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„Influence of weight reduction on the epigenetic regulation and gene expression of inflammatory mediators and DNA repair in metabolic syndrome "

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List of abbreviations

BMI	Body mass index
Bp	Base pairs
cDNA	copy-DNA
CpG	dinucleotide cytosine guanine
Ct	threshold cycle
DNA	Desoxyribonucleinacid
DNMT1	DNA-(cytosine C5)-methyltransferase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL-6	Interleukin-6
LINE-1	Long interspersed element-1
MetS	metabolic syndrome
MLH1	MutL-homologe-1
MMR	mismatch repair system
mRNA	messenger RNA
PBMC's	peripheral blood mononuclear cells
RNA	Ribunucleinacid
RNAi	RNA interference
ROS	reactive oxygen spezies
SAM	S-Adenosylmethionine
VAT	visceral adipose tissue
WHO	World Health Organisation

1. Abstract

Overweight, obesity and metabolic syndrome (MetS) are multifactorial disorders that result from a long-term imbalance of diet, physical activity and also genetic predisposition. The adipose tissue as a highly metabolic organ influences several metabolic pathways including epigenetic regulation. The adipokines lead to a higher production of so called reactive oxygen species (ROS), which have an influence on genome wide hypomethylation and promoter hypermethylation.

The aim of this work is to investigate the influence of weight loss in metabolic syndrome patients on the epigenetic regulation (DNA-methylation) and gene expression of IL-6, DNMT1, MLH and LINE-1 using high resolution melt curve analysis and qPCR.

Mean weight loss after intervention was 8.0±4.5kg. Weight loss did not significantly influence gene expression of DNMT1 (fold-change: 0.95) or MLH1 (fold-change: 1.06), whereas after weight reduction the expression of IL-6 was about 74% higher than before (fold-change: 1.74).

Due to the poor DNA-quality, the methylation level of MLH1 was not determinable. DNMT1 methylation was about 0.2% lower (p=0.157) after weight loss and LINE-1 methylation was about 2.2% lower at the second point of time (p=0.181).

In this study neither the DNA-methyltransferases, nor the DNA mismatch repair system are affected by the loss of weight in obese patients. In case of the pro-inflammatory cytokine IL-6, the recent available data suggest, that a weight loss intervention in metabolic syndrome patients leads to an elevated expression. A weight loss intervention in metabolic syndrome patients may have an impact on the methylation level of LINE-1 and furthermore on the whole genome methylation.

2. Zusammenfassung

Als Adipositas, Übergewicht oder Metabolischem Syndrom werden Erkrankung bezeichnet, die durch verschiedene Faktoren, wie einer unausgeglichenen Ernährung, geringe physische Aktivität oder genetischer Veranlagung beeinflusst werden und die neben gesundheitlichen Folgen auch Einfluss auf epigenetische Regulationen haben können. Das Fettgewebe ist jedoch nicht nur ein Energiespeicher, sondern auch ein metabolisch sehr aktives Organ, welches bioaktive Peptide, sogenannte Adiponektine produziert. Diese Adiponektine führen zu einer erhöhten Produktion von reaktiven Sauerstoffspezies (ROS), welche sowohl bei der genomweiten Hypomethylierung als auch bei der Promoterhypermethylierung eine Rolle spielen. Da weltweit die Anzahl an adipösen und Patienten die an metabolischen Syndrom leiden, stetig steigt, ist die Untersuchung des Zusammenhangs zwischen MetS und Epigenetik besonders interessant.

Das Ziel dieser Masterarbeit ist es, zu untersuchen, wie sich eine Gewichtsreduktion bei Personen mit metabolischen Syndrom auf die epigenetische Regulation (DNA-Methylierung) und Genexpression auswirkt. Dazu wurde für die Gene IL-6, DNMT1 und MLH1 eine Genexpressionsanalyse mittels qPCR durchgeführt. Die Methylierungsanalyse wurde für DNMT1, MLH1 und LINE-1 mittels high resolution melt curve (HRM) durchgeführt.

Die durchschnittliche Gewichtsabnahme der Probanden nach der Intervention betrug 8,0±4,5kg. Die Reduktion des Gewichts hat weder die Expression von DNMT1 (fold-change: 0,95) noch von MLH1 (fold-change: 1,06) signifikant verändert. Die Expression von IL-6 wurde jedoch folglich der Gewichtsreduktion um 74% erhöht (fold-change: 1,74).

Die Bestimmung des Mehylierungslevels für MLH1 war leider aufgrund der schlechten DNA-Qualität nicht möglich. Die Methylierung von DNMT1 wurde durch die Gewichtsreduktion um 0,2% gesenkt (p=0,157) und die von LINE-1 um 2,2% (p=0,181).

In dieser Studie wurden weder DNA-Methyltransferasen noch das DNA-mismatch repair system durch den Gewichtsverlust in adipösen Patienten beeinflusst. In Bezug auf das pro-inflammatorische Zytokin IL-6 lassen die Ergebnisse dieser Studie darauf schließen, dass eine Gewichtsabnahme bei Personen mit Metabolischen Syndrom zu einer erhöhten Expression führt. Des Weiteren könnte eine Gewichtsreduktion in MetS Patienten einen Einfluss auf den Methylierungsgrad von LINE-1 und folglich auf die Gesamtmethylierung des Genoms haben.

3. Scientific background

3.1. Metabolic syndrome and obesity

Obesity is defined as a body mass index (BMI) ≥30kg/m² and occurs due to an imbalance of energy intake, energy expenditure and genetic predisposition. According to the World Health Organisation, the occurrence of obesity has more than doubled since 1980. In 2014 more than 1.9 billion adults were overweight and from them about 600 million were obese. The rate of obese children is also alarmingly high (WHO). As obesity is a consequence of poor eating habits and low physical activity it often occurs with other co-morbidities, that can be summarized as metabolic syndrome.

The term 'metabolic syndrome' defines a cluster of metabolic disorders, which are risk factors for the development of cardiovascular diseases. Furthermore, the increased amount of adipose tissue in these patients leads to a higher production of proinflammatory mediators such as Interleukin-6 (1). The association between metabolic syndrome with mortality, morbidity and adverse cardiovascular diseases is well established (2). For example, metabolic syndrome leads to a higher occurrence of ischemic stroke (3), mortality (2) and predisposition of the patients to the development of diabetes mellitus (1). In recent years people suffering from metabolic disorders and further from metabolic syndrome are ever-expanding. This leads to a high financial burden of our health care system.

There are many different diagnose criteria available, but the most commonly used ones are the criteria from the American Heart Association/ National Heart, Lungs and Blood Institutes (AHA/NHLBI). According to them, metabolic syndrome is diagnosed, when a patient has three or more out of the following five diagnostic criteria:

- Abdominal obesity (accumulation of the fat especially in the area of the abdomen)
- High triglyceride levels (≥ 150mg/dl blood)
- Fasting glucose ≥100mg/dl
- Systolic blood pressure ≥130mmHg or diastolic blood pressure ≥85mmHg
- HDL cholesterol ≤40mg/dl in men and ≤50mg/dl in women (4)

Independently from the diagnosis criteria, the prevalence from metabolic syndrome rises with age and differs between age and ethical background (5). In the USA about 7% of the persons between 20 and 29 years are diagnosed with metabolic syndrome, whereas the prevalence in the category 60 years and older is about 40%. Especially women with Afro-American roots seem to be a high risk group (5).

The prevalence of MetS in Europe is similar: an analysis of ten big cohort studies conducted by Van Vliet-Ostaptchouk et al in 2014, shows, that 24-65% of women and 43-78% of men are suffering from MetS (6).

This data show, that the number of people with metabolic syndrome worldwide is alarmingly high and still rising. Just because of this, it is quite important to identify the whole complexity of this disease to ensure an adequate therapy. However, MetS does not only lead to cardiovascular consequences, but studies have shown, that obesity directly leads to epigenetic changes in the human body. Wahl et al. could show, that in obese patients the methylation levels of around 280 CpG sites were altered, indicating, that obesity influences epigenetic regulation (7). Furthermore, Milagro et al observed a change in methylation patterns between low and high responders in weight loss. According to this study, methylation patterns of specific genes may be used as epigenetic markers for the response to an weight loss intervention (8).

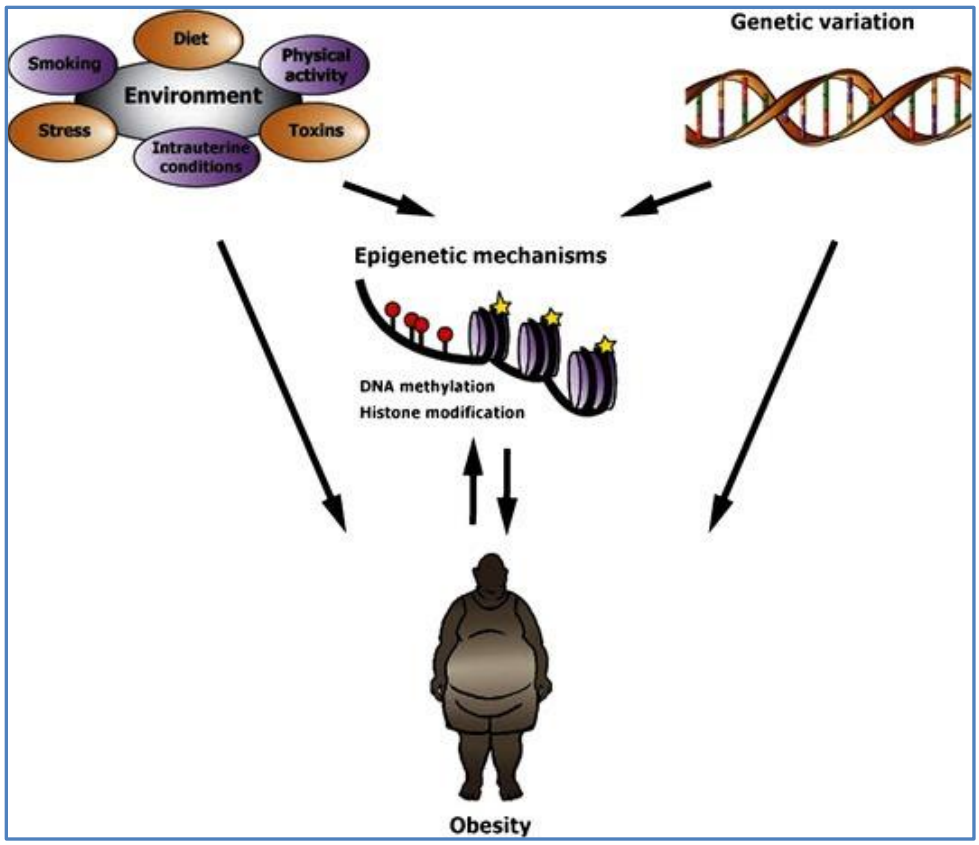


Figure 1: Interaction between environmental/genetic factors and epigenetic changes in the development of obesity (9)

3.2. Epigenetics

The term epigenetics literally means ‘outside conventional genetics’ and defines information, that is transmitted during cell division, but is not based on DNA mutation itself. Epigenetic changes are heritable changes in the DNA, that are not based on the change of the DNA-sequence, but origin from changes in gene expression or activity. Epigenetic changes can be influenced by different external factors as for example diet, lifestyle, environmental status, and it seems, that epigenetic mechanisms allow the organism to respond to different environmental changes with alterations in the gene expression (10).

There are three main epigenetic mechanisms: histone modifications, RNA interferences and DNA-methylation. All these mechanisms can lead to a stable modification of the gene expression (11).

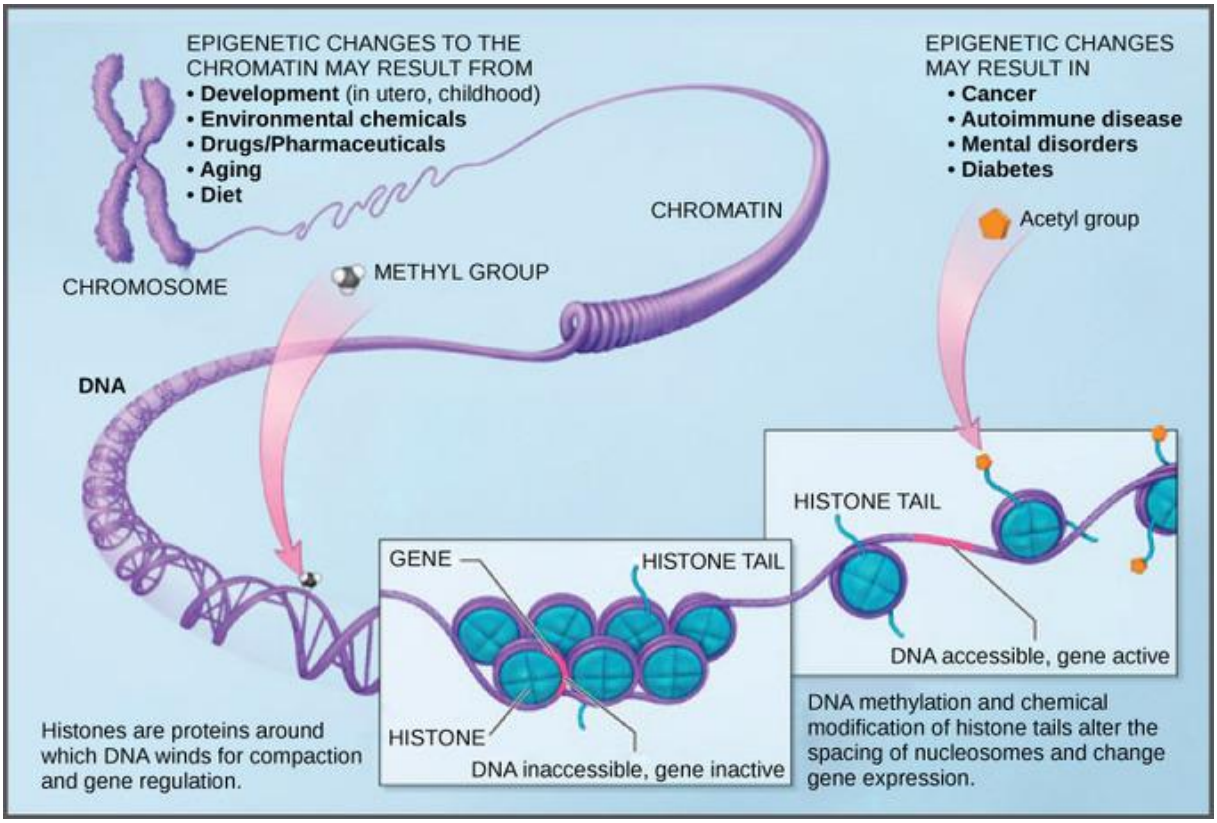


Figure 2: Epigenetic mechanisms: DNA-Methylation and histone modification (https://www.boundless.com/biology/textbooks/boundless-biology-textbook/gene-expression-16/eukaryotic-gene-regulation-113/epigenetic-control-regulating-access-to-genes-within-the-chromosome-459-11682/)

3.2.2. Histone modifications

In eukaryotic organisms the DNA is wrapped around small globular proteins, the so called histones, and form in this way structures called nucleosomes, which look like beads on a string. Four different isoforms of histone proteins are known: H2A, H2B, H3 and H4. Each nucleosome exists out of two copies from each isoform together with 146 basepairs (bp) long DNA. Depending on the formation of the histones either DNA is accessible and can be read and transcripted, or not. The dense, not accessible formation is called heterochromatin and the accessible form in called euchromatin (figure 3).

Histone proteins can be epigenetically modified on their unstructured ends and also at the globular parts (12). Modification of the histones due to methylation leads to a tight formation of the nucleosomes and as a result the DNA cannot be transcripted and expressed.

