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„Epigenetic methylation of *LINE-1* and *sirtuins* as markers of health and nutrition"

verfasst von / submitted by

Magdalena Klimesch, BSc

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ADP	Adenosine diphosphate
BMI	Body mass index
bp	Base pair
CpG	Cytosine-phosphatidyl-guanine
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxy nucleoside triphosphate
ELOVL2	Elongation of very long-chain fatty acids-2
HPLC	High-performance liquid chromatography
HRM	High-resolution melt
IL-6	Interleukin 6
LINE-1	Long interspersed nuclear element 1
miRNA	microRNA
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
ncRNA	Non-coding RNA
NFW	Nuclease free water
ORF	Open reading frame
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
SAM	*S*-Adenosyl-L-Methionine
SIRT6	Sirtuin 6

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V. Summary

Background: Retarding or reversing the biological effects of aging is an ageless dream of the human population. Our metabolism constantly causes damage in our organism. This damage accumulates and eventually causes pathology. Therefore, aging is considered as the main risk factor for diseases. Until now scientists pursued intensive research on this topic. It is broadly acknowledged that lifestyle and diet play a crucial role in the aging process. Research in the field of epigenetics tries to clarify specific mechanisms in this multifactorial, complex process.

Objectives: To gain a better understanding of how lifestyle and diet have an impact on aging and age-related diseases it is necessary to have reliable markers of aging. The aim of this this master thesis was to establish three epigenetic biomarkers that are linked to aging and to figure out connections with lifestyle and dietary factors. Candidate biomarkers used in this study were promoter DNA methylation marks of the genes *LINE-1*, *SIRT6* and *ELOVL2*.

Methods: For now, only one of the three genes selected could be established sufficiently for analysis. Methylation levels of *LINE-1* were measured in the capillary blood of 135 subjects by using high-resolution melt analysis. Afterwards methylation levels were correlated with age, lifestyle factors and dietary habits.

Results: *LINE-1* methylation and age exhibited a highly significant correlation. The older the participants the lower were the methylation levels of *LINE-1*. The intake of whole grains and vegetables and fruits was associated with decreased levels of *LINE-1* methylation. Women tended to have lower methylation levels than men and furthermore exhibited a significant lower methylation status when consuming dietary supplements or probiotics. A positive correlation was found between methylation of *LINE-1* and *IL-6*. No associations were found between methylation levels and lifestyle factors.

Conclusion: Until now study results were inconsistent regarding methylation of *LINE-1* as a biomarker for age. In this master thesis a strong correlation was detected. Further research is needed to confirm this outcome. A reliable biomarker would be a helpful tool to get a better understanding of how lifestyle and diet influence the aging process and may enable early interventions.

VI. Zusammenfassung

Hintergrund: Die biologischen Auswirkungen des Alterungsprozesses zu verlangsamen oder rückgängig zu machen ist ein sehr alter Traum der Menschheit. Während wir leben verursacht unser Metabolismus ständig Schäden in unserem Organismus. Diese Schäden akkumulieren und werden mit der Zeit pathologisch. Daher kann man Altern als den hauptsächlichen Risikofaktor für Krankheiten ansehen. Bis dato forschen Wissenschaftler intensiv an diesem Thema. Bekanntlich spielen Ernährung und Lebensstil wichtige Rollen im Alterungsprozess. Die Forschung im Bereich der Epigenetik setzt sich mit den spezifischen Mechanismen in diesem multifaktoriellen, komplexen Prozess auseinander.

Zielsetzung: Um den Einfluss von Lebensstil und Ernährung auf Alterung und altersbedingte Krankheiten besser verstehen zu können, sind verlässliche Altersmarker notwendig. Diese Masterarbeit hatte zum Ziel drei epigenetische mit Alterung verknüpfte Biomarker zu etablieren und zusätzlich nach Verbindungen mit Lebensstilfaktoren Ausschau zu halten. Diese möglichen Biomarker sind DNA Promotor Methylierungsmarker der Gene *LINE-1*, *SIRT6* und *ELOVL2*.

Methoden: Von den drei ausgewählten Genen konnte nur eines für die Analyse etabliert werden. Methylierungsgrade von *LINE-1* wurden im Kapillarblut von 135 Personen anhand der High Resolution Melt Analyse gemessen. Anschließend wurden die Methylierungswerte mit dem Alter, Lebensstilfaktoren und Ernährungsgewohnheiten korreliert.

Ergebnisse: *LINE-1* Methylierung und Alter wiesen eine hochsignifikante Korrelation auf. Je älter die Teilnehmer, umso niedriger waren die gemessenen Methylierungsgrade von *LINE-1*. Der Verzehr von Vollkornprodukten und Obst und Gemüse war mit verminderten *LINE-1* Werten assoziiert. Frauen neigten zu geringeren Methylierungswerten als Männer und zeigten außerdem einen signifikant niedrigeren Methylierungsstatus wenn Nahrungsergänzungsmittel oder Probiotika konsumiert wurden. Eine positive Korrelation zwischen den Methylierungswerten von *LINE-1* und *IL-6* wurde festgestellt. Zwischen Methylierungswerten und Lebensstilfaktoren wurden keine Assoziationen gefunden.

Schlussfolgerung: Bezüglich der Frage, ob die Methylierung von *LINE-1* als Alterungs-Biomarker geeignet ist, widersprechen sich die Studienergebnisse. In dieser Masterarbeit wurde eine starke Korrelation festgestellt. Um dieses Ergebnis zu bestätigen ist jedoch noch weitere Forschungsarbeit notwendig. Ein zuverlässiger Biomarker wäre ein wichtiges Instrument um ein besseres Verständnis darüber zu erlangen, wie Lebensstil und Ernährung den Alterungsprozess beeinflussen und könnte eine frühzeitige Intervention ermöglichen.

1. Introduction

1.1. Aging and epigenetics

Aging is associated with numerous changes at the organismal, tissue as well as cellular level. In general, aging is a time-dependent functional decline combined with an impaired ability to repair those damages. López-Otín *et al.* describes nine hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (López-Otín *et al.,* 2013). Among those hallmarks, epigenetic alterations represent one mechanism that got lots of attention in the field of aging research in the past years. The term epigenetics was defined by C.H. Waddington in 1942 when he described the causal interactions interfering along the path from the genotype to the phenotype (Waddington, 2012). A more current definition was presented by Goldberg *et al.*, that epigenetics refer to the phenomenon that changes the final outcome of a locus or chromosome without changing the underlying DNA sequence (Goldberg *et al.,* 2007). Those mechanisms are reversible, heritable and include modifications of DNA through methylation, modifications of histone proteins and transcription of non-coding RNAs (Pal and Tyler, 2016) (figure 1).

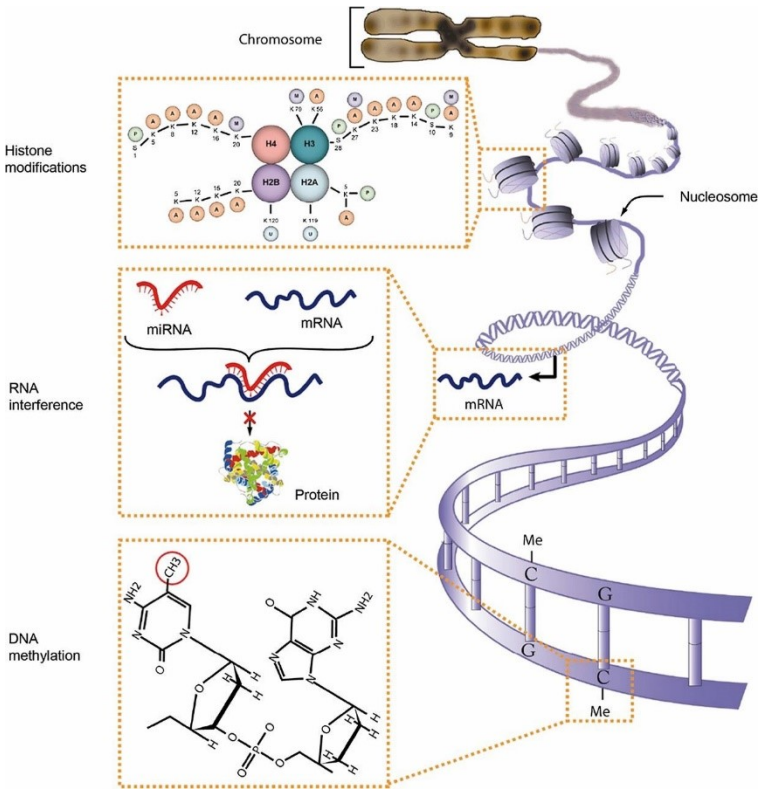

Figure 1: The primary mechanism of epigenetic regulation (Sawan *et al.*, 2008)

1.1.1. DNA methylation

The best characterized epigenetic modification is DNA methylation. DNA methylation is usually located on the cytosine residues of cytosine-phosphatidyl-guanine (CpG) dinucleotides. Regions with a high density of CpGs are so called CpG-islands (Goll and Bestor, 2005).

CpG methylation within promoter regions correlates with transcriptional repression, as methylation leads to a more compact structure of chromatin, which is called heterochromatin. It was observed that in younger cells the majority of CpGs have cytosine methylation, which results in the silencing of certain genes. (Pal and Tyler, 2016)

By comparing monozygotic twins, it was reported that epigenetic modifications diverge with ongoing age due to environmental factors (Fraga *et al.,* 2005).

The enzymes that are responsible for establishing or maintaining DNA methylation are the DNA methyltransferases (DNMTs) that transfer the methyl group from the cofactor *S*-adenosyl-L-methionine (SAM) to the cytosine residues. The resulting 5-methylcytosine (5mC) can be converted into 5-hydroxymethylcytosine (5hmC) by oxidation of the methyl group (figure 2). 5hmC was recently discovered as a new epigenetic mark. DNMT1 is the methyltransferase that is responsible for maintaining the methylation patterns across replication cycles. In contrast, DNMT3A and DNMT3B establish new methylation patterns. (Jurkowska *et al.,* 2011; Zampieri *et al.*, 2015)

Expression of DNMT1 and DNMT3B correlates with age, thus resulting in altered methylation patterns and – as one consequence – eventually leading to epigenetic instability (Ciccarone *et al.,* 2016).

