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„DNA methylation status of *MAOB* as biomarker for diagnosis of Alzheimer's disease"

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CONTENTS

1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common type of age-related dementia. Nearly 24 million people are affected by AD and the number will double in the next 20 years (1).

Alois Alzheimer described 1906 the neuropathological and clinical features of a patient who died of a dementing disease. The illness is clinically characterized by progressive loss of memory and cognitive abilities. Neuropathological hallmarks are the formation of neurofibrillary tangles (NFTs) and senile plaques (SPs), and a massive loss of neuronal cells (2).

To make a definitive diagnosis of AD, the brain must contain a sufficient number of NFTs and SPs, which only can be examined post-mortem (3).

1.1 Late onset Alzheimer's disease

There are two types of AD: early-onset Alzheimer's disease (EOAD) and late-onset Alzheimer's disease (LOAD). EOAD is diagnosed before the age of 65 and about 5% of all AD patients are affected from this type. Mutations in genes, e.g. *Presenilin 1 (PSEN1)* and *Beta secretase 1 (BACE1),* seem to be the cause of EOAD (4, 5). In contrast, LOAD occurs sporadically above the age of 65. LOAD is the most prevalent type of AD. Several environmental and genetic factors contribute to the development of this disease. Having the ε4 allele of the Apolipoprotein E gene (APOE) increases the risk of AD about fourfold. Furthermore, epigenetic modifications contribute to the susceptibility for LOAD (5).

1.2 Epigenetic modifications

The term 'epigenetics', that literally means 'above the genetics', was defined by Conrad Waddington in 1939. Irreversible genetic changes, such as gene mutations, are causing many types of human disease. However, it is known, that also heritable reversible epigenetic alterations play an important role in disorder onset and progress. Therefore, epigenetic changes have become very important in biomedical research and may become useful biomarkers for disease diagnostics. In contrast to genetic alterations, epigenetic mechanisms do not change the primary DNA sequence (6-8).

Evidence suggests that epigenetic modifications can be inherited from generation to the next. However, epigenetic changes also occur because of environmental influences, nutrition or exposure to stress (9, 10).

DNA methylation and histone modifications, e.g. histone acetylation and phosphorylation, are two epigenetic modifications. Their synergistically acting role influences gene expression (11). Furthermore, the accumulation of non-coding microRNAs results in an alteration in translation of mRNA (12).

1.2.1 DNA methylation

DNA methylation is the addition of a methyl group to a DNA base. In mammals, it is most commonly the cytosine, which is methylated. The reaction is catalyzed by DNA methyltransferase enzymes (DNMTs) that are using S-adenosylmethionine (SAM) as the methyl donor. Typically, the methylation of cytosine occurs in a CpG dinucleotide. Clusters of CpG dinucleotides are called CpG islands. These islands occur mainly in the promoter regions of genes (13, 14).

A role of DNA methylation is the regulation of gene expression. Unmethylated promoters are commonly accessible for the binding of transcription factors. Thus, the gene is active and gene expression is promoted. In contrast, methylated promoters commonly inhibit the binding of transcription factors. Furthermore, specific methyl-CpG binding proteins and transcriptional repressors bind to the methylated DNA. Therefore, the gene activity is reduced. Figure 1 shows the effects of DNA methylation on gene expression (15).

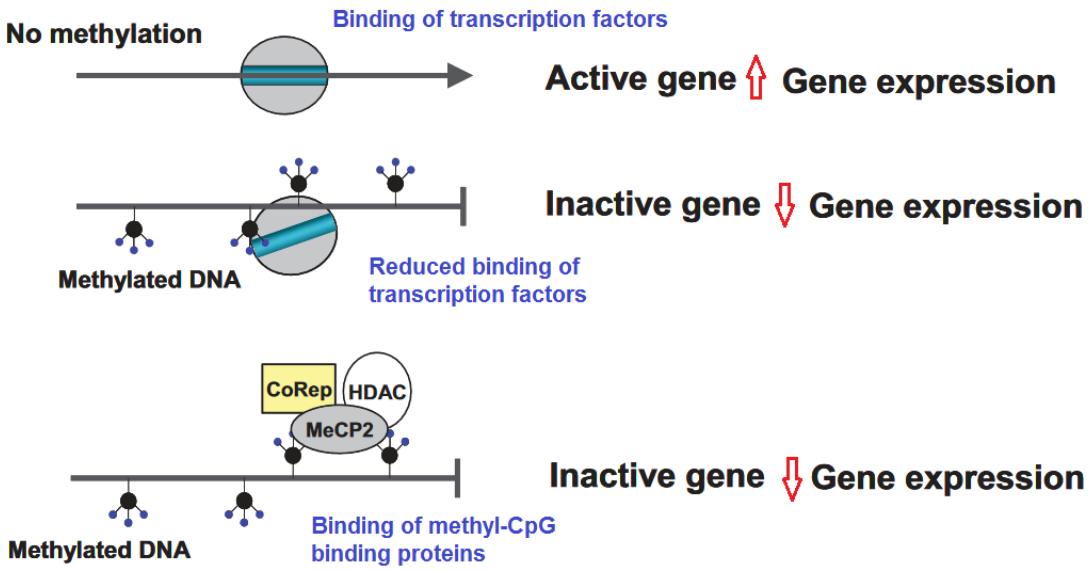


Figure 1: Effects of DNA methylation on gene expression; from (16), modified

The mechanism of DNA methylation is linked to the folate/methionine/homocysteine metabolism. By absorption of folate, tetrahydrofolate (THF) is converted to 5,10-methylenetetrahydrofolate (5,10-MTHF) and subsequently to 5-methyltetrahydrofolate (5-MTHF). Enzymes catalyzing these reactions are vitamin B6 and vitamin B2 dependent.

Homocysteine (Hcy) utilizes 5-MTHF as methyl donor for the re-methylation to methionine. Methionine is converted to S-adenosylmethionine (SAM) and acts as the methyl donor for the methylation of the DNA, catalyzed by DNMTs. By releasing the methyl group, SAM is converted to S-adenosylhomocystein (SAH), which is hydrolyzed to Hcy and adenosine. Hcy and adenosine must be removed to maintain the balance of the metabolism. On the other hand, global hypomethylation of DNA can occur since SAH reduces the activity of DNMTs (17-19).

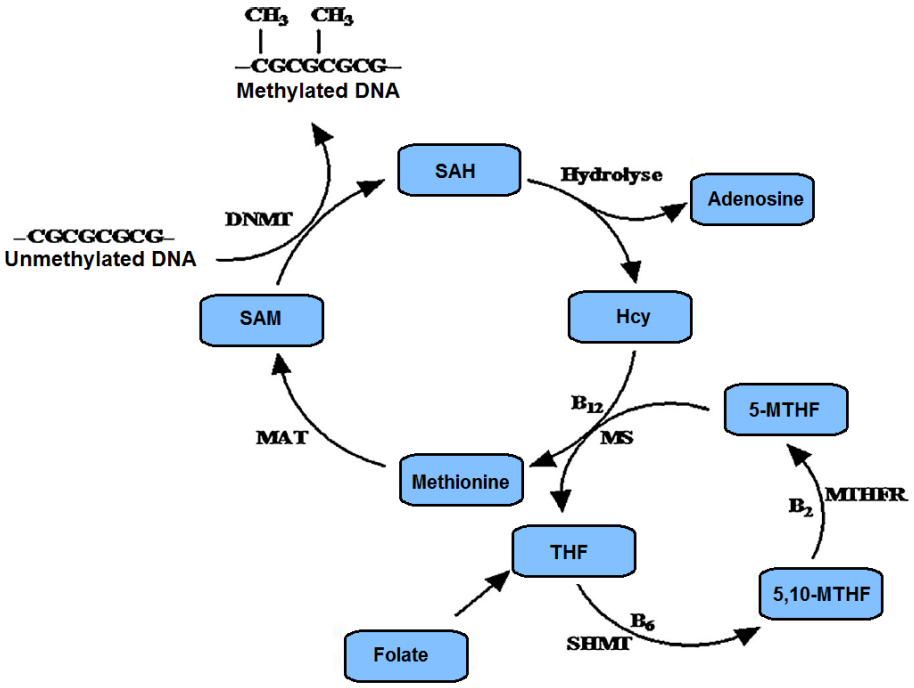


Figure 2: Folate metabolism, from (17), modified

Several studies report that the methylation status is significantly decreasing with ageing and the manifestation of AD (19, 20). A possible reason is that most LOAD patients have increased plasma homocysteine levels and decreased folate concentrations. Furthermore, the concentration of SAM in brain is reduced whereas the SAH concentration is increased. In conclusion, all these factors together may lead to a hypomethylation of the genome (17, 21).

1.3 Senile plaques

As mentioned above, senile plaques are one of the pathological characteristics of AD. Such plaques are formed from the amyloid precursor protein (APP). APP is an integral membrane protein that is mostly produced in the synapses of neurons. Several isoforms ranging from 695 to 770 amino acids have been observed. The cleavage of APP by β- secretase (BACE) and γ-secretase leads to the formation of amyloid β proteins (Aβ) with a length of 40 and 42 amino acids (Aβ40 and Aβ42). Aβ proteins are neurotoxic, in contrast to the neuroprotective secreted alpha amyloid precursor protein (APPs-α), which is formed by the cleavage with α-secretase.

The production of Aβ40/42 leads to deposition and subsequently to formation of senile plaques (SPs) in the grey matter of the brain. Thus, cognitive dysfunction occurs (3, 22-24).

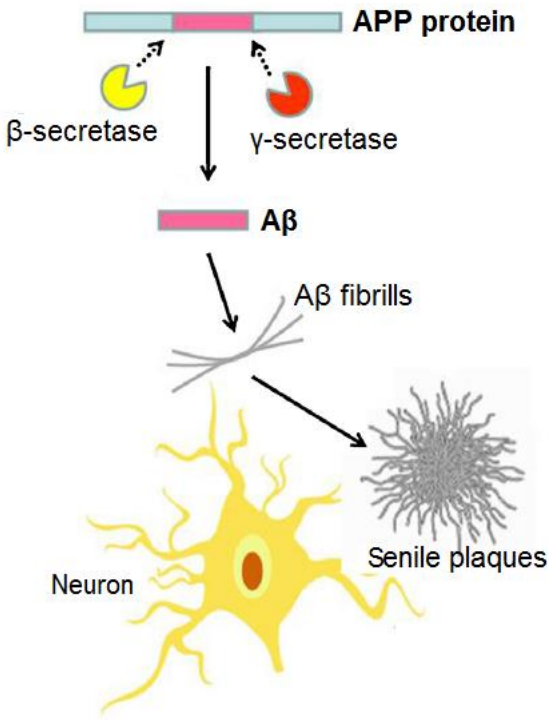


Figure 3: Schematic view of the formation of senile plaques; from (17), modified

Gamma secretase is a protease complex, which consists of the proteins anterior pharynx-defective 1 (APH-1), presenilin 1 (PS1), nicastrin (NCT) and presenilin enhancer 2 (PEN-2) (Figure 4) (25).

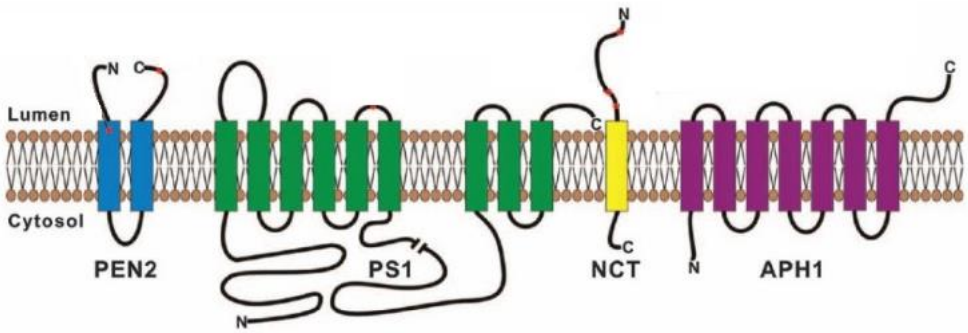


Figure 4: Schematic view of a γ-secretase complex; from (26), modified

In vitro, it has been shown that a folate deprivation leads to overexpression of *BACE* and *PS1* genes caused by hypomethylation of the promoter region of these genes (27). In mice, a folate addition to the diet resulted in up-regulation of PS1 and BACE and enhanced accumulation of Aß (28). From these findings it can be assumed, that the frequent folate deficiency in AD

patients alters the methylation levels of *PS1* and *BACE* promoters and upregulates these genes, which may enhance the production of Aß.

1.4 Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are another pathological hallmark of AD. Since the amount of NFTs is linked to the degree of AD (29), it is supposed, that there is a direct correlation between the formation of NFTs and neuronal dysfunction. The formation mechanism of NFTs is not fully understood yet, but it is already known that NFTs are formed from hyperphosphorylated tau proteins. Normally, tau protein functions by stabilizing microtubule in neurons of the central nervous system. However, excessive phosphorylation leads to bundles of paired helical filaments (PHFs), thus tau proteins are not able to bind microtubules, leading to neuronal death (30-32).

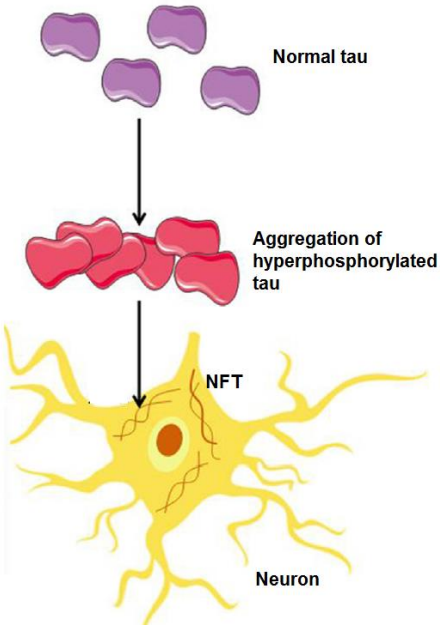


Figure 5: Schematic view of the formation of neurofibrillary tangles; from (17), modified

1.5 Monoamine oxidase B and Alzheimer

Monoamine oxidase B (MAOB) is a mitochondrial enzyme, that catalyzes the oxidative deamination of monoamine neurotransmitters, such as serotonin, dopamine and epinephrine. Rapid deamination ensures correct synaptic neurotransmission and brain function. However, an excessive activity of MAOB can lead to neurodegenerative disorder, since the byproducts of the deamination reaction are neurotoxic (33, 34).

