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„Influence of diet and lifestyle on epigenetic regulation of telomeres and ageing markers“

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IV. List of abbreviations

36B4	single copy gene
ASPA	aspartoacylase
BMI	body mass index
Caco-2	carcinoma colon cell line
CpG	Cytosin-phosphatidyl-Guanin
dNTPs	deoxynucleotide triphosphates
dsDNA	double-stranded DNA
EGCG	epigallocatechingallate
HRM	high resolution melting
ITGA2B	integrin subunit alpha-IIB
miRNA	micro RNA
NAA	N-acetyl-L-aspartic acid
NFW	nuclease free water
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NTC	no template control
PCR	polymerase chain reaction
piRNA	piwi-interacting RNA
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
ssDNA	single-stranded DNA
T0	time point before intervention
T1	time point after intervention

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Publication

The results of this master thesis have been published in the following article, which can be found in the appendix of this thesis.

EGCG Containing Combined Dietary Supplement Affects Telomeres and Epigenetic Regulation

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Journal of Nutrition & Food Sciences, 2017

1 Summary

Background: “Healthy ageing” is a current desirable goal of the society and focus of numerous research groups. The cytosin-methylation levels at specific genomic sites as well as length of telomeres, are associated with age and therefore serve as age-related biomarkers. These parameters can be influenced by lifestyle, including diet, physical activity and smoking status. Biomarkers of healthy ageing are also under development to monitor consequences of nutrition or lifestyle interventions.

Hypothesis: Telomeres and epigenetic markers correlate with nutrition and lifestyle.

Objective: In order to detect these impacts, the analyses of biomarkers (*ASPA, ITGA2B, c-Myc* as well as the telomere length) were performed in a group of 82 participants who attended a six-month intervention with a dietary supplement.

Methods: For the six-month intervention study, a preparation based on various bioactive phytochemicals (e.g., EGCG) was administered and the capillary blood of the 82 participants was analysed. DNA methylation was identified by PCR followed by high resolution melting curve analysis. For determining absolute telomere length a quantitative real-time PCR, using specific oligomer standard (telomere and 36B4) dilutions with known concentrations, was done.

Results: *ASPA* methylation and telomere length showed significant correlations with age. After the intervention, DNA methylations of the genes of interest showed a slight decrease in *ASPA* and *ITGA2B* (not significant), *c-Myc* methylation nearly remained unchanged. However, telomere length showed an increase (from 132,93kb ± 60,97 at T0 to 136,99kb ± 64,87 at T1), but not significant. All examined parameters could be influenced by diet, lifestyle or both.

Conclusion*:* *ASPA* methylation and telomere length have the potential to determine the state of ageing in blood. This in turn could play an important role in the early detection or even prevention of nutrition-associated diseases, which should be proved by further scientific studies. Lifestyle can also be an important tool for modulating unwanted processes.

2 Zusammenfassung

Hintergrund: „Gesundes Altern“ ist ein aktuelles Ziel, das mit großem Interesse der Gesellschaft verfolgt wird und mittlerweile wissenschaftlicher Fokus vieler Forschungsgruppen weltweit ist. Die Cytosin-Methylierungen an bestimmten genomischen Stellen, ebenso wie die Länge der Telomere, sind mit dem Alter assoziiert und können daher als Alterungsmarker herangezogen werden. Diese Parameter können durch den Lebensstil, einschließlich Diät, körperliche Aktivität und Raucherstatus beeinflusst werden. Alterungsbiomarker helfen die Konsequenzen von Ernährungs- und Lebensstilinterventionen zu überwachen.
Hypothese: Telomerlänge und epigenetische Marker korrelieren mit Ernährung und Lebensstil.
Zielsetzung: Um diese Effekte ermitteln zu können, wurden die Analysen von Biomarkern (*ASPA, ITGA2B, c-Myc* Methylierungen sowie die Telomerlängen) in einer Gruppe von 82 Teilnehmern durchgeführt, die an einer sechsmonatigen Intervention mit einem pflanzlichen Nahrungsergänzungsmittel teilnahmen.
Methoden: Für die 6-monatige Interventionsstudie wurde ein Präparat, basierend auf verschiedener bioaktiver, sekundärer Pflanzenstoffe (z. B. EGCG), verabreicht und das Blut von 82 Teilnehmer analysiert. Die DNA-Methylierung wurde mittels PCR, gefolgt von einer hochauflösenden Schmelzkurvenanalyse, analysiert. Zur Bestimmung der absoluten Telomerlänge wurde eine quantitative Echtzeit-PCR unter Verwendung spezifischer Oligomer-Standards (Telomere und 36B4) als Verdünnungsreihen bekannter Konzentration durchgeführt.
Ergebnisse: Die Methylierung von *ASPA* und die Telomerlänge zeigten signifikante Korrelationen mit dem Alter. Nach der Intervention konnte eine leichte Abnahme der DNA-Methylierungen von *ASPA* und *ITGA2B* beobachtet werden (nicht signifikant), die *c-Myc*-Methylierung blieb nahezu unverändert. Die Telomerlänge jedoch zeigte einen Anstieg (von 132,93kb ± 60,97 bei T0 auf 136,99kb ± 64,87 bei T1), der jedoch nicht signifikant war. Alle untersuchten Parameter konnten durch Diät, Lebensstil oder beides beeinflusst werden.
Schlussfolgerung: *ASPA*-Methylierung und Telomerlänge haben das Potential, den Zustand des Alterns im Blut zu bestimmen. Dies könnte wiederum eine wichtige Rolle bei der Früherkennung oder sogar bei der Vermeidung von ernährungsbedingten Krankheiten spielen und sollte deswegen durch weitere

wissenschaftliche Studien bestärkt werden.
Auch der Lebensstil kann ein wichtiges Instrument sein, um modulierend in unerwünschte Prozesse eingreifen zu können.

3 Introduction

3.1 Epigenetic mechanisms

Epigenetic mechanisms describe heritable alterations on a molecular level, which can influence gene regulation with effect on phenotype, morphology and pathobiology without changing the DNA sequence (Lee et al. 2014). The different mechanisms are connected in a narrow network and can influence each other. The three main mechanisms are reflected in figure 1.

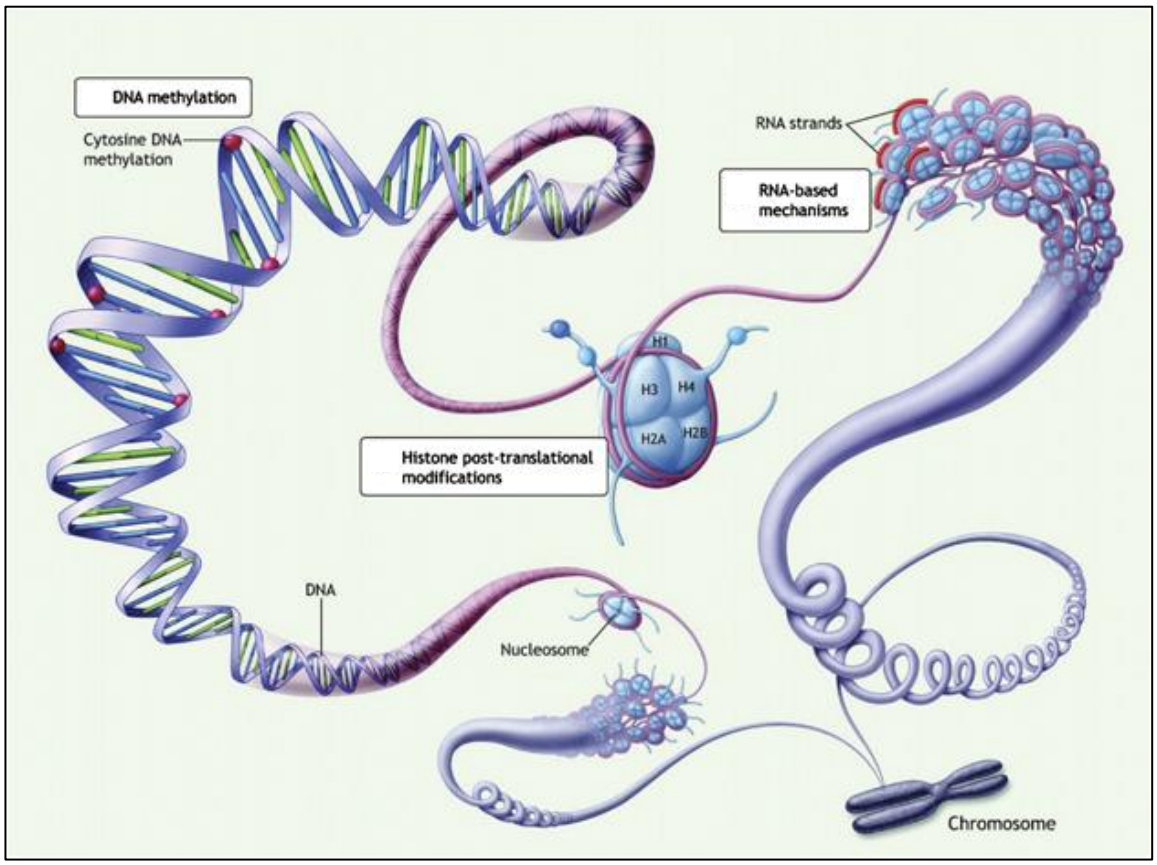

Figure 1: Overview of three most important epigenetic mechanisms (Lee et al. 2014)

3.1.1 Histone modification

DNA is packed into gen-specifically distributed nucleosomes, which consist of eight histone-proteins (figure 1) named H2A, H2B, H3 and H4 (each one occurs twice) wrapped around by about 150 bp of DNA. H1, another histone protein, ties the nucleosomes together to further tighten the nucleosomes. These complexes of DNA and histones form the chromatin. (Zhou et al. 2011)

Histone modifications occurring posttranslational involve acetylations, methylations, phosphorylations, ubiquitinations or sumolations depending on the locus in the nucleosome. The most common sites of the modifications are the histone tails (Biswas et al. 2011).
These alterations have their specific impact on gene regulation, both gene activation as well as repression can be induced. A decisive factor for this is whether or not the modification unfolds the chromatin for example by changing histone charge. Beside gene regulation there are some other processes like maturation and splicing of RNA, DNA replication and repair that can be influenced or controlled by histone modifications. (Corpet & Almouzni 2009; Kouzarides 2007)

3.1.2 Non-coding RNA

The majority of the human genome is transcribed into RNA but only less than two percent (<20.000 genes) are encoding proteins (Ezkurdia et al. 2014).

Another possibility of epigenetic control mechanisms is the regulation of chromatin structure as well as silencing of genes and transcripts by non-coding regulatory RNA. These processes can be mediated transcriptional and post-transcriptional. (Costa 2008)

Some important representatives are differentiated on the one hand into short noncoding RNA, which are fewer than 200 nucleotides long. These are including piwi-interacting RNA (piRNA) that pathway is functional in germ cells and invertebrate neurons and plays a role in control of transposons (Nandi et al. 2016) and micro RNA (miRNA) that can influence gene regulation by incomplete base pairing and small interfering RNA (siRNA).

On the other hand there are long noncoding RNA (lncRNA) (Taft et al. 2010).

Especially dysregulations of miRNA and lncRNA, known as significant regulators in tumour suppressor and oncogenic pathways, can lead to cancer (Huang et al. 2013) and other neurodegenerative, inflammatory and cardiovascular diseases (Esteller 2011).

3.1.3 DNA methylation

DNA methylation is one of the most studied epigenetic mechanisms. In this biological process a methyl group is added mostly on the 5th position of a cytosine. The target cytosines occur predominantly in so-called CpGs sites, cytosine-guanine dinucleotides with a phosphate in-between. Regions, rich in CpGs are also known as CpG islands and are often linked with the promoter region of a gene. In healthy humans CpGs within in a CpG island are for the most part unmethylated, a notable exception, however, are for example retrotransposons (such as long interspersed nuclear elements), where the hypermethylated promoter region is important for silencing the gene to prevent genomic instability due to inducing mutations. (Deaton & Bird 2011)

It is generally accepted that promoter hypomethylation is associated with increased expression, whereas hypermethylation is linked with reduced or silenced gene activity. One exception though is the *hTERT* gene, in which an increasing methylation is accompanied by an increase in expression (Rahat et al. 2014).
Diseases such as cancer are often connected with altered methylation patterns: promoters of tumour suppressor genes are hypermethylated, which leads to a decreased or even inhibited expression of these genes and results in promotion of malignant transformations (Hu & He 2013).

For implementation of DNA methylation the family of DNA methyltransferases (DNMTs), performing different tasks, is of particular relevance. While DNMT3a and DNMT3b are dealing with new methylations (de novo methylations), occurring mainly during embryogenesis and germ cells development, the responsibility of DNMT1 is to maintain methylation patterns after the event of DNA replication due to remethylation of the daughter strand. For this reason DNMT1 is mainly active in proliferating cells.
The cofactor *S*-adenosyl-L-methionine (SAM) serves as the donor of the transferred methyl group. In losing the methyl group, catalysed by DNMT, SAM converts into *S*-adenosyl-L-homocysteine (SAH) before forming homocysteine (figure 2). (Jurkowska et al. 2011)

3.1.3.1 Relationship between Lifestyle, Nutrition and DNA Methylation

The term lifestyle describes the way in which someone lives his life and covers a variety of aspects, which influence epigenetic marks such as DNA methylations. Regular exercise, sports and body weight management are just as part of the lifestyle as factors such as stress and smoking status. (Mathers et al. 2010)

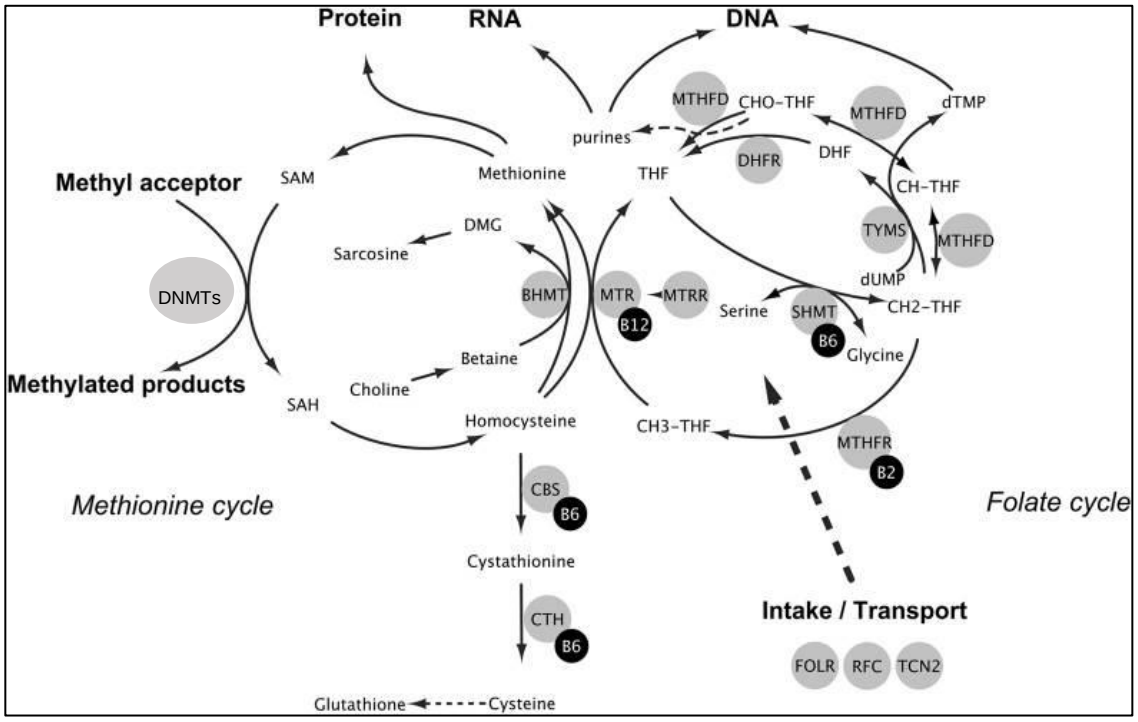

Figure 2: Overview of the one-carbon metabolism, modified (Myte et al. 2017)

A well-balanced nutrition is one of the most important factors of a healthy lifestyle. Especially a focus on a plant-based diet is recommended to prevent several diet-related diseases such as Diabetes mellitus type 2, obesity, hypertension and arteriosclerosis as well as liver diseases among others. (Fardet & Boirie 2013)

An important link between diet and DNA methylation is the one-carbon metabolism (figure 2), consisting of folate and methionine cycle. The methyl group donor SAM is formed via several substrates and precursors, which have to be absorbed partly by diet. Nutrients, which are essential and directly influence the balance of one carbon metabolism include folate, methionine, choline, betaine and vitamins of the B family. Folic acid is one of the most important methyl - donors for the remethylation of S-adenosylhomocysteine to S-adenosylmethionine via homocysteine and methionine and thus influences the

availability of SAM.
Folic acid, which is absorbed by the food, is metabolized to 5-Methyltetrahydrofolate (CH_3-THF) which is further, after cellular uptake, converted into Tetrahydrofolate (THF) by methionine synthase reaction, which is dependent on vitamin B12. Therefore, a methyl group is released and used for the conversion of homocysteine into methionine. Subsequently, THF becomes methyleneTHF (CH2-THF), due to the vitamin B6-dependent serine hydroxymethyltransferase (SHMT). This process is followed by irreversible reduction to methylTHF catalyzed by vitamin B2-linked methylenetetrahydrofolate reductase (MTHFR). (Stanger 2002)
Pauwels et al. observed also an association between maternal intake of methyl group donors during periconception period and the DNA methylation in metabolic and growth-related genes of their infants (Pauwels et al. 2017). An inadequate intake can affect the DNA methylation negatively. (McKay & Mathers 2011)

Apart from nutrients involved in the one carbon metabolism a number of other phytochemicals can have an influence on the methylation of DNA. In particular, various polyphenols not only have a high antioxidant potential due to their OH-groups-containing structures, they also may have a strong impact on epigenetic mechanisms. (Zhang 2015)
A prominent representative is epigallocatechingallate (EGCG), which occurs mainly in green tea and has shown a high free radicals' scavenging activity (Colon & Nerín 2016), is able to inhibit cell cycles of cancer cells and induces apoptosis in them (Shankar et al. 2007). In addition, EGCG can block receptors of tumour cells and thus limit or prevent tumour growth. Moreover, this green tea polyphenol is said to impair the activation of NFκB (critical for the emergence of inflammation), which in turn leads to inhibition of nitrite oxide production caused by lipopolysaccharides. (Singh et al. 2011)
Furthermore, EGCG influences the DNA methylation due to a direct inhibition of DNMT1 (Yiannakopoulou 2015).

Sulforaphane, an isothiocyanate which is mainly contained in cruciferous plants such as broccoli and cabbage, is not only known for its antioxidative effect. This phytochemical also shows to have an impact on the epigenome by inhibition of

DNMT1 and 3A leading to a down-regulation of *hTERT* in cancer cells. This process is associated with the suppression of telomerase in malignant cells, resulting in apoptosis of these. Other bioactive plant ingredients that have a cancer preventive effect include resveratrol, curcumin and genistein, presented in grapes, turmerics and soybeans. (Tollefsbol 2014)

3.2 Ageing Marker

Healthy ageing is a current desirable goal and focus of several research groups. So-called ageing markers offer the possibility to detect unwanted processes at an early stage due to alterations in DNA methylations, in order then to influence epigenetic mechanisms through diet and lifestyle leading to positive modifications in the ageing process.
Therefore, based on the literature, a combination of the following ageing marker described in the next chapters was selected.

3.2.1 ASPA

Figure 3 is presenting the genomic location of *ASPA* on chromosome 17.

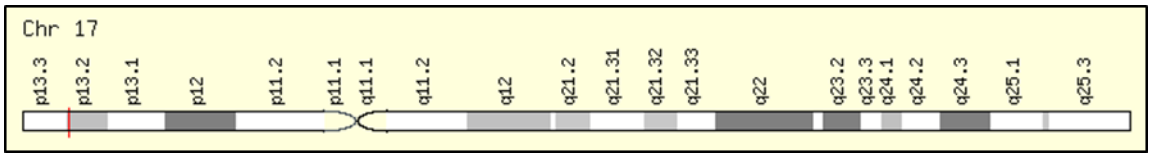

Figure 3: Genomic location of ASPA gene (Database 2017a)

ASPA gene encodes aspartoacylase enzyme, which catalyses the transformation of N-acetyl-L-aspartic acid (NAA) to aspartate and acetate. Without this mechanism there would be an accumulation of NAA in the central nervous system which is also highly detectable in urine. (Gessler et al. 2017)
A dysfunction, inter alia due to inactivation of aspartoaclyase caused by mutations in *ASPA* gene, results in Canavan disease, a lethal pediatric leukodystrophy, which is related to difficulties controlling the head due to the degenerating white matter in the brain, abnormalities in the muscle tone and also problems in sensory organs can occur. (NIH 2011)

ASPA methylation was chosen as one target of interest due to its previously reported age correlation, the association between a specific loci of the gene and ageing. (Freire-Aradas et al. 2016; Huang et al. 2015; Weidner et al. 2014)

3.2.2 ITGA2B

Integrin subunit alpha 2B gene is gnomically located on chromosome 17. (figure 4)

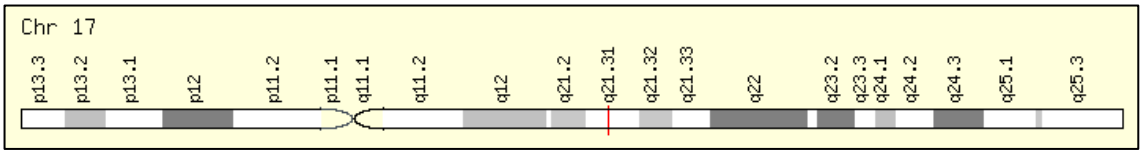

Figure 4: Genomic location of ITGA2B gene (Database 2017a)

ITGA2B encodes for a platelet glycoprotein, which is an essential element for the fibrinogen receptor complex GPIIb/IIIa. A dysfunction can lead to impaired platelet aggregation and bleeding disorders. (Jallu et al. 2010)

Several studies suggested methylation levels of specific CpGs in *ITGA2B* gene as relevant markers for ageing. (Huang et al. 2015; Weidner et al. 2014)

3.3 c-Myc

c-Myc alias V-Myc Avian Myelocytomatosis Viral Oncogene Homolog is located on Chromosome 8 (figure 5).

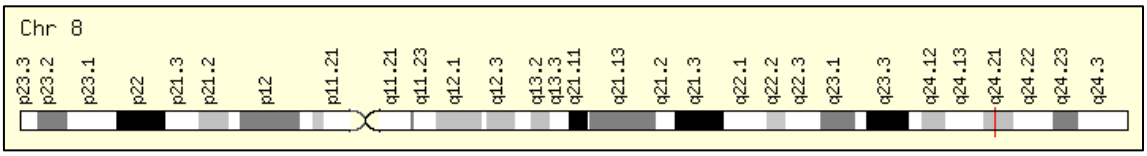

Figure 5: Genomic location of c-Myc gene (Database 2017b)

c-Myc is a well-studied proto-oncogene that is known for encoding nuclear transcription factor with a basic helix loop helix leucine zipper domain. c-Myc forms heterodimers with its partner protein MAX (myc-associated factor X). Due to this heterodimerization they are able to bind to the enhancer box sequence CACGTG of specific genes, amongst other target DNA sequences (Fernandez et al. 2003) to stimulate the transcription, if an open chromatin context is present. (Koh et al. 2016)

c-Myc has an important impact on several cellular and molecular processes. It influences not only cell proliferation and growth but also apoptosis, energy and anabolic metabolism and other biosyntheses such as DNA replication or RNA metabolism. (Kress et al. 2015)

The abnormal activation as well as overexpression of c-Myc leads to a deregulated cell cycle and malignant transformation of cells.(Qiu & Simon 2015; Miller et al. 2012)

c-Myc is also known as a transcription factor influencing the regulation of telomerase, the key enzyme for the extension of telomeres. (Rahat et al. 2014; Zhao et al. 2014) Therefore the methylation of *c-Myc* was also considered to be analysed due to its ageing link.

3.4 Telomeres

Telomeres are short tandem hexameric repeats with sequence $(5'\text{-}TTAGGG\text{-}3')_n$ on the G-rich strand, bound on a six-protein complex termed shelterin (de Lange 2005; Palm 2008), which cap the chromosomes to ensure genome stability. Due to the built of 3' ssDNA overhangs (figure 6A) DNA breaks would be detected erroneously, followed by a DNA damage response as well as the possibility of end to end fusions of chromosomes, which is prevented by shelterin (figure 6B) (Rajavel et al. 2014).