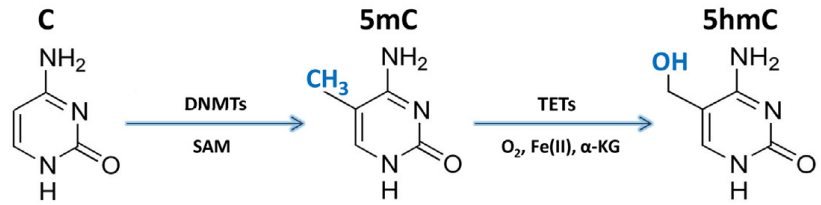

Figure 2: Conversion of cytosine to 5mC by DNMT enzymes and further oxidation of 5mC and formation of 5hmC. (Zampieri *et al.*, 2015)

1.1.2. Histone modification

Chromatin is built by histone proteins that wrap DNA around an octamer of histones to form nucleosomes. The degree to which chromatin is packed depends on numerous factors and can be influenced by post-translational modifications of histone proteins. Such modifications include lysine acetylation, lysine and arginine methylation, arginine citrullination, lysine ubiquitination, lysine sumoylation, ADP ribosylation, proline isomerization and serine/threonine/tyrosine phosphorylation. (Rothbart and Strahl, 2014) For example acetylation and phosphorylation are able to alter the charge of the basic histone proteins which changes the accessibility of DNA (Goldberg *et al.,* 2007). Sirtuins are one important family of histone deacetylases that affect multiple pathways not only in the aging process (Haigis and Sinclair, 2010). There are seven mammalian sirtuins that all act NAD^+-dependent and have multiple enzymatic activity. Histone deacetylation results in gene silencing due to chromatin inaccessibility (Watroba *et al.,* 2017).

1.1.3. Non-coding RNAs

Non-coding RNAs (ncRNA) describe RNAs that are not translated into proteins and can be distinguished in long and small ncRNAs. The most intensively studied small ncRNAs constitute the microRNAs (miRNA) that regulate gene expression post-transcriptionally. This miRNA-mediated regulation functions through inhibiting translation of specific target mRNAs. (Bürkle *et al.,* 2015)
They are characterized as 21-24 nucleotide RNAs and are transferred between cell membranes in exosomes. Through this transfer they represent an important mechanism of intercellular communication including cellular senescence. (Xu and Tahara, 2013)

1.2. *LINE-1*

LINE-1 belongs to the autonomously replicating retrotransposable elements that lack the long terminal repeated sequences. In humans, LINEs (Long Interspersed Nuclear Elements) constitute about 21% of the genome whereby only the *LINE-1* family is still active. Its fraction accounts for 17% of the genome. The majority of the *LINE-1* copies is inactive and not able to retrotranspose due to accumulation of mutations or truncation. Full-length copies of *LINE-1* are 6-8 kb long and replicate via reverse transcriptase of an RNA intermediate. (Lander *et al.,* 2001)

LINE-1 contains two open reading frames (ORFs) and encodes for two proteins: one protein for RNA binding (ORF1) and one with endonuclease and reverse transcriptase activity (ORF2) (Martin, 2006).

As *LINE-1* constitutes for such a large portion of the human genome and interspersed repetitive elements are known to contain numerous CpG dinucleotides, it is consequent that *LINE-1* methylation might serve as an indicator for global methylation.

Weisenberger *et al.* confirmed this theory by correlating MethyLight-based *LINE-1* methylation levels to global HPLC-based DNA methylation. Their data suggests that methylation of interspersed repeats can be used as a surrogate marker for estimating global DNA methylation levels. (Weisenberger *et al.,* 2005)

In normal tissues, repetitive elements are known to be highly methylated. Loss of methylation in repetitive elements might account for most of the global hypomethylation which is a common event in the aging process. This hypomethylation is also linked to human cancers as it plays a role in genomic instability or indirectly in the expression of genes (Ehrlich, 2002).

1.3. *SIRT6*

The human *SIRT6* gene is 8,427 bp long and is localized to chromosome 19p13.3. It is transcribed into a 1,636 bp long mRNA, which is further translated into a 355-amino acid protein. The SIRT6 protein is widely expressed, mainly in the cell nucleus. (Mahlknecht *et al.*, 2006)

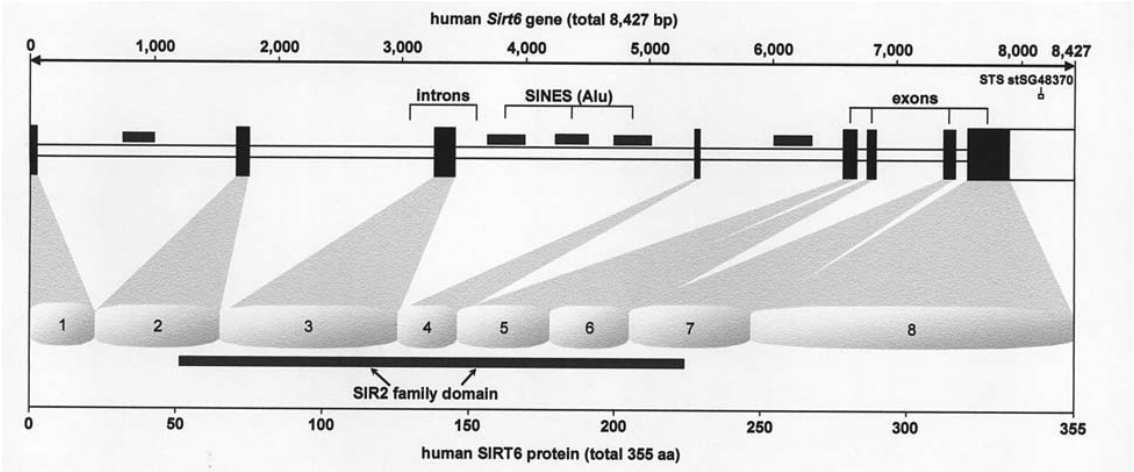

Figure 3: Genomic organization of the human *SIRT6* gene. (Mahlknecht *et al.*, 2006)

In mice, absence of SIRT6 leads to an accelerated development of age-associated degenerative processes and a shortened life span. SIRT6 knock-out mouse cells show genomic instability and a greater susceptibility to DNA damage. It is suggested that SIRT6 is a chromatin-associated protein and plays a significant role in DNA repair and genomic stability. (Mostoslavsky *et al.,* 2006)

Michishita *et al.* showed that SIRT6 functions as a NAD^+-dependent histone deacetylase and is highly site-specific for histone H3 lysine 9. By deacetylating this histone tail residue, SIRT6 leads to a more compact and thus stabilized chromatin structure at mammalian telomeres. This is essential for telomere metabolism and function. (Michishita *et al.,* 2008)

SIRT6 also plays a vital role in repairing DNA damage. McCord *et al.* reported that SIRT6 can form a complex with a DNA-dependent protein kinase at chromatin, mobilizes its catalytic subunit and thus promotes the repair of DNA double-strand breaks (McCord *et al.,* 2009).

SIRT6 is possibly another crucial factor in the aging process and the question arises if epigenetic alterations, such as promoter methylation, may serve as aging marker.

Sahin *et al.* correlated *SIRT6* promoter methylation levels with human longevity. The age of the study group ranged from 9-95 years and promotor methylation was highest between 20 and 79 years. However, there was no significant correlation with longevity detectable. (Sahin *et al.,* 2014)

1.4. *ELOVL2*

Another gene of interest in this study is *ELOVL2*. This protein coding gene has its chromosomal location on 6p24.2 and encodes for a 296-amino acid peptide that is particularly expressed in brain, liver, placenta and testis. The official full name is ELOVL Fatty Acid Elongase 2 and already provides a clue on its function. This transmembrane protein is involved in the elongation of 20- and 22-carbon long-chain polyunsaturated fatty acids (PUFAs). (Fagerberg *et al.,* 2014; Leonard *et al.,* 2002)

In a genome-wide association study *ELOVL2* was identified as a genetic contributor of plasma PUFA concentrations. This suggests *ELOVL2* as a genetic marker that may be useful in the examination of the interrelationship between diet, genetics and diseases. (Tanaka *et al.,* 2009)

As PUFAs are commonly known to play a crucial role in inflammation, maintenance of cell integrity and energy production it is plausible that *ELOVL2* methylation may play a role in the aging process. Garagnani *et al.* were the first to link *ELOVL2* with aging. They

compared the methylation levels of *ELOVL2* in whole blood DNA of mother-offspring couples and discovered a strong correlation between methylation levels and age of the subjects (Garagnani *et al.,* 2012). Others suggest that *ELOVL2* in combination with additional markers can serve as a predictor of human age in forensic applications (Zbieć-Piekarska *et al.,* 2015).

2. Objectives

For a long time, aging was considered as irrevocable. Though, meanwhile there are numerous attempts to slow down or even reverse the process of aging, most of those attempts hardly lead to any results. The aim of this master thesis is to establish further epigenetic markers that may help to unravel the complex multifactorial biological process which aging presents. Those markers are epigenetic alterations in the methylation levels of certain genes. Therefore, DNA methylation levels in the blood from a study group were investigated and then related to age. Additionally, they were associated with further information regarding lifestyle, nutrition and diseases.

3. Material and methods

3.1. Study design

In this study DNA samples from 143 participants - 61 male and 82 female - were analyzed. The age ranged from 22-79 years. Protein saver cards for the blood samples and a food frequency and lifestyle questionnaire (see appendix 8.3.) were provided by our laboratory. The aim was to establish the methylation analysis for *LINE-1*, *SIRT6* and *ELOVL2*. However, only the methylation levels of *LINE-1* were measured and analyzed in the end.

3.2. Exclusion criteria

In the first place, DNA extraction and analysis were carried out for all samples of the participants. Participants had no severe illness. But before starting statistical analysis, the influence of metabolic diseases on the methylation levels of *LINE-1* was assessed. As persons with thyroid dysfunction showed significant distinct levels, compared to people with no metabolic disease, they were excluded from the data set. Finally, 135 participants were included in the statistical analysis (60 male and 75 female).

3.3. Dried blood spots

To obtain DNA from our participants they were asked to take capillary blood samples by themselves and apply it on Whatman® protein saver cards (Sigma-Aldrich). Capillary blood is collected from the fingertip by puncturing the skin with a single-use lancet. Blood drops are transferred to the circles on the filter card. Afterwards blood spots are left to air dry. Filter cards were sent to us and stored at room temperature.

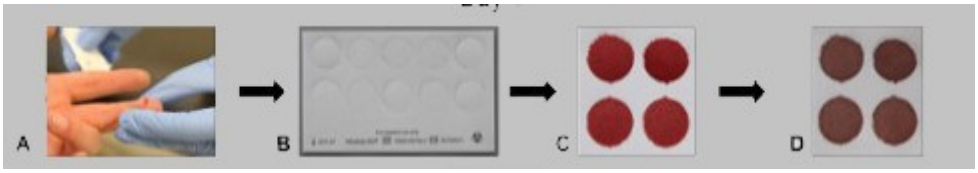

Figure 4: Collection of blood and preparation of blood spots, modified (Grüner *et al.*, 2015)

3.4. DNA extraction

Extraction of genomic DNA was done by using the QIAamp DNA Mini Kit for purification from dried blood spots. As shown in Figure 5, cells are broken down through proteinkinase K in the first place. Application of the lysate to the mini spin columns is followed by several washing steps in order to purify DNA. The eluate was stored at -20°C. The exact protocol provided by the manufacturer was slightly adapted and can be found in the appendix (see appendix 8.1.).