It was reported that the concentration of MAOB in blood platelets from AD patients (n=40) was about one third higher compared to those from healthy persons (n=26) (35). Blood platelets are often used to study neuronal pathways, since they use similar cellular machinery as monoaminergic neurons (36).

Our cooperation partners (Ass. Prof. Dipl. Ing. Dr. Maria Zellner and Mag. Dr. Ellen Umlauf) recently investigated the MAOB protein expression in blood platelets of AD patients (n=34) and healthy controls (n=34). They found out that MAOB expression increased with increasing age in the healthy control group (from 55 until 104 years). In contrast, in AD patients MAOB protein expression was not correlated with age. In general, AD patients showed higher MAOB levels than healthy controls. AD patients between the age of 60 and 75 showed similar MAOB levels as cognitively healthy persons between 96 and 104 years (33). Figure 6 shows the correlation between MAOB expression levels and the age of healthy individuals and AD patients.

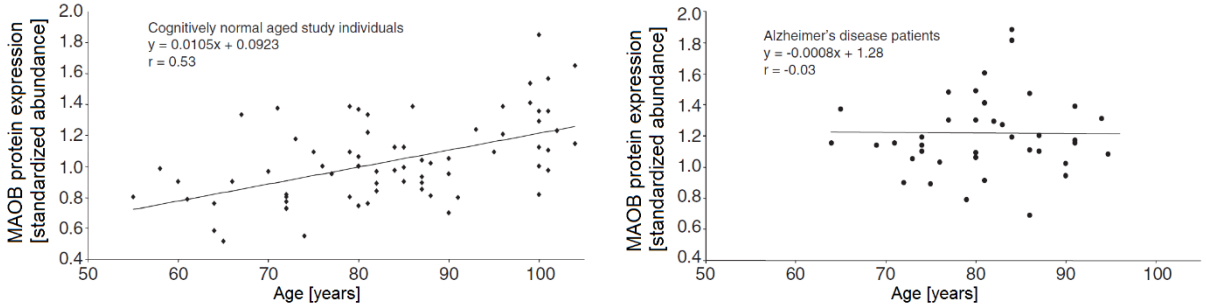


Figure 6: Correlation between MAOB protein expression and age, left: healthy controls, right: AD patients; from (33), modified

1.5.1 DNA methylation status of *MAOB* in LOAD patients

In preliminary experiments the DNA methylation status of the promoter region of *MAOB* in five healthy women and five women who suffered from LOAD was determined by our cooperation partners (Ass. Prof. Dipl. Ing. Dr. Maria Zellner and Mag. Dr. Ellen Umlauf). The DNA was isolated from whole blood and analysis was performed by bisulfite sequencing. Figure 7 shows the DNA sequence of the promoter region and the 48 CpG dinucleotides which were analyzed. The sequence of the forward primer used is shaded in blue. The underlined nucleotides show the annealing position of the reverse primer.

A significant difference in the methylation status between LOAD patients and controls was found for one CpG (shaded pink in Figure 7).

GGGACTCTTTGAAGTCCTAGGTGACCTCTCCGCCCAGGCACCCGCCCTCCCCGGGCCTGGGCTGCAGAGC
TGCGGGCGGGGGCGGTGGCCCTGCGCTGCACCCGGCCTGCCTGCGCGTAGGCGGGCGGGCGGGGCTGCGC
GTCCGGGCTCCCGGGGCTGGTAATATAGCGGCTCGCCGAGGCGCTGGTGCACGGGGGCAGCGCGCAGCAG
GCCGGCGGGCAGGCGGGCGGGCTGGCTGGCAGGCAGGACTGGGATCGAGGCCCAGAAAACGGAGCAGCGG
GCACCAGGGAGGCCTGGAACGGGCGAGCGCCATGAGCAACAAATGCGACGTGGTCGTGGTGGGGGGCGG
CATCTCAGGTTAGTCGCGGCTGTGCGCCCTCCTTCCTCTTCACGCGCTCGGACAGGTGGCTGCCTGGGGG
AGACGCGGGGGGCGGCCGTGGGGCAGGGGCTGACCCTGGAGTC

Figure 7: Sequence of MAOB (promoter region) analyzed in preliminary experiments; blue shaded: forward primer; underlined: annealing position of the reverse primer; green shaded: 48 CpG dinucleotides that were examined; pink shaded: CpG that showed a significant difference in DNA methylation status between AD patients and healthy controls

1.6 MAOB as a biomarker for early diagnosis of Alzheimer's disease

Biomarkers are defined as indicators, which are objective, quantifiable characteristics of biological and pathogenic processes. They are used e.g. for early diagnosis, prognosis or examination of pharmacological responses to therapeutics (37, 38).

Biomarkers can be molecules or genes that are measured in blood or tissue samples. In most cases they indicate a change in protein expression levels that correlate with the progression of a pathological situation. Epigenetic modifications, like DNA methylation, are an early event in diseases, especially in cancer and neurodegenerative disorders. Therefore, the development of potential DNA methylation biomarkers could be interesting for the early diagnosis of Alzheimer's disease. (37, 39).

As already mentioned in 1.2.1, the DNA methylation status in the promoter of genes is frequently associated with the protein expression level. Thus, the higher MAOB expression in blood platelets of AD patients (compared to healthy controls) (33) could be caused by hypomethylation of the *MAOB* gene. Hypomethylation of the *MAOB* gene could therefore be a marker for early diagnosis of AD.

2 Aim of the master thesis

Alzheimer's disease (AD) is the most common form of age-related dementia. It is characterized by cognitive dysfunction and progressive memory loss. So far, AD can only be diagnosed post mortem. Currently, many studies are looking for biomarkers allowing early diagnosis of AD before irreversible changes, e.g. neuronal cell loss, plaque deposition and neurofibrillary tangles, occur.

There is increasing evidence that late-onset Alzheimer's disease (LOAD), the most prevalent type of AD, is mediated by aberrant epigenetic modifications. AD patients frequently show high homocysteine and low folate levels in plasma, both leading to global hypomethylation of the genome. Since hypomethylation is as an early event in AD, aberrant DNA methylation levels are potential biomarkers for the diagnosis of AD.

The main objective of the present master thesis was to investigate if the DNA methylation status of *monoamine oxidase B* (*MAOB*) is suitable as biomarker for early diagnosis of AD. MAOB catalyzes the breakdown of many neurotransmitters, e.g. dopamine. In the brain of AD patients, MAOB is frequently found to be up-regulated. The aim of this analysis was to determine whether there are differences between AD patients and healthy persons, so that MAOB could be used as a potential biomarker for early diagnosis of LOAD.

In preliminary studies of our cooperation partners Ass. Prof. Dipl. Ing. Dr. Maria Zellner and Mag. Dr. Ellen Umlauf (Institute of Physiology, Department of Physiology and Pharmacology, Medical University of Vienna) the DNA methylation levels of 48 CpGs in the promoter region of *MAOB* were determined. One CpG showed a significant difference in the methylation status between AD patients and healthy persons. Since the used bisulfite sequencing method was too time-consuming and expensive, the methylation status in only five LOAD patients and five healthy individuals was determined.

In the present study, a larger number of individuals should be examined. Thus, a pyrosequencing (PSQ) method should be developed that targets the CpG for which a significant difference had been found. The method should then be applied to determine the methylation status in DNA from whole blood samples of 40 patients suffering from LOAD and 40 healthy individuals serving as control. However, before pyrosequencing, the DNA had to be treated with sodium bisulfite and amplified by the polymerase chain reaction (PCR). Methylation levels obtained by pyrosequencing should be subjected to statistical tests in order to find out, if there is a significant difference in the methylation status between AD patients and healthy controls.

3 Theoretical background

Determination of the methylation status by PSQ involves several steps: conversion of the DNA with sodium bisulfite, amplification of the bisulfite converted DNA by PCR, followed by PSQ. Bisulfite treatment is necessary because the PCR polymerase does not distinguish between methylated and unmethylated cytosine.

3.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is an effective method to copy a specific DNA sequence *in vitro*. This procedure was developed in 1983 by Kary B. Mullis and ten years later he was awarded the Nobel Prize for his work. The PCR is now an important analytical technique in many laboratories. This method enables an amplification (multiplication) of very low amounts of nucleic acids from different samples (40).

3.1.1 Principle of PCR

For carrying out the PCR, the following reagents are needed: template DNA, forward and reverse primer, mixture of all deoxynucleosid triphosphates (dNTPs), DNA polymerase, Mg^{2+} ions and reaction buffer (41).

Most commonly, for DNA polymerase the *Taq DNA polymerase*, which is isolated from the bacterium *Thermus aquaticus*, is used. The *Taq polymerase* is thermostable and can withstand high temperatures such as 95°C. Furthermore, this polymerase is cheap and has a high processivity and robustness. The reaction buffer provides the ideal conditions for the PCR reaction. The concentration of Mg^{2+} ions influences the efficiency and specificity of the PCR. The required concentration varies for each reaction. Low concentrations usually lead to low and high concentrations to high amplicon yields, but too high concentrations of Mg^{2+} ions cause the formation of unspecific PCR products. The dNTPs are the substrates and building blocks for a new DNA strand. By removing pyrophosphate and water, the complementary nucleotide is linked to the DNA strand which is going to be synthesized. Four different dNTPs are used: deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine trisphosphate (dCTP) and deoxythymidine triphosphate (dTTP). The two synthetic oligonucleotide primers are complementary to the 3’ ends of the sense and antisense strand of the template DNA, respectively (40).

A PCR process consists of 30 to 50 cycles for the amplification of one specific DNA sequence. Each cycle consists of three steps: the denaturation of the double stranded DNA (dsDNA),

annealing of the primers and elongation by the polymerase. Figure 8 shows a schematic flow of the DNA amplification (40).

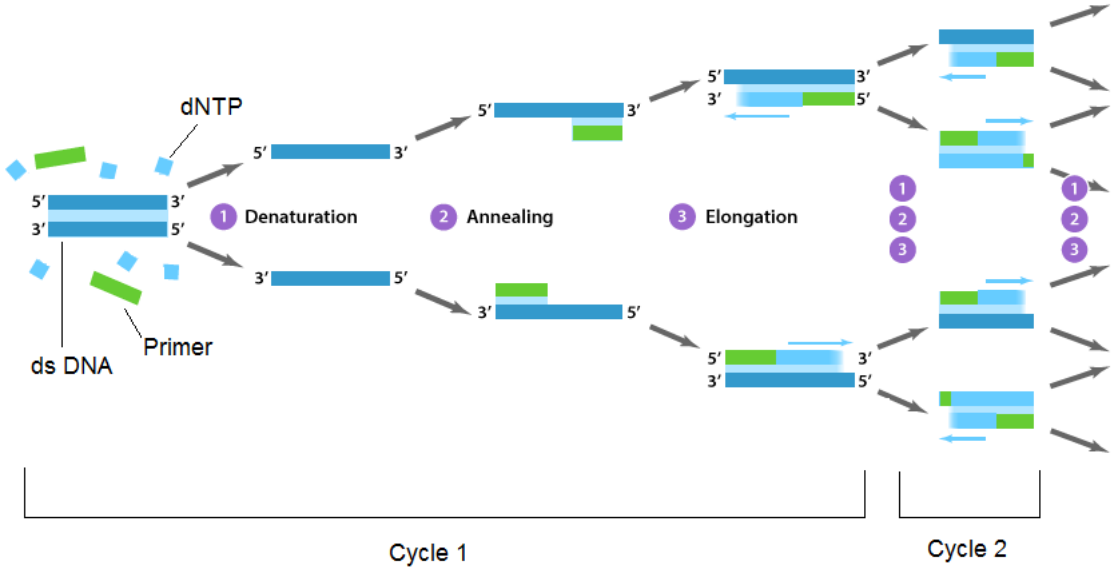


Figure 8: Schematic flow of a PCR reaction, 2 cycles shown; from (42), modified

In the first step of the PCR, the temperature is increased within the reaction tube to 95°C for denaturation. The dsDNA is separated by breaking the hydrogen bonds between the complementary bases. Therefore, the DNA becomes single stranded, forming the DNA template. Secondly, in the annealing step, the temperature is lowered to a primer specific annealing temperature (Ta), which is in general 5°C below the melting temperature (Tm) of the primers. At this temperature the primers hybridize to the template. In the third step, the temperature is increased to 72°C, which is the optimum working temperature for the DNA polymerase. In this so called elongation step, the DNA is synthesized from the 5' to 3' end (40, 43).

3.1.2 Kinetics of PCR

After the first cycle, the number of copies is doubled and the amplified DNA is used as new target DNA. Theoretically, this means that the concentration of amplicons is exponentially increasing after each cycle, if PCR achieves 100% efficiency. However, the actual efficiency is most commonly lower. One cause for a lower efficiency is that the lower concentrations of primers and deoxynucleotides is decreased at higher cycle number. Another reason is that the polymerase loses its activity due to recurring heating steps (44).

Figure 9 shows an amplification curve and its three phases. The product concentration is plotted against the number of cycles. In the first phase there is only a slight increase of the amplification curve, because the concentration of the template is low and the primers are searching for the template. In the second phase the concentration of template is sufficient and

so the product concentration is increasing exponentially. In the third phase the curve flattens and reaches a plateau because of the limiting factors mentioned above (44).