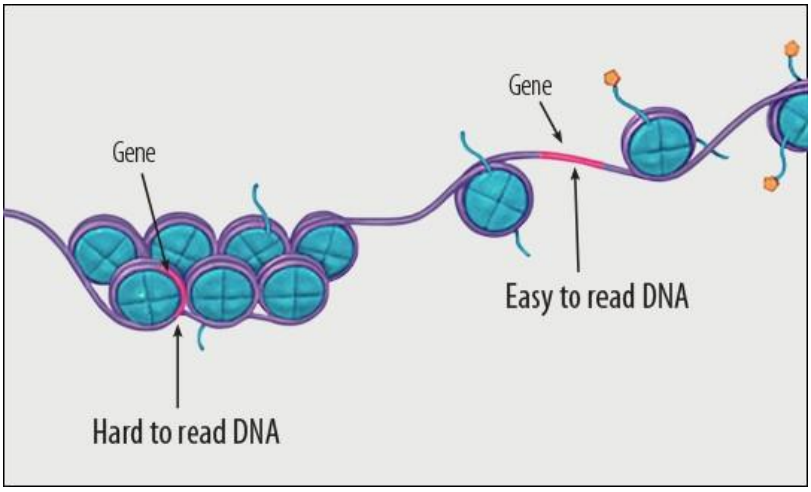


Figure 3: Histonmodifikation- open chromatin versus closed chromatin (https://www.nichd.nih.gov/health/topics/epigenetics/conditioninfo/Pages/default.aspx)

3.2.3. RNA Interference

RNA interference (RNAi) is a method for gene silencing, that limits the level of transcription by activating a RNA degradation process. Micro-RNA formations were shown to be a natural occurring RNAi process in eukaryotic organisms (13). Furthermore, these non-coding RNAs might be able to influence gene expression by causing heterochromatin formation and promoting DNA-methylation and histone modifications.

3.2.1. DNA-Methylation

DNA-methylation is one of the best investigated and important epigenetic mechanism. Our DNA contains the four different bases adenine, guanine, cytosine and thymine. Especially cytosine of the dinucleotide CpG is amenable for methyl-groups. In the mammalian genome about 70% of the CpGs are methylated and the distribution of CpGs is not random. The genome regions with a high content of CpGs are called CpG islands (14). After binding of the methyl group on the 5th position of a cytosine a 5-methylcytosin is generated (figure 4). This modification does not change the DNA sequence per se, but it has been shown, that it influences the DNA transcription and stability. Furthermore, it is also associated with transcriptional silencing and plays an important role in the development of mammalian organisms (15). In most cases the methyl groups origin from a diet high in methyl donors, for example folic acid. The DNA-methyltransferases (DNMTs) are responsible for the transfer of the methyl groups to cytosine (11). In mammalian organisms three different isoforms of DNA-methyltransferases have been identified so far. DNMT3A und DNMT3B, which are responsible for *de novo* methylation of DNA and DNMT1, which is responsible for the maintenance of methylation patterns during replication (16). Thereby DNMT catalyses the transfer of the methyl group from S-Adenosylmethionine (SAM) to the 5th carbon position of cytosine. For this reaction SAM is needed as a methyl donor (17).

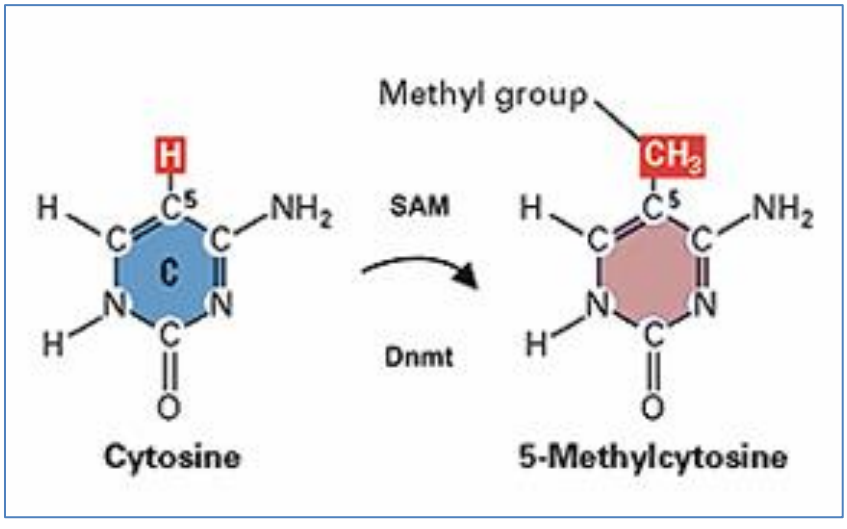


Figure 4: DNA-methylation: conversion of cytosine to 5-methylcytosine by DNMT. (https://card2brain.ch/box/affekacke_proteinbiosynthese)

Aside of epigenetic regulation, it has been proven, that DNA-methylation plays an important role in different cellular processes like proliferation, differentiation and senescence by changing the transcription of genes (18). The methylation of the promotor region of a gene leads to a transcriptional repression thereof (19). Also the formation of chromatin structures depends on DNA-methylation (20).

Furthermore, the DNA-methylation is required for the differentiation of stem cells in the small intestine: with loss of DNA-methylation, the gene expression becomes inappropriate and the differentiation of cells is delayed (21).

Abnormal methylation patterns can lead to dysfunctions in the human body. Other studies show, that an insufficient global DNA-methylation has an impact on adverse health effects during ageing (18).

3.3. Genes of interest

3.3.1. DNMT1

As already mentioned, DNMTs play an important role in the process of DNA-methylation. DNMT1 is an abbreviation for DNA-(cytosine C5)-methyltransferase and it is the most abundant DNMT in adult cells (14). Three different DNA methyltransferases have been identified in mammalian organisms so far. Whereas two of them, namely DNMT3a and DNMT3b, are responsible for the establishment of DNA-methylation patterns, DNMT1 has the ability to stabilize these methylation patterns and thus is called maintenance-DNA-methyltransferase. After cell replication the parenteral DNA strand remains methylated while the newly synthesized daughter strand is unmethylated. In this case, DNMT1 binds on the new synthesized strand and methylates the cytosines according to the parenteral strand (14).

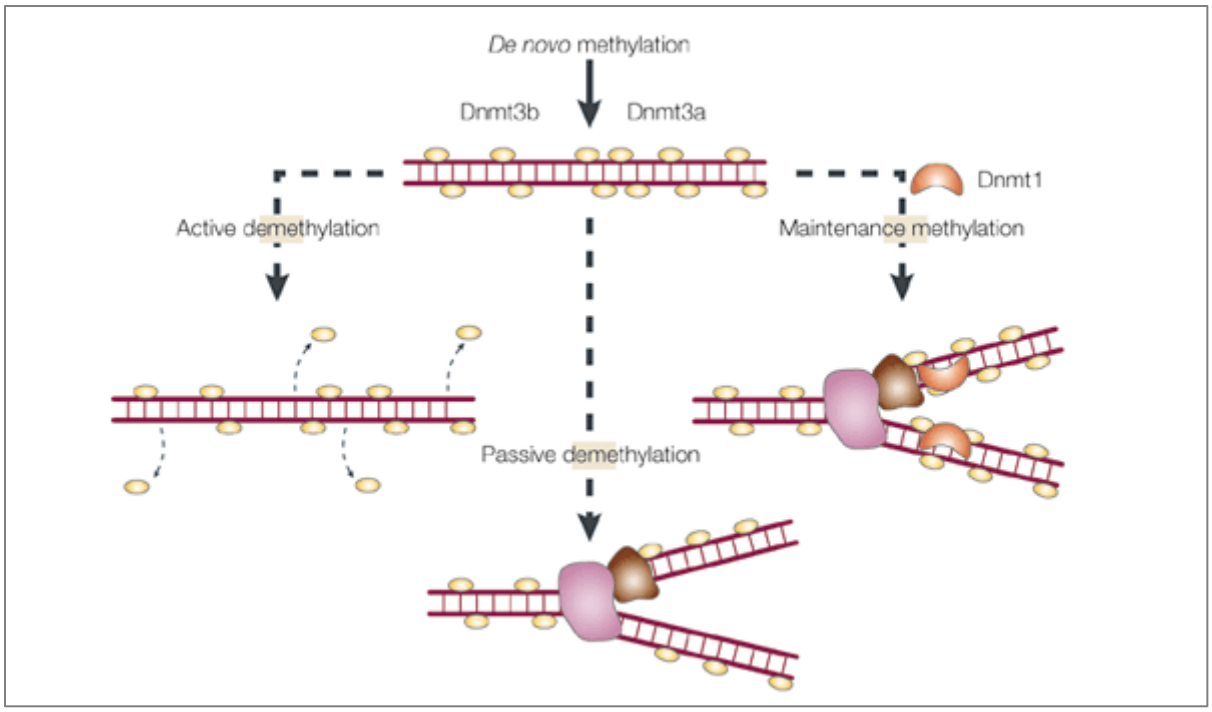


Figure 5: Different functions of mammalian DNA-methyltransferases (17)

It has been proven, that there is an interaction between IL-6 and DNMT1: Hodge et al showed, that a treatment with high concentration of IL-6 in results in a higher expression of DNMT1 (22). According to several studies, an overexpression of DNMT1 can be found in colon tumour cells. Alterations in DNMT1 methylation lead to an altered expression of DNMT1 in cells.

3.3.2. MLH1

The repair protein MLH1 or MutL homolog 1 is part of the DNA mismatch repair system (MMR) (23). The MMR is responsible for elimination and correction of mismatches of the newly synthesized DNA strands and in this way protects the genome from mutations. Since mutations in somatic or germline cells can alter the cellular phenotype and lead to dysfunctions or diseases, a proper and accurate repair is very important for the genetic stability (23). Consequently, defects in the mismatch system lead to a higher number of spontaneous mutation rate (24). Therefore, the DNA damage or mismatch has to be recognized. This happens by the mismatch protein MSH2, which is able to recognize base-base mismatches or deletion/insertion mispairs, which are characterized by one or two unpaired nucleotides (24). After binding of MSH2 on the incorrect strand, MLH1 is recruited and also binds on the location to be renewed. Now the MLH-complex recruits the enzyme exonuclease,

which cuts off only the false synthesized part of the DNA strand and at last the strand is resynthesized by DNA polymerase (figure 6) (25).

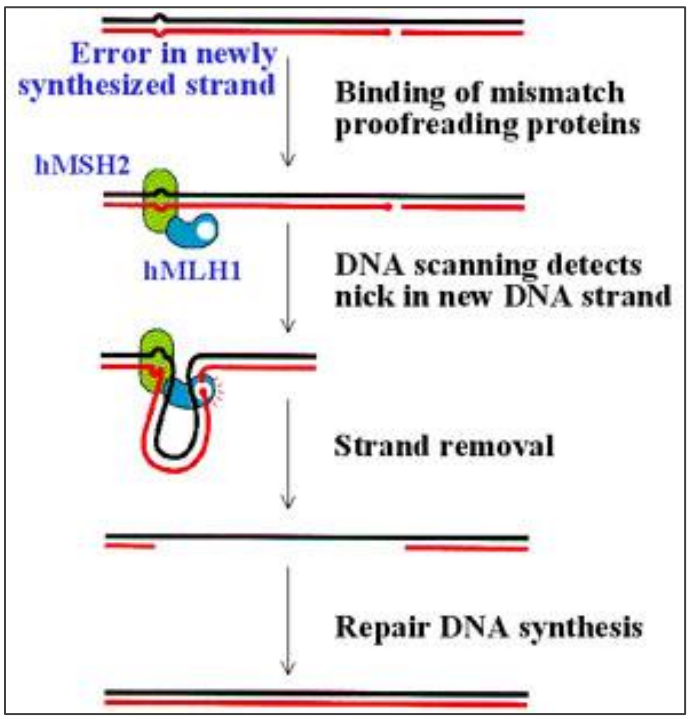


Figure 6: DNA mismatch repair system (http://www.dxline.org/medic/term/mismatch-repair/)

3.3.3. IL-6

Interleukin-6 is a cytokine, which is produced by macrophages and monocytes as an answer to infectious inflammation. Furthermore, IL-6 is one of the major inflammatory mediators in obese individuals and is produced by adipose tissue. As it was shown, that in metabolic syndrome patients, especially in obese ones, a persistent low-grade-inflammatory process is present, IL-6 is also an interesting target for the epigenetic analysis (26).

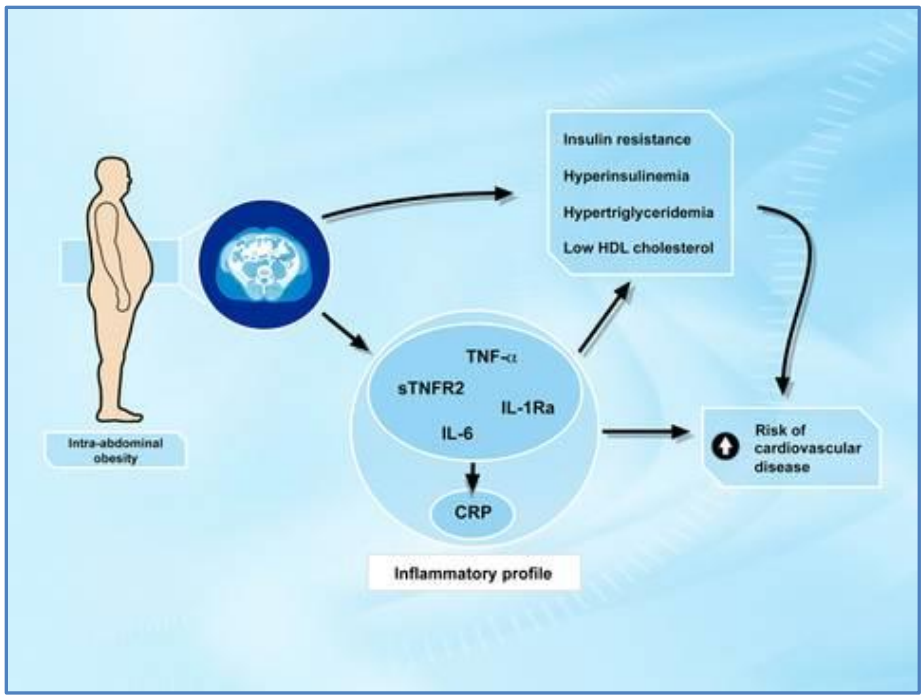


Figure 7: Connection between obesity and inflammatory processes.

3.3.4. LINE-1

About 45% of the human genome persists of transposable elements. These transposons are one kind of mobile genetic elements, who are transposable and do not have a strict position in the DNA, but are able to change their location within the genome. Because of this property, they are also called jumping genes and are able to create or reverse mutations and they can alter a cell's genome size. However, most of the transposons in the human genome have lost their ability to change their locations because of mutations (27). According to their mechanism of transposition, they can be classified into two groups: either copy-and-paste or cut-and-paste (28) (figure 8).