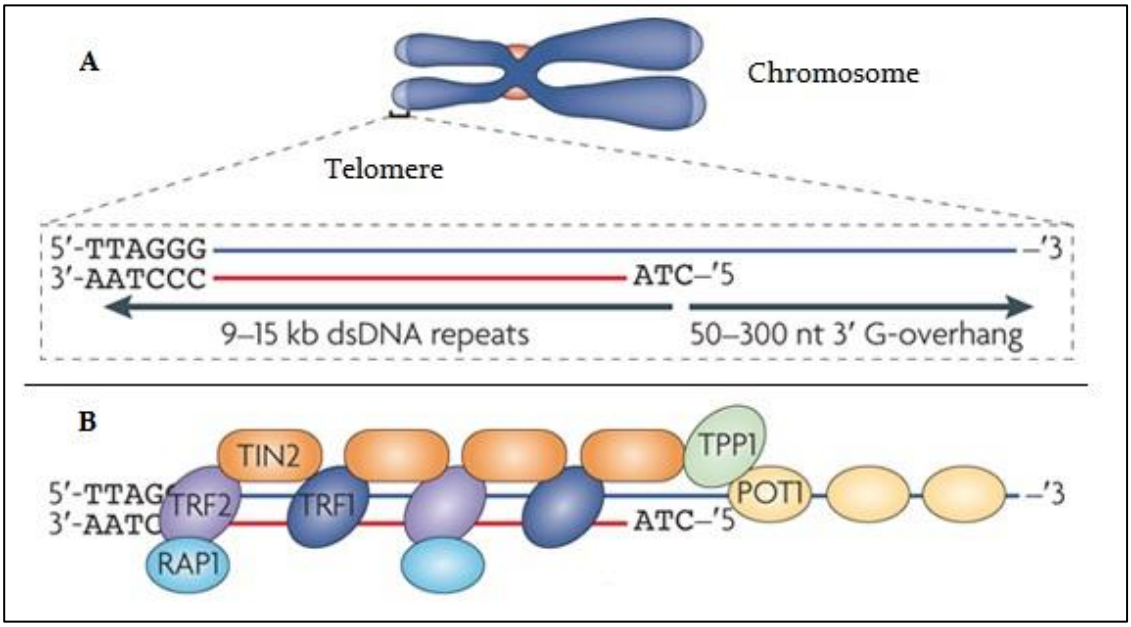

Figure 6: A. Location and structure of telomere; B. telomere bound by shelterin, modified (O'Sullivan & Karlseder 2010)

A possible explanation for this is given by the ability of telomeres to form so-termed t-loops, which are specific telomeric figures by TRF2 (shelterin complex) among other factors. For this purpose, a displacement loop (D-loop) is also generated (Griffith et al. 1999).

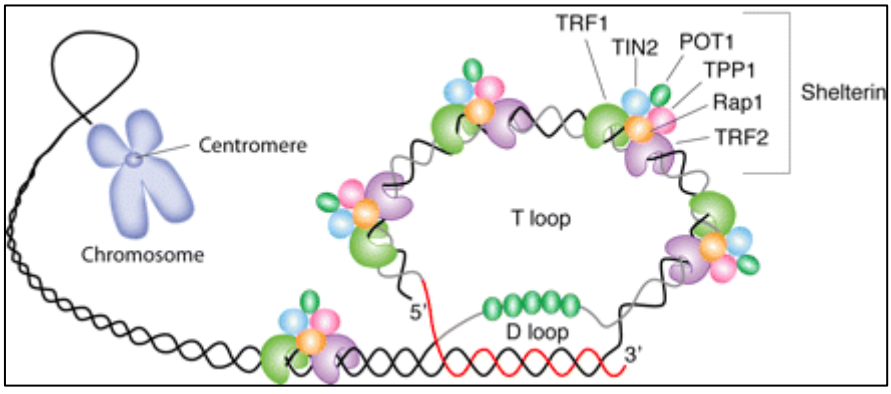

Figure 7: Formation of the t-loop (Calado & Young 2008)

The free end of 3' telomeric overhang of the single strand is threaded into the double strand (figure 7), resulting in a triplex structure. This configuration seems to protect telomeric DNA ends from fusion, actions of telomerase and DNA repair activities. (de Lange 2004)

Telomere length varies between species, individuals, tissues and cells (Takubo et al. 2010). In normal somatic cells of human, which have normally a telomere length of 10-15 kb at birth, telomeres are shortening by each cell division (about 20-60 bp/year) because of the end replication problem (Zhang et al. 2016). The DNA at the very end of the chromosome cannot be fully synthesized in each round of replication because of unidirectional 5'-3'-synthesis of the DNA polymerase, resulting in a loss of bases at the 5' end of each template strand and further in a gradual shortening of the chromosome. Shortening rates vary. One of highest losses was found in the liver of 94 persons with an average of 55bp/ year (Takubo et al. 2000).
The yearly reduction rate of peripheral lymphocytes, which was analysed in 123 participants, aged 2-95 was valued on 31bp (Slagboom et al. 1994).

After a certain critically low telomeric length has been reached, the cell becomes senescent. This observation confirms the "Hayflick limit" described in the 1960s, which states that a normal cell has a limited capacity of replication (Hayflick & Moorhead 1961).

The regulation of the telomere length maintenance at the end of the chromosomes is dominantly controlled by an enzyme termed telomerase whose activity can be influenced. Telomerase is consisting of a ribonucleoprotein complex including hTERT (human telomerase reverse transcriptase), which is encoded by *TERT gene*, located on the chromosome 5 and an enzyme termed hTERC (human telomerase RNA component) produced by *TERC gene*. While hTERT is responsible for the synthesis and maintaining of the telomeres, hTERC is essential by serving as a template for the telomere repeat. (Ozturk et al. 2017)

Telomerase activity can hardly be observed in normal proliferating somatic cells due to a silenced *hTERT* promoter. However in germ cells, stem cells, lymphocytes as well as cancer cells an overexpression of hTERT is common,

leading to a high activity of telomerase. Cancer cells, where the telomerase is reactivated are linked to an immortality, nevertheless, telomeres are often shorter than in other cells but are maintained at these lengths. (Hiyama & Hiyama 2007)

As mentioned before the expression of the transcription factor c-Myc is associated with the telomerase activity due to the stimulation of TERT-expression (Wu et al. 1999). A higher expression of c-Myc is correlated with a higher expression of hTERT resulting in an increased telomerase activity (Xu et al. 2008).

Accelerated telomeric shortening, which in turn leads to accelerated ageing, can be influenced by different triggers. It was observed that gender (Fitzpatrick et al. 2011), age (Wolkowitz et al. 2017), BMI (Njajou et al. 2012), genetic determinants (Graakjaer et al. 2003; Njajou et al. 2007), nutrition (Freitas-Simoes et al. 2016) and lifestyle habits such as smoking (Weischer et al. 2012), psychological stress (Epel et al. 2004) as well as inflammations (Weng 2008) and diseases affect telomere length.
Although exact mechanisms involved in the telomere shortening of lifestyle factors are not clear yet, studies suggest inflammation and oxidative stress to be key factors here.

There are several studies reporting effects of natural compounds such as curcuminoids (Taka et al. 2014) as well as extracts of green tea, red wine, chia seeds and broccoli seeds on the activity of telomerase (Ait-Ghezala et al. 2016).

The previously mentioned EGCG may also have an effect on telomere length by its ability to inhibit telomerase observed in cancer cell lines, which could be a promising approach for cancer therapeutics (Berletch et al. 2008).

4 Objective

The epigenetic regulation of several physiological processes plays an important role not only for the maintenance of health, but also during the normal aging process.

The possibility to intervene in these epigenetic mechanisms through diet and lifestyle modifications results, in promising approaches, among others, to disease prevention.

On the one hand, the aim of this master thesis was to evaluate the effect of an EGCG-containing intervention supplement, after a 6-month ingestion period, on DNA methylations of the ageing markers *ASPA*, *ITGA2B and c-Myc* by High Resolution Melting Method*,* furthermore, to investigate the impact of the intervention preparation on the participants' telomere lengths.

On the other hand, the goal of the study was to identify nutritional and lifestyle factors that correlate with DNA methylations of certain regions of the genes of interest, as well as the length of the telomeres. Therefore, a Food Frequency and Lifestyle questionnaire was used to evaluate associations statistically.

5 Materials and Methods

5.1 Study design

For the study 82 participants, aged 31-72 of whom 55 were women and 27 men, were analysed. Samples were collected at two time points: time point 0 (T0) where the sample was taken before the intervention and time point 1 (T1) after a six month period of intake of an EGCG and other phytochemicals containing preparation (Appendix: 7.4) were used for analysis. Furthermore, a food frequency questionnaire, collecting data about food and lifestyle parameters was evaluated (Appendix: 7.3).

5.1.1 Exclusion criteria

Due to various studies, which prove the adverse influence of smoking on DNA methylation (Tsaprouni et al. 2014) and telomere length (Song et al. 2010), smokers were excluded of the study.
Participants who were taking medicines against thyroid dysfunction, hypertension, reflux and depressions were also omitted from analysis because of the possibility of a disturbed uptake and bioavailability of the ingredients of the intervention preparation.

5.2 Dried Blood Spots

For sample collection dried blood spots on protein saver cards (Protein Saver Cards, 903®, Whatman™, VWR, Radnor Pennsylvania) were taken at T0, before starting the intervention and at T1, after intervention period. Samples were stored at room temperature until analyses were conducted.

5.3 DNA Isolation

DNA was extracted using QIAamp® DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol with minor adaptions (see appendix 7.1). After extraction DNA was immediately stored at -20°C.

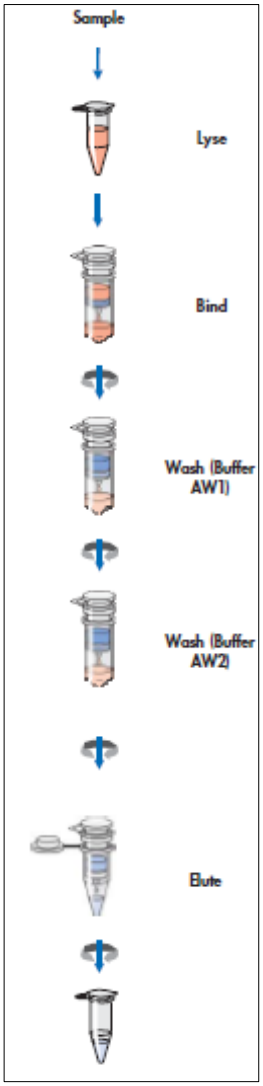

Figure 8: Procedure of DNA extraction (Qiagen 2016)

Figure 8 presents an overview of the procedure. For lysis Proteinase K and buffer AL was added to the tube containing the punched dried blood spot and buffer ATL. Ethanol was added following an incubation step.
After binding the DNA on a silica membrane, placed on a spin column, several washing steps were necessary to ensure the removal of contaminants that otherwise could have influenced further following analyses.
In the last step the purified DNA was eluted from the spin column into a microcentrifuge tube and stored at -20°C.

5.4 Bisulfite Conversion

Bisulfite conversion of extracted DNA was carried out with EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany). Bisulfite conversion is used to „conserve" DNA methylation by chemically modified unmethylated cytosines to uracil, to differ between methylated or unmethylated status after PCR. The procedure is separated into: DNA bisulfite conversion followed by a clean-up step.

Due to a bisulfite thermal cycling program (Appendix 7.2) the isolated DNA was denatured to get single strands. So the Bisulfite Mix containing Sodium Bisulfite that was added before, was able to start the reaction. Figure 9 shows the chemical conversion schematically.

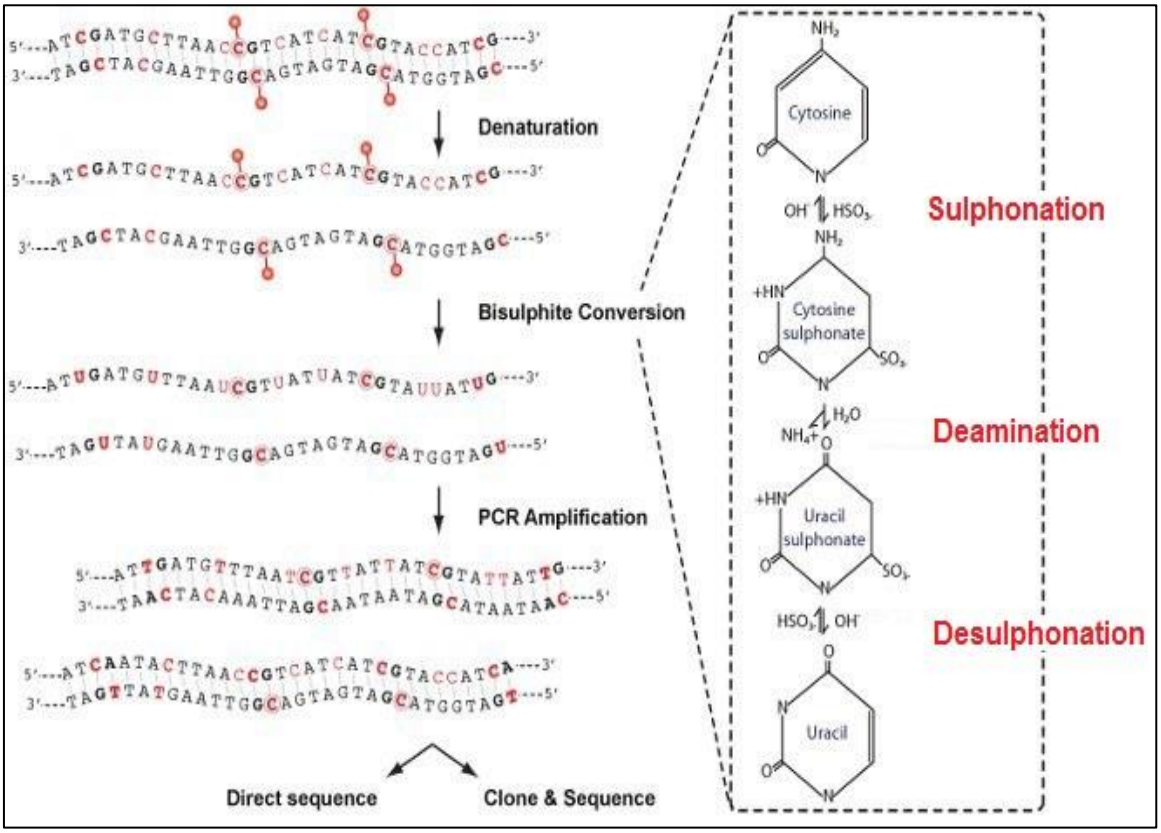

Figure 9: Schematic bisulfite conversion, modified (Patterson et al. 2011)

Bisulfite is binding on the 6th position of the unmethylated cytosine ring (sulphonation). The resulting cytosine sulphonate is converted into uracil sulphonate by hydrolytic deamination. (Patterson et al. 2011)

Afterwards DNA was transferred and bonded to a spin column membrane for purification and desulphonation, which led to modified uracil.

In the subsequent PCR uracil was treated as thymine. This event enabled a distinction of methylated or unmethylated cytosines. The converted samples were stored at -20°C.

5.5 High Resolution Melting

5.5.1 HRM principle

HRM analysis is based on the different melting behaviour of DNA depending on the DNA sequence. A HRM analysis includes an initial amplification of bisulfite converted DNA via PCR which is followed by a dissociation of the PCR product due to a gradual increase of temperature. Whilst temperature is increasing the fluorescence level of intercalating dye such as EvaGreen ® is measured. Intercalating dyes are able to bind to double-stranded DNA, therefore while dissociation of products into single strands fluorescence decreases continuously.

The melting temperature of the PCR product, where dsDNA is melted to 50% into single-stranded DNA, is indicated by a sharp drop of fluorescence (figure 10) because EvaGreen has no binding affinity to ssDNA and is released. (Wojdacz et al. 2010; Wojdacz et al. 2008)
Depending on their sequence, different melting temperatures occur. Thus CG rich sequences require higher temperatures to solve the triple bond than AT rich, in which only one double bond has to be solved.

HRM analyses were performed in a Rotor Gene® Q (Qiagen, Hilden, Germany). For the PCR-HRM reaction a 2x EpiTect® HRM PCR Master Mix (Qiagen, Hilden, Germany) containing HotStarTaq® Plus DNA Polymerase, a PCR Buffer with EvaGreen® (a fluorescent dye) and a nucleotide mix was used, as well as specific pairs of oligonucleotide primers (table 1, 4, 7) in adjusted concentrations (see table 3, 6, 9). For each sample 9,5µl (for c-Myc: 9,0µl) of this mix and 0,5µl (c-Myc: 1µl) of the sample or standard was pipetted into a 0,1ml tube for the PCR.
After PCR amplification (chapter 3.5.2) high resolution melting analysis started with an increasing temperature from 60-65° (depending on gene) to 90°C.

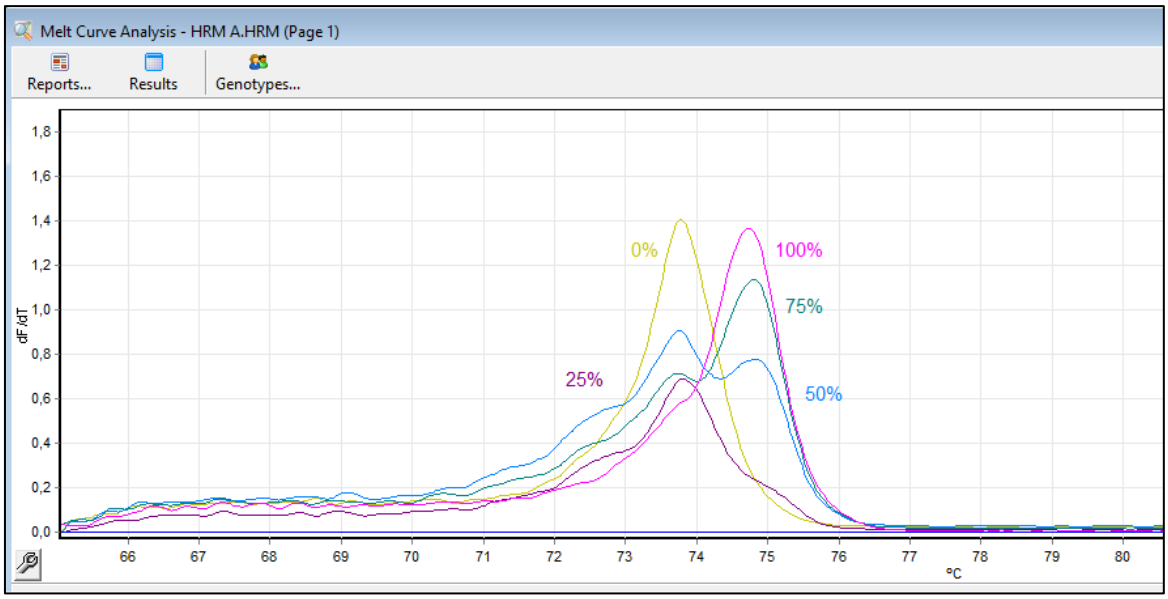

Figure 10: Standard peaks indicating melting temperature

In order to ensure a comparability of the curves, which differ from each other due to small differences in the quantity of amplified PCR products, the start and end of the fluorescence curves were normalized (figure 11).

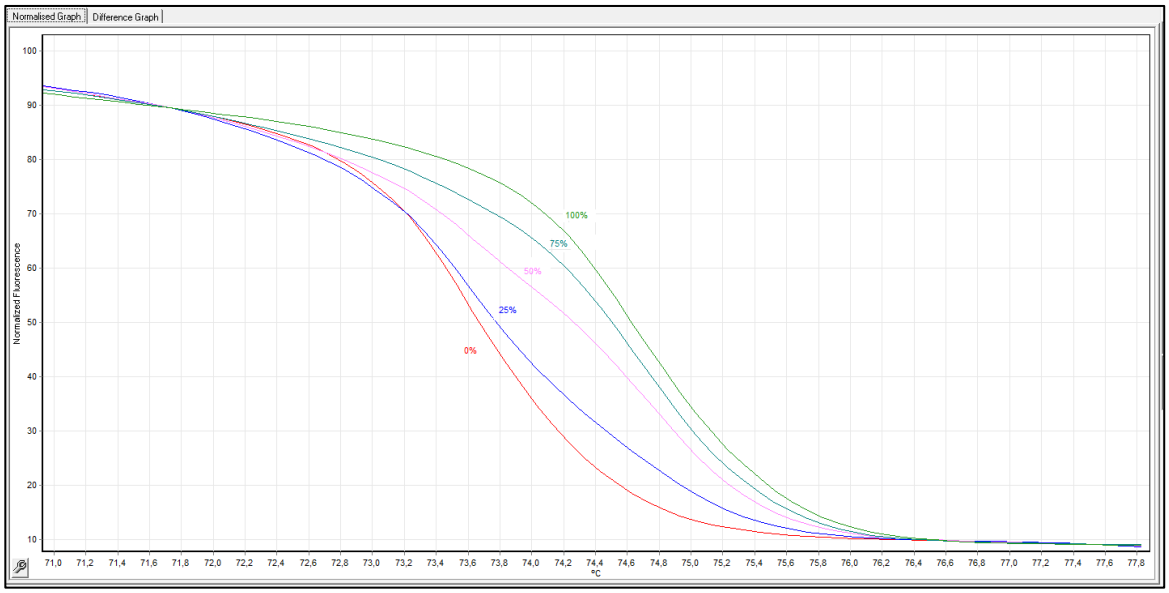

Figure 11: Normalized melt curve of standards

To appraise the methylation levels of the samples, standards containing Caco-2 cells with known methylation status (0% and 100%) were used. They were diluted into 25%, 50%, and 75% for *ASPA* and *ITGA2B* and 6,25%, 12,5%, 18,75% and 25% for *c-Myc*.

Also a no template control (NTC), containing all reaction components (Master

Mix, Primers, NFW) except DNA was used to diagnose possible contaminations. All samples and standards were analysed as a dual approach.

5.5.2 PCR process and conditions

As already mentioned before a PCR is performed in the first step of HRM to amplify a specific sequence of a target gene. The most important components that are needed for this purpose are the following:

- **primers**:

A specific pair of primers (forward and reverse), consisting of oligonucleotides, that are complementary to the DNA fragment of interest, is used.

- **DNA Taq Polymerase**:

This enzyme, used in a heat-stable form, is responsible for synthesizing the new dsDNA. It is inactive at temperatures below 70°C

- **deoxynucleotide triphosphates** (dNTPs):

Deoxyadenosine triphosphate (dATP), Deoxythymidine triphosphate (dTTP), Deoxyguanosine triphosphate (dGTP) and Deoxycytidine triphosphate (dCTP) are required as DNA building blocks by polymerase.

After an initial PCR activation step (5 minutes at 95°C), where the Polymerase is activated, the most common three step thermal cycling process (figure 12) starts

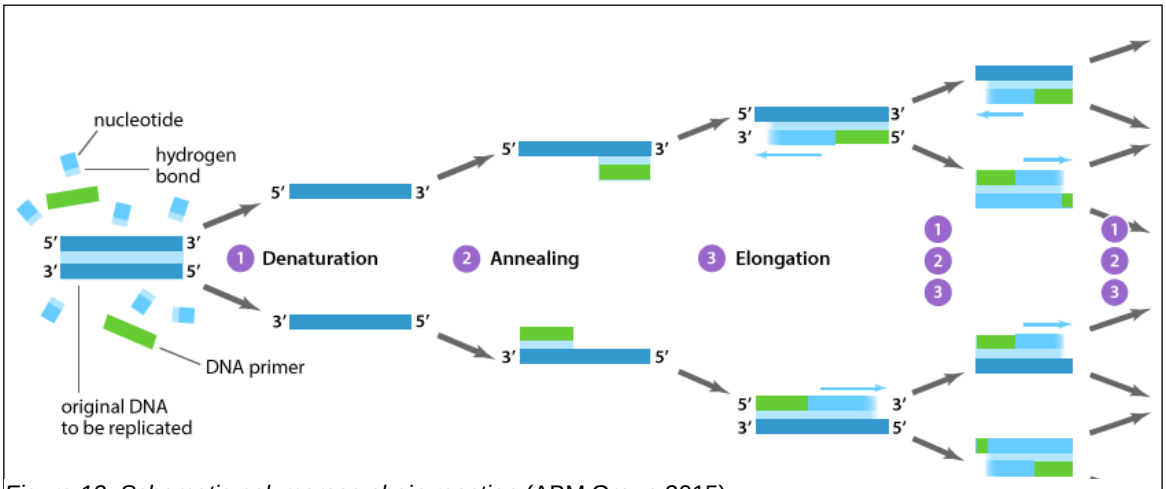

Figure 12: Schematic polymerase chain reaction (ABM Group 2015)

with a denaturing phase at high temperature (93-96°C).

During this time the dsDNA is melting into ssDNA due to the splitting of hydrogen bonds.
This process enables the annealing of the primer pair to ssDNA, which is the second step of the cycle after cooling (55-65°C). The single-stranded primers can bind to the ssDNA due to their complementary of their base sequences, hydrogen bonds are built, which leads to a primer hybridization.
In the elongation phase, the last step of the cycling, the polymerase extends the primer through an addition of dNTPs from 5′ to 3′, while reading the template from 3′ to 5′. (Gadkar & Filion 2013; Rodriguez-Lazaro & Hernandez 2013; Valasek & Repa 2005)

5.5.2.1 *ASPA* PCR specifications

***ASPA* forward HRM**	***ASPA* reverse HRM**
5‘-tgg agg aat tta tgg gaa tga gtt a-3‘	5‘-ttt tac ctc caa ccc tat tct cta aat ct-3‘
Size: 25bp	Size: 29bp
PCR product length: 97bp	
PCR product CpGs: 2	

Table 1: ASPA Primer specifications

Table 1 informs about the sequence of used *ASPA* primer pair, as well as size of product and melting temperature.
The conditions of PCR for *ASPA* are listed in table 2. The initial PCR activation was followed by 45 times repeated cycles, each containing a denaturation step (95°C), annealing (50°C) and elongation (72°C). After two holding steps HRM process was started (65°C-90°C).