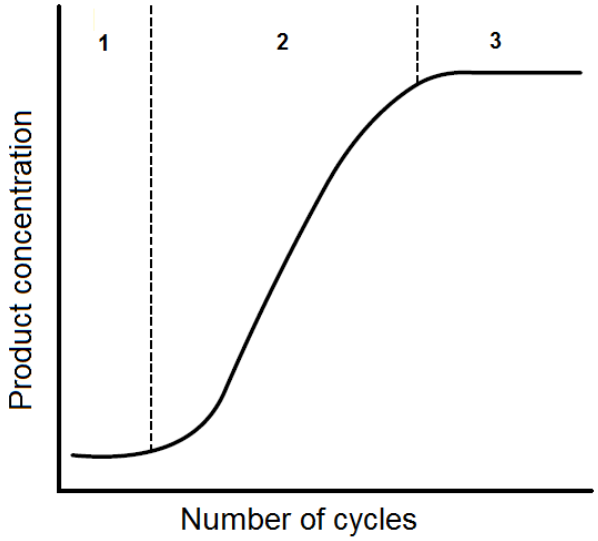


Figure 9: Three phases of an amplification curve; from (44), modified

3.1.3 Primer design

There are some general guidelines for primer (forward and reverse) design, which should be followed: (44)

- The primers should have a length between 16 and 30 nucleotides to bind specifically to the target sequence. The primers should not bind to other regions of the genome.
- The primers should have approximately the same length.
- Their Tm should be approximately the same.
- They should not form primer homo- or heterodimers or secondary structures.
- Especially the 3' end of the primers should perfectly match to the template, since this is the end which is extended.

If the primers are designed for bisulfite converted DNA, the following guideline should be followed too:

- In order to avoid a PCR bias, the primer should not contain CpG dinucleotides.

3.2 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used for separating, purifying or identifying nucleic acids. Agarose, which is extracted from seaweed, is a natural polysaccharide polymer. It consists of D-galactose and 3,6-anhydro-L-galactopyranose, which is alternately linked by

(1/4) and (1/3) bounds. After heating in a buffer and cooling, it forms polysaccharide double helices and gelation occurs. Thus, this kind of gels have a large pore size and are suitable for separating large DNA molecules. Another advantage is that agarose gel is a nontoxic medium and the gels are easy and quick to cast (45-47).

3.2.1 Principle of gel electrophoresis

In electrophoresis, molecules, such as DNA, are separated by their size with the aid of an electric field. Therefore, an electrophoresis chamber connected to a power source is needed. The chamber has a positively and a negatively charged electrode and is filled with buffer. After the prepared agarose gel is hardened, it is put on a gel caster in the electrophoresis chamber. Figure 10 shows a schematic agarose gel chamber setup (47-49).

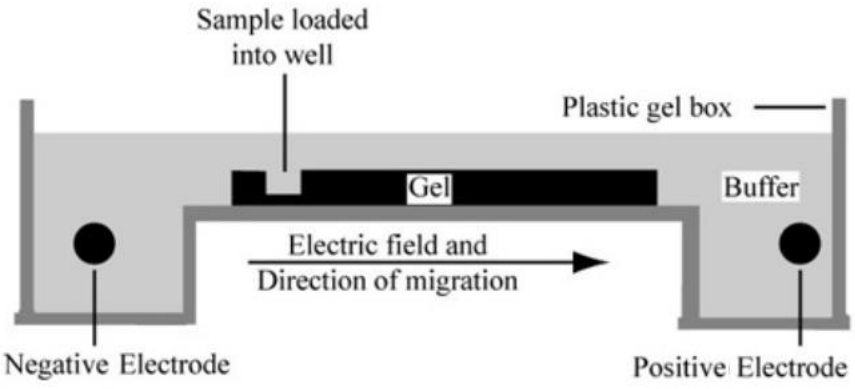


Figure 10: Schematic setup of an agarose gel chamber, from (50), modified

Nucleic acids are negatively charged, because of their phosphate backbone. The negative charge increases proportionally with the length of the nucleic acid strand. However, the charge density remains the same. Therefore, nucleic acids are separated based on their molecular weight. By using standards, the molecular weight of samples can be determined (47-49).

As already mentioned, DNA is negatively charged and so after the electric current is applied DNA migrates in direction of the positively charged electrode as shown in Figure 11 (47).

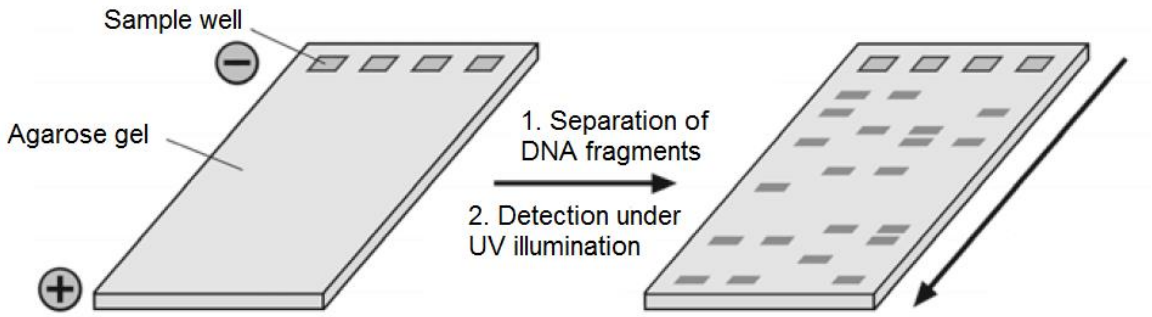


Figure 11: Schematic image of an agarose gel before and after separation of DNA fragments; from (47), modified.

After separating the DNA fragments, they have to be visualized with the help of an intercalating dye. Ethidium bromide is commonly used to detect nucleic acids, because it intercalates in

double stranded nucleic acids. When exposed to ultraviolet (UV) light, it fluoresces and DNA fragments can be detected. Figure 12 shows the structural formula of ethidium bromide (47).

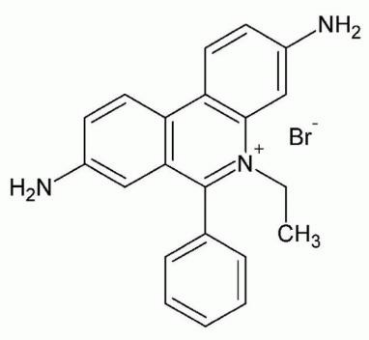


Figure 12: Structural formula of ethidium bromide, from (51)

Ethidium bromide is very toxic and acts like a mutagen, since it deforms DNA by intercalation. There are, however, less toxic alternatives, e.g. SYBR-based dyes (52, 53).

3.3 Bisulfite conversion

In the bisulfite conversion, cytosine is converted to uracil, whereas 5-methylcytosine is left unchanged. The mechanism of the deamination of cytosine to uracil consist of three steps, which are shown in Figure 13. In the first step cytosine is sulfonated by nucleophilic addition. In the second step, cytosine sulfonate is deaminated to uracil sulfonate. Finally, uracil is formed by the release of hydrogen sulfite (54, 55).

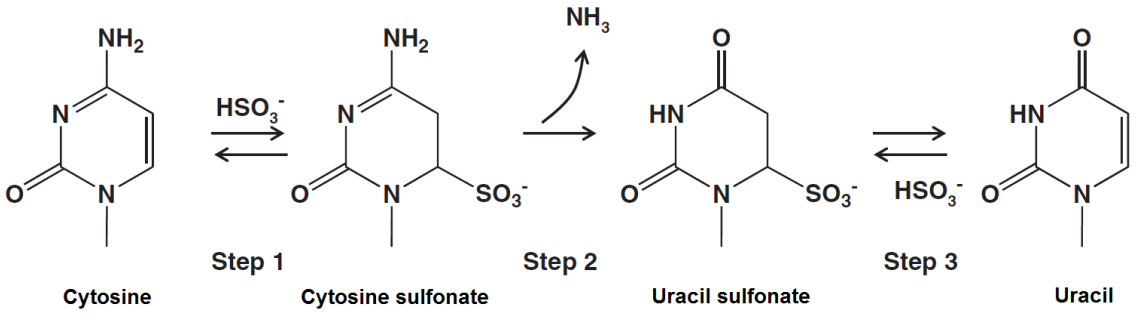


Figure 13: Mechanism of bisulfite conversion: formation of uracil by deamination of cytosine, from (55), modified

When bisulfite converted DNA (bcDNA) is amplified, uracil (U) is read as thymine (T) by the DNA polymerase. Thus, uracil pairs with adenine (A) of the newly synthesized daughter strand. In contrast 5-methylcytosine pairs with guanine (G). Figure 14 shows the flow of amplification of bcDNA. Because of the conversion two non-complementary DNA strands are formed, which leads to two different dsDNA products after PCR (56).

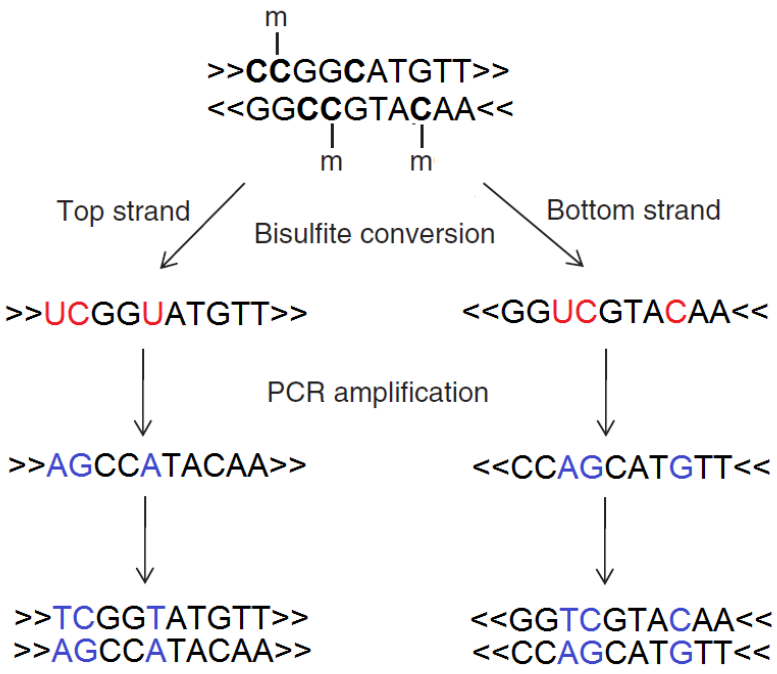


Figure 14: Flow of PCR amplification after bisulfite conversion; m-C: 5-methylcytosine; from (56), modified

3.4 Pyrosequencing

Currently, pyrosequencing is an important method used to study biological systems. The method can be used for sequencing of DNA fragments or amplicons, methylation studies, SNP analysis and microbial identifications. Comparing to traditional Sanger sequencing, pyrosequencing has a higher throughput (more than tenfold) because several sequences (from different genes) can be read at the same time (57, 58).

3.4.1 Principle of pyrosequencing

Pyrosequencing is a sequencing by synthesis technique, that is detecting pyrophosphate (PPi) which is released during DNA synthesis (59). At the end of an enzymatic cascade, visible light is emitted, the intensity of which is proportional to the amount of released PPi (60).

Before the PSQ reaction, the target region has to be amplified. One of the two PCR primers has to be biotinylated in order to obtain a biotinylated template. By denaturation and washing steps single stranded DNA (ssDNA) is isolated. The PSQ reaction starts with binding of the sequencing primer. During the reaction the four different dNTPs (dATP, dGTP, dCTP, dTTP) are injected one after another. The DNA polymerase catalyzes the incorporation of the nucleotide that is complementary to the template strand, resulting in the release of PPi (57). PPi is then converted into ATP by ATP sulfurylase. ATP is then used by the enzyme luciferase to catalyze the oxidation of luciferin to oxyluciferin. The photons can be detected by a

illuminometer or a charge coupled device camera (61). By plotting the light intensity against the added nucleotides, a pyrogram is obtained. If the same nucleotide is incorporated twice in a row, the peak heights is doubled.

Figure 15 shows the reaction cascade after injecting dNTPs.

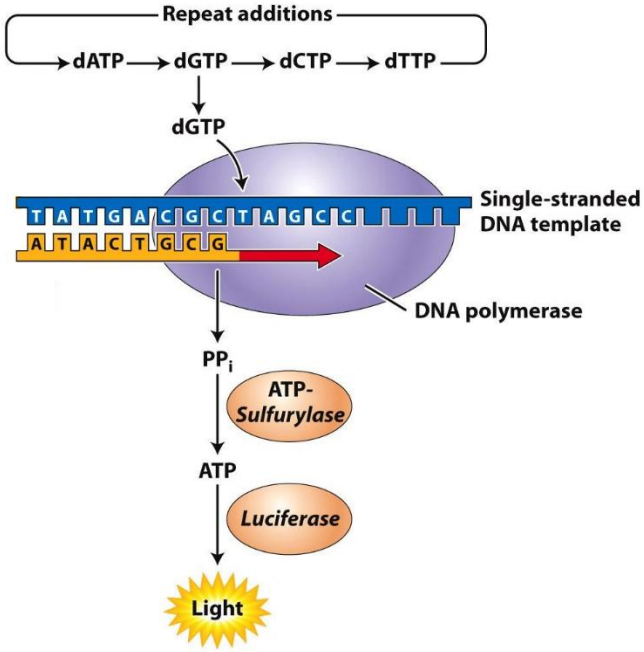


Figure 15: Reaction cascade of pyrosequencing ; from (62), modified

Before the next nucleotide is added and the stepwise synthesis of DNA is continued, excess dNTPs have to be removed.

There are two different pyrosequencing strategies: solid phase and liquid phase pyrosequencing. Solid phase pyrosequencing uses immobilized DNA. By using a biotinylated forward or reverse primer, the PCR product becomes biotinylated and binds to streptavidin coated magnetic beads. Three enzymes are used for solid phase pyrosequencing: DNA polymerase, ATP sulfurylase and luciferase. After each nucleotide addition step the excess components of the reaction are removed by a washing step. Liquid phase pyrosequencing utilizes an additional enzyme, the apyrase. Apyrase is an enzyme which catalyzes the degradation of ATP and nucleotides that have not been incorporated. Thus, no washing steps are needed. Liquid phase pyrosequencing can be used for high-throughput analyses, because it can be performed in an automated system in contrast to solid phase pyrosequencing (59).

3.4.2 Deoxyadenosine alpha-thiotriphosphate (dATPαS)

Since dATP is a substrate of the luciferase system, is cannot be used as building block for the synthesis of the new strand. Most commonly, deoxyadenosine alpha-thiotriphosphate (dATPαS), a modified form of dATP, is used (63).

