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EGCG Containing Combined Dietary Supplement Affects Telomeres and Epigenetic Regulation

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Abstract

Objective: *In vitro* and *in vivo* studies in rodents have demonstrated many health promoting properties of individual phytochemicals including antioxidative and chemopreventive effects. Recently combination of substances is claimed to enhance activity.

The objective of this study was to investigate health benefits of a daily consumption of a combination of a large variety of phytochemicals (TimeBlock®). To assess potential changes we analyzed specific biomarkers that are associated with aging, oxidative stress and DNA stability: Methylation of *LINE-1, c-Myc, IL-6, MLH1, DNMT1, ITGA2B* and telomere length.

Methods: For this study 110 healthy participants of both sexes between 31-76 years were recruited, 101 subjects were included in further analysis. A small reference group (n=20) without intervention within the same age interval served as control. Participants received a plant based dietary supplement (TimeBlock®) for 6 months by oral administration. Ingredients included extracts from green tea (EGCG), wheatgrass (tocotrienols), barley grass (folic acid), tomatoes (lycopene), tagetes (zeaxanthin, lutein), algae, shiitake mushrooms (vitamin D) and grape seeds (resveratrol). Capillary blood samples were collected from all participants before administration and within 6 days after the end of the study period following DNA extraction, bisulfite conversion and qPCR as well as high resolution melting curve analysis addressing analysis of *LINE-1, c-Myc, IL-6, MLH1, DNMT1, ITGA2B* and telomere length. Nutrition, lifestyle and health status were assessed with a standardized food and lifestyle questionnaire.

Results and discussion: Our results confirmed the positive effect of plant derived antioxidants on telomeres and inflammation frequency. An age-specific drift of analyzed markers could be observed. While methylation of *c-Myc*-a key factor in telomerase regulation-was not affected by administration, total telomere length showed a significant increase, which we suggest to be linked with an increased cell turnover and accelerated apoptosis of senescent or mutated cells without enhancing telomerase activity. Further, methylation of mismatch repair protein gene *MLH1* showed a strong negative correlation with telomere length, supporting the influence of MMR on telomere regulation.

Conclusion: The results of the present study indicate that a combined administration of a variety of phytochemicals can be a potential preventive and therapeutic agent, as each substance exhibits different modes of action and in combination, health promoting effects could be potentiated. Addressing different mechanisms of aging, specific phytochemicals could be used as new therapeutic approach against age-related diseases.

Keywords: EGCG; Telomere length; LINE-1; c-Myc; IL-6; MLH1; DNMT1; ITGA2B; DNA methylation; aging

Introduction

Research of the last decades has shown that understanding the interaction of nutrition and health plays a substantial role in disease prevention and therapy and consequently healthy aging. Numerous trials and meta-analyses have already demonstrated that a diet comprising a rich variety of vegetables and fruits is strongly associated with a reduced risk of various chronic and age-related diseases including diabetes mellitus, cardiovascular or neurodegenerative disorders and cancer [1-5]. It is considered that the health promoting properties are in particular attributable to non nutritive plant compounds such as vitamins and phytochemicals like polyphenols, carotenoids or glucosinolates which include multiple mechanisms to improve human health [6] and as discussed more recently, may delay the onset of aging and age-related disorders [7,8]. To understand different modes of action of phytochemicals in this context it is necessary to focus on the mechanisms of aging.

Mechanisms of aging

Aging is a multifactorial and tissue-specific process involving diverse alterations regarded as the "hallmarks of aging" by Lopéz-Otín 2013, which include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intracellular communication [9].

Several theories of aging are discussed covered by two prominent mechanisms: Damage-based theories of aging state that aging is mainly due to interactions with the environment and/or a result of damage from chemical reactions. On the other hand, programmed theories imply that aging is a predetermined process influenced by genetic factors. However, it is considered highly probable that several

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different molecular pathways overlap based on changes in gene expression, defects in DNA repair and accumulating DNA damage. It is well established, that over the course of time, the genomic landscape as well as the gut microbiota composition is subject to ongoing changes. While being hugely affected by external factors like environment, lifestyle and diet, these processes result in a greater susceptibility to a wide variety of age-related diseases.

One crucial factor in aging is the reduced proliferative potential of cells leading to accelerated aging in elderly persons. As the body ages and the cells divide, a small portion of DNA is lost with each cell division at the end of our chromosomes. Telomeres, specific DNA–protein structures comprised of tandem repetitions of a nucleotide sequence (TTAGGG) constitute and protect the ends of the chromosomes. The telomere protein system is essential for genomic stability and chromosomal integrity. As the body ages, telomeres shorten with each cell cycle. When telomeres get critically short, cells undergo senescence and/or apoptosis. Thus, telomere length may serve as a biological clock to determine the lifespan of an organism or cell.

A critically determining factor of telomere length is the enzyme telomerase that has the capacity to slow telomere attrition by synthesizing telomeric repeat DNA and therefore maintaining telomere length. Telomerase contains two core components, a catalytic unit called the Human Telomerase Reverse Transcriptase (hTERT) and an RNA template (hTERC) in addition to associated proteins. In adult humans most somatic cells have a very low telomerase activity in contrast to cells with high replicative demands including fetal epithelial cells, lymphocytes and hematopoietic cells. c-Myc, a proto oncogene essential for cell growth regulation has been shown to regulate telomere length [10,11]. *c-Myc* hypomethylation and overexpression were associated with various types of tumors [12,13].