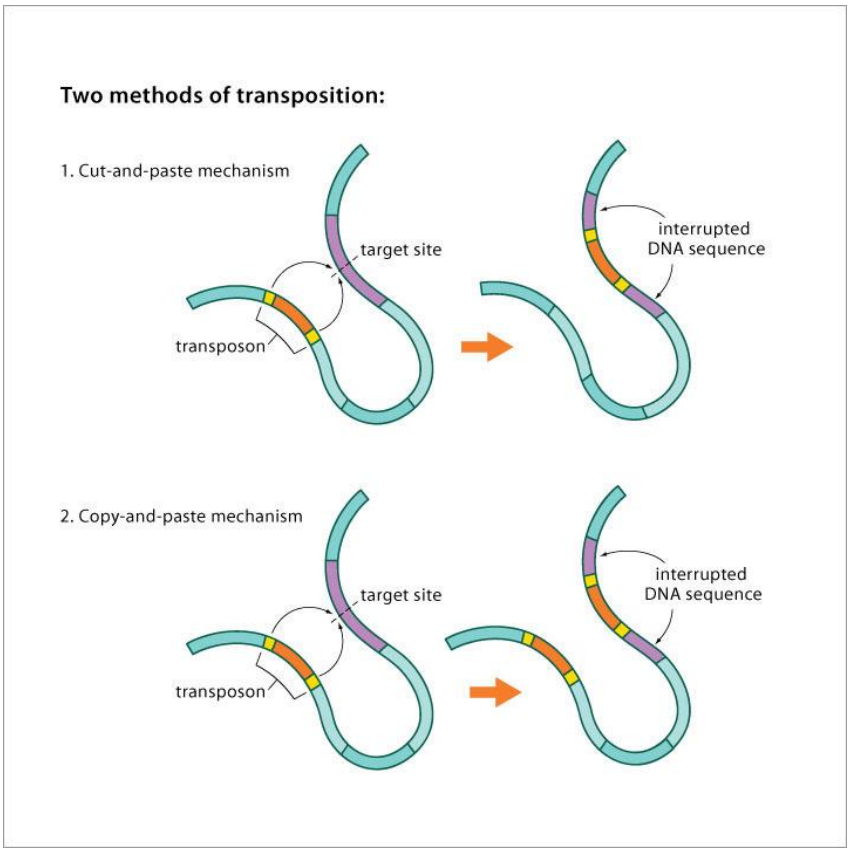


Figure 8: Possible mechanisms of transposition of transposons (https://www.broadinstitute.org/files/news/stories/full/transposons_720x720_v2.jpg)

Long Interspersed Element–1 (LINE-1) is one of these transposons and refers to the first class of transposons, also called retrotransposons. With up to 17%, LINE's make up a large part of our DNA. Because of the high occurrence, LINE-1 became a good predictor for the global methylation and for DNA-stability in our genome. The higher the methylation of LINE-1, the more stable it is and the less transpositions it makes, which could result in interrupting other genes and blocking their activities (29).

4. Objectives

Metabolic syndrome and obesity are associated with a persistent low-grade inflammation and a higher production of so called reactive oxygen species (ROS). This association has already been shown by our group *in vitro* and in a mice model. It seems, that these metabolic disturbances originate mainly from the increased adipose tissue, which is an important source of ROS and inflammatory mediators. Furthermore, these mediators can affect the other tissues as well. Epigenetic mechanisms, especially DNA-methylation may influence the regulation of inflammatory processes (IL-6), DNA repair mechanisms (MLH1) and also in the development of oxidative stress. As different studies have shown a connection between obesity and alteration in epigenetic regulation processes, we decided to investigate the gene expression and methylation levels of DNMT1, MLH1, IL-6 and LINE-1.

Following objectives will be addressed in my thesis:

1. Effects of metabolic syndrome on the epigenetic regulation and gene expression of DNA repair mechanisms and maintenance of DNA-methylation especially MLH1, DNMT1 and LINE-1
2. Effects of weight loss intervention in accordance with DACH references on the epigenetic regulation and gene expression of DNA repair and maintenance of DNA methylation especially MLH1 and DNMT1
3. Effects of weight loss intervention in metabolic syndrome patients on gene expression of genes involved in inflammatory process, especially IL-6
4. Effects of weight loss intervention on the epigenetic regulation of LINE-1

5. Materials and Methods

5.1. Overview

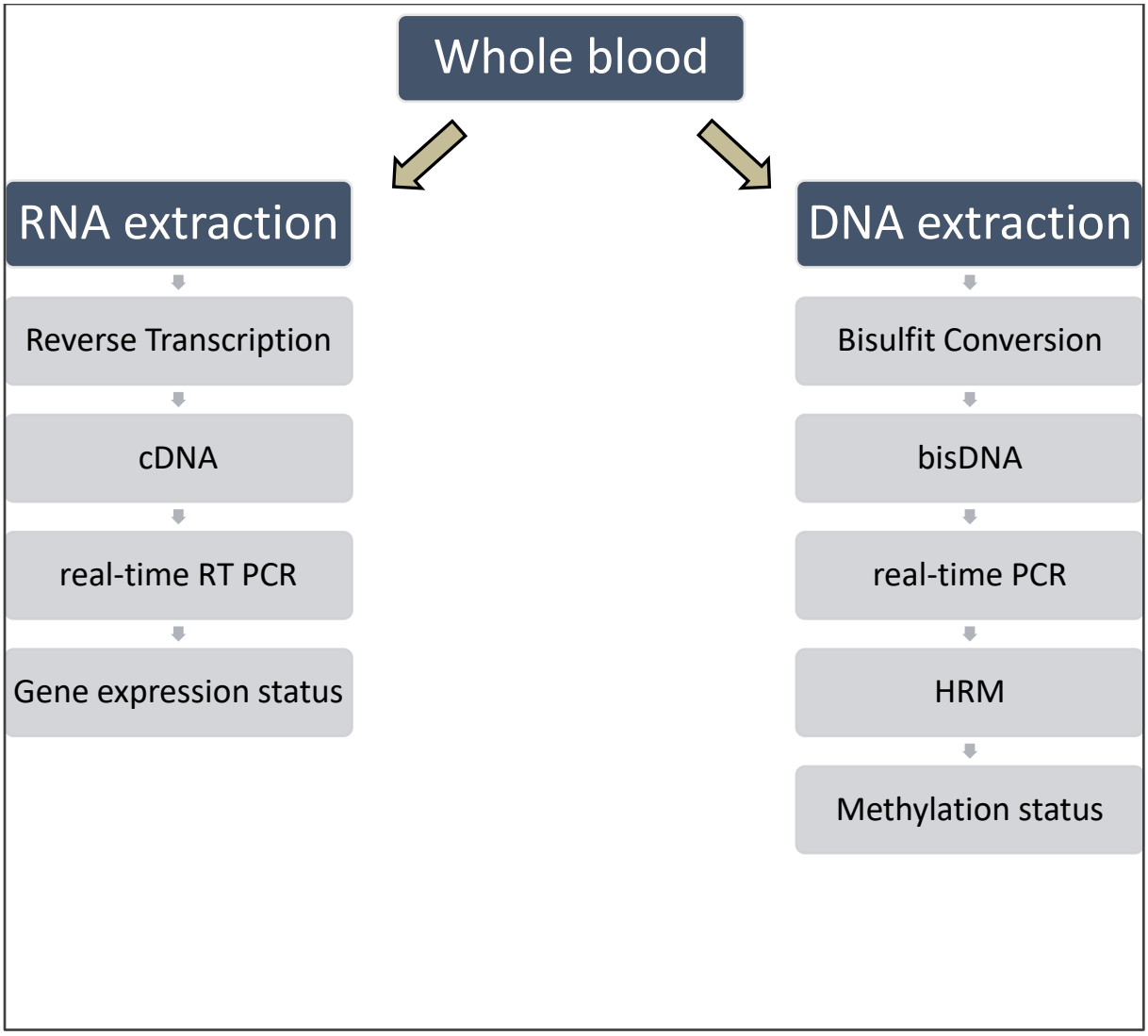


Figure 9: Overview of the performed analyses

5.2. Study design

The samples for my analysis derive from a study, that was conducted in 2012 in cooperation with Dr. Petra Rust and the clinic in Eggenburg, Lower Austria, Austria. In total 33 obese individuals were enrolled for the study with the aim of weight loss by individual dietary intervention in accordance with DACH references and an additional physical activity plan under medical guidance in the clinic in Eggenburg. All study participants gave written consent

for the use of the data from the food frequency questionnaire (appendix) and blood samples, in accordance with the declaration of the Viennese Human Ethics committee.

Inclusion criteria:

- BMI ≥ 30kg/m² without additional side effects
- Weight loss ≥5% of initial body weight during intervention

Exclusion criteria:

- Gastrointestinal diseases,
- Type 2 diabetes,
- The use of antibiotics, probiotics or prebiotics before and during the whole intervention period

At the end 20 of the study participants were enrolled for gene expression and methylation analysis. Mean weight at the beginning of the study was 125±30kg, mean BMI was 45±10kg/m².

5.3. Questionnaire about eating habits and life-style

To assess the participants eating and life-style habits, a questionnaire was filled out at the beginning and at the end of the study. The questionnaire contained questions about:

- Eating habits: amount and type of food that is consumed normally
- Smoking habits
- Intake of dietary supplements and fortified foods
- Drinking habits and amount of consumed alcohol and caffeine
- Physical activity like sports frequency and exercise

5.4. Sample collection

During the intervention study, human whole blood was sampled by venous puncture at three different time points:

-T1: at the beginning of the study (bevor any intervention)

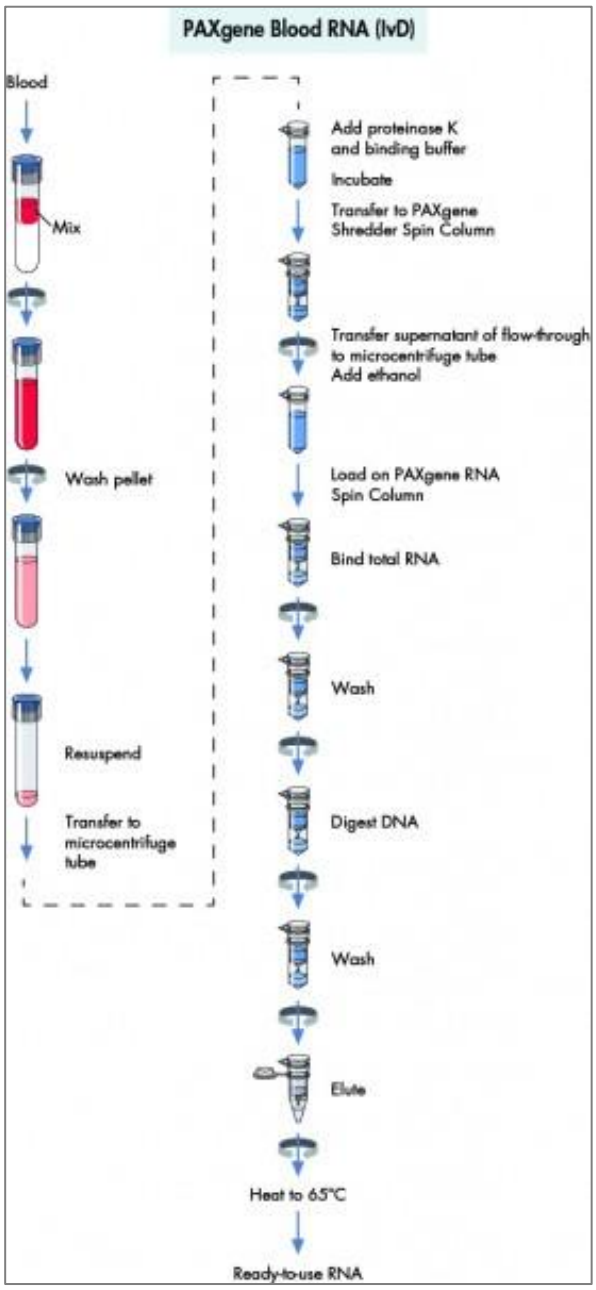
-T2: during the study

-T3: at the end of the study

Figure 10: Timetable of whole blood collection

For the analysis of gene expression and methylation status the samples of T1 and T3 were used, because the biggest effect was expected after the longest period of time. Human whole blood was collected with PAXgene blood RNA tubes (for gene expression analysis) and in PAXgene blood DNA tubes (for analysis of methylation status) and stored at -20°C until use.

5.5. RNA extraction



For gene expression analysis the RNA was extracted with Blood RNA kit (PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's protocol.

As there has been no gene expression analysis done with the samples before, the RNA had to be extracted out of PAXgene Blood RNA tubes.

The mechanism of RNA extraction is based on the following steps at figure 11

5.6. DNA extraction

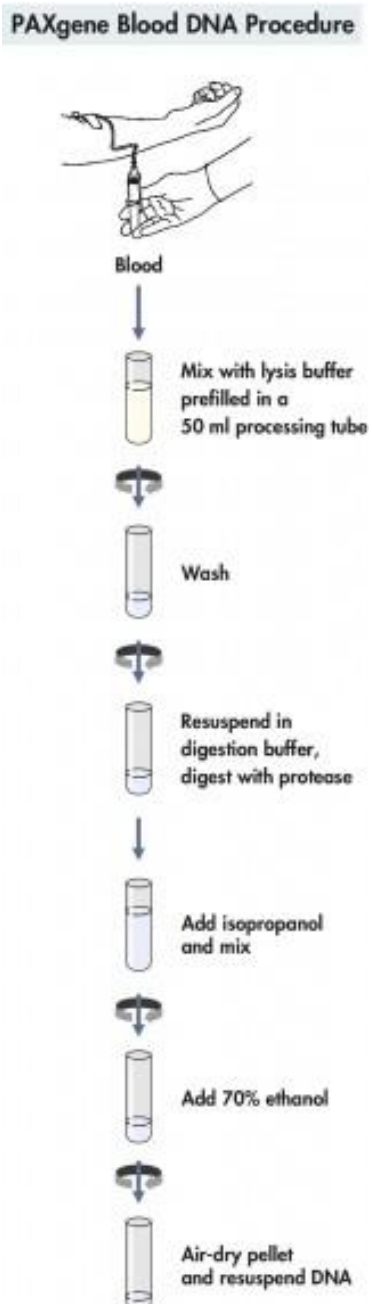


Figure 12: Protocol for DNA extraction

For methylation analysis the DNA was extracted of whole blood, collected with PAXgene blood DNA tubes, using PAXgene Blood DNA Kit (Qiagen, Germany). The extraction was performed following the instruction of the manufacturers protocol (figure 12).

5.7. Quantification of the samples

After whole blood extraction, the DNA and RNA contents were measured using a UV/Vis spectrometer. For determining the purity and concentration of DNA and RNA the absorbance was measured with Pico100 (Picodrop, Hinxton, United Kingdom) at 260nm.

5.8. Sample processing

5.8.1. Gene expression analysis

The following figure shows the systematic workflow, which is necessary to perform the gene expression analysis with qPCR.