3.4.3 Methylation analysis

To determine the DNA methylation status of a CpG dinucleotide, the ratio between cytosine and thymine incorporation is measured. Bisulfite conversion creates a polymorphic position at each methylation site (YG). During pyrosequencing, for each YG dCTP and dTTP are added in succession. From the ratio of the light intensities, the ratio between C and T can be calculated. Thus, the methylation status of single CpG dinucleotides is determined (60).

3.4.4 Primer design

For pyrosequencing, the design of a forward and a reverse PCR primer and one sequencing primer is required. Since the amplicon is immobilized on streptavidin coated beads during the preparation for solid phase pyrosequencing, one of the two PCR primers (forward/reverse) has to be biotinylated on the 5' end. The guidelines for designing the amplification primers have been summarized in chapter 3.1.3. In addition, it is important that the sequencing primer does not hybridize at any other position in the amplicon. Due to decreasing enzyme efficiency the binding position of the sequencing primer should be close to the region to be sequenced (60).

4 Results and discussion

The aim of the present master thesis was to investigate if the DNA methylation status in the promoter region of *monoamine oxidase B* (*MAOB*) is significantly different. At the beginning, a PSQ method allowing the determination of the methylation status was developed. The method was then applied to DNA extracts from whole blood of AD patients and healthy controls. In addition to DNA extracts of individual AD patients and controls, DNA extract pools were analyzed. These pools were obtained by mixing DNA extracts of individual persons.

4.1 Development of a PSQ method for *MAOB*

The primer set, consisting of a forward, reverse and sequencing primer, was designed for the *MAOB* gene with the use of the *PyroMark Assay Design 2.0* software. The primer set was designed following all guidelines given in chapter 3.1.3.

As described in chapter 1.5.1, the DNA methylation status of *MAOB* in AD patients has already been determined in preliminary experiments by our cooperation partners. Since a significant difference between AD patients and healthy persons was found at one CpG dinucleotide, the task was to design a primer set targeting a region containing the specific CpG. Thus, the possibilities were limited and we had to make some compromises with regard to fulfilling the criteria for primer design. Details on primer design and PCR optimization are given in chapter 6.3.2 and 6.3.4, respectively.

4.1.1 Primer set

The DNA sequence of *MAOB* (NG_008723.1) was found in the database *National Center for Biotechnology Information* (NCBI) (64). The promoter region (FP022608) of this gene was taken from *The Eukaryotic Promoter Database (EPD)* and inserted in the *PyroMark Assay Design 2.0* software (65). The software displayed the sequence automatically in the bisulfite converted form.

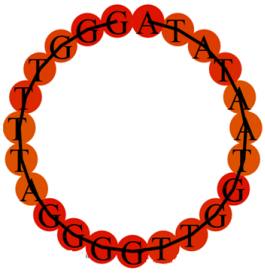
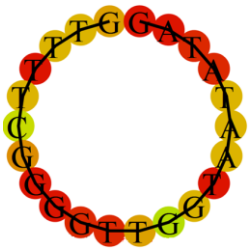
In Figure 16 a part of the promoter region of *MAOB* is shown. The forward primer is shaded in blue and the sequencing primer is framed black. The underlined sequence shows the position, where the reverse primer binds to the complementary strand. The *sequence to analyze* is shaded in grey and the CpGs are shaded in green. The CpG shaded in pink is the CpG that should be targeted due to results obtained in preliminary experiments (chapter 1.5.1).

TTTTTTTAGTCGTTAAGTTTGATAGTGTATTAGGGAGGTTGATGGGAAAATATCGTGTAGAAGGTTGAGGAGT
GGGGATTTTGGGGCGGGATTTTTTGAAGTTTTAGGTGATTTTTTCGTTTAGGTATTCGTTTTTTTCGGGTTTGGG
TTGTAGAGTTGCGGGCGGGGGCGGTGGTTTTGCGTTGTATTCGGTTTGTTTGCGCGTAGGCGGGCGGGCGGG
GTTGCGCGTTCGGGTTTTCGGGGTTGGTAATATAGCGGTTCGTCGAGGCGTTGGTGTACGGGGGTAGCGCGT
AGTAGGTCGGCGGGTAGGCGGGCGGGTTGGTTGGTAGGTAGGATTGGGATCGAGGTTTAGAAAACGGAGTA
GCGGGTATTAGGGAGGTTTGGAACGGGGCGAGCGTTATGAGTAATAAATGCGACGTGGTCGTGGTGGGGGG
CGGTATTTTAGGTTAGTCGCGGTTGTGCGTTTTTTTTTTTTTTTACGCGTTCGGATAGGTGGTTGTTTGGGGGA
GACGCGGGGGGCGGTCGTGGGGTAGGGGTTGATTTTGGAG

Figure 16: Fragment of the promoter region of MAOB and illustration of the target region; blue shaded: forward primer; underlined: position, where the reverse primer binds to the complementary strand; black framed: sequencing primer; grey shaded: sequence to analyze; green shaded: CpG dinucleotides, that were examined; pink shaded: CpG, that showed a significant difference in DNA methylation status between AD patients and healthy controls in preliminary experiments

In Table 1 the characteristics of the primer set and the amplicon are summarized. Due to the limited possibilities in primer design it was not possible to avoid that the primers contained a CpG (underlined in Table 1) in their sequence. No secondary structures were found by using the web server *RNAfold* (66). The melting temperature (Tm) given by *Sigma Aldrich* (distributor) and the Tm assessed by using the webserver *Oligo Calc* (67) deviated. Thus, the annealing temperature (Ta) had to be optimized experimentally.

Table 1: Characteristics of the primer set and the amplicon

	Forward primer	**Reverse primer**	**Sequencing primer**
Sequence (5'→3')	GGGTTTTAGGGGT TGGTAATATA	TTCTAAACCTCCA TCCCAATCCT	GTTTTCGGGGTT GGTAATATAG
Length [nt]	23	23	22
Secondary structures			
Tm [°C] according to *Sigma Aldrich*	60.6	65.5	55.1
Tm [°C] calculated witch *Oligo Calc* (67)	59.2	60.9	58.4
Characteristics of the amplicon			
Amplicon size [bp]		123	
Sequence to analyze [bp]		61	
Number of CpGs		11	

4.1.2 Optimization of the PCR method

First of all, the PCR method was optimized by applying a temperature gradient (4 different temperatures: 59.7°C, 57.9°C, 56.4°C, 54.0°C) and different primer concentrations (200 µM and 400 µM). The primer set was tested with fully methylated and fully unmethylated bisulfite treated control DNA. Details on method optimization are given in 6.3.4. Figure 17 shows the results obtained by loading the PCR products onto an agarose gel. As it can be seen, both the Ta and the primer concentration influenced PCR amplification. Box 7 (arrow) in Figure 17 shows the optimal PCR conditions, resulting in the highest fluorescence intensity and thus the highest amount of PCR product. The Figure also indicates that no unspecific products or primer dimers were formed.

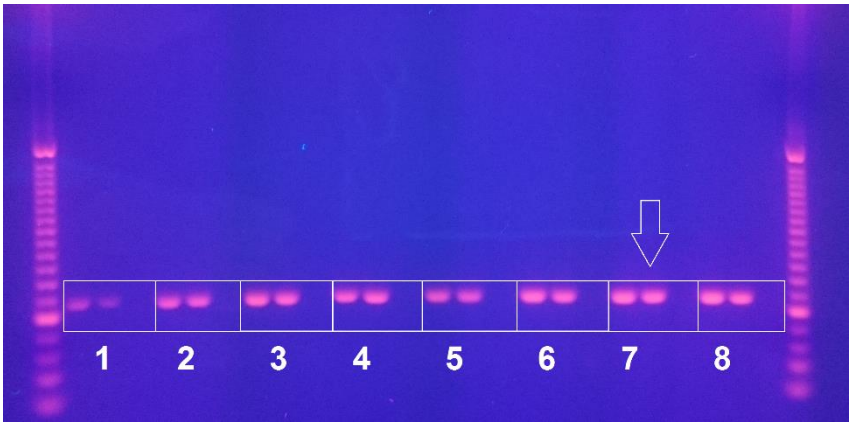


Figure 17: Agarose gel showing the results of PCR optimization. Each box represents a pair of fully unmethylated and methylated control DNA and a no template control, performed under different conditions. The following annealing temperatures and primer concentrations were applied: 1 = 59.7°C, 200 µM; 2 = 57.9°C, 200 µM; 3 = 56.4°C, 200 µM; 4 = 54.0°C, 200 µM; 5 = 59.7°C, 400 µM; 6 = 57.9°C, 400 µM; 7 = 56.4°C, 400 µM; 7 = 54.0°C, 400 µM. A 25 bp DNA ladder was used. The arrow indicates the optimal PCR conditions

The optimal conditions for the PCR method were a Ta of 56.4°C and a primer concentration of 400 µM.

4.2 Repeatability of the PSQ method

4.2.1 Repeated analysis of control DNA

The repeatability of the PSQ method was tested by determining the DNA methylation status of fully unmethylated and fully methylated bisulfite treated control DNA in each run. Over a longer period (several weeks), the results for the control DNA were rather similar. In Table 2, methylation levels obtained by analyzing control DNA (0% methylated and 100% methylated) on different days are summarized.

Table 2: Results obtained by determining the DNA methylation status of fully unmethylated control DNA (0%) and fully methylated control DNA (100%) on different days

Methylation status [%]												
	CpG											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	
0% methylated control DNA	*	*	*	*	*	*	*	5	*	*	*	Day 1
	*	*	*	*	*	*	*	*	*	*	*	Day 2
	*	7	8	8	*	*	*	*	*	*	*	Day 3
	*	14	*	*	*	*	*	*	*	*	*	Day 4
100% methylated control DNA	97	100	96	99	89	91	92	99	83	82	91	Day 1
	94	100	90	89	88	90	85	100	79	83	88	Day 2
	98	100	100	99	95	96	95	99	93	92	92	Day 3
	83	100	99	86	83	81	88	100	79	79	79	Day 4

* DNA methylation status < 5%, the limit of quantification (LOQ) of the method

Figure 18 and Figure 19 show representative pyrograms for fully unmethylated and methylated control DNA, respectively.

The positions highlighted in blue indicate the polymorphic YG position, the methylations levels are given in the boxes shown above. In each run, an internal bisulfite conversion control was performed (orange bar).

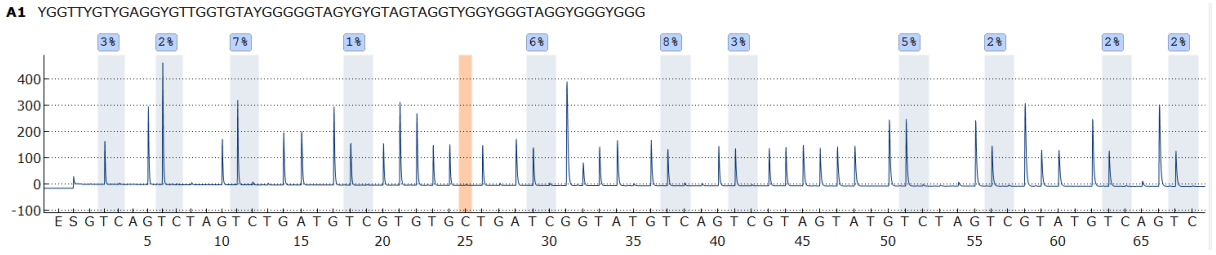


Figure 18: Representative pyrogram for a 0 % methylated control DNA

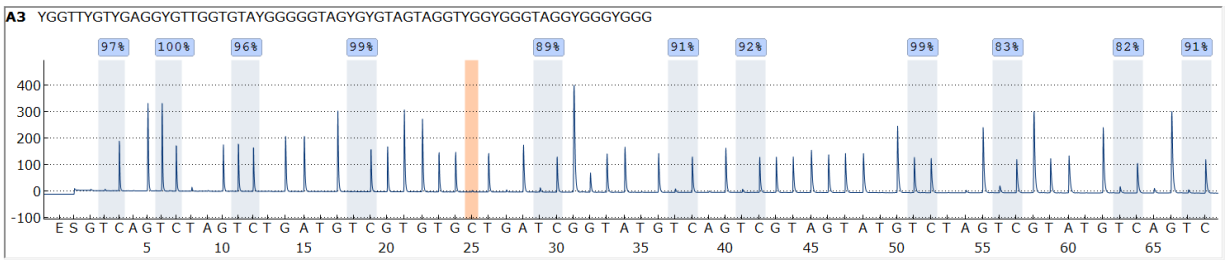


Figure 19: Representative pyrogram for a 100 % methylated control DNA

4.2.2 Repeated analysis of DNA extracts

DNA extracts from whole blood of AD patients and healthy controls were analyzed in replicates (n=3-5). Table 3 summarizes the methylation levels obtained for sample A163 (representative sample) on four different days. The coefficient of variation (CV) depended on the CpG and was in the range from 6 to 34%.

Table 3: DNA methylation status of MAOB in a representative sample (A163); determined on different days

	Methylation status [%]											
	CpG											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	
A163	30	25	43	20	34	20	31	38	24	17	15	Day 1
	28	48	55	41	42	20	37	36	29	15	16	Day 2
	26	51	60	45	35	33	31	51	37	20	28	Day 3
	28	41	49	31	33	23	28	25	14	19	24	Day 4
	29	35	44	27	29	21	26	32	21	23	18	
CV [%]	6	26	14	31	14	22	14	26	34	16	27	

4.3 Formation of unspecific products

From time to time unspecific products were formed during PCR amplification. The formation of unspecific products was independent of the DNA sample. Unspecific products were formed in no template controls in which no DNA was added (Figure 20). However, they were also obtained in the presence of template DNA (Figure 21). In both cases, the peak pattern deviated from the expected one shown in the histograms. Due to these deviations, the warning message “failed analysis” was obtained.

