

Article

Vitamin E Modifies High-Fat Diet-Induced Increase of DNA Strand Breaks, and Changes in Expression and DNA Methylation of *Dnmt1* and *MLH1* in C57BL/6J Male Mice

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Received: 20 April 2017; Accepted: 30 May 2017; Published: 14 June 2017

Abstract: Obesity is associated with low-grade inflammation, increased ROS production and DNA damage. Supplementation with antioxidants might ameliorate DNA damage and support epigenetic regulation of DNA repair. C57BL/6J male mice were fed a high-fat (HFD) or a control diet (CD) with and without vitamin E supplementation (4.5 mg/kg body weight (b.w.)) for four months. DNA damage, DNA promoter methylation and gene expression of *Dnmt1* and a DNA repair gene (*MLH1*) were assayed in liver and colon. The HFD resulted in organ specific changes in DNA damage, the epigenetically important *Dnmt1* gene, and the DNA repair gene *MLH1*. Vitamin E reduced DNA damage and showed organ-specific effects on *MLH1* and *Dnmt1* gene expression and methylation. These results suggest that interventions with antioxidants and epigenetic active food ingredients should be developed as an effective prevention for obesity—and oxidative stress—induced health risks.

Keywords: *MLH1*; *Dnmt1*; DNA damage; gene expression; DNA methylation; SCGE assay

1. Introduction

Obesity is associated with a positive energy balance, an abnormal increase of adipose tissue and weight gain that impairs health [1]. Genetic factors such as single nucleotide polymorphisms, the environment, social status, dietary behavior, metabolism, microbiota, and physical activity are proposed to influence its development [2]. However, the adipose tissue is not merely an energy depot: it is also a highly active metabolic and endocrine organ. Various bioactive peptides, called adipokines, are involved in energy homeostasis, lipid and glucose metabolism, inflammation, fibrinolysis, coagulation, and blood pressure. Among others, they include cytokines such as interleukin-6 (*IL-6*) and tumor necrosis factor α (*TNF α*), leptin, monocyte chemoattractant protein-1 (*MCP-1*), plasminogen activator inhibitor-1 (*PAI-1*), adiponectin, and resistin [3,4].

In addition to adipocytokines and free fatty acids (FFAs), an increase of reactive oxygen species (ROS) production and increased oxidative stress are reported [5,6]. In obese mice, the ROS production increases in adipose tissue along with an elevated expression of nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase and decreased expression of antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) as well as altered production of adipocytokines in accumulated fat. Together with a positive correlation of biomarkers for systemic oxidative stress, it is likely that adipose tissue is the main source of elevated plasma ROS [7]. Oxidative stress is involved in both genome-wide hypomethylation and promoter hypermethylation of the DNA [8].

Increased levels of oxygen radicals are involved in DNA damage including base modifications, deletions, strand breaks, and chromosomal rearrangements, which interfere with DNA methylation [9,10]. Guanine within CpG dinucleotides is the favored base for oxidative damage, resulting in the production of 8-oxoguanine (8-oxoG). Substitution of guanine to 8-oxoG reduces the binding capacity of transcription repressor proteins causing persistent transcription of affected genes. However, the methyl group of 5-methylcytosine is similarly accessible to oxidation, forming 5-hydroxy-methylcytosine [10]. Another product of oxidative damage is 8-hydroxy-2-deoxyguanosine within CpG dinucleotides, leading to strong inhibition of cytosine methylation [9]. On the other hand, ROS-induced oxidative stress contributes to hypermethylation of normally unmethylated promoter regions, resulting in transcriptional silencing of key antioxidant enzymes as well as tumor suppressor genes [11].

O⁶-methylguanine-deoxyribonucleic acid methyltransferase (MGMT) is a DNA repair enzyme, which is able to repair O⁶-methylguanine by eliminating mutagenic and cytotoxic alkyl groups [12,13]. Either the repair protein MutL homolog 1 (*MLH1*) is part of the DNA mismatch repair (MMR) system [14,15]. This correction of replication errors involves recognition of mismatches and selective removal of the affected DNA region. If the repair of DNA lesions is not accurate, an increase of DNA mutations is the consequence and can cause cellular dysfunctions and diseases including sporadic and hereditary human cancers. In patients with hereditary non-polyposis colorectal cancer, as well as a wide variety of other cancers, increased mutations in microsatellite sequences, known as microsatellite instability (MSI), are associated with defects in MMR system [16]. MSI is often associated with promoter hypermethylation, resulting in inactivation of *MLH1* [15,16].

The methyl donor S-Adenosylmethionin (SAM) and the expression of *DNA methyltransferase 1* (*Dnmt1*) are also impaired due to oxidative stress [17] and may promote health disorders: for example, in cancer, an increased expression has been mentioned [18]. In a mouse model of asthma disease *Dnmt1* was down-regulated [19]. Hodge et al. (2001, 2005, 2007) extensively studied the connection between *Dnmt1* and the inflammatory cytokine: IL-6. Treatment of cells with IL-6 increased the expression and the activity of *Dnmt1* due to transcriptional activation in the promoter [20–22]. Furthermore, the elevated expression of *Dnmt1* coordinated by IL-6 is negatively correlated with the expression level of tumor suppressor gene p53. It is suggested that IL-6 has the ability to induce p53 promoter methylation through up-regulation of *Dnmt1* [21]. Thus, chronic exposure to low-grade inflammation, especially IL-6, might induce dysregulation of the *Dnmt1* gene.

Nutritional interventions, e.g., with antioxidants, may improve DNA methylation and nucleotide biosynthesis reactions and as a consequence DNA repair. We reported previously that a diet rich in antioxidants (e.g., EGCG) [23] and vitamins (in particular folate) alters DNA methylation and ameliorates ROS induced epigenetic lesions [24]. It was shown that vitamin E plays a key role in antagonizing oxidative stress [25] as a direct scavenger of toxic free radicals, induction of antioxidant enzymes, enhancing inflammatory/immune response, modulation of DNA repair systems, and of signal transduction pathways [26]. In the colon, tocopherols react with lipid soluble peroxy radicals and quench the further propagation of free radicals [27]. In the liver, oxidative stress is involved in the pathogenesis of various diseases such as non-alcoholic fatty liver disease (NAFLD). NAFLD is the hepatic manifestation of metabolic syndrome and frequently associated with diabetes, hyperlipidemia, and obesity. Antioxidant treatment of NAFLD with vitamin E is a well-established pharmacological approach [28].

Several earlier findings with rodent and humans indicate that obesity induces low-grade inflammatory processes in the following genomic instability [29–31]. One of the strategies to prevent

adverse effects of obesity and co-morbidities may be nutritional interventions. In the present study, we investigated the impact of vitamin E intake on genomic instability, DNA methylation, and gene expression of *Dnmt1*, and of the DNA repair molecule *MLH1*, assayed in liver and colon of C57BL/6J mice. The colon plays an important role in nutrient absorption, while the liver has important impact on glucose and insulin metabolism as the main organ for insulin clearance from blood. DNA damage was measured in single cell gel electrophoresis (SCGE) experiments which are based on the determination of DNA migration in an electric field: the extent of the size of “comets” reflects formation of single and double strand breaks and apurinic site [32]. DNA methylation was analyzed with bisulfite converted DNA in a Pyromark, and the expression of candidate genes was assayed from reverse-transcribed complementary DNA (cDNA).

