper, Pogribny et al. developed a "cytosine extension assay", This method combines *Hpa*II and *Msp*I endonucleases with single nucleotide extension using radio-labeled [3H]dCTP, so that incorporation of [3H]dCTP is inversely correlated with DNA methylation. In the original report this assay was employed to show that human hepatocellular carcinoma DNA is hypomethylated relative to normal liver (Pogribny et al., 1999). A modified non-isotopic cytosine extension assay using biotinylated dCTP was described later (Fujiwara & Ito, 2002), and DNA methylation was defined as the *Hpa*II/*Msp*I ratio. If the DNA is completely unmethylated, the *Hpa*II/*Msp*I ratio would be 1.0, and if the DNA is 100% methylated, the *Hpa*II/*Msp*I ratio would approach zero. In the current thesis study the radio active single nucleotide extension assay was replaced by Pyrosequencing reaction which is also based on a polymerase extension assay and the new assay was called "LUMA".

The LUMA is based on DNA cleavage by methylation sensitive or insensitive restriction enzymes followed by a luminometric polymerase extension assay to quantify the extent of restriction cleavage. The CpG methylation sensitive restriction enzyme *Hpa*II (recognition sequence CCGG) and its methylation insensitive
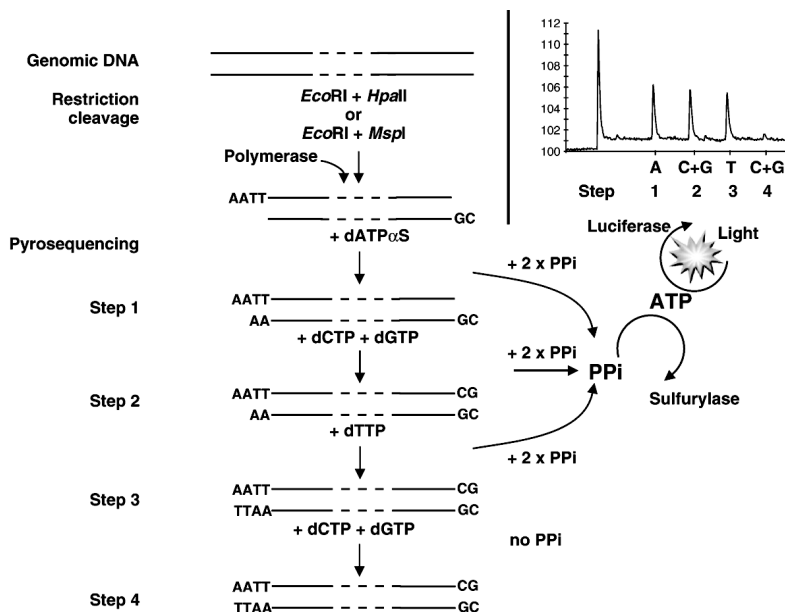
Figure 3. Analysis of global DNA methylation with the LUMA assay. Genomic DNA of the test sample is first digested with two combinations of restriction enzymes, either *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI. The enzymatic cleavage is then determined by a polymerase extension assay based on Pyrosequencing reaction in four step. After each nucleotide incorporation, inorganic pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase. Then ATP is used by Luciferase to activate Luciferin to produce a proportional amount of visible light which is detected by a CCD camera. The amount of light is proportional to the number of overhangs produced by the respective restriction enzymes. The A and T peaks correspond to Step 1 and Step 3 in Pyrosequencing reaction, reflecting the *Eco*RI cleavage and should be equal. The C + G peak resulting from pyrosequencing Step 2 reflects *Hpa*II or *Msp*I cleavage. The second C + G peak originating from Step 4 is an internal control peak that should be close to zero.

isoschizomer *Msp*I were used in parallel reactions. *Eco*RI (recognition sequence GAATTC) was 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 during the stepwise dispensation of dNTPs on the Pyrosequencing™ platform. Following successful extension by 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 (Ronaghi, 2001). 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) dispensations 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 a control for the completion of Step 2. The corresponding peak is expected to be zero or close to zero. The schematic principle of LUMA is illustrated in Figure 3.

3.1.1 Practical protocol for LUMA

Reagents
I. Restriction enzymes *Hpa*II, *Msp*I and *Eco*RI, from New England Biolabs (Beverly, MA).
II. The Tango® buffer (33 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA) from Fermentas (Fermentas Scandinavia, Stockholm)
III. PSQ™ 96 SNP reagents for pyrosequencing from Biotage (Biotage AB, Uppsala, Sweden).

Procedure

I. Digestion reactions (Final volume 20 μl):
- Mix A (*Hpa*II+*Eco*RI):
  Tango buffer 2 μl
  > *Eco*RI 0.5 μl (10U/μl)
  > *Hpa*II 0.5 μl (10U/μl)
  > dH$_2$O 12 μl

- Mix B (*Msp*I+*Eco*RI):
  > Tango buffer 2 μl
  > *Eco*RI 0.5 μl (10U/μl)
  > *Msp*I 0.5 μl (10U/μl)
  > dH$_2$O 12 μl

- Add 250-500 ng genomic DNA (in 5μl) to each digestion mix. DNA digestion can be performed either in 0.5 ml tubes or in 96-well Pyrosequencing plates. If 96 well plates are used, a plastic cover should be used to avoid evaporation.
- Incubate at 37°C for 4 hrs.
- Add 20 μl of Pyrosequencing™ supplied "Annealing Buffer" to each digestion reaction. If genomic DNA has been digested in 0.5 ml tubes, transfer the whole reaction to 96-well Pyrosequencing plates.