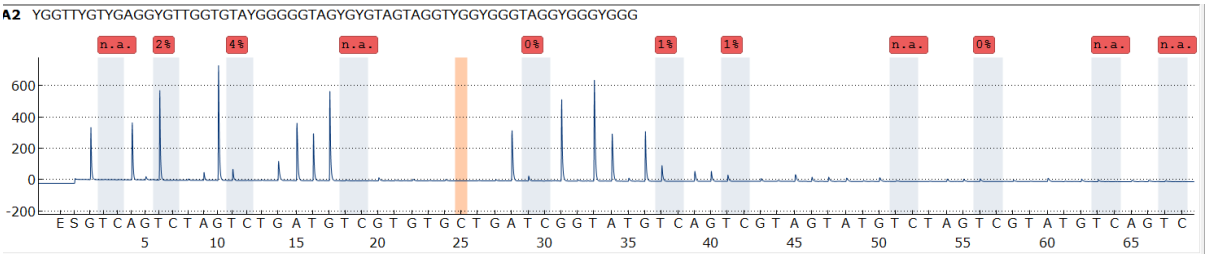


Figure 20: Representative pyrogram for a no template control, indicating the formation of an unspecific product

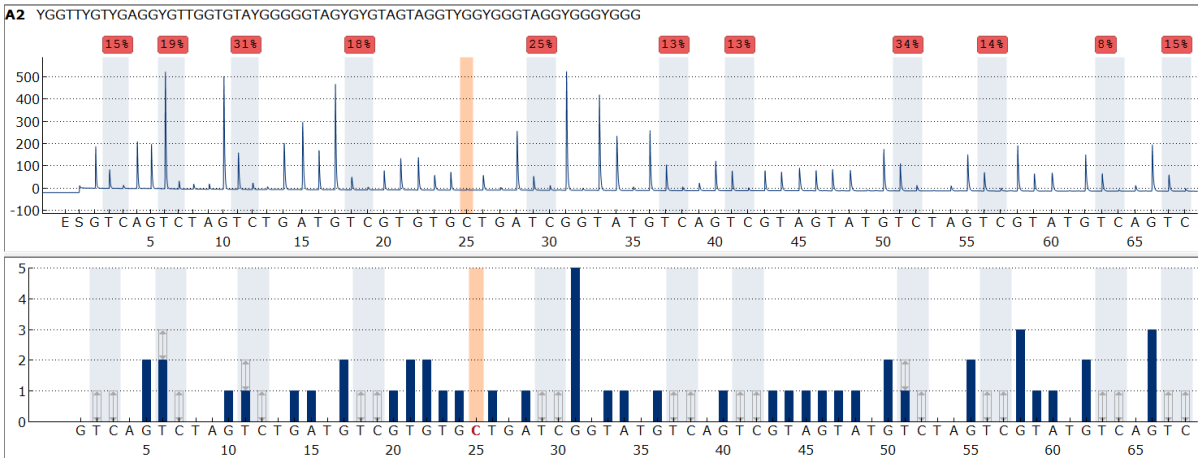


Figure 21: Representative pyrogram for a sample, indicating the formation of an unspecific product; red values indicate that the analysis failed. The histogram at the bottom shows the expected peak pattern

Unspecific products may be formed during PCR amplification, because of unspecific annealing of PCR primers. The formation of unspecific products could not be eliminated by increasing the annealing temperature. In order to save time and costs, all PCR products were loaded to an agarose gel before sequencing. Samples for which more than one band was obtained were not subjected to PSQ.

4.4 Determination of the DNA methylation status in whole blood samples from Alzheimer's disease patients and healthy persons

DNA has already been isolated from whole blood samples of 40 patients suffering from LOAD (sample name beginning with "A", see Table 6) and 40 healthy individuals serving as control (sample name beginning with "K", see Table 7). The isolated DNA was obtained from Ass. Prof. Dipl. Ing. Dr. Maria Zellner and Mag. Dr. Ellen Umlauf, Institute of Physiology, Department of Physiology and Pharmacology, Medical University of Vienna.

The LOAD patients and healthy individuals were females between 65 and 94 years old. All persons were nonsmokers and both groups were age-matched. The age of the persons can be found in APPENDIX B.

After treatment with sodium bisulfite, the DNA was amplified by PCR. Experimental details on sodium bisulfite conversion and PCR amplification are given in chapters 6.2 and 6.4, respectively. Then the DNA methylation status of 11 CpGs was determined by the PSQ method developed in course of the master thesis. Information on PSQ analysis is given in chapter 6.6.

According to the manufacturer of the pyrosequencing instrument, the limit of quantification (LOQ) of the PSQ method is 5%. Methylation levels below 5% are listed as ”<LOQ” and handled as described in chapter 6.8.2.

The DNA methylation status of *MAOB* in DNA extracts was determined repeatedly (n=3-5). All raw data are listed in APPENDIX A. The DNA methylation status could be determined for 36 AD patients and 40 healthy controls. In case of samples A030, A036, A049 and A052 no amplicon was obtained within 50 cycles.

In Table 4 and Table 5 the coefficients of variation (CV) of the DNA methylation status obtained by repeatedly (n=3-5) analyzing DNA extracts from AD patients and healthy controls are given, respectively.

Since methylation levels obtained by repeatedly measuring one and the same sample showed a rather high variability, data were not subjected to an outlier test. The medians and not the means were used for statistical analyses. Scattering of the data is most probably caused by the biological variability within the samples. Whole blood, from which DNA was extracted, consists of different blood cell populations, each of them showing distinct methylation patterns. DNA extracts are therefore heterogeneous mixtures of DNA strands differing in their methylation status. Thus, aliquots of one and the same DNA extract may therefore differ in their composition.

Table 6 and Table 7 show the medians of the DNA methylation status obtained by repeatedly (n=3-5) analyzing DNA extracts from AD patients and healthy controls, respectively. In all extracts, the methylation status was found to be > LOQ.

Table 4: CVs of the DNA methylation levels obtained by repeatedly analyzing (n=3-5) DNA extracts from AD patients; details on the patients given in APPENDIX B

CV [%]											
	CpG										
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A031	41	24	17	15	21	44	35	25	18	69	44
A032	23	13	17	26	14	56	20	33	73	31	39
A033	54	26	27	28	57	20	33	12	40	39	23
A034	22	39	40	79	47	40	40	15	18	44	10
A038	31	32	10	46	54	35	13	51	38	49	76
A041	49	2	26	20	2	32	22	16	20	16	9
A042	45	36	38	59	62	35	48	22	44	55	50
A043	32	22	20	51	15	13	4	6	12	64	22
A044	40	37	21	10	43	51	29	26	37	40	48
A055	46	35	7	24	15	22	30	47	46	67	6
A060	17	17	10	29	81	28	15	22	4	16	34
A064	32	10	19	22	12	52	37	49	13	22	17
A066	8	35	4	21	12	47	27	8	17	37	13
A069	52	16	15	25	14	42	30	10	21	68	19
A070	23	21	22	20	9	53	73	16	30	17	58
A072	9	27	5	14	47	23	12	60	29	42	10
A081	12	19	24	34	3	18	7	29	31	49	20
A111	9	31	6	53	50	63	49	26	44	59	12
A114	20	31	39	18	10	26	17	36	21	29	56
A117	17	29	21	13	23	10	19	24	73	60	41
A134	10	56	30	9	26	25	22	26	25	22	40
A148	19	12	29	13	21	15	10	20	46	20	46
A155	14	22	34	28	73	9	34	2	93	31	80
A159	24	38	38	30	51	43	18	75	21	63	82
A161	47	26	32	57	30	84	61	30	41	22	80
A163	6	26	14	31	14	22	14	26	34	16	27
A172	63	44	9	33	41	39	63	37	61	28	29
A174	78	54	26	50	70	72	36	59	14	61	73

Table 4 continued from previous page

CV [%]											
	CpG										
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A175	4	8	32	14	2	22	42	15	21	12	36
A179	12	29	14	17	35	21	56	33	10	20	19
A184	14	20	9	13	24	74	15	19	99	16	33
A187	11	37	39	70	44	3	16	39	43	55	35
A192	25	16	29	14	11	39	11	25	27	26	32
A199	35	9	28	25	35	21	25	12	42	14	61
A214	50	79	10	88	29	11	57	22	68	32	2
A249	33	52	31	37	39	30	17	18	68	30	26

Table 5: CVs of the DNA methylation levels obtained by repeatedly analyzing (n=3-5) DNA extracts from healthy controls; details on the patients given in APPENDIX B

CV [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
K146	5	85	16	42	47	43	58	11	59	19	86
K148	84	80	43	64	51	100	58	49	57	59	47
K149	28	44	14	21	45	53	58	51	39	33	40
K150	19	26	26	43	44	28	5	31	28	24	11
K152	38	24	13	63	50	47	29	27	18	15	73
K153	18	16	19	6	24	25	10	11	7	10	25
K155	20	14	9	2	20	7	6	15	33	22	31
K157	6	28	9	2	23	15	11	19	13	57	26
K158	77	25	11	6	24	6	28	10	48	20	39
K159	16	44	27	29	31	45	38	28	42	44	26
K177	72	74	29	63	82	66	70	32	81	59	74
K184	43	33	36	102	66	32	33	17	58	79	82
K185	17	31	12	31	20	41	46	32	42	70	64
K200	9	4	14	29	44	45	12	13	53	42	24
K203	75	76	36	73	85	34	20	75	70	60	108

Table 5 continued from previous page

CV [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
K206	36	35	17	31	22	17	27	11	21	76	33
K210	17	29	7	54	38	51	16	24	67	87	60
K214	21	26	21	29	10	30	17	29	60	36	45
K217	53	43	45	57	111	80	57	90	93	57	49
K221	28	55	20	65	123	81	35	47	108	76	44
K230	24	45	29	8	25	18	31	8	14	25	14
K257	22	5	36	37	53	58	49	65	31	52	62
K263	9	20	22	18	40	22	4	24	9	21	28
K268	14	36	29	22	31	16	3	19	29	11	45
K293	16	31	10	10	29	38	32	38	21	75	69
K305	80	64	36	66	19	10	7	11	16	49	47
K307	39	10	13	41	50	62	31	15	47	9	51
K317	18	10	13	16	19	15	23	16	31	11	12
K319	44	78	50	45	27	41	26	47	80	60	92
K320	29	32	27	44	13	66	16	21	46	16	39
K324	29	1	19	24	15	82	11	32	38	10	11
K329	29	19	21	24	43	46	33	18	25	68	53
K336	17	16	8	21	9	12	30	16	13	26	18
K337	49	34	22	20	20	47	40	29	52	19	40
K348	46	45	30	28	10	53	41	16	74	27	75
K357	32	35	37	19	31	44	29	15	12	65	69
K361	13	13	4	3	9	13	20	15	26	23	6
K374	17	35	16	16	16	13	43	21	15	30	33
K378	24	26	11	20	7	19	23	18	23	43	7
K447	57	53	25	35	8	5	21	40	37	26	45

Table 6: Medians of the DNA methylation levels obtained by repeatedly analyzing (n=3–5) DNA extracts from AD patients; details on the patients given in APPENDIX B

Methylation status [%]											
	CpG										
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A030*	-	-	-	-	-	-	-	-	-	-	-
A031	37	35	57	42	48	29	34	46	31	25	36
A032	28	40	44	31	34	27	27	43	37	36	18
A033	24	28	43	27	20	22	19	34	19	26	31
A034	32	26	40	20	20	17	25	33	21	19	13
A036*	-	-	-	-	-	-	-	-	-	-	-
A038	17	15	39	17	24	13	26	25	18	24	19
A041	40	29	44	25	35	23	36	40	26	19	27
A042	26	41	40	26	21	29	29	32	20	14	12
A043	23	29	47	25	28	28	25	40	29	17	16
A044	26	36	43	25	20	23	24	26	20	13	15
A049*	-	-	-	-	-	-	-	-	-	-	-
A052*	-	-	-	-	-	-	-	-	-	-	-
A055	32	14	40	41	42	23	38	20	20	14	11
A060	46	19	51	13	11	34	25	23	12	18	21
A064	25	37	47	10	27	16	23	33	12	22	12
A066	20	18	34	21	16	9	20	27	21	12	15
A069	23	67	59	21	44	17	15	43	18	12	14
A070	33	32	43	33	11	13	14	20	9	28	11
A072	32	27	47	32	20	16	25	30	21	15	21
A081	26	25	37	21	22	30	29	37	15	21	12
A111	18	19	30	17	14	14	18	29	16	16	19
A114	19	19	25	18	34	17	19	22	11	9	8
A117	32	27	55	27	31	24	29	38	11	20	16
A134	23	13	48	28	23	23	28	39	24	23	17
A148	42	25	62	22	27	50	54	37	11	15	19

Table 6 continued from previous page

Methylation status [%]											
	CpG										
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A155	37	59	42	38	35	35	40	53	16	35	17
A159	24	14	37	30	18	29	26	22	16	11	13
A161	22	39	50	8	19	8	13	22	12	12	12
A163	28	41	49	31	34	21	31	36	24	19	18
A172	34	19	42	22	21	22	28	32	23	34	20
A174	41	58	79	54	53	45	33	58	11	39	19
A175	32	35	33	27	30	18	27	33	22	20	13
A179	23	34	61	36	18	19	15	30	21	27	25
A184	55	66	55	60	19	18	18	18	10	33	22
A187	28	23	55	33	21	26	29	32	23	14	13
A192	22	39	43	26	30	18	24	36	23	25	15
A199	20	23	37	19	17	16	21	27	13	15	11
A214	21	28	53	24	30	29	31	42	6	27	18
A249	25	27	39	25	18	17	28	28	15	24	14

* no amplification product was obtained within 50 cycles

Table 7: Medians of the DNA methylation levels obtained by repeatedly analyzing (n=3-5) DNA extracts from healthy controls; details on the patients given in APPENDIX B

Methylation status [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
K146	40	19	61	25	33	27	23	34	21	28	20
K148	28	24	42	18	31	13	34	45	20	20	14
K149	28	29	42	33	24	25	18	33	17	27	14
K150	32	37	39	29	43	41	34	44	39	25	31
K152	27	28	46	18	19	18	25	36	17	24	14
K153	29	32	47	21	35	32	37	37	30	27	17
K155	39	45	51	42	50	38	37	31	15	16	20
K157	32	32	43	30	17	19	25	35	19	19	16
K158	25	36	53	26	30	26	26	39	18	23	14
K159	46	48	57	40	30	24	43	32	38	31	33
K177	36	25	50	23	27	26	30	37	21	24	11
K184	14	28	44	18	29	17	24	31	9	11	4
K185	47	24	63	30	38	30	28	45	28	14	17
K200	27	49	40	21	20	12	20	25	12	11	20
K203	18	42	40	18	18	19	23	16	12	13	9
K206	20	25	42	29	37	23	26	33	27	13	19
K210	28	37	46	18	24	19	36	24	13	5	22
K214	35	38	53	33	31	29	37	33	15	25	18
K217	14	13	20	14	10	11	35	15	9	9	8
K221	32	16	35	20	14	21	30	40	14	19	22
K230	36	22	35	21	29	19	25	26	23	20	19
K257	31	35	45	20	18	17	23	32	13	8	14
K263	38	20	52	19	15	22	32	26	30	12	16
K268	26	17	39	25	23	37	41	39	18	26	24
K293	22	29	46	18	27	13	29	29	24	11	16
K305	31	31	52	15	14	38	41	44	36	24	19

Table 7 continued from previous page

Methylation status [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
K307	36	47	60	35	27	24	26	37	20	31	23
K317	21	36	32	16	22	18	22	44	21	25	27
K319	34	34	31	21	29	33	32	38	22	13	11
K320	43	34	51	37	29	18	24	34	21	21	28
K324	24	35	48	27	25	5	20	38	24	14	17
K329	21	42	46	17	15	10	18	16	9	7	12
K336	25	28	44	20	30	25	21	36	16	27	25
K337	25	31	42	32	25	19	26	47	22	21	25
K348	22	15	38	18	19	22	22	32	15	18	13
K357	34	32	45	24	17	22	30	37	17	17	14
K361	35	24	52	31	30	29	33	42	34	32	21
K374	33	26	36	23	36	20	36	39	17	16	19
K378	42	32	61	36	35	36	37	37	25	31	28
K447	24	19	35	24	18	25	23	43	20	25	13

Figure 22 shows the distribution of the medians of the DNA methylation levels for each CpG.