2. Materials and Methods

The animal experiment was approved by the Ethical Committee of the Medical University of Vienna (BMFWF-66.009/0329-WF/V/3b/2014). Six-week-old C57BL/6J male mice ($n = 60$, 15 mice/group, Janvier Labs, France) were used for the animal experiment. Three animals were kept per cage (Macrolon type III, Techniplast GmbH, Hohenpeißenberg, Germany) under standard conditions (24 ± 1 °C, humidity $50\% \pm 5\%$, 12 h light/dark cycle); food and water were provided ad libitum. After 14 days of acclimatization with CD (control diet, EF R/M Control, 11 kJ % fat, ssniff Spezialdiäten GmbH, Soest, Germany) mice were divided into four groups (time point T1: start of intervention): (i) a CD group; (ii) a CD plus vitamin E group (CD + E; 4.5 mg/kg body weight per day); (iii) a HFD group (high fat diet: 54 kJ % fat ssniff EF acc.D12492 (I) mod., ssniff Spezialdiäten GmbH, Soest, Germany); and (iv) a HFD plus vitamin E group (HFD + E; 4.5 mg/kg body weight per day).

The drinking water of animals was supplemented with vitamin E: “Aqua E” [33] containing 20 IU d- α -tocopherol, 15 mg other tocopherols, and 2 mg tocotrienols per mL. Aqua E has been used to guarantee an equivalent Vitamin E absorption: according to Papas et al. (2007), Aqua E showed a better bioavailability in malabsorbing patients compared to conventional based supplements [33]. Body weight and food intake were measured weekly, water/vitamin E uptake as an average per cage daily. Animals were sacrificed by cervical dislocation after 4 months (T4: end of intervention).

2.1. SCGE (Single Cell Gel Electrophoresis) Assay

DNA migration was studied in hepatocytes and colonocytes of mice in SCGE assay. These experiments are based on the measurement of DNA migration in an electric field [34]. Cells from livers and colons were collected according to the method developed by Sasaki et al., (2000) [35]. Briefly, 1.0 g liver tissue was homogenized by use of a Potter Elvehjem-type (B. Braun, Melsungen, Germany) at 400 rpm in 4.0 mL chilled homogenization buffer (pH 7.5). Subsequently, the homogenates were centrifuged (800 g, 10 min, 4 °C). Colon cells were isolated by scratching from the colon mucosa and kept on ice in 2.0 mL homogenization buffer. The nuclei were re-suspended in LMPA (low melting point agarose, 0.5%, Gibco, Paisley, UK) and transferred to slides which were pre-coated with NMPA (normal melting point agarose, 1.0%, Gibco, Paisley, UK).

The experiments were carried out according to international guidelines for SCGE experiments [36]. After lysis (pH 10.0) and electrophoresis (30 min, 300 mA, 25 V, at 4 °C, pH > 13), the gels were stained with ethidium bromide (20 μ g/mL, Sigma-Aldrich, Vienna, Austria). Three slides were prepared per experimental time point and 50 cells were evaluated from each slide. Slides were examined under a fluorescence microscope (Nikon EFD-3, Tokyo, Japan) using 25-fold magnification. DNA migration was determined with a computer-aided comet assay image analysis system (Comet Assay IV, Perceptive Instruments, Bury St Edmunds, UK).

2.2. Gene Expression Analysis

Colon and liver samples were stored at $-80\text{ }^{\circ}\text{C}$. RNA and DNA were isolated from liver and colon using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The concentration were measured respectively purity controlled with a Picodrop100 (Picodrop, Hinxton, UK). Complementary DNA (cDNA) was synthesized from $1\text{ }\mu\text{g}$ of total RNA by reverse transcription using RT² First Strand Kit (Qiagen, Hilden, Germany). cDNA was analyzed in real-time PCR using qPCR Primer Assays (Qiagen, Hilden, Germany) and RT² SYBR Green Mastermix (Qiagen, Hilden, Germany) according to protocol. PCR conditions were as follows: initial step of $95\text{ }^{\circ}\text{C}$ for 10 min, followed by 40 cycles of $95\text{ }^{\circ}\text{C}$ for 15 s and $60\text{ }^{\circ}\text{C}$ for 1 min, ending with melting curve analysis (gradient melting of the products was performed at $0.5\text{ }^{\circ}\text{C}/10\text{ s}$ from $65\text{ }^{\circ}\text{C}$ to $95\text{ }^{\circ}\text{C}$). Each sample was analyzed in duplicate, with normalization to the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as an internal control.

2.3. Methylation Analysis

Two micrograms of genomic DNA was bisulfite converted with EpiTect[®] Fast Bisulfite Conversion kit (Qiagen, Hilden, Germany) and amplified by PCR using the PyroMark PCR Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions with primers for *Dnmt1* and *MLH1* designed by PyroMark Assay Design SW 2.0 Software (Table 1).

The PCR was carried out in a total reaction volume of $25.0\text{ }\mu\text{L}$, containing $12.5\text{ }\mu\text{L}$ Pyromark 2 \times PCR master mix, 10 pmol (*Dnmt1*) or 7 pmol (*MLH1*) of each primer, $2.5\text{ }\mu\text{L}$ Coraload Concentrate 10 \times (Qiagen, Hilden, Germany), and 10.0 ng (*Dnmt1*) or 15.0 ng (*MLH1*) bisulfite converted DNA. Thermocycling started with initial denaturation at $95\text{ }^{\circ}\text{C}$ for 15 min, followed by 45 cycles at $94\text{ }^{\circ}\text{C}$ for 30 s, $55.5\text{ }^{\circ}\text{C}$ for 45 s, $72\text{ }^{\circ}\text{C}$ for 45 s and a final extension at $72\text{ }^{\circ}\text{C}$ for 10 min. PCR product quality was examined with agarose gel-electrophoresis. Analysis of CpG methylation was performed with a Pyromark Q24 MDx (Qiagen, Hilden, Germany).

Table 1. Sequence to analyze and primers for CpG methylation analysis.

Gene	Primer	Sequence (5' → 3')	Size (bp)	GC%
<i>DNMT1</i>	FW	Biotin-GTA GGT TGT AGA AGA TAG AAT AGT TTT GA	29	31
	RW	CCC ACT CTC TTA CCC TAT ATA ATA CAT	27	37
	Seq	CCC CTC CCA ATT AAT TTC	18	44.4
Sequence ID: gb AH009208.2 <i>DNMT1</i> : at reverse strand of chromosome 9: 20907205–20959888 (52684 bp).				
Sequence to analyze		7104-CGCGCGCGCGAAAAAGCCGGGTCTCGT-7131	27	7 CpGs
<i>MLH1</i>	FW	AGG GTA TTT TAG TTT TTA TTG GTT GGA GA	29	31
	RW	TTA CAC CTC AAT TCC TAA AAT CTC TAT CCC-Biotin	30	37
	Seq	TTT AGT TTT TAG AAA TGA GTT AAT A	25	16
Sequence ID: ref XR_379849.3 <i>MLH1</i> : at reverse strand of Chromosome 9: 111228228–111271786 (43559 bp)				
Sequence to analyze		19-GAAGAGCGGACCGTGAACCTTTGACGCGCAAGCGCG TTGCCTTCTA-GCCTGGTGTCCGGCCGCTG-82	64	8 CpGs

2.4. Statistical Analyses

In SCGE assays, statistical analyses were performed using GraphPad Prism 5.02 (GraphPad Software, La Jolla, CA, USA). The means and SD of percent DNA in the comet tails of the nuclei from the different treatment groups were calculated. Group means were compared using Student's *t*-test based on the means of three slides.