Another crucial factor of aging is the epigenetic makeup of the cells. Epigenetics refers to modifications in the DNA without changing the underlying DNA sequence resulting in a different DNA accessibility and chromatin structure and consequently, an altered pattern of gene activity and expression. Multiple epigenetic mechanisms have been identified including DNA methylation and histone modifications, as well as non-coding RNAs with recent studies revealing an intense crosstalk between these pathways [14,15]. Epigenetic processes are essential for normal development and metabolism. Therefore interference of these natural pathways can have notable consequences and is associated with aging and cancer [16]. However, regulation of the epigenetic landscape can turn specific genes on and off in a reversible manner [17,18]. Particularly DNA methylation patterns are suggested to change in an age dependent manner including local hypermethylation and global hypomethylation [19-21]. Latter notably emerges at repetitive DNA sequences and thus is believed to be responsible for reactivating retro transposon elements during age resulting in a higher incidence of cancer [22,23]. This decrease in DNA methylation can be measured by the DNA methylation of the repetitive element *LINE-1* which is spread throughout the genome [24]. Apart from global methylation patterns local DNA methylation of very specific DNA sites can also be correlated with the age of individuals [19,21,25]. Weidner et al. could identify a set of three age-related CpGs-located in the genes *ITGA2B, ASPA* and *PDE4C*- which correlated very precisely with a variety of physiological parameters of biological aging [25].

Furthermore, while aging the immune system is subject to alterations. Chronic inflammation strongly affects the pathogenesis of chronic and age-related diseases. With increasing age there is an enhanced incidence of a low level chronic inflammation in the absence of infection which is called inflammaging [26,27]. Among other cytokines, in particular interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-alpha) levels are elevated in this state. Therefore they are widely used markers for the presence of chronic inflammation and consequently, indicators of inflammaging [26,28]. Systemic low-grade inflammation is a key mechanism of aging and can result in persistent oxidative stress causing DNA damages, telomere attrition, genetic or coding errors, epigenetic abnormalities, and impaired regulation of gene expression since processes like DNA methylation and repair as well as transcription and translation are susceptible to free radicals [29-31].

Phytochemicals: Modes of action

Many studies have indicated that the potential effects of dietary phytochemicals are associated with their intrinsic antioxidant activity meaning the scavenging ability of Reactive Oxygen Species (ROS) [32]. Due to their chemical structure comprising aromatic rings, polyphenols are the main kind of antioxidant phytochemicals abundant in human diet [32,33]. Their antioxidant capacities are able to combat an overproduction of oxidants with its resulting damages to DNA, lipids or proteins that are responsible for the development of several diseases including cancer. Oxidative stress is still of one the most debated mechanisms of aging [34]. However, antioxidative nutrients are discussed as potential anti-aging agents [35,36]. In this context particular polyphenols have evoked special interest. For instance, epigallocatechingallate (EGCG), the main polphenol in green tea, was shown as a strong antioxidant *in vitro* as well as in regulating age-related oxidative damage in rodents [37,38].

Many antioxidant phytochemicals not only possess strong free radical scavenging abilities but also anti-inflammatory action providing the basis for health promoting properties such as inhibition of prostaglandin, influence on cytokine production, and regulation of nuclear factor-κB activity [38,39].

Research on the various modes of action of phytochemicals has developed significantly in the past years and it has become clear that their effectiveness goes beyond the regulation of oxidative stress. Particularly awareness of how phytochemicals act at the molecular level affecting gene expression has evoked special interest. When investigating nutrigenomics-the relationship between nutrients and our genome-epigenetics has turned out to be a promising new field and a rapidly growing area of research.

Phytochemicals such as EGCG are capable of affecting aberrant epigenetic events by various mechanisms including inhibition of DNA methyltransferase (DNMT)–the enzyme responsible for adding methylgroups to DNA, modulation of histone acetylation via histone deacetylase (HDAC), histone acetyltransferase (HAT) inhibition or influence on noncoding RNA expression [40-44]. Thus, dietary phytochemicals exhibiting epigenetic properties such as EGCG could prevent disease development and premature aging [44,45]

Furthermore, especially nutrients involved in the metabolism of methyl groups such as methionine, choline, vitamin B12 and folic acid are suggested to play a central role in maintaining DNA methylation patterns while aging [46].

There is growing evidence that epigenetic mechanisms affecting DNA methylation and histone status also modulate genomic instability and DNA damage response. By impacting the acetylation status of histone and non-histone proteins HDAC inhibitors like EGCG are able to silence DNA repair pathways [40]. Furthermore it has been shown that EGCG also acts as a HAT inhibitor suppressing

transcription factor p65 acetylation, and consequently inhibiting interleukin 6 (IL-6), nuclear factor kappa B (NFκB), and downstream target genes [41]. In addition, Fang et al. demonstrated that EGCG *in vitro* caused a reversal of hypermethylation of *retinoic acid receptor beta* (*RARbeta*), *p16* (*INK4a*), *O(6)-methylguanine methyltransferase* (*MGMT*), and *human mutL homologue 1* (*hMLH1*) genes in cancer cells with a concurrent effect on the expression of mRNA of these genes [42]. Gene silencing and promoter methylation of mismatch repair (MMR) genes *MLH1* and *MGMT* was shown to be associated to the development of microsatellite instability (MSI) which itself is involved with various human malignancies like cancer [47]. Furthermore, MMR proteins were reported to interact with silencing epigenetic modifiers such as DNMTs when damages exceed the repair capacity [48].