Temperature	**Time**	
95°C	5‘	
95°C	**10‘‘**	**X45**
50°C	**30‘‘**	
72°C	**10‘‘**	
96°C	1‘	
45°C	1‘	
65°C – 90°C	HRM	

Table 2: ASPA PCR cycling conditions

The PCR was carried out in a 10µl reaction mix. Therefore the following amounts of reagents (table 3) were needed per reaction:

Component	**Volume/reaction**
MasterMix (EpiTect®)	5 µl
Primer forward (10 µM)	0,375 µl
Primer reverse (10 µM)	0,375 µl
NFW	3,75 µl
+ Template DNA	0,5 µl
	10 µl

Table 3: ASPA reaction composition

5.5.2.2 *ITGA2B* PCR specifications

For PCR and additional HRM analysis of *ITGA2B* the primers described in table 4 were used.

***ITGA2B* forward HRM**	***ITGA2B* reverse HRM**
5‘-agg agt ttt gtt ttt aag gga ttt at-3‘	5‘-ttt acc taa aaa aac ctt ccc taa ct-3‘
Size: 26bp	Size: 26bp
PCR product length: 137bp	
PCR product CpGs: 4	

Table 4: ITGA2B Primer specifications

Before HRM analysis was started, the amplification of target DNA was done by a three step thermal cycling process (table 5).

Temperature	**Time**	
95°C	5‘	
95°C	**10‘‘**	
58°C	**30‘‘**	**X45**
72°C	**10‘‘**	
96°C	1‘	
45°C	1‘	
65°C – 90°C	HRM	

Table 5: ITGA2B PCR cycling conditions

Table 6 represents the composition of the reaction mix.

Component	**Volume/reaction**
MasterMix (EpiTect®)	5 µl
Primer forward (10 µM)	0,375 µl
Primer reverse (10 µM)	0,375 µl
NFW	3,75 µl
+ Template DNA	0,5 µl
	10 µl

Table 6: ITGA2B reaction composition

5.5.2.3 *c-Myc* PCR specifications

***c-Myc* forward HRM**	***c-Myc* reverse HRM**
5‘-tga gga ttt tcg agt tgt gtt gt-3‘	5‘-ctt ctc gaa aca aaa cca aaa-3‘
Size: 23bp	Size: 27bp

Table 7: c-Myc Primer specifications

HRM analysis was performed with the primer pair described in table 7. Following PCR conditions (table 8) were used:

Temperature	**Time**	
95°C	10‘	
95°C	**15‘‘**	**X45**
60°C	**1‘**	
95°C	10‘‘	
60°C	1‘	
60°C – 90°C	HRM	

Table 8: c-Myc PCR cycling conditions

MeltDoctor™ HRM Master Mix (Applied Biosystems, Waltham, Massachusetts) was used for reaction. Magnesium Chloride with a final concentration of 1,5mM was added (table 9).

Component	**Volume/reaction**
MasterMix (MeltDoc™)	5µl
PrimerMix* (10pmol/µl/Primer)	0,25µl
$MgCl_2$ (25mM)	0,6µl
NFW	3,15µl
+ Template DNA	1µl
	10µl

Table 9: c-Myc reaction composition

5.6 Telomere length measurement

In our study absolute telomere length was measured by a quantitative real-time polymerase chain reaction using specific oligomer standards in different dilutions of known quantities (tables 10, 11) to calculate a standard curve. Furthermore, single copy gene (36B4) standards were used to infer absolute telomere length per genome due to the calculation of Telomere-to-Single Copy Gene (T/S) ratio. (O'Callaghan & Fenech 2011)

Also a no template control (NTC), containing all reaction components (Master Mix, Primers, NFW) except DNA was used to diagnose possible contaminations. All samples and standards were analysed as a dual approach and were run on an ABI StepOne Real-Time Cycler (Applied Biosystems, Framingham, MA, USA) using 96-well PCR-plates.

5.6.1 Oligomeres used for measuring telomere length

5.6.1.1 Standards

Standards	***Sequence (5'-3')***
Telomere standard	$(TTAGGG)_{14}$
Amplicon size	84 bp
36B4 (single copy gene) standard	CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAAGGCCAGGACTCGTTTGTACCCGTTGATGATAGAATGGG
Amplicon size	75 bp

Table 10: Standards used for telomere length measurement

5.6.1.1.1 Standard dilutions

The standards were diluted according to the following schemes:

Telomere standards	**dilution ratio**	**concentration**
Ax1	1:20	50ng/µl
Ax2	1:10	5ng/µl
Ax3	1:10	0,5ng/µl
A1	1:10	50pg/µl
A2	1:10	5pg/µl
A3	1:10	0,5pg/µl
A4	1:10	50fg/µl
A5	1:10	5fg/µl

Table 11: Dilutions of telomere standards. Ax1-Ax3 are pre-dilutions

36B4 (single copy gene) standards	dilution ratio	concentration
Bx1	1:20	50ng/µl
Bx2	1:10	5ng/µl
Bx3	1:10	0,5ng/µl
Bx4	1:10	50pg/µl
Bx5	1:10	5pg/µl
Bx6	1:10	0,5pg/µl
B1	1:10	50fg/µl
B2	1:10	5fg/µl
B3	1:10	500ag/µl
B4	1:10	50ag/µl
B5	1:10	5ag/µl

Table 12: Dilutions of 36B4 standards. Bx1-Bx6 are are pre-dilutions

5.6.1.2 Primers

PCR primers	***Sequence (5'-3')***
telo forward	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
telo reverse	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
Amplicon size	>76 bp
36B4 forward	CAGCAAGTGGGGAAGGTGTAATCC
36B4 reverse	CCCATTCTATCATCAACGGGGTACAA
Amplicon size	75 bp

Table 13: PCR primers used for telomere length measurement

5.6.2 PCR reaction composition

A mastermix for PCR is produced, one with the specific primers for telomeres and one with those for single copy gene.

Component	**Volume/reaction**
LightCycler® MasterMix (Roche Diagnostic)	5 µl

Primer forward (2 µM)	0,5 µl
Primer reverse (2 µM)	0,5 µl
NFW	2,0 µl
+ Template DNA	2,0 µl
	10 µl

Table 14: Composition of the master mix for telomeres and 36B4

5.7 Statistical analysis

For statistical analysis the IBM® SPSS® Statistics 24 software (Armonk, New York) was used.

To test if the data were normally distributed, a Kolmogorov-Smirnov test was performed.
Paired t-test or Wilcoxon signed-rank test, depending on the distribution, were applied for examining an effect of intervention. Pearson's correlation analysis was applied as well as Spearman's Rho analysis to analyse possible relationships among metric parameters. The correlations of categorical variables were assessed by Kendall's Tau analysis. The significance level was set at $p < 0,05$. Unless otherwise stated, all given data are represented as mean values ± standard deviation.

6 Results

6.1 Effects of intervention

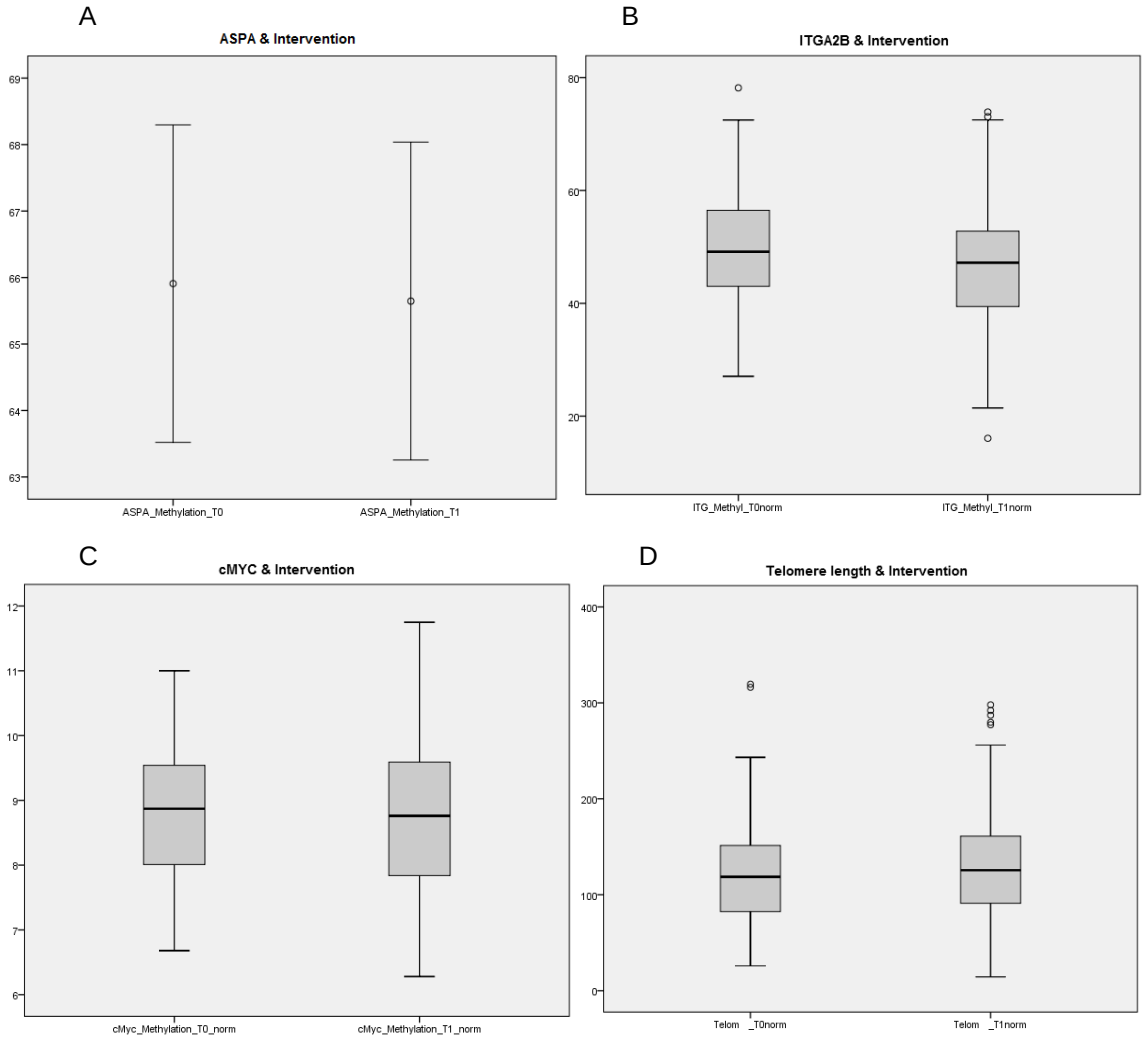

Figure 13: Effects of interventionA: methylation of ASPA gene, B: methylation of ITGA2B gene, C: methylation of c-Myc gene, D: telomere length

Figure 13 represents the influence of intervention on the different genes or telomere length. The first graphs each shows the methylation status in percentages at time point 0 (T0), the second bar illustrates the methylation after intervention period (T1).

(Figure 13A) Mean *ASPA* methylation was slightly decreased after six month of taking the preparation (T0: 65,91% ± 10,45 vs T1: 65,65% ± 10,46).
Similarly, methylation of *ITGA2B* gene (figure 12B), was reduced from 49,35% ±

9,80 (T0) to 47,00% ± 12,89 (T1). Figure 12C illustrates that the mean methylation level of *c-Myc* (T0: 8,81% ± 1,04) nearly remained unchanged after intervention (T1: 8,74% ± 1,17).
Mean telomere length increased from 132,93kb ± 60,97 at T0 to 136,99kb ± 64,87 at T1 (figure 13D).
There were no significant effects observed in any of the targets.

6.2 Results of *ASPA*

6.2.1 Correlations and trends

ASPA	**Correlations**	
	significant (✓) trend (×)	**variable/methylation**
age	✓	↑/↓
height	✓	↑/↑ (male)
BMI	×	↑/↓
liquids	✓	↑/↓ (male)
coffee	✓	↑/↑
cereals	×	(↑/↓)
soft/hard cheese	✓	↑/↓ (male)
leafy vegetables	✓	↑/↑ (female)

Table 15: Overview about correlations and trends of ASPA methylations and variables of the questionnaire The first column describes the individual variables.The last column reveals the direction of methylation in case of an increase of the variables.

In table 15 the results of analysed *ASPA* methylation and the associations with lifestyle and nutritional habits of the study participants are summarized. In the following chapters the correlations and trends are described in detail.

6.2.2 Gender distribution

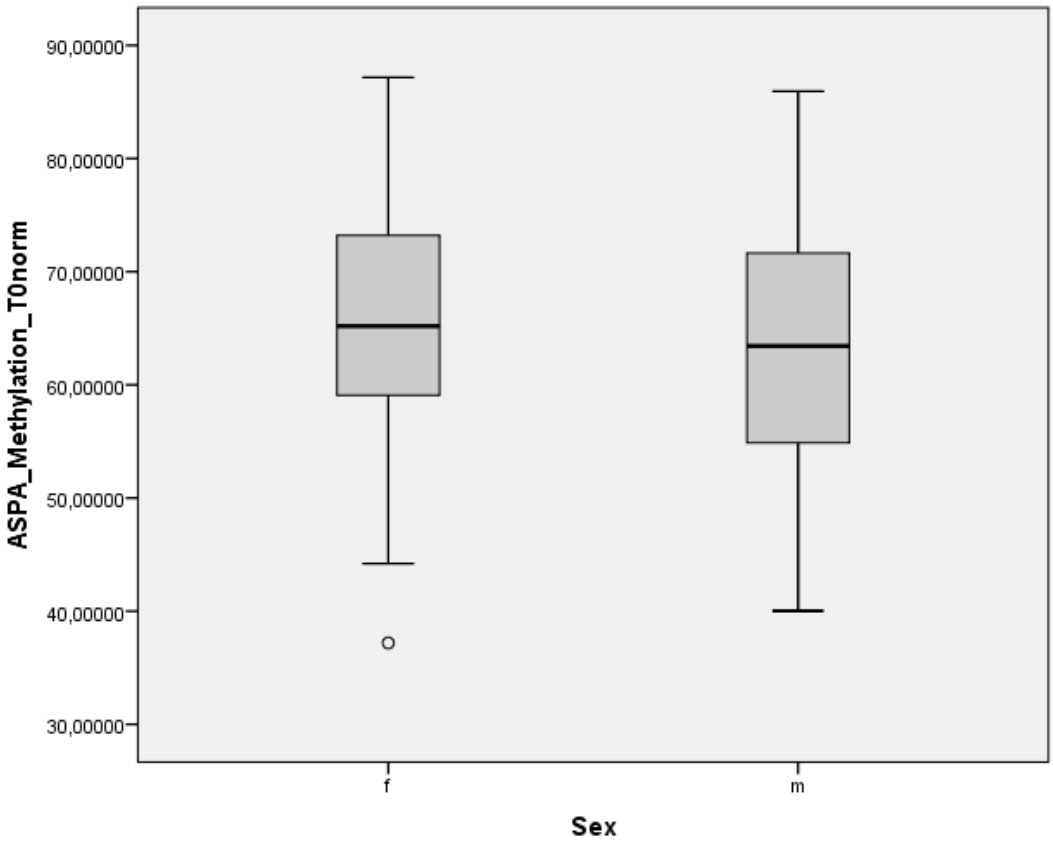

Figure 14: ASPA methylation & gender distribution (f-female; m-male)

Figure 14 shows the *ASPA* methylation differences by gender.
Mean *ASPA* methylation is slightly higher in women (n: 54; 64,90% ± 10,63) than in men (n: 27; 62,40% ± 12,39). The highest methylation level of 87,17% was found in a female. The range of *ASPA* methylation stretched from 40,02% to 85,94% within men.

6.2.3 *ASPA* methylation & age

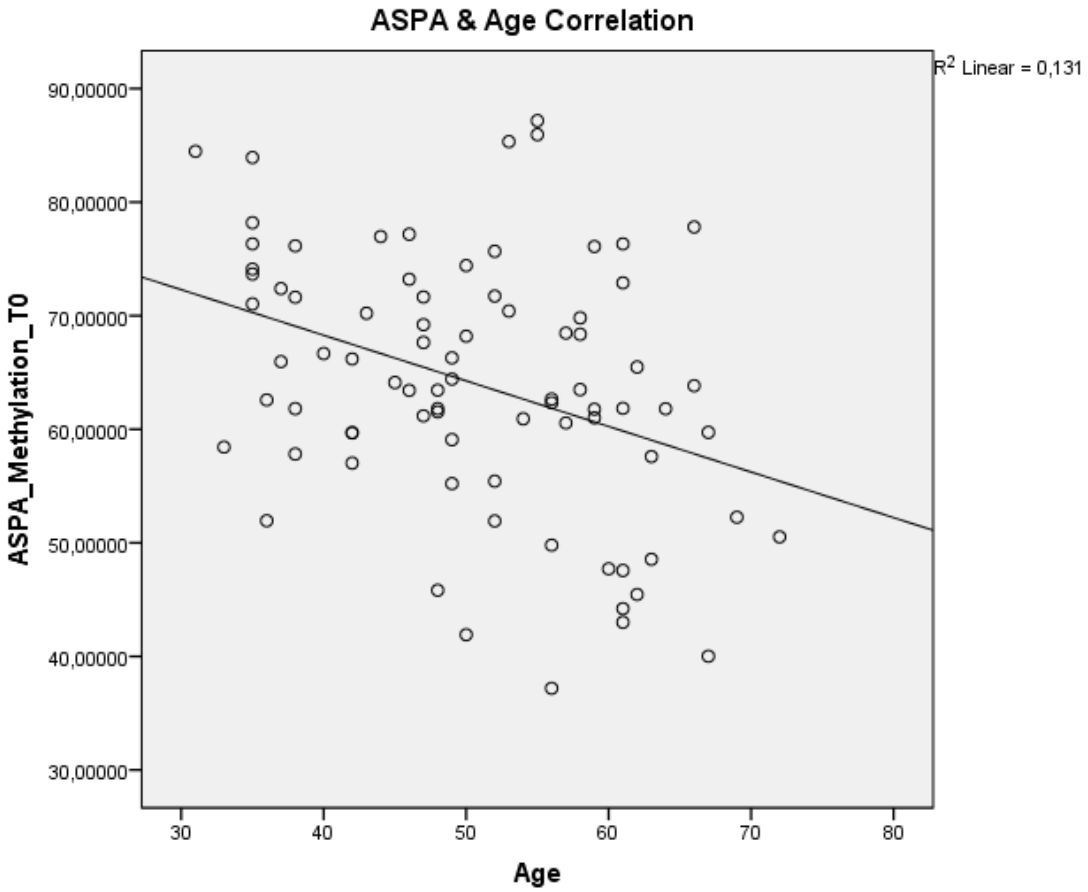

Figure 15: ASPA methylation & age correlation

Figure 15 shows a significant decrease of *ASPA* methylation with increasing age (N: 81; Spearman's rho: -0,347; p: 0,002). Younger participants of the study had higher methylation levels of *ASPA* than older ones.

6.2.4 *ASPA* methylation & height

In 27 men a positive correlation between *ASPA* methylation (figure 16) and height could be observed (n: 27; Spearman's rho: 0,389; p: 0,045). A higher *ASPA* methylation was found in taller men. In women this effect was not detected.

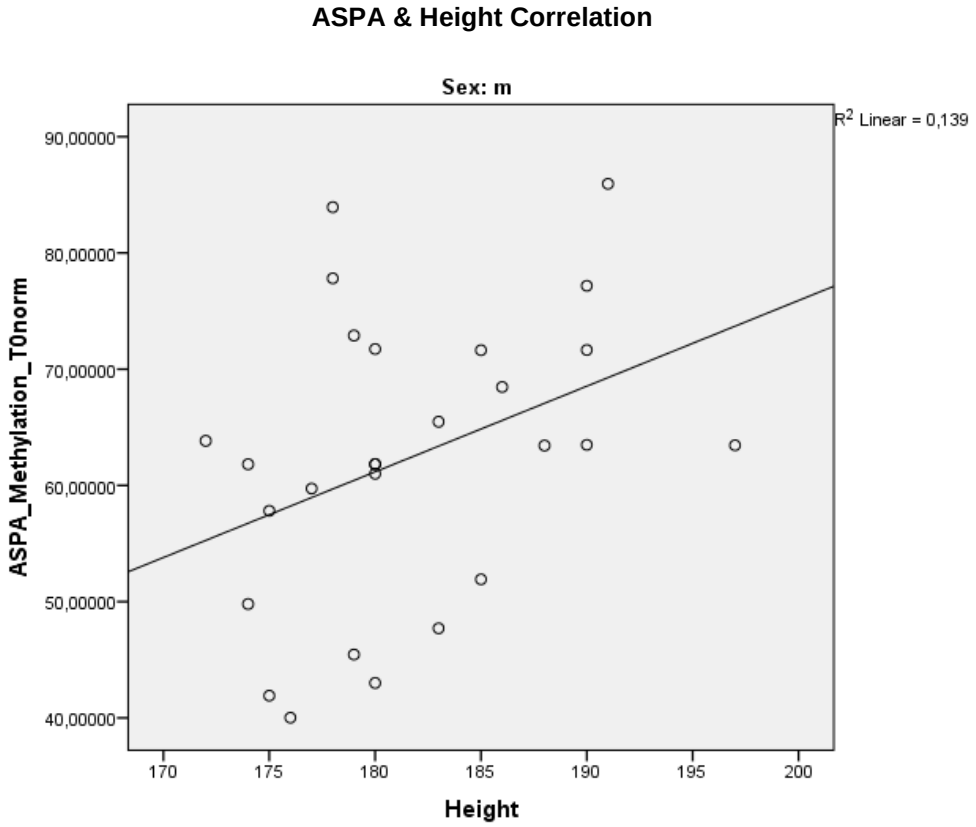

Figure 16: ASPA methylation & height correlation within men

6.2.5 *ASPA* methylation & BMI

BMI and *ASPA* methylation showed a negative correlation (see figure 17). An increased BMI (kg/m^2) is linked to a decreased methylation. Study participants with a lower BMI were associated with a higher *ASPA* methylation.

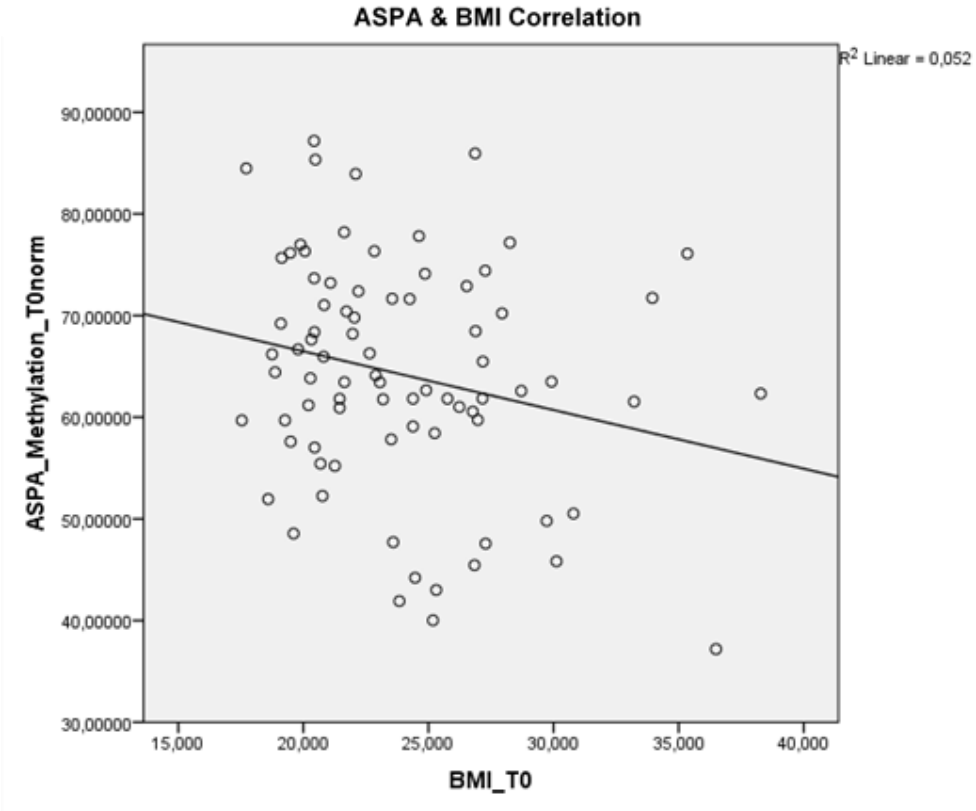

Figure 17: ASPA methylation & BMI correlation

6.2.6 *ASPA* methylation & nutrition

6.2.6.1 Coffee

Statistical analysis determined a significant positive correlation between *ASPA* methylation and the consumption of coffee (N: 79; Kendall's tau: 0,178; p:0,043). People who had drunk more cups of coffee per day, demonstrated a higher level of *ASPA* methylation (Figure 18).

6.2.6.2 Cereals

Figure 19 represents a possible negative trend about the frequency of eating cereals and the corresponding *ASPA* methylation. It could be observed that

people who ate cereals once a day presented a lower methylation status of *ASPA* than these who consumed cereals one to five times per week.

6.2.6.3 Cheese

A significant negative correlation between consumption of cheese and *ASPA* methylation was found but only within the group of men (n: 26; Spearman's rho: -0,399; p: 0,044). Therefore, men who had reported to consume more cheese were associated with lower *ASPA* methylation. (Figure 20)

6.2.6.4 Leafy vegetables

In figure 21 the strong significant positive correlation concerning the consume of leafy vegetables and methylation of *ASPA* (for women n: 53; Spearman's rho: 0,455; p: 0,001) is illustrated. Accordingly to this finding females with a higher intake of leafy vegetables showed increased levels of *ASPA* methylation compared to those who ate lower amounts.