All statistical analyses of gene expression and methylation analyses were performed using IBM SPSS Advanced Statistics 20.0 (SPSS, Standford, CA, USA). All data are shown mean \pm SD.

Δ CT values of each gene were calculated by normalization to the housekeeping gene GAPDH (Δ CT = CT-Target – CT-GAPDH). The $\Delta\Delta$ CT value shows the difference between the two groups. The Δ CT value of the control group was deducted from the Δ CT value of the vitamin E group ($\Delta\Delta$ CT = Δ CT-Vitamin E – Δ CT-Control). Relative changes in gene expression between the intervention and control group are determined by the $2^{-\Delta\Delta$ CT} equation (fold change = $2^{-\Delta\Delta$ CT). The Kolmogorov–Smirnov-Test was used to test the normality of the data distribution. To examine significant relationships, Mann–Whitney-U Test was used. The interaction between DNA damage and mean methylation was tested by Spearman correlation test. For all difference-of-mean and correlation tests p -values ≤ 0.05 were considered as significant.

3. Results

3.1. Body Weight, Food Intake and Vitamin E Uptake

Body weight and food intake were measured weekly and water/vitamin E uptake as an average per cage daily. As shown in Table 2 food intake and total water consumption did not differ between the groups. The body weight of mice fed a HFD (T1: 32.57 ± 2.09 g; T4: 47.09 ± 0.83 g) and a HFD + E (T1: 32.77 ± 2.3 g; T4: 47.67 ± 0.49 g) increased significantly in comparison to CD-fed mice (T1: 24.66 ± 0.75 g; T4: 28.31 ± 0.24 g) and the CD + E group (T1: 24.94 ± 0.77 g; T4: 28.63 ± 0.14 g) over study period ($p < 0.01$, Figure 1). The body weight increase over study period was significant in all groups ($p < 0.01$, Figure 1).

Mean Vitamin E uptake was 3.90 ± 0.14 μ L of Aqua E in the CD group and 5.89 ± 0.11 μ L of Aqua E in the HFD group of each mouse per day. The α -tocopherol intake was 0.08 IU (CD) and 0.12 IU (HFD) per day and mouse.

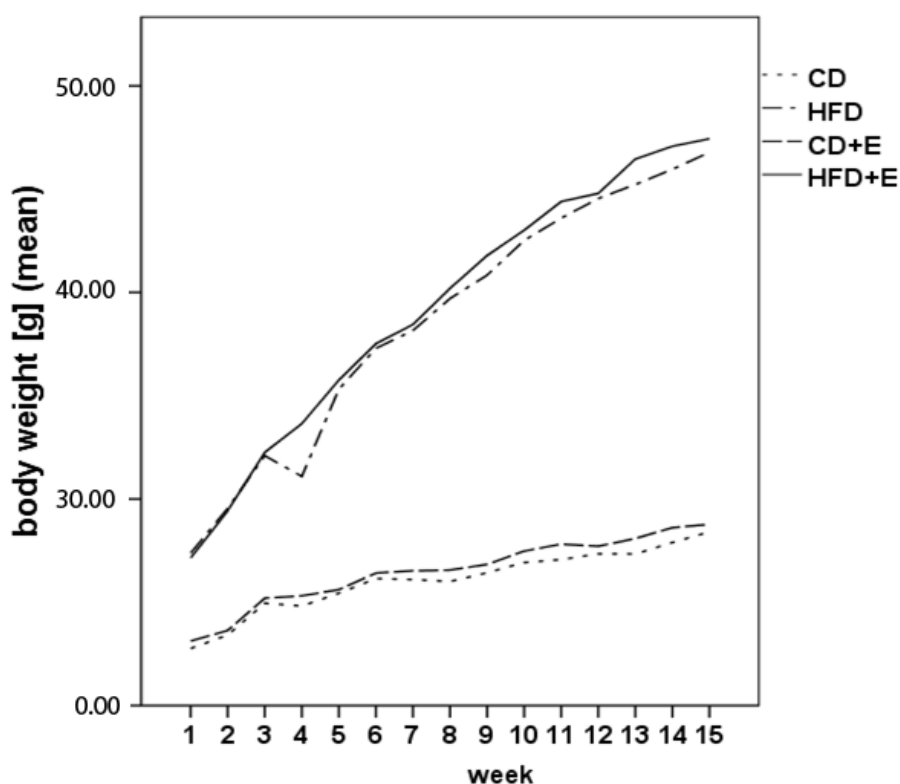


Figure 1. Body weight gain of C57BL/6J male mice over four months ($n = 15$) (CD = control diet; HFD = high fat diet; CD + E = control diet plus vitamin E; HFD + E = high fat diet plus vitamin E).

Table 2. Body weight, food and water intake of C57BL/6J male mice over a period of four months.

Month	Chow Intake (g)				Water Intake (mL)				Weight (g)				
	1	2	3	4	1	2	3	4	1	2	3	4	
Intervention	CD	2.64 ± 0.07	2.11 ± 0.01	2.08 ± 0.04	2.06 ± 0.03	5.58 ± 0.21	5.29 ± 0.22	5.39 ± 0.28	4.95 ± 0.56	24.66 ± 0.75	26.17 ± 0.16	27.17 ± 0.18	28.31 ± 0.24
	CD + E	2.70 ± 0.08	2.70 ± 0.05	2.76 ± 0.06	2.76 ± 0.51	5.80 ± 0.21	5.76 ± 0.15	5.97 ± 0.17	5.55 ± 0.27	24.94 ± 0.77	26.58 ± 0.15	27.75 ± 0.19	28.63 ± 0.14
	HFD	2.56 ± 0.04	2.59 ± 0.02	2.60 ± 0.02	2.56 ± 0.06	5.34 ± 0.20	4.93 ± 0.24	5.10 ± 0.14	5.01 ± 0.18	32.57 ± 2.09	39.00 ± 1.37	43.97 ± 1.02	47.09 ± 0.83
	HFD + E	2.51 ± 0.02	2.45 ± 0.06	2.50 ± 0.05	2.54 ± 0.01	5.21 ± 0.20	4.69 ± 0.06	5.05 ± 0.04	5.09 ± 0.11	32.77 ± 2.3	39.49 ± 1.64	44.67 ± 1.23	47.67 ± 0.49

3.2. SCGE Experiments from Colon and Liver Cells

HFD caused significant induction of DNA damage in both organs compared to CD (Figure 2). The extent of DNA migration was more pronounced in the colon (2.6-fold) than in the liver (2.3-fold). In HFD + E, the extent of DNA migration was significantly decreased by 17% ($p \leq 0.05$) in the colon while no effect was seen in the liver compared to HFD fed mice (Figure 2). Supplementation with vitamin E in CD group caused significant DNA migration in both organs (1.7-fold in colon and 1.3-fold in liver).

