DIPLOMARBEIT

EPIGENETIC INFLUENCE OF SPECIFIED FOOD COMPONENTS ON DNA METHYLATION, HISTONE ACETYLATION, AND GENE EXPRESSION OF INTERLEUKIN 8

Epigenetischer Einfluss von bestimmten Nahrungsinhaltsstoffen auf die DNA Methylierung, Histonacetylierung und Genexpression von Interleukin 8

angestrebter akademischer Grad

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Verfasserin:	Eva Aumüller
Matrikel-Nummer:	a0100555
Studienrichtung /Studienzweig (lt. Studienblatt):	A474 Ernährungswissenschaften
Betreuer:	Univ.-Doz. Dr. Alexander G. Haslberger

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BPA: bisphenol A

BSP: bisulfite sequencing PCR

cDNA: complementary DNA

DNMT: DNA-methyltransferase

dNTP: deoxynucleotidtriphosphat

EGCG: epigallocatechin-3-gallate

ELISA: enzyme-linked immunosorbent assay

ERα: estrogen receptor alpha

FOXO: fork head transcription factor

HAT: histone acetyltransferase

HDAC: histone deacetylase

HRP-streptavidin: horseradish peroxidase-streptavidin

IGF2: insulin-like growth factor 2

IL-8: interleukin 8

MBD: methyl-CpG-binding domain protein

miRNA: microRNA

MSP: methylation specific PCR

MTHFR: methylenetetrahydrofolate reductase

NF-κB: nuclear factor kappa B

PARP: pol-(ADP-ribose)polymerase

PCR: polymerase chain reaction

PI3K: phosphatidylinositol 3-kinase

PTEN: phosphatase and tensin homolog

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SIR: silent information regulator

TNF-α: tumor necrosis factor alpha

TSA: trichostatin A

X$_i$: inactivated X chromosome

1. <u>INTRODUCTION</u>

1.1. The Epigenome

1.1.1. <u>History and Definition</u>

The term epigenetic was defined differently over time according to the development of epigenetic science. It was first used in the 1940s by the developmental biologist Conrad Waddington to explain "the interactions of genes with their environment, which bring the phenotype into being". In the 1970s chemical DNA modifications were proposed to elucidate Waddington's hypothesis. Several years later the heritable nature of epigenetic mechanisms was described. Since the 1990s epigenetic is defined as *changes in gene expression that occur without a change in DNA sequence*. This altered gene expression is often heritable. (Dolinoy et al., 2007) These changes occur via several mechanisms, which will be presented in the following.

1.1.2. <u>DNA methylation</u>

The adherence of a methyl group to the DNA can only occur on specific sites. These loci are cytosines followed by a guanine in the DNA sequence. On this so called CpG-sites methyl groups can be transferred enzymatically from the methyl-group-donor S-adenoslymethionine (SAM) to the carbon-5 position of the cytosine ring (figure 1). The resulting 5-methylcytosine has an altered steric requirement of space than cytosine and because of that acts as an fifth base with different functional characteristics. This modification occurs after DNA-replication. The methyl group extends into the major groove of DNA and several ways are known by which cytosine methylation can regulate gene expression. 5-methylcytosine can inhibit or hinder the association of some transcriptional factors with their cognate DNA recognition sequences. Another mechanism is the binding of methyl-CpG-binding domain proteins (MBDs) to methylated cytosines mediating a repressive signal. MBDs can interact with chromatin forming proteins modifying surrounding chromatin, linking DNA methylation with chromatin modification. (Grafi et al., 2007) These interferences lead to an altered (mostly inhibited) transcription of the affected gene.

CpG dinucleotides are underrepresented in the human genome and their appearance does not accord with the random distribution of other nucleotides. This could be explained by the
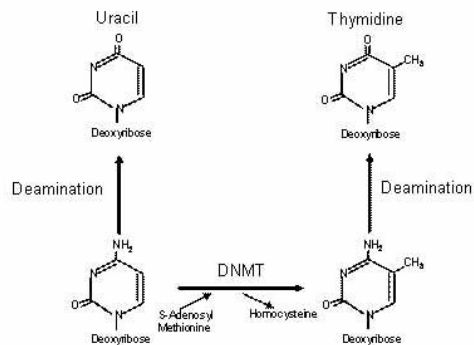
Figure 1: Correlation between Cytosine, 5-Methylcytosine, Uracil, and Thymidine (Bryan et al., 2008)

evolutionary spontaneous deamination of 5-methylcytosine to thymine (figure 1) CpG-islands, defined as regions containing 50 % or more CpG content and a length of 500 to 2000 base pairs, are often located within or near gene promoters or first exons of housekeeping genes. CpGs in CpG-islands are predominantly unmethylated and can be found in half of all human genes. (Agrawal et al., 2007; Dolinoy et al., 2007) A global

hypermethylation usually leads to a silencing of the affected gene but many exceptions of this generalization are known by now. An increased methylation on certain CpGs with a regulatory effect could raise gene expression and a hypomethylation on other CpGs can be responsible for an over expression of a gene even though a global hypermethylation exists. (Kim, 2004b)

The role of the majority of all methylated CpGs is host defense against transposons which are rich in CpGs. (Bestor, 2000) Transposons are small repeating DNA sequences which could move within the genome of a cell between different genes. (Geurts et al., 2003) Transcripted transposons interfere with the regulation of the expression of the host gene and destabilize the genome through insertion and recombination. Due to the repressive effect of cytosine methylation the expression of the transposons is inhibited. The conversion from 5-methylcytosine to thymine provides long-term protection against transposition by permanent inactivation through sequence alteration.

Most dividing cells express DNA methyltransferases (DNMTs), which are responsible for DNA methylation at position five of cytosines. Mammalian DNMTs can be divided into two general classes. The DNMT1 enzyme is responsible for the maintenance of global methylation patterns on DNA. It preferentially methylates CpGs on hemimethylated DNA (CpG methylation on one of both DNA strands), therefore guaranteeing transfer of methylation marks through the cell cycle in eukaryotic cells. The DNMT1 enzyme is directly incorporated in the DNA replication complex. The *de novo* methyltransferases DNMT3a and DNMT3b establish methylation patterns at previously unmethylated CpGs. These enzymes

seem to act only on specific loci on the genome. But the mammalian DNMTs are not sequence specific and thus a strict separation of their functions is not possible. It was shown that DNMT1 is also involved in *de novo* methylation. DNMT3L is a DNMT related enzyme, which associates with DNMT3a/3b. It influences its enzymatic activity while lacking one of its own. Finally, for the DNMT2 enzyme a biological function in mammals remains to be demonstrated. A role in some aspects of centromer function seems possible. (Bestor, 2000)

1.1.3. Histone modification

The principle structure of a nucleosome core particle contains a histone octamer, two copies of each H2A, H2B, H3 and H4 and 146 base pairs of DNA wrapped around. This arrangement forms a highly conserved structure which is the same in nearly all eukaryotes. But the possibilities of posttranslational histone modifications lead to an enormous variability of these conserved formation – the so called "histone code". These modifications take place on the N-terminal tails of the histone-protein, which are exposed on the nucleosome surface. (Turner, 2000) Modifications can be (mono-, di-, and tri-) methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, hydroxylation, glycosylation and others. Adherence of the listed groups can take place on the amino acid residues
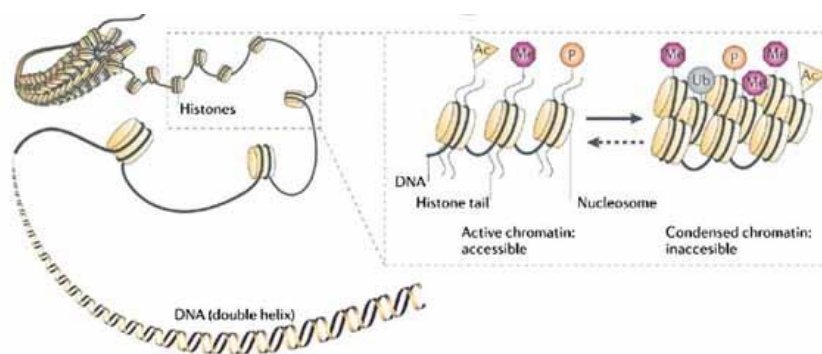
Figure 2: Nucleosomes and histone modifications (Nature Publishing Group)

of serine, lysine, arginine, histidine, glutamic acid, proline, and threonine. (Delage and Dashwood, 2008; Turner, 2000)

The resulting modified nucleosome can cause a shift in gene expression if modifications occur on a regulatory region, e.g. the promoter region. Through modifications the affinity of the histone proteins to the DNA is loosened or tightened depending on the modification. This means that the packing of the chromatin becomes either more or less dense and Euchromatin is shifted to heterochromatin or vice versa. Transcription factors and other for important mechanisms for gene expression can only work if the chromatin packing allows

their binding. A shift from eu- to heterochromatin thus leads to an alteration of gene expression.

Histone acetylation is normally associated with an increase in gene expression because chromatin packaging is relaxed. The effect of histone methylation cannot easily be predicted because it depends on the kind of histone and the amino acid residue modified. (Dolinoy et al., 2007) An overview of the functions of histone modifications is given in table 1.

Histone	Modification	Effect
H1	Phosphorylation	Chromatin condensation, gene specific condensation and repression.
	Ubiquitination	Transcriptional activation
H2A	Acetylation	Transcriptional activation
	Ubiquitination	Elusive
H2B	Ubiquitination	Prerequisite of H3 methylation
	Phosphorylation	Chromatin condensation
	Acetylation	Chromatin remodeling
	Methylation	Chromatin stabilization
H3	Methylation (H3-K4, R17)	Transcriptional activation
	Methylation (H3-K9, K79)	Transcriptional repression
	Acetylation	Transcriptional activation
	Phosphorylation	Chromatin condensation; transcriptional activation
	Ubiquitination	Nucleosome loosening
H4	Acetylation	Transcriptional activation
	Methylation (H4-K20)	Transcriptional repression
	Methylation (H4-R3)	Transcriptional activation

Table 1: Consequences of defined histone modifications

Histone acetylation of the ε-amino group of defined lysine residues is the most abundant and therefore been the most investigated histone modification. The state of histone

acetylation results from the dynamic equilibration of enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs). (Turner, 2000)

HATs transfer acetyl groups from Acetyl-Coenzyme A to lysine residues in the N-termini of the core histones. (Tanner et al., 2000) This leads to a local expansion of the chromatin and allows regulatory proteins of gene transcription to access the DNA. Gene transcription is mostly activated by histone acetylation.

HDACs remove acetyl groups from the ε-amino group of lysine residues and so counteract the activity of HATs. Because of this contrary mechanism to the HATs, it was assumed that HDAC activity results in transcriptional repression. In fact this assumption is correct in most of the cases but the transcription of some genes can even be activated by the activity of HDACs. There are several mechanisms which could explain this dual function of HDACs: If a gene coding for a transcriptional repressor is silenced by histone deacetylation, genes acting as substrate for this repressors get activated. Or the altered chromatin density changes the activity of transcriptional repressors and therefore increases expression of defined genes. Finally some transcription factors like p53, E2F, and GATA are known as substrates for HDACs. The deacetylation could result in an activation or repression of the transcription factor and so gene expression is altered depending on the gene and transcription factor. 18 human HDACs have been identified until now and they are divided into three classes depending on their homology to yeast *Saccharomyces cerevisiae* HDACs. Class I HDACs are expressed in nearly all human cells and tissues and are located in the nucleus. HDAC1, HDAC2, HDAC3, and HDAC8 belong to this class. HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 can shuttle between the nucleus and the cytoplasm and form the class II. The HDAC11 could not relate to class I or II because the sequence is not homolog to any of these classes. The structure of class III HDACs is highly distinct from class I or II. Because of their homology to the yeast protein Sir2 (silent information regulator 2) they are named SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7. Their special enzymatic mechanism needs NAD^+ as a co-factor. (Mottet and Castronovo, 2008) ADP-ribose residue of the NAD^+ accepts the acetyl group, which is then separated from the histone or protein. (Jackson et al., 2003)

1.1.4.   Small RNAs

Small RNAs consist of 21 to 30 nucleotides and they play a regulating role in a broad range of biological pathways including regulation of gene transcription. For example some prevent

the jumping of transposons, are important stem cells and tissues development, regulate cell division and control insulin secretion. (Zamore and Haley, 2005) Small RNAs can be divided in several classes: microRNAs (miRNAs), small interfering RNAs (siRNAs), repeat-associated small interfering RNAs (rasiRNAs), and Piwi-interacting RNAs (piRNAs). (Kloosterman and Plasterk, 2006) Together with associated proteins small RNAs establish RNA silencing pathways that regulate transcription, chromatin structure, genome integrity, and mRNA stability. Associated proteins are double-stranded RNA (dsRNA)-specific endonucleases, dsRNA binding proteins, and small RNA binding proteins. (Zamore and Haley, 2005) siRNAs can be derived from double-stranded RNA and lead to mRNA degradation. Therefore a perfect complementarity is necessary. rasiRNAs are associated with genomic repeats and retrotransposons. piRNAs are 29 to 30 nucleotides long and they are germ-line specific. They bind to P-element induced wimpy testis (Piwi) proteins which are important for the cell division of germ line cells. (Kloosterman and Plasterk, 2006)

The most prominent and most investigated class of small RNAs by now is that of the miRNAs.
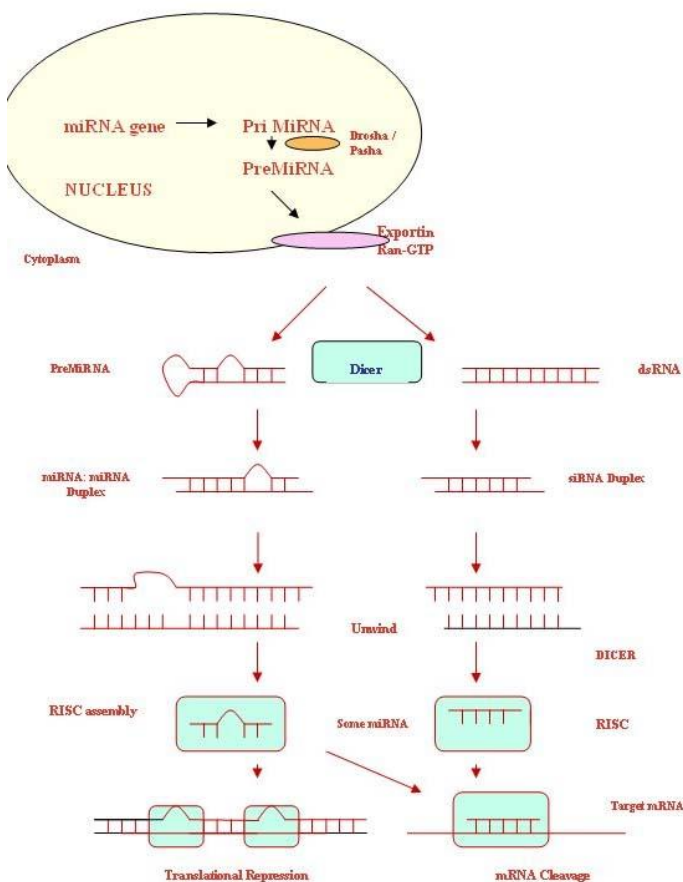
Figure 3: miRNA interference (Khanna D. et al., 2007)

The product of transcription of micro RNA-genes are so called pri-miRNAs up to several kilobases long. These pri-miRNAs are shaped like an imperfect hairpin on which the mature miRNA is located on one arm. In the nucleus of mammalian cells a complex is located where the maturation of the miRNA starts. It consists of the RNase III enzyme Drosha and the double stranded RNA-binding protein DGCR8. This microprocessor cleavage yields a 65 to 75 nucleotide pre-mi RNA, which is then exported to the cytoplasm. The RNase III enzyme Dicer trims the pre-miRNA to its mature 21 to 23

nucleotides length. One strand of the miRNA is loaded to the RNase Argonaute2 (Ago2). The reference of the loaded strand matches the reference of the target mRNA on the 3´-untranslated region (3´UTR). Together with other proteins the RNA-induced silencing complex (miRISC) is formed. Given perfect complementarity between the miRNA and the mRNA, the mRNA is cleaved by Ago2 between bases 10 and 11 measured from the 5´ end of the miRNA. If the complementarity is incomplete, miRISC causes translational repression and destabilization of the targeted mRNA. (Ku and McManus, 2008) There are evidences that this decay of mRNA takes place in a certain dynamic compartment of the cytoplasm called P-body. The miRNA interference pathway establishes a very complex regulatory system because a single miRNA can repress translation of up to over 100 mRNA and a single mRNA can be targeted by many miRNA. (Bruno and Wilkinson, 2006) These multiple target sites are features of the so-called switch targets that lead to a strong repression. Fine-tuned mRNAs possess only one binding site for one miRNA. They have in only a weak regulatory capacity. The majority of miRNA regulated processes belong to the fine-tuning type. It has its great significance when the target gene expression is low. Gene products of housekeeping genes have shorter 3´UTR preventing them be targeted by miRNAs. During development miRNAs are expressed in a tissue specific manner. Thus, miRNAs are important for the development and maintenance of specific tissues. The importance of miRNAs was demonstrated in the mouse model: mice lacking miRNAs were nonviable and died during embryonic phase. miRNAs are not only regulators of the developmental timing but they are also involved in signaling pathways. Apoptosis and metabolism are under the influence of miRNAs. (Kloosterman and Plasterk, 2006) For example a fly miR-14 mutant showed obesity and increased triacylglycerol levels. MiRNAs appear to play a role in fat metabolism and energy homeostasis. (Teleman et al., 2006) A silencing interference is also involved in mygenisis, carcinogenesis, and brain functions. Tumors show altered patterns of miRNAs expression but it is still unclear whether this is a cause or a consequence of cancerogenesis. (Kloosterman and Plasterk, 2006)

1.2. Epigenetic phenomena in mammals

1.2.1. Genomic imprinting

Genomic imprinting is a gene silencing on one allele by epigenetic mechanisms. Thus only one out of two homologous genes is expressed in a parent-of-origin manner. Imprinted

genes act functionally haploid and therefore they are not protected from recessive mutations like diploid genes usually are. (Dolinoy et al., 2007) Three mechanisms have been suggested to cause this effect: DNA methylation, chromatin modification and noncoding RNAs. 90 imprinted genes are identified in humans by now and most of them are tissue specific or affect developmental timing. They are often found in clusters and are controlled by an imprinting control region (ICR). (Ideraabdullah et al., 2008) Paternally expressed genes tend to promote growth of the offspring; maternally expressed genes tend to suppress growth. In an evolutionary context, genomic imprinting might have evolved to handle mother's resources during pregnancy and breastfeeding, known as the kinship theory. (Stoger, 2008) Imprinted syndromes are functional disorders caused by mutations or dysregulation of imprinted genes. Three genetic mechanisms are suggested: (i) large deletion or duplication of imprinted regions, (ii) DNA mutations in imprinted genes or their imprinting control center, (iii) uniparental disomy (two copies of a chromosome from one parent and none from the other). So called epimutations lead to epigenetic disorders of gene silencing. Hypermethylation or loss of methylation can occur and lead to a defective gene expression. (Amor and Halliday, 2008) Prader-Willi syndrome, Angelman syndrome and Beckwith Wiedemann syndrome are examples for imprinting disorders. (Dolinoy et al., 2007) Many imprinted genes show aberrant gene expression in cancer cells leading to the assumption that imprinting disorders could increase the susceptibility to cancer. (Murphy and Jirtle, 2003) Loss of imprinting and aberrant expression of H19 (a long noncoding RNA) and insulin-like growth factor 2 (IGF2) are associated with somatic overgrowth, embryonic tumors and are linked to more than 20 types of cancer. (Ideraabdullah et al., 2008)

1.2.2. X chromosome inactivation

In female mammals including humans one of the two X chromosomes is inactivated by epigenetic mechanisms. The affected X chromosome is chosen at random early in embryonic development and becomes the inactivated X chromosome (X_i). The transcription silencing occurs by DNA methylation and histone acetylation. The X_i is hypermethylated on CpG-islands and gene promoter regions and hypoacetylated on histone H4. (Dolinoy et al., 2007) The inactive state is clonally transmitted throughout cell division. A X-chromosomal inactivation center (XIC) controls the X-inactivation specific transcript genes Xist and Tsix, which help in the silencing process. These genes encode long noncoding RNAs.