II. Pyrosequencing assay:
  Programming the Pyrosequencing instrument:
- Run the Pyrosequencer machine in SNP mode,
- Define the assay sequence as; AC/TCGA (This is not a SNP analysis but the machine is programmed according to SNP principles and has to beprogrammed with the same logical rules).
- Define nucleotide addition as: ACTCGA
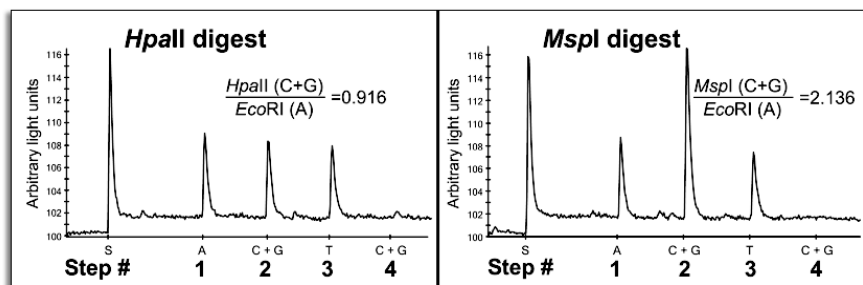Preparing Pyrosequencing reagents:

Figure 4. Typical LUMA results for human genomic DNA sample. The graphs show the luminometric output from two representative LUMA runs of human lymphocyte DNA, using *Hpa*II + *Eco*RI, and *Msp*I + *Eco*RI. The A and T peaks denote the additions of dATPaS and dTTP, respectively, the nucleotides of which are substrates for filling in all *Eco*RI generated T and A overhangs. These correspond to Pyrosequencing Step 1 and Step 3, thus reflecting the *Eco*RI cleavage and should be equal. The C+G peak resulting from Pyrosequencing Step 2 denotes *Hpa*II or *Msp*I cleavage. The second C+G peak corresponding to Step 4 is a control for completion of step 2, and should be close to zero. The peak designated "S" is the substrate peak representing the starting point of Pyrosequencing reactions.

- Dissolve enzyme mix from the SNP kit in 620 μl H2O and add to the "E"chamber of the Pyrosequencing cartridge.
- Dissolve Substrate mix in 620 μl and add to the "S" chamber of the Pyrosequencing cartridge
- Mix 50 μl dATPaS with 50 μl H2O and add to "A" chamber of the Pyrosequencing cartridge.
- Mix 50 μl dTTP with 50 μl H2O and add to the "T" chamber of the Pyrosequencing cartridge.
- Mix 50 μl dCTP with 50 μl dGTP and add to the "C"chamber of the Pyrosequencing cartridge.
- Add 100 μl H_2O to the "G" chamber of the Pyrosequencing cartridge.
- Place the cartridge in place and start the run.
- A typical run should look like (Figure 4).

III. Data Analysis:
- When the run is finished, go to "data analysis mode"
- Analyze data in "AQ" mode.
- Collect the peak height results from the "peak height" menu.
- Calculate the ratio of (peak "C")/(peak "A"). This indicates the *Hpa*II/*Eco*RI for digestion mix A and *Msp*I/*Eco*RI for digestion mix B.
- Calculate *Hpa*II/*Msp*I for each sample as (*Hpa*II/*Eco*RI)/(*Msp*I/*Eco*RI).

3.2 FLOURESCENCE CORRELATION SPECTROSCOPY

The principles of fluorescence correlation spectroscopy (FCS) was developed around 30 years ago by Magde et al, who for the first time applied this method to measure diffusion and chemical dynamics of DNA-intercalating drugs (Koppel et al., 1976; Magde et al., 1974). The assay was then improved over time, both concerning the theoretical basis and development of the instruments. However, it was not until the advent of high-quality optics and photon-detection technology in the 1990s that the real power of the technique was finally realized. The final breakthrough was achieved at Karolinska Institutet, Stockholm when Rudolf Rigler and his colleagues combined the principles of the FCS technique with confocal detection (Reviewed by Vucojević et al. (Vukojevic et al., 2005)). The original technique is now further developed to several sub-techniques such as fluorescence photobleaching recovery, multiphoton FCS and multiphoton microscopy. FCS can be summarized as a sensitive single molecule detection assay to study fast dynamic interaction processes in living cells. FCS has both the temporal and spatial resolution to determine the properties of ligand–receptor complexes within live cells. In addition, it has the ability to quantify the concentrations of both free and bound ligand, which is a prerequisite to the determination of potency and affinity of the ligands. It also has a resolution high enough to localize measurements to areas of the cell as small as some membrane microdomains.

The technique of FCS is based on measuring fluctuations in fluorescence intensity as fluorescently labelled particles diffuse through a small defined detection volume, which is approximately 0.25 fl in size (roughly the same volume as a mitochondrion). Statistical calculation of these fluctuations using autocorrelation analysis gives information about both the diffusion coefficient of the fluorescent particles and their concentration.

FCS uses a small detection volume which is achieved by focussing laser beams to a diffraction-limited spot using a microscope objective lens with a high numerical aperture. By positioning of a pinhole in the confocal image plane, a Gaussian-shaped detection volume of approximately 0.25–0.5 fl is created. As fluorescent molecules diffuse through this volume, they are excited by laser and the emitted photons are detected in a time-correlated manner using a single photon-counting device. Over time, this leads to fluctuations in the detected fluorescent intensity which is caused by translational diffusion of the fluorescent species through the volume, by photo-physical events within the fluorophore or by changes in its fluorescent properties, such as quantum yield or interstate conversions. Based on this observation and by considering the shape and dimensions of the detection volume, the absolute concentration of the detected species can be calculated readily. 'N' and 'τ_D' are obtained from the autocorrelation function by non-linear curve-fitting of the data to an appropriate biophysical model, with the choice of model (e.g. 2D vs 3D, free vs anomalous diffusion) being crucial for the correct quantification and interpretation of the data which is an important aspect of FCS analysis (Vukojevic et al., 2005). With modern hardware and detection technology, FCS can provide useful information over a large range of fluctuation times (1 μs–500 ms) and has thus led to its application in
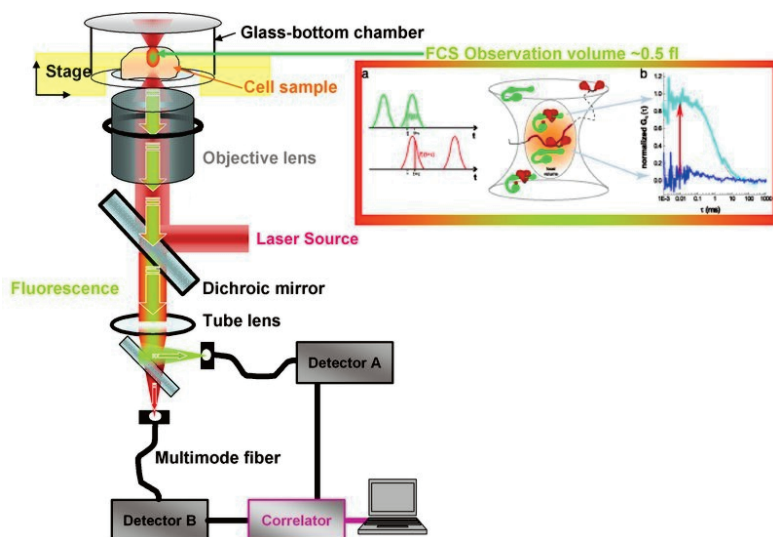
Figure 5. Schematic design of FCS instrumentation: A parallel laser beam for one-photon excitation or two-photon excitation is coupled into the microscope (red beam path) via a dichroic mirror epi-illuminating the objective lens. Fluorescence is generated in a small, diffraction-limited focal spot which serves as the FCS observation volume. Fluorescence light is collected via the same objective lens, passed through the dichroic mirror (green fluorescence path), and finally projected onto a high-sensitivity detector (single-photon counting module). The digital photon signal is sent to a hardware correlator. Respective correlation curves are displayed and stored on a computer for subsequent analysis. (Adapted from Nature Methods, 2007, 4:963-973.)