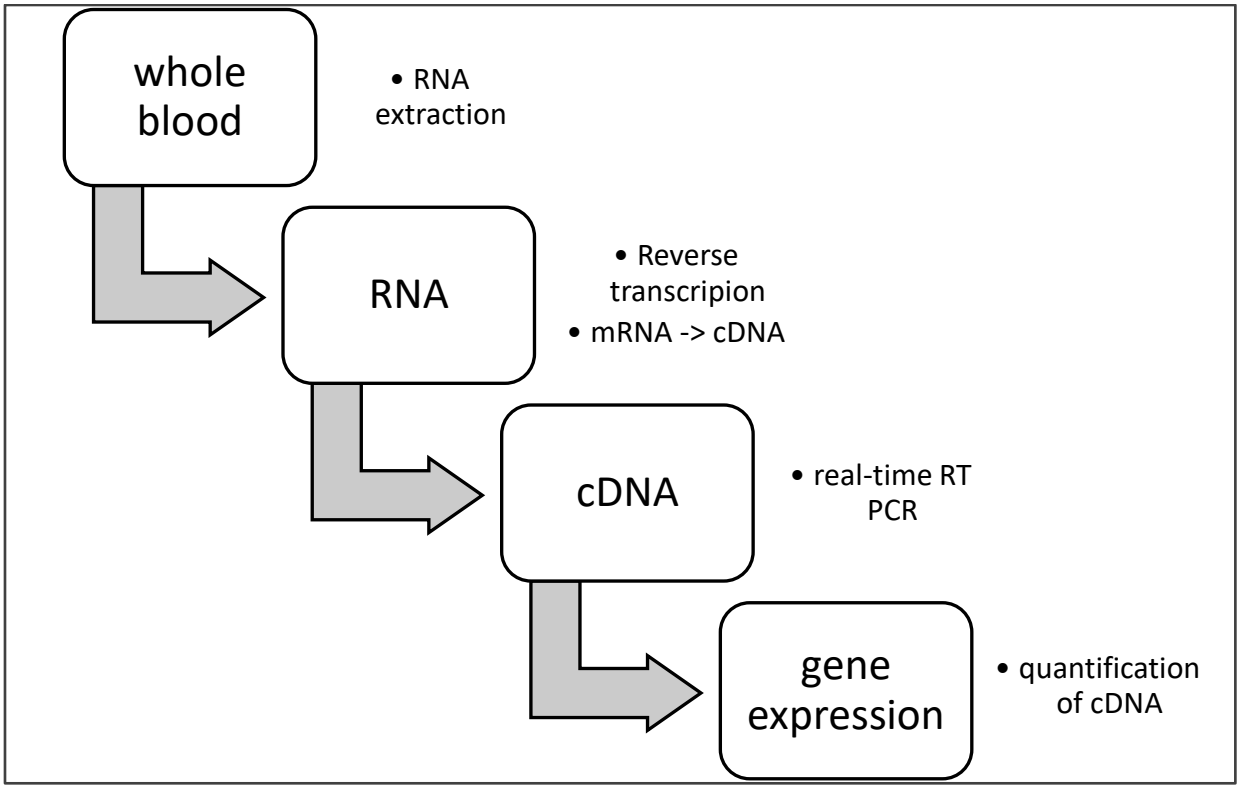


Figure 13: Overwiew: Sample processing for gene expression analysis

5.8.1.1. Reverse transcription

Since mRNA is very unstable, it is necessary to convert it into more stable copy-DNA(cDNA) using the mechanism of reverse transcription. For this the RT² First Stand Kit (Qiagen, Germany) with SYBR Green Mastermix was used. The reverse transcription was performed in order with the manufacturer's protocol.

After short centrifugation of the reagents, the genomic DNA elimination mix was prepared for each RNA sample according to the following table:

COMPONENT	AMOUNT (1 REACTION µL)
RNA	25ng-5µg
BUFFER GE	2µl
RNASE-FREE WATER	Variable
➔ **TOTAL VOLUME**	10µl

Table 1: DNA elimination mix

After incubation of the DNA elimination mix for 5 min at 42°C the samples were immediately cooled on ice for at least 1 minute.

Next the reverse transcription mix was prepared for each sample according to the following table. In total, a surplus of 1 reaction was added.

COMPONENT	VOLUME FOR 1 REACTION
5X BUFFER BC3	4µl
CONTROL P2	1µl
RE3 REVERSE TRANSCRIPTASE MIX	2µl
RNASE-FREE WATER	3µl
➔ **TOTAL VOLUME**	10µl

Table 2: Reverse transcription mix

10µl of each sample containing genomic DNA was prepared in a suitable tube. Now 10µl of the prepared reverse transcription mix was added to each sample.

After incubation for exactly 15 minutes at 42°C the reaction was stopped by incubating the samples at 95°C for 5 minutes.

Next, 91µl RNase-free water was added to each sample before giving them into the thermal cycler under following conditions:

Conditions thermal cycler:

CYCLES	DURATION	TEMPERATURE
1	10min	95°C
40	15s	95°C
	1min	60°C

Table 3: Conditions thermal cycler for performing reverse transcription

As we used an Applied Biosystems thermal cycler, the recommended conditions for this system were used, according to the manual's protocol.

The samples were stored at -20°C until use.

5.8.1.2. Primers for MLH1, DNMT1 and IL-6

RT² qPCR PrimerAssay (Qiagen, Germany) were used for analysing the genes MLH1, DNMT1 and IL-6.

	GENE	CATALOG NUMBER
RT²QPCR PRIMER ASSAY FOR HUMAN	GAPDH	PPH00150F
RT²QPCR PRIMER ASSAY FOR HUMAN	MLH1	PPH00196F
RT²QPCR PRIMER ASSAY FOR HUMAN	DNMT1	PPH01055F
RT²QPCR PRIMER ASSAY FOR HUMAN	IL-6	PPH00560C

Table 4: PrimerAssays used for the gene expression analysis

5.8.1.3. Real-time reverse transcription polymerase chain reaction

The gene expression analysis was made by using a StepOne Plus real-time PCR system (Applied Biosystems). This method allows the continuous monitoring of the amplification process of the gene of interest. Therefore, the assay is based on the measurement of the added fluorescent dye, which correlates to the amount of DNA inside the sample. For the later

analysis of the gene expression data the threshold cycle (Ct) is important. This is the PCR cycle with whom the signal rises over the threshold background level. The higher the messenger RNA (mRNA) of the gene of interest, the lower the Ct value (30).

The principle of the method is based on three steps:

1. The conversion of the mRNA into more stable cDNA by reverse transcription
2. Amplification of the DNA sequence of interest
3. Quantification and detection of the amount of amplicons

The real-time reverse transcription polymerase chain reaction was performed for the genes DNMT1, MLH1 and IL-6. As a reference gene the housekeeping gene GAPDH was used. The total reaction volume was 25µl and qPCR was performed as followed:

COMPONENT	**VOLUME FOR 1 REACTION (µL)**
-RT SYBR GREEN MASTERMIX	12,5
-CDNA SYNTHESIS REACTION	1
-RT² QPCR PRIMER ASSAY	1
-RNASE FREE WATER	10,5
→ **TOTAL VOLUME**	25µl

Table 5: Real time reverse transcription PCR

5.8.1.4. Calculation of the gene expression.

After running the gene expression analysis the expression levels of the genes were evaluated by using the ΔΔ$C_T$methode. At first the C_T-values of GAPDH, MLH1, DNMT1 and IL-6 were determined by qPCR. Next, the ΔC_T and the ΔΔC_T-values were calculated as followed:

ΔC_T(control):	C_T(gene of interest) - C_T(housekeeping gene)
ΔC_T(experimental):	C_T(gene of interest) - C_T(housekeeping gene)
ΔΔC_T:	ΔC_T(experimental)- ΔC_T(control):
Fold-change	$2^{-\Delta\Delta CT}$

Figure 14: Control: before weight loss; experimental: after weight loss; housekeeping gene: GAPDH; gene of interest: DNMT1, MLH1, IL-6.

5.8.2. Methylation analysis

For the analysis of the methylation level of the samples, a high resolution melting curve was conducted. With this method the global methylation level of all CpG's in one gene can be measured. Therefore, the extracted DNA was used, which had to be bisulfite converted before the analysis.

5.8.2.1. Bisulfite conversion

Before the methylation status of the samples can be analysed, the DNA has to be bisulfite converted by using EpiTect Fast DNA Bisulfite Kit (Qiagen, Germany). During this process the samples are treated with high sodium bisulfite concentrations in the presence of low ph, which leads to the conversion of the unmethylated cytosine into uracil. This change allows the easy detection of the methylation pattern of the gene of interest (31).

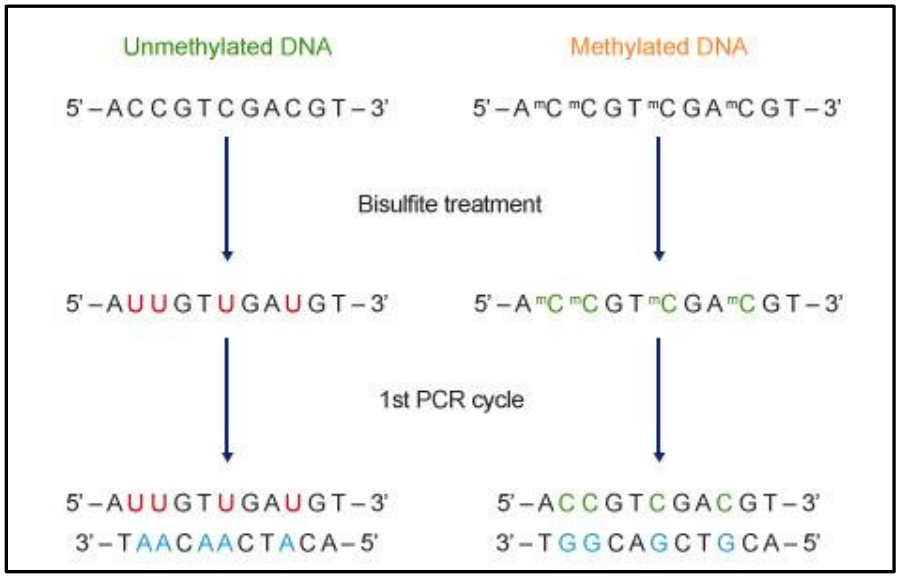


Figure 15: Difference between methylated and unmethylated DNA (http://www.abcam.com/epigenetics/dna-methylation-a-guide)

5.8.2.2. Primers for MLH1, DNMT1 and LINE1

MLH1:

Assay	Sequence to analyse 5'-3'	Size (bp)	GC-content (%)
MLH1 forward	ttttttttaggagtgaaggagg	21	38,0%
MLH1 reverse	aacrccactacraaactaaa	20	35,0%

DNMT1:

Assay	Sequence to analyse 5'-3'	Size (bp)	GC-content (%)
DNMT1 forward	ggtatcgtgtttatttttttagtaa	24	25,0%
DNMT1 reverse	acgaaaccaaccatacccaa	20	45,0%

LINE1:

Assay	Sequence to analyse 5'-3'	Size (bp)	GC-content (%)
LINE1 forward	ttttgagttaggtgtgggatata	23	34,0%
LINE1 reverse	aaaatcaaaaaattccctttc	21	23,0%

5.8.2.3. High resolution melting curve methylation analyses

5.8.2.4. Principle of the method

High resolution melting curve is a relatively new, post-pcr analysis, that allows the measurement of the global methylation status of the genes of interest. Additionally, this method provides further information of the analysed samples as for example purity and amount of DNA. A further advantage of this method is the small DNA amount that is needed.

By adding a double helix-detecting dye before performing the PCR, the measurement of the melting DNA is possible. As melting is a fundamental property of DNA, the heating of the

samples during the analysis leads to the separation of the double-helix into two single strands. During this separation the added dye gets free and leads to a measurable fluorescent signal (32). By adding methylation standards with different methylation levels, it is possible to calculate a standard curve with whose help the methylation of the samples can be estimated.

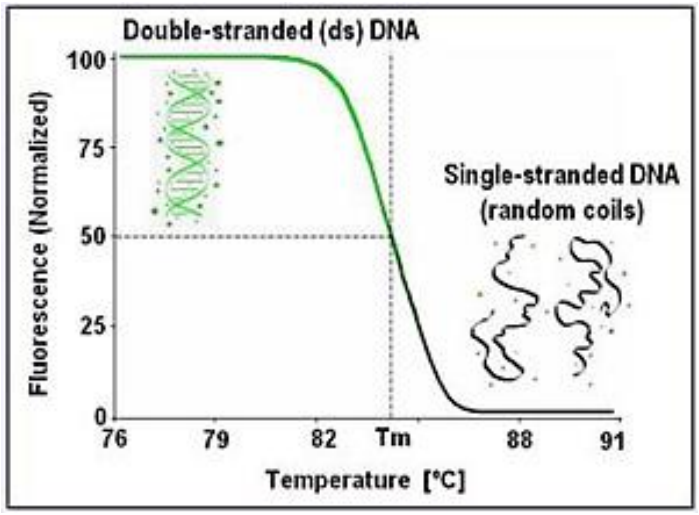


Figure 16: Overview high resolution melting curve (https://www.dna.utah.edu/Hi-Res/TOP_Hi-Res%20Melting.html)

5.8.2.5. Performance of HRM

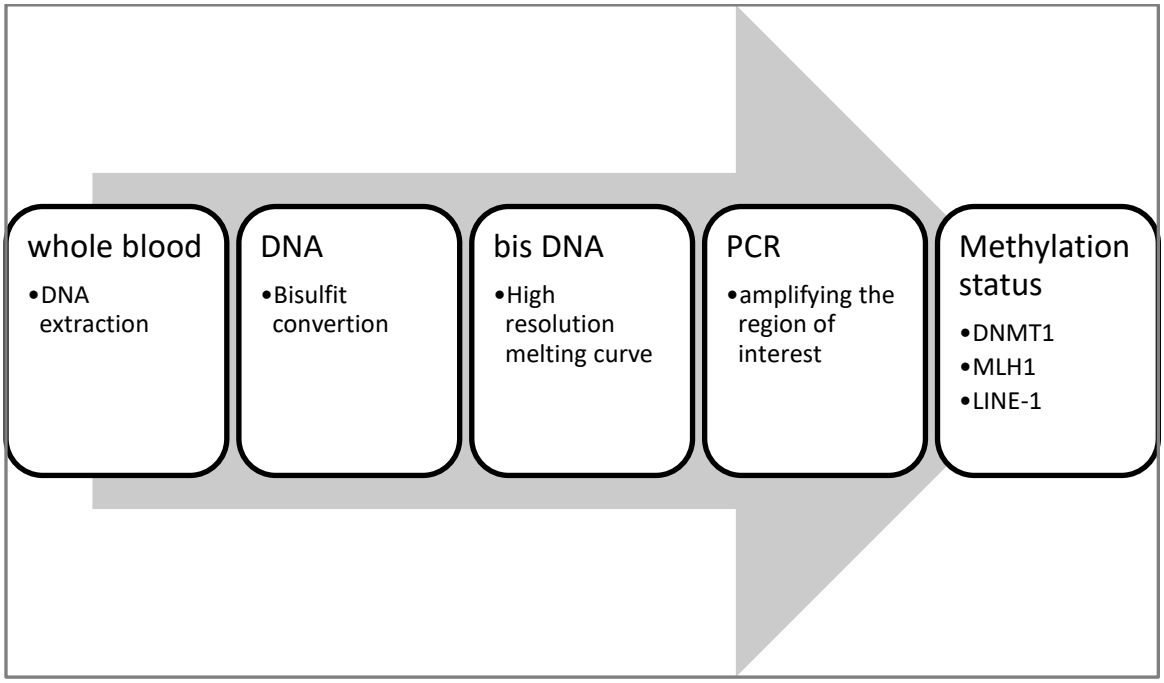


Figure 17: Overview: schematic procedure for the methylation analysis

For the analysis of the global methylation levels a high resolution melting curve was performed with the Rotor Gene Q (Qiagen, Germany). For all 3 genes a total reaction volume of 10µl was used. For every analysis a surplus of 10% was calculated and added. For the conduction of the analysis a HRM PCR mastermix, primer forward, primer reverse and nfH2O were necessary and mixed as described in the following tables:

5.8.2.5.1. DNMT1:

COMPONENT	**VOLUME FOR 1 REACTION (µL)**
-HRM PCR MM	5
-PRIMER FORWARD	0,075
-PRIMER REVERSE	0,075
-NFH_2O	3,734
- Mg_2Cl (25MM)	0,116 +1ul DNA template

Table 6: Pipetting scheme of DNMT1- Methylation analysis

Cycling conditions DNMT1:

Cycling conditions DNMT1

95°C	5min	
95°C	15s	
50°C	30s	40 cycles
72°C	20s	
95°C	1min	
40°C	1min	

Table 7: Cycling conditions DNMT1- Methylation analysis

The high resolution melting curve (HRM) was performed at 55°C-95°C, 0,2°C steps, 2s waiting after each step and gain optimisation.

The normalisation range was set between 74,06-74,65°C; 82,76-83,33°C.