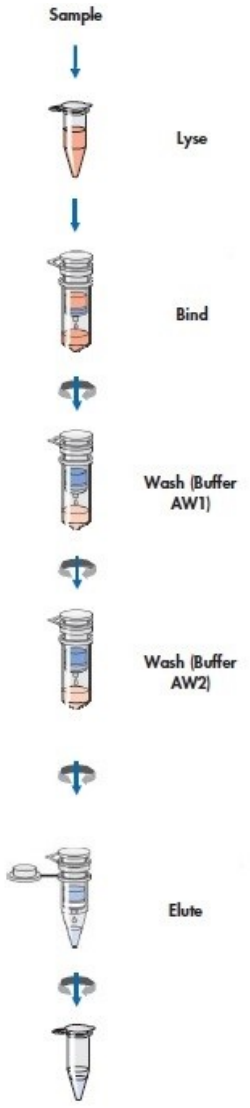

Figure 5: DNA extraction procedure (Qiagen, 2016)

3.5. Bisulfite conversion

Bisulfite conversion followed by a polymerase chain reaction (PCR) is a frequently used method to determine the methylation status of a DNA sequence. As it is not possible to amplify the methyl groups of a DNA sequence it is necessary to perform a bisulfite conversion beforehand. The basic principle behind this method is to conserve the 5-methylcytosines of a specific DNA sequence, whereas unmethylated cytosines are converted into uracil. This change is the basis for further analyses, in this case the high-resolution melt analysis.

The bisulfite conversion of the extracted DNA was done by following the protocol for sodium bisulfite conversion of unmethylated cytosines in DNA and by using the EpiTect Bisulfite Kit from Qiagen. The essential steps are presented in Figure 6 and are described in detail in the appendix (see appendix 8.2.).

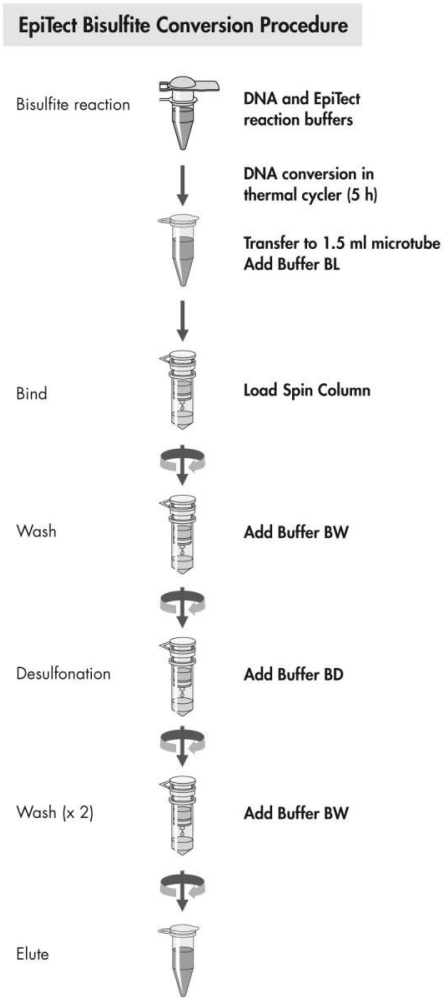

Figure 6: Bisulfite conversion procedure (Qiagen, 2014)

3.6. High-resolution melt analysis

3.6.1. HRM principle

High-resolution melt analysis is a PCR amplification method followed by a temperature gradient. This temperature gradient ensures a characterization of DNA samples according to their melting behavior. The melting behavior of a DNA strand is highly dependent on its content of guanine-cytosine pairs (GC pairs) since a GC pair is bound by three hydrogen bonds, while AT pairs are held together by only two hydrogen bonds. So, the higher the GC-content, the higher the temperature for melting the double stranded DNA. That is why HRM is a method suitable to analyze the methylation status of a specific DNA strand of bisulfite converted samples.

Analysis was done with the EpiTect HRM PCR Kit (Qiagen) on the Rotor-Gene Q (Qiagen). The kit includes the 2x EpiTect HRM PCR Master Mix which contains: HotStar Taq Plus DNA Polymerase, EpiTect HRM PCR Buffer (containing EvaGreen dye) and dNTPs.

For PCR a pair of primers is necessary to mark the start- and endpoint of the DNA synthesis. The aim was to establish HRM analysis for the genes *LINE-1*, *SIRT6* and *ELOVL2*. Therefore, several primer pairs were tested for each gene. Primer sequences were both taken from literature and self designed with the Methyl Primer Express Software v1.0. The sequences were produced by biomers.net GmbH, Ulm, Germany. Suitability of all primer sets was tested under different conditions. Attempts were made to optimize the melt analysis of each primer pair by changing different parameters like the annealing temperature or elongation time, additionally giving different $MgCl_2$ concentrations to the reaction mixture or using various primer concentrations. During the period of the master thesis project it was not possible to establish adequate HRM assays for *SIRT6* and *ELOVL2*. Either there was no correct fractionation of the standards, samples were located far outside the range of the standards, or duplicates of standards and samples differed heavily.

To assess the methylation levels of the samples, standards were used. Therefore, a CpGenome™ 5-mC DNA standard which is a linear, double-stranded DNA containing approximately 100% 5-methylcytosine (Merck, Darmstadt, Germany) and an unmethylated DNA standard (Qiagen) were bisulfite converted and combined to obtain 5 standards with 0%, 25%, 50%, 75% and 100% methylation levels. In Figure 7 the melting curve of the standards according to their methylation level is shown. In order to balance the small differences between the different initial fluorescence of individual samples the start- and endpoints of the melting curves were normalized.

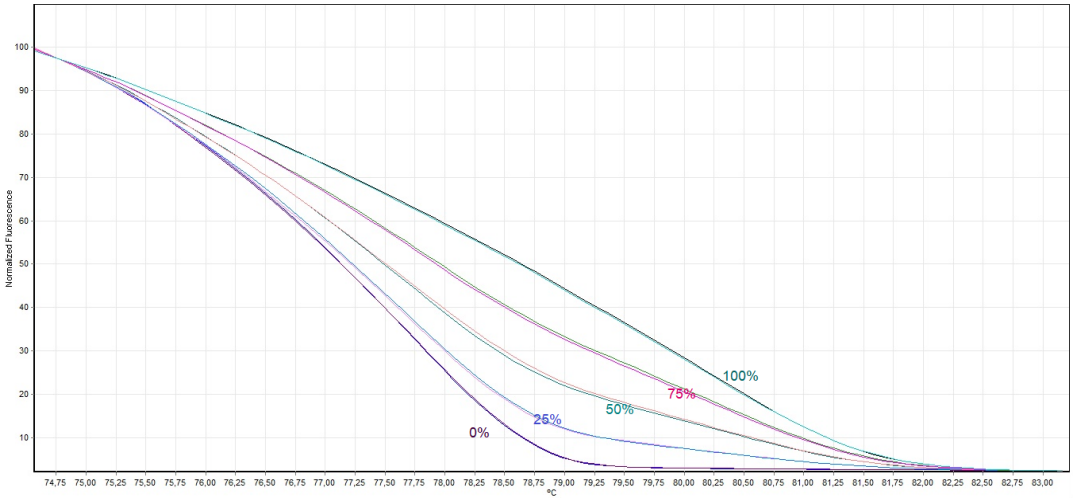

Figure 7: Normalized standard melting curve

3.6.2. HRM analysis setup *LINE-1*

We succeeded to optimize the melt analysis of one *LINE-1* primer pair. The fractionation of the standards first showed a shift to the higher methylated side. To counteract this effect the reverse primer was modulated to bias the shift to the opposite direction. Combining the two reverse primers in equal parts solved this problem. (table 1)

	Sequence
forward	5´tgt tag ata gtg ggt gta ggt t 3´
reverse 1:1 mixture	5´aat aca tcc gtc acc cct tt 3´
	5´aaa tac atc cat cac ccc ttt 3´

Table 1: Primer *LINE-1*

Master Mix, primers, RNase-free water, standards, template DNA and control DNA were thawed on ice. Components were pipetted in PCR tubes according to Table 2. In each experimental procedure a negative control (no template control), a positive control and a set of 5 standards (0%, 25%, 50%, 75%, 100%) was included. Furthermore, each sample or control was determined in duplicates. The cycling protocol for HRM is presented in table 3.

Component	**Volume per reaction**
EpiTect HRM PCR Master Mix	5 µl
Primer forward (10 pmol/µl)	0,25 µl
Primer reverse (10 pmol/µl)	0,25 µl
NFW	3,5 µl
Bisulfite converted template DNA or standards	1 µl
	= 10 µl

Table 2: Pipetting scheme *LINE-1* for one reaction

	Time	Temperature	
Initial PCR activation step	5 min	95°C	
Denaturation	10 sec	95°C	x 50
Annealing	30 sec	63°C touchdown for 7 cycles (1°C/cycle)	
Extension	10 sec	72°C	
Hold	1 min	95°C	
Hold	1 min	40°C	
HRM	0,1°C/2 sec	50-95°C	

Table 3: Optimized cycling protocol for *LINE-1*

3.7. Statistical analysis

HRM data was analyzed with the Rotor-Gene Q Series Software 2.3.1. DNA methylation percentage of the participants was determined by a standard curve and a standard formula using Microsoft Excel 2016. Statistical analysis was carried out with IBM SPSS Statistics Version 24.

Normal distribution of data was confirmed with Kolmogorov-Smirnov test and significant if p-value two-sided >0,05. Pearson´s correlation analysis and Spearman´s Rho analysis were used to correlate metric parameters. For analysis of categorical variables Kendall´s Tau test or Mann-Whitney U test was performed. Significance level for correlations was set to p<0,05 and a p-value below 0,01 was considered highly significant.