several fields, ranging from photo-physical investigations and chemical reaction monitoring to protein diffusion in live cells (Briddon & Hill, 2007). A schematic illustration of FCS instrumentation is presented in Figure 5.

In a very interesting review, different applications of FCS analysis in DNA and RNA studies at single molecule level are very nicely presented by Dr. K.Greulich (Greulich, 2005). A broad range of applications are discussed in the paper including folding of RNA and DNA, chromatin assembly, DNA-protein complexes, DNA replication and DNA repair, transcription and RNA polymerase activity. A step-by-step guide to the application of FCS in cellular systems has been recently published, where FCS methods are discussed and practical points provided for minimizing artifacts, optimizing measurement conditions and obtaining parameter values. This paper provides useful and relevant information for researchers who are willing to apply FCS technique in their research (Kim et al., 2007). Application of confocal microscopy techniques in epigenetic research is very well illustrated by Schermelle *et al.*, where they have analysed the interaction of DNA methylation inhibitors and DNMT1 in live cells (Schermelleh et al., 2005).

3.2.1 Analysis of DNA methyltransferases by FCS

One aim of this study was to analyze DNMTs properties in live cells before and after treatment with the HDAC inhibitor TSA. Fluorescent-tagged DNMTs were expressed in Hep3B cells and FCS analyses were performed after 24 hrs of TSA treatment.

Instrumental setup: Studies were carried out using the confocal microscope unit at Center for Molecular Medicine at Karolinska university Hospital. This unit consists of a unique system for FCS/CLSM analysis with special design by the Carl Zeiss company (Carl Zeiss, Jena, Germany). All mobility measurements for DNMTs were performed on the ConfoCor3 instrument consisting of an epifluorescence inverted microscope (Axiovert 200 M); a VIS-laser module comprising the Ar/ArKr (458, 477, 488 and 514 nm), HeNe 543 nm and HeNe 633 nm lasers; scanning module LSM 510 META modified to enable detection using silicon avalanche photodiodes (SPCM-AQR-1X; PerkinElmer, USA). The C-Apochromat 40×/1.2 W UV-VIS-IR objective was used throughout the study.

Fluorescence intensity fluctuations were recorded in arrays of 10 consecutive measurements, 10 seconds each. The non-linear least-square fitting of the autocorrelation curve was performed using the Levenberg-Marquardt algorithm. The FCS data were evaluated by residuals analysis as described before (Vukojevic et al., 2005).

FCS Results: Assuming that a fraction of investigated proteins is freely moving in the nucleus, whereas the other fraction is involved in protein complexes bound to chromatin, the experimental autocorrelation curves were analyzed using the following model:

$$G(\tau) = 1 + \frac{1}{N} \cdot \left(\frac{1 - f_2}{\left(1 + \frac{\tau}{\tau_{D1}}\right)\sqrt{1 + \frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_{D1}}}} + \frac{f_2}{\left(1 + \frac{\tau}{\tau_{D2}}\right)} \right) \cdot \left[1 + \frac{T}{1 - T}\exp(-\frac{\tau}{\tau_T}) \right]$$

In this equation, N is the average number of particles in the observation volume element; τ_{D1} is the diffusion time of the free protein; τ_{D2} is the diffusion time of the bound protein; f_2 is the fraction of the bound protein; T is the average equilibrium fraction of molecules in the triplet state; τ_T is the triplet correlation time, related to rate constants for intersystem crossing and the triplet decay. Based on this analysis method, FCS analysis revealed significant mobility changes for DNMT1, DNMT3a and DNMT3b after TSA treatments.

Overall results of the FCS study are presented in Table 1. Based on FCS analysis, it was shown that TSA treatment appears to increase DNMT3a binding to the nuclear components. In control cells a large fraction (85%) of unbound DNMT3a molecules was observed, whereas a rather small fraction was bound and associated into small aggregates. Upon TSA treatment, the mobility of DNMT3a decreased and pronounced aggregation of the protein was observed by Confocal laser scaning microscope.

Analysis of the autocorrelation curves showed a TSA-induced reduction in free DNMT3a, whereas the slowly moving fractions increased and even a third component with rather slow diffusion could be observed (Table 1).

TSA treatment appears to induce similar effects on DNMT3b as on DNMT3a, but to a somewhat lesser extent. FCS analysis indicated that DNMT3b binding to the chromatin is similar to DNMT3a. TSA treatment slowed DNMT3b mobility of the bound fraction indicating formation of larger aggregates (Table 1).