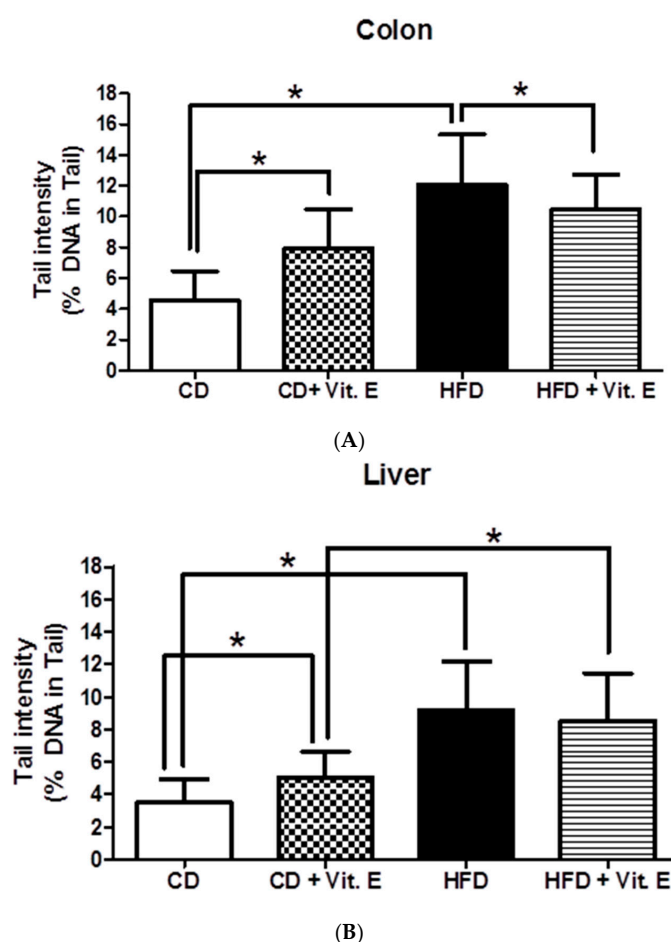


Figure 2. Impact of vitamin E supplementation on DNA damage in colon (A) and liver (B) of C57BL/6J male mice ($n = 15$). Bars indicate means \pm SD of results obtained with 15 animals per group. From each sample, three slides were made and 50 cells were evaluated per slide. (CD = control diet; HFD = high fat diet; CD + E = control diet plus vitamin E; HFD + E = high fat diet plus vitamin E; * p -value ≤ 0.05).

3.3. Relative Gene Expression (Figure 3) and CpG Methylation (Figure 4) of *Dnmt1* in Colon and Liver Cells

In colon cells, the relative expression of *Dnmt1* decreased for 61% in HFD compared to CD ($p \leq 0.01$). With vitamin E supplementation *Dnmt1* relative gene expression was significantly lower (86%) in HFD + E in comparison to CD + E ($p \leq 0.01$). The relative gene expression of *Dnmt1* in colon cells of HFD showed no significant differences in comparison to HFD + E ($p = 0.394$). Relative to CD mice, the vitamin E supplementation (CD + E) resulted in 87% higher expression of *Dnmt1* ($p \leq 0.01$; Figure 3). In the liver, a significantly lower expression of *Dnmt1* in HFD compared to CD was shown with a reduction of 61% ($p \leq 0.01$). CD compared to CD + E (79%) and HFD compared to HFD + E (68%) showed both a lower gene expression of *Dnmt1* in the liver ($p \leq 0.01$). The relative gene expression of *Dnmt1* in liver cells was 25% lower in HFD + E compared to CD + E ($p \leq 0.01$, Figure 3).

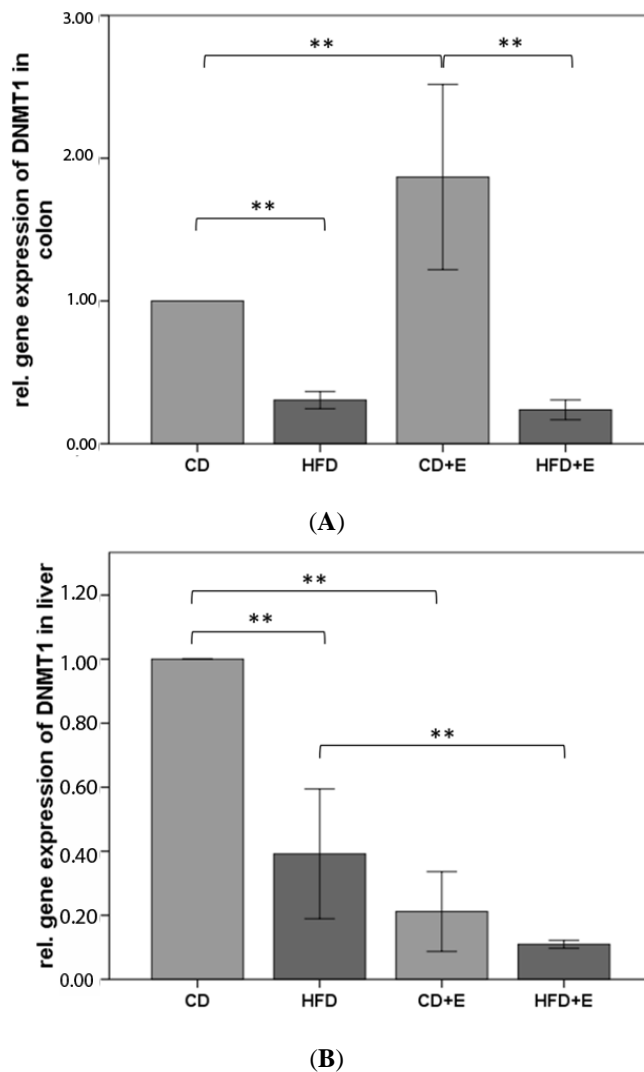


Figure 3. Relative gene expression of *Dnmt1* in colon (A) and liver (B) of C57BL/6J male mice ($n = 15$). Gene expression data were calculated relative to CD-data and normalized to the house keeping gene GAPDH. The error bar represents a 95% confidence interval. (CD = control diet; HFD = high fat diet; CD + E = control diet plus vitamin E; HFD + E = high fat diet plus vitamin E; ** p -value ≤ 0.01).

Four CpGs were analyzed in the promoter region of *Dnmt1* in liver and in colon (Figure 4). In colon cells, significant differences in methylation status were measured in CpG 3 and 4. In CD + E, CpG 3 showed 33.67% lower methylation ($p \leq 0.01$) when compared to CD, and 25.67% lower when compared to HFD + E. Moreover, a significant decrease in methylation of both CpG 3 (17.13%, $p \leq 0.01$) and CpG 4 (21.57%, $p \leq 0.05$) was indicated in HFD + E in comparison to HFD. By comparing HFD with CD, a slight increase in methylation levels was observed in HFD, however, no significant difference was detected between those two groups ($p = 0.394$, Figure 4A). In CD a negative correlation between DNA damage and the mean methylation of *Dnmt1* in colon was seen ($r^2 = -0.837$, $p \leq 0.05$).

In liver cells, CpG 3 showed the highest relative methylation among all intervention groups. When compared to CD animals, increased methylation levels of CpG 3 were found in HFD, CD + E and HFD + E. Particularly in CD + E, vitamin E supplementation caused a significant hypermethylation of CpG 3 (59.67%, $p \leq 0.01$). No significant difference in *Dnmt1* methylation in liver was observed between the HFD and HFD + E group (Figure 4B). Furthermore, in HFD DNA damage correlated positively with the mean methylation of *Dnmt1* in the liver ($r^2 = 0.956$, $p \leq 0.01$).