4. Results

4.1. *LINE-1* methylation

LINE-1 methylation ranged from 73,49% to 81,25% with an average value of 76,98%. Methylation data was normally distributed. (figure 8)

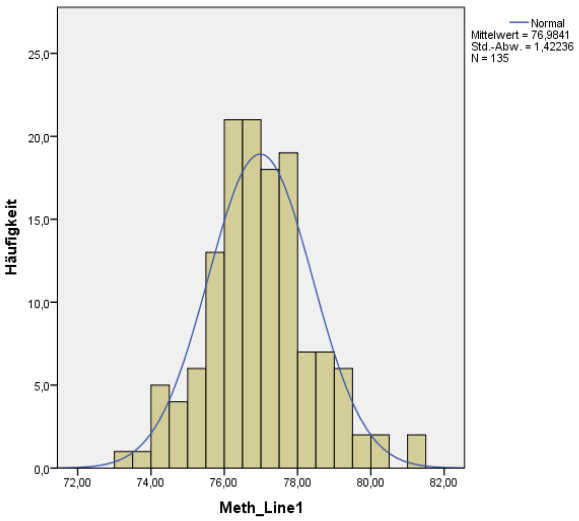

Figure 8: Distribution of *LINE-1* methylation levels

4.2. *LINE-1* methylation and age

Participants´ age ranged from 22 to 79 years with an average age of 49 years. Data set for age was normally distributed according to Kolmogorov-Smirnov test. Figure 9 shows a decrease in methylation with increasing age. Correlation was highly significant with p<0,01. (Pearson: -0,245; p: 0,004; N: 135)

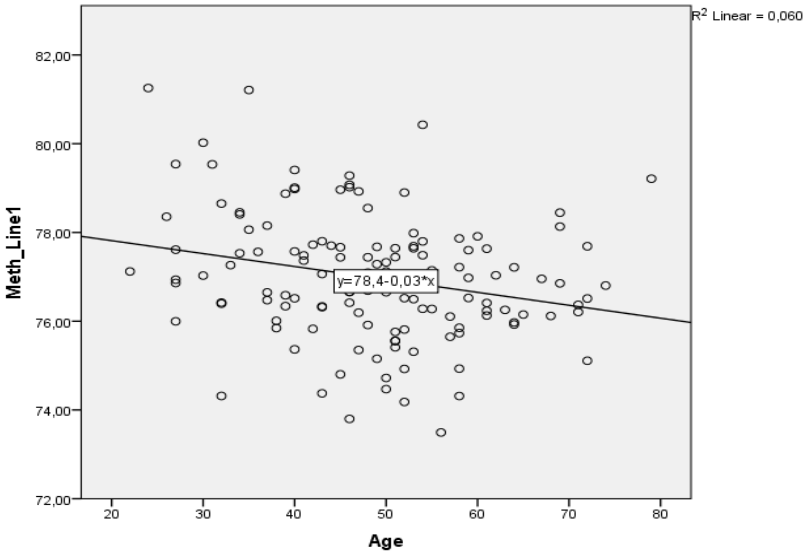

Figure 9: Methylation of *LINE-1* and age correlation.

4.3. *LINE-1* methylation and weight/BMI

Average BMI was 26,2 kg/m² for male and 22 kg/m² for female participants. Weight and BMI were not normally distributed according to Kolmogorov-Smirnov test. (p-value: 0,000 (BMI) and 0,003 (Weight); N:133) Therefore, Spearman´s correlation was done. Although not significant, weight as well as BMI increased slightly with decreasing methylation status. (figure 10)

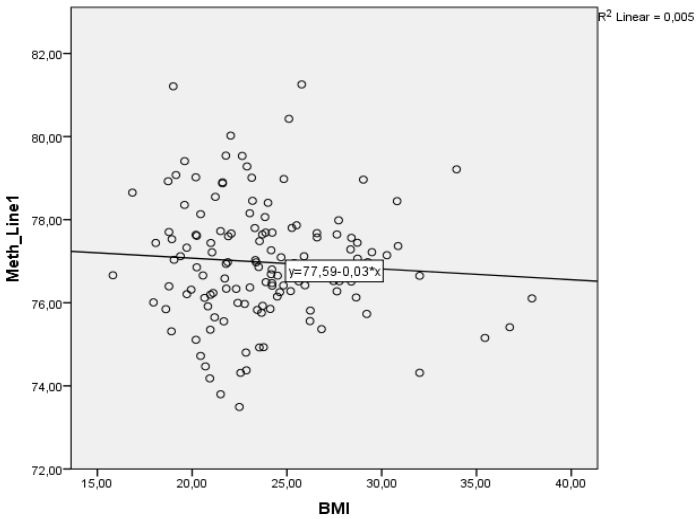

Figure 10: Methylation of *LINE-1* and BMI correlation

This trend becomes clearer when participants are divided by sex. (male: Spearman´s rho: -0,213; p: 0,106; N: 59; female: Spearman´s rho: -0,157; p: 0,182; N: 74) (figure 11 and 12)

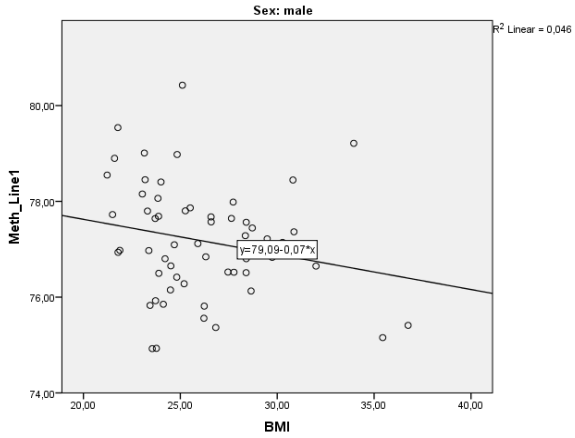

Figure 11: Methylation of *LINE-1* and BMI correlation within men

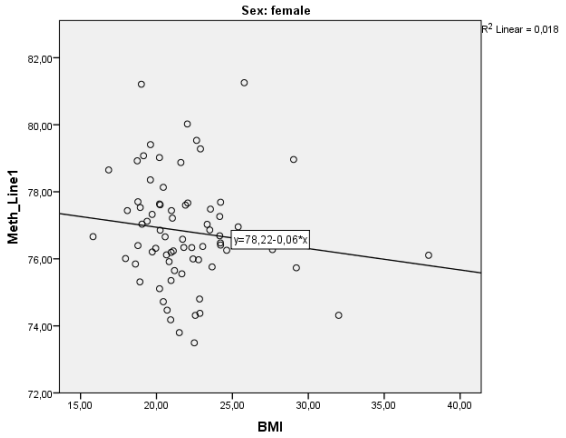

Figure 12: Methylation of *LINE-1* and BMI correlation within women

4.4. *LINE-1* methylation and inflammation

Participants that stated an existing inflammation had no significant difference in the methylation status than those with no existing inflammation. (Mann-Whitney U z: -1,322; p: 0,186; N: 124)

4.5. *LINE-1* methylation and sex

The study group consisted of 60 male and 75 female participants. Although not significant, female participants had lower methylation levels than men as illustrated in figure 13. (p: 0,065; N: 135)

LINE-1 methylation is normally distributed in both sexes according to Kolmogorov-Smirnov test.

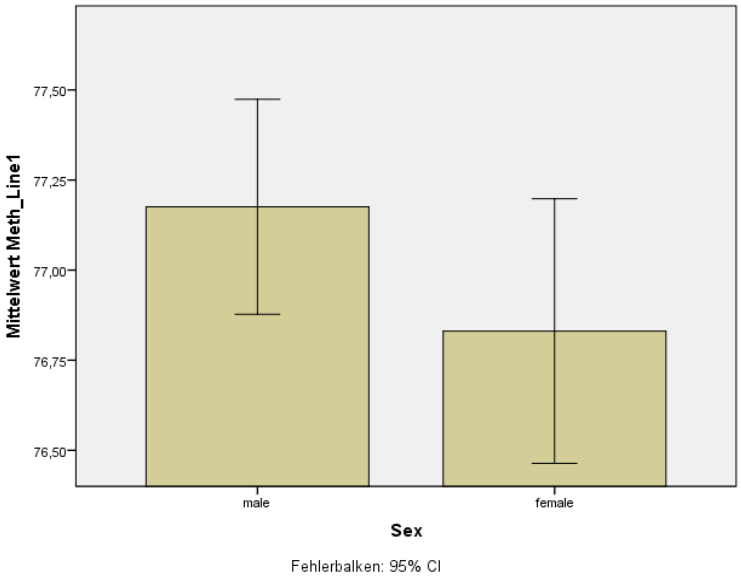

Figure 13: Methylation of *LINE-1* and sex

4.6. *LINE-1* methylation and nutrition

4.6.1. Whole grains

A significant negative correlation between the intake of whole grains and the methylation status was found. (Kendall-Tau-b: -0,148; p: 0,028; N: 134) (figure 14)
When divided according to sex the significance was only present in the male group. (Kendall-Tau-b: -0,246; p: 0,016; N: 59)

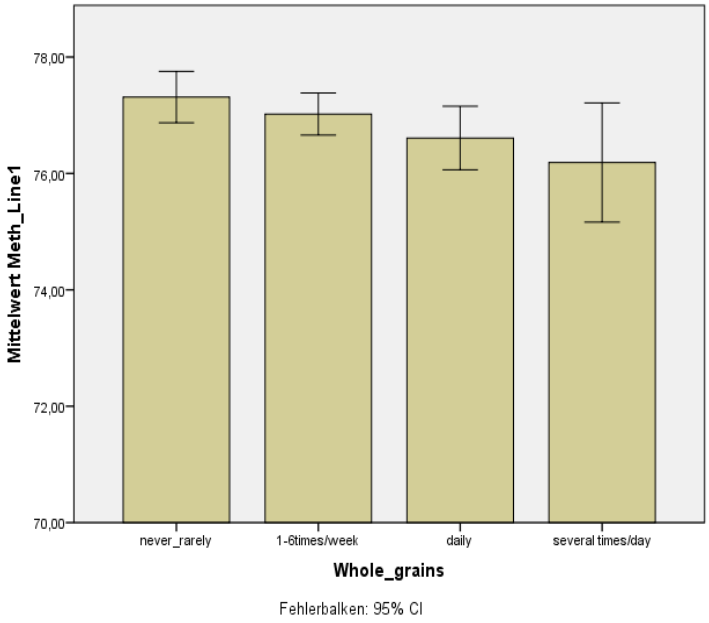

Figure 14: Methylation of *LINE-1* and consumption of whole grains

4.6.2. Vegetables and fruits

Participants consuming vegetables and fruits more frequently had a lower *LINE-1* methylation status. This correlation was not significant. (Kendall-Tau-b: -0,122; p: 0,63; N: 135) (figure 15)

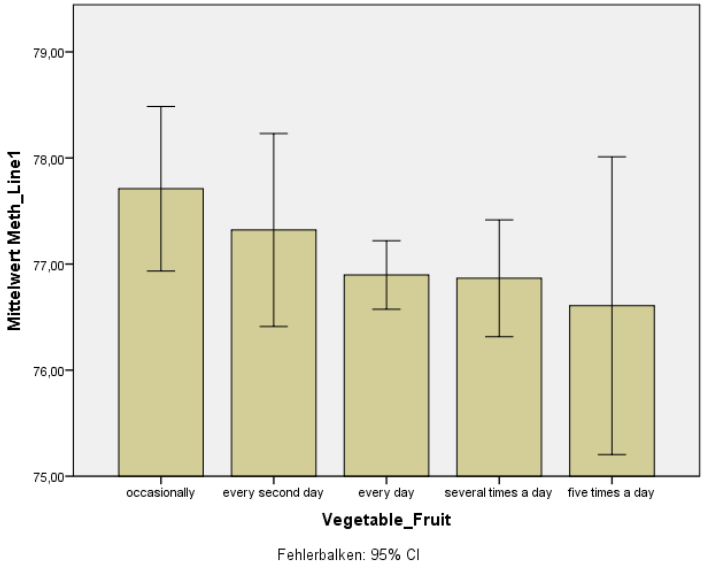

Figure 15: Methylation of *LINE-1* and consumption of vegetables and fruits

4.6.3. Dietary supplements

Female participants consuming dietary supplements had significant lower methylation levels of *LINE-1* than female non-consumers. (Z: -2,379; p: 0,017; N: 75) (figure 16)