5.8.2.5.2. LINE-1

COMPONENT	VOLUME FOR 1 REACTION (µL)
-HRM PCR MM	5
-PRIMER FORWARD	0,075
-PRIMER REVERSE	0,075
- NFH_2O	3,85
	+1µl DNA template

Table 8: Pipetting scheme of LINE-1- Methylation analysis

Cycling conditions LINE-1:

Cycling conditions LINE-1

95°C	5min	
95°C	15s	45 cycles
54°C	40s	
72°C	25s	
95°C	1min	
45°C	1min	

Table 9: Cycling conditions LINE-1- Methylation analysis

The high resolution melting curve (HRM) was performed at 65°C-95°C, 0,2°C steps, 2s waiting after each step and gain optimisation.

The normalisation range was set between 70,5-71°C; 79-79,5°C.

5.8.2.5.3. MLH1

COMPONENT	VOLUME FOR 1 REACTION (µL)
-HRM PCR MM	5
-PRIMER FORWARD	0,05
-PRIMER REVERSE	0,05
- NFH_2O	3,9
	+1ul DNA template

Table 10: Pipetting scheme of MLHT1- Methylation analysis

Cycling conditions MLH1:

Cycling conditions MLH1		
95°C	5min	
95°C	15s	
50°C	30s	40 cycles
72°C	20s	
95°C	1min	
40°C	1min	

Table 11: Cycling conditions MLH1- Methylation analysis

The high resolution melting curve (HRM) was performed at 55°C-95°C, 0,2°C steps, 2s waiting after each step and gain optimisation. Unfortunately, the DNA-quality of the used samples was too poor for a successful methylation analysis. This was confirmed by a 2% agarose gel electrophoresis.

5.8.2.5.4. Controls for HRM

For the analysis of the methylation level of the samples, unmethylated and fully methylated control samples are necessary for creating a standard curve. 100% methylated and 0% unmethylated control samples were generated by our laboratory. The 25%, 50% and 75% methylated controls were made by mixing 0% methylated control with 100% as following:

STANDARD (METHYLATION)	PARTS OF 0% METHYLATION STANDARD	PARTS OF 100% STANDARD
25%	3	1
50%	1	1
75%	1	3

Table 12: Pipetting scheme for the Methylation standards

The standards were used with a concentration of 5 ng/µl.

The real methylation levels of the standards were assessed by analysis of the methylation with PyroMark Q24 (Qiagen Germany):

0%: 8,7% methylation

100%: 80% methylation

According to this, the standard curve for each gene was generated with the following data:

METHYLATION STANDARD	REAL METHYLATION
0%	8,7%
25%	26,5%
50%	44,4%
75%	62,2%
100%	80%

Table 13: Real methylation levels of the methylation standards

The data were analysed with the RotorGene Q series 2.1.3. software. Next the data were exported into Excel where they were evaluated. For generating the standard curve, the real methylation values were used. Each sample was normalised by using a positive control sample.

5.9. Statistical analysis

The statistical analysis was done with SPSS Statistics Version 23 (IBM, New York). All samples were analysis as duplicate and the mean values and standard deviation will be calculated.

Comparison within a group between the time-points will be done with hypothesis test for dependent samples. Statistical significance was considered at $p \leq 0,05$.

6. Results

6.1. Mean weight loss during the intervention

The mean weight loss during three to four month of dietary intervention was 8±4,5kg. The mean BMI changed from 45,1 kg/m² before intervention to 42,3 kg/m² after intervention.

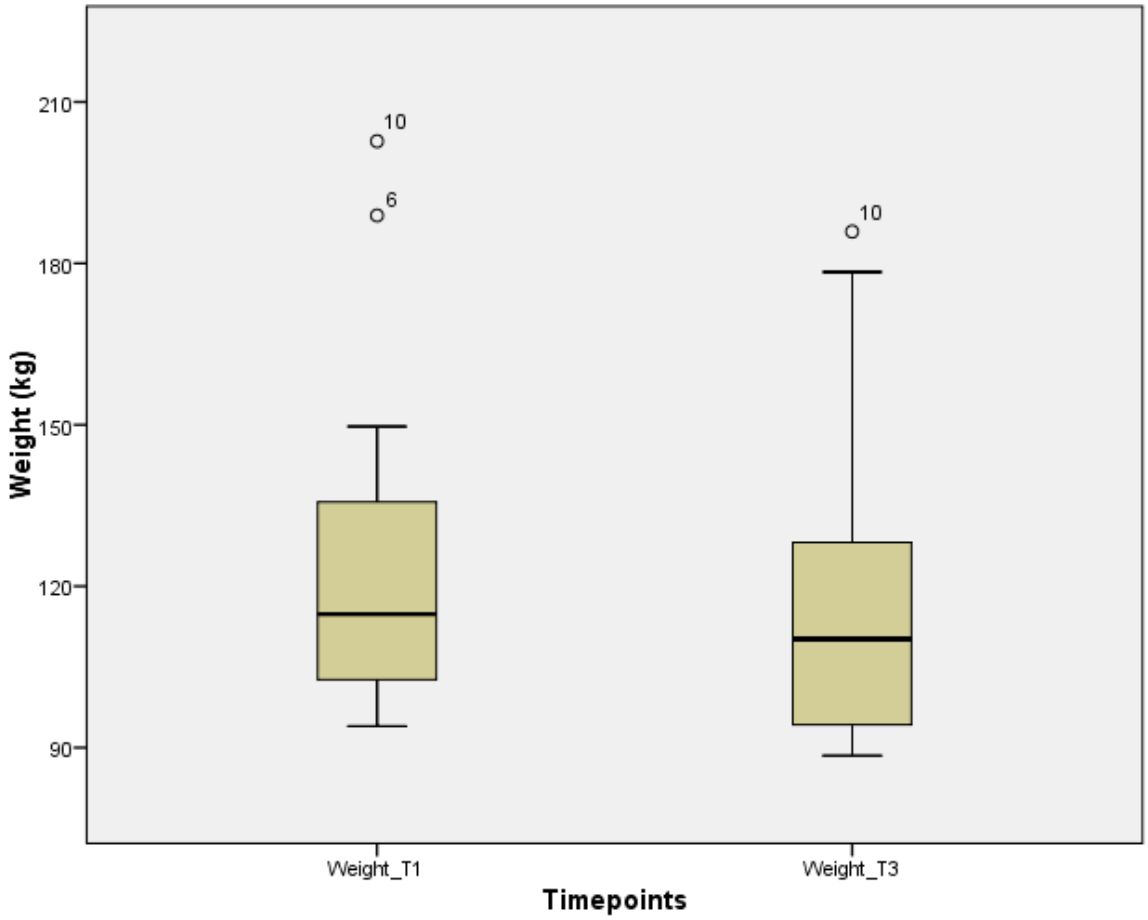


Figure 18: Boxplot body weight before and after intervention

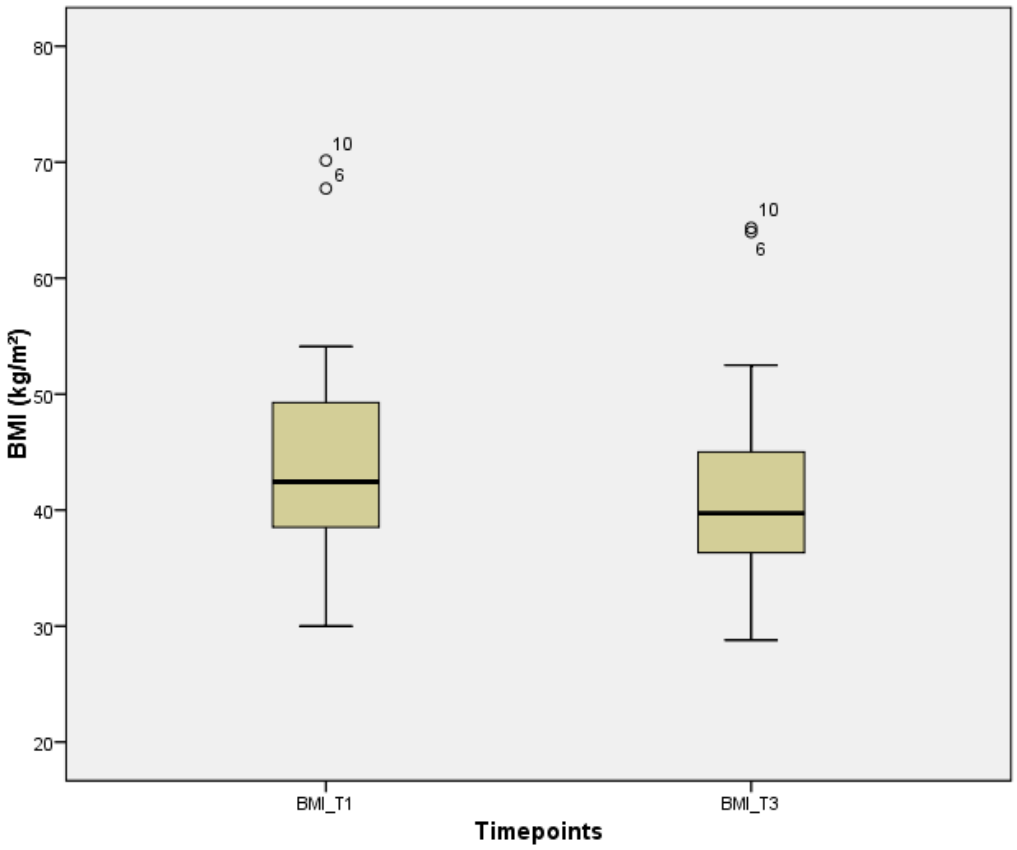


Figure 19: Boxplot BMI before and after intervention

6.2. Gene Expression

6.2.1. Picodrop RNA concentration

Mean RNA concentration of the samples was 34.7±16.9 ng/µl.

6.2.2. DNMT1 gene expression

The mean ΔC_T-values differ between the two timepoints. ΔC_TT1:5.14, ΔC_TT3:5.4.

The fold change is 0.956, which means, that the expression of DNMT1 after the weight loss period is about 5% lower than before weight loss. This result is statistically not significant (p=0.192).

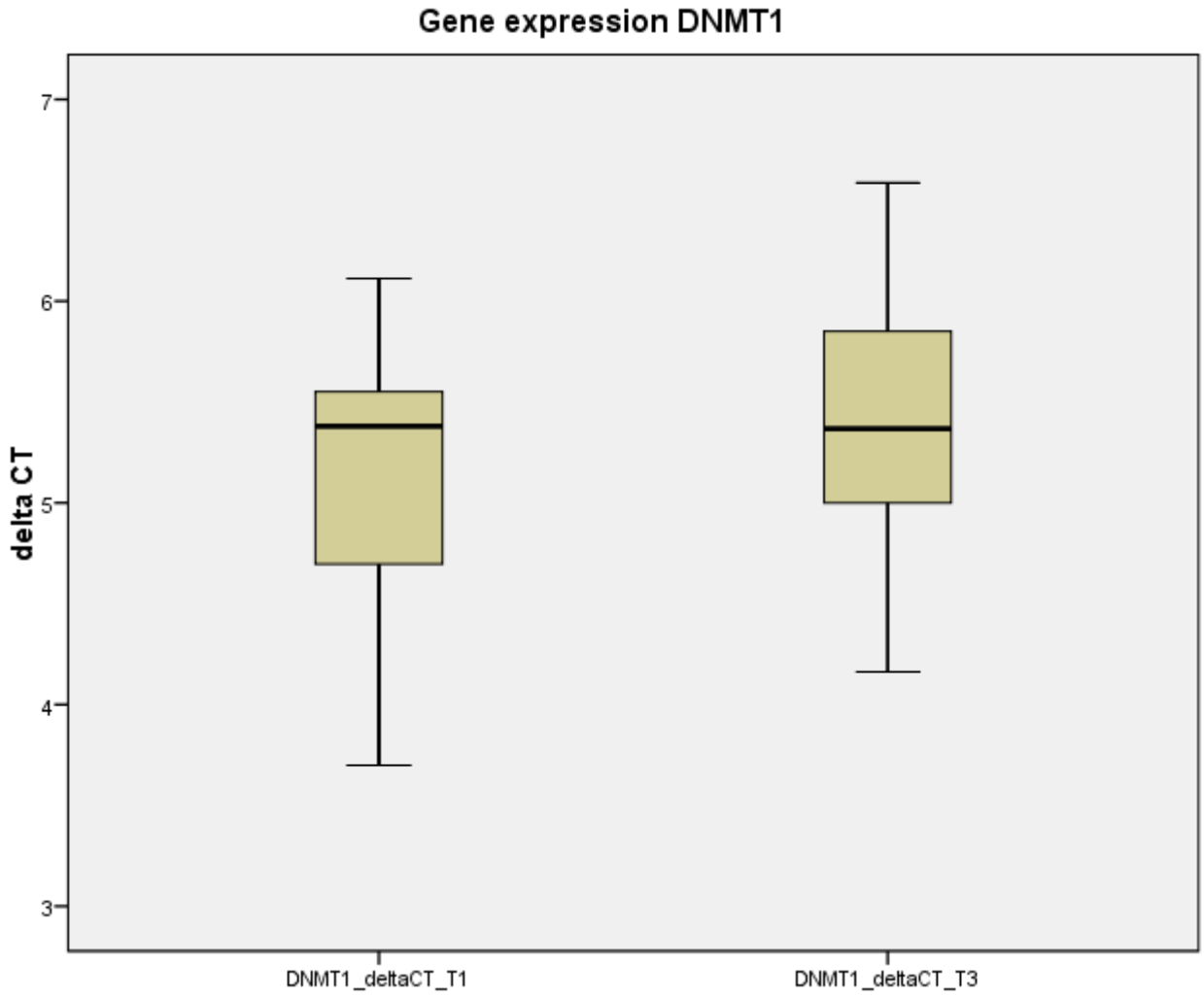


Figure 20: Result: Gene expression DNMT1

6.2.2. MLH1 gene expression

In case of MLH1 expression there is no difference between the two timepoints. The mean ΔC_T-values do not differ between the two timepoints: ΔC_TT1:3.84, ΔC_TT3:3.93

The fold-change is 1.06%, which means, that MLH1 after weight loss is about 0.6% higher expressed. This result is statistically not significant (p=0.351).