(Ideraabdullah et al., 2008) Xist RNA represses the Tsix expression by coating the chromosome on the inactive chromosome and Tsix RNA avoids Xist expression on the active chromosome. Xist is hypermethylated on the active X and unmethylated on X_i. (Dolinoy et al., 2007)

1.2.3. The Agouti mouse model

The wild type agouti gene encodes a paracrine signaling molecule that produces either black eumelanin or yellow phaeomelanin. The transcription of both is initiated from a developmentally regulated promoter, which is hair cycle specific. During a certain stage of hair growth phaeomelanin is expressed resulting in a sub-apical yellow band on each black hair. This phenomenon causes the brown coat color of the wild-type mice. Through the insertion of a retrotransposon the metastable epiallele A^{vy} is formed, including a cryptic promoter. Metastable epialleles are identical alleles that are variably expressed through epigenetic modifications early in development. This additional promoter leads to ectopic Agouti transcription and affects all cells. The phenotype of these mice is altered to yellow fur, obesity, diabetes, and increased susceptibility to cancer. DNA methylation correlates inversely with ectopic Agouti expression. Different degree of methylation causes a wide variation in coat color ranging from yellow (unmethylated) to dark brown (pseudoagouti, methylated).

Figure 4: Agouti mice (Dolinoy et al., 2007)

The variable methylation state depends on maternal nutrition and environmental exposure during early phases of pregnancy.

For example the epigenetic influence of bisphenol A (BPA) was studied using the agouti mouse model. BPA is used in the manufacture of polycarbonate plastics and epoxy resins and it can be found in many commonly used items including food and beverage containers, baby bottles, and dental sealants. To investigate the effects of BPA to the fetal epigenome, 50 mg BPA/kg was added to the mother´s diet 2 weeks prior to mating and throughout gestation and lactation. This exposure shifted the coat color of the offspring carrying the A^{vy} epiallele toward yellow and decreased the methylation on nine CpGs in the A^{vy} cryptic promoter. The effect of BPA is not gene

specific and hypomethylation was also induced on other epigenetically labile genes. Supplementation of maternal diet with methyl donors (e.g. folic acid, betaine, vitamin B_{12}, choline) or the phytoestrogen genistein prevented the loss of methylation and so protected the offspring from obesity, diabetes and cancer. The phenotype resulting from epigenetic modifications in early embryonic development persist transgenerationally despite lack of continued exposure in subsequent generations. This example demonstrates how simple changes in diet or environment can affect the epigenome and the phenotype. (Dolinoy, 2008)

1.3. Epigenetic and nutrition

The agouti mouse model illustrates the influence nutrition has on epigenetic mechanisms and how epigenetic mechanisms can affect body composition. Several ongoing research projects aim at investigating the mostly unexplored complex associations between nutrition and epigenetics.

As seen in the metastable Agouti-epiallele, DNA methylation plays an important role in the interaction between nutrition and epigenetics. (Dolinoy et al., 2007) As mentioned above (SAM) is the universal methyl donor for DNMTs and other enzymes involved in methylation reaction. It is exclusively provided by folate-mediated one-carbon metabolism and is a metabolite of the methionine-homocysteine cycle. (Delage and Dashwood, 2008) High abundance of methyl donor enhances the production of SAM. Such methyl donors appear naturally in the diet, e.g.: folic acid, vitamin B_{12}, choline and betaine. (Dolinoy et al., 2007) A diet rich in these nutrients can increase the methylation of CpGs in an unspecific manner. (Dolinoy, 2008) Other nutritional components like catechols, phytochemicals and metals can also interfere with the methionine-homocysteine cycle. They alter methyltransferases activity, affect the SAM/S-adenosylhomocysteine (SAH) ratio and DNA methylation. A diet low in methyl donors and genetic polymorphisms in folate metabolism have been associated with aberrant DNMT expression, global DNA hypomethylation, and increased cancer risk.

A number of natural food compounds can influence the activity or expression of HDACs and therefore the acetylation status of histones. Another epigenetically active compound is the short chain fatty acid butyrate, which is a fermentation product of the gastrointestinal microbiota. Other dietary substances that influence HATs and/or HDACs activity are found in garlic (organosulfur compounds e.g. diallyl disulfide) and cruciferous vegetables (e.g.

isothiocyanates, sulforphane and 6-methylsulfinylhexyl). HDAC modulating agents are objectives of several research projects due to their potential as cancer therapeutic. (Delage and Dashwood, 2008)

As described in the Agouti mice the mother´s diet has a great impact on the epigenome of her offspring. (Dolinoy, 2008) The most critical time points for epigenetic reprogramming are the fetal period and the early postnatal development. (Junien, 2006) This altered epigenome is heritable and transgenerationally maintained. It was reported in some human population studies that the nutritional condition of the grandparents could affect the phenotype of the grandchildren. Genetic mutations cannot explain the observed effects and so epigenetic inheritance was assumed. (Feil, 2006)

One of the most popular examples to study the effect of maternal nutrition on the unborn is the Dutch hunger winter 1944-45. Individuals who were exposed to this famine prenatally can be traced because the health care system worked during this clearly defined period and the official food rations were documented. The methylation of the IGF2, which plays a crucial role in growth and development, was investigated in individuals exposed to the famine periconceptionally. However, the birth weights of the participants were within the normal range because they were delivered after the end of this hunger period. All CpG sites except one were significantly less methylated than unexposed controls. Contrarily no differences were found in participants born in or shortly after the famine. Their exposure was in late gestation. These findings introduce that epigenetic programming takes place in very early phases of development. (Heijmans et al., 2008)

Data collected in Sweden add evidence that the cardiovascular disease mortality of the grandchildren depended on the food availability of their grandparents. The risk for diabetes and CVD was low, if the paternal grandfather was exposed to poor nutrition during the years before adolescence, the so called slow growth period. Contrarily diabetes mortality increased if the availability of food was high. This phenomenon could not be explained by genetic variations, so it was assumed that epigenetic mechanisms lead to these differences. Alteration in genomic imprinting is unlikely to be the reason, because genomic imprinting happens in the very early embryonic development. Thus other epigenetic mechanisms involving DNA or histone modulation or miRNA interference, which are inherited down the male line, might explain these interesting findings. (Kaati et al., 2002)

1.4. The Interleukin 8 gene (IL-8)

IL-8 belongs to the inflammatory mediators and act as a chemokine. In this function it is inducing chemotaxis, which is the directed migration of cells to a site of inflammation. Neutrophils, which indicate an acute inflammatory response, follow an IL-8 gradient towards higher concentrations and are activated by this chemokine. Monocytes and macrophages are the main cellular sources of IL-8 but all nucleated cells of the body can produce it. The secretion is induced by multiple stimuli including lipopolysaccharide, living bacteria, and other cytokines such as tumor necrosis factor and IL-1. Its production takes place early in the inflammatory process but IL-8 persists over a prolonged period of time. The gene expression of IL-8 can be regulated by oxidative or anti-oxidative agents respectively. (Remick, 2005) Minute quantities of chemokines can cause enormous reactions of immune response. The expression of these genes is tightly regulated, primarily at the transcription level. Without stimuli the transcription of inflammatory mediators needs to be silenced to prevent improper immune reactions. Aberrant expression and activity are the reasons for a variety of immune disorders. The basal transcription of IL-8 also depends on the grade of cell differentiation. The human colon carcinoma cell line Caco-2 expresses IL-8 in the preconfluent state at a high level. After differentiation the IL-8 expression is silenced. (Wen and Wu, 2001)

Some breast cancer cell lines and other cancer cells show enhanced IL-8 expression due to aberrant histone acetylation. These observations suggest that differential acetylation of histones and transcription factors play an important regulatory role in neoplastic process. (Chavey et al., 2008) Transfection studies with reporter genes showed that the elements in the IL-8 gene required to silence expression in postconfluent Caco-2 cells are not located within the immediate 5´-flanking region of the gene. Furthermore the transfection with marked mini-genes lead to the conclusion that the entire immediate IL-8 gene locus lacks elements required to silence IL-8 expression. Inhibiting HDACs by trichostatin A (TSA) or butyrate could reduce IL-8 expression. But the silencing of the basal activity occurs in a HDAC independent manner. (Wen and Wu, 2001) Another study proofed that a mutation on the NF-κB binding site in the IL-8 promoter abolished the response to TSA. This result introduces that the regulation of the IL-8 transcription by histone deacetylases activity is mediated by the NF-κB and tumor necrosis factor α (TNFα) pathway. (Chavey et al., 2008) Downstream

NF-κB the activation of the MAP-Kinase pathway is known to modulate IL-8 expression in various cell systems. These mechanisms influence the phosphorylation of histone H3, which is responsible for accessibility of NF-κB to target promoters. Further acetylation at lysine 9 and tri-methylation at lysine 4 reflect positive modulation of transcription. (Raymond et al., 2009) Also variants of histones play a regulatory role in IL-8 expression. MacroH2A is a vertebrate specific H2A variant for example enriched on the inactive X chromosome. It is structurally different to other histone variants and so it is suggested that incorporation of macroH2A into nucleosomes could confer an epigenetic mark for gene expression. Agelopoulos et al. reported that the content of macroH2A in the nucleosomes of different cell types determines the expression of IL-8 and how much it can be stimulated. MacroH2A masks the binding site of NF-κB in the promoter region inhibiting its binding to the DNA. (Agelopoulos and Thanos, 2006)

Not only histone modification but also DNA methylation plays an important role in the epigenetic regulation of IL-8 expression. Investigating different breast cancer cell lines it was reported that metastatic cancer types express high levels of IL-8 whereas non-metastatic cancers do not. These results corresponded to the methylation of two CpG-sites 1.2 kb upstream the promoter region. The methylation at these sites correlated positively with the mRNA levels. This finding is contrary to the common assumption of gene silencing through high methylation states. In addition no regulatory effects of CpG-sites within or near the promoter region of the IL-8 gene could be observed. Several mechanisms might explain these findings. For example the methylated cytosines inhibit the binding of a repressor within the vicinity of the two CpGs. Alternatively, the regulatory sites may serve as a center around which a protein complex forms and remodels chromatin structure leading to an up-regulation of IL-8. (De Larco et al., 2003)

1.5.   Characterization of the used epigenetically active agents
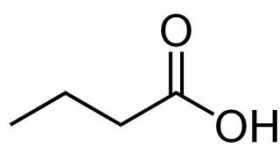
1.5.1. Butyrate

Figure 5: Structure of butyric acid

Short chain fatty acids such as butyric acid are produced by the gastrointestinal microbiota through the breakdown of non-digestible fibers. Butyric acid serves as the preferred energy source for colonocytes and about 95 % is transported across the

epithelium. Concentrations in portal blood are undetectable because of the rapid utilization. (Pryde et al., 2002)

The epigenetic effect of butyrate is mainly caused by the non-competitive inhibition of HDACs, especially class I and II, leading to a histone hyperacetylation. Theoretically silenced genes should be reactivated by this mechanism, but several studies indicate that a high number of genes are down-regulated. An up-regulation of tumor suppressor genes after treatment with butyrate has been reported and an increased histone acetylation in the promoters of these genes was detected. This mechanism could explain the growth arrest and/or apoptosis properties of butyrate and its anti-tumourogenic properties. (Rada-Iglesias et al., 2007) But only the expression of 2 % of all mammalian genes is influenced through the HDAC inhibitoring activity of butyrate. These findings suggest the existence of a butyrate response element in the promoter regions of the affected genes. The transcription factors Sp1 and Sp3 are located within the butyrate response element binding sites. These transcription factors can act as activators or repressor of transcription. For example the promoter region of the gene $p21^{Waf1/Cip1}$, which plays an important role in the cell cycle, contains six Sp1-binding sites. Butyrate induces expression of $p21^{Waf1/Cip1}$ and prevents the cell to enter S phase. The cell is arrested to the G1 phase and undergoes differentiation or apoptosis. (Davie, 2003) Other genes involved in cell cycle are also regulated by butyrate. (Della Ragione et al., 2001) Treatment of the liver cell carcinoma cell line HepG2 with butyrate lead to an increase global histone acetylation as expected but also to a decreased histone acetylation at the promoter region of about 1 % of all genes. This loss of acetylation was reversible upon withdrawal of the drug and could also be seen with other HDAC inhibitors like trichostatin A. CpG islands were significantly more common in deacetylated regions. This finding points towards a mechanistic link between DNA methylation and histone acetylation. But it is not yet clear if the histone deacetylation is the reason or the consequence of gene silencing in these cases. It also seems possible, that these epigenetic alterations are responses to stress caused by the butyrate treatment of the cells. (Rada-Iglesias et al., 2007) Other SCFAs such as acetate or propionate also have epigenetic effects but to a less extent. (Pryde et al., 2002)

In concentration from 5 to 100mM butyrate shows apoptotic activity. This property involves the caspase-3 pathway. Butyrate causes cleavage of the pro-enzyme caspase-3 to its active form. Also pro-apoptotic proteins like bak increased after butyrate treatment whereas the

expression of the anti-apoptotic proteins like bcl-2 was not altered. Cycloheximide, which inhibits protein synthesis, could prevent butyrate induced apoptosis. This shows that additional intermediate steps requiring protein synthesis must be involved. The caspase-3 inhibitor zVAD-FMK could also prevent apoptosis. This shows that the pro-apoptotic activity of butyrate depends on the caspase-3 pathway. (Ruemmele et al., 1999)

The anti-inflammatory properties of butyrate result from inhibited activation of the transcription factor NF-κB. (Pryde et al., 2002)

1.5.2. Genistein
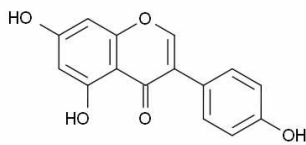
Figure 6: Structure of genistein

Genistein (4´,5,7-trihydroxyflavone) is an isoflavonoid naturally occurring in soy. It is a potential chemopreventive agent against various types of cancer. Its anit-cancerogenous effect might be attributable to several mechanisms: prevention of DNA mutation, reduction of cancer cell proliferation, inhibition of angiogenesis, and induction of differentiation. (Li et al., 2009) Genistein has been shown to have estrogenic properties, anti-neoplastic activity in several tumors, and is an inhibitor of protein tyrosine kinases, which play key roles in cell growth and apoptosis. These characteristics may be the results of an epigenetic impact of genistein. The activation of nuclear factor kappaB (NF-κB) is inhibited, which could be mediated via the AKT signaling pathway. The serine/threonine protein kinase AKT functions downstream of phosphatidylinositol 3-kinase (PI3K) in response to mitogen or growth stimulation. Activation of AKT prevents apoptosis by multiple mechanisms and is associated with the development and metastasis of several kinds of cancer. Additionally genistein modulates the activity of the transcription factors NF-κB and the fork head transcription factors (FOXO) influencing apoptosis indirectly. FOXO is induced by genistein and acts downstream the phosphatase and tensin homolog (PTEN) tumor suppressor, which down-regulates the activity of AKT. NF-κB is involved in proliferation, apoptosis, inflammation, and immune response. The activities of both mentioned transcription factors are regulated by the Sirtuin SIRT1, which belongs to the atypical class III HDACs. SIRT1 was identified to play a role in anti-apoptosis, neuronal protection, cellular senescence, aging, and longevity. A variety of transcription factors is down-regulated by SIRT1 and is not inhibited by TSA like other HDACs. It might promote tumorgenesis because an up-regulation of SIRT1 was observed in tumor cells. A study from

Kikuno et al. aimed at the mechanistic pathway of genistein. The following explorations were made: Genistein inactivates the AKT-pathway via inhibition of PI3K and increases the mRNA levels of PTEN, the tumor suppressor gene p53, and FOXO3a. The nuclear translocation of NF-κB is blocked and so its DNA-binding activity is decreased. Genistein lowers the expression of SIRT1 and so increases the histone acetylation of H3K9 in several gene promoter regions like PTEN and FOXO3a. The methylation of these sites decreases parallel. These effects result in enhanced expression of these genes. (Kikuno et al., 2008)

The cancer preventive properties of genistein could also be explained via its impact on telomerase activity. Telomerases add a repeating DNA sequence (the telomere) to the end of chromosomes at each DNA replication, which guarantees stability. The telomere is normally shortened during each cell cycle finally resulting in apoptosis once the telomere is consumed. In multiple cancer cells the activity of telomerase is increased and apoptosis prevented. Genistein was shown to repress telomerase activity in cancer and precancer cell lines and may thus prevent the development of tumors. (Li et al., 2009)

1.5.3. Folic acid
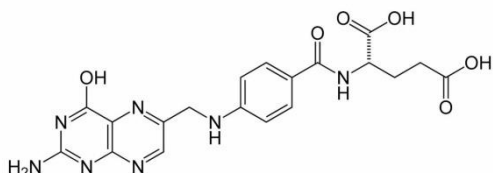
Figure 7: Structure of folic acid

Folic acid is a water soluble vitamin and is essential for numerous body functions because it acts as one-carbon donor. It exerts three major functions related to DNA metabolism. For the conversion from deoxyuridine to thymidylate folic acid serves as methyl donor. Deficiency leads to a missincorporation of uracil instead of thymidine into DNA. DNA then becomes susceptible to DNA strand breaks and therefore for tumorigenic transformation of cells becomes more likely. A folate metabolite also serves as substrate for the synthesis of the purines adenine and guanine. Thus a deficiency of folic acid may inhibit DNA synthesis by the lack of both purines. The third mechanism how folic acid acts in DNA metabolism is the most relevant for epigenetic research: 5,10-methylenetetrahydrofolate is converted into 5-methyltetrahydrofolate by the enzyme methylenetetrahydrofolate reductase (MTHFR). 5-methyltetrahydrofolate is the methyl donor for the conversion of homocysteine to methionine, which is activated by ATP to form SAM.
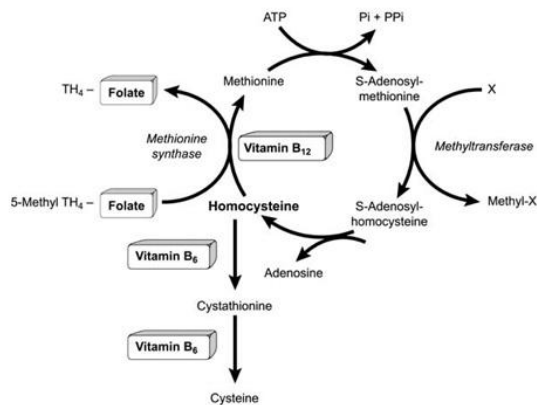
Figure 8: The methionine-homocysteine-cycle (Oregon State University)

SAM is known as methyl donor in many methylation reactions including DNA and histone methylation. Since cancer cells have aberrant DNA methylation pattern folate intake and blood levels in an individual might modify its susceptibility to cancer. Many cancer cell lines are characterized by a global DNA hypomethylation and localized regions of hypermethylation, leading to a shift of gene expression.(Miller et al., 2008) In addition some known mutations and SNPs can affect SAM availability. For example the MTHFR SNP C677T is a well-described and common variant that increases the dietary folate requirement. Polymorphisms and dysfunctions of the choline metabolism interfere with the folate metabolism and can so alter the availability of SAM. These associations influence the methylation state of the DNA. (Zeisel, 2009)

1.5.4. Zebularine
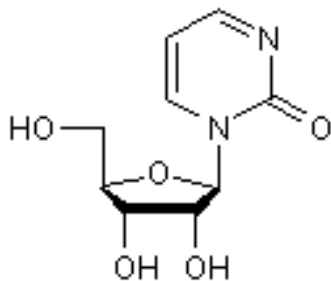
Figure 9: Structure of zebularine (Tocris Bioscience)

Zebularine is a cytidine analog and was originally developed as a cytidine deaminase inhibitor. It acts as DNMT inhibitor by forming tight covalent complexes between DNMT proteins and zebularine-substituted DNA. Studies in breast cancer cell lines confirmed this mechanism. It was seen that depletion of DNMT proteins took place instead of changes in mRNA levels of the DNMTs. The advantages of zebularine compared to other DNMT inhibitors are its high stability and its low toxicity in most tested cell lines. Through incorporation into DNA, the agent leads to growth inhibition and increased expression of cell cycle regulatory genes in cancer cells. (Billam et al., 2009) It reactivates abnormally silenced methylated genes, e.g. genes for cancer related antigens. Therefore it is evaluated as a possible therapeutic agent in cancer. Zebularine is incorporated into DNA at a linear velocity. The incorporation rate was higher in cancer cells than in normal fibroblasts. In preliminary experiments the response to the treatment was higher in cancer cells. The enzyme uridine/cytidine kinase may determine the amount of zebularine that incorporates into DNA.