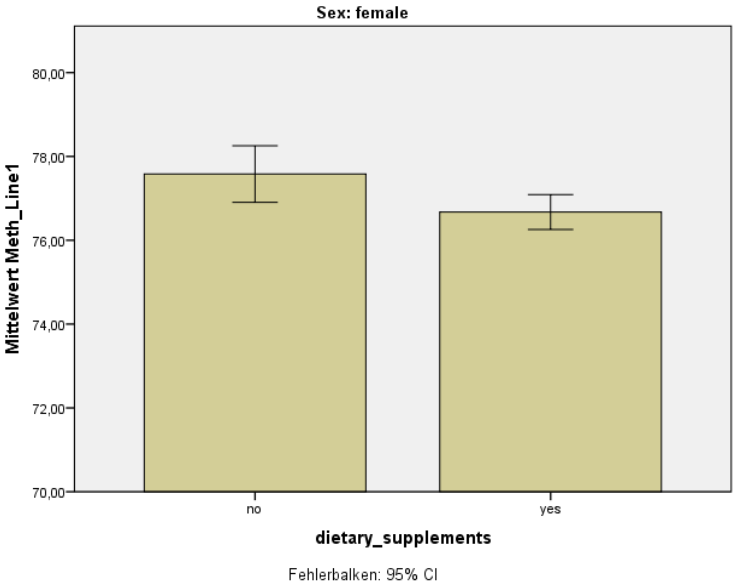

Figure 16: Methylation of *LINE-1* and consumption of dietary supplements within women

4.6.4. Probiotics

Similarly, women had a significant lower *LINE-1* methylation level if consuming probiotics as displayed in figure 17. (Z: -2,025; p: 0,043; N: 72)

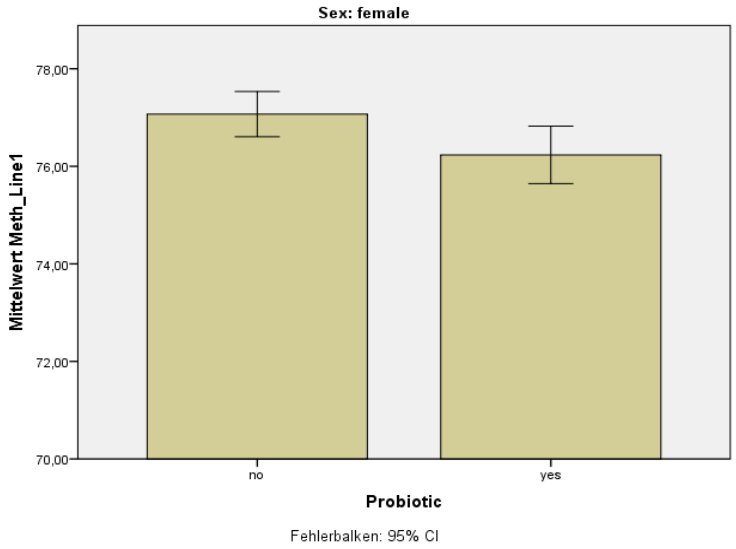

Figure 17: Methylation of *LINE-1* and consumption of probiotics within women

4.6.5. Folic acid

Furthermore, women who supplement folic acid showed a trend towards a lower *LINE-1* methylation. (Z: -1,748; p; 0,081; N: 73) (figure 18)

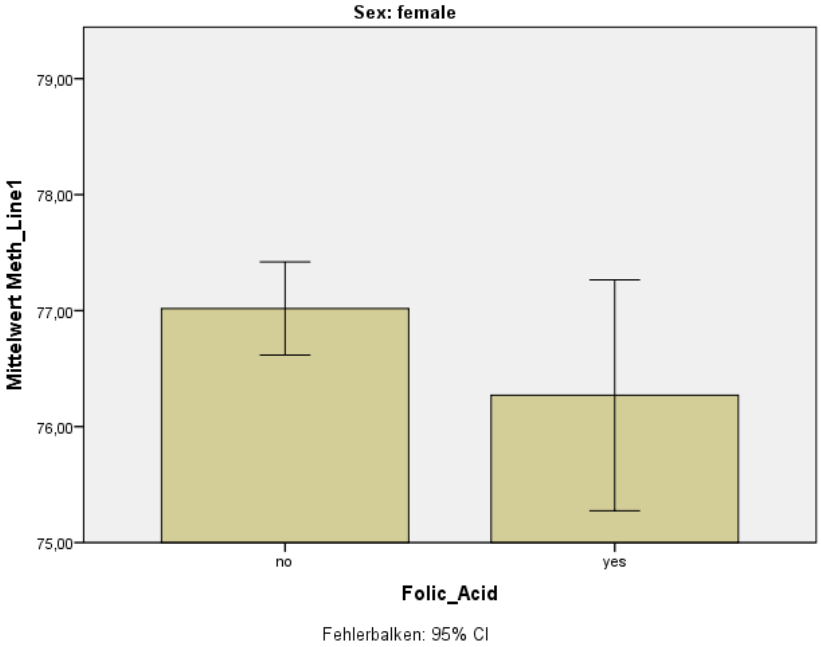

Figure 18: Methylation of *LINE-1* and consumption of folic acid within women

4.7. *LINE-1* methylation and lifestyle

No associations between lifestyle factors like smoking habits, physical activity and sports, drinking alcohol or perceived stress levels and the methylation status of *LINE-1* were found in this thesis.

4.8. *LINE-1* methylation and methylation of *IL-6*

Simultaneously HRM-analysis of the gene *Interleukin 6* (*IL-6*) was performed by a fellow colleague with the same DNA samples. Although not significant, methylation levels of both genes seem to correlate positively, as displayed in Figure 19. (Pearson´s r: 0,129; p: 0,176; N: 111)

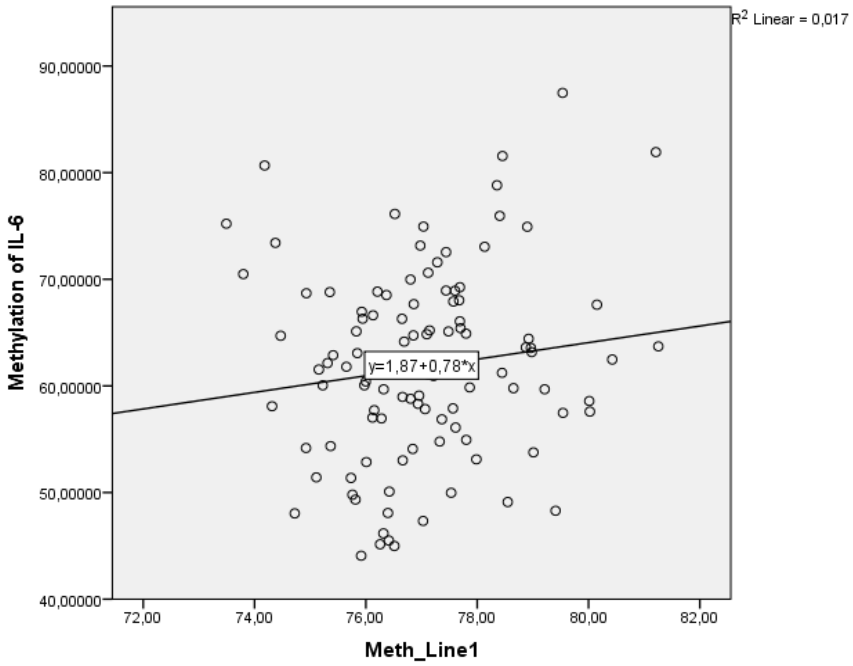

Figure 19: Methylation of *LINE-1* and *IL-6*

4.9. *LINE-1* methylation and total cholesterol

Participants with lower total cholesterol level tended to have higher methylation levels than participants with a high total cholesterol level. (Kendall-Tau-b: -0,231; p: 0,70; N: 40) (figure 20)

Within women, correlation was significant. (Kendall-Tau-b: -0,358; p: 0,46; N: 21)

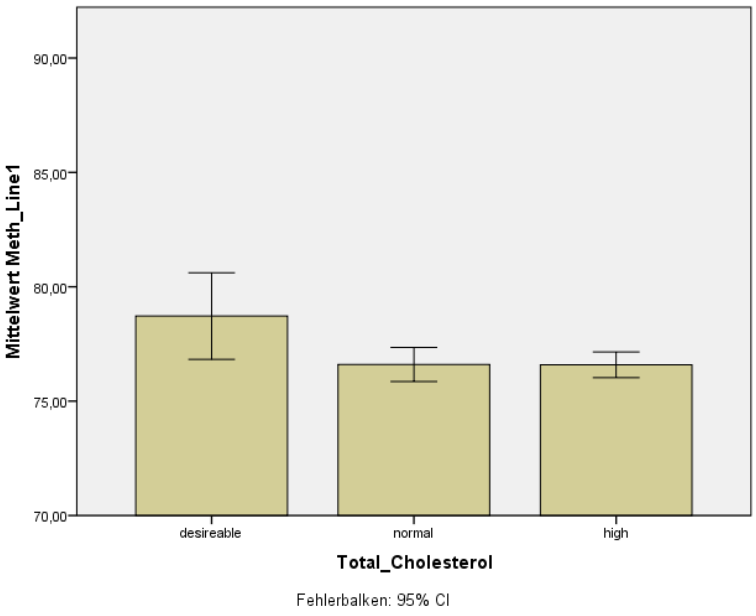

Figure 20: Methylation of *LINE-1* and total cholesterol

5. Discussion

5.1. Age

Global hypomethylation is a common epigenetic event occurring not only in the aging genome but also in cancer cells. As repetitive elements constitute a considerable proportion of the genome, it seems plausible that aging comes along with a loss of *LINE-1* methylation.