In vitro studies have demonstrated many positive effects of single phytochemicals. However, it proved difficult to elucidate the health effects of any single phytochemical *in vivo* because it is unclear whether such effects are impact of an individual phytochemical or as a consequence of interaction of components, that are working synergistically, additively or inhibitory in a matrix of nutrients within a food. Furthermore, bioavailability can vary widely between substances.

Thus, one of the key questions of this research has been whether a purified phytochemical is able to show similar health promoting properties as a diet rich in these component. However, results were inconsistent. Recently, combination of substances is claimed to enhance activity and specific plant ingredients such as EGCG, resveratrol or lycopene are in the center of research interest, because of their promising results *in vitro*. Addressing the different mechanisms of aging, specific phytochemicals could be used as new therapeutic agents against age-related diseases. In this context, it must be considered that bioavailability is critical for the biological properties of phytochemicals. Gut microbiota is essentially involved in the uptake, conversion and degradation of these components and thus, regulates their activity.

The objective of this study was to investigate health benefits of a daily consumption of a combination of extracted phytochemicals and vitamins that roughly reflect a diet rich in fruit and vegetables. Therefore, we chose a dietary supplement containing a large variety of phytochemicals (Time Block®) that is readily available to consumers in many countries worldwide, and administered it to a group of healthy individuals for a period of 6 months. To assess potential changes we analyzed specific biomarkers that are associated with aging, oxidative stress and DNA stability: Methylation of *LINE-1, c-Myc, IL-6, MLH1, DNMT1*, *ITGA2B* and telomere length.

Material and Methods

Study population

For this study 110 participants were recruited. Exclusion criteria were chronic diseases, acute inflammation at time points of sampling and smoking. Due to acute inflammation or pregnancy, 9 participants were excluded. 101 subjects of both sexes between 31 and 76 years were included in the further analysis (Table 1). For age-specific correlations all samples from T0 were analyzed.

Participants received TimeBlock® for 6 months oral administration. Participants had to fill out a food frequency questionnaire regarding their diet, health status and lifestyle before and after the study period. A small reference group (n=20) without intervention within the same age interval served as control.

Intervention

TimeBlock® is a plant based dietary supplement. Ingredients include extracts from green tea (EGCG), wheatgrass (tocotrienols), barley grass (folic acid) in Telomer Complex Day® and tomatoes (lycopene), tagetes (zeaxanthin, lutein), algae, shiitake mushrooms (vitamin D) and grape seeds (resveratrol) in Telomer Complex Night®, further Q10, Vitamins B1, B2, B6, B12, C, K, D, biotin, selen, zinc and magnesium (TimeBlock®, 2016). Each capsule of Telomer-Complex Day® contains 90 mg of EGCG and 600 µg folic acid (TimeBlock® 2016, https://www.time-block.com/en/). Participants were advised to take two capsules a day.

Sampling

Capillary blood samples were collected from all participants before administration and within 6 days after the end of the study period. Blood samples were collected on Whatman Protein Saver Cards (Sigma-Aldrich, Austria) and stored at room temperature until extraction.

DNA extraction and bisulfite conversion

DNA extraction was carried out using the QIAamp® DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocol for DNA Purification from Dried Blood Spots. DNA was stored at -20°C until analysis was conducted.

Bisulfite conversion was carried out with EpiTect® Fast Bisulfite Conversion Kit (Qiagen) following the manufacturer's protocol using a thermocycler. DNA concentrations were determined with Picodrop Pico100 UV/VIS spectrophotometer.

HRM analysis of DNA methylation

Promoter region CpG methylation analysis of chosen target genes was carried out by Methylation-Sensitive High Resolution Melting (MS-HRM). This real-time PCR-based technique can differentiate sequences on the basis of their melting behaviour dependent on GC content. MS-HRM was performed according to the EpiTect® HRM™ PCR handbook (Qiagen) with the Rotor-Gene® Q (Qiagen) including a 72-well rotor. Reaction mix for PCR contained 5 µl 2x EpiTect HRM PCR Master Mix (*ITGA2B, LINE-1, IL-6, DNMT1, MLH1*) or MeltDoctor™ HRM Master Mix *(c-Myc),* 5-10 pmol/µl of each primer, 5-30 ng bisulfite converted DNA, 0–2 mM $MgCl_2$ and RNase-free water. PCR conditions were established for each primer set. Methylation standard curves were used for analysis, 0% and 100% methylation standards were acquired from Qiagen (EpiTect control DNA). For primer sequences see supplementary material.

Telomere length measurement by real-time qPCR

Telomere length was measured using a real time quantitative PCR according to O'Callaghan method [49]. Complementary primers to the telomere sequence 5'TTAGGG'3 repeats were used. In order to obtain genome copies per sample, oligomer standards with known length and molecular weight are needed. For calculation of absolute telomere length, relative telomere length has to be normalized to a single copy gene reference. 36B4 and Albumin were used for this purpose. Standard curves were created by serial dilution of known quantities of the synthesized oligonucleotids. LightCycler Mastermix with SYBR Green Dye from Roche and AB StepOnePlus™ were used to perform PCR under following cycling conditions: 60°C/30 s, 95°C/10 min, 40 cycles: 95°C/15 s, 60°C/1 min, followed by a holding stage (60°C/30 s).