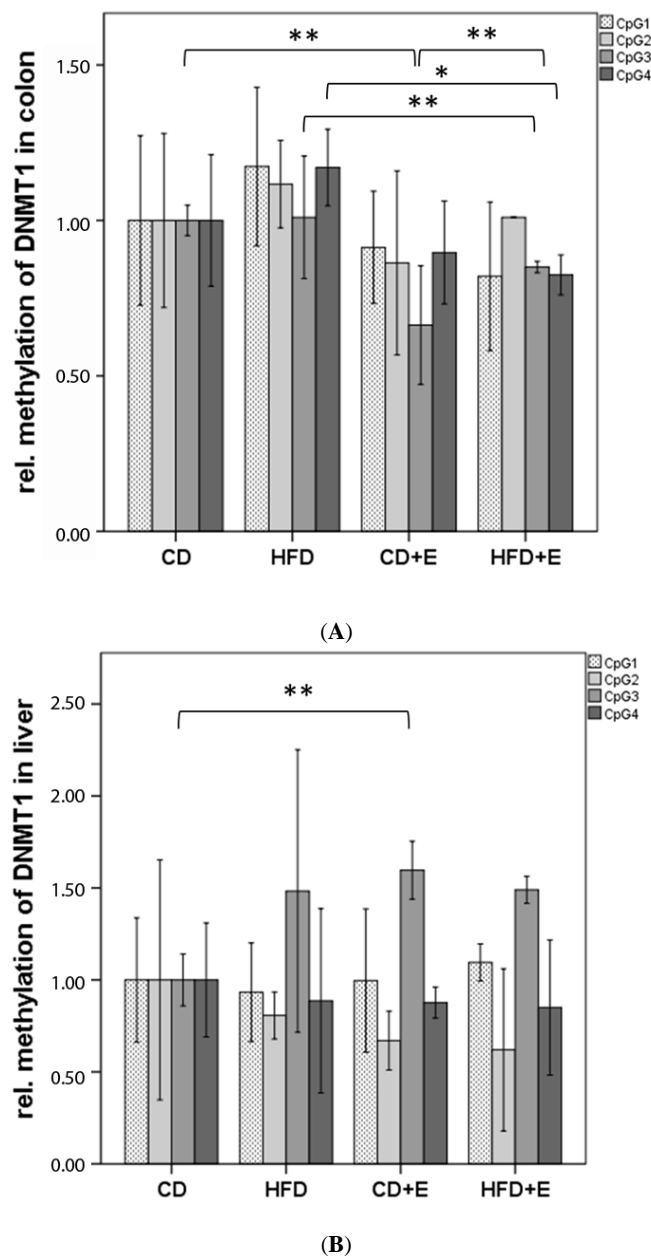


Figure 4. Relative CpG methylation status in promoter region of *Dnmt1* in colon (A) and liver (B) of C57BL/6J male mice ($n = 15$). All methylation data are relative to CD. The error bar represents a 95% confidence interval. (CD = control diet; HFD = high fat diet; CD + E = control diet plus vitamin E; HFD + E = high fat diet plus vitamin E; * p -value ≤ 0.05 ; ** p -value ≤ 0.01).

3.4. Relative Gene Expression (Figure 5) and CpG Methylation (Figures 6 and 7) of *MLH1* in Colon and Liver Cells

The relative gene expression of *MLH1* in colon did not result in significant differences between CD and HFD animals ($p = 0.659$). However, vitamin E supplementation induced a higher gene expression of *MLH1* in CD + E in comparison to CD (36%, $p \leq 0.01$). In contrast vitamin E supplementation in HFD caused a significant reduction of 72% compared to HFD ($p \leq 0.01$, Figure 5). The relative gene expression of *MLH1* in liver was significantly lower in the HFD animals (49%) compared to CD and HFD + E (53%) compared to HFD. Vitamin E supplementation induced a significant (58%) lower expression of *MLH1* in liver compared to CD ($p \leq 0.01$; Figure 5).

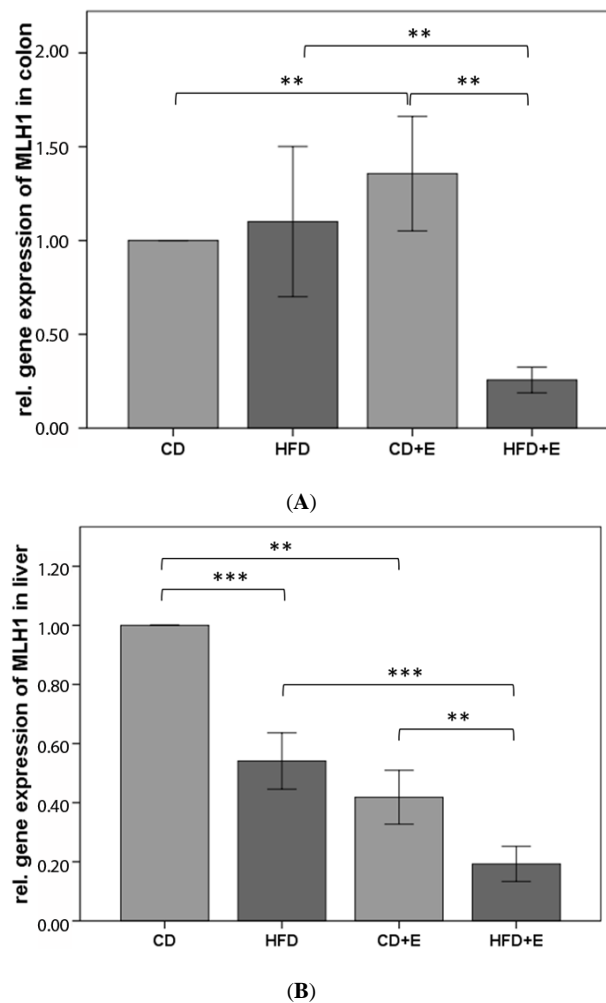
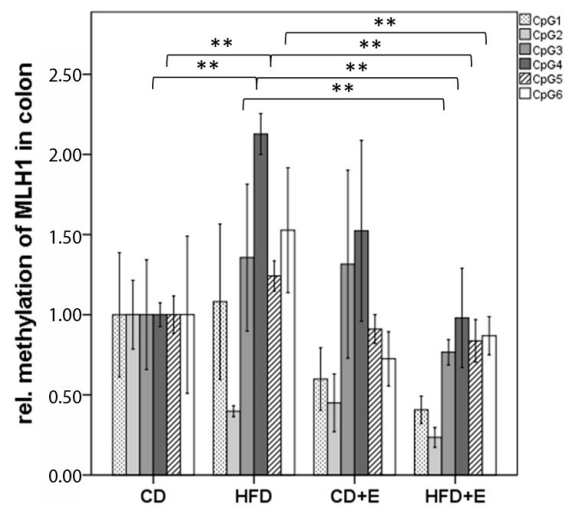
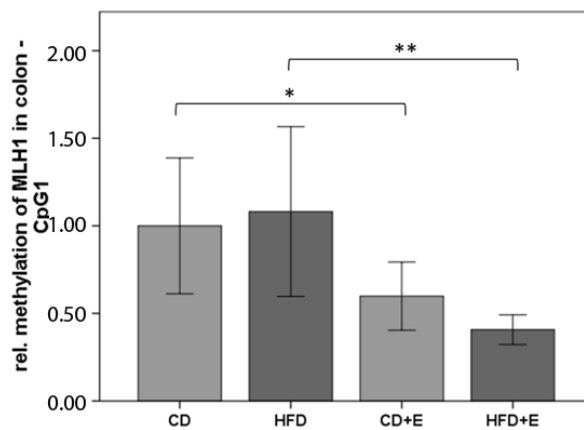


Figure 5. Relative gene expression of *MLH1* in colon (A) and liver (B) of C57BL/6J male mice ($n = 15$). Gene expression data were calculated relative to CD-data and normalized to the house keeping gene GAPDH. The error bar represents a 95% confidence interval. (CD = control diet; HFD = high fat diet; CD + E = control diet plus vitamin E; HFD + E = high fat diet plus vitamin E; ** p -value ≤ 0.01 ; *** p -value ≤ 0.001).