In a study from Cho *et al.*, the association between *LINE-1* hypomethylation and age was investigated. The results indicate that DNA hypomethylation and age both predict centromere positive micronuclei which serve as biomarker for age-dependent genomic instability. (Cho *et al.,* 2015)

The question arises if *LINE-1* may serve as a biomarker for aging. However, only a few studies found an evidence to support this thesis. Huen *et al.* reported that global DNA methylation in fetal cord blood is higher than in blood from 9-year-old children (Huen *et al.,* 2014). Several studies found no relationship between age and *LINE-1* methylation (Jintaridth and Mutirangura, 2010; El-Maarri *et al.,* 2011). Perng *et al.* even observed a slightly positive correlation between age and *LINE-1* methylation (Perng *et al.,* 2014). There can be several reasons for those contrary results. The methods of measuring methylation levels differ between the studies. Pyrosequencing methods or high-resolution melt analysis are only two examples of techniques that are currently used. Using different primer sequences of *LINE-1* is an additional factor that makes it difficult to compare the results. With the HRM method and the primer sequences that were used in this master thesis a highly significant association between *LINE-1* and age was found. Further research is needed to confirm this outcome but maybe this result helps to provide a promising *LINE-1* biomarker for aging.

5.2. Sex

Although difference was not significant, it was measured that men have higher *LINE-1* methylation levels than women. This confirms the findings of other studies and may be due to hormonal factors or to lower folate levels in women. (Marques-Rocha *et al.,* 2016; Tajuddin *et al.,* 2013; Perng *et al.,* 2014).

5.3. BMI/Weight

Regarding BMI and weight there was no significant correlation identifiable, but one can assume a slight trend towards an inverse correlation of *LINE-1* methylation and both parameters. Literature shows inconsistent results. Some studies cannot identify associations between *LINE-1* methylation and BMI as well (Tajuddin *et al.,* 2013). However, elevated BMI was associated with significant lower levels of *LINE-1* methylation (Zhang *et al.,* 2012), whereas other working groups reported a positive correlation between *LINE-1* methylation and BMI (Perng *et al.,* 2014). Although Marques-Rocha *et al.* found a positive correlation between body weight and *LINE-1* methylation, indicators of adiposity were lower among subjects with higher *LINE-1* methylation (Marques-Rocha *et al.,* 2016). On the contrary, Delgado-Cruzata *et al.* described that a decrease in body fat comes along with a decreased *LINE-1* methylation (Delgado-Cruzata *et al.,* 2015).
This explains why there is an ongoing discussion about the interaction of DNA methylation and weight and if *LINE-1* methylation can also serve as a biomarker of weight loss.

5.4. Inflammation

Although not significant, a trend towards a positive correlation between the methylation levels of *IL-6* and *LINE-1* was observed. The results from Marques-Rocha *et al.* are in line with this finding. In their study, correlation was significant and furthermore, they found an inverse association between *IL-6* methylation in white blood cells and relative expression of the inflammatory marker IL-6 (Marques-Rocha *et al.,* 2016). These findings suggest a linkage between methylation and inflammation in the aging process. No difference between the mean methylation levels of participants suffering frequently from inflammation or infections and those who do not was observed in this study. But this data relies only on self-evaluation and was not confirmed by any inflammation biomarkers.

5.5. Diet

Regarding dietary intake of certain food groups there was only one significant correlation detectable. Subjects consuming whole grains more often exhibited lower levels of *LINE-1* methylation. Literature search yielded no studies dealing with this association. Interestingly, participants who consume vegetables and fruits more frequently tend to

have a lower methylation status. This effect does not correspond with the findings of other studies. For example, Delgado-Cruzata *et al.* reported that increasing the frequency of eating fruit resulted in elevated *LINE-1* methylation levels (Delgado-Cruzata *et al.,* 2015).

Similarly, in a study by Zhang *et al.*, two dietary patterns were associated with genomic DNA methylation. They highlighted that a high adherence to a “prudent” diet, which is rich in vegetables and fruits, is associated with a reduced risk of DNA hypomethylation. Especially the consumption of dark green vegetables showed a positive correlation with global DNA methylation. (Zhang *et al.,* 2011)

The results in the present study rather indicate a negative effect of dietary habits that are considered healthy, like eating more whole grains or vegetables and fruits, on the methylation levels of *LINE-1*. But one has to keep in mind that this data regarding food intake relies on a self-reporting questionnaire which always bears the risk of bias. Furthermore, the association between dietary intake of certain food groups and DNA methylation could be confounded by other lifestyle factors.

Female participants that are consuming dietary supplements and probiotics had a significantly lower *LINE-1* methylation status. Similarly, women supplementing folic acid were associated with a lower *LINE-1* methylation status. However, this association was not significant. In contrast, several studies found a correlation between low intake of folate, or folate deficiency, and *LINE-1* hypomethylation (Agodi *et al.,* 2015; Zhang *et al.,* 2011) or reported a positive correlation between folate intake from fortified food and *LINE-1* methylation levels (Zhang *et al.,* 2012). As folate acts as a methyl donor and is thus necessary in DNA methylation, it appears likely that a deficiency in folate or other methyl donors can contribute to global DNA hypomethylation. Still, findings of the present study indicate lower *LINE-1* methylation if supplementing folic acid. Several factors could have contributed to this factor. First, measuring serum folate or assessing total folate intake may be more suitable methods to investigate the relationship between folate and DNA methylation. In this study participants were only asked if they are supplementing this vitamin or not, which involved too many confounders for reliable statements. Furthermore, the folate status of the subjects may play a crucial role. Pusceddu *et al.* showed that supplementation of vitamins does play a role in DNA methylation but depends on the kind of vitamin and even on the CpG sites that are examined (Pusceddu *et al.,* 2016).

5.6. Lifestyle

No associations between lifestyle factors and methylation of *LINE-1* were discovered in this thesis. Possibly methylation of the investigated *LINE-1* sequence is not dependent on lifestyle factors or the sample size was too small to detect any associations.

6. Conclusion

The main goal of the present study was to find reliable aging markers. Such biomarkers can contribute to a better understanding how diet and lifestyle have an impact on aging and age-related diseases. Additionally, they may play a role in personalized health care. Due to their ability to measure biological age against chronological age, age-related biomarkers may help detecting anomalies in the epigenomic landscape and enable early intervention. In this thesis, methylation levels of the repetitive element *LINE-1*, which serves as a surrogate marker for global methylation, exhibited a strong correlation with the age of the subjects. Diet and lifestyle are crucial factors that have an impact on physiological and pathological processes. But they are highly interrelated, which makes it challenging to identify specific effects of nutrients, dietary habits or lifestyle choices. This study had some limitations describing such effects. First, a self-reported questionnaire is prone to response bias. Second, impacts of factors like inflammation or folate status should be measured by means of established biomarkers. Therefore, further studies are needed to gain a better understanding of how lifestyle and diet are related to epigenetic alterations and aging.

7. References

Agodi A, Barchitta M, Quattrocchi A*,* Maugeri A, Canto C, Marchese AE, Vinciguerra M (2015) Low fruit consumption and folate deficiency are associated with *LINE-1* hypomethylation in women of a cancer-free population. *Genes and Nutrition* 10.

Bürkle A, Moreno-Villanueva M, Bernhard J, Blasco M, Zondag G, Hoejimakers JH, Toussaint O, Grubeck-Loebenstein B, Mocchegiani E, Collino S, *et al.* (2015) MARK-AGE biomarkers of ageing. *Mech Ageing Dev* 151: 2-12.

Cho YH, Woo HD, Jang Y, Porter V, Christensen S, Hamilton RF, Chung HW (2015) The Association of *LINE-1* Hypomethylation with Age and Centromere Positive Micronuclei in Human Lymphocytes. *PLoS One* 10: e0133909.

Ciccarone F, Malavolta M, Calabrese R, Guastafierro T, Bacalini MG, Reale A, Franceschi C, Capri M, Hervonen A, Hurme M, *et al.* (2016) Age-dependent expression of DNMT1 and DNMT3B in PBMCs from a large European population enrolled in the MARK-AGE study. *Aging Cell* 15: 755-65.

Delgado-Cruzata L, Zhang W, McDonald JA, Tsai WY, Valdovinos C, Falci L, Wang Q, Crew KD, Santella RM, Hershman DL, Greenlee H (2015) Dietary modifications, weight loss, and changes in metabolic markers affect global DNA methylation in hispanic, african american, and afro-caribbean breast cancer survivors. *Journal of Nutrition* 145: 783-790.

Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. *Oncogene* 21: 5400-13.

El-Maarri O, Walier M, Behne F, van Uum J, Singer H, Diaz-Lacava A, Nusgen N, Niemann B, Watzka M, Reinsberg J, Reinsberg J, van der Ven H, Wienker T, Stoffel-Wagner B, Schwaab R, Oldenburg J (2011) Methylation at global *LINE-1* repeats in human blood are affected by gender but not by age or natural hormone cycles. *PLoS One* 6: e16252.

Fagerberg L, Hallstrom BM, Oksvold P*,* Kampf C, Djureinovic D, Odeberg J, Habuka M, Tahmasebpoor S, Danielsson A, Edlund K, *et al.* (2014) Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics* 13: 397-406.

Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, *et al.* (2005) Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* 102: 10604-9.

Garagnani P, Bacalini MG, Pirazzini C, Gori D, Giuliani C, Mari D, Di Blasio AM, Gentilini D, Vitale G, Collino S, *et al.* (2012) Methylation of *ELOVL2* gene as a new epigenetic marker of age. *Aging Cell* 11: 1132-4.

Goldberg AD, AllisD and Bernstein E (2007) Epigenetics: a landscape takes shape. *Cell* 128: 635-8.

Goll MG and Bestor TH (2005) Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 74: 481-514.

Grüner N, Stambouli O and Ross RS (2015) Dried Blood Spots - Preparing and Processing for Use in Immunoassays and in Molecular Techniques. *Journal of Visualized Experiments : JoVE* 52619.

Haigis MC and Sinclair DA (2010) Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* 5: 253-95.

Huen K, Yousefi P, Bradman A, Yan L, Harley KG, Kogut K, Eskenazi B, Holland N (2014) Effects of age, sex, and persistent organic pollutants on DNA methylation in children. *Environ Mol Mutagen* 55: 209-22.

Jintaridth P and Mutirangura A (2010) Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. *Physiol Genomics* 41: 194-200.

Jurkowska RZ, Jurkowski TP and Jeltsch A (2011) Structure and function of mammalian DNA methyltransferases. *Chembiochem* 12: 206-22.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature* 409: 860-921.

Leonard AE, Kelder B, Bobik EG, Chuang LT, Lewis CJ, Kopchick JJ, Mukerji P, Huang YS (2002) Identification and expression of mammalian long-chain PUFA elongation enzymes. *Lipids* 37: 733-40.

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The Hallmarks of Aging. *Cell* 153: 1194-1217.

Mahlknecht U, Ho AD and Voelter-Mahlknecht S (2006) Chromosomal organization and fluorescence in situ hybridization of the human Sirtuin 6 gene. *Int J Oncol* 28: 447-56.

Marques-Rocha JL, Milagro FI, Mansego ML, Mourao DM, Martinez JA, Bressan J (2016) *LINE-1* methylation is positively associated with healthier lifestyle but inversely related to body fat mass in healthy young individuals. *Epigenetics* 11: 49-60.