Statistical analysis

To calculate the methylation percentage of the unknown samples, a standard curve and standard equation were created using Microsoft®

Excel® 2010. All data was then analyzed with IBM® SPSS® Statistics Version 20. Q-Q plots were generated to check the normal distribution of data.

In order to determine if there are changes in the lifestyle or nutrition behavior of the participants between start point of the study and after the intervention (over the 6 months of intervention) T Student Test (for metric data) and Wilcoxon signed rank Test (for non-parametric, categorical variables) were carried out. To compare if the administration of TimeBlock® had any influence on the selected epigenetic markers, again T Student Test was used. Correlation between age and methylation was analyzed with Pearson's correlation.

Results

LINE-1

Methylation of *LINE-1* was positively correlated with age (Figure 1). Mean methylation percentage of *LINE-1* in the study population before intervention (T0) was 75.10% ± 6.33% compared to 74.40% ± 6.84% after the intervention (T1) (Figure 2). After the intervention period there was a decrease in methylation of *LINE-1* between the two sampling points. No significant sex-specific differences could be established through the intervention.

ITGA2B

Age correlation analysis revealed that *ITGA2B* methylation tends to increase with age (Figure 1). *ITGA2B* methylation showed a decrease (p=0.081) after intervention with 48.88% ± 11.86% at T0 and 45.94% ± 12.83% at T1 (Figure 2). Female participants showed a significant decrease (p=0.025) after intervention which was not apparent in male participants.

c-Myc

c-Myc showed a trend towards a higher methylation in age (Figure 1). *c-Myc* displayed a mean methylation of 8.87% ± 1.02% in the beginning of the study and 8.73% ± 1.11% at T1 (Figure 2). Intervention showed no significant sex-specific differences.

MLH1

Methylation analysis of *MLH1* showed a trend towards a higher methylation with increasing age (Figure 1). Mean methylation percentage of MLH1 at starting point of the study was 13.80% ± 1.81% compared to 13.66% ± 2.09% after 6 months (Figure 2). No significant sex-specific differences could be established through the intervention.

DNMT1

DNMT1 was positively correlated with age (Figure 1). Participants showed a mean methylation of 11.60% ± 1.50% before and 11.35% ± 1.23% after intervention (Figure 2). After intervention participants showed a slight increase in methylation with no apparent sex-specific differences.

IL-6

IL-6 methylation was negatively correlated with age (Figure 1). Intervention showed no changes in methylation (T0=11.40% ± 3.74, T1=11.40% ± 4.6) (Figure 2) as well as no significant sex-specific differences.

Telomere length

Results of telomere length showed a high significant correlation between age and telomere length (Figure 3). With increasing age the telomeres shorten significantly (p=0.008). After the 6 month intervention period there was a 17.77% significant increase in telomere length (p=0.024) (Figure 3). Significant sex-specific differences could not be established through the intervention.

Correlation between markers

Pearson's correlation showed a strong negative relationship between telomere length and *MLH1* methylation (r=−0.505, p<0.01) (Table 2). Further, a positive correlation with methylation levels of *ITGA2B* could be observed (r=−0.251, p<0.05). Methylation of *c-Myc* exhibited a strong positive correlation with *ITGA2B* (r=−0.320, p<0.01) (Table 2).

Questionnaire

We assessed the participants' dietary and lifestyle habits using a food frequency questionnaire at the beginning and end of the study period. Further, we asked for well-being and frequency of inflammations of participants. All study participants were omnivores. Analysis showed differences in stress levels. The mean age of female participants was significantly lower than the age of male participants (p=0.009). Mean Body Mass Index (BMI) of the male subjects was significantly higher than the mean BMI of the females (p=0.001) (Table 1). Regarding lifestyle and diet, no significant differences between both sexes were found. Analyses of diet changes during the study period revealed that meat and cereal consumption were significantly higher (p=0.035, p=0.046) and sweets intake lower (p=0.009) at the final sampling time point. No further significant changes in diet were discovered. The BMI showed no significant changes. After intervention period absolute number of reported inflammations decreased.

Discussion

In the last years the field of epigenetics has been rapidly growing and with it the knowledge that external influences like lifestyle, diet and environment can directly interact with our genes and induce epigenetic alterations. It has been reported multiple times that gene expression and silencing can be altered by epigenetic modifications [50-52]. DNA methylation is one of the most investigated epigenetic modifications and within epigenetic research, one of the most studied and well characterized associated diseases is cancer. Along with that aging and other age-related disorders are in the center of interest.