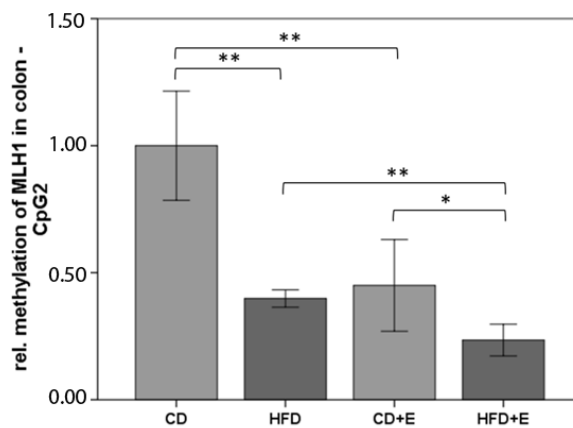
In the *MLH1* promoter region, the relative methylation was analyzed for six CpGs in colon (Figure 6) and liver (Figure 7). In colon cells, vitamin E supplementation significantly reduced methylation of CpG 1 (CD + E: 40.17%, $p \leq 0.05$; HFD + E: 59.30%, $p \leq 0.01$) and 2 (CD + E: 55.00%, $p \leq 0.01$; HFD + E: 76.53%, $p \leq 0.01$) in comparison to CD (Figure 6A,B). The same effect has been observed over all six CpGs in HFD + E compared to HFD (all $p \leq 0.01$). Significantly different methylation levels between CD and HFD were found in CpG 2, 4 and 5 (all $p \leq 0.01$). In general, HFD showed a higher relative methylation over all CpGs with exception of CpG 2, where *MLH1* methylation decreased by 60.17% in comparison to CD ($p \leq 0.01$) (Figure 6A,C). In liver cells, similar to the colon, hypomethylation of CpG 1 was found in both supplementation groups (CD + E: 59.63%; HFD + E: 60.63%; all $p \leq 0.01$) in comparison to CD, and the methylation of CpG 1 was reduced by 56.63% in HFD ($p \leq 0.01$) (Figure 7B). On the contrary, the HFD showed significant hypermethylation of CpG 4 (72.11%, $p \leq 0.01$) and CpG 6 (11.93%, $p \leq 0.05$) when compared to CD. In comparison to HFD, vitamin E treatment (HFD + E) significantly decreased the methylation levels of CpG 1 (9.23%, $p \leq 0.05$) and 6 (35.37%, $p \leq 0.01$), whereas methylation of CpG 3 significantly increased by 27.57% ($p \leq 0.05$) (Figure 7A).



(A)



(B)



(C)

Figure 6. Relative CpG methylation status in promoter region of *MLH1* in colon of C57BL/6J male mice ($n = 5$): (A) mean methylation status for *MLH1* in colon is shown as an overview; (B) the methylation status of CpG 1; and (C) the methylation status of CpG 3 is specified. All methylation data are shown relative to CD. The error bar represents a 95% confidence interval. (CD = control diet; HFD = high fat diet; CD + E = control diet plus vitamin E; HFD + E = high fat diet plus vitamin E; * p -value ≤ 0.05 ; ** p -value ≤ 0.01).

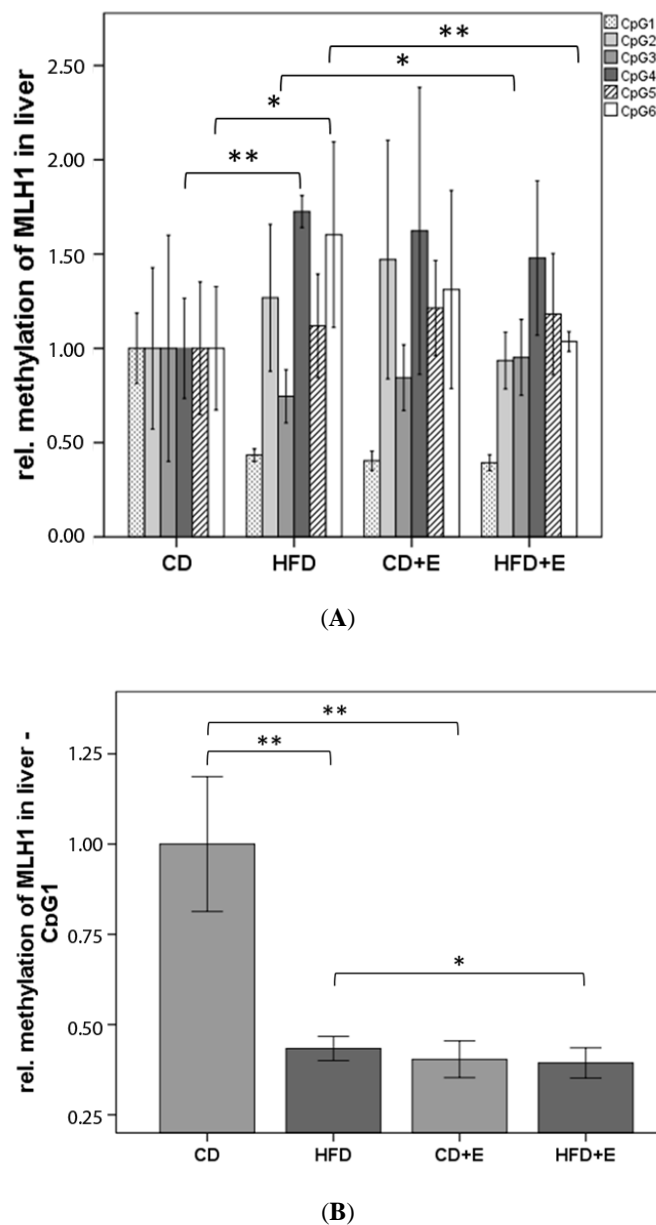


Figure 7. Relative CpG methylation status in promoter region of *MLH1* in liver ($n = 15$). Mean methylation data are shown for: *MLH1* in the liver as an overview (A) and CpG 1 (B). All methylation data are shown relative to CD. The error bar represents a 95% confidence interval. (CD = control diet, HFD= high fat diet, CD + E = control diet plus vitamin E, HFD + E = high fat diet plus vitamin E, * p -value ≤ 0.05 , ** p -value ≤ 0.01).

4. Discussion

HFD induced a significant increase of DNA damage in liver and colon compared with CD in mice. *Dnmt1* relative gene expression decreased significantly in both organs, whereas methylation status showed a slight increase in HFD compared to CD. The relative gene expression of *MLH1* in colon did not show significant differences between the two different diets, while, in liver, significantly lower *MLH1* expression was shown in HFD compared to CD. The methylation status of all CpGs was generally higher in HFD in comparison to CD.