In contrast to the effect on DNMT3a and DNMT3b, TSA treatment seems to induce opposite effects on DNMT1, increasing its mobility in the nucleus. FCS analysis indicates that in control cells DNMT1 is less mobile, as compared to DNMT3a or DNMT3b. TSA treatment increased DNMT1 mobility, suggesting that DNMT1 binding to the nuclear components is reduced by the TSA treatment.

Table 1: FCS analysis of TSA treatment on DNMTs in Hep3B cells

	Fractions in untreated cells	Fractions in treated cells	Change in mobility
DNMT3a	$f_1 = 0.85 \pm 0.05$, $\tau_{D1} = (250 \pm 50)$ µs $f_2 = 0.15 \pm 0.05$; $\tau_{D2} = (5 \pm 2)$ ms	$f_1 = 0.50 \pm 0.05$, $\tau_{D1} = (250 \pm 50)$ µs $f_2 = 0.35 \pm 0.05$, $\tau_{D2} = (5 \pm 2)$ ms $f_3 = 0.15 \pm 0.05$; $\tau_{D3} = (20 \pm 5)$ ms	Decreased
DNMT3b	$f_1 = 0.75 \pm 0.05$, $\tau_{D1} = (250 \pm 50)$ µs $f_2 = 0.25 \pm 0.05$, $\tau_{D2} = (5 \pm 2)$ ms	$f_1 = 0.70 \pm 0.05$, $\tau_{D1} = (250 \pm 50)$ µs $f_2 = 0.30 \pm 0.05$, $\tau_{D2} = (20 \pm 5)$ ms	Decreased
DNMT1	$f_1 = 0.65 \pm 0.05$, $\tau_{D1} = (250 \pm 50)$ $f_2 = 0.35 \pm 0.05$, $\tau_{D2} = (20 \pm 5)$ ms	$f_1 = 0.70 \pm 0.05$, $\tau_{D1} = (100 \pm 20)$ µs $f_2 = 0.30 \pm 0.05$, $\tau_{D2} = (9 \pm 2)$ ms	Increased

4   RESULTS AND DISCUSSION

4.1   PAPER I:

LUMA: A high throughput method to the analysis of genomic DNA methylation

Conclusion: A novel assay to estimate genome-wide DNA methylation was developed referred to as LUminometric Methylation Assay (LUMA). In this method, DNA cleavage by methylation-sensitive restriction enzymes is coupled to polymerase extension assay by Pyrosequencing™. The method is quantitative, highly reproducible and easy to scale up. LUMA was proposed as a useful method to analyze genome-wide DNA methylation for a variety of physiological and pathological conditions including etiologic, diagnostic and prognostic aspects of cancer.

Since available global methylation assays were not suitable for high throughput studies we aimed to develop a high through-put technique to measure genomic DNA methylation at the global level under different physiological and pathological conditions. LUMA was thus developed as a new quantitative DNA methylation assay suitable as a routine method in DNA methylation studies of large numbers of samples. Since LUMA is performed in a 96-well plate format, it can be readily scaled up and automated. Analysis of DNA methylation using LUMA is based on DNA cleavage with methylation sensitive restriction enzymes followed by a luminometric polymerase extension assay using Pyrosequencing™ platform.

The method is highly sensitive and versatile, does not require base modifications and permits accurate studies of DNA methylation in 6 hours. In addition, the assay requires only 200–500 ng of genomic DNA which is very important when working with clinical samples. Processing of different amounts of pBlueScript plasmid DNA, however, showed that the assay is linear and reproducible over more than 10-fold difference in DNA quantity (125–2,000 ng pBlueScript plasmid). To assure complete cleavage, 10- to 20-fold excess of restriction enzymes is used. The accuracy and linearity of LUMA were verified by *in vitro* methylated lambda DNA. Methylated and unmethylated Lambda DNA were mixed in different proportions ranging from 0 to 100%. LUMA data showed a good inverse correlation between the methylation level and *Hpa*II/*Msp*I ratio ($R^2 = 0.98$). In addition, DNA methylation levels were assessed by LUMA in cell lines treated with a DNA methyltransferase inhibitor (5-AzaCytidine). A dose dependent and highly reproducible DNA hypomethylation was found after 5-AzaCytidine treatment. Therefore, LUMA may provide a new method for drug screening to evaluate potential DNA demethylating agents.

LUMA is also unique in having an internal control for DNA input. Normalization of genomic DNA input has been recognized as a challenging problem in most DNA methylation assays based on restriction enzyme cleavage. LUMA circumvent this problem by including *Eco*RI as an internal indicator for DNA input and digestion. The exact determination of DNA concentration is unnecessary when *Eco*RI is used as an

internal control. According to LUMA data, the *Hpa*II/*Eco*RI ratio can be used instead of the *Hpa*II/*Msp*I ratio, which simplifies the assay. In addition, the amount of DNA needed is reduced in half, and the sensitivity is increased since the *Hpa*II/*Eco*RI ratio can vary in the range of 0.0–3.0 for human samples, while the *Hpa*II/*MSP*I range is 0.0–1.0. By incorporation of *Eco*RI, sites for *Hpa*II and *Msp*I can be investigated separately, thus LUMA is also suitable for analysis of DNA methylation in plants where most of the methylation has been reported to be non-CpG methylation which can be investigated by *Msp*I cutting (Kutueva et al., 1996).

Nevertheless, it is important to note that by using the *HpaII*/*MSPI* isoschizomers, all CpG sites in a genome are not covered. The use *EcoRI*, however, makes LUMA a flexible assay, and various methylation-sensitive restriction enzymes can be used if they leave suitable 5′-overhangs. LUMA has been used in several studies and proven to be useful for the analysis of DNA methylation in different pathological and physiological conditions.

4.2   PAPER II:

Impact of inflammation on epigenetic DNA methylation – a novel risk factor for cardiovascular disease?

Conclusions: It was shown, for the first time, that global DNA hypermethylation is correlated with systemic inflammation in CKD patients. Moreover, it was demonstrated that there is an association between global DNA hypermethylation and outcome in patients starting dialysis treatment. The study revealed a correlation between genomic DNA hypermethylation and cardiovascular disease. Therefore DNA methylation could become useful as a novel prognostic marker in these patients. In addition, it can also open up new therapeutic options for treatment of cardiovascular disease.