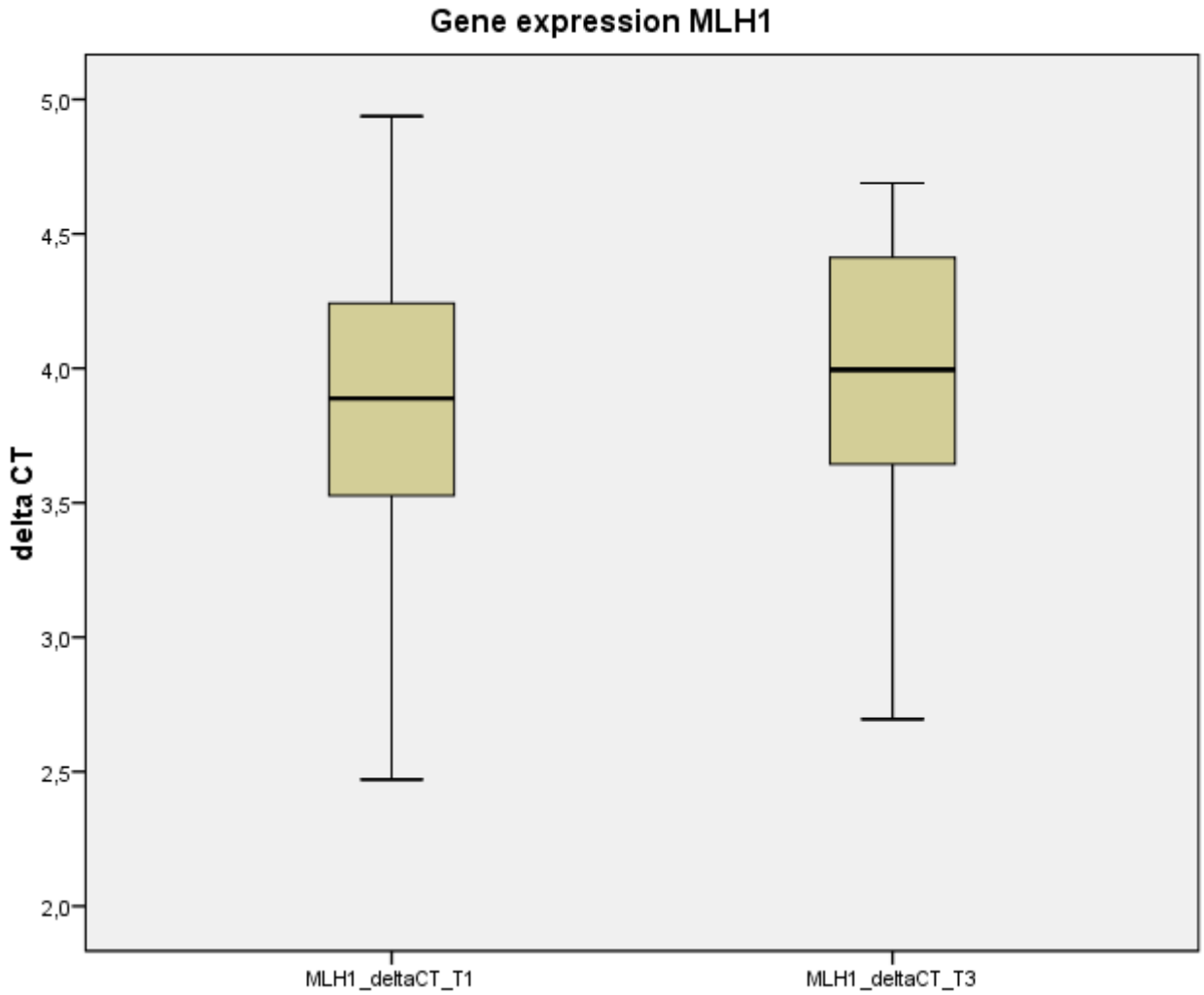


Figure 21: Result: Gene expression MLH1

6.2.3. IL-6 gene expression

Only 24 of the 40 samples showed evaluable results after conducting the qPCR others were under the detection limit.

The fold change of IL-6 expression between the two different points of time is 1.74, which means, that the expression of IL-6 after weight loss is about 74% higher.

The mean ΔC_T-values between the two points of time do not differ significantly: ΔC_TT1:10.82, ΔC_TT3:11.22 (p=0.53).

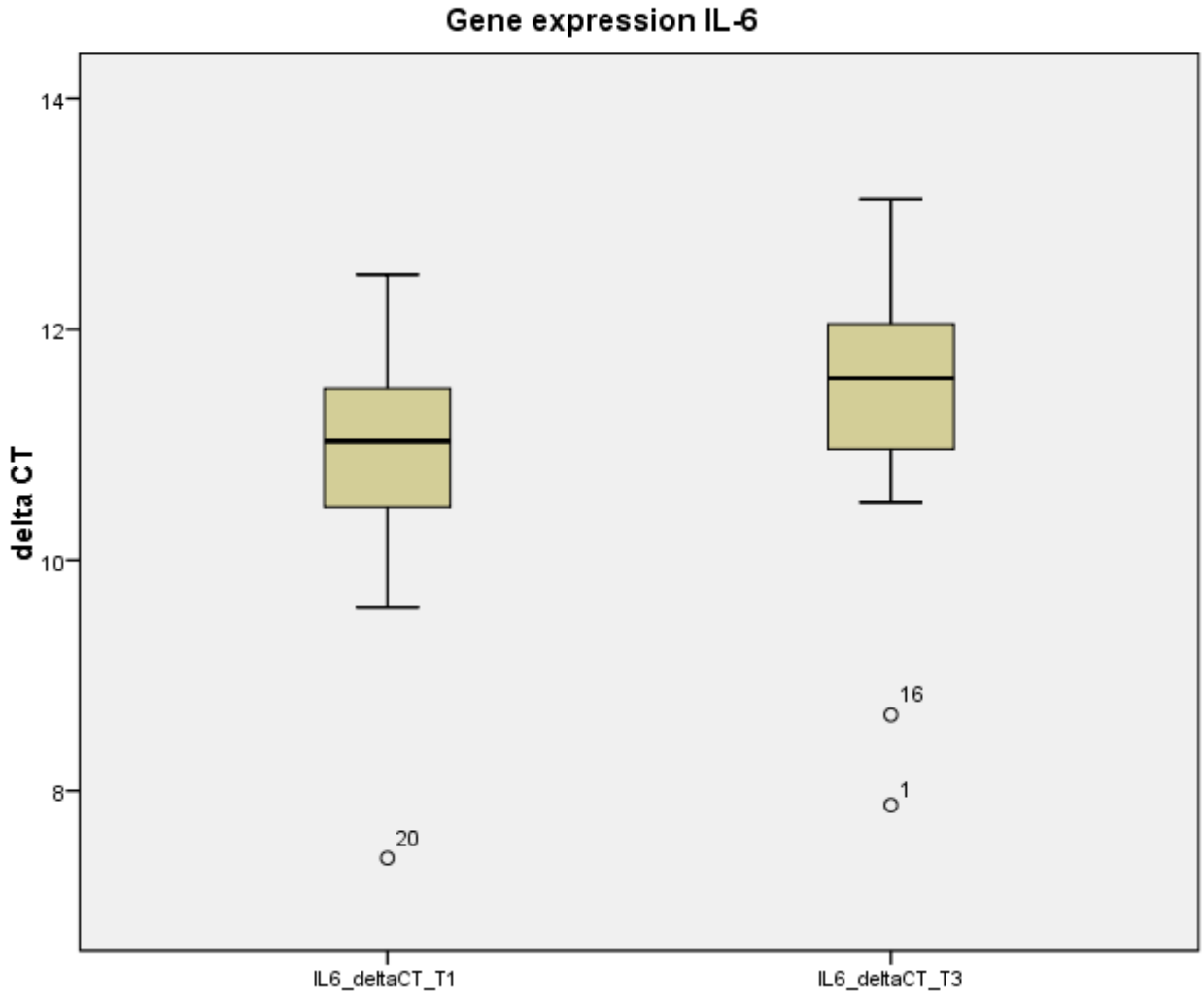


Figure 22: Result: Gene expression IL-6

6.3. Methylation analysis

6.3.1. Picodrop DNA concentration

Mean DNA concentration of the samples was 286.3±153.1 ng/µl.

6.3.2 DNMT1 methylation

In comparison of the two points of time T1 (before weight loss) and T3 (after weight loss), the mean methylation of DNMT1 was 0.20% lower. This result shows a non-statistically significant difference before and after weight reduction (p=0.157).

The mean methylation before intervention was 8.6±1.1% and after intervention 8.4±1.5%.

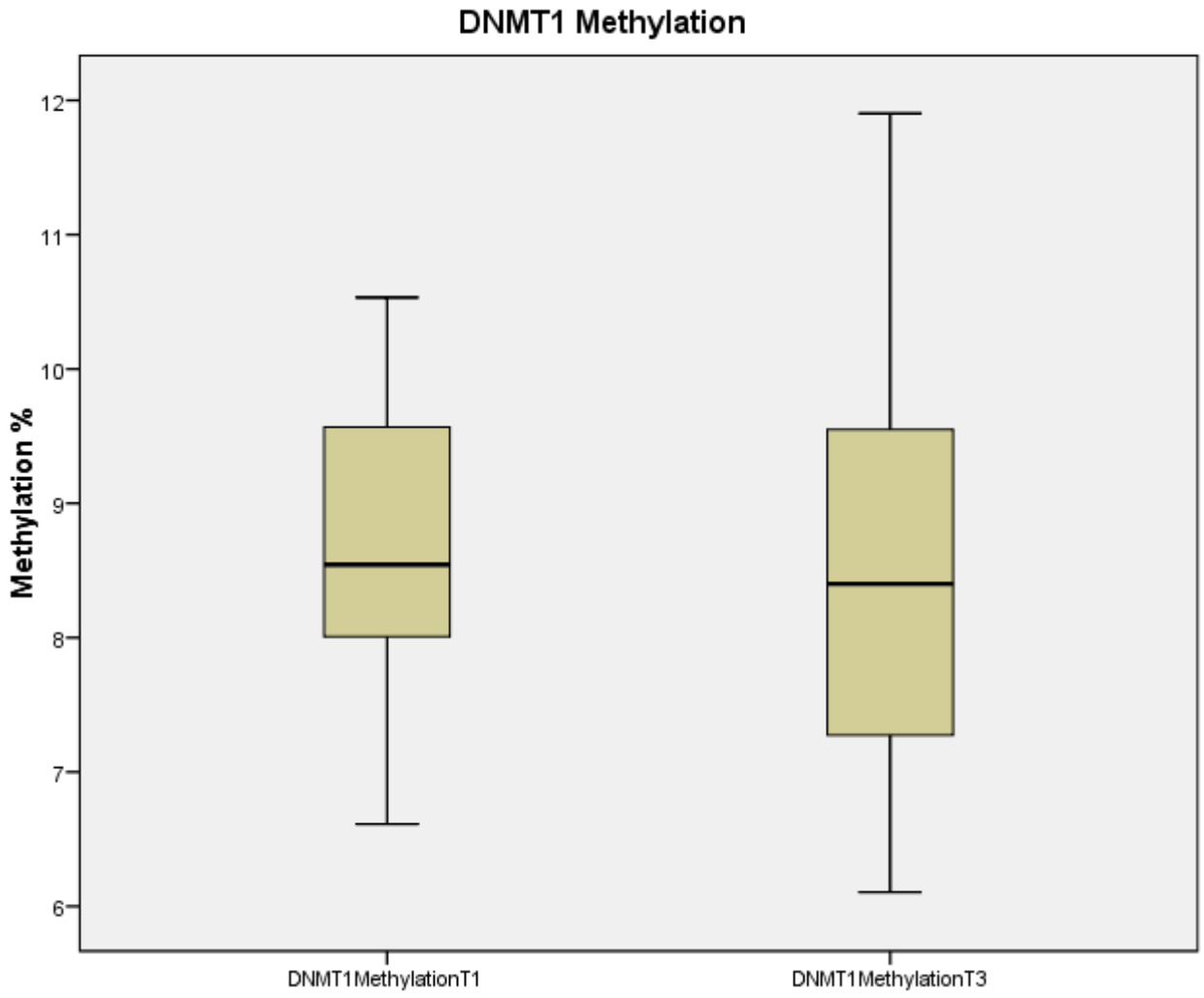


Figure 23: Result: Methylation analysis DNMT1

6.3.3. LINE-1 methylation

In comparison of the two points of time T1 (before weight loss) and T3 (after weight loss), the mean methylation of LINE-1 was after weight reduction 2.2% lower.

The mean methylation before intervention was 38.2±6.1% and after intervention 36.0±4.8%.