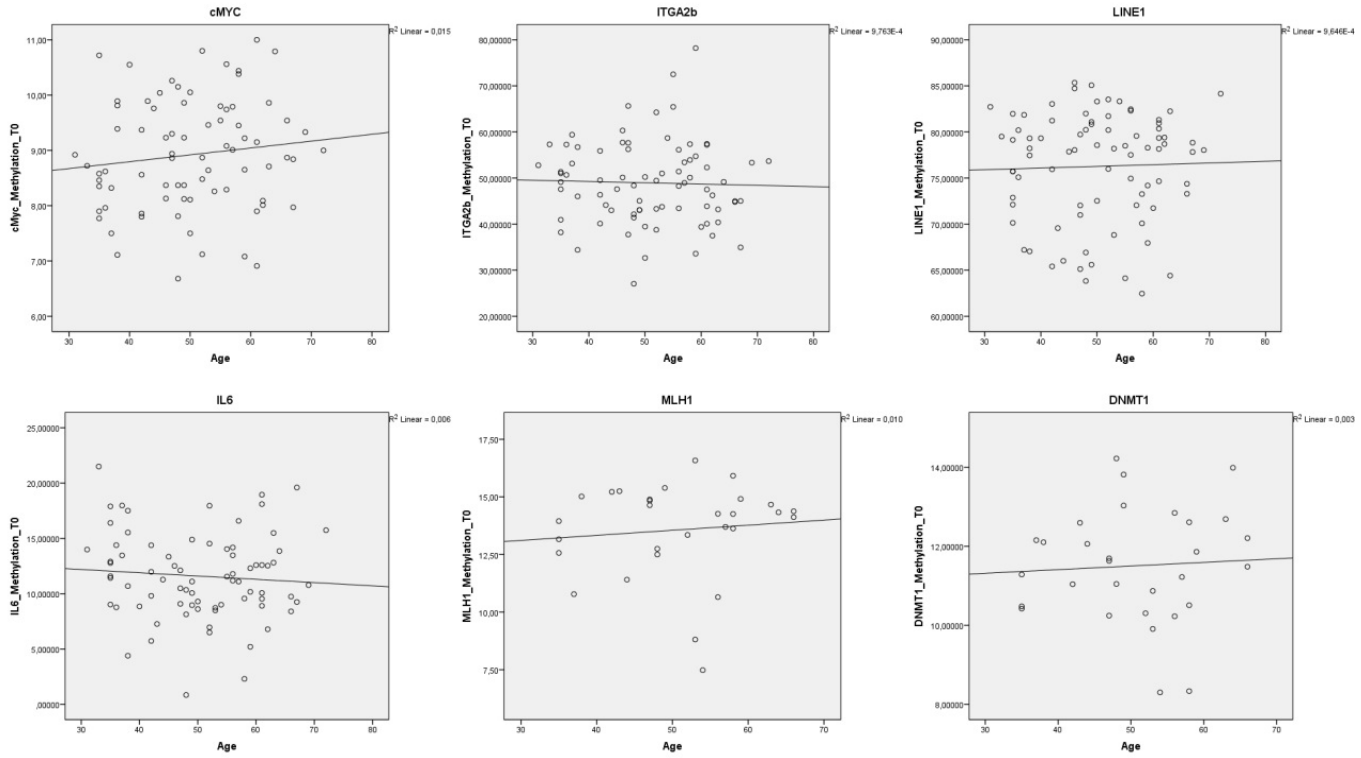
Particular nutrients and bioactive food compounds as well as lifestyle factors such as smoking or increased sugar consumption have been associated with altered DNA methylation and telomere length respectively [53,54]. Further, DNA methylation and telomeres are linked to various diseases such as cardiovascular disorders, T2DM and cancer [55-58]. Unhealthy lifestyle and diet can induce numerous diseases through epigenetic mechanisms; therefore investigating the link between them bears a great potential to identify and establish prevention opportunities.

Studies to date suggest that particular dietary compounds may influence genomic and gene-specific DNA methylation levels in

	Male n=35 Mean (SD)	Female n = 66 Mean (SD)	Total n = 101 Mean (SD)
Age (years)	54.3 (8.3)	48.98 (10.3)	50.8 (9.96)
Age range	35-67	31-76	31-76
BMI (kg/m^2)	25.71 (2.83)	22.67 (4.39)	23.66 (4.19)

Table shows the characteristics of the population studied, noting that participants are relatively similar regarding their health status, diet and lifestyle.

Table 1: Characteristics of the study population.



Scatter-plots display age correlations of promoter DNA methylation of MLH1, DNMT1, ITGA2B, LINE-1, IL6, c-Myc before administration (T0) as analysed by linear regression analysis.

Figure 1: Age-associated methylation changes..