The aim of this study was to evaluate peripheral blood cell DNA methylation (defined as *Hpa*II/*Msp*I ratios in LUMA) in clinically well-defined cohorts of renal disease patients. By this approach, it was possible to investigate the association between renal function, hyperhomocysteinaemia, inflammation and aberrant DNA methylation. In addition, the independent impact of DNA methylation on patient survival both in all-cause and cardiovascular mortality was assessed by multivariate regression analysis in incident patients starting renal replacement therapy.

During this study, DNA methylation was analyzed in peripheral blood leucocytes from three different groups of chronic kidney disease (CKD) populations (37 CKD stages 3 and 4 patients, 98 CKD stage 5 patients and 20 prevalent haemodialysis patients) and 36 healthy control subjects. Clinical characteristics (diabetes mellitus, nutritional status and presence of clinical CVD), inflammation and oxidative stress biomarkers, homocysteine and global DNA methylation in peripheral blood leucocytes (defined as *Hpa*II/*Msp*I ratio by LUMA) were evaluated. CKD stage 5 patients (N = 98) starting dialysis treatment were followed for a period of 36 ± 2 months.
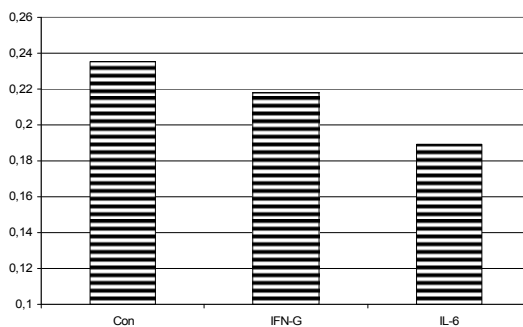
Figuer 6. Effect of IFN-gama and IL-6 on DNA methylation of blood lymphocytes. Blood lymphocytes from a healthy donor was treated for 24 hrs by IFN-gamma or IL-6 followed by extraction of genomic DNA and LUMA. The *Hpa*II/*Msp*I values are presented.

Results from this study revealed a strong association between surrogate markers of inflammation and DNA methylation in peripheral blood leucocytes. Whereas CKD patients without signs of overt inflammation had *Hpa*II/*Msp*I ratios comparable with age- and gender-matched controls, inflamed CKD stage 5 patients and prevalent HD patients exhibited DNA hypermethylation. A possible explanation is that inflammation may cause aberrant DNA methylation and a significant increase in global DNA methylation was actually observed when human lymphocytes were treated with IL-6 (1ng mL^{-1}) for 24h (Fig 6). It was suggested that persistent inflammation may cause aberrant DNA methylation, which via an inhibition of SOCS further exaggerates IL-6 signalling.

A novel finding of this study was the association between DNA hypermethylation on both all-cause and cardiovascular mortality in incident CKD stage 5 patients (Figure 7). As ageing and atherosclerosis are considered major independent risk factors for cardiovascular morbidity and mortality both in the nonrenal and renal patient population, it was found remarkable that the association between CVD and mortality in univariate analysis was lost when we adjusted for the impact of DNA methylation. This observation may suggest that aberrant DNA methylation is a determinant, or marker, of atherosclerotic CVD in this population. Consequently, epigenetic modifications may be more important in the pathogenesis of atherosclerosis than genetic polymorphisms. It was speculated that aberrant DNA methylation may contribute to accelerated atherosclerosis through upregulation of atherosclerosis-susceptible and down-regulation of atherosclerosis-protective genes. Taken together, the results of the present and previous studies indicate that aberrant DNA methylation is an important process through which novel factors for atherosclerosis, including pro-inflammatory cytokines and hyperhomocysteinaemia, affect the function of the genome.
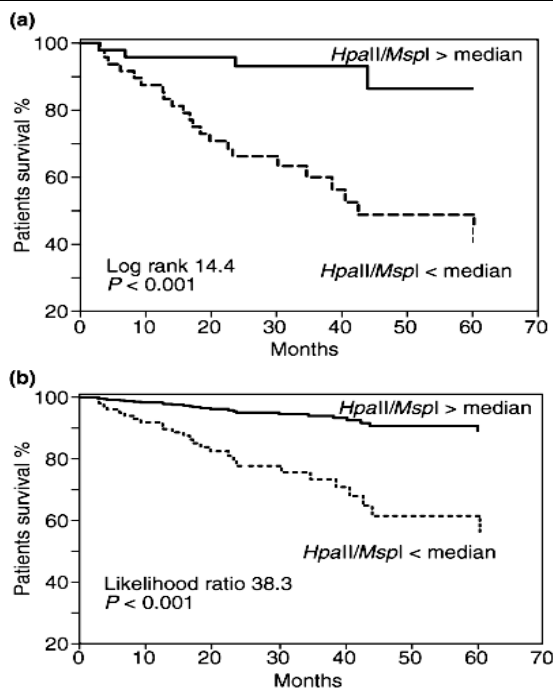
Figure 7. Unadjusted (a) and adjusted (b) all-cause mortality in 98 CKD stage 5 patients starting renal replacement therapy. A significant correlation between genomic DNA methylation and survival is evident.

4.3 PAPER III:

Genomic DNA hypomethylation by HDAC inhibition involves DNMT1 release from nuclear components

Conclusion: In this paper changes in DNA methylation by TrichostatinA (TSA) treatment was investigated in Hep3B cells. To address this, the effect of TSA on DNA methylation was studied at gene specific and global levels, as well as protein levels of DNA methyltransferases and histone deacetylases. It was shown that TSA treatment results in genomic hypomethylation by affecting DNA methyltransferases both at the protein level and by changes in the DNMTs mobility in the nucleus. These data are interesting since Hep3B is a p53 negative cell line. Therefore, HDAC inhibitors are promising drugs to induce p21^{waf1} and other epigenetically silenced tumor suppressor genes in p53 negative tumors. This is an important aspect of HDACI drugs which should be investigated in more details.