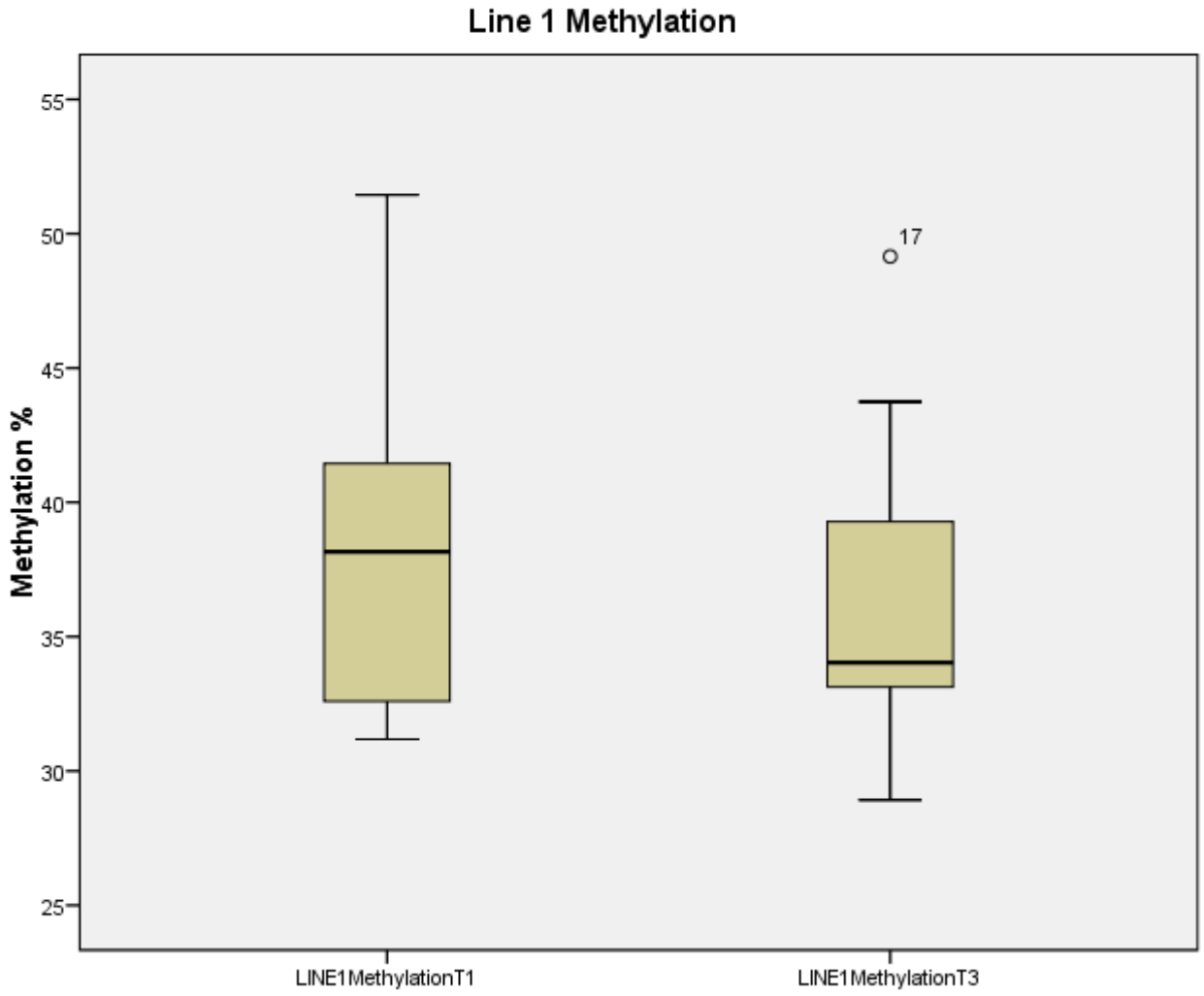


Figure 24: Result: Methylation analysis LINE-1

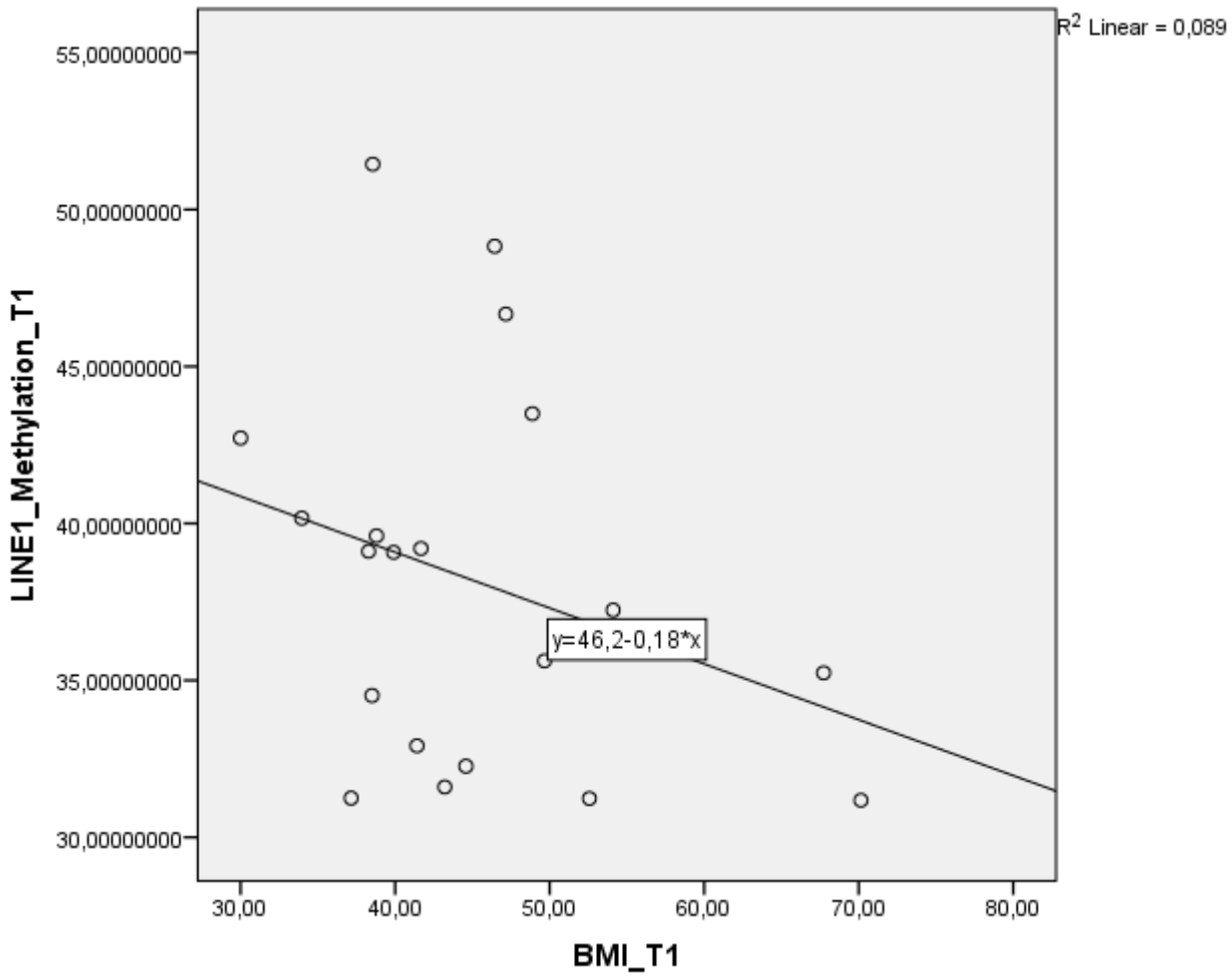


Figure 25: Correlation between LINE-1 and BMI

Furthermore, this study could observe, that the LINE-1 methylation decreases with increasing BMI. This observation has also already been shown by several other studies.

6.3.4. MLH1 methylation

Due to the poor DNA-quality of the samples, it was not possible to perform a successful methylation analysis for MLH1. After 2 unsuccessful runs with the HRM, a 2% agarose-gel electrophoresis for genomic DNA was conducted for confirmation of the insufficient DNA-quality.

7. Discussion

7.1. Weight loss did not influence gene expression and methylation level of DNMT1, MLH1 and LINE-1 significantly

There was no statistical significant difference of gene expression and methylation level before and after weight loss intervention for the genes MLH1, DNMT1 and LINE-1 detectable. In case of DNMT1 a higher methylation level of the promoter region causes a lower expression of this gene. The methylation analysis of DNMT1 did not show any change, but anyway the gene expression decreased after the intervention. Although the results of this study are not statistically significant, the results indicate the opposite of earlier findings from other studies.

As other studies conducted in a mouse model reported a decrease of DNMT1 methylation after weight gain, the opposite, namely an increased methylation on this gene was expected in this study (33).

Other studies showed, that there is a connection between DNMT1 and the DNA mismatch repair system: Loughery et al investigated the connection between these two compounds and came to the conclusion, that a deficiency of DNMT1 also triggers defects in the human mismatch repair system (34).

Furthermore, Xia et al showed, that the methylation level of human DNMTs may be connected to the expression of leptin during the development of obesity (33).

The expression of the repair gene MLH1 after weight loss was about 6% higher than before (fold-change: 1.06). This result suggests that the human body is after weight loss intervention in a higher need of repair mechanisms due to changes in the body composition. A sufficient operating mismatch repair system is very important, as it has been shown, that a loss of MLH1 activity appears in many patients suffering from endometrial cancer (35). On the contrary, an overexpression of MMR is linked to prostate cancer and may lead to poor outcome, like Wilczak et al showed in a recent study (36).

As the methylation analysis did not improve after new bisulfite conversion of the samples, we suggest, that the DNA double helix of most of the samples was broken in-between our region

of interest for the gene MLH1. The gel electrophoresis showed an intense band in a low kbp region, which would prove our assumption.

LINE-1 methylation levels were about 2.2% lower after the intervention, which indicates, that this gene became more unstable and may furthermore play a role in interrupting other genes. A new study conducted by Garcia-Lacarte et al. investigated the eligibility of LINE-1 as a possible biomarker for the individual response to a hypocaloric diet. As a result, they could observe, that high responder (lost more than 8% of the bodyweight due to the weight loss intervention) had a significantly higher LINE-1 methylation levels as the low responders. Furthermore, they concluded, that a methylation level >84.15% may be used as a predictive threshold above which individuals may response positively to a weight loss intervention. As the methylation level in this analysis are much lower, the findings of Garcia-Lacarte et al. can only be assigned in a limited way. A possible explanation may be, that Garcia-Lacarte et al. used peripheral blood mononuclear cells (PBMC's) compared to our whole blood sample (38). Another study from Turcot et al observed that the LINE-1 methylation levels in visceral adipose tissue (VAT) are associated with the risk of development of metabolic syndrome. They came to the conclusion, that lower levels of global methylation are a risk factor for the development of MetS in obese patients. However, in this study the mean methylation levels of the samples were higher (mean methylation level: 75%) than the one measured in our experiment (mean methylation level:38%) (39).

Our results are conform with the ones from Druggan et al, which also did not observe a statistically significant difference in the LINE-1 methylation level in peripheral blood leucocytes in overweight women after weight loss (40).

7.2. Weight loss induced a higher expression of pro-inflammatory IL-6

Only 24 of the 40 samples showed evaluable results after qPCR, the other samples were under the detection limit. The mean fold-change of these samples was 1.74. This result means, that the expression of IL-6 after weight-loss intervention was about 74% higher than before. This findings accord to ones from Marques et al, where the mean fold-change also rose after an 8 week weight loss intervention (1). The methylation levels of different IL-6 CpGs have already been determined in earlier investigations by our laboratory. As a result, the methylation of three CpGs was higher after weight loss intervention.

A recent study from Na et al also showed the connection between obesity an IL-6 methylation level: They divided the study population into three groups, namely normal weight, overweight and obese. The obese group showed the highest methylation levels of all groups (37).

8. Limitations

The main limitation of this analysis is the age and the inadequate storage of the whole blood samples inside the PAXGene Blood RNA tubes, which influenced the DNA-quality. As the blood was collected 5 years ago, the tubes were stored in different refrigerators and temperatures and remained until the extraction for my analysis inside the tubes.

Furthermore, the sample size of the study is very small and for significant results insufficient.

9. Conclusion

The results of this study suggest that a weight loss intervention has no significant influence on the gene expression of DNMT1 and MLH1. This means, that neither the DNA-methyltransferases, nor the DNA mismatch repair system are affected by the loss of weight in obese patients.

In case of the pro-inflammatory cytokine IL-6, the recent available data suggest, that a weight loss intervention in metabolic syndrome patients leads to an elevated expression.

A weight loss intervention in metabolic syndrome patients may have an impact on the methylation level of LINE-1 and furthermore on the whole genome methylation. But therefore further research is needed.

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11. Appendix

11.1. Manuals

11.1. DNA Extraction

PAXgene Blood DNA Kit

Protocol: Purification of Genomic DNA from Human whole Blood Collected into PAXgene Blood DNA Tubes (status: May 2016)

The PAXgene Blood DNA Kit is used for purification of genomic DNA from 8.5 ml of human whole blood, collected in PAXgene Blood DNA Tubes (cat. no. 761115 [USA and Canada only], 761105 [Japan only] or 761125 [all other countries]). PAXgene Blood DNA Tubes and the PAXgene Blood DNA Kit are an integrated system for collection of whole blood and isolation of genomic DNA.

Important note before starting

All centrifugation steps should be carried out at room temperature (15-25°C) in a swing-out rotor.

Things to do before starting

- Thaw frozen PAXgene Blood DNA Tubes in a wire rack at ambient temperature (18-25°C) for approximately 2 hours or at 37°C in a water bath for approximately 15 minutes. Carefully invert the thawed PAXgene Blood DNA Tubes 10 times before beginning the procedure.
- Heat a heating block or water bath to 65°C for use in steps 8 and 17.
- Add 1.4 ml Buffer BG4 (resuspension buffer) to lyophilized PreAnalytiX Protease. Dissolved PreAnalytiX Protease should be stored at 2-8°C or in aliquots at -20°C (see “Storage”, page 4).
- For every sample, mix 5 ml Buffer BG3 (digestion buffer) and 50 µl reconstituted PreAnalytiX Protease. For example, to process 10 samples, mix 50 ml Buffer BG3 with 500 µl PreAnalytiX Protease. The Buffer BG3-PreAnalytiX Protease mixture should be prepared immediately before the start of the procedure.

Procedure

1. Pour all the blood from one PAXgene Blood DNA Tube into a Processing Tube containing 25 ml Buffer BG1. Close the tube. To avoid cracking the blue lids of the Processing Tubes, do not overtighten them. Tighten the lid only until the first sign of resistance is felt. Mix by inverting the tube 5 times. If the blood in the PAXgene Blood DNA Tube has separated into plasma and red blood cells, invert the tubes carefully 10 times to homogenize the sample.
2. Centrifuge for 5 min at 2500 x g in a swing-out rotor.
3. Carefully discard the supernatant and place the tube in a rack. In rare cases the pellet may be loose, so pour slowly.
4. Add 5 ml Buffer BG2, close the tube, and wash the pellet by vortexing vigorously for 5 s.
5. Centrifuge for 3 min at 2500 x g in a swing-out rotor.
6. Carefully discard the supernatant and place the tube back in the rack. In rare cases the pellet may be loose, so pour slowly.
7. Add 5 ml Buffer BG3/PreAnalytiX Protease (see “Things to do before starting”), close the tube, and vortex for 20 s at high speed. Vortexing for 20 s is essential to dissolve the

pellet completely. Shorter vortexing times may lead to incomplete resuspension of the pellet and reduced DNA yield or purity.
After this step, samples can be stored for at least 7 days at 2-8°C. After storage, resume the procedure at step 8.

8. Place the tube in a heating block or water bath and incubate at 65°C for 10 min. The sample changes color from light red to light green, indicating that protein digestion has occurred.
9. Vortex again for 5 s at high speed.
10. Add 5 ml isopropanol (100%) and mix by inverting the tube at least 20 times until the white DNA strands clump visibly together. Complete mixing with isopropanol is essential to precipitate the DNA and should be checked by inspection. Only tightly clumped DNA strands can be efficiently pelleted by centrifugation. Do not vortex as this might reduce DNA yield.
11. Centrifuge for 3 min at 2500 x g.
12. Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for 1 min. In rare cases the pellet may be loose, so pour slowly. Inverting the tube onto absorbent paper minimizes backflow of isopropanol from the rim and sides of the tube onto the pellet.
13. Add 5 ml 70% (v/v) ethanol and vortex for 1 s at high speed.
14. Centrifuge for 3 min at 2500 x g.
15. Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for at least 5 min. In rare cases the pellet may be loose, so pour slowly. Inverting the tube onto absorbent paper minimizes backflow of ethanol from the rim and sides of the tube onto the pellet.
16. Carefully dab the tube onto absorbent paper to remove ethanol from the rim, and leave it inverted for a further 5 min to allow the DNA pellet to dry. Avoid overdrying the pellet, since overdried DNA is very difficult to dissolve.
17. Add 1 ml Buffer BG4 and dissolve the DNA by incubating for 1 h at 65°C in a heating block or water bath, followed by incubation overnight at room temperature. Highly concentrated, high-molecular-weight genomic DNA samples may not redissolve completely after an incubation of 1 h at 65°C, therefore an additional overnight incubation at room temperature is recommended.

11.1.2. RNA extraction

Things to do before starting

- Blood must be collected in PAXgene Blood RNA Tubes (BRT) according to the instructions in the *PAXgene Blood RNA Tube Product Circular*. If necessary, see Appendix C (page 62) for recommendations on handling PAXgene Blood RNA Tubes (BRT).
- Ensure that the PAXgene Blood RNA Tubes (BRT) are incubated for at least 2 hours at room temperature after blood collection to ensure complete lysis of blood cells. Incubation of the PAXgene Blood RNA Tube (BRT) overnight may increase yields. If the PAXgene Blood RNA Tube (BRT) was stored at 2–8°C, –20°C or –70°C after blood collection, first equilibrate it to room temperature, and then store it at room temperature for 2 hours before starting the procedure.
- Read the safety information on page 9.
- Read the guidelines on handling RNA (Appendix A, page 59).

- Ensure that instruments, such as pipets and the shaker–incubator, have been checked and calibrated regularly according to the manufacturer's recommendations.
- A shaker–incubator is required in steps 5 and 20. Set the temperature of the shaker–incubator to 55°C.
- Binding buffer (BR2) may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- Wash buffer 2 (BR4) is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.
- If using the RNase-Free DNase Set for the first time, prepare DNase I stock solution. Dissolve the solid DNase I (RNFD; 1500 Kunitz units)* in 550 µl of the DNase resuspension buffer (DRB) provided with the set. Take care that no DNase I (RNFD) is lost when opening the vial. Do not vortex the reconstituted DNase I (RNFD). DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- Current data shows that reconstituted DNase I (RNFD) can be stored at 2–8°C for up to 6 weeks. For long-term storage of DNase I (RNFD), remove the stock solution from the glass vial, divide it into single-use aliquots (use the 1.5 ml microcentrifuge tubes [MCT] supplied with the kit; there are enough for 5 aliquots), and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- When reconstituting and aliquoting DNase I (RNFD), ensure that you follow the guidelines for handling RNA (Appendix A, page 59).

Procedure

1. Centrifuge the PAXgene Blood RNA Tube (BRT) for 10 minutes at 3000–5000 x *g* using a swing-out rotor.

Ensure that the blood sample has been incubated in the PAXgene Blood RNA Tube (BRT) for a minimum of 2 hours at room temperature (15–25°C), in order to achieve complete lysis of blood cells. The rotor must contain tube adapters for round-bottom tubes. If other types of tube adapter are used, the tubes may break during centrifugation.

2. Remove the supernatant by decanting or pipetting. Add 4 ml RNase-free water (RNFW) to the pellet, and close the tube using a fresh secondary BD Hemogard closure (supplied with the kit).

If the supernatant is decanted, take care not to disturb the pellet, and dry the rim of the tube with a clean paper towel.

3. Vortex until the pellet is visibly dissolved, and centrifuge for 10 minutes at 3000–5000 x *g* using a swing-out rotor. Remove and discard the entire supernatant.