Martin SL (2006) The ORF1 protein encoded by *LINE-1*: structure and function during L1 retrotransposition. *J Biomed Biotechnol* 2006: 45621.

McCord RA, Michishita E, Hong T, Berber E, Boxer LD Kusumoto R, Guan S, Shi X, Gozani O, Burlingame AL, Bohr VA, Chua KF (2009) *SIRT6* stabilizes DNA-dependent protein kinase at chromatin for DNA double-strand break repair. *Aging (Albany NY)* 1: 109-21.

Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, Kusumoto R, Kawahara TL, Barrett JC, *et al.* (2008) *SIRT6* is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* 452: 492-6.

Mostoslavsky R, Chua KF, Lombard DB, Pang WW, Fischer MR, Gellon L, Liu P, Mostoslavsky G, Franco S, Murphy MM, *et al.* (2006) Genomic instability and aging-like phenotype in the absence of mammalian *SIRT6*. *Cell* 124: 315-29.

Pal S and Tyler JK (2016) Epigenetics and aging. *Sci Adv* 2: e1600584.

Perng W, Villamor E, Shroff MR, Nettleton JA, Pilsner JR, Liu Y, Diez-Roux AV (2014) Dietary intake, plasma homocysteine, and repetitive element DNA methylation in the Multi-Ethnic Study of Atherosclerosis (MESA). *Nutrition, Metabolism and Cardiovascular Diseases* 24: 614-622.

Pusceddu I, Herrmann M, Kirsch SH, Werner C, Hubner U, Bodis M, Laufs U, Wagenpfeil S, Geisel J, Herrmann W (2016) Prospective study of telomere length and *LINE-1* methylation in peripheral blood cells: the role of B vitamins supplementation. *Eur J Nutr* 55: 1863-73.

Rothbart SB and Strahl BD (2014) Interpreting the language of histone and DNA modifications. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1839: 627-643.

Sahin K, Yilmaz S and Gozukirmizi N (2014) Changes in human sirtuin 6 gene promoter methylation during aging. *Biomed Rep* 2: 574-578.

Sawan C, Vaissiere T, Murr R, Herceg Z (2008) Epigenetic drivers and genetic passengers on the road to cancer. *Mutat Res* 642: 1-13.

Tajuddin SM, Amaral AFS, Fernández AF, Rodríguez-Rodero S, Rodríguez RM, Moore LE, Tardón A, Carrato A, García-Closas M, Silverman DT, *et al.* (2013) Genetic and non-genetic predictors of *LINE-1* methylation in leukocyte DNA. *Environmental Health Perspectives* 121: 650-656.

Tanaka T, Shen J, Abecasis GR, Kisialiou A, Ordovas JM, Guralnik JM, Singleton A, Bandinelli S, Cherubini A, Arnett D, Tsai MY, Ferrucci L (2009) Genome-wide association study of plasma polyunsaturated fatty acids in the InCHIANTI Study. *PLoS Genet* 5: e1000338.

Waddington CH (2012) The epigenotype. 1942. *Int J Epidemiol* 41: 10-3.

Watroba M, Dudek I, Skoda M, Stangret A, Rzodkiewicz P, Szukiewicz D (2017) Sirtuins, epigenetics and longevity. *Ageing Res Rev* 40: 11-19.

Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW (2005) Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res* 33: 6823-36.

Xu D and Tahara H (2013) The role of exosomes and microRNAs in senescence and aging. *Adv Drug Deliv Rev* 65: 368-75.

Zampieri M, Ciccarone F, Calabrese R, Franceschi C, Bürkle A, Caiafa P (2015) Reconfiguration of DNA methylation in aging. *Mech Ageing Dev* 151: 60-70.

Zbieć-Piekarska R, Spolnicka M, Kupiec T, Makowska Z, Spas A, Parys-Proszek A, Kucharczyk K, Ploski R, Branicki W (2015) Examination of DNA methylation status of the *ELOVL2* marker may be useful for human age prediction in forensic science. *Forensic Sci Int Genet* 14: 161-7.

Zhang FF, Morabia A, Carroll J, Gonzalez K, Fulda K, Kaur M, Vishwanatha JK, Santella RM, Cardarelli R (2011) Dietary patterns are associated with levels of global genomic DNA methylation in a cancer-free population. *J Nutr* 141: 1165-71.

Zhang FF, Santella RM, Wolff M, Kappil MA, Markowitz SB, Morabia A (2012) White blood cell global methylation and *IL-6* promoter methylation in association with diet and lifestyle risk factors in a cancer-free population. *Epigenetics* 7: 606-14.

8. Appendix

8.1. DNA extraction

Protocol: DNA purification from dried blood spots (QIAamp DNA Mini Kit)

Procedure

1. **Place 1 punched-out circle from a dried blood spot into a 1.5 ml microcentrifuge tube and add 180 µl of Buffer ATL.**

   Cut 3 mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper puncher.

2. **Incubate at 85°C for 10 min. Briefly centrifuge to remove drops from inside the lid.**
3. **Add 20 µl proteinase K stock solution. Mix by vortexing, and incubate at 56°C for 1 h. Briefly centrifuge to remove drops from inside the lid.**

   Note: The addition of proteinase K is essential.

4. **Add 200 µl Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at 70°C for 10 min. Briefly centrifuge to remove drops from inside the lid.**

   To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.

   Note: Do not add proteinase K directly to Buffer AL.

   A white precipitate may form when Buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5. **Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.**

   It is essential that the sample and ethanol are mixed thoroughly.

6. **Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.***

   Close each QIAamp Mini spin column to avoid aerosol formation during centrifugation.

7. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.***
8. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.**
9. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

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

10. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 150 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.**

8.2. DNA bisulfite conversion

Protocol: Sodium bisulfite conversion of unmethylated cytosines in DNA

Procedure

Bisulfite DNA conversion

1. **Thaw DNA to be used in the bisulfite reactions. Dissolve the required number of aliquots of Bisulfite Mix by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min.**

   Note: If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C and vortex again.

   Note: Do not place dissolved Bisulfite Mix on ice.

2. **Prepare the bisulfite reactions in 200 µl PCR tubes according to Table 2, page 18. Add each component in the order listed.**

   Note: The combined volume of DNA solution and RNase-free water must total 20 µl.

Table 2. Bisulfite reaction components

Component	Volume per reaction (µl)
DNA solution (1 ng – 2 µg)	Variable* (maximum 20 µl)
RNase-free water	Variable*
Bisulfite Mix (dissolved), see step 1	85
DNA Protect Buffer	35
Total volume	**140**

* The combined volume of DNA solution and RNase-free water must total 20 µl.

3. **Close the PCR tubes and mix the bisulfite reactions thoroughly. Store the tubes at room temperature (15–25°C).**

   Note: DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

4. **Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 3.**

   The complete cycle should take approximately 5 h.

   Note: If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available.

Table 3. Bisulfite conversion thermal cycler conditions

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.

5. **Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.**

   IMPORTANT: Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. It is important to use PCR tubes that close tightly.

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

Cleanup of bisulfite converted DNA

6. **Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.**

   Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

7. **Add 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA (see "Things to do before starting", page 16) to each sample. Mix the solutions by vortexing and then centrifuge briefly.**

   Note: Carrier RNA is not necessary when using >100 ng DNA.

8. **Place the necessary number of EpiTect spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube in step 7 into the corresponding EpiTect spin column.**
9. **Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.**
10. **Add 500 µl Buffer BW to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.**
11. **Add 500 µl Buffer BD to each spin column, and incubate for 15 min at room temperature (15–25°C).**

    If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

    IMPORTANT: The bottle containing Buffer BD should be closed immediately after use to avoid acidification from carbon dioxide in the air.

    Note: It is important to close the lids of the spin columns before incubation.

12. **Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.**
13. **Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.**
14. **Repeat step 13 once.**
15. **Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.**
16. **Recommended: Place the spin columns with open lids into clean 1.5 ml microcentrifuge tubes (not provided) and incubate the spin columns for 5 min at 56°C in a heating block.**

    This step enables evaporation of any remaining liquid.
17. **Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Dispense 20 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15,000 x g (12,000 rpm).**

    Note: To increase the yield of DNA in the eluate, dispense an additional 20 µl Buffer EB to the center of each membrane, and centrifuge for 1 min at maximum speed.

    Note: If the purified DNA is to be stored for up to 24 h, we recommend storage at 2–8°C. For storage longer than 24 h, we recommend storage at –15 to –30°C. At –15 to –30°C, DNA converted and purified using the EpiTect Bisulfite Kit can be stored for at least 3 years* without decrease of quality or conversion.

8.3. Food frequency and lifestyle questionnaire

Vorname / First Name

Nachname / Surname

Geburtsdatum / Date of birth

Geschlecht / Gender

Gewicht/ Weight ☐ kg ☐ lbs

Grösse / Height ☐ cm ☐ ft

Strasse / Hausnummer / Street / House No.

PLZ / Ort / Postal code / City

Telefon / Phone

Email / Email

Datum / Date

Ethnie / Ethnicity

☐ Afrikanisch (Subsahara) oder Afro-amerikanisch
African (sub-saharan) or Afro-american

☐ Kaukasisch ("weiß")
Caucasian ("white")

☐ Nordasiatisch
Northern Asian

☐ Nordostasiatisch und Arktisch
Notheast Asian and Arctic

☐ Südasiatisch (inkl. Südostasiatische Inseln)
Southern Asian (incl. insular southeast Asia)

☐ Pazifische Inseln
Pacific Islands

1.a Leiden Sie oft an Entzündungen und/ oder Infektionen? Do you often suffer from inflammation and/or infections?

- [ ] Ja / Yes
- [ ] Nein / No
- [ ] Weiß nicht / I don't know

1.b Wenn ja, welche Art von Entzündungen und wo treten diese auf? If yes, what type of inflammation and where do they occur?

- [ ] Grippale Infekte / Flu virus
- [ ] Entzündungen der Haut und oder Schleimhaut / Inflammation of the skin and or mucus
- [ ] Entzündliche Erkrankungen im Magen-/Darmbereich / Inflammatory diseases in the gastrointestinal region
- [ ] Muskel- oder Gelenksentzündungen / Muscle- or joint inflammation
- [ ] Blasenentzündung / Cystitis
- [ ] Pilzinfektionen im Genitalbereich / Fungal infections in the genital area
- [ ] Pilzinfektionen von Haut oder Füssen / Fungal infections of the skin or feet
- [ ] Sonstiges / Other _____

2. Was sind Ihre vordringlichsten Probleme, weshalb Sie Ihren Arzt/Therapeuten konsultieren? Welche Symptome traten auf? What are the main issues which cause you to consult a doctor or therapist?