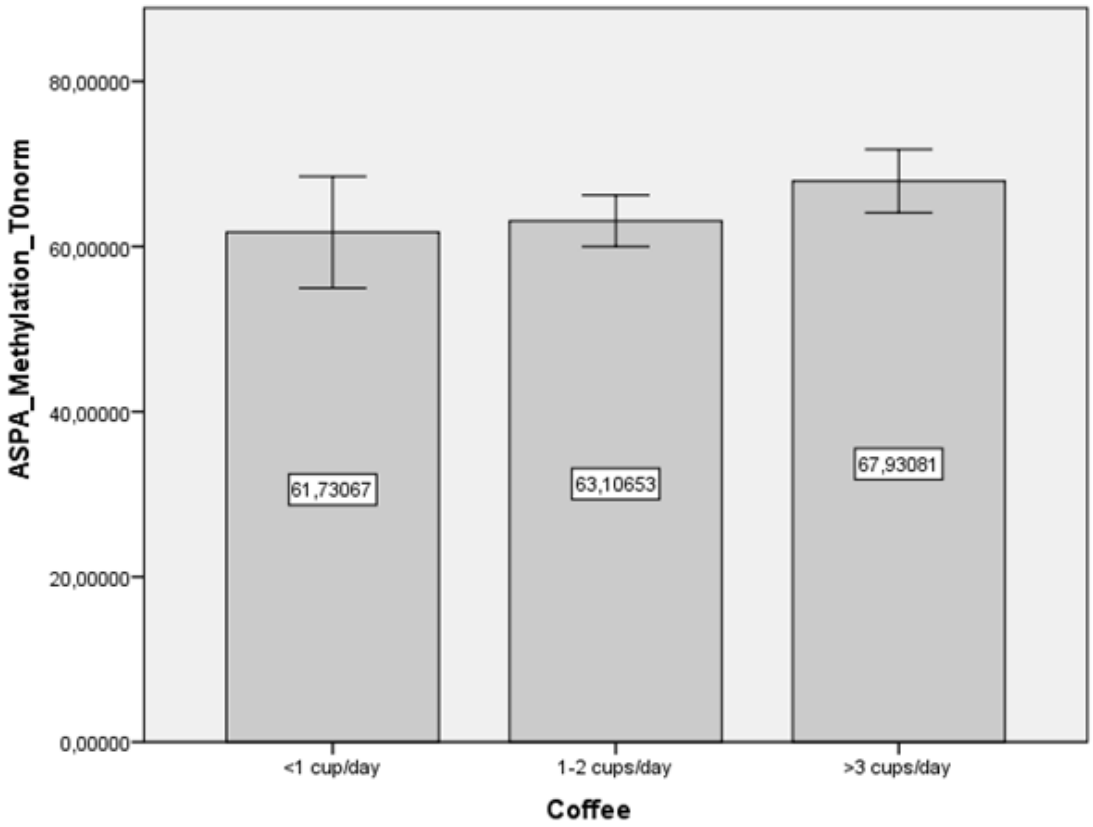

Figure 18: ASPA methylation & coffee correlation

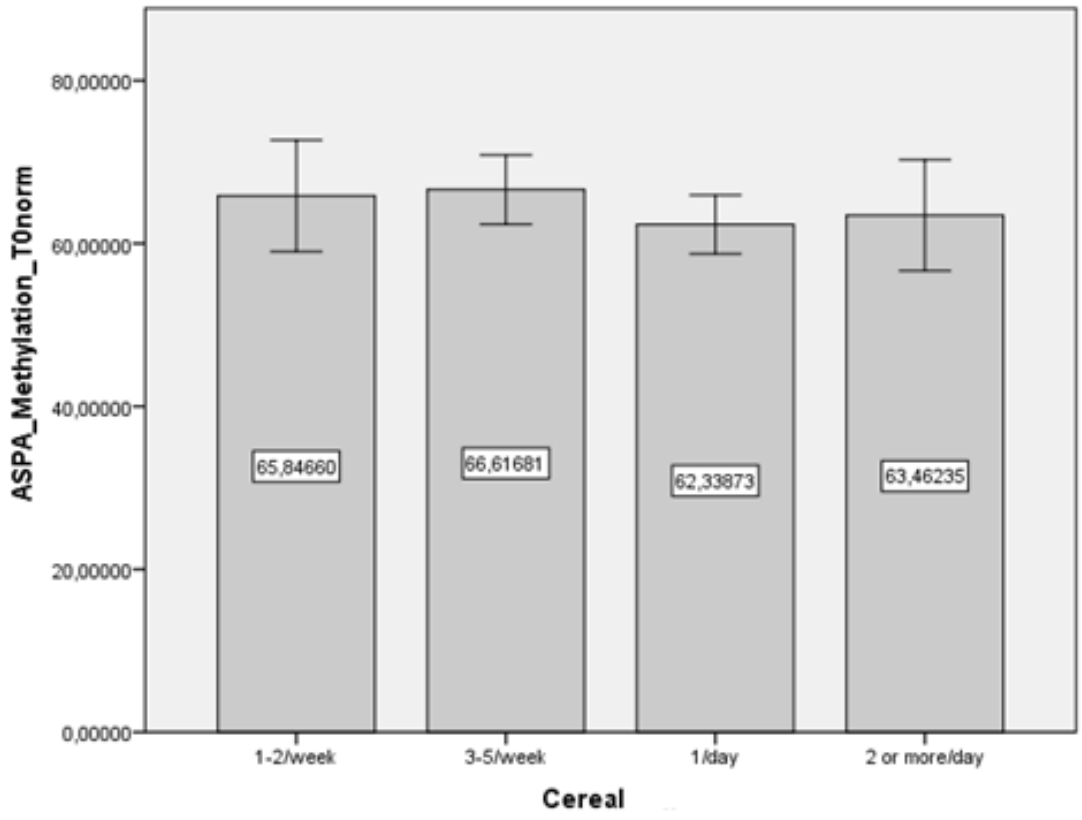

Figure 19: ASPA methylation & cereals correlation

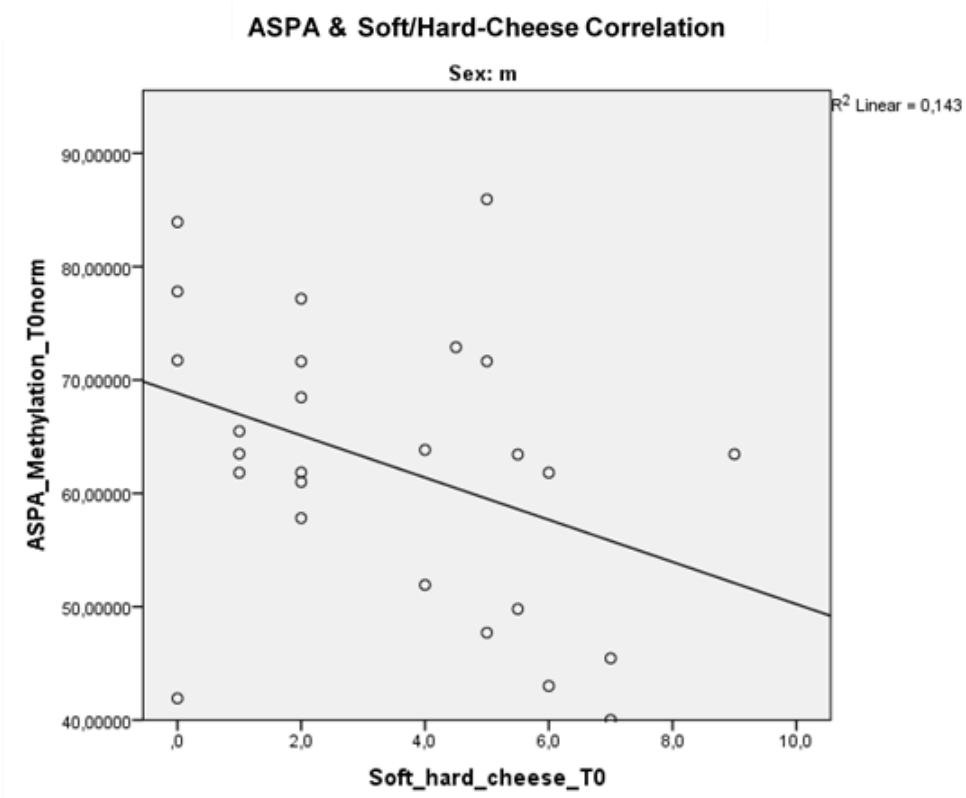

Figure 20: ASPA methylation & cheese correlation within men

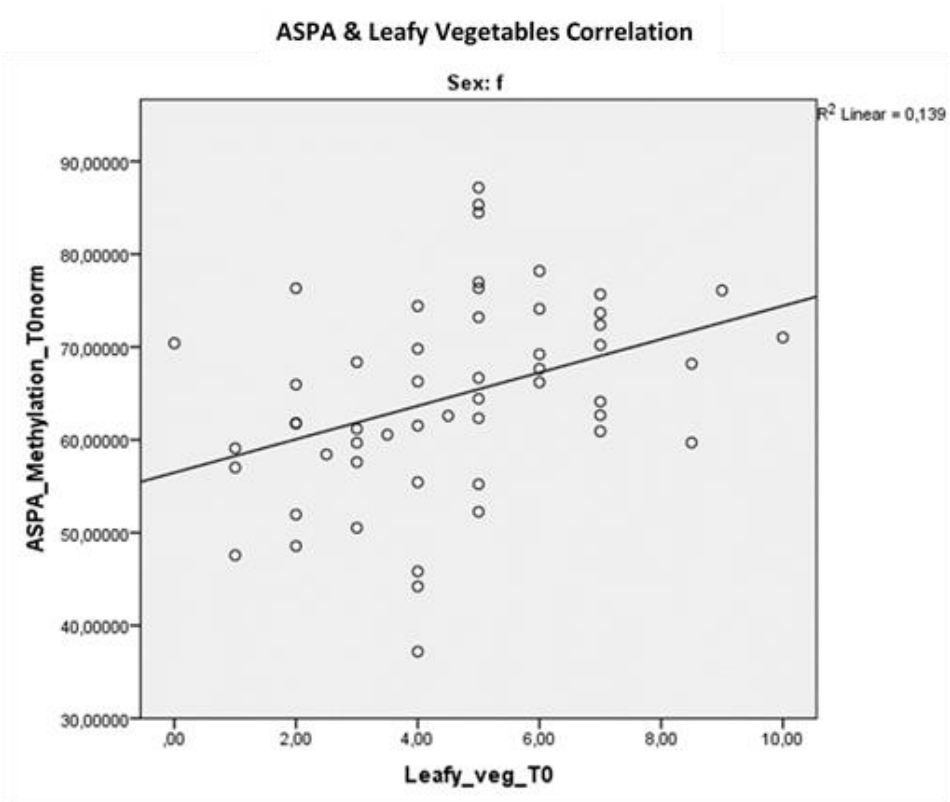

Figure 21: ASPA methylation & leafy vegetable correlation within female

6.3 Results of *ITGA2B*

6.3.1 Correlations and trends

ITGA2B	**Correlations**	
	significant (✓) trend (×)	**variable/methylation**
age	×	(↑/↓)
BMI	×	(↑/↑)
movement	✓	several times/d -> highest mean (female)
liquids	×	(highest consume -> highest mean)
vegetables	×	*ITGA2B* increasing till 1x/d; >2x ↓
fruits	×	*ITGA2B* increasing till 1x/d; >2x/d ↓
dairy products	×	*ITGA2B* increasing till 1x/d; >2x/d ↓

Table 16: Overview about correlations and trends of ITGA2B methylations and variables of the questionnaire

Table 16 represents the results of the relationships of *ITGA2B* methylation and the various variables of the questionnaire including eating habits and lifestyle parameters which are described in the following chapters.

6.3.2 Gender distribution

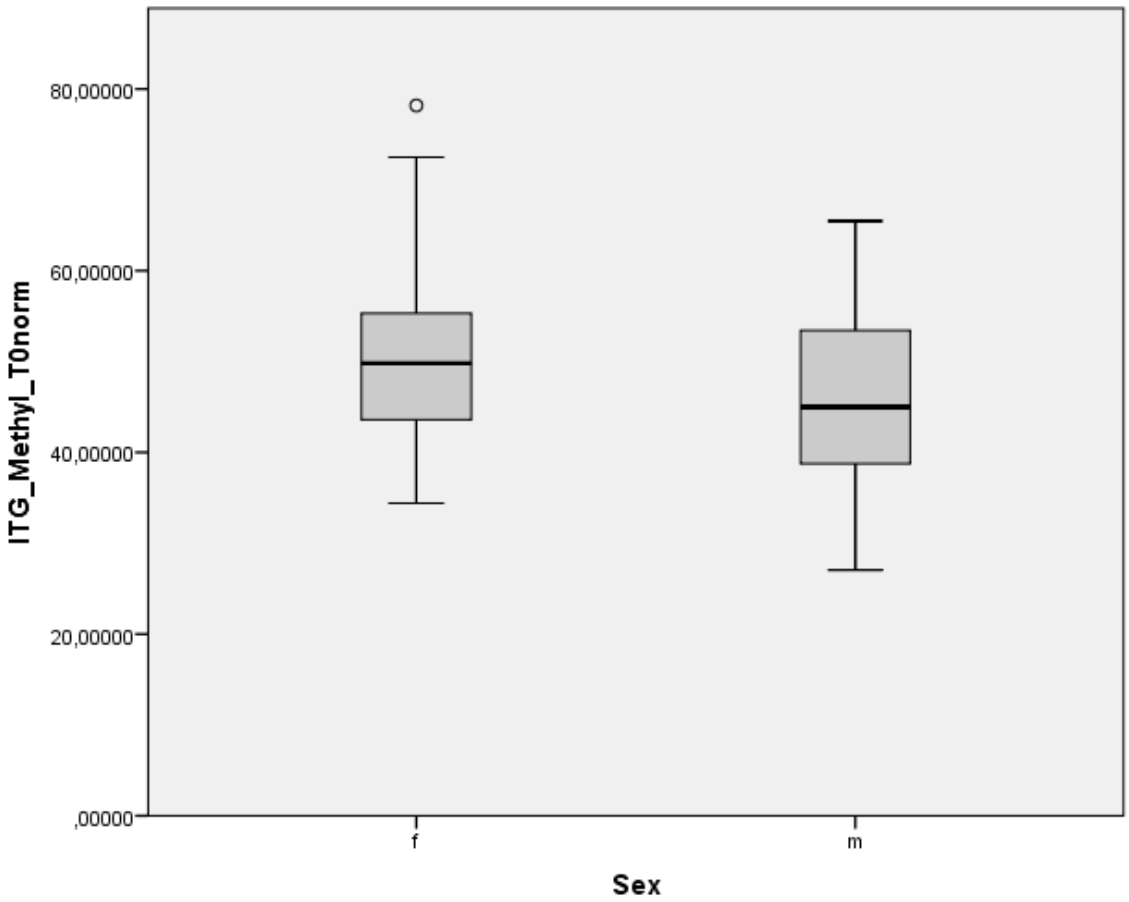

Figure 22: ITGA2B methylation & gender distribution (f-female; m-male)

Figure 22 shows the distribution of *ITGA2B* methylation between genders. The first boxplot describes *ITGA2B* methylations of females, next to it the values of men. In general analysed mean methylation of this gene was higher in women than in men (n: 52; 50,46% ± 8,38 vs n: 45,94% ± 9,60). The minimum methylation of female participants amounts to 34,40% (maximum 78,19%). Range of methylation stretches from 27,06% to 65,46% in the group of men.

6.3.3 *ITGA2B* methylation & age

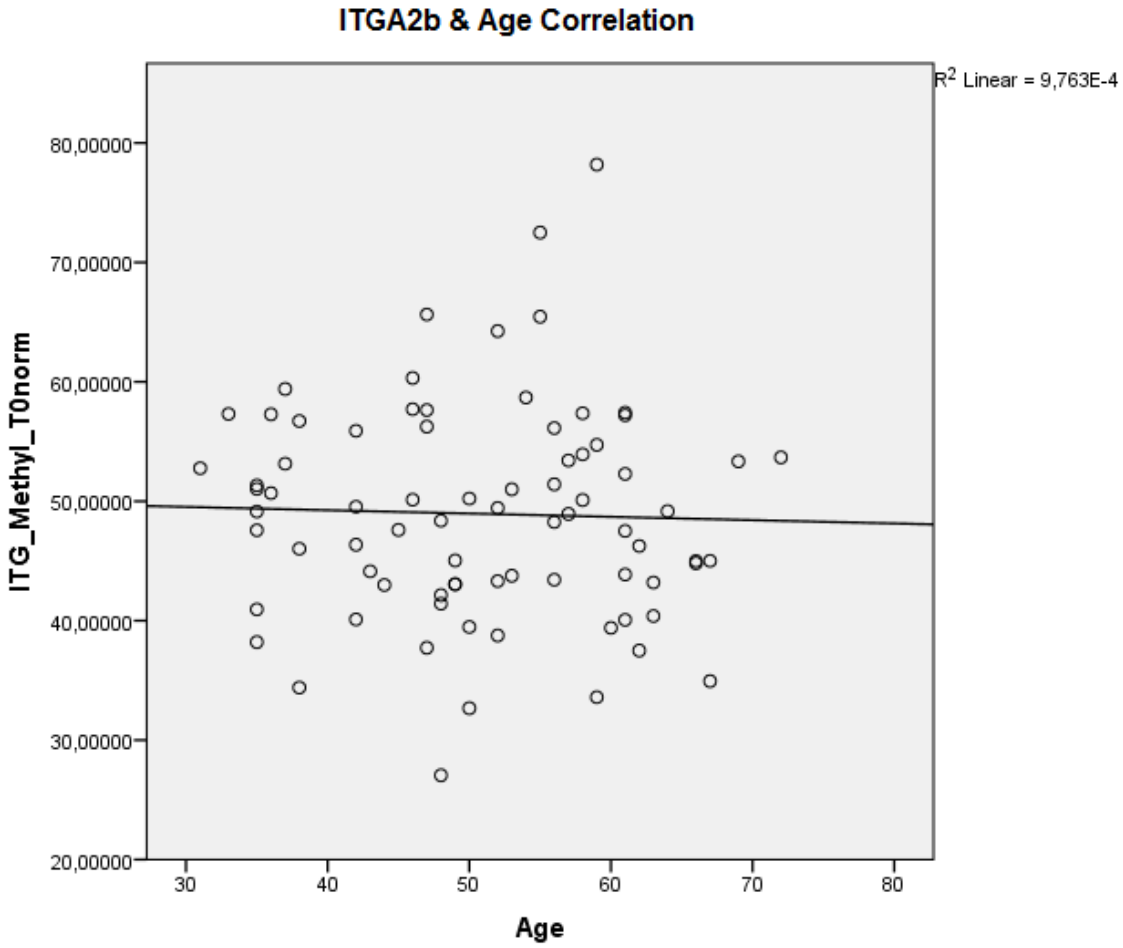

Figure 23: ITGA2B methylation & age correlation

A slight negative correlation between *ITGA2B* methylation and age could be observed, greater age led to a lower methylation (figure 23).
ITGA2B was rather higher methylated in younger than in older participants.

6.3.4 *ITGA2B* methylation & BMI

There was a trend towards a higher *ITGA2B* methylation with an increasing BMI. This observation could be seen both in female (figure 24) and male participants (figure 25).

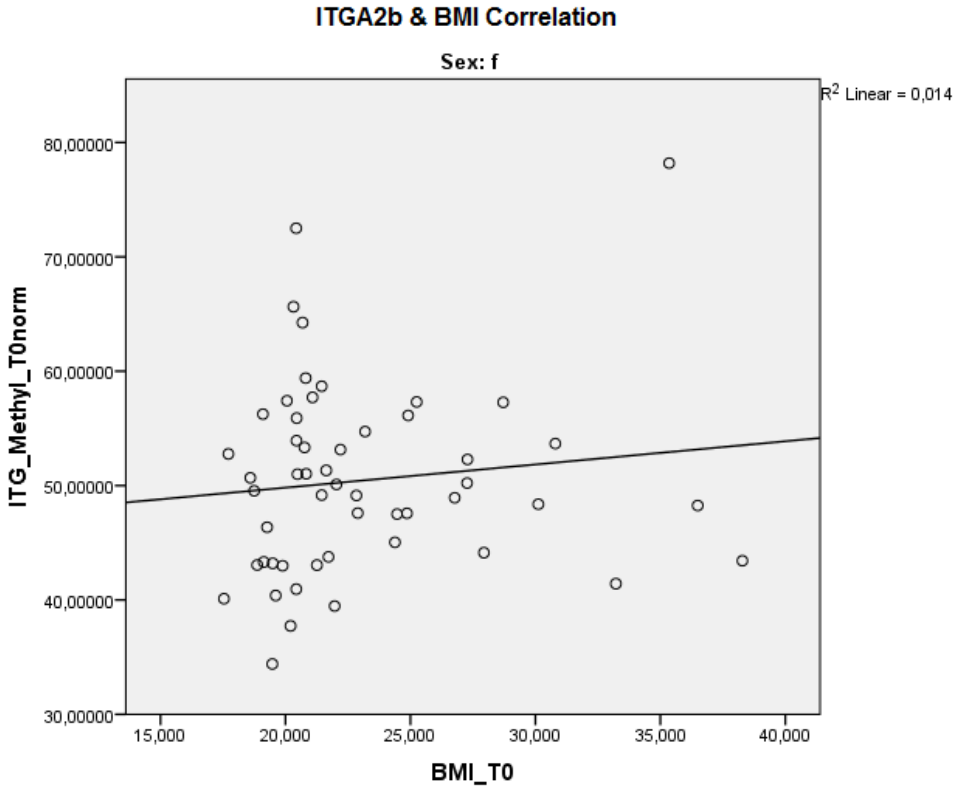

Figure 24: ITGA2B methylation & BMI correlation within female

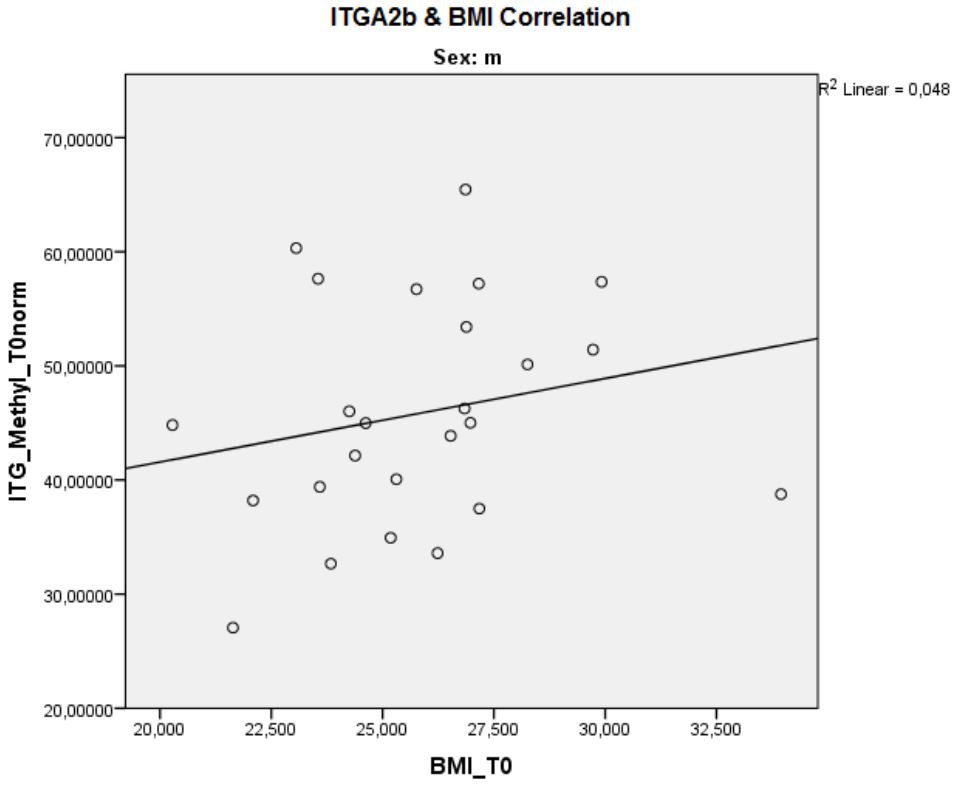

Figure 25: ITGA2B methylation & BMI correlation within men

6.3.5 *ITGA2B* methylation & lifestyle

6.3.5.1 Movement

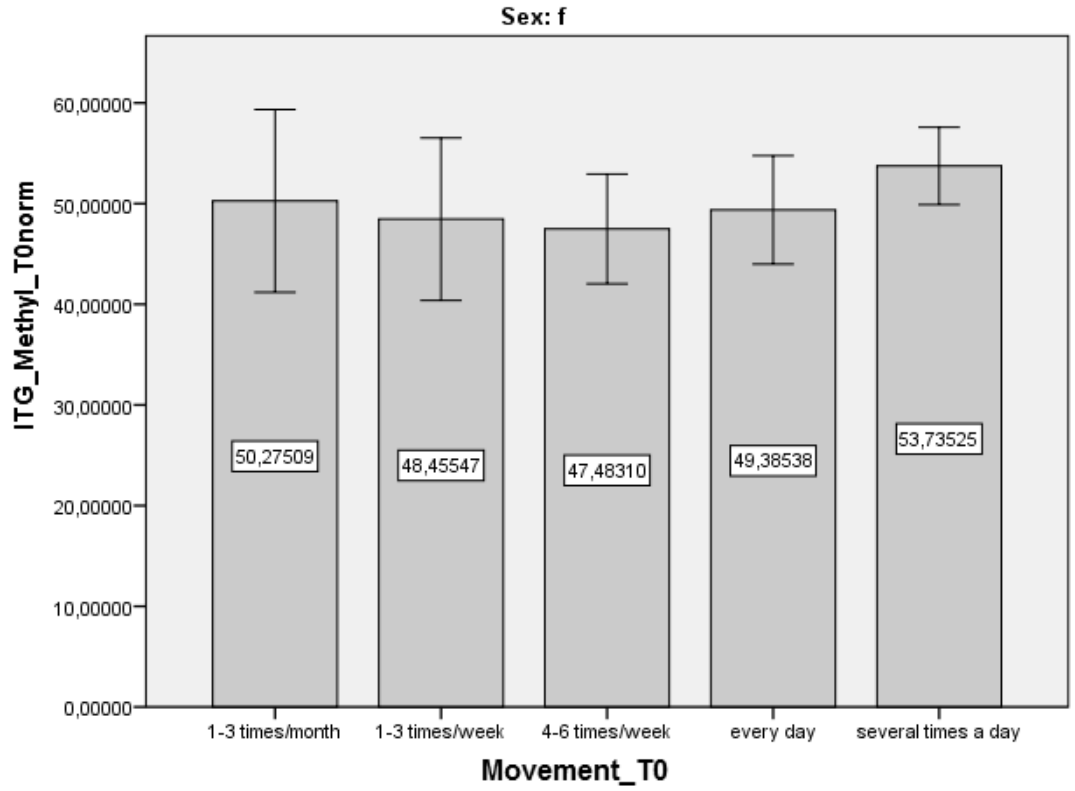

Figure 26: ITGA2B methylation & movement correlation within women

Statistical analysis revealed a significant positive correlation between *ITGA2B* methylation and daily movement of female participants (n: 51; Kendall's tau: 0,238; p: 0,025). (Figure 26)

Women who had reported the highest frequency of daily movement, showed the highest mean *ITGA2B* methylation (n: 16; 53,74% ± 7,22)

6.3.6 *ITGA2B* methylation & nutrition

6.3.6.1 Liquids

A slight positive trend concerning the amount of drunken liquids and *ITGA2B* methylation level could be observed.