Since lower levels of this enzyme are present in normal cells, this could explain the enhanced response of cancer cell lines to zebularine treatment. But it cannot be excluded that the incorporation of zebularine into RNA is responsible for the observed effects. (Cheng et al., 2004) In addition to its low toxicity zebularine induced apoptotic mechanism in breast cancer cell lines. In some breast cancer cell lines the expression of the estrogen receptor is silenced via epigenetic mechanisms. Treatment with zebularine caused a re-expression of this gene. (Billam et al., 2009)

1.5.5. Epigallocatechin-3-gallate (EGCG)
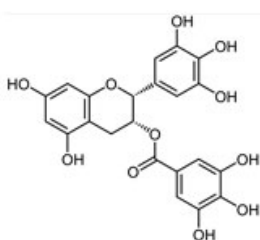
Figure 10: Structure of EGCG (Alibaba.com)

EGCG is the major polyphenol of green tea accounting for more than 50 % of all green tea polyphenols. Several studies show that EGCG exhibits the ability to suppress cancer cell growth, induce apoptosis, and inhibit tumor angiogenesis. One of the mechanisms which are responsible for these effects is the inhibition of DNMT1. (Berletch et al., 2008) It was suggested that EGCG acts both in a competitive and in a noncompetitive way. SAM methylates EGCG on two methylation sites and is so transformed to SAH. Because SAM is also the substrate for DNMTs, this is the competitive pathway of the inhibition. (Fang et al., 2007) Like zebularine EGCG can reactivate methylation silenced genes through this pathway. But for example the gene coding for the telomerase hTERT is down-regulated even though hypomethylation takes place. This could be explained by the enhanced binding of the methylation-sensitive transcription suppressor E2F-1. (Berletch et al., 2008) EGCG also significant reduced acetylation of histone due to its action as HAT inhibitor. But it does not bind to the active sites of histones and none competitively inhibits HAT activity. EGCG quenches HATs in a specific and global manner not influencing other enzymes for which histones serve as substrate. The balance and the interplay between HATs and HDACs are altered by this polyphenol. (Choi et al., 2009) Cancer cells treated with EGCG showed enhanced apoptosis. This could be caused perhaps through an oxidant pathway or up-regulation of pro-apoptotic genes. Different response in different cancer cells leads to the assumption that EGCG interferes with multiple pathways. (Berletch et al., 2008) Another mechanism induced by EGCG is the suppression of the transcription factor NF-κB, which is involved in inflammatory processes. The histone acetylation on the NF-κB binding site on the

IL-6 gene is shifted by EGCG leading to an altered binding and activity of the transcription factor. Because of that EGCG was seen to act as an anti-inflammatory agent in the mouse model. (Choi et al., 2009)

1.5.6. Chrysin
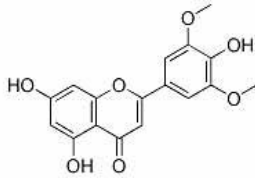
Figure 11: Structure of chrysin (chemicalbook.com)

Chrysin is a flavonoid naturally occurring in many plants, honey, and propolis. Chrysin was shown to possess antioxidant, anti-allergic, anti-inflammatory, anti-cancer, anti-estrogenic, and anxiolytic properties. It also can inhibit enzymes like tyrosinase and aromatase and prevents estradiol-induced DNA synthesis. In a study in rats with ethanol induced liver disorders chrysin elevated the activity of enzymes relevant for oxidative balance like superoxide dismutase (SOD) and chloramphenicol acetyltransferase (CAT). Lipid peroxidation decreased significantly. (Sathiavelu et al., 2009) No epigenetic investigations of chrysin have been published by now. But due to the manifold effects shown by chrysin epigenetic pathways and gene regulation e.g. for antioxidative genes seem possible.

2. <u>OBJECTIVES</u>

The aberrant expression of chemokines such as IL-8 can cause an overreaction of the human immune system and therefore lead to multiple disorders. (Remick, 2005) For example an over-expression of IL-8 also corresponds with the metastatic potential of various cancer types especially breast cancer cell lines. (De Larco et al., 2003) A shifted and elevated pattern of chemokines plays a critical role in chronic inflammatory bowel diseases including Crohn´s disease and colitis ulcerosa. (Monteleone and MacDonald, 2000) A lot of attention is paid to these disorders in nutritional sciences because their development, progression and therapy depend on nutrition. It is known that diet and special nutrients can alter the appearance and concentration of inflammatory mediators and may alter symptoms of disease. For example the diet-dependent fatty acid pattern can smooth acute inflammation phases of some disorders. (Lucendo and De Rezende, 2009)

It was shown in many cell cultures or animal studies, that the expression of IL-8 and other cytokines is - among others - regulated by epigenetic mechanisms. These include DNA methylation, histone modification and micro RNAs. Less is known how miRNAs influence cytokines but both DNA methylation and histone modification have been objectives in several studies investigating the regulation of inflammatory mediators. Diet and specified nutrients can have epigenetic effects and can shift gene expression patterns. (Dolinoy et al., 2007)

The aim of this thesis was to investigate the influence of specified nutrients on the inflammatory mediator IL-8 and the epigenetic mechanisms behind the effects. The Caco-2 cell line was chosen as a model for the human gut because of its similar properties to mature enterocytes. (Sambuy et al., 2005) The analyses included DNA methylation and histone acetylation of the cells after treatment with well defined agents to explore the effects and mechanisms behind. Together with other similar studies these findings might have multiple impacts: Firstly, they can serve as a start point for further research on individualized diet for the prevention of inflammatory processes. Secondly they emphasize the increasing importance of epigenetic mechanisms as targets for drug development.

3. <u>MATERIALS AND METHODS</u>

3.1. Polymerase Chain Reaction (PCR) and real-time PCR analysis

Real-time PCR follows the same principle like end-point PCR: DNA is amplified which makes a very little amount of DNA detectable. Unlike an end-point PCR in real-time PCR the monitoring of the PCR-product takes place while the reaction is proceeding (real time) and not afterwards. Because of the sensitivity of the different detection systems the DNA amount can be quantified.

For a PCR reaction following components are necessary:

- <u>Deoxynucleotidetriphophats</u> (dNTPs) of the four DNA-bases Adenine, Guanine, Cytosine, and Thymine as building blocks for the new synthesized DNA fragments.
- <u>DNA-Polymerase</u> is the enzyme which builds DNA out of dNTPs.
- <u>Magnesium chloride</u> as a co-factor of the DNA-Polymerase.
- <u>Primers</u> are short oligonucleotides of about 15 to 40 base pairs length which function as start signal for the DNA-Polymerase.
- <u>Fluorescent reporter</u> to detect and quantify the synthesized DNA. (only in real time PCR)

3.1.1. <u>Primer</u>

Primers act as start signals for the DNA-polymerase in the PCR. Primers are essential not only for the PCR reaction but also for DNA-replication because DNA-polymerase is not able to initiate the replication but only to elongate already existing DNA-fragments. It is necessary to apply a pair of primers, a forward and a reverse one to amplify double stranded DNA. The short oligonucleotides bind to the DNA sequence complementary to their own sequence. Through the choice of the sequence specific gene – or promoter regions of interest can be amplified.

3.1.2. <u>The PCR reaction</u>

A PCR consists of 30 to 40 cycles of denaturation, annealing and elongation.

- *Denaturation:* In this first step the DNA is denatured at a temperature of 90°C to 95°C.
- *Annealing:* In this step the primers bind to their target DNA sequence. The temperature depends on primer sequence, usually between 50°C to 70°C.

- *Elongation:* At a temperature of 72°C the polymerase builds a DNA strand starting at the annealed primer.
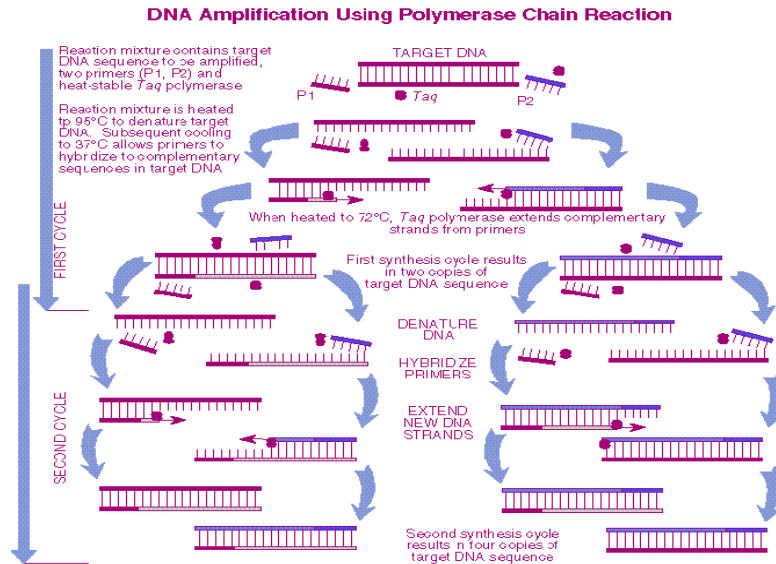
Figure 12: Principle of a PCR reaction (Torak et al.)

The amount of DNA copies is doubled during each cycle and can be formulated mathematically as follows:

$$N = N_0 \times 2^n$$

N: number of DNA copies

N_0:   initial number of DNA-molecules

n: number of cycles

2:   optimal efficiency, which means that after every cycle 2 DNA molecules resulted out of one. This optimal efficiency is hardly ever reached.

3.1.3. Real time PCR detection systems

Hydrolysis probe technique

In addition to the two primers a third oligonucleotide, the probe, is used. At the 3´and 5´ ends of the probe a fluorescent dye (fluorochrome) and a quencher are attached. The quencher absorbs the fluorescence of the reporter as long as the probe is intact. The probe anneals to the single stranded DNA between the two primers and gets destroyed during the DNA elongation due to the exonuclease activity of polymerases. So the fluorochrome and the quencher get separated and the fluorescence can be detected. During each cycle of the

PCR reaction more probe is hydrolyzed and the signal increases exponentially. Techniques that rely on this principle are TaqMan, Beacons, and Scorpions. (van der Velden et al., 2003)

Figure 13: Principle of Sybr Green (van der Velden et al., 2003)
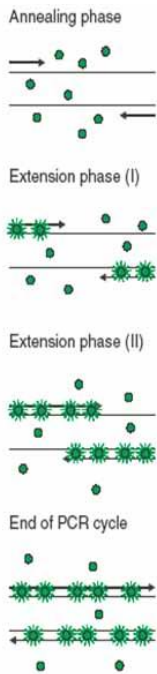
Hybridization probe technique

For this system two oligonucleotides binding direct next to each other on the DNA are necessary. One probe is labeled with a donor fluorochrome and the other one with an acceptor fluorochrome. If both probes anneal to the DNA, the emitted light of the donor will excite the acceptor whose emitted fluorescence can be detected during the annealing phase. The signal is increasing after each cycle because more DNA copies are available to bind for the probes. (van der Velden et al., 2003)

DNA-binding agents

A fluorescent agent binds to double stranded DNA after the elongation phase and so the signal increases with an increasing amount of DNA. One of the most popular dyes for this technique is Sybr Green, which is also used in these experiments. (see Figure 13) (van der Velden et al., 2003)

Figure 14: Example for result curves of a real-time PCR reaction

3.2. Cell culture conditions and treatment

For this experiment the human colon carcinoma cell line Caco-2 was used. The cells were grown in RPMI 1640 media, which was supplemented with 10 % heat-inactivated fetal bovine serum, 4 mM glutamine, and 100 units each of streptomycin and penicillin. After about 14 days of growth, cells were treated as listed in table 2 for 48 hours.

substances	concentration
butyrate	5 mmol/l
genistein	200 µmol/l
folic acid	200 µmol/l
zebularine	100 µmol/l
EGCG	100 µmol/l
chrysin	50 µmol/l

Table 2: Added agents and concentrations

The cell media was stored in aliquots to use for ELISA. The cells were washed twice with ice-cold PBS before proceed to DNA methylation analysis and ChIP.

3.3. Enzyme-linked immunosorbent assay (ELISA)

To get a fast overview whether the treatment of the cells altered the IL-8 gene expression an ELISA assay for the IL-8 protein in the cell supernatant was performed.

3.3.1. Principle of the method

A 96 well microtiter plate is coated with an antibody against IL-8, which binds to the ground and walls of the wells (capture antibody). An IL-8 standard curve and the samples are applied to the plate. During incubation the IL-8 proteins bind to the immobilized antibody. For detection another antibody (detection antibody) is added to each well which is conjugated to an enzyme. Through adding a substrate for the enzyme color development takes place depending on the antigen concentration. An UV plate reader detects the different intensities of the color. Standard dilutions allow quantification of IL-8 in the samples. (Porstmann and Kiessig, 1992)

3.3.2. Procedure

For this experiment the IL-8 Eli-pair kit from Diaclone was used.

Coating

- Per plate 50 µl of the capture antibody was diluted in 10 ml of coating buffer.

- 100 µl of the solution were pipette into each well of the plate.

- The plate was sealed and incubated overnight at 4°C.

- On the next day the wells were emptied and washed twice with 400 µl of wash buffer.

- 250 µl of saturation buffer were applied to the wells and incubated for 2.5 hours at room temperature.

- After that the saturation buffer was discarded and the plate dried one day at room temperature.

Sample application

- A standard curve was diluted following the scheme below using the standard stock solution and the standard diluent buffer:

standard	concentration [pg/ml]	volume standard	volume buffer
1 – stock solution	2000	–	–
2	1000	210 µl standard 1	210 µl
3	500	210 µl standard 2	210 µl
4	250	210 µl standard 3	210 µl
5	125	210 µl standard 4	210 µl
6	62,5	210 µl standard 5	210 µl

Table 3: Concentrations of the standards

- 100 µl of each standard and each sample were applied to the wells in duplicate. Two wells contained only standard diluent buffer as blanks.

- The plate was incubated for two hours at room temperature.

- Following the incubation the wells were emptied and washed three times with 400 µl of wash buffer.

- The detection antibody solution was made out of 100 µl biotinylated detection anti-IL-8 and 5 ml of biotinylated antibody diluent buffer.

- 50 µl of the prepared solution were distributed to each well and incubated for one hour at room temperature.
- The plate was emptied and washed three times with 400 µl of wash buffer.

Color development

- 5 µl of horseradish peroxidase-streptavidin (HRP-streptavidin) were diluted in 500 µl of HRP-streptavidin diluents buffer and 150 µl of this solution was again diluted in 10 ml of the same buffer.
- 100 µl of the HRP-streptavidin were dispensed to each well and incubated 20 minutes at room temperature.
- For color development 100 µl of 3,3´,5,5´-Teramethylbenzidine was applied to each well and the color was allowed to develop for eight minutes.
- The color reaction was stopped by adding 100 µl of 1 molar sulfuric acid to the wells.
- The absorbance was measured immediately at 450 nm.

Analysis

The standard curve was drawn in Microsoft Office Excel 2007 and the concentrations of the samples were calculated with the determined formula.

3.4. Gene expression

3.4.1. Principle of the method

Messenger RNA (mRNA) is a product of DNA transcription. The amount of mRNA is directly proportional to the gene activity. To quantify mRNA it is extracted and converted into more stable complementary DNA (cDNA) through reverse transcription. For reverse transcription three types of primers can be used:

- *Random hexamer primers:* These are small primers of six base pairs length, which bind randomly to the all mRNAs existing in the sample.
- *Anchored-oligo(dT) primer:* These primers consist only of Thymine and bind to the poly-A-tail of mRNAs.
- *Sequence specific primers:* With this kind of primers only specific genes or part of genes are converted during transcription reaction.

The resulting cDNA is than quantified by real-time PCR.

3.4.2. Procedure

mRNA extraction

For the mRNA extraction the NucleoSpin RNA II from Macherey-Nagel was used and followed the instruction applied.

Reverse transcription

To convert the mRNA into cDNA the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) was used.

- For each sample agents were mixed as following:

Transcriptor reverse transcriptase reaction buffer	4 µl
Deoxynucleotide mix	2 µl
Protector RNase inhibitor	0.5 µl
Anchored-oligo(dT)$_{18}$ primer	1 µl
Transcriptor reverse transcriptase	0.5 µl
water	6 µl
RNA	6 µl
total volume	**20 µl**

Table 4: Reaction mix for reverse transcription

- The mixes were set at 50°C for 60 minutes for transcription
- The samples were heated up to 85°C for 5 minutes to inactivate the transcriptase.

real-time PCR

The primers, sample composition, and cycling conditions for the gene expression detection are listed in the tables below.

Primer	Sequence	Annealing temp	concentration
IL-8 sense	ATG ACT TCC AAG CTG GCC GTG GCT	66°C	5 pmol/µl
IL-8 antisense	TCT CAG CCC TCT TCA AAA ACT TCT C	66°C	5 pmol/µl

Table 5: Primer sequences, annealing temperatures and primer concentrations for gene expression

Sample composition:

SensiMix	5 µl
water	2.8 µl
primer	1 µl
Sybr Green	0.2 µl
DNA	1 µl
total volume	**10 µl**

Table 6: PCR reaction mix for gene expression

Cycling conditions:

Polymerase activation	10 min	95°C
Cycling	35 times	
Denaturation	45 sec	95°C
Annealing	60 sec	66°C
Elongation	60 sec	72°C
Melt curve	0.5°C every 5 sec	50°C to 95°C

Table 7: Cycling conditions for gene expression PCR

3.5. DNA methylation detection

3.5.1. Principle of the method

For the detection of the methylation pattern on specific DNA-sites the whole genomic DNA is extracted from the cells.

Bisulfite conversion

The genomic DNA is treated with a reagent containing bisulfite (HSO_3^-). During the reaction all cytosines which are not methylated are converted into uracil. The methylated cytosines stay unchanged because the methylation group acts as a protector. (Hayatsu, 2008)
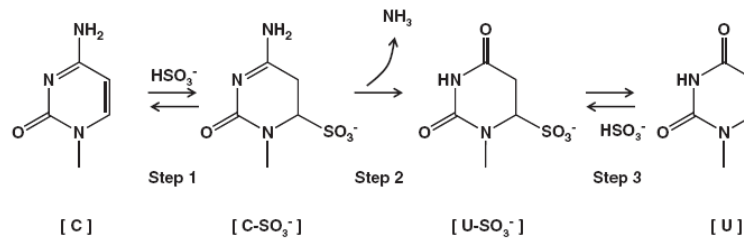
Figure 15: Bisulfite conversion of cytosine (Hayatsu, 2008)

PCR

After bisulfite conversion specific genes or part of genes are amplified in a PCR reaction. For converted DNA special primers are necessary because the sequence was changed during conversion. Nested PCRs are often used in the methylation detection. This means that two consequent PCR reactions with different primer pairs are performed. The first pair of primers amplifies a piece of DNA which is a little longer than the part of interest. The primers for the second PCR amplify the sequence of interest. This approach increases the specificity of the reaction.

Bisulfite Sequencing PCR (BSP)

After bisulfite conversion a PCR reaction is performed to amplify the interesting part of the genome DNA. The PCR-product is purified and sequenced. If there is still a C in the interesting CpG-site this means that the C was methylated. If a T is found instead it means that the CpG was not methylated.

Methylation specific PCR (MSP)

For this method two pairs of primers are necessary. The primers bind exactly to the CpG of interest. One pair of primers matches to the bisulfite converted DNA that has been formerly methylated and the other one to the formerly unmethylated DNA. It is possible to read out the how much of the DNA had been methylated.

3.5.2. DNA-Extraction

The DNA was extracted from cells applying the QIAamp DNA Mini Kit from Qiagen following the protocol of the manufacturer (see chapter protocols).

3.5.3. Bisulfite Conversion

The bisulfite conversion was done with the EpiTect Bisulfite Kit from Qiagen following the protocol of the manufacturer (see chapter protocols).

3.5.4. <u>BSP</u>

A nested BSP was performed to determine the methylation status of the CpG-sites -1342 and -1412 base pairs upstream the promoter region of the IL-8 gene. The primer sequences and PCR conditions are shown in the table 8. The PCR mastermix was purchased from Promega. The sample composition (table 9) was the same for both PCRs but for the internal reaction the product of the external PCR was diluted 1:100. Cycling conditions are shown in table 10.