_____

_____

_____

3. Nehmen Sie derzeit Medikamente zu sich? Are you currently taking medication?

- [ ] Nein / No
- [ ] Ja, folgende / Yes, the following

_____

4. Rauchen Sie oder haben Sie mal geraucht? Do you smoke or have you smoked in the past?

- [ ] Nein / No
- [ ] Früher (vor weniger als 10 Jahren) / In the past (less than 10 years ago)
- [ ] Früher (vor mehr als 10 Jahren) / In the past (more than 10 years ago)
- [ ] Zur Zeit weniger als eine Schachtel am Tag / Currently less than 1 pack per day
- [ ] Zur Zeit mehr als eine Schachtel am Tag / Currently more than 1 pack per day

5. Halten Sie eine bestimmte Ernährungsweise oder Diät ein (z.B.: vegetarisch, low-carb)? Do you adhere to a specific long-term diet? (e.g. vegetarian, low-carb)?

_____

6. An wie vielen Tagen in der Woche essen Sie sehr deftige Speisen oder Fertiggerichte? How many days a week do you eat processed foods or ready made meals?

- [ ] Jeden Tag in der Woche / Every day
- [ ] Mehrmals die Woche / Several times a week
- [ ] Selten / Rarely
- [ ] Nie / Never

7.a An wie vielen Tagen in der Woche essen Sie Gemüse und Obst? How many days a week do you eat vegetable and/or fruit?

- [ ] Hin und wieder / Occasionally
- [ ] Jeden zweiten Tag / Every second day
- [ ] Jeden Tag / Every day
- [ ] Mehrmals am Tag / Several times a day
- [ ] Fünf Mal am Tag / Five times a day

7.b Welche Obst und Gemüsesorten essen Sie am häufigsten? Which fruit and vegetable do you eat the most?

_____

7.c In welcher Form konsumieren Sie Ihr Gemüse hauptsächlich? In what form do you mainly consume your vegetable?
Mehrfachnennungen möglich / Multiple answers possible

- ☐ Roh / Raw
- ☐ Gekocht / Boiled in water
- ☐ Gebraten / Fried
- ☐ Gegart / Steamed
- ☐ Gedünstet / Stewed
- ☐ Frittiert / Deep-fried

8.a Vertragen Sie Milch und Milchprodukte? Do you tolerate milk and dairy products?

- ☐ Nein / No
- ☐ Ja / Yes
- ☐ Weiß nicht / I don't know

8.b Wenn ja, an wie vielen Tagen in der Woche konsumieren Sie Milch und Milchprodukte? If yes, how many days per week do you consume milk or dairy products?

- ☐ Nie / Never
- ☐ Gelegentlich bis jeden zweiten Tag / Occasionally, up to every other day
- ☐ Jeden Tag / Every day
- ☐ Mehrmals am Tag / Several times per day

9. Wie oft in der Woche essen Sie Fisch? How many times a week do you eat fish?

- ☐ Selten bis nie / Rarely, almost never
- ☐ Einmal pro Woche / Once per week
- ☐ 2-3 Mal pro Woche / 2-3 times per week
- ☐ Vier und mehr Mal die Woche / Four times and more per week

10. Wie oft in der Woche essen Sie Fleisch und Wurst? How many times a week do you eat meat and processed meat products?

- Selten bis nie / Rarely, almost never
- 1-6 Mal pro Woche / 1-6 times per week
- Täglich / Daily
- ☐ Mehrmals täglich / Several times per day

11.a Vertragen Sie Weizen bzw. Gluten? Do you tolerate wheat and gluten?

- ☐ Nein / No
- ☐ Ja / Yes
- ☐ Weiß nicht / I don't know

11.b Wenn ja, Wie oft in der Woche essen Sie Vollkornprodukte? If yes, how many times a week do you eat whole grain products?

- ☐ Selten bis nie / Rarely, almost never
- ☐ 1-6 Mal pro Woche / 1-6 times per week
- ☐ Täglich / Daily
- ☐ Mehrmals täglich / Several times per day

12. Nehmen Sie Nahrungsergänzungsmittel zu sich? Wenn Ja, welche Nahrungsergänzungsmittel haben Sie im letzten halben Jahr eingenommen? Do you take dietary supplements? If yes, what kind did you take in the last six months?

- ☐ Nein / No
- ☐ Vitamin C / Vitamin C
- ☐ Vitamin D / Vitamin D
- ☐ Kalzium / Calcium
- ☐ L-Carnitin / L-Carnitin
- ☐ Knoblauchpräparate / Garlic powder
- ☐ Ballaststoffe / Dietary fibre
- ☐ Biotin / Vitamin B7
- ☐ Magnesium / Magnesium
- ☐ Folsäure / Folic acid
- ☐ Cobalamin / Cobalamin
- ☐ Multivitamin / Multi-Vitamins
- ☐ Probiotische Kapseln / Probiotic capsules
- ☐ Sonstiges / Other

13.a Wie oft in der Woche sind Sie körperlich aktiv? (Einkäufe zu Fuß, Spazieren, Gartenarbeiten...) How many times a week are you physically active (walks, gardening, shopping...)?

- ☐ Selten bis nie / Rarely, almost never
- ☐ Einmal pro Woche / Once per week
- ☐ 2-5 Mal pro Woche / 2-5 times per week
- ☐ 6-7 Mal die Woche / 6-7 times per week

13.b Wie oft in der Woche machen Sie Sport? (Bewegung mit Schwitzen über 30min...)
How many times a week do you exercise intensively for more than 30 min.?

- ☐ Selten bis nie (max. 2 Mal pro Monat) / Rarely, almost never (max. twice a month)
- ☐ 1-2 Mal pro Woche / 1-2 times per week
- ☐ Mind. 2 Mal pro Woche / Minimum twice per week

14.a Wie viel Flüssigkeit nehmen Sie täglich zu sich?
How much liquid do you drink per day?

- ☐ Weniger als 1 Liter / Less than 1 liter
- ☐ 1-2 Liter / 1-2 liters
- ☐ 2-3 Liter / 2-3 liters
- ☐ Mehr als 3 Liter / More than 3 liters

14.b Wie viele Tassen Kaffee trinken Sie täglich?
How much coffee do you drink per day?

- ☐ Weniger als 1 Tasse / Less than 1 cup
- ☐ 1-2 Tassen / 1-2 cups
- ☐ 3-5 Tassen / 3-5 cups
- ☐ Mehr als 5 Tassen / More than 5 cups

14.c Wie oft trinken Sie Alkohol?
How often do you drink alcohol?

- ☐ Nie / Never
- ☐ Nur zu Anlässen / Only socially
- ☐ 1-2 mal im Monat / 1-2 times per month
- ☐ 3-5 mal im Monat / 3-5 times per month
- ☐ 1-2 mal die Woche / 1-2 times per week
- ☐ Täglich / Daily
- ☐ Mehrmals am Tag / Several times a day

14.d Welche Getränke nehmen Sie hauptsächlich zu sich? What kind of beverages do you mainly drink?
Mehrfachnennungen möglich / Multiple answers possible

- ☐ Energiegetränke / Energy drinks
- ☐ Tee (schwarz, grün,..) / Tea (black, green, ...)
- ☐ Tee gesüßt mit Zucker / Tea with sugar
- ☐ Tee (Kräuter) / Herbal Tea
- ☐ Tee (Kräuter) mit Zucker / Herbal tea with sugar
- ☐ Wasser / Water
- ☐ Limonade / Soft drinks
- ☐ Kaffee / Coffee
- ☐ Cola / Coke
- ☐ Kaffee gesüßt mit Zucker / Coffee with sugar
- ☐ Dicksaft / Syrup
- ☐ Nektar / Nectar
- ☐ Saft / Juice
- ☐ Verdünnte Säfte / Diluted juices
- ☐ Sonstige / Other _____

15. Wie viele Portionen der angegebenen Süßigkeiten/Süßspeisen konsumieren Sie wöchentlich?
How many servings of the sweets/desserts indicated do you consume per week?

Eine Portion entspricht ungefähr 4 Stück Schokolade, 200g Pudding oder 1 Stück Kuchen.
A serving is about 4 pieces of chocolate , 200g pudding or 1 piece of cake.

	< 1	1-3	3-5	5-10	10-15	> 15
Schokolade / Pralinen / Chocolate						
Kuchen / Gebäck / Cakes and pastries						
Kekse / Biscuits						
Gummibären / Gummisachen / Gummi bears / Jellybabies						
Bonbon / Zuckerl / Sweets						
Eis / Ice cream						
Puddings / Cremes / Puddings / Cream puddings						

Sonstiges / Other _____

16. Wann nehmen Sie meistens Ihre Hauptmahlzeiten zu sich? When is your main meal?

Mehrfachnennungen möglich / Multiple answers possible

- ☐ Frühstück / Breakfast
- ☐ Mittag / Lunch
- ☐ Abend / Dinner
- ☐ Irgendwann / Every so often

17.a Gab es im letzten Jahr einen oder mehrere Stoffe, auf die Sie allergisch oder mit Unverträglichkeiten reagiert haben? Were there one or more substances to which you had an allergic reaction or intolerance in the last year?

- ☐ Ja / Yes
- ☐ Nein / No
- ☐ Weiß nicht / I don't know

17.b Wenn ja, auf welche Substanzen reagierten Sie allergisch? Auch nicht vom Arzt überprüfte Substanzen If yes, which substances are you allergic to? includes also substances not checked by a doctor

Folgende: / The following:

_____

_____

18. Kennen Sie Ihre Cholesterinwerte? Do you know your cholesterol level?

- ☐ Nein / No
- ☐ Ja, folgende / Yes, the following

HDL	mg/dl
LDL	mg/dl
Gesamt Cholesterin / Total cholesterol level	mg/dl

19. Haben Sie eine der folgenden Stoffwechselkrankheiten? Do you have one of the following metabolic diseases?

- ☐ Diabetes mellitus Typ 1 / Diabetes mellitus Type 1
- ☐ Diabetes mellitus Typ 2 / Diabetes mellitus Type 2
- ☐ Schilddrüsendysfunktion / Thyroid dysfunction
- ☐ Pankreasleiden / Disease of the pancreas
- ☐ Gicht / Gout
- ☐ Nein / No

Gibt es diesbezügliche Befunde?
Are there any referring medical findings?

_____

20. Wie hoch würden Sie Ihre derzeitige Stressbelastung einschätzen? How high would you rate your current stress level?

- ☐ Gering / Low
- ☐ Mäßig / Moderate
- ☐ Hoch / High
- ☐ Sehr hoch / Very high

21. Versuchen Sie den ursächlichen Anteil bei der Entstehung Ihres Stressproblems in Prozent zu schätzen. z.B. Arbeit 45%, Freizeit 20%, Familie 35% Try to estimate the cause of your stress level in percent. e.g. Work 45% , leisure 20% , family 35%

Freizeit / Leisure	%
Familie / Partner / Family / Partner	%
Arbeit / Work	%