A higher consumption of liquids was linked to an increased methylation. (Figure 27)

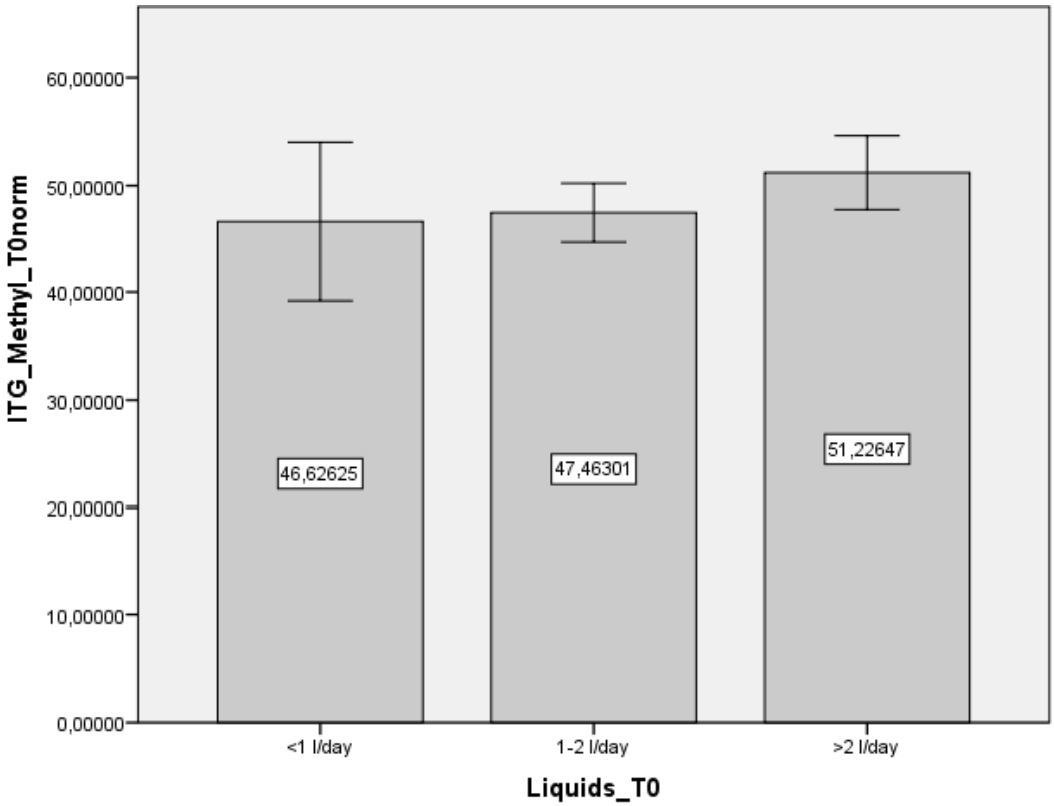

Figure 27: ITGA2B methylation & liquids correlation

6.3.6.2 Fruits

Analysis revealed a soft increase of *ITGA2B* methylation following a fruit consumption frequency of once per day (n: 31; 50,11% ± 11,95) as seen in figure 28. This correlation could not be further strengthened by a higher intake: Participants who ate fruits two or more times a day, had lower mean methylation than all other groups (n: 9; 47,18% ± 6,31).

6.3.6.3 Vegetables

A similar pattern was seen among methylation levels of *ITGA2B* in context to vegetable intake (figure 29). There was an observed trend towards a higher mean methylation with increasing frequency of vegetable intake with the highest value within the once a day group (n: 24; 51,31% ± 10,39).

6.3.6.4 Dairies

Regarding the results of the influence of diary eating habits on *ITGA2B* methylation a positive trend was also visible (figure 30). *ITGA2B* methylation showed higher mean values in participants who had reported a rare consumption

of dairies (once to twice per week: n: 15; 47,28% ± 7,78) than in those with a consumption frequency of once a day (n: 21; 51,05%± 10,63).

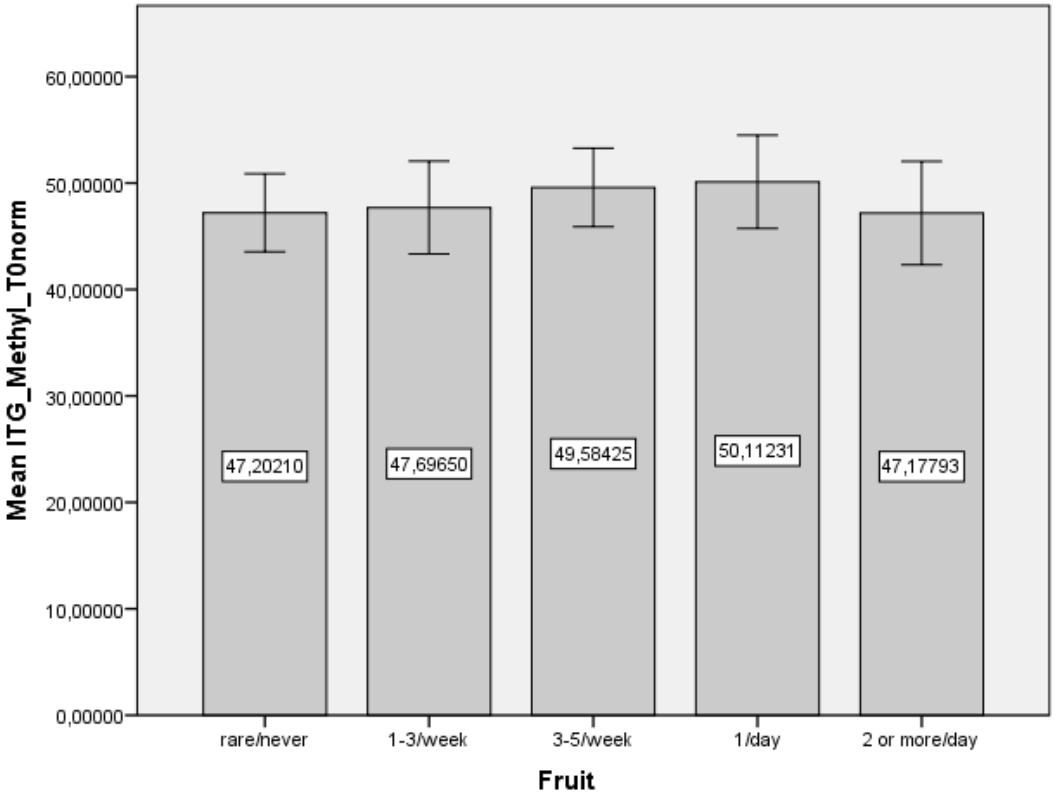

Figure 28: ITGA2B methylation & fruit correlation

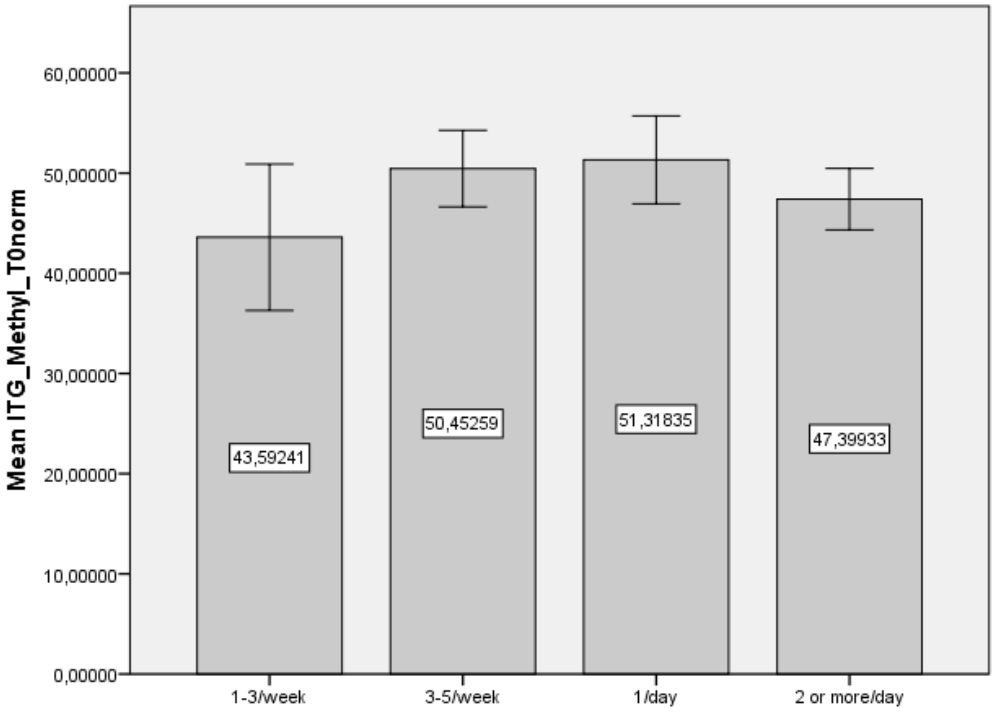

Figure 29: ITGA2B methylation & vegetable correlation

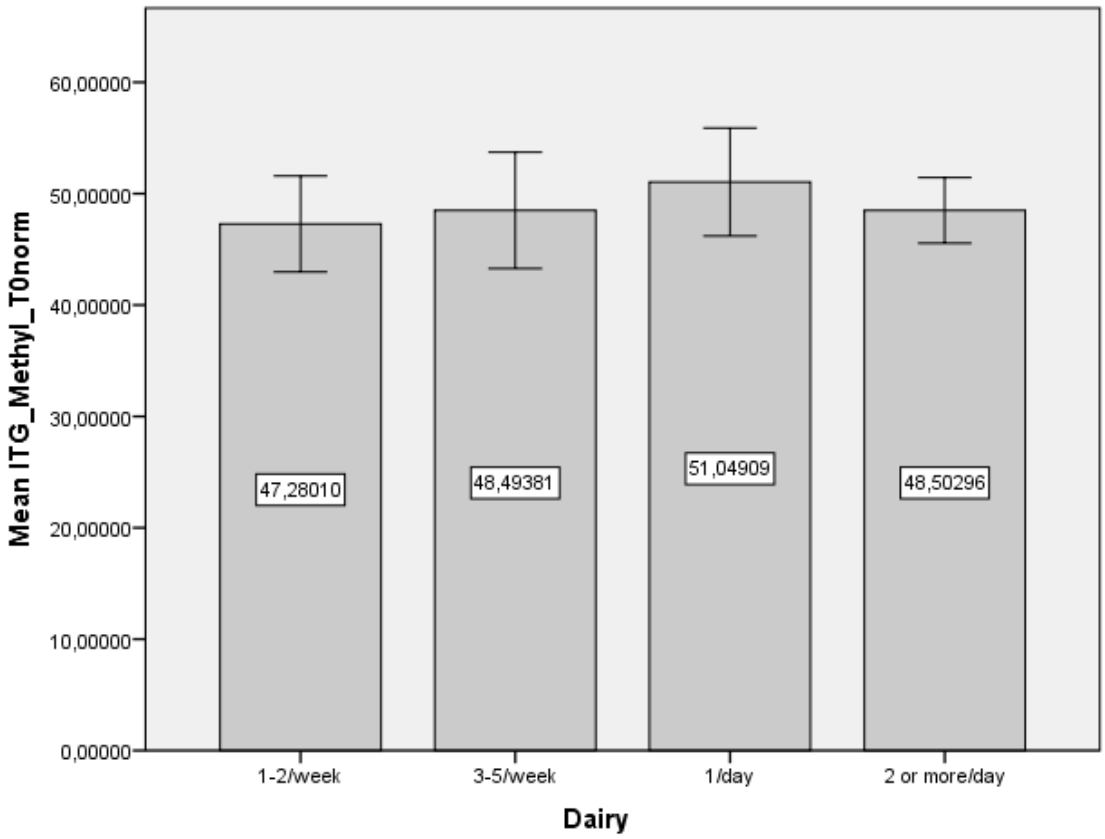

Figure 30: ITGA2B methylation & dairy correlation

6.4 Results of *c-Myc*

6.4.1 Correlations and trends

c-Myc	**Correlations**	
	significant (✓) trend (×)	**variable/methylation**
age	×	↑/↑
sport	×	↑/↑
fitness	✓	↑/↑ (female)
stress	×	↑/↓
meat	×	highest consume -> highest mean
vegetable	✓	↑/↓
snacks	✓	↑/↑

Table 17: Overview about correlations and trends of c-Myc methylations and variables of the questionnaire

Table 17 contains a summary of the results of analysed *c-Myc* methylations and their associations with lifestyle and nutritional habits of the study participants. In the following chapters the correlations are described in detail.

6.4.2 Gender distribution

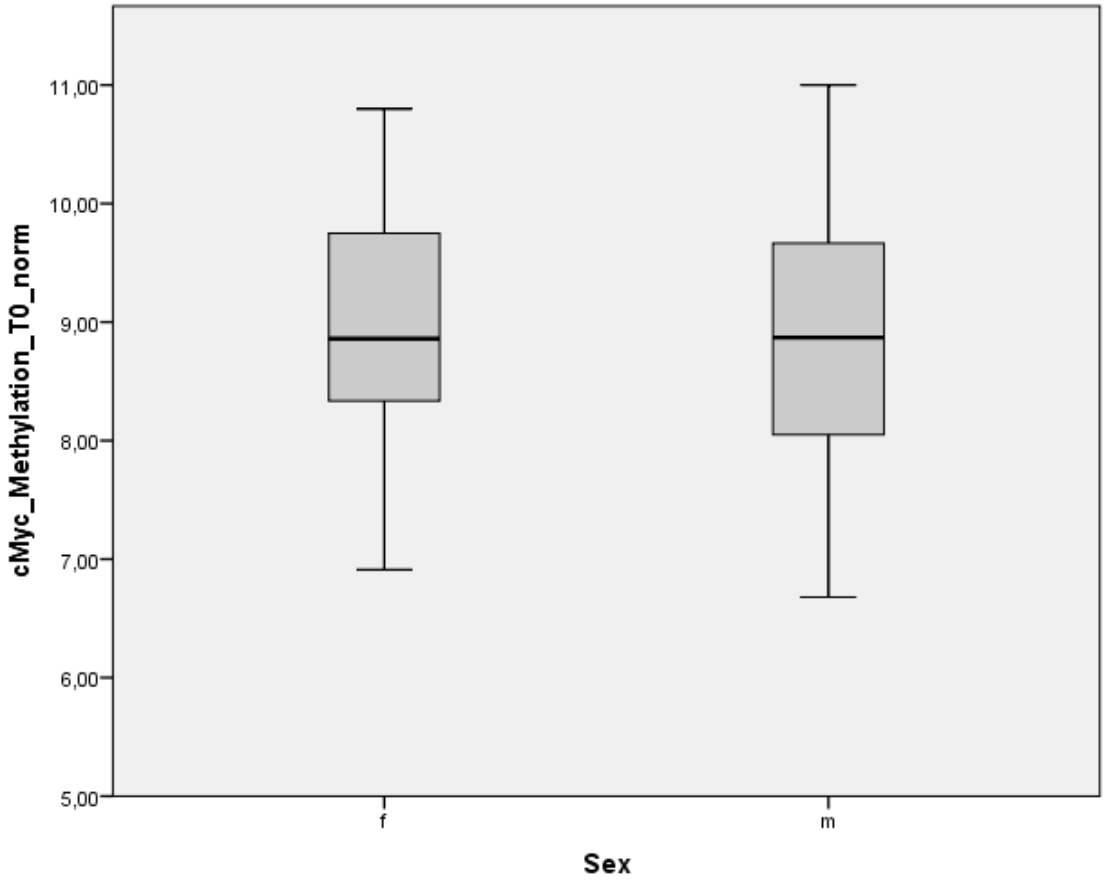

Figure 31: c-Myc methylation & gender distribution (f-female; m-male)

Both sexes showed similar mean levels of *c-Myc* methylation as seen in figure 30. For women a mean methylation of 8,97% ± 0,95 (n: 55) was analysed while for men it was 8,80% ± 1,15 (n: 23), giving an overall mean of 8,92% ± 1.01 (N: 78). Both lowest (6,68%) and highest (11,00%) *c-Myc* methylations were found in the male group.

6.4.3 *c-Myc* methylation & age

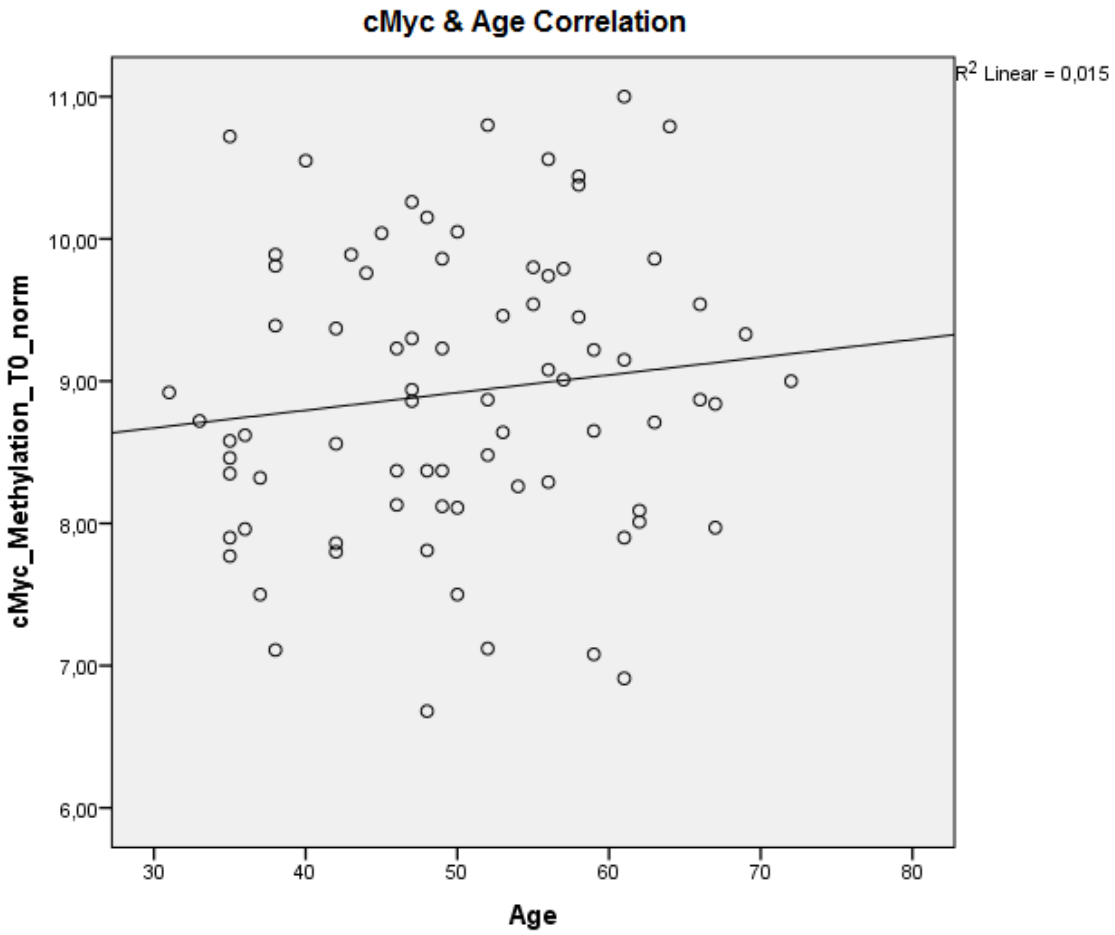

Figure 32: c-Myc methylation & age correlation

There was no significant correlation between *c-Myc* methylation and age, however, a slight positive tendency could be observed (figure 32).
An increasing age was linked to a higher methylation of *c-Myc* meaning younger participants rather had lower methylation values of *c-Myc* than elders.

6.4.4 *c-Myc* methylation & lifestyle

6.4.4.1 Sport

A slight positive trend between *c-Myc* DNA methylation and sport was observed (figure 33). Exercising more often led to a higher mean of methylation.

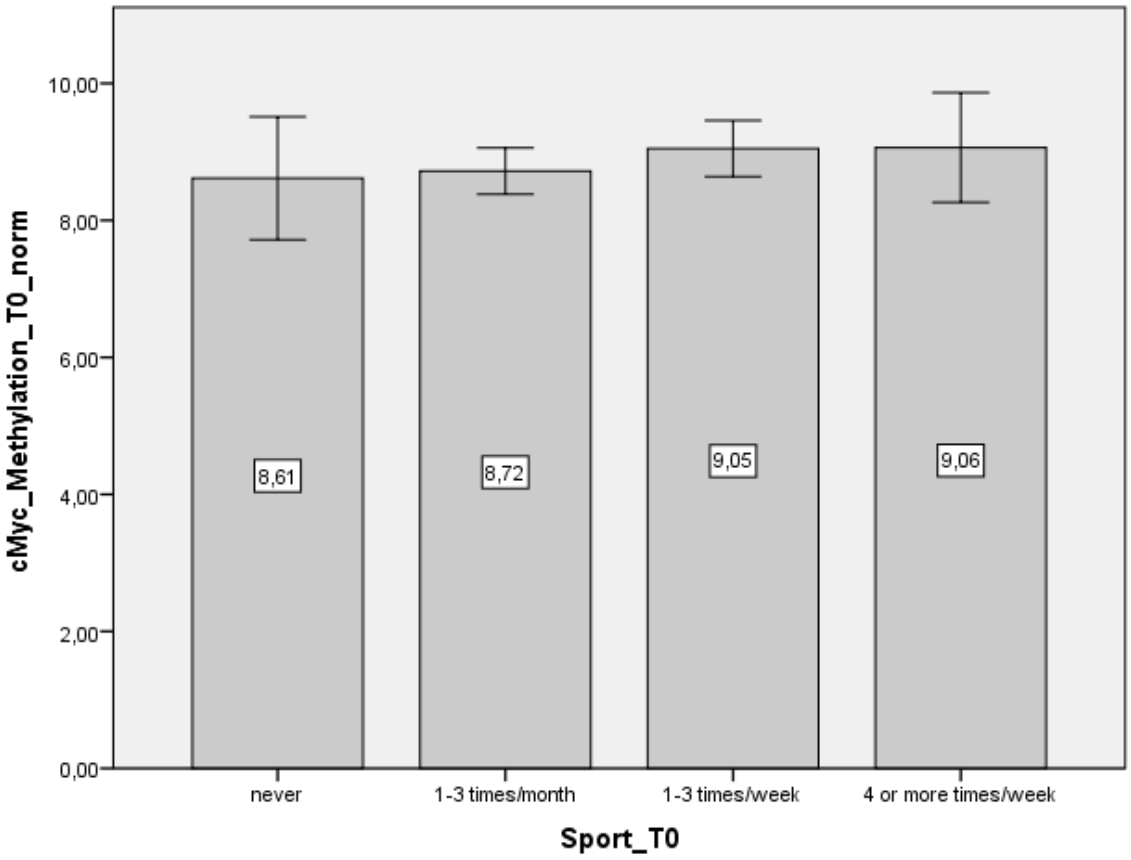

Figure 33: c-Myc methylation & sport correlation

6.4.4.2 Fitness

c-Myc methylation was found to be significant positively correlated with doing fitness in women (n:55; Kendall's tau: 0,204; p: 0,05). The more frequent doing fitness was reported, the higher was the analysed mean methylation.