Primer	Sequence	Annealing temp	concentration
external PCR			
sense	GGT TGG AGA AAG ATA ATA AAA AGA A	59°C	59 pmol/µl
antisense	AAC ACC AAA TCT AAC CTC TAA AAA C	59°C	59 pmol/µl
internal PCR			
sense	AGT GTG TTT ATA GTG TGG GTA AAT T	58°C	58 pmol/µl
antisense	AAA AAT TTC AAC AAA TCA TAA AAA A	58°C	58 pmol/µl

Table 8: Primer sequences, annealing temperatures and primer concentrations for methylation PCRs

Sample composition for internal and external PCR:

	external PCR	internal PCR
Mastermix	5 µl	25 µl
water	3 µl	15 µl
primer	1 µl	5 µl
DNA	1 µl	5 µl
total volume	**10 µl**	**50 µl**

Table 9: PCR reaction mix for methylation PCRs

Cycling conditions:

Polymerase activation	10 min	95°C	
Cycling	35 times	**internal PCR**	**external PCR**
Denaturation	30 sec	95°C	
Annealing	50 sec	59°C	58°C
Elongation	60 sec	72°C	

Table 10: Cycling conditions for methylation PCRs

After the amplification the product of the second PCR reaction was cleaned up with the QIAquick PCR purification kit from Qiagen and controlled by gel-electrophoresis. The purified product was sequenced by DNA Confidence, Vienna.

3.6. Chromatin immunoprecipitation (ChIP)

3.6.1. Principle of the method

The ChIP procedure is suitable to investigate the interaction between DNA and proteins like transcription factors and histones. Because of the treatment of the whole living cells without extracting the DNA or chromatin it is possible to explore the structure and the dynamic changes in the DNA-protein interactions in their native chromatin environment. ChIP shows at which locus of the DNA a protein of interest binds and also modifications are detectable. The DNA-protein bonds and protein modifications or interactions affect the structure of the chromatin. This changes the transcription of specific genes.

The DNA-proteins bonds are fixed in the living cells with crosslinking agents such as formaldehyde or UV. The use of formaldehyde as crosslinking agent dates back to the 1960s, when it was firstly used to consolidate protein/protein, protein/DNA, and protein/RNA interactions. The treatment with formaldehyde fixes bonds between the exocyclic amino and endocyclic imino groups of the DNA bases and side chain nitrogens of lysine, arginine, and histitidine as well as α-amino groups of all amino acids of the protein. These complexes are then enriched by precipitation with antibodies which are specific to the protein, a component or a modification of the protein. After the enrichment of the DNA-protein complex the crosslinking can be reversed and the DNA as well as the protein of interest can be investigated or quantified. ChIP has been applied to map the bonds of modified histones along target genes, the cell-cycle regulated assembly of origin-dependent replication and centromer specific complexes, and the *in vivo* position of transcription factors. (Kuo and Allis, 1999)

Figure 16: Principle of the ChIP procedure (Kuo and Allis, 1999)

Caco-2 cells were treated with a crosslinking mix containing formaldehyde. The immunoprecipitation took place with an antibody specific for histone H3 which is acetylated at the lysine residue in position 9 (H3K9).

3.6.2. <u>Procedure</u>

Crosslinking

- 1.5 ml crosslinking mix were added to each flask containing the cells.
- The flasks were rotated for 10 minutes.
- 1.5 ml of glycine solution (c=1.25 mol/l) were added to each flask to quench the crosslinking reaction.
- 1 ml of lysis buffer was spread over the cells and the flasks were incubated for 5 minutes at 4°C.
- The cells were scrapped from the grounds of the flasks and transferred into a 15 ml falcon tube.

Sonication

To find out how many pulses of sonication are necessary for the appropriate fragment length the untreated cell control was used.

- One impulse of 20 seconds and 50 % power was applied to the lysate.
- An aliquot of 20 µl lysate was diluted with 80 µl of water.
- 5 µl Proteinase K was added to a final concentration of 500 µg/ml.
- The aliquot was incubated for 30 minutes at 37°C and over night at 65°C to reverse the crosslinking.
- The DNA was purified with the QIAquick PCR purification kit from Qiagen following the instructions provided (see chapter protocol).
- To control the length of the DNA a gel electrophoresis was run on a 2 % agarose gel with 4 µl sample at 100 V.
- The gel was colored in an ethidium bromide solution with a concentration of 0.5 µg/ml.

The points above were repeated until the DNA of the untreated cells had a length between 200 and 600 base pairs. When this was achieved all samples were treated as following:

- 8 impulses of 20 seconds and 70 % power to each lysate.

- An aliquot of 20 µl of each sample was taken and 80 µl of water and 5 µl of proteinase K were added.

- The aliquots were incubated for 30 minutes at 37°C and over night for 65°C.

- On the next day the DNA was purified with the QIAquick PCR purification kit from Qiagen.

- A gel electrophoresis on a 2 % agarose gel under conditions mentioned above showed the length of the DNA.

Immunoprecipitation

As negative controls one additional aliquot of each sample was treated as described following but without adding the antibody.

- Two times 100 µl of each sample were diluted with 900 µl dilution buffer and 100 µl lysis buffer.

- 8 µl of the specific antibody was applied to each sample which is up to 4 µg of the antibody.

- The tubes are fixed on an overhead rotator and stored over night at 4°C

Simultaneously the protein beads were prepared for the further procedure:

- For each sample 0.2 g of protein A beads are diluted in 1 ml of a mixture of dilution buffer and lysis buffer in a proportion of 9:1.

- 120 µl of bovine serum albumin (BSA) which is up to a final concentration of 500 µg/ml and 60 µg of sheared salmon sperm DNA were added.

- The beads rotated over night at 4°C.

- On the next day the beads were centrifugated at 2000 rpm for 1 minute and supernatant was discarded.

- A wash solution was prepared from dilution buffer and lysis buffer in a proportion 9:1.

- The beads were washed twice with approximately 2 ml of wash solution and centrifugated carefully at 2000 rpm for 1 minute between the washes.

- Finally the beads were resuspended in 1 ml of the buffer mixture.

- 100 µl of the resuspended beads were added to each tube (samples and negative controls)

- The tubes rotated for two hours at 4°C.

- After centrifugation at 2000 rpm for one minute the supernatant was discarded.

- The protein beads with the absorbed immune complexes were washed four times with 1 ml of wash buffer. The tubes were rotated for 10 minutes at 4°C containing the wash buffer and then centrifugated at 2000 rpm for 1 minute between the washes.

- A fifth wash was performed using 1 ml of final wash buffer.

- After centrifugation under conditions used before the supernatant was removed carefully and discarded.

- 150 µl of elution buffer, 15 µl of proteinase K, and 15 µl of RNase A leading to a final concentration of proteinase K and RNase of 500 µg/ml were added to all tubes containing samples, negative controls, and inputs.

- All tubes were warmed to 37°C for 30 minutes and afterwards incubated at 65°C over night to reverse the crosslinking.

- On the next day the DNA was purified with the QIAquick PCR purification kit from Qiagen and eluted with 100 µl of elution buffer.

To analyze the DNA after the ChIP procedure a Sybr Green real-time PCR system was used. Primers and annealing temperature used are listed in table 14

Primer	Sequence	Annealing temp	concentration
IL-8 sense	GAA GTG TGA TGA CTC AGG	62°C	5 pmol/µl
IL-8 antisense	GAA GCT TGT GTG TGC TCAG C	62°C	5 pmol/µl

Table 11: Primer concentrations, annealing temperature and primer concentrations for ChIP PCR

Sample composition:

SensiMix	5 µl
water	2.8 µl
primer	1 µl
Sybr Green	0.2 µl
DNA	1 µl
total volume	**10 µl**

Table 12: PCR reaction mix for ChIP PCR

Cycling conditions:

Polymerase activation	10 min	95°C
Cycling	35 times	
Denaturation	45 sec	95°C
Annealing	60 sec	62/67°C
Elongation	60 sec	72°C
Melt curve	0.5°C every 5 sec	50°C to 95°C

Table 13: Cycling conditions for ChIP PCR
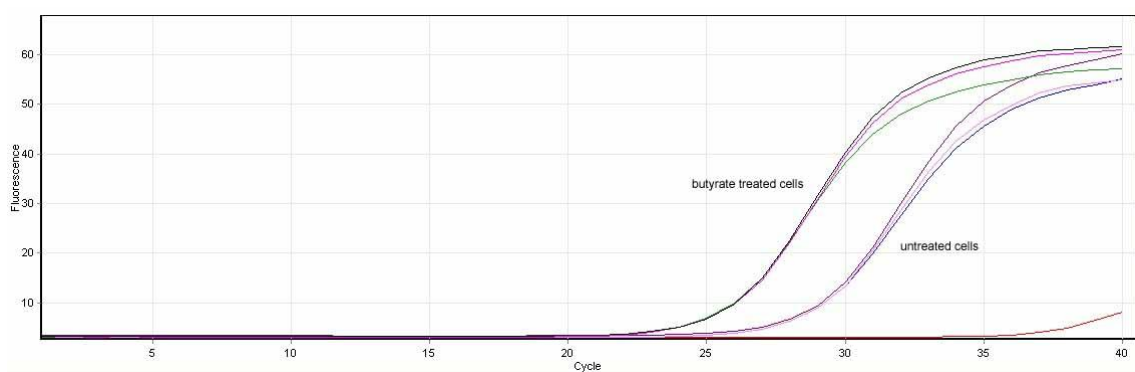
Figure 17: Real-time PCR curves of the ChIP reaction

3.7. Data and Statistical analysis

The results of the real-time PCR were analyzed with the software Rotor-Gene Version 6.1 belonging to the Corbett Rotor Gene 300 real time cycler. Hypothetic concentrations were calculated by the software and the amplification efficiency was adjusted between individual samples. Average and the standard deviations were calculated with Microsoft Office Excel 2007. A two tails t-test was used to determine the significance of the results, which also was performed in Microsoft Office Excel 2007. Results were regarded as significantly different at $p < 0.05$.

4. <u>RESULTS</u>

4.1. IL-8 ELISA
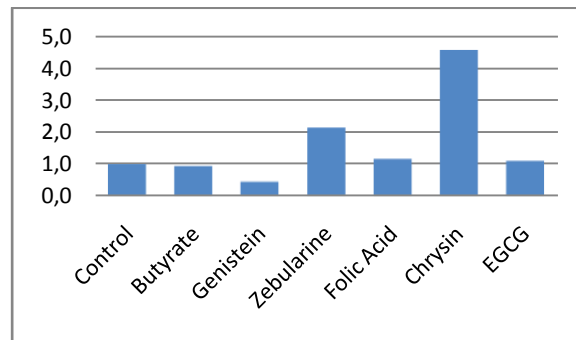
Figure 18: IL-8 ELISA

Compared to the untreated cells the treatment with Zebularine and Chrysin for lead to a significant 2.1 and 4.5 fold increase in IL-8 production ($p<0.01$). Genistein decreased the IL-8 concentration of the cell supernatant to halve compared to the untreated cells ($p=0.01$). Butyrate (0.9), folic acid (1.2), and EGCG (1.1) did not alter the IL-8 concentration significantly.

4.2. IL-8 gene expression
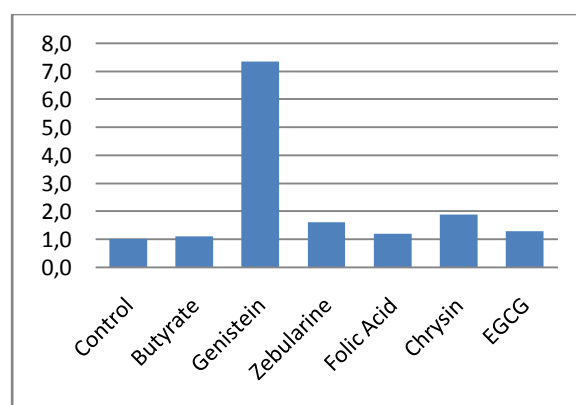
Figure 19: mRNA gene expression of IL-8

In contrast to ELISA results gene expression analysis on the mRNA level showed a 7.4 fold enhanced IL-8 expression after cell treatment with genistein. Zebularine (1.6) and Chrysin (1.9) lead to a slight increase of IL-8, but no changes could be observed for butyrate (1.1), folic acid (1.2), and EGCG (1.3).

4.3. DNA-Methylation on CpGs -1342 and -1412
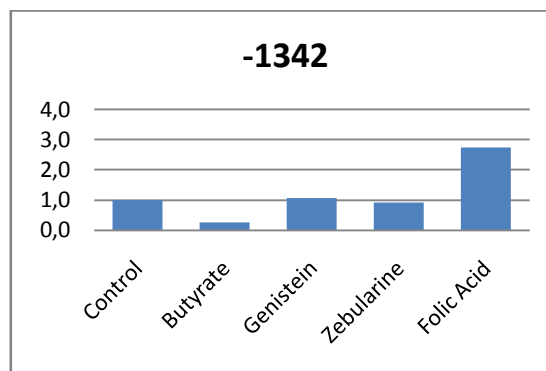
Figure 20: DNA methylation on CpG -1342
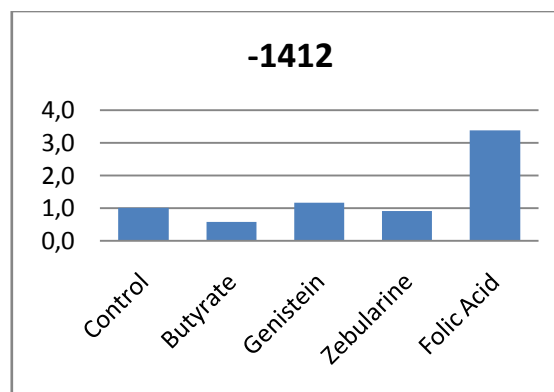
Figure 21: DNA methylation on CpG -1412

A clear 2.8 fold/3.4 fold hypermethylation was observed after treatment with folic acid on CpG-sites -1342/-1412 compared to the untreated cells. Butyrate led to a decrease of the methylation grade on the site -1342 to 0.3 and on -1412 to 0.6. Genistein (-1342: 1.1; -1412: 1.2) and zebularine (-1342: 0.9; -1412: 0.9) showed only slight changes in the methylation pattern of both CpG-sites.

4.4.   ChIP
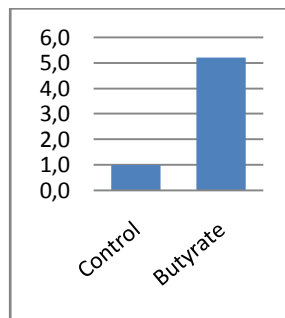
Figure 22: ChIP of H3K9

ChIP was performed with cells after treatment with the HDAC inhibitor butyrate. The acetylation of H3K9 was 5.2 fold increased, this was significant ($p < 0.01$).

5. <u>DISCUSSION</u>

5.1. Butyrate

Butyrate is known as HDAC inhibitor. Thus no changes in the grade of DNA methylation could be expected. However, a significant decrease of the methylation state of the two CpG-sites on the IL-8 gene was observed. One possible explanation for that might be the apoptotic effect of butyrate involving the caspase-3 pathway. Activated caspase-3 cleaves several substrates including the DNA repair enzyme pol-(ADP-ribose)polymerase (PARP) leading to DNA breakdown. The decrease in methylation might be due to a beginning degradation of the DNA. (Ruemmele et al., 1999)

Only few studies were made to investigate the influence of butyrate on the methylation pattern of genes. In one of these a hypermethylation of the retinoblastoma gene in the human colon tumor cell line HT29 was observed. This confirms our findings, which also show an effect of butyrate on DNA methylation. (Gope and Gope, 1993) The present findings suggest a hypomethylation of the IL-8 gene through butyrate treatment. Further investigation will be necessary to prove these results.

The histone acetylation on H3K9 significantly increased after treatment with butyrate. This result was expected because butyrate is known as HDAC inhibitor. Acetylation of the chosen site was shown to correlate with the nucleosome structure. Changes in acetylation have previously been linked to a change in gene expression. (Michishita et al., 2008; Strasak et al., 2009) The lysine residue 9 is located at the end of the histone. That is why modifying enzymes can easily access this site. The impact of agents on histone acetylation can thus be observed on lysine 9 more clearly than on others. Even though only about 2 % of all human genes are responsive to butyrate treatment (Davie, 2003) IL-8 is known to be regulated by this SCFA. (Chavey et al., 2008; Davie, 2003; Della Ragione et al., 2001). Butyrate acts as a non-competitive HDAC inhibitor particularly affecting class I and II HDACs. (Rada-Iglesias et al., 2007)

The IL-8 expression did not show significant changes after butyrate treatment on mRNA or protein level in spite of increased histone acetylation. It has been reported that an increased mRNA level after butyrate treatment is not only caused by altered histone acetylation state

but also by post transcriptional events (Hirsch and Bonham, 2004). It is a transient event. However, Peart et al. observed that HDAC inhibitor treatment results in an early response of gene activation, but gene repression increased over time. After 16 hours of HDAC inhibitor treatment more genes were repressed than activated. (Peart et al., 2005) Considering these results, it might be possible that peak activation had already been over and thus remained undetected after 48 hours.

Butyrate enhances the rate of apoptosis in a dose and time depending manner. Apoptotic effects might have rendered impossible to identify changes in gene expression. At a concentration of 5mM, which was used in this experiment, the activity of apoptotic enzyme caspase 3 was significantly increased. (Ruemmele et al., 1999) Even though butyrate should show decreased IL-8 expression due to its anti-inflammatory properties the opposite was reported when the Caco-2 cells were immune activated before the treatment. (Wen and Wu, 2001) So although other results have been expected, the recent findings can be explained by the reasons mentioned above.

5.2. Genistein

Treatment with 200 µmol/l genistein did not result in significant changes in methylation pattern of the two investigated CpGs. Li et al. explored the influence of genistein on breast cancer cell lines and linked it to telomerase activity. They found a decreased expression of DNMTs leading to a hypomethylation of several genes associated with telomerase activity and cancer. But this altered methylation pattern could not be seen in all genes determined. (Li et al., 2009)

Another hint of the methylating potential of genistein was shown in the agouti mouse model. Supplementation of genistein to the mother´s diet shifted the coat color of the offspring towards pseudoagouti. The methylation grade of the investigated CpGs increased. (Dolinoy, 2008)

Genistein is an anti-inflammatory agent. Decreased IL-8 level as shown by ELISA confirmed several previous studies that found a decreased expression. But the mRNA level of IL-8 increased significantly after genistein treatment for 48 hours. Genistein was seen to slow down cell growth in cancer cell lines and hinder DNA synthesis. The increased IL-8 expression could thus be a symptom of altered cell cycle and beginning apoptosis. (Handayani et al.,

2006) Another possible explanation might be a generalized inflammatory response of the Caco-cells due to the addition of genistein to their media in excess concentrations. Genistein regulates gene expression in a very complex way and so the manner how transcription is influenced is hard to predict. Despite the effect on the expression of the HDAC SIRT1, which is lowered by genistein (Kikuno et al., 2008) and the decreased expression of DNMTs (Li et al., 2009) the estrogenic impact of genistein can also alter the gene expression. It binds to estrogen receptor alpha (ERα), which acts as a transcription factor upon activation. The activated ERα increases the activity of HATs introducing another epigenetic mechanism. (Hong et al., 2004) So even though genistein was objective in many studies until now, further investigations will be necessary to understand the holistic mechanism by which genistein influences human gene expression.