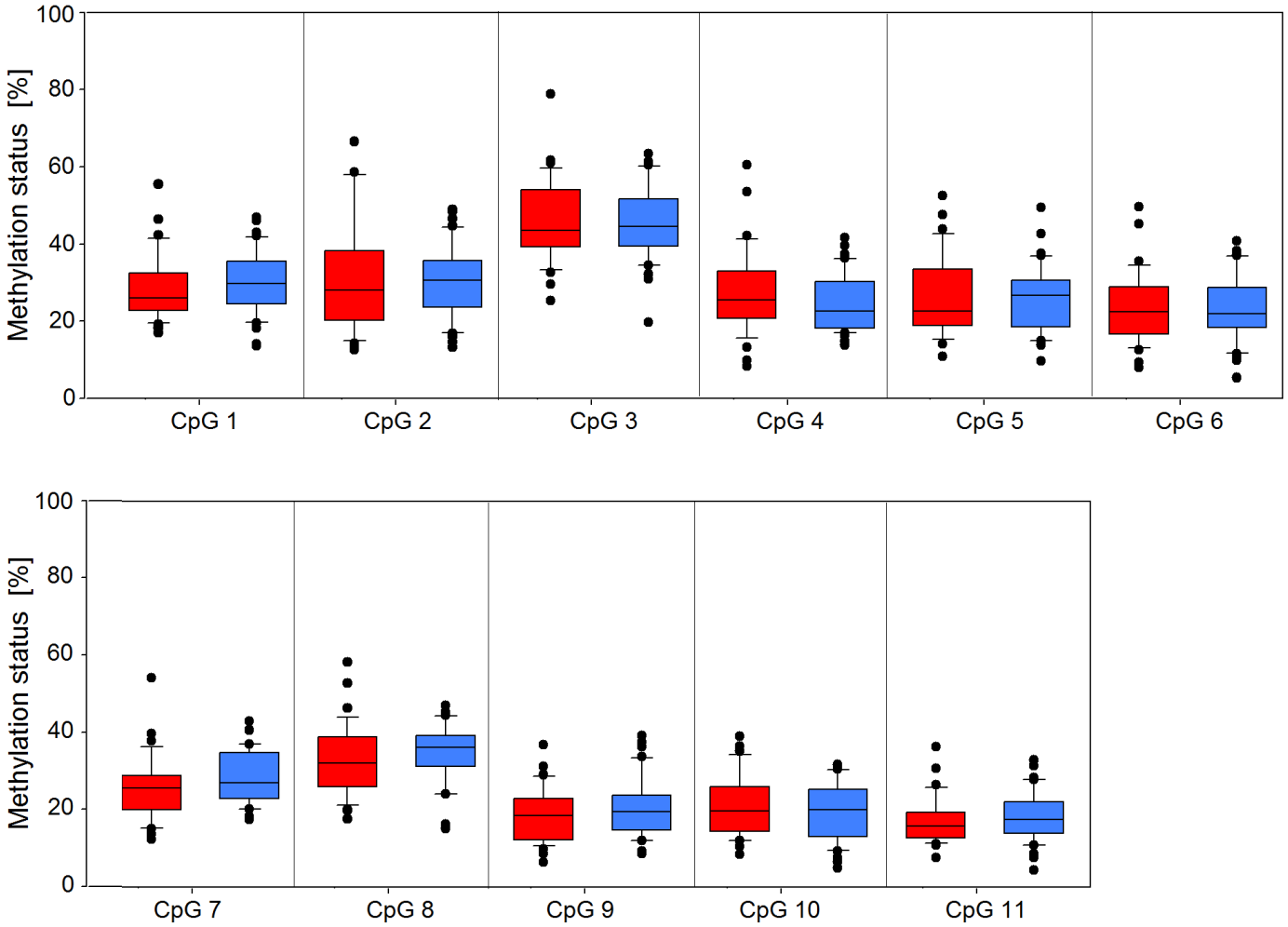


Figure 22: Distribution of the medians of the DNA methylation levels obtained by repeatedly analyzing DNA extracts from AD patients and controls; red=AD patients, blue=controls; the horizontal line inside the boxes indicates the median

4.4.1 Testing for significant differences between AD patients and controls

A t-test was performed to examine if there is a significant difference in the DNA methylation status between AD patients and healthy persons. For the t-test, the medians given in Table 6 Table 7 were used.

In Table 8 the p-values obtained by performing the t-test are summarized.

Table 8: P-values obtained by testing for significant differences in the DNA methylation status between 36 AD patients and 40 controls; the t-test was performed for each CpG

CpG	P-value
1	0.524
2	0.699
3	0.688
4	0.199
5	0.972
6	0.858
7	0.145
8	0.439
9	0.206
10	0.464
11	0.393

No significant difference in the DNA methylation status of CpG 1-11 of *MAOB* between the two groups (36 AD patients and 40 controls) was found.

4.4.2 Comparison with previous studies

Launay *et.al.* investigated the DNA methylation status of *MAOB* in smokers, former smokers and nonsmokers. The DNA was isolated from peripheral blood mononuclear cells (PBMC) and the methylation status of 22 CpG dinucleotides in the promoter region of *MAOB* was determined. The study showed that smoking induced hypomethylation of CpGs and that this effect persisted after quitting smoking (68). Figure 23 shows the DNA methylation status in smokers (S), former smorkers (FS) and nonsmokers (NS). Since our samples were from nonsmokers, we compared the methylation status of nonsmokers from the Launay study to our results. CpG 15-22, shown in orange in Figure 23, correspond to CpG 1-8 targeted by the PSQ method which was developed in course of the master thesis.

In order to be able to compare the results, for each of the CpGs 1-8 we calculated the mean of the medians obtained for the AD patients (Table 6) and the healthy controls (Table 7). The mean values are listed in Table 9.

Table 9: Mean of the methylation levels determined for the 36 AD patients and 40 healthy controls examined in course of the present study. Mean values were calculated from the medians given in Table 6 and Table 7

CpG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
AD patients								
Mean methylation status [%]	29	31	46	27	26	23	26	33
Controls								
Mean methylation status [%]	30	30	45	25	26	23	29	35

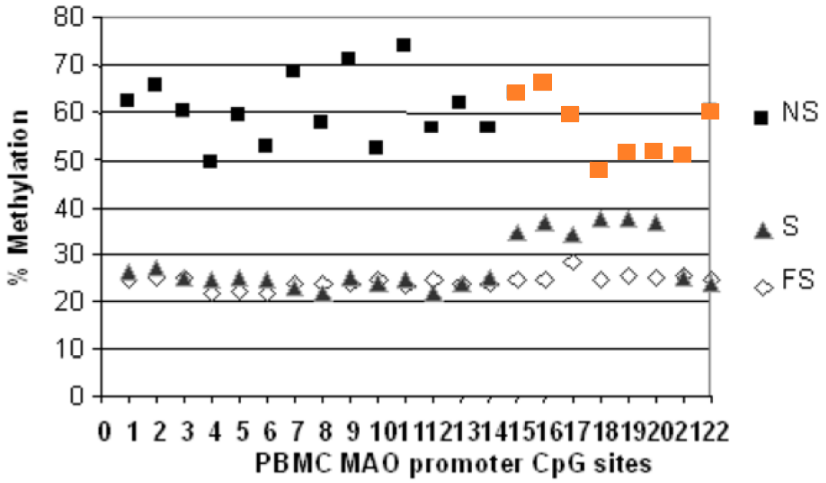


Figure 23: DNA methylation status of MAOB in PBMC from smokers (S), former smokers (FS) and nonsmokers (NS), 22 CpGs were analyzed, CpG 15-22, shown in orange correspond to CpG 1-8 targeted by the PSQ method developed in course of the master thesis; from (68), modified

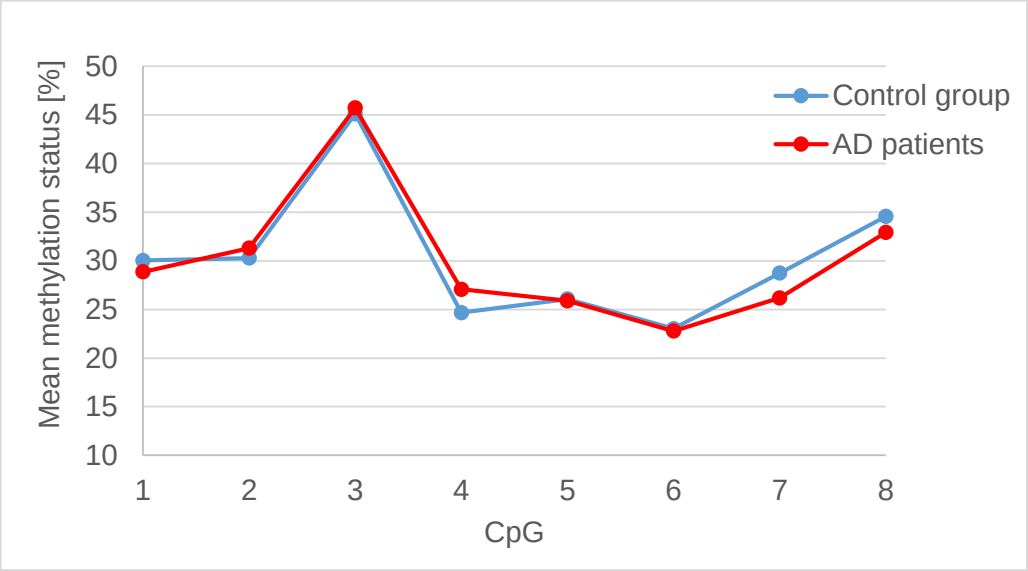


Figure 24: Mean methylation status of CpG 1-8 in 36 AD patients and 40 healthy controls. Mean values were calculated from the medians given in Table 6 and Table 7

Figure 24 indicates that the methylation levels determined in the present study are in line with those obtained by Launay et. al. In the present study, CpG 4-7 showed a lower methylation status than CpG 1-3 and CpG 8. This finding is in line with the study of Launay et.al., in which the methylation status of CpG 18-21 was lower than that of CpG 15-17 and CpG 22. In general, the methylation levels determined in course of the master thesis were lower than those reported by Launay et al. This difference is most probably caused by the fact that in the present study, DNA was isolated from whole blood, whereas in the study of Launay et al. DNA was derived from PBMC.

4.4.3 Correlation of the DNA methylation status with characteristics of AD patients and healthy controls

Our cooperation partners kindly provided information on the persons/samples examined in course of the master thesis. Details are given in APPENDIX B.

For each of the CpGs, statistical analyses were carried out to investigate if there is a correlation between the methylation status and one of the following parameters:

- MAOB expression levels in platelets
- Age of individuals
- Age of samples
- Number of white blood cells in whole blood (WBC in WB)
- Number of red blood cells in whole blood (RBC in WB)

We did not find a correlation between the methylation status and the parameters tested.

4.5 Determination of the DNA methylation status in pooled DNA extracts

In course of the master thesis we learned that the bisulfte conversion kit which was used for all DNA extracts had not been stored at 4°C as suggested by the supplier but at -20 °C. We thus re-checked each of the pyrograms with regard to the bisulfite control. In none of the pyrograms, the bisulfite control indicated that bisulfite conversion was not complete. In addition, bisulfite conversion was repeated by using a new bisulfite kit that had been stored as suggested by the supplier. However, in contrast to the experiments described above, mixtures of DNA extracts (“pooled” DNA extracts) and not DNA extracts from individual persons were used. The main reason for using mixtures was to save time and costs.

In total, nine pools were produced by mixing DNA extracts from individual AD patients. In addition, nine pools were obtained by mixing DNA extracts from individual healthy controls. Details on the preparation and composition of the pools are given in chapter 6.1 and APPENDIX C, respectively. Each pool was analyzed four times. Table 10 lists the medians of the methylation levels, Table 11 the CVs.