6.4.4.3 Stress

Figure 34 reflects the negative tendency concerning the relationship of *c-Myc* methylation and having stress. People who had indicated they were suffering from high stress, showed the lowest methylation values (n: 34; 8,79% ± 0,90).

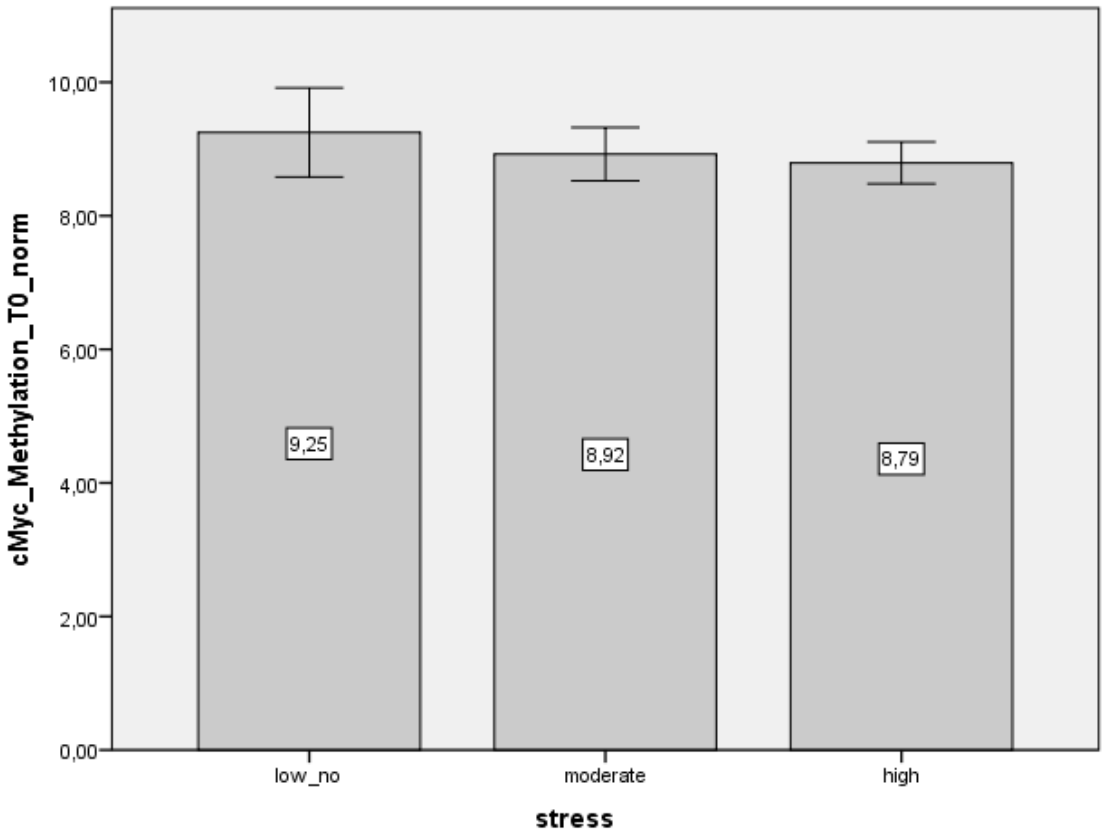

Figure 34: c-Myc methylation & stress correlation

6.4.5 *c-Myc* methylation & nutrition

6.4.5.1 Meat

A slight positive correlation trend could be found between the consumption frequency of meat and methylation level of *c-Myc*.

As seen in figure 35 participants who had reported to consume meat every day showed the highest mean methylation (n: 6; 9,32% ± 0,88) compared with those whose meat consumption was never or rarely (n: 13; 8,65 ± 0,96).

6.4.5.2 Vegetables

The Kendall's correlation analysis revealed a significant negative relationship between consumption patterns concerning vegetables and influence of *c-Myc* methylation (figure 36).

A more frequent eating was associated with a lower methylation (N: 75; Kendall's tau: -0,179; p: 0,043).

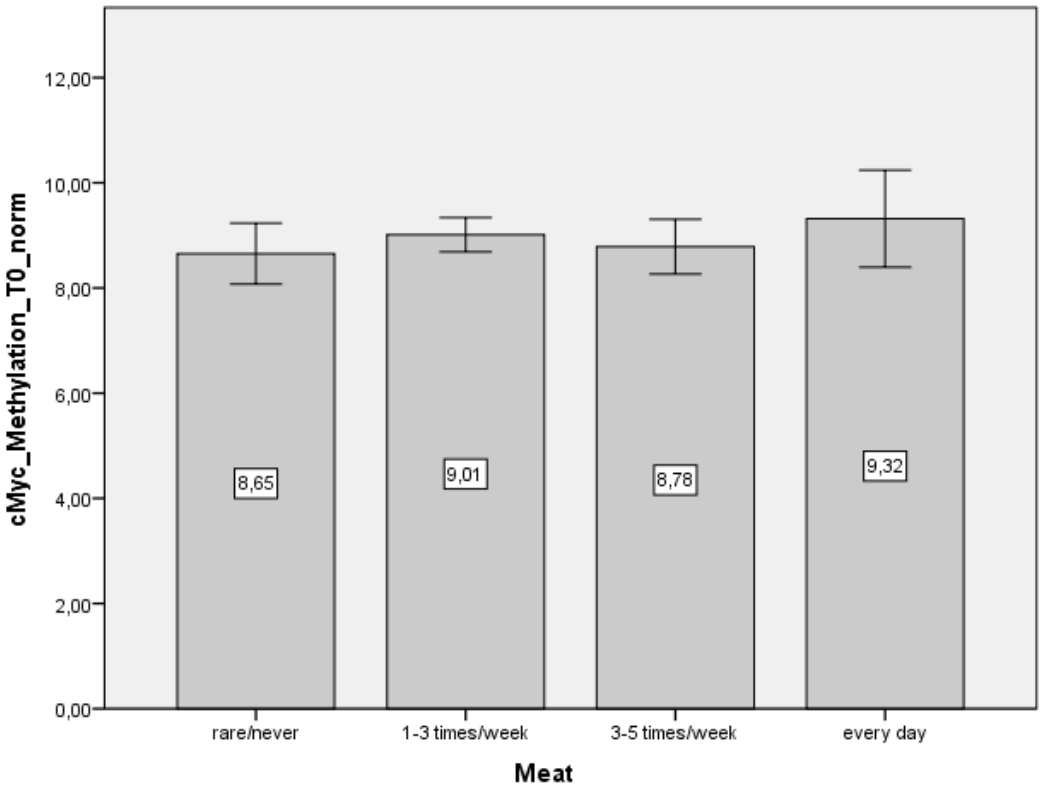

Figure 35: c-Myc methylation & meat correlation

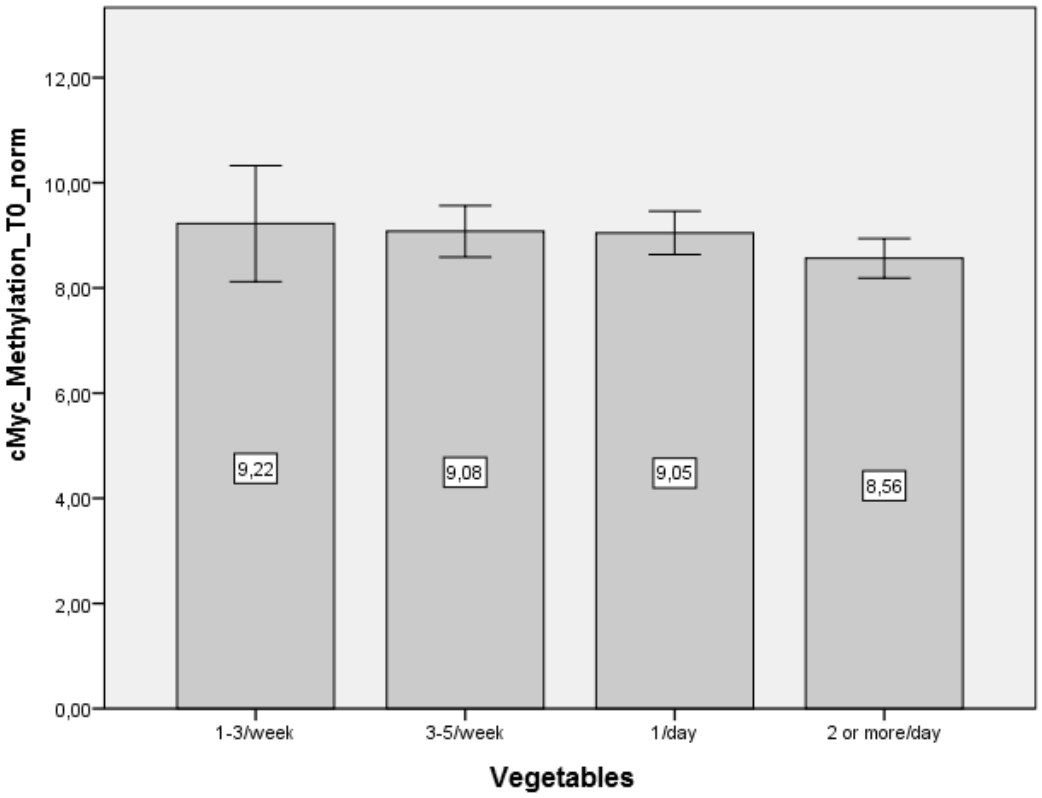

Figure 36: c-Myc methylation & vegetable correlation

6.4.5.3 Snacks

Figure 37 illustrates the impact of eating snacks such as crisps and salty sticks but also nuts and seeds on methylation of *c-Myc*. A significant positive correlation was observed (N: 77; Kendall's tau: 0,187; p: 0,032).

A snacking of more than three times a week showed a higher methylation (n: 18; 9,25% ± 1,30) than never or rare (n: 19; 8,63% ± 0,82).

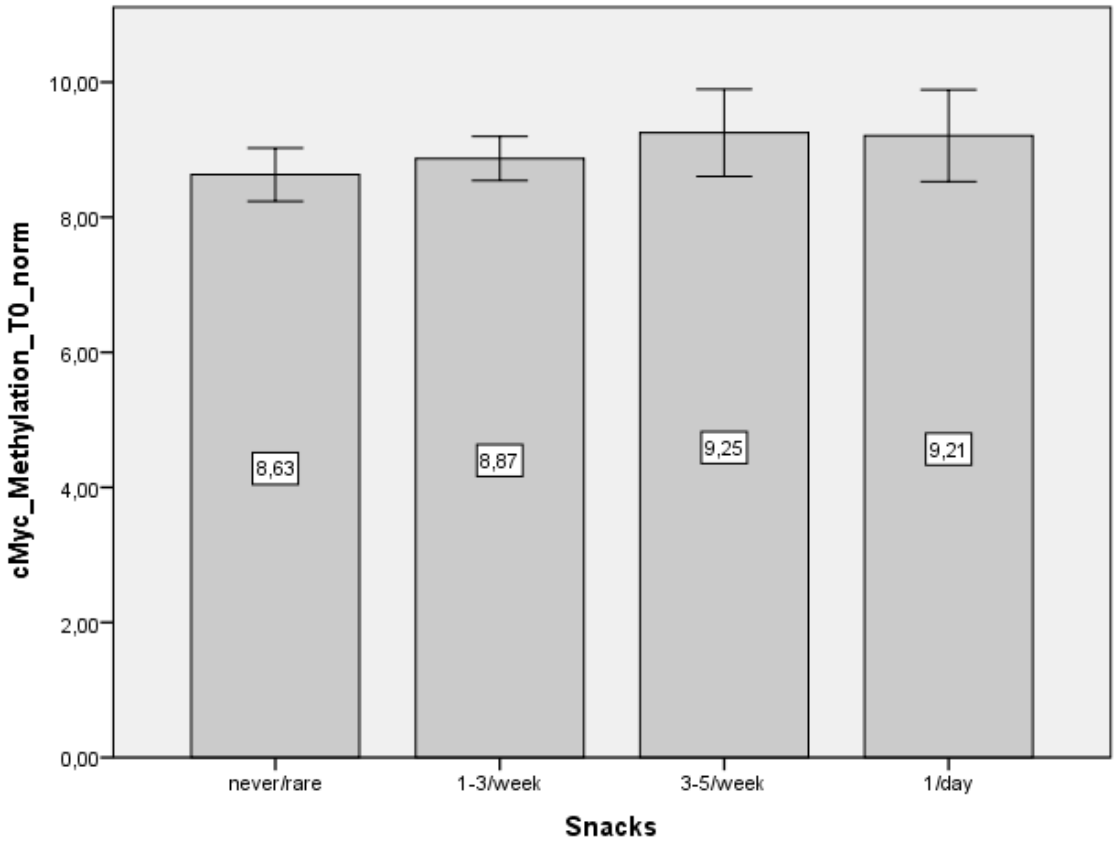

Figure 37: c-Myc methylation & snack correlation

6.5 Results of Telomeres

6.5.1 Correlations and trends

Telomere length	Correlation	
	significant (✓) trend (×)	**variable/methylation**
age	✓	↑/↓
BMI	×	↑/↓
movement	×	↑/↑ (female)
sport	×	↑/↑ (female)
inflammation	×	↑/↓ (female)
coffee	×	lowest consume (female) → max mean
alcohol servings	✓	↑/↓
vegetable	×	↑/↑
dairy	×	↑/↑
leafy vegetable	✓	↑/↑
white flour products	✓	↑/↓
vegetable oil	✓	↑/↑
coconut-/palmoil	✓	↑/↑ (female)

Table 18: Overview about correlations and trends of telomere length and variables of the questionnaire

The trends and significances of telomere length and several variables of the questionnaire are listed in table 18. In the following chapters the results are described in detail.

6.5.2 Gender distribution

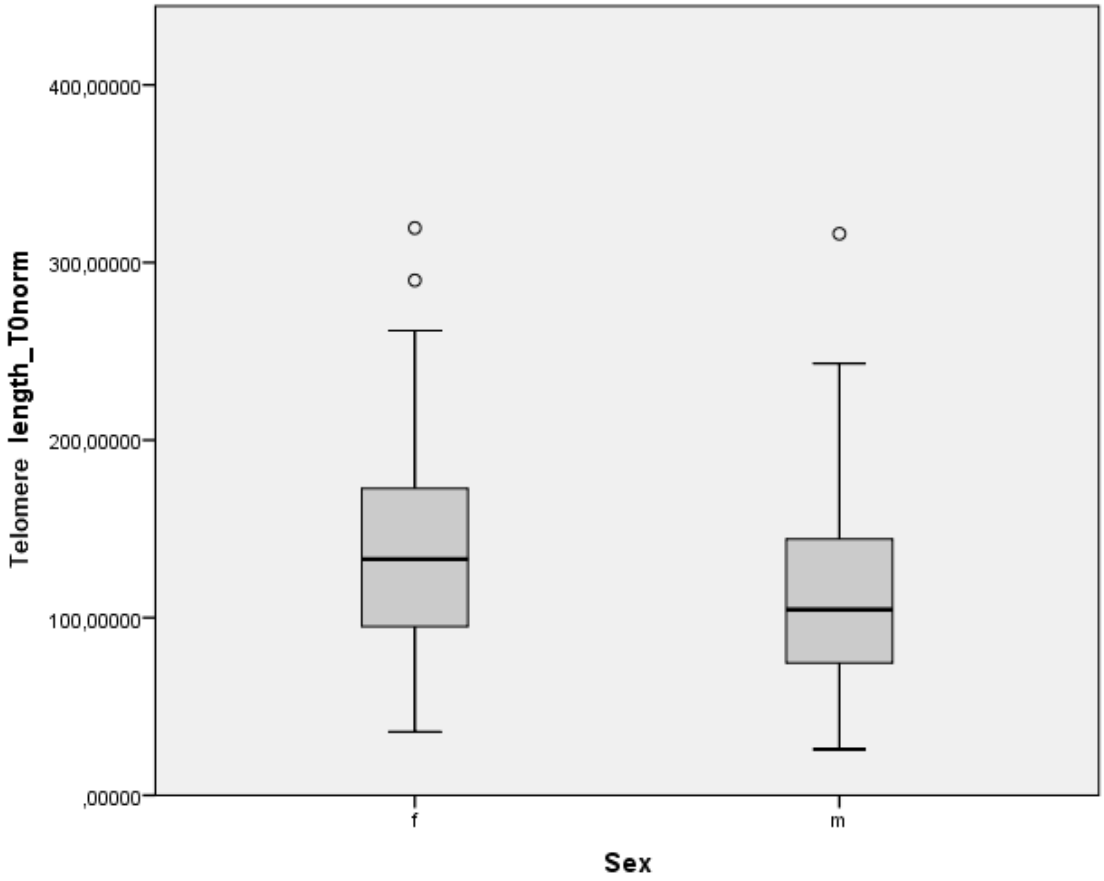

Figure 38: Telomere length & gender distribution (f-female; m-male)

Figure 38 reveals the telomere lengths of 80 participants divided by gender. The telomeric length shown, indicates the absolute telomere length in kilobases per human diploid genome. The mean telomere length of females (n: 54; 140,78kb ± 62,62) was higher than that of men (n: 26; 114,88kb ± 62,96).
A general wide range of telomere length, which stretched from 25,85kb (in men) up to 319,46kb (in women) was detected.

6.5.3 Telomere length & age

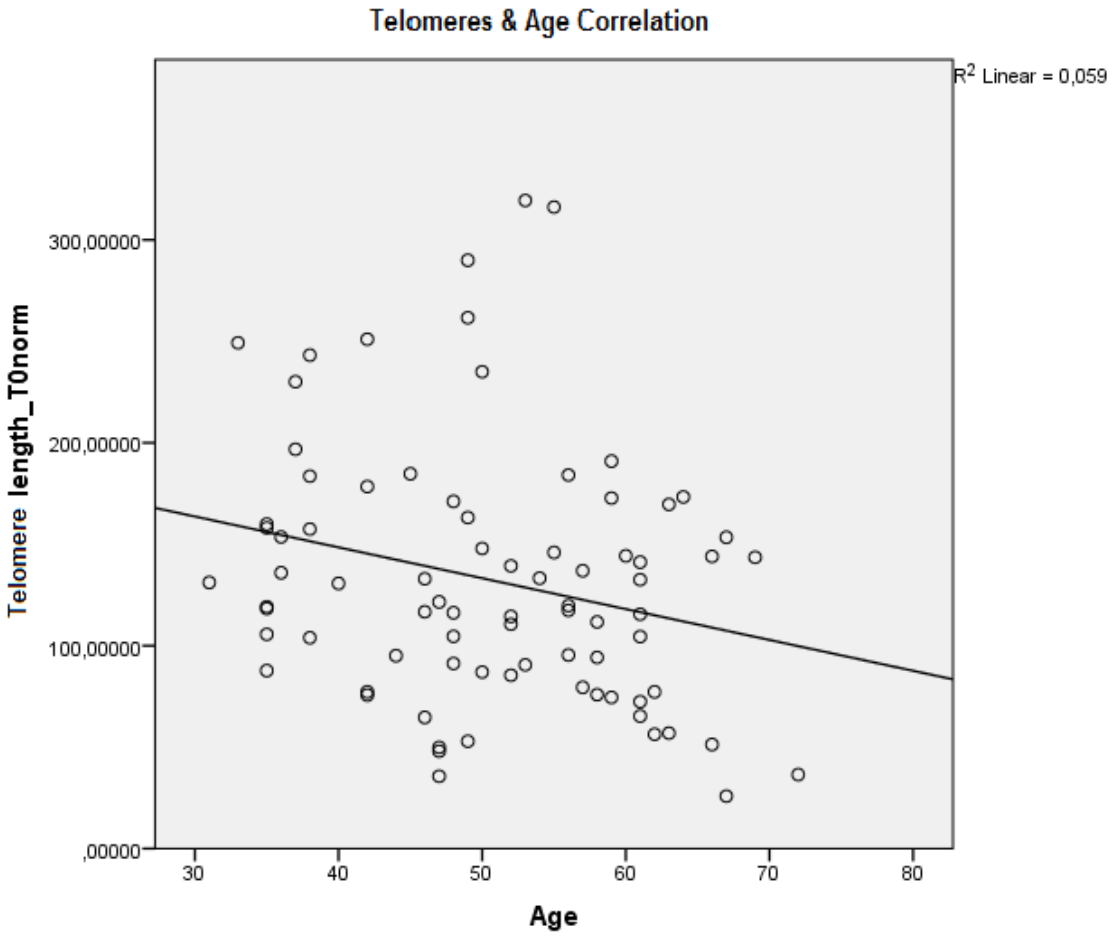

Figure 39: Telomere length & age correlation

Spearman's correlation analysis revealed a significant negative association between telomere length and age (N: 80; Spearman's rho: -0,232; p: 0,038). Figure 39 reveals the decrease of telomere length with increasing age, older participants presented shorter telomeres compared with younger ones.

6.5.4 Telomere length & BMI

Figure 40 shows the relationship between telomere length and BMI. There was a slight negative trend of telomere length with increasing BMI meaning a higher body mass index was linked to shorter telomeres.

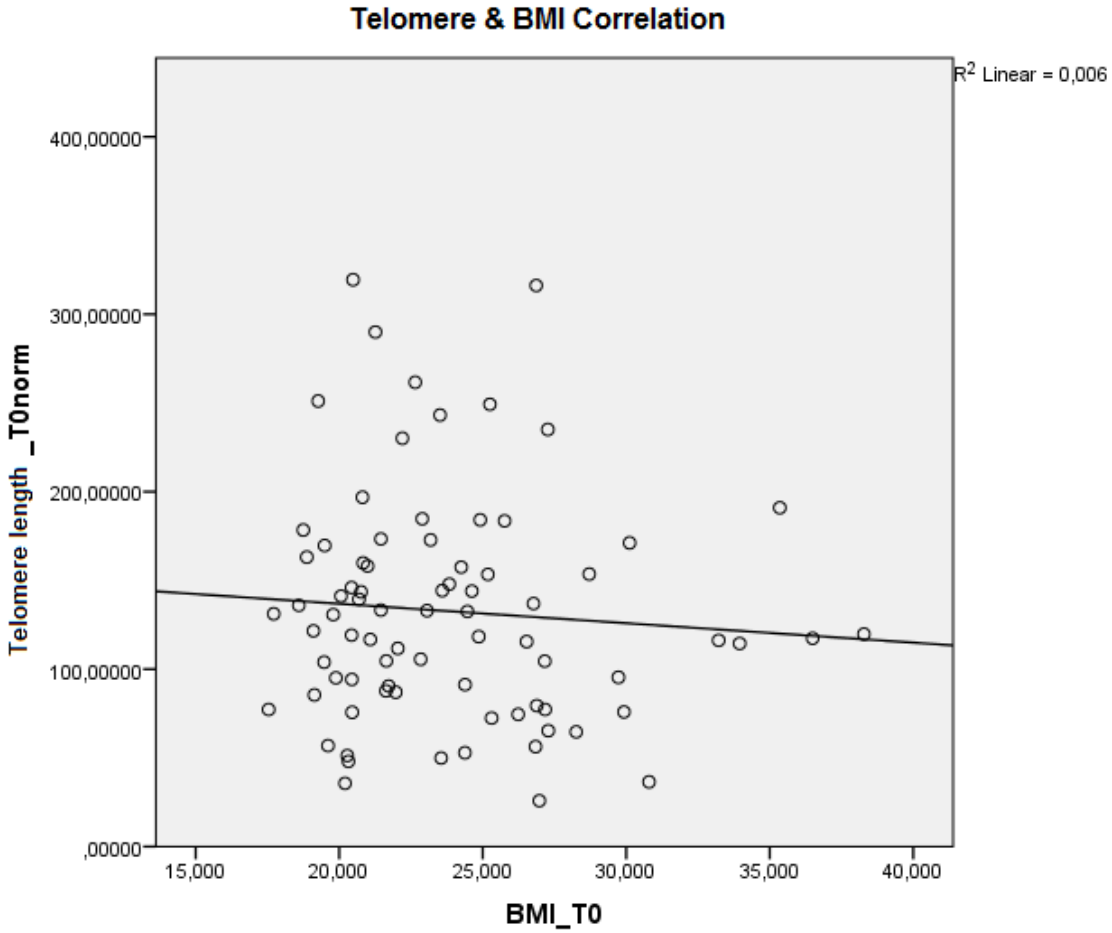

Figure 40: Telomere length & BMI correlation

6.5.5 Telomere length & lifestyle

6.5.5.1 Movement

Analysis showed a positive influence of daily movement on telomere length (figure 41). A more frequent movement resulted in longer telomeres.

6.5.5.2 Sport

A similar output was given considering sports/exercise in the group of women, which can be seen in figure 42. Female participants who had reported never doing sports presented much shorter telomeres (n: 5; 111,84kb ± 56,11) than those exercising a lot (n: 7; 153,45kb ± 67,42).