Although IL-6 expression was below the detection limit in our experiment, low-grade inflammation is known as a major cause of obesity caused by the release of FFAs from adipocytes,

related to the increased amount of adipose tissue. FFAs are usually stored as triglycerides or provide energy through β -oxidation by cell's mitochondria. Minor products, ROS, are potentially harmful for cellular functions. A complex antioxidant system provides protection, although an imbalance due to obesity results in oxidative stress, potentiating comorbidities. Antioxidant therapies have been shown to reduce the oxidative stress, reduce susceptibility of low-density lipoprotein (LDL) to oxidation, inhibit secretion of pro-inflammatory cytokines [37], improve insulin signaling in vitro [38], and improve glycemic control in individuals with type 2 diabetes [39–41]. In vivo, vitamin E no longer improves insulin sensitivity [42], only transient improvements were shown [41,43].

Vitamin E interrupts lipid peroxidation due to the presence of the phenolic hydroxyl group on the chroman ring of the molecule resulting in tocopheroxyl radicals, which are regenerated by means of hydrogen donors. However, due to incomplete reduction, tocopheroxyl radicals can also induce oxidative stress by reaction with polyunsaturated fatty acids in the LDL particles. Thus, Aqua E has been used in concentrations in accordance to recommended daily allowance, and further studies testing different concentrations would be of interest.

Natural vitamin E comprises four tocopherols and four tocotrienols. Therefore, we used a mixture of tocopherols and tocotrienols, which reflects the human diet more accurately than pure α -tocopherol, used in previous animal and human studies. All forms of the vitamin E family are absorbed and delivered to the liver. Only α -tocopherol accumulates in this organ, whereas the other isoforms are rapidly metabolized and excreted. The accumulation of mainly α -tocopherol in hepatic tissue is the consequence of the expression of a cytosolic protein (α -tocopherol transfer protein, α -TTP) with high selectivity for α -tocopherol and low or very low affinity for the other tocopherols [44]. α -TTP and other bound vitamin E forms are prevented from being catabolized in the liver. Among the isoforms γ -tocopherol is slightly less efficient than α -tocopherol as a scavenger of oxygen radicals, but it is an efficient scavenger of reactive nitrogen species [45]. In addition, tocotrienols have more pronounced cancer protective effects than tocopherol [46], and tocotrienols are notably also more effective in NF- κ B inhibition than tocopherols [47].

4.1. Vitamin E Protects DNA Damage Caused by HFD

We found a significant increase of DNA damage with HFD in both organs. Vitamin E supplementation decreased DNA damage. Bardowell et al (2012) estimated that unmetabolized tocopherols and tocotrienols are discarded via biliary excretion in feces [48]. This observation may provide an explanation for more pronounced protective effects in the colon. Ju et al. [49] evaluated 32 animal studies published since 1980 with regard to cancer-preventive activities of tocopherols and tocotrienols. Only 12 studies focused on colon tumorigenesis and aberrant crypt foci formation, and only two out of twelve studies showed a protective effect of vitamin E family compounds in colon. Vitamin E has also been shown to protect against liver damage induced by oxidative stress in animal experiments [50,51].

Taken together, this study showed that supplementation of Aqua E with HFD for four months significantly improves DNA damage in colon and liver of mice. This identifies vitamin E as an important nutritional factor in the prevention of DNA damage caused by oxidative stress due to obesity although the dosage has to be taken into account.

4.2. Vitamin E Supplementation Affects Specific CpG Sites of *Dnmt1*, Resulting in Altered Relative Gene Expression of *Dnmt1*

As mentioned above, oxidative stress due to obesity is involved in both genome-wide hypomethylation and promoter hypermethylation of the DNA [8] as oxygen radicals impair DNA lesions. These lesions interfere with methylation activity since damaged DNA cannot serve as acceptor for methyl groups, causing global hypomethylation [9,10].

The methyl donor SAM and the expression of *Dnmt1* are also impaired by oxidative stress [17,18]. Both are important in the maintenance of epigenetic modifications by adding methyl groups to the

C5 position of cytosine in CpG dinucleotides at the replication fork and are responsible to copy DNA methylation patterns to newly synthesized daughter strands [18]. Thus, altered expression of *Dnmt1* can lead to hypo- or hypermethylation of other genes, resulting in expression changes of mRNAs or proteins. Altered levels of *Dnmt1* can disrupt cellular mechanisms and may lead to pathological changes of different gene functions [18].

We showed a lower expression of *Dnmt1* in colon of HFD with no changes due to vitamin E supplementation, although in CD animals an increase with supplementation was noted (Figure 3). Methylation status of CpG 3 and 4 in the promoter region of *Dnmt1* in colon is significantly lower in CD + E compared to CD and HFD + E. The same CpGs showed a significantly lower methylation in HFD + E compared with HFD control group. HFD showed a slightly higher promoter methylation status compared to CD (Figure 4). In all intervention groups, a decreased gene expression was noted in liver (Figure 3) although the methylation status of CpG 3 was increased in HFD, CD + E as well as in HFD + E. However, no significant differences were observed between the two HFD groups, while in the CD + E group vitamin E supplementation caused a significant hypermethylation of CpG 3 (Figure 4). Furthermore, in the liver, a positive correlation of *Dnmt1* mean methylation and DNA damage has been observed in liver whereas in CD a correlation has been found in the colon.

Given those observations, it may be concluded that vitamin E induces tissue specific changes in *Dnmt1* gene expression and relative promoter methylation, which presumably depend on the host metabolic state (lean vs. obese). In addition to this statement, different results in colon and liver may be explained by vitamin E metabolism. It is known that all vitamin E isomers undergo intestinal absorption and afterwards are taken up by liver. However, only α -tocopherol can be retained in hepatocytes, which is primarily due to the hepatic α -TTP that preferentially binds to α -tocopherol and prevents its hepatic catabolism [52]. Other forms, in contrary, are fast metabolized and excreted, with about 80% of the total metabolites being eliminated via fecal route [52]. γ -tocopherol and tocotrienols are thought to have anti-inflammatory and anti-oxidative properties not shared by α -form [53]. Furthermore, particular metabolites that may accumulate in colon have been shown to inhibit pro-inflammatory pathways more strongly than unmetabolized vitamin E forms [52].

In addition, some mechanistic studies suggested that vitamin E exerts its anti-oxidative and anti-inflammatory activities by modulating transcriptional factors via *Dnmt1*-dependent route. One of them is NF- κ B, a pro-inflammatory factor activated by ROS [54]. It is reported that NF- κ B stimulates *Dnmt1* through cytokine-dependent pathway, and vice versa, inhibition of NF- κ B led to reduction in *Dnmt1* expression [55]. Tocotrienols and especially α -tocopheryl succinate have been recognized as effective inhibitors of NF- κ B [56], and all of them were supplied by the vitamin E supplement used in our study. However, we did not detect a higher gene expression of *IL-6* between the groups in colon and liver. Upritchard et al. (2012) showed a significant decrease of the inflammatory status in type 2 diabetics due to vitamin E supplementation (800 IU/day), indicated by decreased plasma levels of C reactive protein (CRP) [57]. Inhibition of IL-1 β release decreases the expression of IL-6 and further of CRP [37]. However, an antioxidant-independent effect via a decrease of 5-lipoxygenase activity is suggested [58], although no changes of inflammatory markers and of plasma CRP levels were observed due to vitamin E supplementation (800 IU/day and 1200 IU/day) in overweight individuals [41]. Adverse effects were also shown, such as increased risk for heart failure [59] or increased risk of hemorrhagic stroke [60]. Furthermore, tumor suppressor genes or cell cycle regulation may be affected leading to aberrant cell growth [20]. Thus, a routine vitamin E supplementation due to obesity is not recommended at the present.