Table 10: Medians of the DNA methylation levels obtained by repeatedly analyzing (n=4) pooled extracts from AD patients (P 1-9) and healthy controls (P 11-19); details on the pools given in APPENDIX C

Methylation status [%]											
AD patients											
CpG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P1	27	28	40	26	21	17	22	22	11	15	12
P2	24	15	38	24	21	25	26	27	13	15	15
P3	27	28	34	21	21	21	26	28	18	20	17
P4	19	13	37	14	23	12	18	24	11	9	15
P5	11	25	37	29	33	9	27	18	6	19	22
P6	39	26	40	18	4	24	34	26	17	15	17
P7	28	34	34	22	15	20	24	21	19	17	15
P8	39	36	47	28	27	26	29	27	26	19	23
P9	29	18	44	32	16	23	25	35	26	18	19
Controls											
P11	24	19	40	19	20	23	22	25	15	13	16
P12	30	24	40	19	23	22	25	31	17	22	15
P13	22	17	36	15	20	22	25	33	15	16	8
P14	29	21	32	22	27	13	24	28	18	13	17
P15	21	18	45	20	27	5	38	11	27	33	22
P16	30	21	42	22	26	17	22	31	20	13	13
P17	24	24	44	28	21	18	23	25	15	15	15
P18	23	21	39	25	28	26	27	24	18	20	14
P19	18	23	38	21	22	26	20	26	17	15	16

Table 11: CVs of the DNA methylation levels obtained by repeatedly analyzing (n=4) pooled extracts from AD patients (P 1-9) and healthy controls (P 11-19); details on the pools given in APPENDIX C

CV [%]											
AD patients											
CpG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P1	26	48	20	16	14	7	20	33	14	22	40
P2	28	48	17	16	20	28	32	29	44	28	21
P3	38	32	11	53	45	25	15	17	36	9	31
P4	35	56	51	70	17	68	44	45	52	49	50
P5	118	58	33	37	13	110	59	60	53	64	67
P6	23	55	28	48	138	66	22	51	64	60	74
P7	51	37	29	31	47	30	44	38	59	40	44
P8	21	41	28	42	38	21	45	30	19	77	28
P9	24	42	12	19	36	39	22	19	21	27	26
Controls											
P11	21	42	18	36	33	28	35	31	38	15	35
P12	24	18	35	20	21	32	22	23	25	34	22
P13	34	33	23	33	28	21	34	6	10	30	58
P14	29	27	12	22	27	33	33	17	32	28	33
P15	64	88	29	78	51	150	27	134	48	29	60
P16	19	40	11	28	20	78	22	20	26	69	52
P17	18	26	13	15	38	23	13	20	13	28	41
P18	23	17	10	23	22	17	32	14	19	10	34
P19	24	28	13	16	28	7	34	10	16	23	39

Figure 25 shows the distribution of the medians of the DNA methylation levels for each of the CpGs.

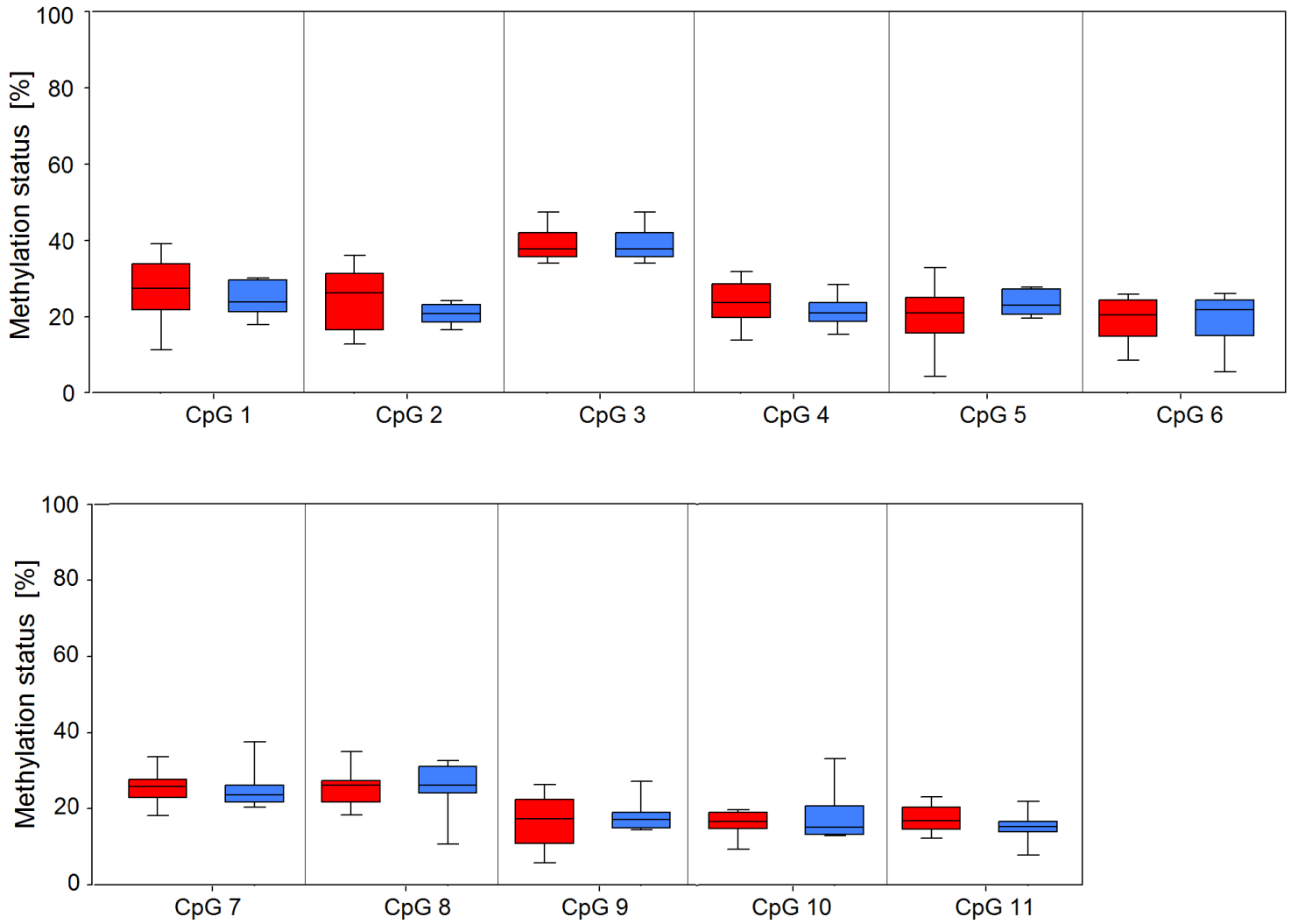


Figure 25: Distribution of the medians of the DNA methylation levels obtained by repeatedly (n=4) analyzing pooled extracts from AD patients and controls; red=AD patients, blue=controls; the horizontal line inside the boxes indicates the median

4.5.1 Testing for significant differences between pooled extracts from AD patients and healthy controls

Medians of the methylation levels (Table 10) were subjected to a t-test to test for significant difference in DNA methylation levels of pooled extracts between AD patients and healthy persons.

In Table 12 the p-values of the t-test are summarized.

Table 12: P-values obtained by testing for significant differences in the DNA methylation status of pooled extracts between AD patients and controls; the t-test was performed for each CpG

CpG	P-value
1	0.465
2	0.196
3	0.781
4	0.267
5	0.226
6	0.856
7	0.812
8	0.808
9	0.530
10	0.563
11	0.235

No significant difference in the DNA methylation status of CpG 1-11 of pooled extracts between AD patients and healthy controls was found.

4.6 Comparison of DNA methylation levels obtained for DNA extracts from individual persons and those obtained for the respective pooled extracts

4.6.1 Correlation between pool and individual samples

Among the 18 pooled extracts prepared, pools 1-4 and pools 11-14 consisted of individual extracts the methylation status of which was determined in course of the master thesis (see 4.5, APPENDIX C, Table 6 and Table 7)

In order to be able to compare the results for individual extracts with results obtained by analyzing the pooled extracts, we calculated the mean of the methylation levels obtained for the extracts which were contained in the respective pool.

The results are summarized in Table 13. In Figure 26 and Figure 27, the methylation levels obtained for the pooled extracts were plotted against the mean values calculated from the methylation levels obtained by analyzing the individual extracts contained in the pools.

Table 13: Methylation levels obtained for the pooled extract in comparison to the methylation levels obtained by analyzing the individual extracts contained in the pools. "P" stands for pooled, "I" for individual extracts. Methylation data for pooled extracts are the medians of the DNA methylation levels obtained by repeatedly (n=4) analyzing pooled extracts. Methylation data for individual extracts are the means of the methylation levels obtained by analyzing the individual extracts (Table 6 and Table 7) contained in the pools

Methylation status [%]											
AD patients											
CpG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P1	27	28	40	26	21	17	22	22	11	15	12
I1	31	32	45	32	25	21	26	30	18	23	19
P2	24	15	38	24	21	25	26	27	13	15	15
I2	28	31	49	24	26	25	28	34	17	22	15
P3	27	28	34	21	21	21	26	28	18	20	17
I3	29	34	45	26	27	26	27	36	19	19	15
P4	19	13	37	14	23	12	18	24	11	9	15
I4	27	27	44	28	26	18	23	31	21	18	20
Controls											
CpG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P11	24	19	40	19	20	23	22	25	15	13	16
I11	32	30	49	24	26	24	28	34	19	18	18
P12	30	24	40	19	23	22	25	31	17	22	15
I12	32	30	43	27	25	25	31	37	21	24	20
P13	22	17	36	15	20	22	25	33	15	16	8
I13	30	32	45	23	24	22	28	31	21	16	17
P14	29	21	32	22	27	13	24	28	18	13	17
I14	26	29	43	24	29	21	28	34	20	19	17

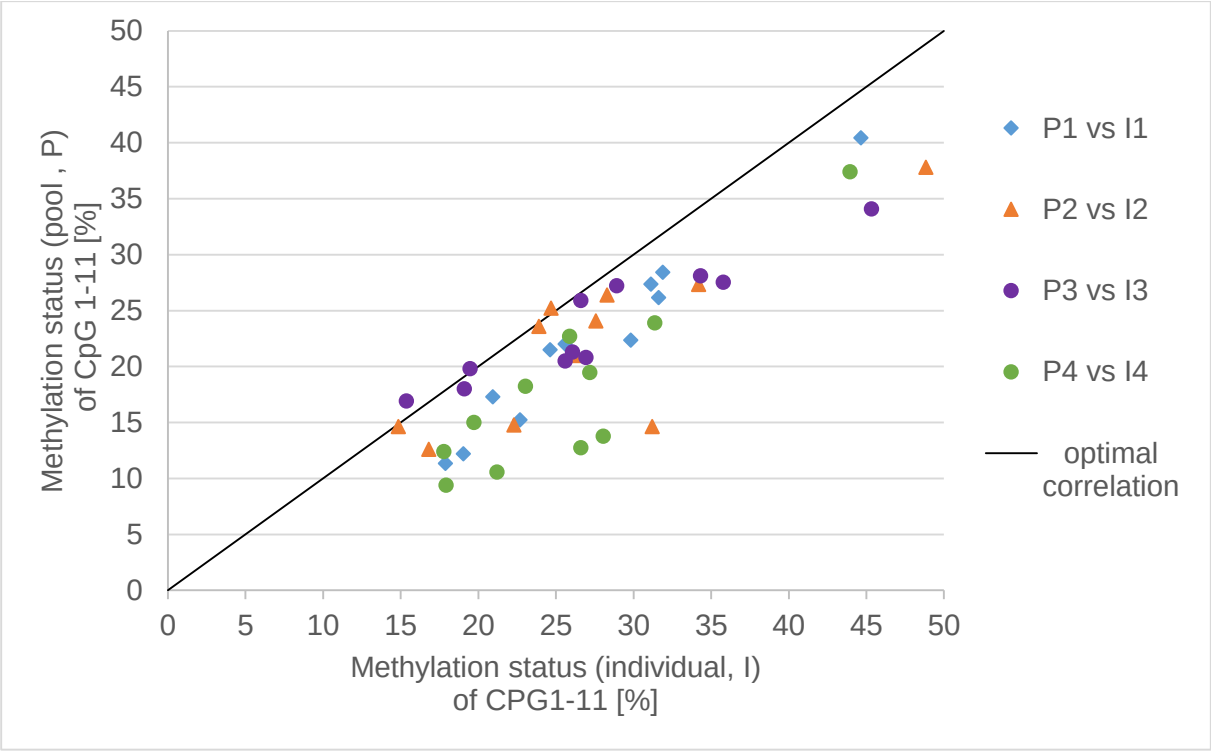


Figure 26: Correlation of the methylation levels obtained by the analysis of pooled extracts from AD patients and those obtained by analysis of the individual extracts contained in the pools. "P" stands for pooled, "I" for individual extracts. Methylation data for pooled extracts are the medians of the DNA methylation levels obtained by repeatedly analyzing pooled extracts. Methylation data for individual extracts are the means calculated from the methylation levels obtained by analyzing the individual extracts contained in the pools

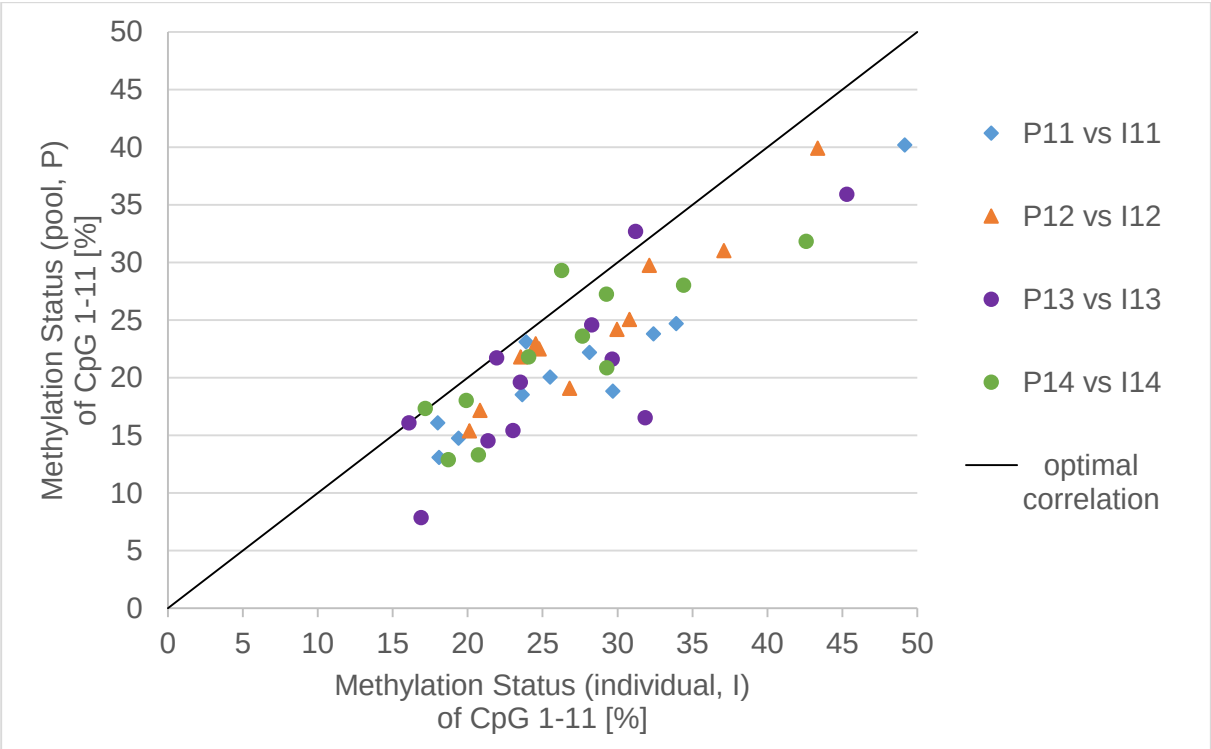


Figure 27: Correlation of the methylation levels obtained by the analysis of pooled extracts from healthy controls and those obtained by analysis of the individual extracts contained in the pools. "P" stands for pooled, "I" for individual extracts. Methylation data for pooled extracts are the medians of the DNA methylation levels obtained by repeatedly analyzing pooled extracts. Methylation data for individual extracts are the means calculated from the methylation levels obtained by analyzing the individual extracts contained in the pools

The figures indicate that the analysis of individual extracts resulted in higher methylation levels than the analysis of the respective pooled extracts.