In this study the effect of TSA treatment on DNA methylation in Hep3B cells was studied. Effect of TSA treatment on HDAC1, HDAC2 and HDAC3 was investigated by Western blotting at first step. Although overall expression of HDAC1 did not change significantly after TSA treatment, there was an apparent translocation of the protein from the nucleus to the cytoplasm. The expression level of the HDAC2 protein did not change after TSA treatment, however, a lower band appeared in both fractions in Western blots, indicating a degradation of HDAC2. The HDAC3 protein showed no

significant changes after TSA treatment, neither by degradation, nor in cellular relocalization.

In the next step, the expression levels of DNMT1, DNMT3a and DNMT3b in Hep3B cells were investigated after TSA treatment. A significant decrease in DNMT1 and DNMT3a levels after 24 hours TSA treatment was revealed. These data were similar to several findings from different groups who have reported decreased DNMTs expression, after HDACI treatment. There was no conclusive data regarding DNMT3b since DNMT3b protein in Hep3B cells was very low.

Next, it was investigated whether reduced DNMT1 and DNMT3A levels are correlated to an effect on genomic DNA methylation. Global genomic DNA methylation was measured by two different methods, LUMA and Nearest Neighbour Analysis (NNA). A general decrease in genomic DNA methylation was evident by both methods. LUMA revealed a dose dependent decrease in genomic DNA methylation after TSA treatment. The *Hpa*II/EcoRI ratio was 1.03 in untreated cells and increased to 1.63 after treatment with 800 nM of TSA indicating considerable loss of methylation at CCGG sequences. NNA also revealed a 15% decrease in genomic methylated cytosine after TSA treatment.

To investigate the genomic hypomethylation at a gene specific level, seven gene promoters were analysed by methylation specific PCR (MS-PCR). Four of these (E-cad, CDH13, DAPK and RASSF1) were significantly demethylated after TSA treatment for 24 hours, while there was no change in the promoters of the remaining three genes. These data indicated that genomic hypomethylation by TSA treatment is indeed a selective phenomenon which occurs at specific genomic loci. This selective promoter hypomethylation can explain the controversial results by different groups where some groups have found hypomethylation following TSA treatment while others have reported no changes.

In addition to changes in expression levels, the effect of TSA on DNMTs intracellular mobility was investigated. Florescence correlation spectroscopy was applied to investigate real time interactions between DNMTs and nuclear compartments in live cells at single molecule level. It was shown that TSA treatment has a direct effect on their nuclear mobility. The mobility of DNMT3a and DNMT3b was attenuated after TSA treatment which means there are more interactions between these enzymes and other nuclear complexes after treatment. In contrast, DNMT1 mobility was increased upon TSA treatment, suggesting that DNMT1 is dissociated from some nuclear complexes. Release of DNMT may be explained in three ways: by DNMT1 acetylation per se or by HSP90 degradation, or by induction of p21^{waf1}.

4.4   PAPER IV:

Human cytomegalovirus causes alteration in DNA methylation by influencing DNA-methyltransferase intracellular localization

Conclusion: In this study, the impact of CMV infection on the host cell DNA methylation machinery was investigated. Using several experimental models it was shown that CMV infection alters the DNA-methylation machinery of the host cell leading to profound global DNA hypomethylation. It was revealed that intracellular relocalization of DNMT1 is changed upon CMV infection. It was also suggested that defects in the DNMT1 function render non-permissive cells to be permissive to CMV infection. These findings may also help to understand the etiology of CMV pathogenesis, develop new therapy, as well as guiding research into new areas of DNA methylation control to discover new epigenetic drugs.

To examine the CMV infection effects on the global state of DNA methylation, genomic DNA from MRC-5 cells infected with the AD169 and VR1814 strains revealed a profound hypomethylation following CMV infection. This was investigated by two different assays for global DNA methylation, LUMA and NNA. The LUMA assay revealed an apparent 30% decrease in global DNA-methylation at 3 dpi by VR1814 strain, while NNA showed a 36% decrease. When the AD169 strain was used, these numbers were 10% and 8%, respectively despite equal infection levels. Inhibition of viral replication by Foscavir treatment resulted in no significant change in DNA methylation, indicating that the main source of hypomethylated DNA is the viral genome.

In the next step, to further investigate the mechanism of CMV induced hypomethylation, the expression pattern of DNMTs in infected cells was analyzed by immuno-fluorescence staining. Using confocal microscopy, it was revealed that the interference of the host cell methylation involves a shift in intracellular localization of DNA methyltransferases, DNMT1 and DNMT3b, from the nucleus to the cytoplasm. This would provide the chance for the virus to express viral proteins and replicate in a DNA methylation free environment and avoid formation of suppressing complexes on viral promoters. By Foscavir treatment, it was also shown that this process was most likely accomplished in two steps, where DNMT1 is first concentrated in nuclear bodies and in the second step is exported from the nucleus to the cytoplasm or alternatively degraded in nucleus while synthesized in the cytoplasm. The appearance of cytoplasmatic DNMT1 in infected macrophages as well as in intestinal epithelial cells of IBD patients demonstrates that these observations indeed have clinical relevance.

In addition, Western blot analysis of the IE72 and IE86 expression after infection showed that infection by AD169 only produced IE72 while the VR1814 strain expressed both IE72 and IE86 at relatively equal amounts. High expression levels of IE86 may be responsible for the more hypomethylated DNA following VR1814 infection.

It was also investigated whether CMV infection could affect DNA methylation on specific loci in the genome of the host cell by two array based DNA methylation profiling systems CHARM and Illumina (Illumina infinium, Illumina, SanDiego, CA). Data showed that, from a cell population point of view, there was no methylation changes in any specific genomic locus following infection.

   To further address the inhibitory effect of DNMTs on CMV infection, HCT cell lines lacking the DNMT1, DNMT3b, or both, was infected by CMV AD169. It was observed that AD169 could not infect HCT116wt cells while the HCT-double knock-out cells were permissive to AD169. This implies a role for DNA-methylation in CMV permissiveness.