Recent research indicated the requirement of an ubiquitin interacting motif (UIM) in the N-terminal regulatory domain of *Dnmt1*, which binds to ubiquitinated H3 tails and is essential for DNA methylation in vivo. H3 ubiquitination and subsequent DNA methylation were shown to require UHRF1 (Ubiquitin-like, Containing PHD and RING Finger Domains, 1) PHD (plant homeodomain) binding to H3R2 [61]. In addition, we should consider that the methylation status and gene expression are only snapshots, and cell cycle information is missing. However, Fuks et al. (2000) disclosed the

interaction of *Dnmt1* with histone deacetylase activity and repression of gene transcription in vivo [62]. In mice with diet induced obesity (DIO), the binding of HDACs is increased at the leptin promoter whereas histones H3 and H4 are hypoacetylated, lysine 4 of histone H3 (H3K4) is hypomethylated. The methylation and the binding of DNMTs and methyl-CpG-binding domain protein 2 (MBD2) are increased and RNA Pol II is decreased, resulting in a negative correlation of leptin promoter methylation and leptin gene transcription. These modifications may indicate a feedback loop for the maintenance of leptin concentrations due to obesity [63]. In another DIO mouse model, *Dnmt1* expression and enzymatic activity were elevated in adipocytes, leading to promoter hypermethylation and following decreased adiponectin expression [55].

Potential compensatory effects in response to a lack or oversupply of methyl groups for DNA methylation may also affect *Dnmt1* expression [64]. The sequence [5'-TTTCCGCG-3'] within the genomic methylation analysis (CpG 1 and 2 in our study), was identified as crucial site for the transcriptional regulation of *Dnmt1* by the transcription factor E2F1 [65,66]. However, we were not able to show significant changes on these specific CpG sites. These results disclose the various mechanisms controlling *Dnmt1* activity and the multifaceted interplay between DNA and histone modifications, or even the diverse effects on other CpG methylation of target gene promoters. Thus, we were not able to elucidate how and if *Dnmt1* regulates the MMR system in the current study, although a coherence is shown with *MLH1*.

4.3. Vitamin E Supplementation Affects Specific CpG Sites of *MLH1*, Inducing a Lower Gene Expression of *MLH1* with High-Fat Diet

MLH1 is part of the MMR system that is responsible for ensuring overall DNA integrity [14]. Enhanced oxidative stress, as a consequence of overweight and obesity, can cause elevated DNA damage [67] which in turn requires optimal function of the MMR system, including *MLH1*.

We showed a higher gene expression of *MLH1* in CD + E in comparison to CD and HFD in colon, whereas in HFD + E *MLH1* gene expression decreased (Figure 5). The methylation status of six CpGs in the promoter region of *MLH1* in colon showed a higher methylation over all s in comparison to CD (Figure 6). In addition, vitamin E supplementation induced a lower methylation of specific CpG sites in both supplemented groups, also shown in liver cells. Vitamin E supplementation induced a lower expression of *MLH1* in liver (Figure 5). Both HFD groups also had a lower expression compared to CD. CpG 4 and 6 were significantly hypermethylated in HFD compared to CD. Comparisons between HFD and HFD + E showed significant differences in methylation levels of three CpG sites (Figure 7).

Switzeny et al. (2012) showed a significant higher CpG methylation in two particular *MLH1* promoter regions. The methylation status and DNA strand breaks correlated significantly, although no changes in gene expression of *MLH1* due to dietary intervention with folate in non-insulin dependent diabetes mellitus type 2 was shown [24]. Thus, our results are in accordance with previously published data using antioxidants. Sinicrope et al. (2015) indicated a “less likely” deficient MMR in colon cancers from obese patients, suggesting that obesity-associated colon cancers are predominantly caused by sufficient MMR, a molecular subtype showing chromosomal instability with significantly worse survival rates. However, only the deficient MMR colon cancers are associated with a higher DNA methylation near gene promoter regions of *MLH1*. Higher estradiol levels in both sexes due to obesity might be the cause of the lower frequency of deficient MMR system [68].

In summary, differences in gene expression might indicate tissue specificity, different metabolic pathways, especially as a higher nutrient bioavailability is indicated in colon, main transit organ, but not in the metabolizing organ (liver), with substance dependence in the enterohepatic pathway or the transport system (blood). Differences in methylation status might already indicate an adaption to dietary intervention, although the duration is not sufficient for ROS-dependent defects in gene expression. Differences of methylation status of different CpG-sites in promoter regions are rarely known, although some CpG sites show more profound results between our groups. The involvement of other epigenetic modifications has to be taken also into account: DNA methylation at gene promoters

regulates gene expression through a complicated mechanism involving multiple modifications, including histone modifications and miRNAs. Similar results seen in in vitro experiments with Caco2 cells will be submitted for publication soon.

5. Conclusions

Our study with C57BL/6J male mice fed a HFD or CD with or without supplemental vitamin E shows significant effects of HFD on DNA damage, analyzed in SCGE assays. HFD also resulted in significant organ-specific changes in the epigenetically important *Dnmt1* gene and the DNA repair gene *MLH1*. Vitamin E reduced DNA damage and affected *Dnmt1* and *MLH1* gene expression and methylation, which was also organ specific. These results suggest that intervention with vitamin E, as an epigenetic active food ingredient, can be developed as an effective prevention of obesity-related and oxidative stress-induced health risks.

Acknowledgments: The work was funded by the Austrian Science Fund (FWF; AP2658721). The authors thank Brian Metscher for the English corrections.

Author Contributions: M.R. together with A.H., K.H.W. and S.K. designed and coordinated research. M.R., I.R., M.G., L.M., S.S., S.R. and T.K., F.F., S.T. and N.R. conducted research. S.S. analyzed data. M.R. and F.F. wrote the paper. M.R., A.H. and S.K. had primary responsibility for final content. All authors have read and approved the final manuscript.

Conflicts of Interest: The authors declare to have no actual or potential competing interests that might be perceived as influencing the results or interpretation of a reported study.

Abbreviations

α -TTP	α -tocopherol transfer protein
CAT	catalase
CD	control diet
CD + E	control diet plus vitamin E
cDNA	complementary DNA
CRP	C reactive protein
CYPs	cytochrome P450s
DIO	diet induced obesity
<i>Dnmt1</i>	DNA methyltransferase 1
FFAs	free fatty acids
GAPDH	glyceraldehyd-3-phosphat-Dehydrogenase
GPX	glutathione peroxidase
H	histone
HFD	high fat diet
HFD + E	high fat diet plus vitamin E
IL-6	interleukin-6
LDL	low-density lipoprotein
LMPA	low melting point agarose
MBD2	methyl-CpG-binding domain protein 2
MCP-1	monocyte chemoattractant protein-1
MGMT	O6-methylguanine-deoxyribonucleic acidmethyltransferase
<i>MLH1</i>	MutL homolog 1
MMR	DNA mismatch repair
MSI	microsatellite instability
NADPH	nicotinamide adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver diseases
NMPA	normal melting point agarose
Nrf2	Nuclear factor-erythroid 2-related factor 2
PAI-1	plasminogen activator inhibitor-1
PHD	plant homeodomain

ROS	reactive oxygen species
SAM	S-Adenosylmethionin
SCGE	single cell gel electrophoresis
SOD	superoxide dismutase
TNF α	tumor necrosis factor α
T	time point
UHRF1	Ubiquitin-like, Containing PHD and RING Finger Domains, 1
UIM	ubiquitin interacting motif

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