5.3. Folic acid

Folic acid increased the methylation grade of the investigated CpG-sites as expected because of its function as methyl group donor. Jang et al. observed cell cultures under condition of folate depletion and analyzed their methylation state. They determined a global DNA hypomethylation as expected but surprisingly some regions were hypermethylated. For example a 5´-CpG island of the H-cadherin gene showed hypermethylation and down regulation of gene expression. Presumably, this is a compensatory response. (Jang et al., 2005) Due to observations in rat liver tissue it was suggested that a hypo- or hypermethylation introduced by folic acid acts in a site and gene specific manner. Not all responses might be in the same direction. These results were confirmed in cell culture studies including Caco-2 cells. Studies in rats showed that DNA hypomethylation did not occur in colon-cells by folate deficient diet suggesting that the colorectum may be resistant to the hypomethylating effect. It was also suggested that a folate deficiency of a moderate degree or short duration induces DNA hypermethylation (as it was seen in rodent liver tissue) due to compensatory up regulation of DNMTs. In humans a low folate intake decreased the global DNA methylation grade of leukocytes. Colon DNA methylation was positively correlated with serum and red blood cell folate concentration and negatively correlated with plasma homocysteine. (Kim, 2004b)

Although in general an increased methylation leads to a decreased gene expression no significant alteration in the IL-8 expression was seen on protein or mRNA level. This might be

due to too short incubation time. Studies in rodents fed a diet deficient in different combinations of methyl group donors induced genomic and protoonocogene DNA hypomethylation and elevated levels of corresponding mRNAs. (Kim, 2004b) Methylation on specific CpG sites regulates gene expression in differentiated way independent from the global DNA methylation grade. Cell culture studies showed that folate depletion can lead to an up regulation as well as a down regulation of different genes. The expression of genes involved in one carbon metabolism is enhanced to compensate the lack of folic acid. (Jang et al., 2005) The expression of DNMTs is also up regulated. (Kim, 2004b) In animal studies this response to the folate state of an individual depends on age. Depletion or supplementation of folic acid showed an influence of cancerogenisis relevant genes like p53 and ILGF2 associated with age of the laboratory animals. (Jang et al., 2005)

Folic acid increases the availability of SAM which is also methyl donor for histone methylation enzymes. So it seems possible that not only DNA methylation affects gene expression after folate treatment but also epigenetic pathways caused by altered histone methylation. Due to the concentration and duration of folic acid treatment in this experiment this histone effect might abolish the effect of DNA methylation on gene expression.

5.4. Zebularine

Although zebularine is a known DNMT inhibitor, no significant changes of the methylation state on the investigated CpGs could be seen. This might be due to an improper duration of treatment because in several studies alterations of DNA methylation could only be observed after more than two days. (Billam et al., 2009; Marquez et al., 2005) The following mechanism must also be considered: DNMTs are posttranscriptionally trapped by zebularine with DNMT1 as special target. DNMT3a and DNMT3b are just partially reduced. Since DNA is not actively demethylated by zebularine, treated cells may still retain substantial methylation patterns. (Billam et al., 2009)

Even though an increase of IL-8 protein could be observed in the ELISA test, no significant change was found on the mRNA level. This result is not surprising because no change in DNA methylation has been determined. The effect of zebularine an expression of the tumor suppressor gene p16 was found to depend on the investigated cell line. So perhaps even though the Caco-2 cell line is a cancer cell line, its response to zebularine treatment is

weaker compared to other cancer cells. (Cheng et al., 2004) Zebularine does not only alter gene expression via its influence on DNA methylation. It is assumed that zebularine reactivates silenced genes through an enrichment of histone acetylation on promoter regions. (Billam et al., 2009)

5.5.   Epigallocatechin-3-gallate (EGCG)

EGCG can affect gene expression through several epigenetic mechanisms mentioned in introduction. But no significant changes were seen either in the ELISA test or on mRNA level. The reasons could be manifold. The incubation time might be too short to see any effects. In a study of Fang et al. the mRNA levels after treatment with EGCG started to rise after 72 to 144 hours depending on the gene although an altered methylation pattern could already be seen after 48 hours. (Fang et al., 2007) One pathway by which EGCG acts is the demethylation of E2F-1 binding sites and enhanced binding of this transcription repressor. (Berletch et al., 2008) This regulatory pathway might not be involved in the IL-8 gene at all. This might explain unchanged IL-8 gene expression. Due to the inhibition of NF-κB by EGCG gene relevant in inflammatory, e.g. IL-6, processes showed reduced expression after treatment. (Choi et al., 2009) No such effect was seen in this study but the effects of EGCG were described as cell line and gene specific. (Fang et al., 2007)

5.6.   Chrysin

Epigenetic gene regulation by chrysin seems possible due to its manifold effects. It was described as an antioxidant, anti-allergic, anti-inflammatory, anti-cancer, anti-estrogenic, and anxiolytic agent. (Sathiavelu et al., 2009) Chrysin treatment of Caco-2 cells in 50 µmol/l caused a significant increase of IL-8 protein abundance but only a slight increase in mRNA. These results are contradictory to the postulated anti-inflammatory properties of chrysin. Anyhow, anti-inflammatory effects cannot be explained by a single regulator. The reaction of Caco cells to treatment with chrysin might also be due to a slightly toxic effect of the treatment. Since no epigenetic investigations with chrysin were published till now further research might be necessary to determine physiological treatment conditions.

6. <u>SUMMARY</u>

Epigenetic is defined as changes in gene expression without changes in the DNA sequence and can lead to a shift in phenotypes. There are three epigenetic mechanisms: DNA methylation, histone modifications, and interference of small noncoding RNAs. Gene expression and epigenetic marks can be influenced by environmental factors like nutrition and toxins and they are inheritable over generations. Especially mother´s diet during early pregnancy can impact the embryonic epigenome and the phenotype of the offspring.

In this experiment cells of the human colon carcinoma line Caco-2 were treated with butyrate, genistein, folic acid, zebularine, Epigallocatechin-3-gallate (EGCG), and chrysin. Most of these agents are food components and are known to have epigenetic effects on gene regulation. After the treatment the gene expression, methylation state and histone acetylation of specific sites of the interleukin 8 (IL-8) gene were investigated. IL-8 is an inflammatory mediator which induces chemotaxis. Aberrant chemokine expression plays a critical role in many inflammatory disorders including chronic inflammatory bowel disease.

IL-8 is known to be regulated by epigenetic pathways including DNA methylation and histone acetylation. Contrary to the most genes the regulatory methylation sites are not in the promoter region but about 1.2 kilo bases upstream.

The study aimed the investigation of the effect of specified nutrients on inflammatory mediators on the example of IL-8.

Gene expression was analyzed by ELISA and real-time PCR, DNA methylation by bisulfite sequencing PCR (BSP) and histone acetylation by Chromatin immunoprecipitation (ChIP).

The results showed that butyrate decreased the methylation grade and increased the histone acetylation on histone H3 lysine 9 but no changes in the gene expression could be observed. Folic acid enhanced DNA methylation as it was expected but did not alter gene expression. Gene expression was increased by genistein, zebularin and chrysin.

ZUSAMMENFASSUNG

Epigenetik ist definiert als Änderungen in der Genexpression ohne Änderung der DNA-Sequenz und kann zu einer Veränderung des Phänotyps führen. Es gibt drei epigenetische Mechanismen: DNA Methylierung, Histonmodifikationen und Wechselwirkungen mit kleinen, nicht-codierenden RNAs. Genexpression und epigenetische Marker können von Umweltfaktoren wie Ernährung und Toxinen beeinflusst werden. Speziell die Ernährung der Mutter in der frühen Schwangerschaft hat Auswirkungen auf das embryonale Epigenom und den Phänotyps des Nachwuchses.

In diesem Experiment wurden Zellen der humanen Colonkrebslinie Caco-2 mit Butyrate, Genistein, Folsäure, Zebularin, Epigallocatechin-3-gallat (EGCG) und Chrysin behandelt. Die meisten dieser Substanzen sind Nahrungsmittelinhaltsstoffe und haben epigenetisch Effekte auf Gene. Die Genexpression, der Methylierungsgrad und die Histonacetylierung an bestimmten Stellen des Interleukin 8 (IL-8) Gens wurden an den behandelten Zellen untersucht. IL-8 ist ein Entzündungsmediator, der Chemotaxis induziert. Abweichende Expression der Chemokine spielt eine kritische Rolle in vielen entzündlichen Erkrankungen wie z.B. in chronisch entzündlichen Darmerkrankungen. Es ist bekannt, dass IL-8 über epigenetische Mechanismen der DNA Methylierung und Histonacetylierung reguliert wird. Im Gegensatz zu den meisten Genen, befinden sich die regulatorischen Methylierungsstellen nicht in der Promoterregion sondern ca. 1,2 Kilobasen aufwärts.

Diese Studie hatte zum Ziel, Effekte von bestimmten Nahrungsstoffen auf Entzündungsmediatoren am Beispiel von IL-8 zu untersuchen.

Genexpression wurde mittels ELISA und real-time PCR, DNA Methylierung mittels bisulfite sequencing PCR (BSP) und Histonacetylierung mittels Chromatin Immunopräzipitation analysiert.

Die Resultate zeigten, dass Butyrat den Methylierungsgrad herabsetzte und die Histonacetylierung am Histon H3 Lysin 9 steigerte, aber zu keinen Änderungen in der Genexpression führte. Folsäure steigerte wie erwartet die DNA Methylierung aber die Genexpression wurde ebenfalls nicht verändert. Genistein, Zebularin und Chrysin beeinflussten die Genexpression.

7. <u>PUBLICATIONS</u>

7.1. Draft paper

Epigenetic regulation of IL-8 expression by histone acetylation and DNA methylation is modulated by food ingredients in CACO 2 cells

Roman Thaler[1], Eva Aumueller [1], Karolina Kubala[1], Petra Klein [1], Alexander G. Haslberger[1]
Department of Nutritional Sciences, University of Vienna
Althanstrasse 14, A- 1090 Vienna, Austria

Corresponding author:
Alexander G. Haslberger
Institute of Nutritional Sciences of the University of Vienna,
Althanstrasse 14, UZAII
1090 Vienna, Austria
Contact
Tel. +43 1 4277 54996
e-mail: alexander.haslberger@univie.ac.at

Abstract
High ectopic expression of IL-8 associated with changes of methylation in CpG islands or histone modification is seen in cells of various tumors and is correlated with metastatic potential and tumor growth. This study examined the relevance of methylation of defined CpG sites and histone deacetylation as well as food ingredients effecting epigenetic pathways for the expression of IL-8 in undifferentiated, preconfluent CACO 2 cells.
CACO-2 cells were treated with genistein, butyrate, zebularine, folic acid, EGCG, and chrysin for 48 hours. We analyzed gene expression by real time PCR, DNA-methylation by bisulfite sequencing PCR, and histone acetylation by Chromatin immunoprecipitation.
Only IL-8 increased IL-8 expression significantly. Folic acid stimulated the IL-8-DNA-methylation on the sites -1342 and -1412. Butyrate stimulated the histone acetylation on H3 lysine 9 at IL-8 and demethylated both investigated CpGs. Genistein and butyrate also stimulated apoptosis relevant caspase-3. Changes in butyrate producing GI-microbiota (*Clostridium* cluster IX and XIVa) were found due to nutrition.
These results suggest that DNA methylation of the CpGs at - 1342 and - 1412 bp as well as histone modulation participate in the epigenetic control of IL-8 expression and metastatic characteristics. Furthermore, dietary strategies may be effective in the modulation of multiple inflammatory diseases where IL-8 expression plays an important role.

Introduction.

Expression of chemokines such as Interleukine-8 is pivotal for tumourgenesis and in the development of chronic inflammation such as Crohn´s disease and inflammatory bowel diseases (Demetter et al., 2000). Gene expression of chemokines is tightly regulated because of the potent effects induced by even minute quantities of such proteins in an autocrine, paracrine or hormonally derived way (Kitadai et al., 2000; Tuschil et al., 1992; Wen and Wu, 2001). Under normal conditions, IL-8 expression is restricted to a few kinds of cells including monocytes, endothelial cells, epithelial cells and fibroblasts and is stimulated by pro-inflammatory cytokines, stress and viral infections (Elenkov, 2008; Mastronarde et al., 1996). High ectopic expression of IL-8 is seen in several undifferentiated tumour cells and is positively correlated with the metastatic potential and tumour growth of melanoma cells, lung tumours, breast tumour tissues and colon carcinoma cells (Bar-Eli, 1999; Cheng et al., 2004; De Larco et al., 2003; Li et al., 2001; Singh et al., 1999). The CACO-2 cell-line is in the preconfluent proliferative state relatively undifferentiated and shows ectopic IL-8 expression which is inhibited after spontaneous differentiation (Li et al., 2001; Wen and Wu, 2001). Activation of IL-8 expression by immune mediators is primarily controlled by a basal promoter located immediately upstream of the transcriptional start site which includes four cis-regulatory elements: NF-κB at - 82 to - 70, CCAAT/enhancer-binding protein (C/EBP) at - 94 to - 84, activator protein 1(AP1) at - 126 to - 120 as positive acting elements and Oct-1 at - 90 to - 83 as repressor of IL-8 transcription (Fraga et al., 2005; Matsushima et al., 1989). Wen et al (Wen and Wu, 2001) demonstrated that in CACO-2 cells, immune-activated transcription of the IL-8 gene is silenced upon differentiation through epigenetic histone deacetylation by elements located outside of the immediate 5´-flanking region of the gene. The same study showed a basal transcriptional repression of the gene by an epigenetic, but histone deacetylase independent mechanism that requires elements located several kilobases away from the gene locus. In addition modulation of the nuclear factor κB pathway by histone deacetylases has been shown to regulate IL-8 expression (Chavey et al., 2008). In a study (De Larco et al., 2003), comparing high and low metastatic breast carcinoma cell lines, a strong, positive correlation between the methylation status of two CpG sites located at - 1342 and - 1412 bp upstream of the transcriptional start site and ectopic expressed IL-8 mRNA was identified.

The SCFA butyric acid is produced by the gastrointestinal microbiota through the breakdown of non-digestible fibers. The epigenetic effect of butyrate is mainly caused by the non-competitive inhibition of HDACs, especially class I and II, leading to a histone hyperacetylation. An up-regulation of tumor suppressor genes after treatment with butyrate has been reported and an increased histone acetylation in the promoters of these genes was detected. (Rada-Iglesias et al., 2007) Only the expression of 2 % of all mammalian genes is influenced through the HDAC inhibitoring activity of butyrate suggesting the existence of a butyrate response element in the promoter regions of the affected genes. (Davie, 2003) In concentration from 5 to 100mM butyrate shows apoptotic activity involving the caspase-3-pathway. (Ruemmele et al., 1999)

Genistein (4´,5,7-trihydroxyflavone) is an isoflavonoid naturally occurring in soy. (Li et al., 2001) It modulates the activity of the transcription factors NF-κB and the fork head transcription factors (FOXO) influencing apoptosis indirectly. Genistein lowers the expression of SIRT1 and so increases the histone acetylation of H3K9 in several gene promoter regions like PTEN and FOXO3a. (Kikuno et al., 2008)

Folic acid is a water soluble vitamin and is essential for numerous body functions because it acts as one-carbon donor, e.g. for SAM. Since cancer cells have aberrant DNA methylation pattern folate intake and blood levels in an individual might modify its susceptibility to cancer. (Miller et al., 2008)

Zebularine is a cytidine analog and was originally developed as a cytidine deaminase inhibitor. It acts as DNMT inhibitor by forming tight covalent complexes between DNMT proteins and zebularine-substituted DNA. (Billam et al., 2009) The incorporation rate was higher in cancer cells than in normal fibroblasts. In preliminary experiments the response to the treatment was higher in cancer cells. (Cheng et al., 2004)

Epigallocatechin-3-gallate (EGCG) is the major polyphenol of green tea accounting for more than 50 % of all green tea polyphenols. It is known to inhibit the activity of DNMT1. (Berletch et al., 2008) EGCG also significant reduced acetylation of histone due to its action as HAT inhibitor. Another mechanism induced by EGCG is the suppression of the transcription factor NF-κB, which is involved in inflammatory processes. (Choi et al., 2009)

Chrysin is a flavonoid naturally occurring in many plants, honey, and propolis. It was shown to possess antioxidant, anti-allergic, anti-inflammatory, anti-cancer, anti-estrogenic, and anxiolytic properties. No epigenetic investigations of chrysin have been published by now. But due to the manifold effects shown by chrysin epigenetic pathways and gene regulation e.g. for antioxidative genes seem possible.

In the present work we analyzed the impact of food ingredients on the cytosine methylation of the CpGs located at - 1342 and at - 1412 on IL-8 mRNA expression in CACO-2 cells. The effects of diets on epigenetic regulation of gene expression was shown by our group before (Thaler et al., 2008).

Methods.

Cell line and culture conditions. The human colon carcinoma cell line CACO-2 was grown in six well tissue culture plates and cultured in DMEM media supplemented with 10 % heat-inactivated foetal bovine serum, 1 % L-Glutamin (200 mM) and 1 % of penicillin-streptomycin solution (10 000 U penicillin, 10 mg streptomycin/ml, 0,9 % NaCl) at 37°C in a humidified atmosphere of 5 % CO_2 in air. Growth medium was replaced every 2-3 days. The CACO-2 cells were cultured for 6 days to achieve undifferentiated cells. Cell treatments were performed in duplicates with folic acid, vitamin B_{12}, genistein, valproate, valproate plus genistein and zebularine respectively at a concentration of 200 µmol/l for 48 hours. Cells were washed twice with cooled PBS (4°C) before nucleotide extraction.

Genomic DNA isolation and bisulfite modification. DNA was isolated from cells using a DNA Isolation Kit (Qiagen) and bisulfite conversion for detection of unmethylated cytosines was performed using the Epitect Bisulfite Kit (Qiagen). Both kits were used according to the manufacturer's instructions.

Analysis of CpG methylation. Analysis of methylated CpGs was performed by bisulfite genomic sequencing PCR (BSP). To increase specificity of PCR after DNA conversion, a nested PCR approach was used. Characteristics of the diverse primers are listed in table 1. All external PCRs were performed in a 10 µl final volume using the Promega PCR Master Mix, (Wisconsin, USA) with the following PCR-conditions: 95°C for 10 min, 35 cycles at 95°C for 30 sec, 50 sec at a specific temperature and 1 min at 72°C and 5 min at 72°C. For internal PCR, samples were diluted 1:100 and amplified with the same master mix in a 50 µl final volume. After purification samples were subsequently directly sequenced by an ABI sequencing system. Finally, at the interested CpG sites, signal heights were directly compared using the

software Sequence Scanner, version 1.0 from Applied Biosystems. No significant differences were seen between sequencing of cloned samples compared with directly sequenced samples.

Analysis of gene expression. Total mRNA was extracted from cells using the NucleoSpin RNA II Kit (Macherey-Nagel, Dueren, Germany) and reverse transcribed using the single-strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Gene expression was performed in triplicates by Sybr Green real-time PCR using the Corbett Rotor-Gene 3000. The target mRNA expression was normalized to the glucose-6-phosphate dehydrogenase (G6PD) mRNA expression which was found to be stable expressed after the listed cell-treatments. The primers for gene expression are characterized in table 1. The following thermo-cycling conditions were selected: 95°C for 10 minutes followed by 35 cycles at 95°C for 45 sec, further 60 sec 66°C and 1 min at 72°C. All reactions were set-up in a 10 µl final volume using the master mix SensiMix DNA Kit, (Quantace, London). Optimum reaction conditions were obtained with 5 µl 2x PCR master mix, 1 µl of each primer, 2 µl H_2O and 2 µl template (cDNA).

Analysis of histone acetylation by Chromatin Immunoprecipitation (ChIP). Cells were treated with formaldehyde to crosslink DNA and histones. The complexes were sonicated to get fragments of 200 to 600 base pairs length. For immunoprecipitation 4 µg of an antibody specific for acetylation on histone H3 lysine 9 (H3K9) was added to each sample and the immune complexes were enriched at protein A beads. The DNA was purified out of the immune complexes and quantified by real time PCR (primers listed in table 1).

Results.

IL-8 CpG methylation at - 1342 and - 1412 in CACO-2 cells

A clear 2.8 fold/3.4 fold hypermethylation was observed after treatment with folic acid on CpG-sites -1342/-1412 compared to the untreated cells. Butyrate led to a decrease of the methylation grade on the site -1342 to 0.3 and on -1412 to 0.6. Genistein (-1342: 1.1; -1412: 1.2) and zebularine (-1342: 0.9; -1412: 0.9) showed only slight changes in the methylation pattern of both CpG-sites.

Modifications of epigenetic control of IL-8 gene expression

In contrast to ELISA results gene expression analysis on the mRNA level showed a 7.4 fold enhanced IL-8 expression after cell treatment with genistein. Zebularine (1.6) and Chrysin (1.9) lead to a slight increase of IL-8, but no changes could be observed for butyrate (1.1), folic acid (1.2), and EGCG (1.3).

Chromatin Immunoprecipitation of H3K9:

ChIP was performed with cells after treatment with the HDAC inhibitor butyrate. The acetylation of H3K9 was 5.2 fold increased, this was significant (p<0.01).