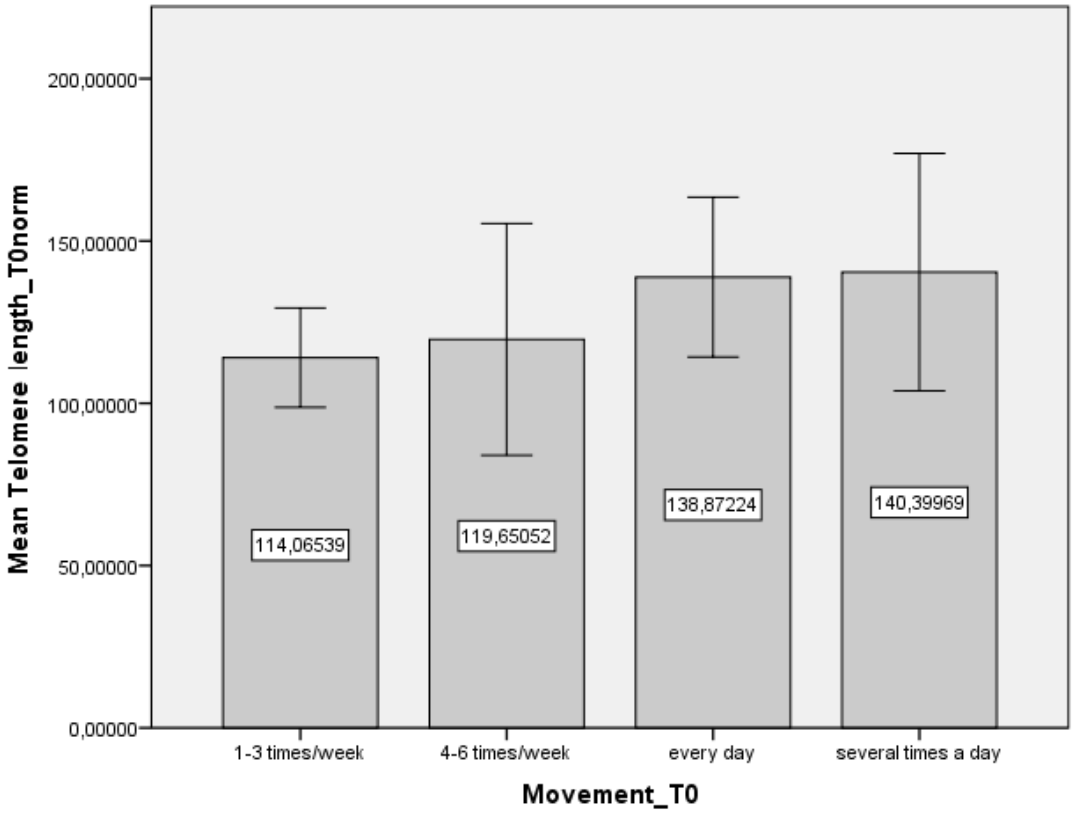

Figure 41: Telomere length & movement correlation

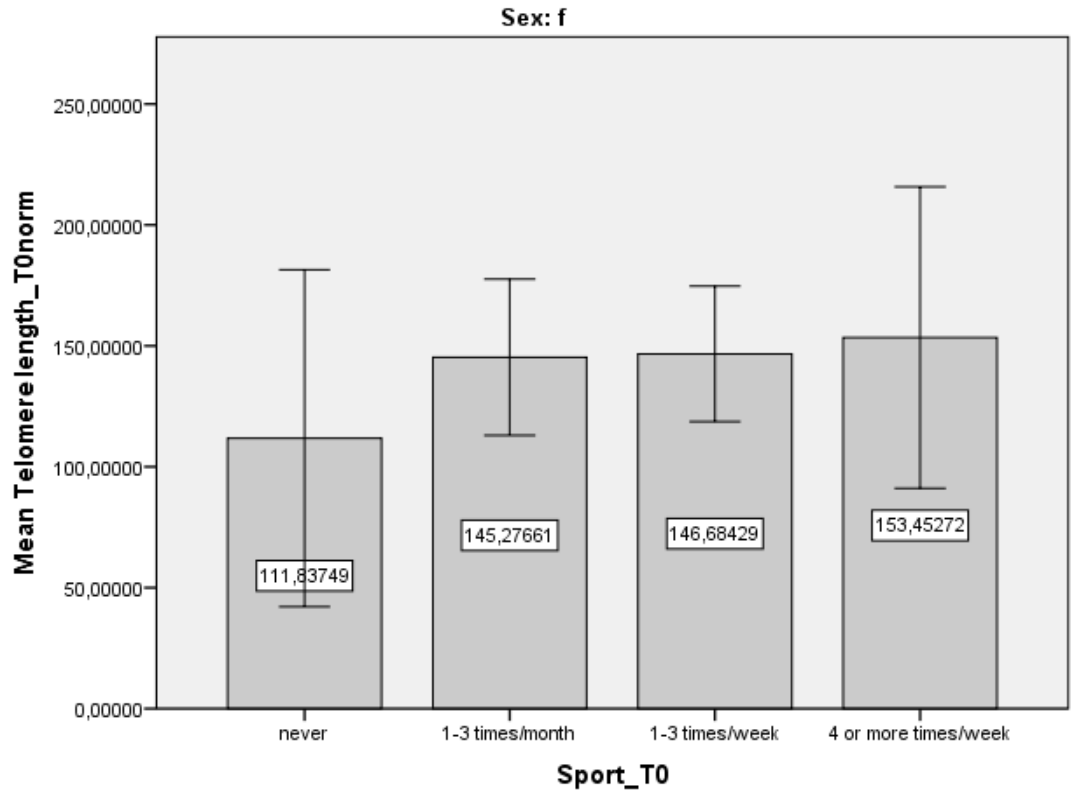

Figure 42: Telomere length & sport correlation within females

6.5.5.3 Inflammations

A negative trend concerning the frequency of inflammations and telomere length was observed in female participants (figure 43). Women who had reported to suffer often from inflammations (n: 4; 124,15kb ± 11,39) also presented lower telomere length compared to those without any inflammations (n: 15; 144,68kb ± 64,89).

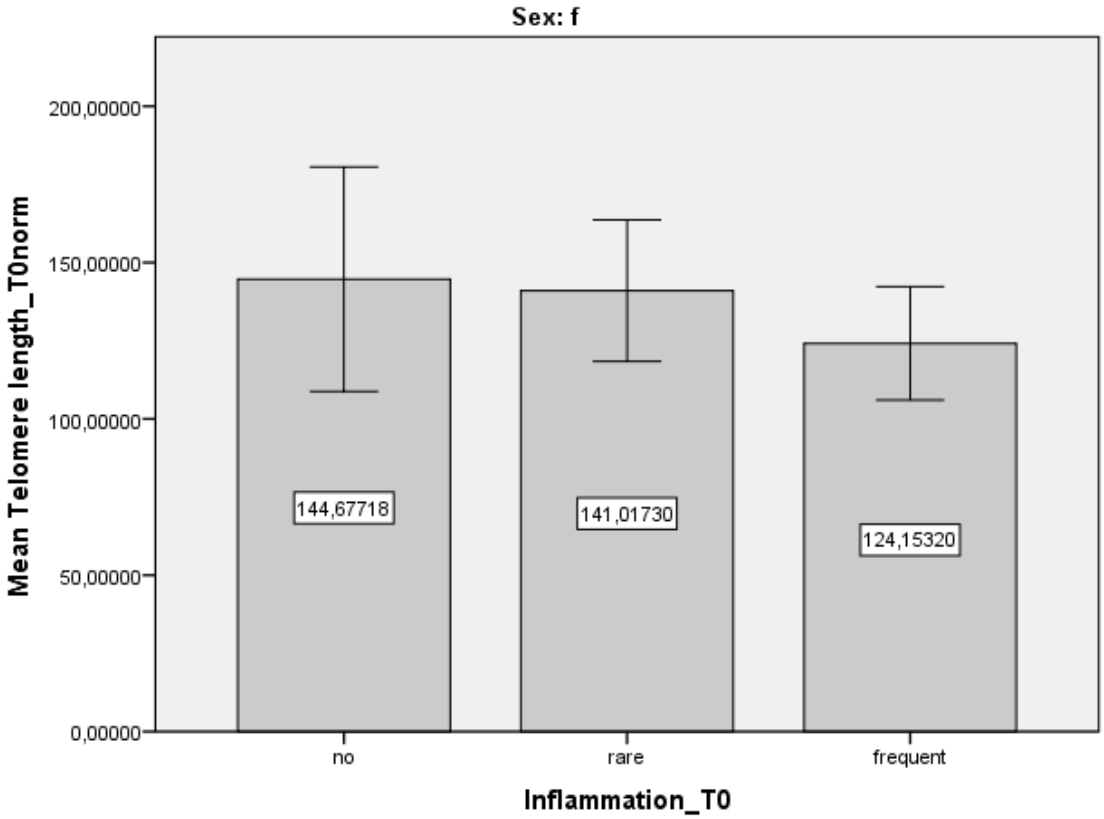

Figure 43: Telomere length & inflammation correlation within women

6.5.6 Telomere length & nutrition

6.5.6.1 Coffee

It was observed that women with an intake of less than one cup of coffee per day had the longest telomeres (n: 12; 157,59kb ± 73,82)

6.5.6.2 Alcohol servings

Figure 44 reveals the negative trend of alcohol servings on the telomere length. It could be shown that a higher alcohol consumption (at least one per day) was linked to shorter telomeres. Participants with 1 or more servings a day had a mean telomere length of 109,65kb ± 65,06 (n: 18) compared to 153,95kb ± 66,97 in participants who reported 1-2 servings per month (n: 14).

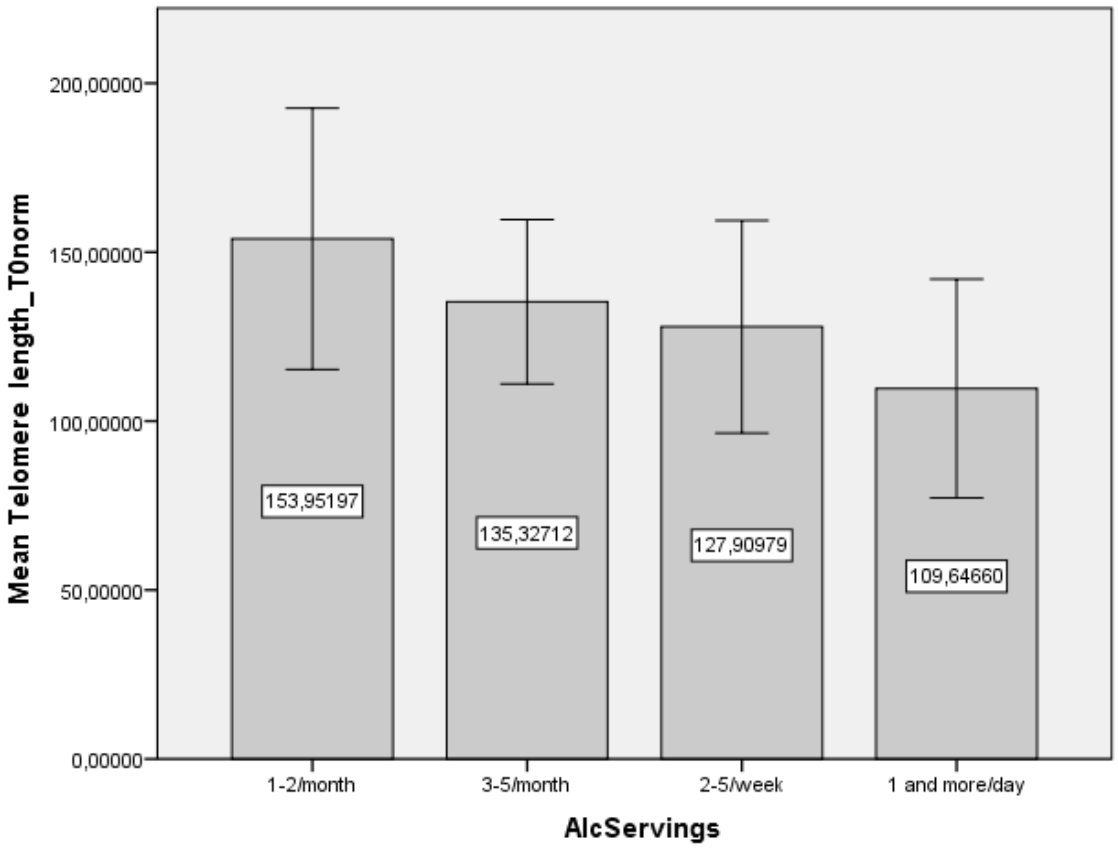

Figure 44: Telomere length & alcohol serving correlation

6.5.6.3 Vegetables

Participants who had reported to eat vegetables once or more a day presented longer telomeres than those who ate less. This trend is illustrated in figure 45.

6.5.6.3.1 Leafy vegetables

As revealed by Spearman's analysis a significant positive correlation between leafy vegetable and length of telomeres (N: 79; Spearman's rho: 0,303; p: 0,007) was found.

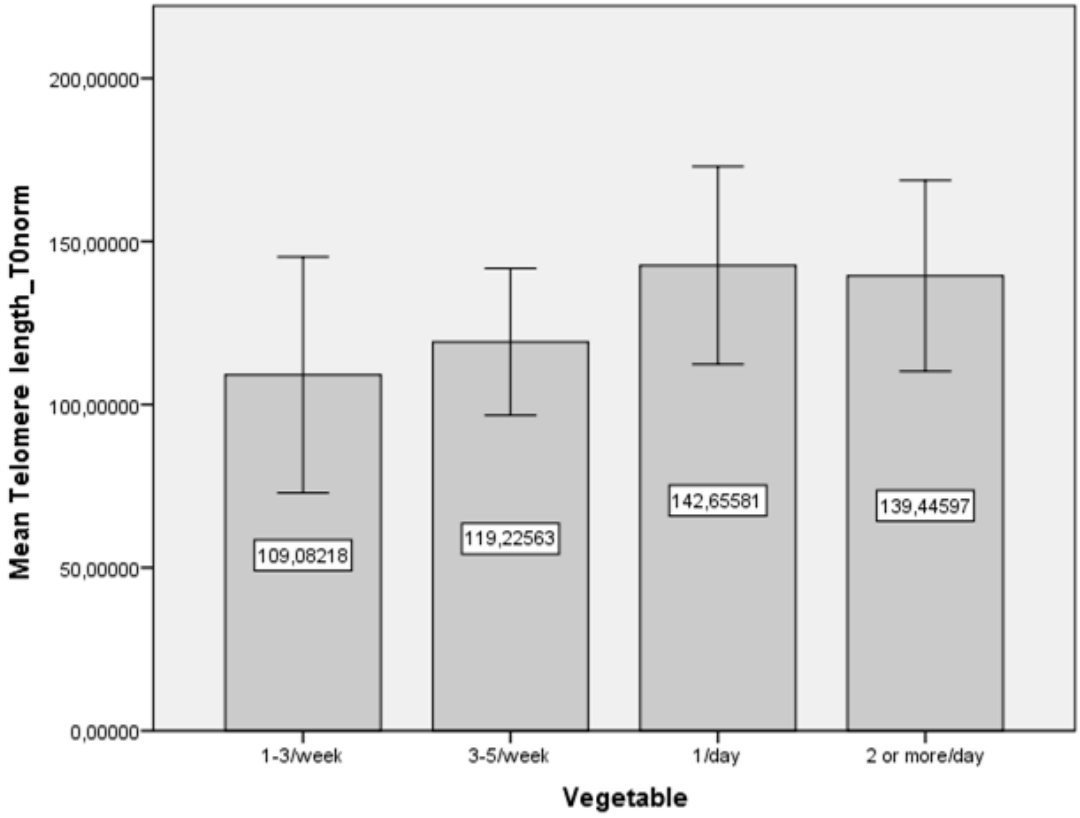

Figure 45: Telomere length & vegetable correlation

6.5.6.4 Dairy

Analysis revealed a raising trend of eating dairies and telomere length. A higher intake of dairy products was associated with longer telomeres (figure 46).

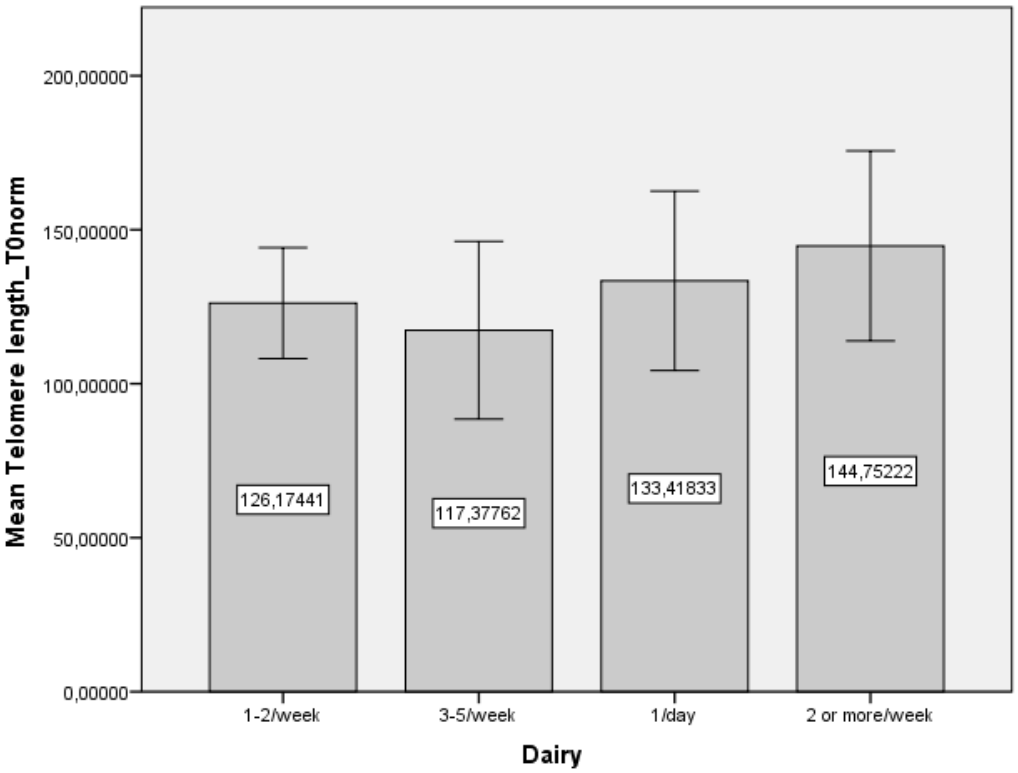

Figure 46: Telomere length & dairy correlation

6.5.6.5 White flour products

Spearman's Correlation analysis showed a significant negative correlation between the frequency of white flour products use and telomere length (N: 74; Spearman's rho: -0,274; p: 0.018).

6.5.6.6 Vegetable oil

There was a significant positive correlation between consumption of vegetable oil and telomere length (N: 78; Spearman's rho: 0,228; p: 0,044). A higher intake of oil was related to longer telomeres.

6.5.6.7 Coconut-/palmoil

Furthermore a significant positive association concerning coconut and/or palmoil was observed in the group of women (n: 52; Spearman's rho: 0,384; p: 0,005).

7 Discussion

7.1 Age & Gender

Ageing is responsible for various alterations in body processes and functions. Investigations also showed a link between ageing and changed DNA methylation of specific genes.

Our study could confirm the outcomes of several researchers (Huang et al. 2015; Freire-Aradas et al. 2016; Bekaert et al. 2015) concerning the negative correlation of *ASPA* methylation and age (N: 81; Spearman's rho: -0,347; p: 0,002). Weidner et al determined to use the methylation levels of *ASPA*, *ITGA2B* and a third age correlated gene (*PDE4C*) to estimate biological age (Weidner et al. 2014).

We could not detect a significant correlation between *ITGA2B* methylation and age, which is discussed controversially in literature (Freire-Aradas et al. 2016; Bekaert et al. 2015). In general *ITGA2B* methylation was higher in women than men, which was also observed by Eipel et al. (Eipel et al. 2016).

As expected we found an inverse relation of telomere length and age (N: 80; Spearman's rho: -0,232; p: 0,038). Telomere length is known to be shortened with each mitotic event, which results in the limitation of the lifespan of somatic cells due to the restricted capacity of replication. Many authors (Hewakapuge et al. 2008; Nordfjäll et al. 2009; Diez Roux et al. 2009) described the telomere shortening during ageing, even more pronounced in men (Wolkowitz et al. 2017; Lapham et al. 2015), which is also seen in our study.

Thomas et al compared age-related changes in telomere length of white blood cells in young (aged 18-26) and old (aged 66-75) participants and found a significant shortening by 21% (Thomas et al. 2008).

7.2 BMI

The body mass index, which reflects the relation of body size and body weight seems to have an influence on the methylation of some genes (Mendelson et al. 2017). In our study methylation of *ITGA2B* showed a tend to be higher in people with an increased BMI, whereas *ASPA* methylation decreased with increasing BMI, however rose with height (n: 27; Spearman's rho: 0,389; p: 0,045). A study, including 336 women, aged 20-48 years dealing with determinants of methylation

biomarkers, revealed similar results. They reported a positive correlation of height and SAM, which is the methyl group donor for DNA methylations. It was also observed that weight was positively associated with higher concentration of SAH, the demethylated form of SAM, which leads to inhibition of the methyltransferases that are responsible for donation process (van Driel et al. 2009).
A higher BMI was also related to shortened telomeres, which is also reported by several other studies (Cui et al. 2013; Lee et al. 2011; Zee et al. 2010). Increased weight and obesity, whose prevalence are increasing globally (Di Cesare et al. 2016), are risk factors for developing disorders such as cardiovascular diseases, arteriosclerosis, hypertension, strokes and Diabetes Mellitus type II (Danaei 2014). Metabolic imbalances in people with heightened BMI can lead to accelerated alteration of telomere length and decreased life expectation. A possible mechanism could be the increased oxidative stress and inflammation caused by obesity (Pou et al. 2007). The result is a persistent unrepaired damage of telomeres, which leads to a shortening of telomeric DNA (Coluzzi et al. 2014).

7.3 Intervention

The administered intervention product contains epigallocatechingallate and other bioactive phytochemicals such as antioxidants and vitamins, which have been previously reported to have several positive effects on health. EGCG, which occurs primarily in green tea and is known as a strong antioxidant (Colon & Nerín 2016) due to its polyphenolic structure, is considered as a modulator of epigenetic processes. Due to experimental trails with different cells it is suggested that this green tea catechin is able to inhibit activity of DNMTs (Meeran et al. 2012; Wong et al. 2011), which can lead to demethylation of genes. This can result in a downregulation of oncogenes and an upregulation of tumour suppressor genes (Nandakumar et al. 2011; Fang et al. 2003), which is jointly responsible for the chemopreventive effect. In our analysis, we could not detect any significant differences concerning the methylation of the target genes (*ASPA, ITGA2B* and *c-Myc*) before and after intervention. A possible explanation for this could be the limited bioavailability of the phytochemicals involved. In addition, most previous studies conducted cell and mouse experiments, so that a transfer of results to humans is not compelling and completely possible.

Furthermore a slight increase of 3,05% in mean telomere length was observed after intervention period. Methylation of *c-Myc* as a main modulator of telomerase activity was not altered after intervention, for this reason we suggest that increase of telomere length was not induced by a heightened telomerase activity.
However, we could not observe a significant correlation between *c-Myc* methylation and telomere length. There are reports about the ability of EGCG to send cells, which are degenerated, in apoptosis (Shankar et al. 2007) or inhibit their telomerase (Berletch et al. 2008). So another approach, to clarify for longer telomeres without targeting telomerase, is that EGCG could induce apoptosis not only in pathologically modified cells, but also in senescent cells, similar to certain drugs that show evidence of the ability to selectively kill senescent cells (Zhu et al. 2015). This would result in an altered cell population with “renewed” cells representing longer telomeres. Besides, the method by which telomeric length was determined, measures an average telomere length of all cells and does not differentiate according to cell types. Therefore, a higher number of young cells result in an average increase in telomere length.

7.4 Nutrition

A balanced and varied diet is essential for functions and processes of the body. Additionally nutrition has an important impact on epigenetic mechanisms such as DNA methylations, which influence the regulation of genes.

7.4.1 Coffee

Coffee, a caffeine-containing drink is known for its range of beneficial ingredients such as chlorogenic acid, a strong antioxidant, caffeine, which has evidence to reduce cognitive declines and neurodegenerative diseases in human and animals (Ritchie et al. 2007; Ullah et al. 2015) and N-methylpyridinium with antithrombotic properties (Kalaska et al. 2014).
In literature an association between consumption of coffee and DNA methylation (Chuang et al. 2017) was revealed. In our study people with a higher intake of coffee presented an increased methylation of *ASPA* (N: 79; Kendall’s tau: 0,178; p:0,043), although a study of Lee at al., observing effects of coffee polyphenols, showed a decreased in vitro methylation due to an inhibition of *DNMT1* caused by an increased level of SAH (Lee & Zhu 2006). A possible reason for this

controversial result could be due to the in vitro approach with isolated coffee substances, without attention on the whole coffee matrix and interaction of the various ingredients of coffee.
An increase associated with coffee consumption and increased telomere length could not be observed in our study, despite the result that was presented by Liu et al in their study including 4780 women of the Nurses' Health Study, who found significantly longer telomeres linearly associated with an higher intake of coffee (Liu et al. 2016). This might be explained by the antioxidative effect of the coffee-containing polyphenols, which leads to a prevention of damage of telomeric DNA due to oxidative stress.

7.4.2 Vegetables

Due to the large number of bioactive secondary plant contents and vitamins as well as dietary fibres, the sufficient intake of vegetables is essential for a balanced diet. Especially leafy vegetables, including kale, cabbage, spinach, chicory, are known for their beneficial effects (Colonna et al. 2016).
We found a significant positive correlation between leafy vegetable consumption and methylation of *ASPA* (n: 53; Spearman's rho: 0,455; p: 0,001). This result could be due to the folic acid contained in green leafy vegetables, which can influence one carbon metabolism. Folate serves as a methyl donor (figure 2) in the transformation of homocysteine into methionine, followed by the conversion into SAM, the key substance for DNA methylation. A dietary deficiency can lead to alterations in DNA methylation.
Moreover a higher intake of leafy vegetables was also linked to longer telomeres (N: 79; Spearman's rho: 0,303; p: 0,007). Carotenoids are known for their antioxidative potential and occur in leafy vegetables in adequate amounts. In a study of Min et al, including 3660 participants aged 20 years and older, it was reported about the observation of increased telomere length in persons with a higher serum carotenoid level by the inhibition of accelerated telomere shortening caused by oxidative stress (Min & Min 2016).