5 CONCLUDING REMARKS

Cytosine methylation is an important epigenetic mechanism to control the vertebrate genome. DNA methylation occurs mainly at cytosines located in CpG dinucleotides in the mammalian genome. CpG-rich regions of 0.5–4 kb in length, known as CpG islands, are found in many gene promoters and are generally protected from methylation.

Methylation of CpG islands in promoter regions is associated with silencing of gene expression, and participate in the regulation of gene expression. Such gene silencing can be observed during differentiation and in the inactivation of one X-chromosome in female mammals. Methylation of specific CpGs is also an important mechanism in mono-allelic silencing of imprinted genes.

Changes in genomic DNA methylation have also been demonstrated in aging, auto-immune diseases and cancer. Aberrant DNA methylation patterns, involving both hypomethylation and hypermethylation, have been observed in cancer and other pathological processes. Hypermethylation of tumor suppressor gene promoters is an important factor for tumor development by silencing of gene expression. On the other hand, genome-wide hypomethylation, especially in centromeric regions, results in genome instability. Therefore, analysis of DNA methylation at the genome-wide level is expected to provide basic information of disease processes as well as molecular markers for diagnostic and prognostic purposes.

The aim in this study was to shed some light on the DNA methylation process and how it is controlled. As a first step LUMA was developed as a high trough-put global DNA methylation assay (Paper I). To facilitate application of LUMA by other researchers, a practical step-by-step protocol was later published by our group entitled as "Using LUMA: a Luminometric-based assay for global DNA-methylation" (Karimi et al., 2006). Since the assay is cheap and quick, it has been used as a screening tool in several projects to reveal DNA methylation changes in different physiological and pathological conditions. Although there were no methylation changes in some conditions which was interesting by itself, significant methylation changes was found in several models which resulted in interesting findings in the field. It was shown for the first time that DNA hypermethylation in blood leucocytes is correlated with systemic inflammation in CKD patients (Paper II). Furthermore, DNA hypermethylation was significantly correlated with survival rates s in end stage renal patients.

In another study we showed that LUMA can be applied in research on epigenetic drugs (Paper III). In this study, the effect of TSA on DNA methylation was studied. The study revealed a significant DNA hypomethylation after TSA treatment in Hep3B cells, which was also associated with changes in DNMTs mobility. These results were also confirmed in a collaborative study that has been published under the title "Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA

demethylation in human cancer cell lines" (Ou et al., 2007). Both studies identified decreased DNA methylation after TSA treatment in different cancer cell lines.

Application of LUMA in pharmacological studies was further demonstrated through another study published as "Hypomethylation and apoptosis in 5-azacytidine-treated myeloid cells" where DNA hypomethylation by 5-AzaCytidine treatment was investigated on myeloid cells (Khan et al., 2008). It was shown that DNA hypomethylation is observed only in apoptotic cells following 5-AzaCytidine treatment. Therefore LUMA is an excellent assay in pharmacological research and drug screening studies, considering that high trough-put screening methods for epigenetic drugs are not so common.

Another interesting field in epigenetic research is the interaction between the epigenetic machinery and viral infections, given that only certain types of cells are permissive to each individual virus. In this study the interaction between CMV infection and DNA methylation machinery was investigated (Paper IV). It was shown that there is a significant hypomethylation in viral infected cells. The observed hypomethylation was accompanied by relocation of DNMT1 to the cytoplasm. This is a very important mechanism in epigenetic control which should be investigated more. It was also suggested that defects in the DNMT1 function render non-permissive cells permissive to CMV infection.

Aberrant DNA methylation is a common finding in cancer, that is generally regarded as involving genome-wide hypomethylation together with hypermethylation of CGI promoters in tumors. To further evaluate this hypothesis, samples of several different types of cancer were analyzed by LUMA. Although there was variations in DNA methylation in different samples, there was no general hypomethylation in tumor samples and no correlation between DNA methylation and malignancy was observed (Geli et al., 2008; Lee et al., 2008). Therefore, based on these data it was suggested that there is no general hypomethylation in cancer samples at least in the samples analyzed in this study.

In another study, brain DNA methylation levels were analyzed by LUMA in a obese hybrid model resulted from an interspecific hybridization in the genus Mus was analyzed by LUMA. It was shown that increased global DNA methylation level in brain is associated with obesity (Singh et al., 2008). This hypermethylation was also correlated to the up-regulation of DNMT3b in the brain.

In conclusion, DNA methylation changes were explored in different physiological and pathological condition and the interaction between DNA methylation and other cellular processes was monitored. The results indicated that the DNA methylation is a very dynamic process that is finely tuned by different cellular and environmental factors, most of which are presently unknown. Based on the combined results of this study, the following facts are suggested as part of current knowledge about DNA methylation:

- Cellular DNA methylation is a dynamic process and is not fixed.

- DNA methylation levels are changed based on cellular responses to environmental factors.
- DNA methylation can be controlled by signaling pathways.
- DNMT1 can be relocated to cytoplasm in some conditions, such as CMV viral infection.
- HDAC inhibitors would decrease DNA methylation levels.
- Global hypomethylation is not a necessary characteristic of cancer.
- Inflammation may lead to DNA hyper-methylation.
- DNA hypermethylation is correlated with cardiovascular disease.
- CMV infection can inhibit the DNA methylation machinery.
- DNA methylation changes may be associated with obesity.

6 FUTURE PERSPECTIVES

The main aim of this study has been to improve our knowledge about DNA methylation. Several interesting findings were revealed whish in turn has generated novel questions that are important to study further to gain a better understanding of this field.

6.1 PAPER I:

In this paper we have presented LUMA as a novel assay to study global DNA methylation assay. The following aspects related to LUMA would be important to pursue:

- LUMA is presented based on *Hpa*II and *Msp*I assay. It should be noted that by using the *Hpa*II/*MSP*I isoschizomers, all CpG sites in a genome will not be covered since these enzymes only recognize the CCGG sequence. LUMA is however, a very flexible assay that may be modified to utilize other methylation-sensitive restriction enzymes provided that they leave suitable 5'-overhangs. Pilot experiments suggest that other methylation sensitive enzyme combinations recognizing other CG-containing sequences could be used in LUMA to increase the sensitivity and specificity of the method.