Discussion

Butyrate

Butyrate is known as HDAC inhibitor. Thus no changes in the grade of DNA methylation could be expected. However, a significant decrease of the methylation state of the two CpG-sites on the IL-8 gene was observed. One possible explanation for that might be the apoptotic effect of butyrate involving the caspase-3 pathway. Activated caspase-3 cleaves several substrates including the DNA repair enzyme pol-(ADP-ribose)polymerase (PARP) leading to

DNA breakdown. The decrease in methylation might be due to a beginning degradation of the DNA. (Ruemmele et al., 1999)

Only few studies were made to investigate the influence of butyrate on the methylation pattern of genes. In one of these a hypermethylation of the retinoblastoma gene in the human colon tumor cell line HT29 was observed. This confirms our findings, which also show an effect of butyrate on DNA methylation. (Gope and Gope, 1993) The present findings suggest a hypomethylation of the IL-8 gene through butyrate treatment. Further investigation will be necessary to prove these results.

The histone acetylation on H3K9 significantly increased after treatment with butyrate. This result was expected because butyrate is known as HDAC inhibitor. Acetylation of the chosen site was shown to correlate with the nucleosome structure. Changes in acetylation have previously been linked to a change in gene expression. (Michishita et al., 2008; Strasak et al., 2009) The lysine residue 9 is located at the end of the histone. That is why modifying enzymes can easily access this site. The impact of agents on histone acetylation can thus be observed on lysine 9 more clearly than on others. Even though only about 2 % of all human genes are responsive to butyrate treatment (Davie, 2003) IL-8 is known to be regulated by this SCFA. (Chavey et al., 2008; Davie, 2003; Della Ragione et al., 2001). Butyrate acts as a non-competitive HDAC inhibitor particularly affecting class I and II HDACs. (Rada-Iglesias et al., 2007)

The IL-8 expression did not show significant changes after butyrate treatment on mRNA or protein level in spite of increased histone acetylation. It has been reported that an increased mRNA level after butyrate treatment is not only caused by altered histone acetylation state but also by post transcriptional events (Hirsch and Bonham, 2004). It is a transient event. However, Peart et al. observed that HDAC inhibitor treatment results in an early response of gene activation, but gene repression increased over time. After 16 hours of HDAC inhibitor treatment more genes were repressed than activated. (Peart et al., 2005) Considering these results, it might be possible that peak activation had already been over and thus remained undetected after 48 hours.

Butyrate enhances the rate of apoptosis in a dose and time depending manner. Apoptotic effects might have rendered impossible to identify changes in gene expression. At a concentration of 5mM, which was used in this experiment, the activity of apoptotic enzyme caspase 3 was significantly increased. (Ruemmele et al., 1999) Even though butyrate should show decreased IL-8 expression due to its anti-inflammatory properties the opposite was reported when the Caco-2 cells were immune activated before the treatment. (Wen and Wu, 2001) So although other results have been expected, the recent findings can be explained by the reasons mentioned above.

Genistein

Treatment with 200 µmol/l genistein did not result in significant changes in methylation pattern of the two investigated CpGs. Li et al. explored the influence of genistein on breast cancer cell lines and linked it to telomerase activity. They found a decreased expression of DNMTs leading to a hypomethylation of several genes associated with telomerase activity and cancer. But this altered methylation pattern could not be seen in all genes determined. (Li et al., 2009)

Another hint of the methylating potential of genistein was shown in the agouti mouse model. Supplementation of genistein to the mother´s diet shifted the coat color of the

offspring towards pseudoagouti. The methylation grade of the investigated CpGs increased. (Dolinoy, 2008)

Genistein is an anti-inflammatory agent. Decreased IL-8 level as shown by ELISA confirmed several previous studies that found a decreased expression. But the mRNA level of IL-8 increased significantly after genistein treatment for 48 hours. Genistein was seen to slow down cell growth in cancer cell lines and hinder DNA synthesis. The increased IL-8 expression could thus be a symptom of altered cell cycle and beginning apoptosis. (Handayani et al., 2006) Another possible explanation might be a generalized inflammatory response of the Caco-cells due to the addition of genistein to their media in excess concentrations. Genistein regulates gene expression in a very complex way and so the manner how transcription is influenced is hard to predict. Despite the effect on the expression of the HDAC SIRT1, which is lowered by genistein (Kikuno et al., 2008) and the decreased expression of DNMTs (Li et al., 2009) the estrogenic impact of genistein can also alter the gene expression. It binds to estrogen receptor alpha (ERα), which acts as a transcription factor upon activation. The activated ERα increases the activity of HATs introducing another epigenetic mechanism. (Hong et al., 2004) So even though genistein was objective in many studies until now, further investigations will be necessary to understand the holistic mechanism by which genistein influences human gene expression.

Folic acid

Folic acid increased the methylation grade of the investigated CpG-sites as expected because of its function as methyl group donor. Jang et al. observed cell cultures under condition of folate depletion and analyzed their methylation state. They determined a global DNA hypomethylation as expected but surprisingly some regions were hypermethylated. For example a 5′-CpG island of the H-cadherin gene showed hypermethylation and down regulation of gene expression. Presumably, this is a compensatory response. (Jang et al., 2005) Due to observations in rat liver tissue it was suggested that a hypo- or hypermethylation introduced by folic acid acts in a site and gene specific manner. Not all responses might be in the same direction. These results were confirmed in cell culture studies including Caco-2 cells. Studies in rats showed that DNA hypomethylation did not occur in colon-cells by folate deficient diet suggesting that the colorectum may be resistant to the hypomethylating effect. It was also suggested that a folate deficiency of a moderate degree or short duration induces DNA hypermethylation (as it was seen in rodent liver tissue) due to compensatory up regulation of DNMTs. In humans a low folate intake decreased the global DNA methylation grade of leukocytes. Colon DNA methylation was positively correlated with serum and red blood cell folate concentration and negatively correlated with plasma homocysteine. (Kim, 2004b)

Although in general an increased methylation leads to a decreased gene expression no significant alteration in the IL-8 expression was seen on protein or mRNA level. This might be due to too short incubation time. Studies in rodents fed a diet deficient in different combinations of methyl group donors induced genomic and protoonocogene DNA hypomethylation and elevated levels of corresponding mRNAs. (Kim, 2004b) Methylation on specific CpG sites regulates gene expression in differentiated way independent from the global DNA methylation grade. Cell culture studies showed that folate depletion can lead to an up regulation as well as a down regulation of different genes. The expression of genes involved in one carbon metabolism is enhanced to compensate the lack of folic acid. (Jang et al., 2005) The expression of DNMTs is also up regulated. (Kim, 2004b) In animal studies this

response to the folate state of an individual depends on age. Depletion or supplementation of folic acid showed an influence of cancerogenisis relevant genes like p53 and ILGF2 associated with age of the laboratory animals. (Jang et al., 2005)

Folic acid increases the availability of SAM which is also methyl donor for histone methylation enzymes. So it seems possible that not only DNA methylation affects gene expression after folate treatment but also epigenetic pathways caused by altered histone methylation. Due to the concentration and duration of folic acid treatment in this experiment this histone effect might abolish the effect of DNA methylation on gene expression.

Zebularine

Although zebularine is a known DNMT inhibitor, no significant changes of the methylation state on the investigated CpGs could be seen. This might be due to an improper duration of treatment because in several studies alterations of DNA methylation could only be observed after more than two days. (Billam et al., 2009; Marquez et al., 2005) The following mechanism must also be considered: DNMTs are posttranscriptionally trapped by zebularine with DNMT1 as special target. DNMT3a and DNMT3b are just partially reduced. Since DNA is not actively demethylated by zebularine, treated cells may still retain substantial methylation patterns. (Billam et al., 2009)

Even though an increase of IL-8 protein could be observed in the ELISA test, no significant change was found on the mRNA level. This result is not surprising because no change in DNA methylation has been determined. The effect of zebularine an expression of the tumor suppressor gene p16 was found to depend on the investigated cell line. So perhaps even though the Caco-2 cell line is a cancer cell line, its response to zebularine treatment is weaker compared to other cancer cells. (Cheng et al., 2004) Zebularine does not only alter gene expression via its influence on DNA methylation. It is assumed that zebularine reactivates silenced genes through an enrichment of histone acetylation on promoter regions. (Billam et al., 2009)

Epigallocatechin-3-gallate (EGCG)

EGCG can affect gene expression through several epigenetic mechanisms mentioned in introduction. But no significant changes were seen either in the ELISA test or on mRNA level. The reasons could be manifold. The incubation time might be too short to see any effects. In a study of Fang et al. the mRNA levels after treatment with EGCG started to rise after 72 to 144 hours depending on the gene although an altered methylation pattern could already be seen after 48 hours. (Fang et al., 2007) One pathway by which EGCG acts is the demethylation of E2F-1 binding sites and enhanced binding of this transcription repressor. (Berletch et al., 2008) This regulatory pathway might not be involved in the IL-8 gene at all. This might explain unchanged IL-8 gene expression. Due to the inhibition of NF-κB by EGCG gene relevant in inflammatory, e.g. IL-6, processes showed reduced expression after treatment. (Choi et al., 2009) No such effect was seen in this study but the effects of EGCG were described as cell line and gene specific. (Fang et al., 2007)

Chrysin

Epigenetic gene regulation by chrysin seems possible due to its manifold effects. It was described as an antioxidant, anti-allergic, anti-inflammatory, anti-cancer, anti-estrogenic, and anxiolytic agent. (Sathiavelu et al., 2009) Chrysin treatment of Caco-2 cells in 50 µmol/l

caused a significant increase of IL-8 protein abundance but only a slight increase in mRNA. These results are contradictory to the postulated anti-inflammatory properties of chrysin. Anyhow, anti-inflammatory effects cannot be explained by a single regulator. The reaction of Caco cells to treatment with chrysin might also be due to a slightly toxic effect of the treatment. Since no epigenetic investigations with chrysin were published till now further research might be necessary to determine physiological treatment conditions.

In conclusion our results suggest that DNA methylation of various CpGs as well as histone modulation participates in the epigenetic control of IL-8 expression and that dietary strategies may be effective in the modulation of multiple inflammatory diseases where IL-8 expression plays an important role.

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7.2. Book chapter

for the book **"Epigenetics and Human Health, Linking hereditary, environmental and nutritional aspects"** (Editor: Dr. Alexander Haslberger)

Interaction of hereditary and epigenetic mechanisms in the regulation of immune and aging relevant gene expression

Roman Thaler, Eva Aumüller, Carolin Berner, Judith Schuster, Alexander G. Haslberger
Department for Nutritional Sciences, University of Vienna

Abstract Hereditary dispositions and environmental factors such as nutrition, natural and societal environment interact on human health. Diet compounds rise increasing interest due to their influence in co-regulating epigenetic gene expression. Nutrition and specific food ingredients have been shown to alter epigenetic markers such as DNA methylation or histone acetylation involving regulation of genes with relevance for fundamental mechanisms such as antioxidative control, cell cycle regulation or expression of immune mediators.

Hereditary dispositions

Interactions between genes and environment are not linear and often include direct and indirect cause of events. Many complex diseases are linked to various heritable dispositions like single nucleotide polymorphisms (SNP) or allelic translocations. Consequently they have become a main focus in modern biomedical research and also started to raise public interest. Single nucleotide polymorphisms are common rather than exceptions. SNP´s may determine the efficiency of gene transcription, gene translation or protein structure, leading to an altered amount of enzyme and/or –activity, thus influencing further metabolic pathways. A SNP can occur within coding sequences of genes, non-coding regions of genes or in intergenic regions. SNPs within a coding sequence do not necessarily change the functional efficiency of the protein (silent mutations) (Nothnagel et al., 2008) (Haiman and Stram, 2008).

Upon completion of the human genome project it was confirmed that 99.9 percent of the human genome's 3 million base pairs are identical in any person. The remaining 0.1 percent, (mainly SNPs) are assumed to play a key role in congenital sensitivity to disease and drug side effects. They also appear to influence peoples' variable responses to pharmaceuticals or different penetrance of the same disease. The International HapMap (haplotype map) Project aimed to study the scope of these SNP variations in different population groups. Clusters of SNPs located on the same chromosome tend to be inherited in blocks. It is expected that the outcomes of this project will provide crucial tools that allow researchers to detect genetic variations with effects on health and disease. In so called genome-wide association studies, researchers compare genomes of individuals with known diseases to a control group of healthy ones in order to detect suspicious tag SNPs which might play a role in development/genesis or course of disease. (HapMap project, http://snp.cshl.org/)

A variety of functional SNPs on several genes, covering almost all aspects for cell viability, is well described in literature. In superoxide metabolism for example, an increase in

superoxide radicals is given by the SNP (rs4880) regarding the mitochondrial Superoxide Dismutase (MnSOD) gene (Taufer et al., 2005). Several kinds of cancer (Kang et al., 2007), Alzheimer disease (Wiener et al., 2007), as well as an accelerated aging process are discussed to be related to this SNP. In opposite, three SNPs in the human forkhead box O3A gene (FOXO3A) were statistically significantly associated with longevity. Polymorphisms in this gene were indeed associated with the ability to attain exceptional old age, the FOXO3A association was considerably stronger in centenarians than in nonagenarians. In nutritional sciences, the methylenetetrahydrofolatereductase (MTHFR) gene is well known as it is essential for folic acid metabolism (Miyaki et al., 2008). The polymorphism C677T in the MTHFR gene is discussed to have an influence in several methylation pathways as for example in the DNA –methylation pathway.

So far, a multitude of ailments have been correlated with their respective SNPs. Yet, the predictability for the development of diseases from the analysis of SNPs still requires larger controlled studies. The present difficulty in utilizing SNPs analysis arises from missing the functional relevance of many SNPs. In addition, the mostly small and variable penetrance of single SNPs leads to statistical limitations and poor reproducibility in many clinical studies associating SNPs and diseases (Janssens et al., 2008).

The combination of an individual's relevant SNPs in addition to environmental influences may define his risk for developing diseases. Sets of candidate SNPs and experiences from the analysis of the work with biobanks need to be evaluated to understand gene – environment interactions (Vineis et al., 2007).

The epigenome

Despite of the genetic code, mammalian cells contain an additional regulatory level which predominates over the DNA code: modification of gene expression by altering chromatin. Thus, due to different chromatin status, same genetic variants might be for example associated with opposite phenotypes depending on different environmental influences. New insights in research clarify the molecular pathways by which, amongst others, nutrition and lifestyle factors influence chromatin packaging and further health status. Indeed several studies clearly correlate the risk for developing multifactorial chronic diseases like cancer, diabetes or obesity with nutritional and lifestyle habits (Jirtle and Skinner, 2007; Johanning et al., 2002; Mann et al., 1999; Tsugane and Sasazuki, 2007).

Measurable consequences of these influences are alterations in the expression of a growing number of genes, caused by remodeling of chromatin and DNA methylation. Methylation of cytosines in DNA, histone modifications as well as alterations in the expression level of micro RNA (miRNA) and short interference RNA (siRNA) are the mechanisms involved in chromatin remodelling. This topic is being studied by the fast evolving research area of epigenetics. The term "epigenome" is used to define a cell's overall epigenetic state. Epigenetic modifications can be stably passed over numerous cycles of cell division. Some epigenetic alterations can even be inherited from one generation to the next (Anway et al., 2006b; Dolinoy et al., 2006b). Studies conducted by the Department of Community Medicine and Rehabilitation of the Umeå University in Sweden showed transgenerational effects due to nutritional habits during a child's slow growth period (SGP), the phase of the prepubertal peak in growth velocity. Clear correlations with descendants' risk of death from cardiovascular disease and diabetes were also seen (Kaati et al., 2002). The finding that monozygotic twins are epigenetically indistinguishable early in life but, with age exhibit substantial differences in the epigenome, indicates that environmentally determined alterations in a cell's epigenetic markers might be responsible (Fraga et al., 2005).

During the development of germ cells and during early embryogenesis DNA is specifically methylated and these markers confer genome stability, imprinting of genes, totipotency, correct initiation of embryonic gene expression and early lineage development of the embryo (Morgan et al., 2005). According to experiments in the Agouti mouse model, early epigenetic programming is alterable through mothers' diet during pregnancy, leading to lifelong modification of selected genes for the offspring (Dolinoy et al., 2007). Thorough knowledge of both epigenetic and classic genetic code is necessary in order to truly understand interactions between the environment and gene expression.

Epigenetic mechanisms

Epigenetic regulation includes DNA methylation, histone modifications and post-transcriptional alteration of gene expression based on microRNA interference.

Methylation

Basic biological properties of segments such as gene density, replication timing and recombination are tightly linked to their guanine –cytosine (CG) content, therefore isochors (DNA fractions > 300 kb on average) of the genome can be classified accordingly (Costantini et al., 2006). CpG islands are defined as genomic regions with a GC percentage greater than 50% and with an observed/expected CpG(cytosine base followed by a guanine base) ratio greater than 60 %. In mammals, CpG islands typically are 200-3.000 base pairs long. CpGs are rare in vertebrate DNA due to the tendency of such arrangements to methylate cytosines to 5-methylcytosines followed by a turn into thymines because of spontaneous deamination. The same mechanism seems to be responsible for the inactivation of retroviruses by changing the virus sequences [17].

The consequences are large DNA -regions low in GC and gene density, clearly visible on isochore maps as "genome deserts". However, some regions escaped large scale methylation and determination during evolution and, therefore, show a high amount of GCs, which is generally parallel to a high CpG island and gene density (16). CpG islands mostly occur in these isochors, at or near the gene's transcriptional start site. Promoters of tissue-specific genes that are situated within CpG islands normally are largely unmethylated in expressing and non-expressing tissues (Bestor, 2000). There are three known ways by which cytosine methylation can regulate gene expression: first 5-methylcytosine can inhibit or hinder the association of some transcriptional factors with their cognate DNA recognition sequences, second methyl-CpG-binding proteins (MBPs) bind to methylated cytosines mediating a repressive signal and third MBPs can interatct with chromatin forming proteins modifying surrounding chromatin, linking DNA methylation with chromatin modification (Klose and Bird, 2006). Mostly DNA methylation cause a repression of mRNA gene expression, however, when CpG methylation blocks a repressor binding site within a gene promoter, this may induce a transcriptional activation as shown for Interleukin-8 in breast cancer (De Larco et al., 2003)

DNA -methylation at position five of CpG-cytosines is conducted by DNA methyltransferases (DNMT's), which are expressed in most dividing cells (Schaefer et al., 2007). Mammalian DNMT's can be divided in two general classes. DNMT1 enzyme is responsible for the maintenance of global methylation patterns on DNA. It preferentially methylates CpGs on hemimethylated DNA (CpG methylation on one site of both DNA strands), therefore guaranteeing transfer of methylation marks through the cell cycle in eukaryotic cells. The

DNMT1 enzyme is directly incorporated in the DNA replication complex. The de novo methyltransferases DNMT3a and DNMT3b establish methylation patterns at previously unmethylated CpGs. DNMT3L is a DNMT related enzyme which associates with DNMT3a/3b. It influences its enzymatic activity while lacking one of its own. Finally, for the DNMT2 enzyme, in mammals a biological function remains to be demonstrated (Bestor, 2000; Schaefer et al., 2007). Specific mutations in the DNMT3b gene were found in patients affected by the autosomal recessive immune disorder ICF.

Most DNMT's contain a sex-specific germline promoter which is activated at specific stages during gametogenesis. Genomic methylation patterns are largely erased during proliferation and migration of primordial germ cells and reestablished in a sex-specific manner during gametogenesis, resulting in a high methylation of the genome. For viability, close regulation of the DNMT genes during these stages and during early embryogenesis is needed (Schaefer et al., 2007). After fertilization a second phase of large epigenetic reprogramming takes place. Upon fertilization, a strong, presumably active DNA demethylation can be observed in the male pronucleus while the maternal genome is slowly and passively demethylated. Imprinted methylation is maintained for both the paternal and the maternal genome. DNA-demethylation occurs until the morula stage, after which de novo methylation is observed (Dean et al., 2003). See figure 1, adapted from Morgan et al 2005 and Dean et al 2003 (Dean et al., 2003; Morgan et al., 2005).

For targeting DNA de-novo methylation, three mechanisms are described. First, DNMT3 enzymes themselves might recognize DNA or chromatin via their conserved PWWP (relatively well-conserved Pro-Trp-Trp-Pro residues, present in all eukaryotes) domain. Mouse experiments show that this domain is necessary for targeting the enzyme to pericentromeric heterochromatin. In humans, mutations in the PWWP domain can cause ICF syndrome Immunodeficiency, Centromere instability and Facial anomalies syndrome). Second, by interaction of DNMT's with site-specific transcriptional repressor proteins DNMT's can be targeted to gene promoter regions. Third, in vitro studies have shown that introduction of double stranded RNA corresponding to the promoter region of the target gene leads to its de-novo DNA methylation and decreased gene expression, suggesting the existence of an RNAi mediated DNA methylation mechanism. However, further efforts are needed to clarify this pathway (Klose and Bird, 2006).