7.4.3 Alcohol

An irresponsive handling of alcohol is associated with diseases and the risk of early death (Rehm et al. 2009). Alcohol abuse may influence regulation of

telomere length. We observed a shortening trend in participants who had reported to drink alcoholic drinks more often. Also Pavanello et al. reported about this effect of alcohol. In this study 457 Italian men were conducted. They found an association between drastically decreased telomere length and increasing alcohol servings per day. It is suggested that alcohol abuse may lead to an increased occurring of oxidative stress resulting in telomeric damaging such as DNA double-strand breaks. (Pavanello et al. 2011)

7.4.4 White flour products

Products made from white flour, such as bread, rolls, pizza, pasta or biscuits, , in contrast to their healthy counterpart made from whole grain, contain much less non digestible polysaccharides, vitamins and minerals. Products made from whole grain are associated with beneficial effects, such as longer saturation, prevention of diarrhoea and constipation or even with decreased total cholesterol and LDL cholesterol (Newby et al. 2007).

Intake of white flour was inversely correlated with telomere length in our study (N: 74; Spearman's rho: -0,274; p: 0.018). In a cross-sectional study including 287 Spanish children and adolescents was found that a higher consumption of white bread negatively influences the length of the telomeres by increased oxidative stress (García-Calzón et al. 2015).

7.4.5 Vegetable oils

Vegetable oil is usually obtained from fruits and seeds and is a good source for polyunsaturated fatty acids, which are known for their cholesterol lowering and anti-inflammatory effect. García-Calzón et al observed a direct correlation between intake of polyunsaturated fatty acids and telomere length (García-Calzón et al. 2015). Moreover oil of vegetables contains antioxidants such as tocopherols and tocotrienols, oryzanols (occurring in rice bran oil) and lignans (in sesame oil) (Dhavamani et al. 2014).

We determined a positive association between frequency of oil intake and telomere length (N: 78; Spearman's rho: 0,228; p: 0,044), which could be explained by the antioxidative effect of vegetable oil ingredients having a preventive effect on oxidative stress, which might otherwise favor a telomeric shortening.

7.5 Lifestyle

Besides a balanced nutrition a healthy lifestyle is characterised by a regular physical activity, an adequate stress level and health care to prevent diseases. Taken together this offers several benefits for the well-being (Prendergast et al. 2016).
Due to this fact, we tried to find connections between our genes of interest as well as telomere length and variables of lifestyle.
For *ASPA* methylation we could not find any results concerning correlations with lifestyle parameters.

7.5.1 Physical activity / sport / fitness

Voisin et al. reviewed 25 papers, revealing an impact of exercising on DNA methylation in a tissue- and gene-specific as well as intensity-dependent manner. The alterations of DNA methylation occurred in metabolic and inflammatory genes as well as in genes of muscle growth and haematopoiesis (Voisin et al. 2015). Our investigation presented a higher methylation of *ITGA2B* in women, who had reported a higher frequency of movement (n: 51; Kendall's tau: 0,238; p: 0,025).

Physical activity seems to influence DNA methylations in a beneficial manner, especially in patients with cancer. A study including female patients with breast cancer, examined after a 6 month long exercising training intervention, detectable changes of methylation in 43 genes in peripheral blood leukocytes. Within these a demethylation of a tumour suppressor gene was observed, which may lead to an inhibition of cancer progression due to an increased expression (Zeng et al. 2012).

Also a significantly higher methylation of proto-oncogene *c-Myc* was observed in persons who had specified to exercise more often (n:55; Kendall's tau: 0,204; p: 0,05). As mentioned earlier, increased *c-Myc* expression can lead to a stimulation of TERT expression, which in turn has resulted in increased telomerase activity as well as longer telomeres.
We could not find a correlation between *c-Myc* methylation and length of telomeres but in our study also telomere length could be improved with increasing exercising, which was also found by other authors (Kingma et al. 2012; Puterman

et al. 2010). It is suggested that moderate exercising is improving the REDOX balance resulting in decreased inflammatory events (Nimmo et al. 2013; Campos et al. 2014).
Also a different mechanism could be another possible explanation for the longer telomeres. Thus, a study (Shlush et al. 2011) found that the increased telomeric length in divers, doing an intensive physical activity, measured in blood, not only occurred due to increased telomerase activity, but also to a repopulation of undifferentiated hematopoietic cells that had longer telomeres.
Another study observed a positive connection between the plasma level of irisin, a hormone, which is produced by exercising skeletal muscles and longer telomeres in 81 healthy, middle-aged participants (Rana et al. 2014).

7.5.2 Stress and inflammations

Stress occurs in all societal strata and ages and can be caused by a variety of triggers such as family-, professional- but also leisure-related stress. At elevated stress levels, certain proteins can be activated, which in turn can stimulate inflammatory processes due to an increased expression of proinflammatory transcription factors and cytokines (Nikkheslat et al. 2015). Tawakol et al. observed a positive association between increased activity of amygdala triggered due to stress and atherosclerotic inflammation (Tawakol et al. 2017). Furthermore it is suggested that a release of hormone cortisol, which is a response of stress, can lead to an inhibition of telomerase activity in T-lymphocytes due to an decreased expression of hTERT resulting in a stress induced alteration of telomere length (Choi et al. 2008).
In our study we observed a trend to an inverse correlation of stress and *c-Myc* promoter methylation. On the basis of the investigation of Epel et al., studying the influence of life stress on telomere length, a shortening of the telomeres could be assumed due to an increase of oxidative stress (Epel et al. 2004). In literature there are even associations between stressful situations in childhood and decreased telomere length (Osler et al. 2016; Verhoeven et al. 2015). In our study we could not find a significant correlation between telomere length and stress.
However, female participants, who had reported a frequent inflammation rate, presented shortened telomeres. A similar outcome was investigated by

Vazirpanah et al., who had detected that patients suffering of gout had shorter telomeres than healthy persons due to an increased cellular senescence (Vazirpanah et al. 2017).

8 Conclusion

A balanced diet and healthy lifestyle considering smoking status, stress level and physical activity, play an important role for wellbeing and “healthy” ageing. These factors trigger interindividual differences between biological and chronological age and associated age-related disorders that are closely linked to genetic and epigenetic alterations. Therefore, age-related biomarkers detecting early alterations in the epigenomic landscape could play an important role in the diagnosis or even prevention of age-associated disorders.

DNA methylation of *ASPA* and telomere length have the potential to determine the state of ageing in blood due to their shown correlation with age.

Furthermore telomeres could serve as indicator for a person’s lifestyle, since longer telomeres were correlated with healthy habits including regular physical activity, reduced alcohol intake, certain dietary patterns including reduced white flour products, more vegetables as well as consumption of vegetable oils.

We could not find any significant correlation between telomere length and promoter methylation of telomerase transcription factor *c-Myc* despite expectation, hence further analyses should also include measurement of c-Myc expression to evaluate a possible effect on the telomere length of participants.

Furthermore, the investigation of the influence of nutritional and lifestyle parameters and the expression of the other analysed genes would be scientifically relevant. Since the study was limited in this respect due to the dried blood spots, this would be an important approach for further studies using whole blood.

Intervention showed a positive effect on telomere length, however significant results could not be found due to high interindividual variations and a rather heterogenous study group. A greater number of participants with similar lifestyle and dietary patterns could verify positive effects.

9 Appendix

9.1 DNA-Extraction

Protocol: DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit)

- Cut off 1-2 dried blood spots (depending on the amount of blood on it) and place into a 1.5 ml microcentrifuge tube
- + 180 µl of Buffer ATL
- Incubation at 85°C (10 min); shortly centrifuge
- + 20 µl proteinase K stock solution; vortex;
- incubation at 56°C (1h); shortly centrifuge
- + 200 µl AL; vortex
- Incubation at 70°C (10 min); shortly centrifuge
- + 200 µl ethanol (96–100%); vortex; shortly centrifuge
- transfer mix to QIAamp Mini spin column; centrifuge (8000 rpm – 1 min)
- put QIAamp Mini spin column onto a new 2 ml collection tube; remove the one containing the filtrate
- + 500 µl Buffer AW1; centrifuge (8000rpm – 1min)
- put QIAamp Mini spin column onto a new 2 ml collection tube; remove the one containing the filtrate
- + 500 µl Buffer AW2; centrifuge at full speed (3 min)
- (Suggestion: put QIAamp Mini spin column onto a new 2 ml collection tube; remove the one containing the filtrate; centrifuge at full speed (1 min))
- put QIAamp Mini spin column onto a new 1,5 ml microcentrifuge tube
- + 150 µl Buffer AE
- incubation at room temperature (1 min)
- centrifuge (8000 rpm -1 min)
- store samples at -20°C

9.2 DNA-Bisulfite Conversion

Protocol: Sodium Bisulfite Conversion of unmethylated Cytosines in DNA

(EpiTect Bisulfite Kit)

➔ first step: Bisulfite DNA conversion

- + 800 µl NFW to each Bisulfite Mix (1 Mix is necessary for 8 conversion reactions); vortex
- Prepare a mix of Bisulfite Mix (85 µl per sample) and DNA Protect Buffer (35 µl per sample); vortex
- pipette 120 µl per sample of this mix into 200 µl PCR tubes
- + 20 µl DNA sample each tube; mix through using a pipette
- close the tubes; shortly centrifuge
- use thermal cycler program according to table 15

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Incubation	175 min (2 h 55 min)	60°C
Hold	Indefinite†	20°C

† Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Table 19: Thermal cycler program for bisulfite conversion

- after performance samples can stay in PCR thermal cycler overnight

➔ 2nd step: Clean-up

- dissolve carrier RNA: + 310 µl NFW to carrier RNA
- mix Buffer BL and carrier RNA depending on sample numbers in a falcon tube according to table 8

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	620 µl	2.5 ml	5 ml	10 ml	15 ml	31 ml
Volume of carrier RNA solution†	6.2 µl	25 µl	50 µl	100 µl	150 µl	310 µl

* The volumes given contain a 10% surplus for pipetting inaccuracies.

† Resulting in a final concentration of 10 µg/ml carrier RNA in Buffer BL.

Table 20: Carrier RNA and BL Buffer volumes

- pipette 560 µl for each sample of the BL/RNA mix (table 16) into 1,9 ml microcentrifuge tubes
- shortly centrifuge the PCR tubes containing converted DNA
- transfer each bisulfite mix completely from PCR tubes into the prepared 1,9 ml microcentrifuge tubes; vortex; shortly centrifuge
- transfer each of these mixtures into EpiTect spin columns
- centrifuge spin columns (maximum speed – 1min)
- pour out filtrate from the collection tubes, place spin columns back into collection tubes
- + 500 µl BW Buffer to each spin column; centrifuge (max speed – 1 min)
- pour out filtrate from the collection tubes, place spin columns back into collection tubes
- + 500 µl BD Buffer to each spin column; incubate (room temperature – 15 min); centrifuge (max speed – 1 min)
- pour out filtrate from the collection tubes, place spin columns back into collection tubes
- + 500 µl BW Buffer to each spin column; centrifuge (max speed – 1 min)
- pour out filtrate from the collection tubes, place spin columns back into collection tubes
- + 500 µl BW Buffer to each spin column; centrifuge (max speed – 1 min)
- remove collection tubes, place spin columns into new collection tubes
- centrifuge (max speed – 1 min)

- (Suggested: put spin columns into new microcentrifuge tubes, open the lids, incubate (56°C – 5min))
- + 20 µl EB Buffer into the middle of membrane; centrifuge (12000 rpm – 1min)
- store samples at -20°C

9.3 Food frequency and lifestyle questionnaire

1. Persönliche Angaben

Alter:	Geschlecht:
Gewicht in kg:	Größe in cm:

2. Lifestyle

Wie oft nehmen Sie Sonnenbäder im Solarium und/oder im Freien?					
	nie	**1-2x/ Monat**	**1-3x/ Woche**	**4-6x/ Woche**	**täglich**
ganzjährig					
saisonal					

Fragen zum Rauchverhalten		✓		✓		✓
Rauchen Sie?	ja		nein			
Haben Sie einmal geraucht?	ja		nein			
Seit wann rauchen Sie nicht mehr?	< 1Jahr		1-5 Jahre		> 5 Jahre	
Sind Sie Passivrauch ausgesetzt?	selten		oft		ständig	

Fragen zu Ihrem Bewegungsverhalten						
	Mehrmals/ Tag	**Täglich**	**4-6x/ Woche**	**1-3x/ Woche**	**1-3x/ Monat**	**nie**
Machen Sie regelmäßig körperliche Bewegung?						

Betreiben Sie regelmäßig Sport im Freien (< 30min mit Schwitzen)? z.B. Wandern, Skifahren, Laufen, Radfahren...						
Betreiben Sie regelmäßig Sport im Fitness-Studio (< 30 min mit Schwitzen)?						

3. Fragen zu Ihren Stressbelastungen

Wie hoch würden Sie Ihre derzeitige Stressbelastung einschätzen?	✓
Null	
Gering	
Mäßig	
Hoch	
Sehr hoch	

Versuchen Sie den ursächlichen Anteil bei der Entstehung Ihres Stressproblems in Prozent zu schätzen (z.B. Arbeit 45%, Freizeit 20%, Familie 35%)	
Arbeit	……%
Freizeit	……%
Familie/Partner	……%

4. Fragen zu Ihrer Gesundheit

Haben Sie altersbedingte Beschwerden?	✓
nein	
zu starke Hautalterung	
zu viele graue Haare	
Antriebs- und Motivationsmangel	
schnelle Ermüdbarkeit	
Muskel- und Knochenschmerzen	
Gedächtnis- und Konzentrationsstörungen	
Sonstiges: _____	

Leiden Sie oft an Entzündungen und/oder Infektionen? z.B.: Hautentzündungen, Pilzinfektionen, Entzündungen im Mundraum/Auge, grippale Infekte, Magen-Darminfekte, Entzündliche Erkrankungen im Magen-/Darmbereich ...	✓
Nie	
Selten	
Häufig	
Sehr häufig	

Neben Sie derzeit Medikamente zu sich?	✓
Ja, folgende:	
nein	

Leiden Sie derzeit an einer systemischen Erkrankung?	✓
Diabetes mellitus Typ 1 oder Typ 2	
Schilddrüsenerkrankung	
Bluthochdruck	
Sonstige:	

5. *Fragen zu Ihren Ernährungsgewohnheiten*

	Selten/ nie	1-3x/ Woche	3-5x/ Woche	1x/ Tag	2-3x/ Tag	>3x/ Tag
Milch- und Milchprodukte						
Weich- und Hartkäse	Portionen pro Woche					
Milch, Joghurt, Molke, Buttermilch	Portionen pro Woche					
Rahmprodukte: Süß- und Sauerrahm, Sahne/Obers, Creme Fraiche, Schmand	Portionen pro Woche					
Topfen, Frischkäse, Hüttenkäse	Portionen pro Woche					
Eier						
Eier	Portionen pro Woche					
Fleisch						
Rind, Schwein, Lamm, Kalb, Schaf	Portionen pro Woche					
Pute, Huhn	Portionen pro Woche					

Fleisch- und Wurstprodukte						
Wurstaufschnitt, Salami	Portionen pro Woche					
Grill-, Brat-, Kochwürste	Portionen pro Woche					
Sonstiges	Portionen pro Woche					

	Selten/ nie	1-3x/ Woche	3-5x/ Woche	1x/ Tag	2-3x/ Tag	>3x/ Tag
Gemüse						
Kartoffel	Portionen pro Woche					
Hülsenfrüchte: Erbsen, Bohnen, Sojabohnen, Kichererbsen	Portionen pro Woche					
Zwiebelgemüse: Lauch, Zwiebel, Knoblauch, …	Portionen pro Woche					
grünes Blattgemüse, Kohlgemüse und Salat	Portionen pro Woche					
Sonstiges: Tomaten, Paprika, Zucchini, Karotten, etc.	Portionen pro Woche					
Obst						
Frischobst	Portionen pro Woche					
Trockenobst	Portionen pro Woche					
Fisch						
Fischfilets frisch oder tiefgefroren, ganz oder Block	Portionen pro Woche					
Verarbeiteter Fisch: Panierter Fisch, Dosenfisch	Portionen pro Woche					

	Selten/ nie	1-3x/ Woche	3-5x/ Woche	1x/ Tag	2-3x/ Tag	>3x/ Tag
Getreideprodukte Brot, Teigwaren, etc.						
Voll- oder Mehrkornprodukte				Portionen pro Woche		
Weißmehlprodukte				Portionen pro Woche		
Süßigkeiten						
Süßigkeiten (Schokolade, Keks, Fruchtgummi…)				Portionen pro Woche		
Mehlspeisen				Portionen pro Woche		
Süße Brotaufstriche: Nutella, Marmelade, etc.				Portionen pro Woche		
Knabbereien						
Salzgebäck (Chips, Soletti…)				Portionen pro Woche		
Nüsse und Samen				Portionen pro Woche		
Öle und Fette						
Tierische Fette: Schmalz, Butter				Portionen pro Woche (1 Portion Öl/Fett entspricht 1 Teelöffel)		
Pflanzliche Fette: Olivenöl Kernöl, Rapsöl, Maiskeimöl, Nussöle, …				Portionen pro Woche (1 Portion Öl/Fett entspricht 1 Teelöffel)		
Kokosfett und Palmöl				Portionen pro Woche (1 Portion Öl/Fett entspricht 1 Teelöffel)		

6. Fragen zu Nahrungsergänzungsmittel

Nehmen Sie Nahrungsergänzungsmittel zu sich? Bitte auch ankreuzen, wenn keine regelmäßige Einnahme **Nahrungsergänzungsmittel:** Nährstoffe in konzentrierter Form, Vitamin-tabletten, Mineralstoffe, Knoblauchkapseln, probiotische Kapseln, …	✓
ja, folgende:	
nein	

7. Fragen zu Ihren Trinkgewohnheiten

		✓		✓		✓		✓
Wie viel Flüssigkeit nehmen Sie täglich zu sich?	< 1 Liter		1-2 Liter		2-3 Liter		>3 Liter	
Wie viele Tassen Kaffee trinken Sie täglich?	< 1 Tasse		1-2 Tassen		3-5 Tassen		> 5 Tassen	
Wie oft trinken Sie Alkohol?	nie		nur zu Anlässe n		1-2/ Monat		2-5/ Monat	
	1-2/ Woche		täglich		mehr-mals/ Tag			
Wie viele Portionen Alkohol trinken Sie? 1 Portion entspricht hierbei 1/3 l Bier, 1/8l Wein/Schaumweine, 1 Schnapsglas (2cl) >15% Alkohol	1-2/ Monat		3-4/ Monat		1-2/ Woche		3-5/ Woche	
	1-2 / Tag		≥3/ Tag					

Welche Getränke nehmen Sie hauptsächlich zu sich? Mehrfachnennungen möglich	✓		✓		✓
Wasser		Kaffee		Früchtetee	
Kräutertee		Schwarztee		Grüntee	
Dicksaft		Limonaden		Energiegetränke	
Verdünnsäfte		Nektar		Frische Säfte	
Sonstiges:					

Wie viele Tassen Grüntee trinken Sie?											
Unregel-mäßig		1-2 Tassen/ Woche		2-5 Tassen/ Woche		1-2 Tassen/ Tag		3-5 Tassen/ Tag		>5 T./ Tag	

9.4 Composition of intervention preparation

The preparation for the intervention contains following ingredients:

Capsule for the day:

- extracts of green tea, barley grass, wheatgrass
- vitamins such as B1 (thiamine), B2 (riboflavin), B3 (nicotinic acid), B6 (pyridoxine), B7 (biotin), B12 (cobalamin), C, D3 (cholecalciferol), K
- selen, zinc, magnesium

Capsule for the night:

- extracts of algae, grapeseed, wheatgrass, tagetes, tomatoes, shiitake

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Research Article OMICS International

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

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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®

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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/m²)	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.

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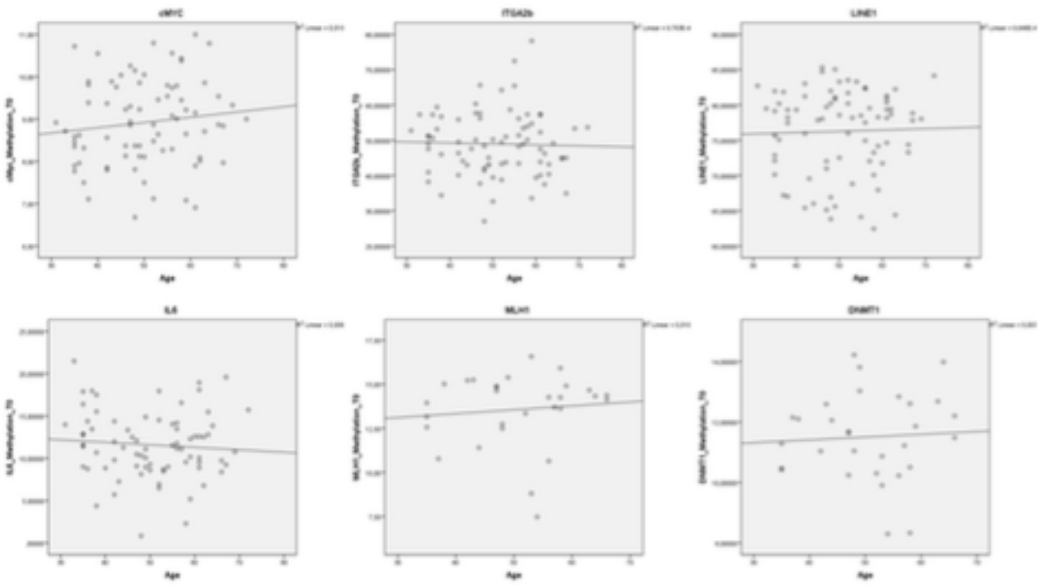

Scatter-plots display age correlations of promoter DNA methylation of MLH1, DNMT1, ITGA2B, LINE-1, IL6, c-Myc before administration (T0) as analysed by linear regression analysis.

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

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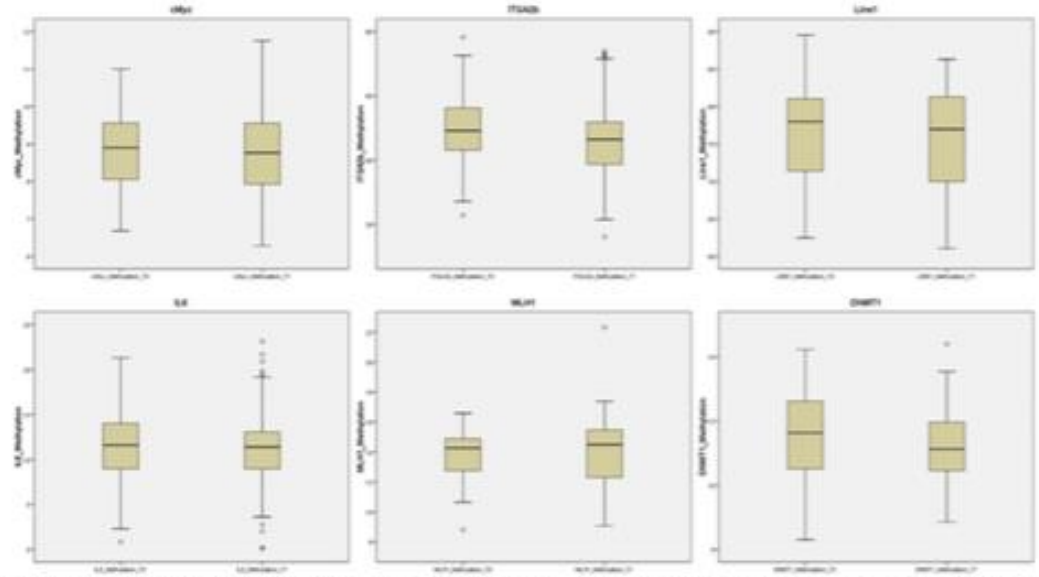
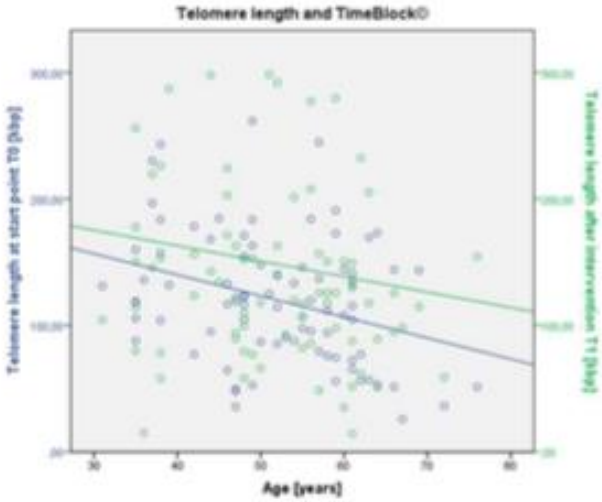


Comparison of mean promoter DNA methylation levels of MLH1, DNMT1, ITGA2B, LINE-1, IL6, c-Myc before (T0) and after (T1) 6 months of administration of plant based dietary supplement Timeblock.

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.

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		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

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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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