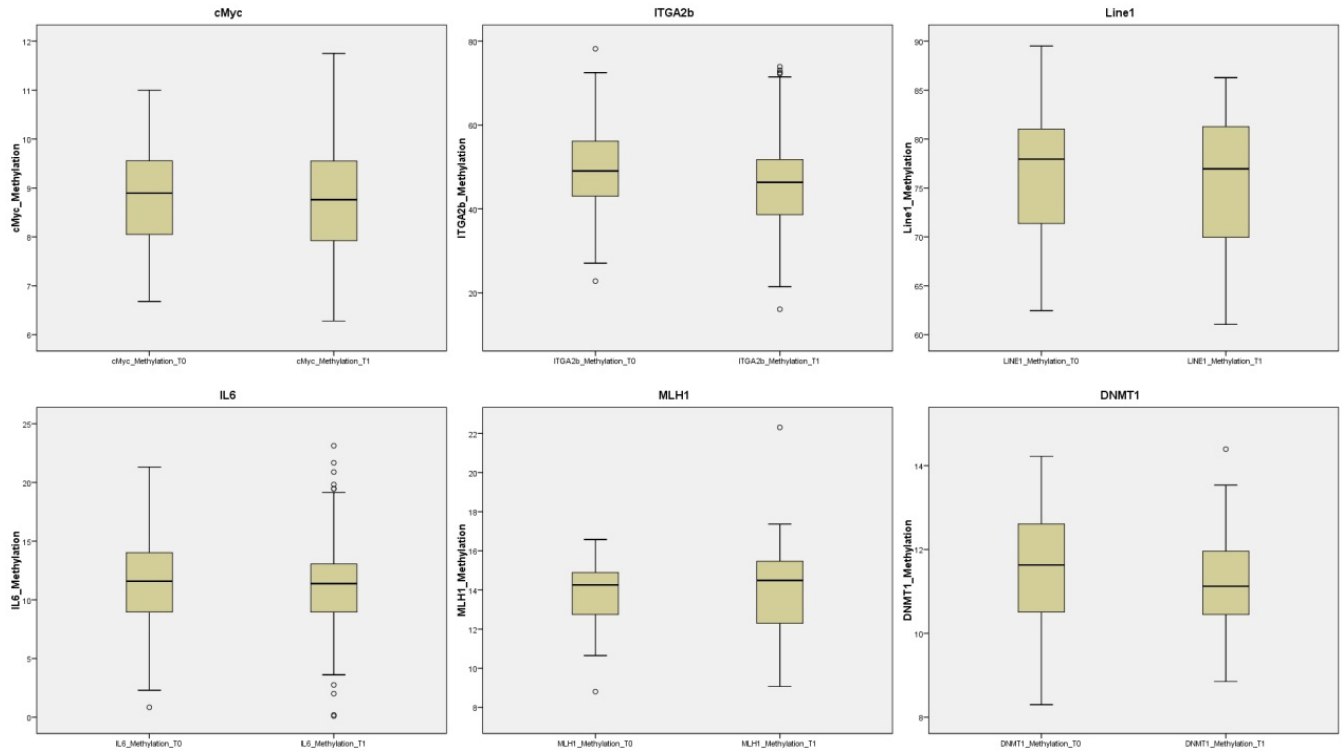
systemic and target tissues, altering genomic stability and transcription of tumor suppressors and oncogenes [8,35,59,60]. Most data and supportive evidence exist for folate, a key nutritional factor in one-carbon metabolism [46]. Other candidate bioactive food components include alcohol and other key nutritional factors of one-carbon metabolism, polyphenols and flavonoids in green tea, phytoestrogens and lycopene.

Considering that cells lose global DNA methylation with increasing age as reported in recent studies and DNA methylation can be altered by certain food components [50,61,62], we analyzed the methylation of *LINE-1* as a global methylation marker and-to reflect gene specific age-correlated methylation drifts-promoter methylation of *ITGA2B* was assessed, which was previously described as an epigenetic marker of age [25]. Age correlation analysis revealed that *ITGA2B* methylation tends to increase with age. After intervention with TimeBlock® *ITGA2B* showed a decrease which was significant in female participants (p=0.025) suggesting a gender specific demethylating effect.

LINE-1 retrotransposable element 1, belonging to the class of Long Interspersed Elements (LINEs) is a highly repetitive sequence making up to 16.89% of the human genome [63]. Due to their widespread throughout the human genome and their rather conserved sequence, *LINE-1* is discussed as a marker for global DNA methylation [64,65]. Furthermore it has been reported that *LINE-1* methylation correlates with age, sex and several lifestyle and environmental factors [66,67]. Moreover global hypomethylation has been linked to chromosomal and genome instability and cancer [68,69]. We found that methylation of *LINE*-1 tends to positively correlate with age, which goes in line with some recent studies observing, that a higher methylation of *LINE-1* was associated with increased risk of renal cell carcinoma [70,71].

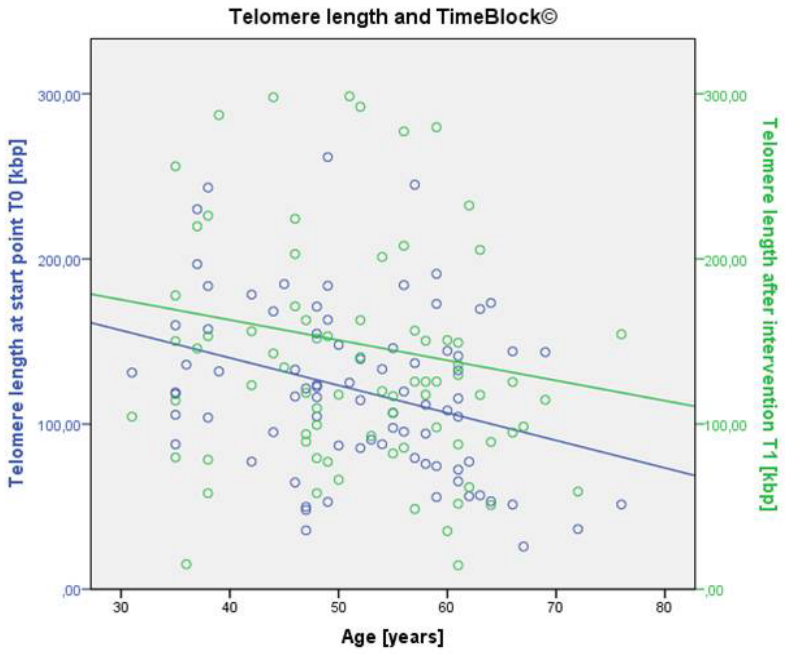
In contrast to that, methylation levels of *LINE-1* repeats were reported to be inversely correlated with CpG-island methylation of the *MLH1* gene, a key component of the DNA mismatch repair [72]. Work by Nakagawa et al. showed that *MLH1* methylation increased with advancing age [73]. Furthermore it was demonstrated, that *MLH1* gene is silenced by promoter methylation in TS1 cells [74]. Defects in DNA Mismatch Repair (MMR) are not only associated with various types of cancer, but also with an elevated telomere shortening [75]. This is also supported by our results, where a strong negative correlation of telomere length and *MLH1* methylation could be identified. Since *MLH1* methylation is directly correlated with a reduced expression and gene silencing [76], *MLH1* deficiency could influence telomere associated proteins and telomerase. Polyphenols like EGCG were shown to be associated with the reactivation of methylation-silenced genes such as *MLH1, p16^{Ink4a} or O6-methylguanine methyltransferase* which appears to correlate with the inhibitory activity on DNMT [42]. However, other pathways like the inhibition of HDACs are also discussed as contributing mechanisms. Switzeny et al. observed an increased *MLH1* promoter DNA methylation in DMT2 subjects following a vitamin and antioxidant rich diet [77]. We could observe that *MLH1* showed a trend towards a higher methylation with increasing age, but methylation levels of *MLH1* were only marginally affected by the administration.