Small debris remaining in the supernatant after vortexing but before centrifugation will not affect the procedure. Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, and therefore affect the conditions for binding RNA to the PAXgene membrane.

4. Add 350 µl resuspension buffer (BR1), and vortex until the pellet is visibly dissolved.

5. Pipet the sample into a 1.5 ml microcentrifuge tube (MCT). Add 300 µl binding buffer (BR2) and 40 µl proteinase K (PK). Mix by vortexing for 5 seconds, and incubate for 10 minutes at 55°C using a shaker–incubator at 400–1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C (for step 20).

Do not mix binding buffer (BR2) and proteinase K (PK) together before adding them to the sample.

6. Pipet the lysate directly into a PAXgene Shredder spin column (PSC; lilac) placed in a 2 ml processing tube (PT), and centrifuge for 3 minutes at maximum speed (but not to exceed 20,000 x *g*). Carefully pipet the lysate into the spin column (PSC) and visually check that the lysate is completely transferred to the spin column (PSC).
To prevent damage to columns (PSC) and tubes (PT), do not exceed 20,000 x *g*. Some samples may flow through the PAXgene Shredder spin column (PSC) without centrifugation. This is due to low viscosity of some samples and should not be taken as an indication of product failure.
7. Carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube (MCT) without disturbing the pellet in the processing tube.
8. Add 350 µl ethanol (96–100%, purity grade p.a.). Mix by vortexing, and centrifuge briefly (1–2 seconds at 500–1000 x *g*) to remove drops from the inside of the tube lid.
The length of the centrifugation must not exceed 1–2 seconds, as this may result in pelleting of nucleic acids and reduced yields of total RNA.
9. Pipet 700 µl sample into the PAXgene RNA spin column (PRC; red) placed in a 2 ml processing tube (PT), and centrifuge for 1 minutes at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT), and discard the old processing tube (PT) containing flow-through.
10. Pipet the remaining sample into the PAXgene RNA spin column (PRC), and centrifuge for 1 minutes at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT), and discard the old processing tube (PT) containing flow-through.
Carefully pipet the sample into the spin column (PRC) and visually check that the sample is completely transferred to the spin column (PRC).
11. Pipet 350 µl wash buffer 1 (BR3) into the PAXgene RNA spin column (PRC). Centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT), and discard the old processing tube (PT) containing flow-through.
12. Add 10 µl DNase I (RNFD) stock solution to 70 µl DNA digestion buffer (RDD) in a 1.5 ml microcentrifuge tube (MCT). Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
If processing, for example, 10 samples, add 100 µl DNase I (RNFD) stock solution to 700 µl DNA digestion buffer (RDD). Use the 1.5 ml microcentrifuge tubes (MCT) supplied with the kit. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube. Do not vortex.
13. Pipet the DNase I (RNFD) incubation mix (80 µl) directly onto the PAXgene RNA spin column (PRC) membrane, and place on the benchtop (20–30°C) for 15 minutes.
Ensure that the DNase I (RNFD) incubation mix is placed directly onto the membrane. DNase digestion will be incomplete if part of the mix is applied to and remains on the walls or the O-ring of the spin column (PRC).
14. Pipet 350 µl wash buffer 1 (BR3) into the PAXgene RNA spin column (PRC), and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT), and discard the old processing tube (PT) containing flow- through.
15. Pipet 500 µl wash buffer 2 (BR4) into the PAXgene RNA spin column (PRC), and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT), and discard the old processing tube (PT) containing flow-through.
Wash buffer 2 (BR4) is supplied as a concentrate. Ensure that ethanol is added to wash buffer 2 (BR4) before use (see “Things to do before starting”, page 46).
16. Add another 500 µl wash buffer 2 (BR4) to the PAXgene RNA spin column (PRC). Centrifuge for 3 minutes at 8000–20,000 x *g*.

17. Discard the processing tube (PT) containing the flow-through, and place the PAXgene RNA spin column (PRC) in a new 2 ml processing tube (PT). Centrifuge for 1 minute at 8000–20,000 x *g*.
18. Discard the processing tube (PT) containing the flow-through. Place the PAXgene RNA spin column (PRC) in a 1.5 ml microcentrifuge tube (MCT), and pipet 40 µl elution buffer (BR5) directly onto the PAXgene RNA spin column (PRC) membrane. Centrifuge for 1 minute at 8000–20,000 x *g* to elute the RNA. It is important to wet the entire membrane with elution buffer (BR5) in order to achieve maximum elution efficiency.
19. Repeat the elution step (step 18) as described, using 40 µl elution buffer (BR5) and the same microcentrifuge tube (MCT).
20. Incubate the eluate for 5 minutes at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chill immediately on ice.
This incubation at 65°C denatures the RNA for downstream applications. Do not exceed the incubation time or temperature.
21. If the RNA samples will not be used immediately, store at –20°C or –70°C.

11.1.3. Adjusted EpiTect Fast Bisulfite Protocol

Things to do before starting
- add 30 ml ethanol (96–100 %) to buffer BW
- add 27 ml ethanol to buffer BD (store at 2–8 °C)
- add 310 µl RNase-free water to lyophilized carrier RNA and dissolve by vortex-ing. If processing fewer samples, split dissolved carrier RNA into aliquots and store at -20 °C
- calculate volume of Buffer BL and dissolved carrier RNA required for number of samples:

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	350 µl	1,4 ml	2,8 ml	5,6 ml	8,4 ml	16,8 ml
Volume of carrier RNA solution	3,5 µl	14 µl	28 µl	56 µl	84 µl	168 µl

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

Bisulfite DNA conversion procedure
1. Thaw DNA, make sure Bisulfite Solution is completely dissolved (if necessary, heat solution to 60 °C and vortex until precipitates are dissolved).
2. Prepare bisulfite reaction in 200 µl PCR tubes according to list below (DNA solu-tion and RNase-free water must total 20 µl). Add each component in the order listed!

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

3. Mix the bisulfite reaction by pipetting. DNA Protect Buffer should turn from green to blue after addition to DNA-Bisulfite Mix!
4. Program for thermal cycler:

Step	Time	Temperature
Denaturation	5 minutes	95°C
Incubation	10–20 minutes	60°C
Denaturation	5 minutes	95°C
Incubation	10–20 minutes	60°C
Hold	Indefinite	20°C

Clean-up of bisulfite converted DNA
5. Briefly centrifuge PCR tubes and transfer to clean 1.6 ml tubes.
6. Add **310 µl** freshly prepared **Buffer BL** containing 10 µg/ml carrier RNA. Mix by vortexing and centrifuge briefly. Carrier RNA is not necessary when using > 100 ng DNA.
7. Add **250 µl ethanol** (96–100%) to each sample. Mix by pulse vortexing for 15 seconds and centrifuge briefly.
8. Place EpiTect spin columns and collection tubes in suitable rack and transfer en-tire mixture from each tube on EpiTect spin column.
9. Centrifuge at maximum speed for 1 minute. Discard flow-through, reuse collection tube.
10. Add **500 µl Buffer BW** and centrifuge at max. speed for 1 minute. Discard flow-through, reuse collection tube
11. Add **500 µl Buffer BD** and incubate for **15 minutes** at room temperature.
12. Centrifuge spin columns at max. speed for 1 min. Discard flow-through, reuse col-lection tube.
13. Add **500 µl Buffer BW** and centrifuge at max. speed for 1 minute. Discard flow-through, reuse collection tube.
14. Repeat step 13.
15. Add **250 µl ethanol** (96–100%) to each spin column and centrifuge for 1 minute.
16. Place spin column into new 2 ml collection tubes. Centrifuge at max. speed for 1 minute to remove any residual liquid.
17. Optional: Incubate spin columns with open lids in a heating block set to 60 °C for 5 minutes to evaporate remaining liquid.
18. Place spin columns into clean 1.6 ml tubes. Add **15 µl Buffer EB** directly onto the centre of each membrane.
19. Incubate the spin columns at room temperature for 1 minute. Elute DNA by centrifugation for **1 minute at 12,000 rpm**
20. Store bisulfite converted DNA up to 24 hours at 2–8 °C, for longer at -20 °C

11.2. Kits and Reagents

DNA and RNA Extraction

PAXgene Blood DNA kit	Qiagen, Germany (Cat. no. 761133)
PAXgene Blood DNA tubes	Qiagen, Germany (Cat. no. 761115)
PAXgene Blood RNA tubes	Qiagen, Germany (Cat. no. 762165)
PAXgene Blood RNA kit	Qiagen, Germany (Cat. no. 762174)

Gene expression analysis

RT2 First Strand Kit	Qiagen, Germany
RT2 qPCR Primer Assay *GAPDH* human	Qiagen, Germany (Cat. no. PPH00150F)
RT2 qPCR Primer Assay *DNMT1* human	Qiagen, Germany (Cat. no. PPH01055F)
RT2 qPCR Primer Assay *MLH1* human	Qiagen, Germany (Cat. no. PPH00196F)
RT2 qPCR Primer Assay *IL-6* human	Qiagen, Germany (Cat. no. PPH00560C)
RT2 SYBR Green qPCR Master Mix	Qiagen, Germany

Methylation analysis

EpiTect Fast DNA Bisulfite Kit	Qiagen, Germany (Cat. no. 59826)
Primer (*DNMT1*, *MLH1, LINE-1*)	Biomers.net, Germany
EpiTect HRM PCR Kit	Qiagen, Germany (Cat. no. 59445)

Further laboratory materials

2 ml reaction tubes	Biozym Scientific, Germany
1.6 ml reaction tubes	Biozym Scientific, Germany
0.2 ml PCR strip tubes and caps	Applied Biosystems, United Kingdom
0.1 ml strip tubes and caps	Qiagen, Germany
96-well plates	Applied Biosystems, United Kingdom
Plastic seal	BioRad Laboratories, USA
Nuclease free water	Qiagen, Germany
Ethanol 96 %	VWR Chemicals, USA

Working Equipment

Pico100	Picodrop, United Kingdom
StepOnePlus	Applied Biosystems, United Kingdom
RotorGene Q	Qiagen, Germany

Software

StepOne Software v2.1	Applied Biosystems, United Kingdom
RotorGene 6 v 2.3.1.	Qiagen, Germany
IBM SPSS Statistics version 23	IBM, USA

11.3. Food frequency questionnaire

ElCoN
Eggenburg Institute for Complex Systems, Health and Neuroscience
Grätzl 5, A-3730 Eggenburg

„Energy"-ID-Nummer:_____
Datum: _____

psychosomatisches zentrum WALDVIERTEL klinik EGGENBURG

Ernährungs- und Bewegungsverhalten

Lebensmittelauswahl	täglich	2-3x pro Woche	1x pro Woche	selten	nie
Obst frisch	O	O	O	O	O
Trocken- und Dosenobst	O	O	O	O	O
Gemüse, Rohkost, Salat	O	O	O	O	O
Weißmehlprodukte (Semmeln, Toast, Nudeln, Knödel, ...)	O	O	O	O	O
Vollkorn (Brot, Nudeln, ...)	O	O	O	O	O
Beilagen (Reis, Nudeln, Kartoffeln, ...)	O	O	O	O	O
Wurst	O	O	O	O	O
Wurst, fettarm	O	O	O	O	O
Huhn, Pute	O	O	O	O	O
Kalb, Rind, Schwein	O	O	O	O	O
Fisch	O	O	O	O	O
Käse	O	O	O	O	O
Käse, fettarm	O	O	O	O	O
Milch, Jogurt, Topfen, ...	O	O	O	O	O
Milch, Jogurt, Topfen, fettarm	O	O	O	O	O
Knabbergebäck, Chips,...	O	O	O	O	O
Mehlspeisen, Torten, Kuchen,	O	O	O	O	O
Schokolade, Pralinen,...	O	O	O	O	O
saure und süße Kaugummis (Gummibärchen, ...)	O	O	O	O	O
Fruchtsaft	O	O	O	O	O
Limonaden	O	O	O	O	O
Light – Limonaden	O	O	O	O	O
Mineralwasser, Wasser, Tee	O	O	O	O	O
Kalorienreduzierte Lebensmittel	O	O	O	O	O
Essverhalten	**täglich**	**2-3x pro Woche**	**1x pro Woche**	**selten**	**nie**
Ich esse regelmäßig	O	O	O	O	O
Ich esse sehr schnell/hastig	O	O	O	O	O
Ich esse noch weiter, auch wenn ich satt bin	O	O	O	O	O
Ich esse mit Unterbrechungen	O	O	O	O	O
Ich esse immer alles auf	O	O	O	O	O
Ich esse häufig ein paar Bissen zwischendurch	O	O	O	O	O
Ich esse nebenbei (beim Lesen, Fernsehen, Autofahren, Telefonieren usw.)	O	O	O	O	O

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„Energy"-ID-Nummer:_____
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psychosomatisches zentrum WALDVIERTEL klinik EGGENBURG

Essverhalten	täglich	2-3x pro Woche	1x pro Woche	selten	nie
Ich esse heimlich bzw. wenn mich keiner beobachtet	O	O	O	O	O
Ich esse, wenn ich Essen rieche oder sehe	O	O	O	O	O
Ich esse, wenn ich plötzlich Heißhunger habe	O	O	O	O	O
Ich esse, weil ich das Verlangen nach bestimmten Lebensmitteln habe	O	O	O	O	O
Ich esse, wenn ich Stress habe	O	O	O	O	O
Ich esse aus Langeweile	O	O	O	O	O
Ich esse aus Kummer, Frust	O	O	O	O	O
Ich esse aus Ärger, Wut	O	O	O	O	O
Ich esse aus Belohnung	O	O	O	O	O
Ich esse außer Haus	O	O	O	O	O
Ich esse nachts	O	O	O	O	O
Ich lasse mich oft von anderen zum Essen oder Trinken überreden	O	O	O	O	O
Ich vermeide in Gesellschaft zu essen	O	O	O	O	O
Wenn ich mit anderen esse, habe ich mich gut unter Kontrolle	O	O	O	O	O
Ich esse immer, wenn ich nach Hause komme	O	O	O	O	O
Ich esse oft im Stehen, Gehen oder direkt aus dem Kühlschrank	O	O	O	O	O
Nach dem Essen habe ich ein schlechtes Gewissen	O	O	O	O	O
Ich verzichte bewusst auf Lebensmittel, Speisen oder Getränke, die dick machen	O	O	O	O	O
Bewegungsverhalten	**täglich**	**2-3x pro Woche**	**1x pro Woche**	**selten**	**nie**
Ich erledige Aufgaben des Alltags wie Einkaufen, Besorgungen, kleine Erledigungen zu Fuß oder mit dem Rad	O	O	O	O	O
Ich bin bei Tätigkeiten im Haus körperlich aktiv (Hausputz)	O	O	O	O	O
Ich bin bei Tätigkeiten im Garten körperlich aktiv (Rasenmähen, Gartenarbeit)	O	O	O	O	O
Ich bin im Alltag körperlich aktiv (Fußwege zwischen Stockwerken,...)	O	O	O	O	O
Ich lege bewusst Pausen in der Arbeit für Bewegungskurzprogramme ein	O	O	O	O	O
Sport / Bewegungsart, Training					
Ich betreibe die Sportart „walken"	O	O	O	O	O
Ich betreibe die Sportart „schwimmen"	O	O	O	O	O
Ich betreibe „Gymnastik"	O	O	O	O	O
Ich betreibe noch weitere Sportarten:	O	O	O	O	O
Ich absolviere regelmäßig ein Ausdauertraining	O	O	O	O	O
Ich absolviere regelmäßig ein Krafttraining	O	O	O	O	O
Ich absolviere regelmäßig ein Beweglichkeitstraining	O	O	O	O	O
Ich absolviere regelmäßig ein Koordinationstraining	O	O	O	O	O