Their catalytic role aside, DNMTs seem to mediate gene silencing by modifying chromatin via protein-protein interactions. They biochemically interact with histone methyltransferases (HAT) and histone deacetylases (HDACs). As mentioned above, binding of MBP's to methylated CpGs mediates silencing of gene expression by the associaton with chromatin remodeling co-repressor complexes. Thus, under certain circumstances, gene silencing by DNA methylation may be attributed directly to chromatin modifications. So far, six different methyl-CpG- binding domains (MBD) are known for the MBPs: MBD1 to 4, MeCP2 and Kaiso. All of them mediate silencing of gene expression by chromatin remodeling (Clouaire and Stancheva, 2008). For example, the MBD1 and a histone H3 methyltransferase enzyme (SetDB1) interact during the cell cycle, linking DNA methylation to rearrangement of chromatin by histone methylation (Klose and Bird, 2006). Even if no active demethylation for DNA is known, gene silencing by DNA methylation is not irreversible. For example, Il-4 expression in undifferentiated T cells is silenced via binding of MBD2 on the methylated

promoter of the gene (Hutchins et al., 2002). After differentiation, TH2 cells express the transcription factor GATA-3 which competes with MBD2 for binding to the IL-4 promoter. In this case epigenetic factors impose a threshold to be overcome in order to achieve efficient gene expression. The binding of MBDs to DNA seems to be for the most domains sequence specific.

In order to achieve DNA methylation, often alterations at the chromatin level must occur before. As previously discussed, DNMTs interact with HATs and HDACs. Small modifications of histones by acetylation, methylation, phosphorylation and ubiquitation on certain amino acids can alter gene expression by chromatin remodeling. The effect of these small modifications on chromatin remodeling depends by type, amount and site of modification as well as the interactions as shown in table 1 (adapted from He and Lehming 2003 (He and Lehming, 2003)).

Histone modifications

Some modifications, including acetylation and phosphorylation, are reversible and dynamic, others, such as methylation, are found to be more stable and involved in long term alterations (Cheung and Lau, 2005; Shahbazian and Grunstein, 2007).

As an attempt to organize the complexity of the different possible modifications, the establishment of a "histone code" is a major focus of epigenetic research. Its necessity is demonstrated in the following example: We know 24 methylation sites on a histone. The possibility of mono-, di-, or trimethylated lysine residues and mono- or dimethylated arginine side chains lead to 3×10^{11} different histone methylation states. The need to distinguish between short-term changes in histone modification associated with ongoing processes and changes that have long term effects still pose a problem to be solved (Turner, 2000; Turner, 2002). The significance of some histone modifications has been identified, especially regarding acetylation and deacetylation of histones (Shahbazian and Grunstein, 2007). Histone acetylation is the major factor regulating the degree of chromatin folding, global loss of monoacetylation and trimethylation at histone H4, all of which are commonly seen in human tumor cells. Histone acetylation also regulates nucleosome assembly.

Over time, the relationship between DNA-methylation and histone modifications will become clearer (Cheung and Lau, 2005; Klose and Bird, 2006). Several factors are involved in the process of gene silencing by DNA-methylation. One prerequisite is attributed to the recruitment of HDACs through the methyl-DNA binding motifs, while methylation at Lys9 on H3 is another prerequisite. Methylation of both DNA and histones seem to have a reciprocative reinforcing effect in gene silencing. Histone variants like macro H2A, accumulated on the inactive X chromosome, have been reported to play a role in gene expression regulation. Their presence in the IL-8 promoter-region is connected to tissue related gene silencing of the IL-8 gene (30). The comprehension of a histone code will be an important step towards in understanding further mechanisms for epigenetic gene expression.

Histone	Modification	Effect
H1	Phosphorylation	Chromatin condensation, gene specific condensation and repression.
	Ubiquitination	Transcriptional activation
H2A	Acetylation	Transcriptional activation
	Ubiquitination	Elusive
H2B	Ubiquitination	Prerequisite of H3 methylation
	Phosphorylation	Chromatin condensation
	Acetylation	Chromatin remodeling
	Methylation	Chromatin stabilization
H3	Methylation (H3-K4, R17)	Transcriptional activation
	Methylation (H3-K9, K79)	Transcriptional repression
	Acetylation	Transcriptional activation
	Phosphorylation	Chromatin condensation; transcriptional activation
	Ubiquitination	Nucleosome loosening
H4	Acetylation	Transcriptional activation
	Methylation (H4-K20)	Transcriptional repression
	Methylation (H4-R3)	Transcriptional activation

Table 1: Consequence of histone modifications on chromatin

Through alteration of gene expression and destabilization of chromatin histone modifications can have an impact on the risk of cancer. Modified cancer pathogenesis is linked to varying activities of both HATs and HDACs. This mechanism is best described for acute promyelocytic leukemias, where a chromosomal translocation leads to inappropriate HDACs activity. A change in HDACs expression was also detected in other types of tumors. For example, in gastric cancer, esophageal squamous cell carcinoma, and prostate cancer an increased HADC1 expression is related to pathogenesis. In contrast, in colon cancer an overexpression of HDAC2 causes a decreased expression of the APC (adenomatous polyposis coli) tumor suppressor gene. Another way of influencing the expression of tumor suppressor genes are abnormal functions of HDACs, for instance by atypical targeting. These observations lead to the current exploration of HDAC inhibitors as anticancer therapeutics. The examined substances aim to shift several cell functions which are known to be down regulated in cancer cells. Among other presumed advantages of the approach show cancer cells' an increased sensitivity for HDAC inhibitors compared to normal cells, thus there may be a possibility to prevent or to decelerate the development of tumours by such substances. Overall, the broad range of function promises efficacy at all stages of tumorigenesis.
Not only HDACs but also HATs influence the risk of developing cancer. Especially in cancer of epithelial origin an overexpression or mutation of HAT genes has been detected. Some lines of lung, breast, and colorectal cancer have in common a mutation which inactivates a specific HAT.

Changes of the methylation status of histones have also been observed in some types of cancer. The loss of the trimethylated form of the lysine 20 residue of the H4 histone is characteristic for cancer cells. Also, the demethylation of lysine 9 on H3 histones increase the formation of B-cell lymphoma in mice significantly because of its links to chromatin silencing. (Davis and Ross, 2007)

Micro RNAs.

In addition to DNA and Histone modifications, micro RNAs are part of the epigenetic gene expression regulatory complex. MicroRNAs are small non-coding RNAs that posttranscriptionally regulate the expression of complementary messenger RNAs and function as key controllers in a countless number of cellular processes, including proliferation, differentiation and apoptosis. Over the last few years, increasing evidence has indicated a substantial number of microRNA genes to be subjected to epigenetic alterations, resulting in aberrant patterns of expression upon the occurrence of cancer (Guil and Esteller, 2009)

Environmental influences

Early life conditions

By now, various findings showing the effects of environmental factors on the epigenetic regulation of gene expression derive largely from mouse experiments. Environmental factors influencing epigenetic gene regulation include more than epigenetically active natural food compounds such as vitamin B12 or genistein. Transgenerational effects are for example caused by environmental toxins such as the endocrine disruptor vinclozolin, a common fungicide used in vineyards. Vinclozolin were seen to disrupt epigenetically the germ line in mice. These effects were transferred through the male germ line for several generations. The described mechanism appears to be attributed to a heritable alteration of epigenetic programming of DNA-methylation in the germ line, which alters the transcriptomes of developing organs, in this case the testis development (Anway et al., 2006a).

Early mammalian development is a crucial period for establishing and maintaining epigenetic markers. Modifications of the epigenome are not limited to the fetal period but extended to the plastic phase of early life. Broad epigenetic reprogramming can be seen after fertilization to achieve totipotency of developing embryo cells, while methylation patterns associated with imprinting are sustained. Epigenetic modifications settled during fetal development are generally stable passed through cell division processes throughout a lifetime.

Prenatal exposure to famine for instance, as shown in an epidemiological study, is also likely to change the epigenetic status. Observed individuals showed less DNA methylation of imprinted IGF-2 gene than their unexposed same sexed siblings (Heijmans et al., 2008). A further, nutrition-related epigenetic alteration have been demonstrated in the viable yellow Agouti (=Avy) mouse model (Dolinoy et al., 2006a). Maternal diet containing bisphenol A (BPA), an estrogenic monomer used in polycarbonate plastic production, significantly decreases offsprings methylation of the Avy gene promoter which induces reversal to a wild-type phenotype. Maternal nutritional supplementation with methyl-donors counteracts the effects from BPA, showing that simple dietary changes may protect against harmful epigenetical effects caused by environmental toxins.

Modified promoter methylation and, accordingly, modified gene expression of the hepatic glucocorticoid receptor and the peroxisome proliferator –activated receptor alpha (PPAR alpha), both important elements in carbohydrate and lipid metabolism regulation, can be seen in the offspring of rats fed a protein restricted diet. The selective methylation of PPAR

alpha in the liver without consequences for the related transcription factor PPAR gamma demonstrates that maternal nutrition and behavior can also influence specific promoter regions rather than being associated with global DNA -methylation alteration. Such changes in gene expression and promoter methylation were also seen to be transmitted to the next generation without further nutritional challenge for the first generation (Gluckman et al., 2007).

Finally, the elucidation of the impact of epigenetic modifications on behaviour and psychic health presents an intriguing challenge. Rodents experiments show that some epigenetic changes can be induced promptly after birth through mother's physical behaviour toward her newborn (Darnaudery and Maccari, 2008; Henry et al., 1994; Zuena et al., 2008) Licked and groomed newborns appear to grow up to be relatively brave and calm. In contrast, neglected newborns grow up to be nervous and hyperactive. The difference in their behaviour can be explained by analyzing specific regions in the brain. The hippocampus of both groups reveal different DNA-methylation patterns for specific genes agreeing with the difference in behaviour. This entails a better developing of the hippocampus in the licked newborns, possibly by releasing less of the stress hormone cortisol. Furthermore, recent research results have demonstrated that complex epigenetic mechanisms have long-lasting effects in mature neurons and that they possibly play a vital role in the etiology of major psychoses, such as schizophrenia or bipolar disorder. Paternal age at conception is a strong risk factor for schizophrenia, explaining about a quarter of all cases. The possible mechanisms for the elevated risk may be de novo point mutations or defective epigenetic regulation of paternal genes. The risk might also be related to paternal toxic exposures, nutritional deficiencies, suboptimal DNA repair enzymes or other factors that influence the reliability of the transfer of genetic information in the constantly replicating male germ line.

Pollution, Toxins

There is now a mounting body of evidence that environmental exposures to toxins, particularly during early development, can induce epigenetic changes, which may be transmitted to subsequent generations or serve as basis for diseases developed later in life. Either way, epigenetic mechanisms will help interpreting toxicogenomic approaches (Reamon-Buettner et al., 2008).

Air pollution, for instance, particular matters and cigarette smoke, appear to have omnipresent toxicological influences on humans. Promotor hypermethylation in early tumorigenesis is likely to have a clinical importance, because dissentient promoter methylation in tumor suppressor genes has been detected in a large percentage of human lung cancers. Noticeably the p16 gene, which expresses an inhibitor of cyclin dependent kinase 4 and 6 consequently, interrupting the cell cycle progression is methylated at its promoter region in 20-65% of the lung tumors. (Hayslip and Montero, 2006) Smoking habits alter the extent of promoter methylation patterns. Increasing methylation of p16 could be seen with increasing smoking duration, packets per years and smoking when a juvenile, whereas methylation decreases gradually over time when a person quits smoking.

Similar effects could be seen in rodents' lung cancer, induced by particulate carcinogens carbon black and diesel exhaust, where the tumor cells showed an overmethylation in the p16 promoter region. Equal circumstances could be found in murine lung tumors, caused by

cigarette smoke. Remarkable effects improvements could be achieved by treating rodents with histone deacetylation inhibitors in a lung cancer mouse model. A greater than 50 % reduction of tumor growth was seen in treated mice.

In addition to well-known carcinogens, air pollution components and particulate matter (PM10), nickel and beryllium compounds have also been shown to also have an impact on histone acetylation and/or altered DNA methylation patterns (Vineis et al., 2006).

Diets

Dietary habits as well as a sedentary lifestyle clearly contribute to today's increasing number of chronic diseases. The influence played by numerous food compounds on the epigenetic machinery is of growing interest. The large number of established and probable epigenetic active compounds found in food will challenge the understanding of how diet may influence epigenetic gene expression. Studies of dietary effects on epigenetic gene regulation are still in their infancy, but first results are shown (Thaler et al., 2008). Food contains compounds influencing both, DNA methylation and histone modifications. However, the consumption of some of these compounds can vary by season, dietary habits, age and environment.

Dietary compounds like vitamin B_{12} or folic acid are implicated in the regulation of the cytosine methylation pathway (Choi et al., 2004; Kim, 2004a; Stempak, 2002). Vitamin B_{12} plays a central role as it acts as co-factor of the methionine synthetase, remethylating homocysteine to methionine. Methionine is further activated to S-adenosylmethionine (SAM), the methyl donor for DNA methylation. SAM converts to S-adenosylhomocysteine (SAH) after DNA-methylation. Reversible hydrolysis of SAH to homocysteine completes the cycle (figure 2, adapted from Kim 2004 (Kim, 2004a)). Folate, cholin or betaine are potent methyl-donors directly implicated in the DNA-methylation pathway. However, under conditions of vitamin B_{12} depletion, cellular folate accumulates in the methylfolate form due to activity of methionine synthetase being blocked, thereby creating a conditional form of folate deficiency. Deficiency in vitamin B_{12} leads also to an accumulation of serum homocysteine. Under conditions of elevated homocysteine concentrations, levels of the potent SAM–inhibitor SAH represses DNA methylation. The role of folate in DNA-methylation seems to be rather complex. Evidence from animal, human, and in vitro studies suggest that folate-dependent DNA methylation is highly complex, gene and site specific(Cravo et al., 1994; Kim, 2004a; Kim et al., 2001; Rampersaud et al., 2000). These studies have shown that the extent and direction of changes in SAM and SAH in cell lines in response to folate deficiency are cell specific and that genomic-site and gene-specific DNA demethylation are not affected by the changes in SAM and SAH induced by folate depletion. Transgenerational studies on a rat model could furthermore demonstrate that folate deficiency during pregnancy impacts on methyl metabolism, but does not effect global DNA methylation in rat foeti (Maloney et al., 2007). A study analyzing the expression of the antioxidative enzyme mitochondrial superoxide dismutase (MnSOD) showed that this gene is expressed differently in vegetarians and omnivores due to different DNA-methylation patterns in the MnSOD promoter region: vegetarians expressed a significant higher amount of MnSOD, showing a low promoter methylation for this gene. Inversely, a lower MnSOD expression by a higher MnSOD promoter methylation was seen in omnivores (Thaler et al., 2008). Several factors which may reduce DNA–methylation machinery in vegetarians are discussed. Studies analyzing the B-vitamin status of vegans and vegetarians compared with

omnivores, showed generally a higher dietary supply of folate and a lower dietary supply of vitamin B_{12} (since vitamin B_{12} intake comes predominantly from animal food products) and methionine for vegetarians and vegans (Majchrzak et al., 2006; Millet et al., 1989). Correlations between plasma folate and vitamin B_{12} values were found and study findings showed that vegetarians and vegans show higher plasma homocysteine concentrations (Majchrzak et al., 2006). Furthermore, DNA hypomethylation due to high homocysteine levels has been reported in vitro and in vivo (Jamaluddin et al., 2007; Jiang et al., 2007). Inhibition of DNA methyltransferase 1 activity by 30% and reduced binding of methyl CpG binding protein 2 have also been seen in this context. A further study (Geisel et al., 2005) comparing vegetarians and omnivores observed a reverse correlation between SAH concentrations and DNA global methylation levels in blood. However, this study was unable to correlate homocysteine concentrations with the degree of DNA global methylation and found no correlation between the degree of CpG methylation of the promoter of the p66Shc gene (involved in oxidative stress) and homocysteine, or SAM or SAH levels.

Other diet derived factors such as diallyl sulfide (Mathers, 2006), an organosulfur compound found in garlic, genisteine, the main flavanoid in soy (Dolinoy et al., 2006b), vitamin D_3 or all-trans-retinoic-acids (Krawczyk and Fabianowska-Majewska, 2006) have been shown to influence DNA methylation by altering histones and chromatin structure. On the histone level, much more food compounds show effects on epigenetic gene regulation. Food compounds acting as histone modifiers are generally weak enzyme ligands and thus are needed in high concentrations to generate a consistent effect and therefore might subtly regulate gene expression (Dashwood et al., 2006). A number of dietary agents are discussed to have a role in epigenetics as HDAC inhibitors. In the literature well described agents in this context are butyrate, diallyl disulfide or sulforaphane. Butyrate is the smallest known HDAC inhibitor, and inhibits histone acetylation at high micromolar or low millimolar concentrations in vitro, levels nonetheless considered to be achievable in the gastro intestinal tract by bacterial metabolism (Dashwood et al., 2006). Intriguing examples for a HDAC inhibitor are conjugated linoleic acids (CLA), which have anticancerogenic and antiatherogenic properties. Interestingly, CLAs decreases Bcl-2 and induces p21, a known HDAC target. Effects of CLAs on HDACs are discussed. Regarding the short chain fatty acids (SCFs) resulting from the microbial fermentation of dietary fibers, acetate, propionate and butyrate are taken up by the colonic mucosa. Butyrate is the preferred energy source for colonocytes and is transported across the epithelium. Butyrate, and to a lesser extent other SCFs, influence gene expression and inflammation primarily through its action as an inhibitor of histone deacetylases. Therefore, diet compounds, toxins or medications interfering with the colonic fermentation may also modulate potential epigenetic effects generated by the microbial flora. By now, there is much work to be done to clarify the influence on histone modifications of a great number of dietary factors, alone or in combination and their tissue specific characteristics. A list of discussed dietary HDAC activity modulators is shown in table 2.

Evidences show that a diet rich in vegetables and fruits might prevent some kind of cancers (Research, 2007). In this context, the role of flavonoids is often discussed. Flavonoids can act either in a pro- or antioxidative manner depending on their structure and characters. Because of the antioxidative properties of some flavonoids, oxidative damage of DNA can be

prevented and cancerogenesis altered. Guarrera et al postulated recently that flavonoids might influence the gene expression of DNA repair genes, which could be a possible explanation for the decrease in tumour development (Guarrera et al., 2007).

In many animal models caloric restriction has been shown to prevent the development of cancer, Thereby the role of sirtuins becomes clearer. In higher eukaryotes, HDACs are grouped in four classes (I-IV). Class I of HDACs are found exclusively in the nucleus, whereas class II of HDACs shuttle between nucleus and cytoplasm. Resveratrol activates the HDAC sirtuin 1 (SIRT1) a HDAC belonging to class III HDACs. Because of their homology to yeast sir2, class III HDACs belong to the sir2 family (Davis and Ross, 2007). SIRT is an abbreviation for silent mating type information regulation two and there are seven groups in humans (Davis and Ross, 2007). Sirtuins are nicotineamide adenine dinucleotide (NAD(+)) dependent deacetylases which exhibit a well-defined regression during aging that is dramatically reverted in transformed cells. .

Another group of diet related epigenetic active compounds comprise short chain fatty acids (SCFs) resulting from the fermentation of dietary fibers. Acetate, propionate and butyrate are taken up by the colonic mucosa. Butyrate is the preferred energy source for colonocytes and is transported across the epithelium. Butyrate, and to a lesser extent other SCFs, influence gene expression and inflammation primarily through its action as an inhibitor of histone deacetylases. Therefore, diets, toxins or medications interfering with the colonic fermentation, possibly by an interaction with the composition of the gastro- intestinal microbiota, may modulate SCFs and their epigenetic effects.

Nutrition and the immune system

Expression of Interleukin 8 and the IL23/IL17 pathway is pivotal in the development of chronic inflammation such as Crohn's disease (CD) or inflammatory Bowel disease.(Li et al., 2004; McGovern et al., 2009)

IL-8, a member of the CXC chemokine family, is an important activator and chemoattractant for neutrophils is primarily regulated at the transcriptional level. IL-8 was shown to be affected by methylation of several CpG islands upstream from the promoter region as well as by histone modification. Compounds, taking part in DNA methylation pathways and histone acetylation such as folic acid, vitamin B12, genistein, zebularine and valproate seem to influence epigenetic modification and gene expression of the IL-8 gene in human CACO-2 colon cancer cells.