Since EGCG is also discussed as a strong chemopreventive compound and was reported to suppress inflammatory processes involved in hyperproliferation, transformation, and initiation of carcinogenesis [78], we analyzed if administration of TimeBlock® influences interleukin 6 (IL-6) as a potential inflammatory marker. IL-6 is an inflammatory cytokine, encoded by the *IL-6* gene. It plays a



Comparison of mean promoter DNA methylation levels of MLH1, DNMT1, ITGA2B, LINE-1, IL6, c-Myc before (T0) and after (T1) 6 months of administration of plant based dietary supplement Timeblock.

Figure 2: Promoter DNA methylation changes after administration of EGCG containing combined dietary supplement..



Scatter-plots display telomere length in kilo basepairs before (T0) and after (T1) 6 months of oral administration of plant based dietary supplement Timeblock as analysed by linear regression analysis, showing a high correlation to age at T0 (p=0.008*).

Figure 3: Changes in telomere length after administration of EGCG containing combined dietary supplement in correlation to age.

		MLH1_ Methylation_ TO	DNMT1_ Methylation_ TO	ASPA_ Methylalation_ TO	ITGA2b_ Methylation_ TO	LINE1_ Methylation_ TO	IL6_ Methylation_ TO	Telomer_TO	cMyc_ Methylation_ TO
MLH1_ Methylation_TO	Pearson Correlation	1	0.211	-0.187	-0.016	-0.284	-0.081	-0.505**	0.347
	Sig. (2-tailed)	-	0.272	0.331	936	0.143	0.687	0.006	0.065
	N	29	29	29	29	28	27	28	29
DNIOT.I_ Methylation_TO	Pearson Correlation	0.211	-	-0.190	-0.271	-0.255	0.317	0.184	.130
	Sig. (2-tailed)	0.272	-	0.313	0.147	0.182	0.100	0.339	0.493
	N	29	30	30	30	29	28	29	30
ASPA_ Methylation_TO	Pearson Correlation	-0.187	-0.190	-	0.251'	-0.166	-0.098	0.178	0.193
	Sig. (2-tailed)	.331	0.313	-	0.027	0.141	0.404	0.117	0.093
	N	29	30	81	78	80	75	79	77
ITGA2b_ Methylation_TO	Pearson Correlation	-0.016	-0.271	0.251*	-	0.053	0.162	0.248*	0.320**
	Sig. (2-tailed)	0.936	0.147	0.027	-	0.649	0.175	0.030	0.006
	N	29	30	78	78	77	72	76	74
LINE1_ Methylation_TO	Pearson Correlation	-0.284	-0.255	-0.166	0.053	1	0.185	-0.064	-0.081
	Sig. (2-tailed)	0.143	0.182	0.141	0.649	-	0.111	0.576	0.481
	N	28	29	80	77	81	75	79	77
IL6_ Methylation_TO	Pearson Correlation	-0.081	0.317	-0.098	0.162	0.185	-	0.156	0.136
	Sig. (2-tailed)	0.687	0.100	0.404	0.175	0.111	-	0.184	0.255
	N	27	28	75	72	75	76	74	72
Telomer_TO	Pearson Correlation	-0.505**	0.184	0.178	0.248*	-0.064	0.156	1	0.044
	Sig. (2-tailed)	0.006	0.339	0.117	0.030	0.576	0.184		0.704
	N	28	29	79	76	79	74	80	77
cMyc_ Methylation_TO	Pearson Correlation	0.347	0.130	0.193	0.320**	-0.081	0.136	0.044	1
	Sig. (2-tailed)	0.065	0.493	0.093	0.006	0.481	255	0.704	-
	N	29	30	77	74	77	72	77	78

**Correlation is significant at the 0.01 level (2 tailed).
*Correlation is significant at the 0.05 level (2-ailed).
Table representing Pearson's correlation coefficient for correlation between promoter DNA methylation of target genes and telomere length. Stars (*, **) indicate statistical significance p<0.05*, p<0.01**

Table 2: Correlation of promoter DNA methylation of MLH1, DNMT1, ASPA, ITGA2B, LINE-1, IL6, c-Myc and telomere length.