- DNA demethylase assay: DNA demethylation is currently an important issue in the DNA methylation field. The main problem in DNA demethylation studies is the lack of a suitable assay to monitor the DNA demethylase activity. It is possible to design a demethylation assay based on LUMA to apply in this field.

- The control of DNA methylation: Some of the unpublished data obtained in this study have suggested out the possibility that cellular DNA methylation level might be controlled by signaling pathways. For instance treatment by IL-6 was found to increase the total DNA methylation level. MAP kinase pathway and TGF-beta signaling are the possible candidates to be investigated. This will help to get a better view on how DNA methylation is controlled during development and cancer.

- Since Pyrosequencing instruments are not generally available to all research labs, it would be interesting to apply LUMA to other platforms. Preliminary data indicate that a 96-well luminometer equipped with an automatic dispenser can substitute the Pyrosequenceing platform. It would also be interesting to use more sensitive systems to reduce the amount of genomic DNA needed in the assay.

6.2 PAPER II

During this study, it was found that global DNA hypermethylation is correlated with systemic inflammation in CKD patients. Moreover, an association between global DNA hypermethylation and survival rate was demonstrated in patients starting dialysis treatment. The main cause of death in this group of patients is cardiovascular disease.

- Based on the data presented in this study, DNA methylation level should be evaluated as a possible diagnostic marker in large and independent cohorts of CKD patients. If the association is well confirmed development of DNA methylation measurements for clinical application as a novel prognostic marker would be important. The relative stability of the necessary samples and the robustness of the methodology speaks in favor of such a development.

- Since inflammed CKD 5 patients had a higher DNA methylation levels it is important to determine whether this is a cause or a consequence of their cardiovascular disease. In the first situation DNA methylation would be an important aspect to study in pathogenesis of cardiovascular disease.

- A putative correlation between DNA hypermethylation and development of cardiovascular disease would open up new therapeutic options for treatment. There are several clinically approved DNA methylation inhibitors available as epigenetic drugs. These drugs could become candidates for evaluation as preventive treatment in CDK patients. In addition, HDAC inhibitors, alone or in combination with DNA metylation inhibitors, are other alternatives for epigenetic treatment trials.

- It is important to fractionate the uremic sera from CKD patients and test different fractions in an epithelial cell culture system to uncover factors which are responsible for DNA hypermethylation.

- Possible correlation between DNA hypermethylation and inflammation and cardiovascular disease should be investigated in other chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and auto-immune diseases.

- Our findings in CKD 5 patients support the concept that hyper-homocysteinaemia may be associated with global hypomethylation. As homocyctein is an inhibitor of DNA methylation cycle, it is important to evaluate if inflammation and hyperhomocysteinaemia have opposing effects on the DNA methylation process.

- Further studies evaluating DNA methylation in response to various nutritional and pharmacological anti-inflammatory treatment strategies are needed to resolve the complex interactions between inflammation and aberrant DNA methylation. It is interesting that procainamide which has long been used as a treatment for cardiovascular disease, has been reported to be a specific inhibitor of DNA methylation (Lee et al., 2005).

- The present association between DNA hypermethylation and inflammation and cardiovascular disease is based on observation at the global genomic level. It would be even more interesting to study DNA methylation of individual genes , particularly in genes that are important in the immune system or cardiovascular system. This can provide more information about the link between DNA methylation and cardiovascular disease.

6.3 PAPER III:

In this study, changes in DNA methylation induced by Trichostatin A (TSA) treatment were investigated in Hep3b cells. It was shown that TSA treatment results in

genomic hypomethylation by affecting DNA methyltransferases both at the protein expression level and by altering the DNMT mobility in the nucleus. This is an important aspect of HDACi drugs that should be investigated in more details.

- The results presented in this paper are based on analyses with TSA. It is therefore necessary to perform similar studies with additional HDAC inhibitors to determine whether DNA hypomethylation is a general effect of HDAC inhibitors or not.
- The mobility of DNMTs in the nucleus was found to change following TSA treatment, suggesting that TSA treatment has affected the interaction between DNMTs and other nuclear factors. This possibility could be further investigated using a proteomics approach with co-immuno precipitation to define the nuclear factors which are interacting with DNMTs before and after TSA treantment. At present very little information is available about proteins interacting with DNMTs.
- Post translation modifications of DNMTs: Since no mechanistic data are available it would be interesting to analyze post translational modifications of DNMTs by using proteomic techniques before and after TSA treatment. This would provide more information regarding how DNMTs are controlled by chemical modifications, especially acetylation *per se.*

6.4 PAPER IV

In this study, the impact of CMV infection on the DNA methylation machinery of the host cell was investigated. It was shown that CMV infection alters the DNA-methylation machinery of the host cell leading to profound global DNA hypomethylation. It was revealed that DNMT1 relocalizes following infection by CMV. It was also suggested that DNMT1 deletion rendered non-permissive cells to be permissive to CMV infection.

- The main focus in this study was on DNA methylation changes. However, it is important to study other epigenetic markers in CMV infected cells to clarify whether other epigenetic changes also occur in CMV infected cells.
- Functional study of CMV proteins: It is also important to evaluate which CMV encoded protein is responsible for the interaction with DNMT1. Gene knock out and SiRNA techniques would be very useful for these analyses.
- Possible alterations of DNA methylation following other other viral infections: Here, inhibition of DNA methylation is reported in association with CMV infection. It is also interesting to study DNA methylation machinery in other viral infections, especially other viruses in herpesviridea family, which has a similar genomic structure to CMV.
- Structural studies on DNMT1: It was shown that DNMT1 is translocated to the cytoplasm following CMV infections. Post translational modifications of DNMT1 and DNMT1-bound proteins should be investigated by proteomics to reveal proteins involved in cytoplasmic re-localization of DNMT1 after CMV infection.

- Cytoplasmic localization of DNMT1: Here cytoplasmic localization of DNMT1 was reported as part of CMV pathogenesis. However, it is also possible that this is a general phenomenon that occurs in other diseases as well. Therefore, the cellular localization of DNMT1 should be investigated in other pathological conditions as well.

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