By contrast to leukemic blasts, where IL-8 is activated by differentiation(Delaunay et al., 2008), immune activated transcription of IL-8 gene may be silenced after differentiation of cells from solid tumors such as breast cancer (Chavey et al., 2008) through histone deacetylation by elements located outside of the immediate 5´flanking region.

The commensal microflora regulates the local expansion of CD4 T cells producing proinflammatory cytokines including IL-17 (Th17 cells) in the colonic lamina propria. (Niess et al., 2008) TH cells, which produce IL-17 and IL-17F, two highly homologous cytokines whose genes are located in the same chromosomal region have been recently identified to promote tissue inflammation. In these cells Histone H3 acetylation and Lysine 4 tri-

methylation were specifically associated with IL-17 and IL-17F gene promoters. (Akimzhanov et al., 2007)

As natural food compounds and diets have been shown to modify the epigenetic regulation of these expression of these mediators (Kikuno et al., 2008; Romier et al., 2008) dietary strategies might be a possibility in modulating inflammatory diseases.

Nutrition and aging

Hereditary disposition, environmental elements, individual lifestyle and nutritional factors are known to interact with the aging process. These impacts on the aging-process can be divided in intrinsic and extrinsic factors. Intrinsic aging (cellular aging) mainly depends on individual hereditary background. Extrinsic aging is generated by external factors like smoking, excessive alcohol consumption and poor nutrition. During aging, epigenetic changes occur, for instance global DNA demethylation decrease contrasting to a CpG island hypermethylation.Progressive loss of global methylation during aging caused by passive demethylation is probably due to increasing DNMT1 enzyme inefficiency. Epigenetic changes can be seen as important determinants of cellular senescence and organism aging. Sirtuins take part in various cellular developments like chromatin remodelling, mitosis, life-span duration and are furthermore involved in the regulation of gene expression, insulin secretion and DNA repair. Since NAD is one of the critical molecules involved in many metabolic pathways, sirtuins activity is NAD(+) dependent and sirtuins control the activity of many other proteins involved in cell growth, it was suggested that sirtuins are involved in the elongation of life-span mediated by caloric restriction. Indeed, in mammals, caloric restriction decreases the incidence or delays the onset of age-associated diseases including cardiovascular diseases, cancer, and osteoporosis as well as neurodegenerative diseases. SIRT1 is known to be down regulated in senescent cells and during aging. Transcription factors like p53, p73, NF-KB, E2F1, p300 and others are also shown to be SIRT1 substrates, suggesting an oncogenetic potential for SIRT 1. In an ongoing process more and more sirtuin inhibitors are being discovered. Recent detections are sirtinol, cambinol, dihydrocoumarin and indole, coumarin and indoles being natural food compounds. Coumarin can be found in dates, dihydrocoumarin naturally in sweet clover and synthetically manufactured in foods and cosmetics. Indole is produced by intestinal bacteria as product of tryptophan degradation. Early findings about this novel class of HDACs are encouraging. Even more intensive research will be necessary to clarify connections between sirtuins, nutrition, aging and cancer genesis.
It has been suggested that sirtuins may be involved in the elongation of life-span mediated by caloric restriction transcription factors, such as p53, p73, NF-KB, E2F1, p300 and others are shown to be SIRT1 substrates. Currently sirtuin inhibitors from natural compounds are being discovered (Akkermans et al., 2007; Kwon et al., 1998). Intensive research will be necessary to clarify any connections between sirtuins, nutrition, aging and cancer genesis.

Conclusion

Epigenetic mechanisms contribute to the control of gene expression as a result of environmental signals due to their inherent malleability. Considerable evidence suggests that nutritional imbalance and metabolic disturbances during critical time windows of development, may have a persistent effect on the health of the concerned individulas and may even be transmitted to the next generation. For example, in addition to a "thrifty genotype" inheritance, individuals with obesity, type 2 diabetes, and metabolic syndrome with an increased risk of cardiovascular diseases may have suffered improper "epigenetic programming" during their fetal/postnatal development due to maternal inadequate nutrition and metabolic disturbances and also during their lifetime, that could even be transmitted to the next generation(s), (Asim K Duttaroy, Evolution, Epigenetics, and Maternal Nutrition, Darwins Day Celebration, 2006).
The balance between genetic and epigenetic impacts in development of malignancy is therefore changing from childhood to later age. Whereas e.g. the majority of childhood tumors are associated with an inherited genetic or epigenetic (e.g., imprinted) burden, this balance shifts in favor of acquired epigenetic and genetic hits in tumors of adults and elderly (Haslberger et al., 2006) (Fig .3).

As predicted by Rupert Riedl working with the so-called "General Systems Theory" the evolution of complex adaptation requires a match between the functional relationships of the phenotypic characters and their genetic representation [60]. Regarding to Riedl "evolvability" results from such a match. If the epigenetic regulation of gene expression "imitates" the functional organization of the traits then the improvement by mutation and selection is facilitated. Riedl predicts that the evolution of the genetic representation of phenotypic characters tends to favor those representations which imitate the functional organization of the characters. Imitation means that complexes of functionally related characters shall be "coded" as developmentally integrated characters but coded independently of functionally distinct character complexes (Wagner and Laubichler, 2004).

Epigenetic mechanisms thus appear as a machinery of recursive feedback which determines the average rate of change of characters at all levels from molecules to cells, organisms and their environment(Haslberger et al., 2006). A better description of role of the epigenetic machinery in the interaction between hereditary and environmental interactions will also add to our understanding of evolution and an improved, individual and preventive health care system.

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7.3. Abstracts for poster presentations

7.3.1. 2nd Congress of the International Society of Nutrigenetics/Nutrigenomics (ISNN),

Geneva, Oct. 6-8 2008

Natural Food Compounds Affect DNA Methyltransferases, Promoter Methylation and Gene Expression of Immune Relevant Genes in the CACO2 Cell-Line

A.G. Haslberger, R. Thaler, V. Sagl, E. Rudolf

University of Vienna, Department for Nutritional Sciences, Vienna, Austria

Natural compounds of foods are under investigation for their effects on the epigenetic control of the expression of genes. This study analyzes the impact of folic acid, vitamin B12 and genistein on the expression of DNA-methyltransferases (Dnmt), histonedeacetylase-2 (HDAC-2) and selected genes under discussion for a potential epigenetic control (IL-8, E-Cadherin). Analysis of gene expression was performed in CACO-2 cell-line after 48 h incubation using Sybr Green rt-PCR. For DNA global methylation ELISA technique was used; gene promoter methylation was analyzed by Bisulfite Genomic Sequencing PCR (BSP) and Methylation Specific PCR (MSP). Incubation of CACO-2 cells with the Dnmt-inhibitor zebularine increased expression of E-Cadherin. The methyldonor folic acid and the methioninesynthetase co-factor vitamin B12 increased the expression of Il-8 as well as the expression of DNMT3a and DNMT3b.Also the HDAC inhibitor Valproate substantially stimulated gene expression of IL-8. In correspondence with gene expression Zebularine could be demonstrated to decrease CpG methylation of promoter regions such as of E-Cadherin as well as DNA-global methylation (-30%). Methylation of CpGs upstream the the IL-8 gene correlated with IL-8 gene expression. A significant increase of the methylation of these sites were seen after treatment with folic acid and vitamin B12. These experiences support evidences for effects of dietary factors on an epigenetic control of expression of genes including genes of the I.S. and encourage strategies for a targeted dietary support in the treatment of immune related diseases with epigenetic control.

7.3.2. NuGOweek 2009, Montecatini Terme, Aug. 31-Sep. 3 2009

EPIGENETIC REGULATION OF IL-8 AND E-CADHERIN GENE EXPRESSION BY BUTYRATE, FOLIC ACID, AND GENISTEIN INCLUDES DNA-METHYLATION AND HISTONE ACETYLATION

E. Aumueller, A. Gnauck, C. Berner, P. Klein, A. G. Haslberger
Department of Nutrition, University of Vienna
Alexander.haslberger@univie.ac.at

The expression of IL-8 and E-Cadherin regulates the interactions between immune cells and epithelia in the GI-tract involving epigenetic mechanisms. Because of the epigenetic activities of the SCF butyrate, produced by intestinal microbiota, as well as genistein and folic acid we analyzed effects on histone acetylation and DNA-methylation on the expression of IL-8 and E-Cadh.

Gene expression was analyzed by real time PCR, DNA-methylation by Methylation Specific PCR (MSP), bisulfite sequencing PCR (BSP) and histone acetylation by Chromatin immunoprecipitation in the CACO-2 cell line model.

IL-8 and E-Cadh expression was increased by genistein, butyrate, folic acid and the DNA methyltransferase inhibitor zebularine. Whereas zebularine decreased DNA methylation of E-Cadh and IL-8 (on the sites -1342 and -1412), butyrate enhanced the histone acetylation on H3 lysine 9 at IL-8. Genistein and butyrate also stimulated the apoptose relevant caspase3. Changes in butyrate producing GI-microbiota (*Clostridium* cluster IX and XIVa) were found due to nutrition.

As the levels of genistein, folic acid, and butyrate in the GI-tract can be modulated by nutrition, nutritional concepts may be feasible to interfere with epigenetic mechanisms in inflammatory GI diseases.

7.3.3. Epigenetic World Congress, Berlin, Sep. 17-18 2009

Food ingredients modulate epigenetic control of expression of estrogen receptor and tumor suppressor genes

Aumueller E.[1], Berner C.[1], Gnauck A.[1], Just A.[2], Haslberger AG[1]

[1]University of Vienna
Department for nutritional sciences

[2]Medical University of Vienna
Department of Obestrics and Gynecology

Estrogen receptor alpha (ESR1) plays vital roles in the development and progression of breast cancer and metabolic disorders. The expression is known to be regulated by epigenetic mechanisms. Also the tumor suppressor genes P15 and P16 are aberrantly methylated in several cancer types.
To evaluate possible effects of dietary compounds on the epigenetic gene expression Caco2-cells were treated with butyrate, resveratrol, genistein, zebularine, folic acid, chrysin, and EGCG, which are known to have estrogenic or epigenetic effects. Gene expression was analyzed for ESR1, P15, and P16 by quantitative real-time-PCR. Methylation of specific CpG-sites was determined by methylation-specific PCR.
Treatment with genistein, folate, EGCG, and resveratrol increased and zebularine and chrysin decreased methylation of ESR1. P15 methylation was enhanced by genistein and folate and decreased by chrysin, zebularine, butyrate, EGCG, and resveratrol. Treatment with folate resulted in P16 hypermethylation whereas all other agents resulted in hypomethylation of this gene. Changes in methylation corresponded with altered gene expression. In contrast to CACO cells human blood cells show less methylation on the investigated genes. These results suggest that food ingredients may influence epigenetic modification of cancer relevant gene expression and might be considered for supplemental treatment and prevention.

7.3.4. 19th International Congress of Nutrition, Bangkok, Oct. 4-9 2009

REGULATION OF IL-8 AND IL-17 EXPRESSION BY BUTYRATE, FOLIC ACID, AND GENISTEIN INCLUDES DNA METHYLATIONAND HISTONE ACETYLATION

E. Aumueller, C. Berner, A. Gauck, P. Klein, A. G. Haslberger
Department of Nutrition, University of Vienna

IL-8 and IL-17 regulate interactions between immune cells and epithelia in the GI-tract, involving epigenetic mechanisms. Because of the epigenetic activities of the SCF (short chain fatty acid) butyrate, produced by intestinal microbiota, genistein and folic acid we analyzed effects on histone acetylation and DNA-methylation on interleukin-8 and -17 genes.

We analyzed gene expression by real time PCR, DNA-methylation by bisulfite sequencing PCR, and histone acetylation by Chromatin immunoprecipitation in the CACO-2 cell line model.

IL-8 and IL-17 expression was increased by genistein, butyrate, and folic acid. Folic acid stimulated the IL-8-DNA-methylation on the sites -1342 and -1412. Butyrate stimulated the histone acetylation on H3 lysine 9 at IL-8. Genistein and butyrate also stimulated apoptosis relevant caspase-3. Changes in butyrate producing GI-microbiota (*Clostridium* cluster IX and XIVa) were found due to nutrition.

As the levels of genistein, folic acid, and butyrate in the GI-tract can be influenced by nutrition, nutritional concepts may be feasible to interfere with IL-8 and IL-17 expression in inflammatory GI diseases.

8. <u>PROTOCOLS</u>

8.1.   RNeasy® Lipid Tissue Handbook (Qiagen)

1. If using the TissueLyser, add one stainless steel bead (5 mm mean diameter) per 2 ml microcentrifuge tube (not supplied). If working with tissues that are not stabilized in RNAlater RNA Stabilization Reagent, place the tubes on dry ice.
2. Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 100 mg. Proceed immediately to step 3. Weighing tissue is the most accurate way to determine the amount. If the tissue sample was stored in RNAlater RNA Stabilization Reagent, remove it from the reagent using forceps and be sure to remove any crystals that may have formed. RNA in harvested tissues is not protected until the tissues are treated with RNAlater RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. 3. Disrupt the tissue and homogenize the lysate using either the TissueRupter (follow step 3a) or TissueLyser (follow step 3b). See "Disrupting and homogenizing starting material", page 12, for more details on disruption and homogenization.
   Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueRupter or TissueLyser generally results in higher RNA yields than with other methods.
3a. Disruption and homogenization using the TissueRuptor:
■ Place the tissue in a suitably sized vessel containing 1 ml QIAzol Lysis Reagent.
   Note: Use a suitably sized vessel with sufficient extra headspace to accommodate foaming, which may occur during homogenization. Generally, round-bottomed tubes allow more efficient disruption and homogenization than conical-bottomed tubes.
■ Place the tip of the disposable probe into the vessel and operate the TissueRuptor at full speed until the lysate is uniformly homogeneous (usually 20–40 s). Proceed to step 4.
   Note: To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in the buffer. Foaming may occur during homogenization, especially of brain tissue. If this occurs, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.
3b. Disruption and homogenization using the TissueLyser:
■ Place the tissues in the tubes prepared in step 1.
■ If the tubes were stored on dry ice, place them at room temperature. Then immediately add 1 ml QIAzol Lysis Reagent per tube.
■ Place the tubes in the TissueLyser Adapter Set 2 x 24.
■ Operate the TissueLyser for 2 min at 20 Hz.
   The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.
■ Rearrange the collection tubes so that the outermost tubes are innermost and the innermost tubes are outermost. Operate the TissueLyser for another 2 min at 20 Hz. Rearranging the tubes allows even homogenization.

■ Carefully pipet the lysates into new microcentrifuge tubes (not supplied).
Proceed to step 4. Do not reuse the stainless steel beads.

4. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.
This step promotes dissociation of nucleoprotein complexes.

5. Add 200 µl chloroform. Securely cap the tube containing the homogenate, and shake it vigorously for 15 s.
Thorough mixing is important for subsequent phase separation.

6. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.

7. Centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C) if the same centrifuge will be used in the later steps of this procedure. After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. For tissues with an especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 600 µl.

8. Transfer the upper, aqueous phase to a new tube (not supplied). Add 1 volume (usually 600 µl) of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 9.
Note: The volume of lysate may be less than 600 µl due to loss during homogenization and centrifugation.
Precipitates may be visible after addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 9.

9. Transfer up to 700 µl of the sample to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) at room temperature (15–25°C). Discard the flow-through.
Reuse the collection tube in step 10.

10. Repeat step 9 using the remainder of the sample. Discard the flow-through.
Reuse the collection tube in step 11.
Optional: If performing optional on-column DNase digestion (see "Important points before starting"), follow steps C1–C4 (page 32) after performing this step.

11. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at _8000 x g (_10,000 rpm) to wash the membrane. Discard the flow-through.
Reuse the collection tube in step 12.
After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
Skip this step if performing optional on-column DNase digestion (page 32).

12. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the membrane. Discard the flow-through.
Reuse the collection tube in step 13.
Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting", page 15).

13. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

14. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.
Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 13.

15. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at 8000 x g (10,000 rpm).

16. Repeat step 15 using another volume of RNase-free water, or using the eluate from step 15 (if high RNA concentration is required).
Reuse the collection tube from step 15.

If using the eluate from step 15, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

8.2. QIAamp® DNA Mini and Blood Mini Handbook (Qiagen)

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x 106 lymphocytes in 200 µl PBS.
    If the sample volume is less than 200 µl, add the appropriate volume of PBS. QIAamp Mini spin columns copurify RNA and DNA when both are present in the sample. RNA may inhibit some downstream enzymatic reactions, but not PCR. If RNA-free genomic DNA is required, 4 µl of an RNase A stock solution (100 mg/ml) should be added to the sample before addition of Buffer AL.
    Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.
3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
    In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. If the sample volume is larger than 200 µl, increase the amount of QIAGEN Protease (or proteinase K) and Buffer AL proportionally; for example, a 400 µl sample will require 40 µl QIAGEN Protease (or proteinase K) and 400 µl Buffer AL. If sample volumes larger than 400 µl are required, use of QIAamp DNA Blood Midi or Maxi Kits is recommended; these can process up to 2 ml or up to 10 ml of sample, respectively.
    Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.
4. Incubate at 56°C for 10 min.
    DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.
5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s.
    After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
    If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally; for example, a 400 µl sample will require 400 µl of ethanol.
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
    Close each spin column in order to avoid aerosol formation during centrifugation.
    Centrifugation is performed at 6000 x g (8000 rpm) in order to reduce noise.
    Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.
    Note: When preparing DNA from buffy coat or lymphocytes, centrifugation at full speed is recommended to avoid clogging.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection

tube containing the filtrate.

It is not necessary to increase the volume of Buffer AW1 if the original sample volume is larger than 200 µl.

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield. A second elution step with a further 200 µl Buffer AE will increase yields by up to 15%. Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation. Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 26). For samples containing less than 1 µg of DNA, elution in 50 µl Buffer AE or water is recommended. Eluting with 2 x 100 µl instead of 1 x 200 µl does not increase elution efficiency.

8.3.   QIAquick® Spin Handbook QIAquick PCR Purification Kit (Qiagen)

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.
   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.
   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.
   Collection tubes are re-used to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.
   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

9. LEBENSLAUF

Kontaktdaten:	
Eva AUMÜLLER, geb. Rudolf geb. 21. Jänner 1983 in Wien Adresse: Lorystraße 83/2/82 1110 Wien Telefon: 0699/126 854 07 E-Mail: a0100555@unet.univie.ac.at	

Familienstand
verheiratet mit Rudolf Aumüller seit 09/2008

Ausbildung:	
Volksschule: Kleistgasse 12, 1030 Wien	1989-1993
Gymnasium: Mater Salvatoris, Kenyongasse 4-12, 1070 Wien	1993-2001
Matura: mit ausgezeichnetem Erfolg	06/2001
Studium:	
Chemie, Universität Wien	09/2001-06/2004
Ernährungswissenschaften, Universität Wien	seit 10/2004
1. Studienabschnitt: mit Auszeichnung abgeschlossen	01/2007
Wahlschwerpunkt im 2. Abschnitt: Psychologie der Ernährung / Ernährungsberatung	

Praktika	
Österreichische Gesellschaft für Ernährung	06/2008

Publikationen
Rudolf E.: Sommerzeit-Eiszeit. Ernährung aktuell, 2/2008
Thaler R., Aumueller E., Berner C., Schuster J., Haslberger A.G.: Interaction of hereditary and epigenetic mechanisms in the regulation of immune and ageing relevant gene expression. In: Epigenetics and Human Health – linking hereditary, environmental and nutritional aspects. (Haslberger A.G., editor), in preparation

Poster
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Aumueller E., Berner C., Gnauck A., Klein P., Haslberger A.G.: Regulation of Il-8 and Il-17 expression by butyrate, folic acid, and genistein includes DNA methylation and histone acetylation. International Congress of Nutrtion, Oct. 4-9 2009, Bangkok
Aumueller E., Gnauck A., Berner C., Klein P., Haslberger A. G.: Epigenetic regulation of IL-8 and E-Cadherin gene expression by butyrate, folic acid, and genistein includes DNA methylation and histone acetylation. NuGOweek 2009, Aug. 31-Sep. 3, 2009, Montecatini Terme

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