crucial role in immune regulation and has numerous other functions, such as differentiation of monocytes, lymphocytes and B-cells. Higher gene expression of *IL-6* protein has been associated with various diseases including cancer, rheumatoid arthritis, insulin resistance and diabetes [51,79,80]. Promoter methylation is one of the regulation mechanisms of *IL-6* gene expression and is correlated to body weight [51,81]. Furthermore, studies revealed an association between elevated mRNA levels of interleukin 6 and a Promoter demethylation [82,83]. IL-6 expression is modulated by the nuclear factor kappa B (NF-KB) whose activation was shown to be blocked by EGCG via inhibition of I kappa B kinase activity in the intestinal epithelial cell line IEC-6 [84]. In the context of regulation of *IL-6* expression various pathways can be targeted by EGCG, pin-pointing the diverse functions in which *IL-6* is involved. Our results showed that *IL-6* methylation was negatively correlated with age, however methylation levels of *IL-6* showed no significant changes over the study period.

EGCG is reported to be involved in cell cycle regulation, and thereby exhibiting strong chemopreventive capacities. Gupta et al. showed that EGCG promotes cell growth arrest and induces apoptosis in prostate cancer cells [85]. Mechanisms involved were reported to be a modulated expression of cell cycle regulatory proteins via activation of killer caspases, and suppression of NFκB activation [86]. Multiple other pathways are discussed to be affected by EGCG, including the Mitogen Activated Protein (MAP), growth factor-mediated pathways, kinase-dependent pathways, ubiquitin/proteasome degradation [60]. Especially impact on c-Myc gene expression has evoked interest recently due to potential effects on telomere length by targeting *hTERT* gene expression [43]. As catalytic subunit of the enzyme telomerase *hTERT* is a crucial factor of its activation. *hTERT* gene Promoter contains a binding site for *c-Myc*, therefore their activity is closely linked. Wang et Lei reported a significant decrease of c-Myc protein level after treatment of EGCG in a malignant cell line, concurrently a reduction in *hTERT* protein levels was observed [43]. As already mentioned, EGCG was reported to block NF-κB activity. Studies showed that NF-κB can upregulate c-Myc and c-Myc is activated by a large number of oncogenic pathways [87]. Targeting c-Myc via NF-κB is one possible pathway of chemotherapeutic effects of EGCG. *c-Myc* dysregulation is discussed as a marker for genomic instability that is linked to tumor initiation [88]. Thus, we analyzed methylation of *c-Myc* with regard to its impact on telomerase regulation via hTERT. Our results showed, that *c-Myc* methylation was hardly influenced by administration of TimeBlock®. Interestingly, after 6 months of administration participants showed a significant increase in telomere length. Since DNA methylation of *c-Myc*-one central telomerase regulating mechanism-was hardly affected through the intervention, we assume

that lengthening of telomeres was not induced by changes in DNA expression of telomerase gene due to altered DNA methylation. EGCG and other natural compounds have been shown to induce apoptosis in many cancer cells and also adipocytes [43,89-91]. Accelerated apoptosis of old or mutated cells can lead a to cell replacement and regeneration depending on the tissue, and thus, to a apoptosis-induced proliferation and tissue regeneration [92,93]. This could result in an increased percentage of young cells with longer telomeres. Since our method of choice for telomere measurement detects the mean telomere length in all cells extracted, this hypothesis could be one possible explanation for a telomere lengthening without addressing telomerase regulation via DNA methylation. Furthermore, oxidative stress and inflammation can induce chromosomal abnormalities and accelerated telomere attrition, and therefore antioxidant phytochemicals play an important role in preventing telomeres from excessive shortening [94]. Apart from polyphenols, positive associations with telomere length have also been reported for Vitamin C, E, D, B12, folate, magnesium, and zinc [94]; all of them are ingredients of the administered food supplement.

Certain phytochemicals such as *Astralagus membranaceus* root are reported for their telomerase activating capacities [95]. Since telomerase activation plays a significant role in cancer development such food supplements have been debated intensely and are still discussed for their potential cancer risk. Thus, we suggest, protecting telomeres without targeting telomerase activation may be a safer alternative.

Conclusion

The present study investigated effects of a combination of extracted bioactive plant compounds on specific markers that are associated with aging, oxidative stress and DNA stability. Our results confirmed the positive effect of plant-derived antioxidants on telomeres and inflammation frequency as well as an age-specific drift of these markers. Total telomeres length showed a significant increase, which we suggest to be linked with an increased cell turnover and accelerated apoptosis of senescent or mutated cells without enhancing telomerase activity. Further, methylation of mismatch repair protein gene MLH1 showed a strong negative correlation with telomere length, supporting the influence of MMR on telomere regulation.

Combination of phytochemicals can be a potential preventive and therapeutic agent, as each substance exhibits different modes of action and in combination, health promoting effects could be potentiated. Addressing the different mechanisms of aging, specific phytochemicals could be used as new therapeutic approach against age-related diseases. However, low absorption and bioavailability rates in the gastrointestinal tract as well as differing metabolic pathways are still limiting factors, explaining differences in effectiveness of *in vivo* and *in vitro* experiments. Still, many underlying mechanisms of health promoting and cancer inhibiting effects of phytochemicals are unknown and are focus of further research.

Ethics Statement

The study was approved by the Viennese Human Ethics committee (3, Thomas-Klestil-Platz 8/2) Votum: EK 14-092-VK_NZ. From all participants involved in the study written consent was obtained.

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