As already mentioned above, the bisulfite kit applied to the individual DNA extracts was not stored as suggested by the supplier. In general, due to incomplete conversion with bisulfite, too high methylation levels would be obtained. However, in none of the pyrograms the bisulfite control indicated that the bisulfite conversion was incomplete.

4.6.2 Comparison of the coefficients of variation

As discussed in section 4.4, the variability of methylation levels obtained by analyzing DNA extracts from individuals was rather high. We were interested if the analysis of pooled extracts resulted in a lower variability. Table 14 compares the CVs obtained by repeatedly analyzing pooled extracts with those obtained when the individual extracts were analyzed repeatedly.

Table 14: Comparison of the CVs obtained by repeatedly analyzing pooled extracts with those obtained when the individual extracts were analyzed repeatedly. "P" stands for pooled, "I" for individual extracts. CVs for pooled extracts were obtained by repeatedly analyzing pooled extracts. CVs for individual extracts are the means of the CVs obtained by analyzing the individual extracts (Table 4 and Table 5) contained in the pools

CV [%]											
AD patients											
CpG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P1	26	48	20	16	14	7	20	33	14	22	40
I1	35	32	15	32	28	39	38	25	48	33	25
P2	28	48	17	16	20	28	32	29	44	28	21
I2	32	26	25	38	32	39	24	34	41	35	47
P3	38	32	11	53	45	25	15	17	36	9	31
I3	24	26	27	27	36	27	24	23	34	42	44
P4	35	56	51	70	17	68	44	45	52	49	50
I4	19	31	20	27	29	34	31	30	19	40	25
Controls											
CpG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P11	24	19	40	19	20	23	22	25	15	13	16
I11	32	31	18	29	31	36	30	20	42	46	46
P12	30	24	40	19	23	22	25	31	17	22	15
I12	33	40	25	36	38	39	27	29	49	32	47
P13	22	17	36	15	20	22	25	33	15	16	8
I13	32	33	24	32	34	31	22	27	25	44	45
P14	29	21	32	22	27	13	24	28	18	13	17
I14	31	37	23	38	45	48	34	35	46	39	44

A t-test was performed to examine if there was a significant difference in the CVs obtained for individual extracts and extract pools.

The p-values obtained from the t-tests can be seen in Table 15 (AD patients) and Table 16 (controls), respectively.

Table 15: P-values obtained by testing for significant differences in the CVs of AD patients between pooled extracts and the respective individual extracts, the t-test was performed for each CpG

AD patients	
CpG	**P-value**
1	0.394
2	0.017
3	0.759
4	0.612
5	0.361
6	0.842
7	0.844
8	0.650
9	0.926
10	0.268
11	0.978

Table 16: P-values obtained by testing for significant differences in the CVs of controls between pooled extracts and the respective individual extracts, the t-test was performed for each CpG

Controls	
CpG	**P-value**
1	0.056
2	0.001
3	0.001
4	0.001
5	0.006
6	0.005
7	0.157
8	0.688
9	0.004
10	0.001
11	< 0.001

Table 15 indicates that in case of DNA extracts from AD patients, with the exception of CpG 2 no significant difference was found. Surprisingly, the analysis of pooled extracts resulted in a higher variability than the analysis of individual extracts.

In case of DNA extracts from healthy controls, a significant difference was found for 8 CpGs. With the exception of one CpG, the analysis of pooled extracts resulted in a lower variability than the analysis of individual extracts.

5 Conclusion

In this master thesis the DNA methylation status of *MAOB* in 36 AD patients and 40 healthy controls was determined. Furthermore, the DNA methylation status of 9 pooled extracts from AD patients and 9 pooled extracts from controls was determined. The methylation levels were analyzed by PSQ with an in house developed method.

No significant difference in the DNA methylation status between AD patients and healthy controls was found, neither in DNA extracts from individuals nor in pooled extracts.

The variability of methylation levels obtained by repeatedly analyzing one and the same extract were rather high. Thus, medians of methylation levels were used for statistical analyses.

We assume that the scattering of the data is caused by the biological variability within the samples. Whole blood, from which DNA was extracted, consists of different blood cell populations. Each type of blood cell may have a distinct methylation pattern. Thus, DNA extracts are heterogeneous mixtures of DNA strands differing in their methylation status. If aliquots of one and the same DNA extract differ in their composition, the repeatability of the results will be low.

In general, the analysis of pooled extracts from healthy controls resulted in a lower variability than the analysis of the respective individual extracts. However, in case of AD patients, we did not find a significant difference in the variability.

In contrast to pooled extracts, the DNA extracts from individual persons were converted with a bisulfite kit that was stored at -20 “C and not at 4°C, as suggested by the supplier. However, in none of the pyrograms the bisulfite control indicated that bisulfite conversion was incomplete.

6 Experimental part

6.1 DNA isolation

DNA was isolated from whole blood samples (of AD patients and controls) by Mag. Dr. Ellen Umlauf, Institute of Physiology, Medical University of Vienna.

The isolation of DNA was performed with a column-based *PerfectPure DNA Blood Kit 0.4 ml*. Purity and concentration of the DNA were determined with *NanoDrop* (Thermo Scientific) spectrophotometer. DNA extracts were diluted with RNase-free water to obtain a concentration of 12.5 ng/µL

In total, DNA extracts from 40 AD patients and 40 controls were obtained. In addition, 18 pooled DNA extracts were prepared by Mag. Dr. Ellen Umlauf, of which nine contained DNA from AD patients (Pool 1-9) and nine contained DNA from controls (Pool 10-19).

Pools consisted of ten individual DNA extracts and were prepared to contain the same amount of DNA from each extract. One pool (Pool 4) consisted of only six DNA extracts. The final concentration of the pooled extracts was 12.5 ng/µL. APPENDIX C gives information on the composition of each pool.

6.2 Bisulfite conversion

Commercially available unmethylated and methylated control DNA and sample DNA were bisulfite treated. Bisulfite conversion was performed with the *EpiTect Fast DNA Bisulfite Kit* from Qiagen according to the manufacturer's protocol (69).

Before starting, 30 mL of ethanol (absolute) were added to the wash buffer (*Buffer BW*) and 27 mL were added to the desulfonation buffer (*Buffer BD*). The bisulfite reaction was set up in 200 µL PCR tubes according to Table 17.

Table 17: Pipetting scheme for high and low concentration sample

	High concentration sample (1 ng – 2 µg DNA)	**Low concentration sample** (1 – 500 ng DNA)
Component	**Volume [µL]**	**Volume [µL]**
DNA	Variable x (0 – 20)	Variable (0 – 40)
RNase free water	Variable (20 minus x)	Variable (40 minus x)
Bisulfite solution	85	85
DNA protect buffer	35	15
Total volume	140	140

The reaction tubes were closed, vortexed and placed in the thermal cycler *Rotor-Gene Q* from Qiagen. The conditions for bisulfite conversion are listed in Table 18.

Table 18: Thermal cycler conditions for bisulfite conversion

Step	**Time [min]**	**Temperature [°C]**
Denaturation	5	95
Incubation	10	60
Denaturation	5	95
Incubation	10	69
Hold	1	25

The PCR tubes were centrifuged briefly at 2000 rpm. Then the reaction mixtures were transferred to clean 1.5 mL Eppendorf reaction tubes and 310 µL loading buffer (*Buffer BL*) were added. After vortexing, the reaction mixtures were shortly centrifuged at 5000-6000 rpm. 250 µL ethanol (absolute) were added, vortexed for 15 s and then briefly centrifuged. The entire content of the PCR tubes was transferred to *MinElute*® DNA spin columns with a corresponding collection tube. After centrifuging the spin columns for 1 min at 14000 rpm, the flow-through was discarded and the spin columns placed back into the collection tubes. 500 µL of *Buffer BW* were added on the spin columns and then it was again centrifuged for 1 min at 14000 rpm. After the flow-through was discarded, the spin columns were placed back into the collection tubes. Then 500 µL of *Buffer BD* were added and incubated for 15 min at room temperature. After this step, the spin columns were centrifuged for 1 min at 14000 rpm and the flow-through was discarded. In each of the next two washing steps, 500 µL *Buffer BW* were added, centrifuged for 1 min at 14000 rpm and the flow-through discarded. Then 250 µL of

ethanol were added on the spin columns and centrifuged again for 1 min at 14000 rpm. Next, the spin columns were incubated with open lids in a heating block for five min at 60°C to evaporate the liquid. After the spin columns were placed into new 1.5 mL collection tubes, it was centrifuged for 1 min to remove any remaining liquid. Then the spin columns were placed again in new 1.5 mL collection tubes and 15 µL of elution buffer (Buffer EB) were added onto the center of the spin columns membrane. After an incubation of 1 min, the spin columns were centrifuged for 1 min at 12000 rpm for elution of the converted DNA. The filtrate was diluted with RNase free water to a concentration of 5 ng/µL and stored at -20°C.

6.3 Development and optimization of the PSQ method

6.3.1 Search for target sequence

For the development of an appropriate PSQ method, the DNA sequence of *MAOB* was taken from the database *National Center for Biotechnology Information (NCBI)* (64). The promoter region was located with the help of the *Eukaryotic Promoter Database (EPD)* (65).

6.3.2 Primer design

With the help of the *PyroMark Assay Design 2.0* software, the primer set (forward, reverse and sequence primer) was designed. Therefore, the promoter region oft *MAOB* was copied in the design software, which also showed the bisulfite converted sequence. In the graphic view the DNA section for sequencing was defined as *Target Region*. After the assay settings were changed and optimized (see Table 19 and Table 20), the software generated several primer sets.

Table 19: PCR primer settings for the primer design

PCR primer settings	
Minimal primer length	18 nucleotides
Maximal primer length	27 nucleotides
Optimal amplicon length from	50 nucleotides
Optimal amplicon length to	100 nucleotides
Maximal amplicon length	400 nucleotides
Allow primer over variable position	yes
Minimal melting temperature	40°C
Maximal melting temperature	72°C
Maximal allowed Tm difference	10°C

Table 20: Sequencing primer settings for the primer design

Sequencing primer settings	
Minimal primer length	15 nucleotides
Maximal primer length	25 nucleotides
Minimal distance from target	0 nucleotides
Maximal distance from target	10 nucleotides
Allow primer over variable position	yes
Minimal melting temperature	29°C
Maximal melting temperature	59°C

Since the software generated several primer sets, they were checked according to the criteria discussed in 3.1.3. The melting temperature was calculated with the help of *Oligo Calc* (67). With the webserver *Oligo Analyzer 3.1* (70) the formation of primer dimers was checked. Possible secondary structures were checked with the webserver *RNA fold* (66).

6.3.3 Primer ordering

The primers were synthesized by Sigma Aldrich. The lyophilized primers were dissolved in RNase free water to obtain a concentration of 10 µM and stored in aliquots (9 µL) at -20°C.

6.3.4 PCR optimization

PCR conditions, in particular the annealing temperature (Ta) and the primer concentration, were optimized. The optimization was carried out with the *iCycler* thermal cycler (BioRad) and the software *iQ5 Optical System 2.1*. The primer set was tested witch 0% methylated and 100% methylated control DNA and no template control (NTC). For the NTC RNase free water was used instead of DNA template.

Ta was estimated (shown in Table 21) by using the melting temperatures (Tm) from Sigma Aldrich and *Oligo Calc* (67). The temperature gradient was set between Ta_1 and Ta_4. In Table 22 the annealing temperature and primer concentrations tested are summarized.

Table 21: Estimation of the annealing temperature

	Forward primer	**Reverse primer**
Sigma Aldrich	Ta_1 (Tm minus 5°C)	Ta_3 (Tm minus 5°C)
Oligo Calc	Ta_2 (Tm minus 5°C)	Ta_4 (Tm minus 5°C)

Table 22: Program settings for the temperature gradient

Temperature [°C]			
59.7	57.9	56.4	54.0
Primer concentration [µM]			
200	400		

6.4 PCR Amplification

The amplification was carried out using the *PyroMark® PCR Kit* (Qiagen) according to the manufacturer's protocol. Both *Rotor-Gene Q* thermal cycler (Qiagen) and *iCycler* thermal cycler (BioRad) were used for the PCR runs (71).

6.4.1 Preparation for the PCR

Following reagents were required for a PCR run:

- *PyroMark* PCR master mix, 2x
- CoralLoad concentrate, 10x
- Primer solutions (forward and reverse) (10 µM)
- Bisulfite converted DNA solutions of the samples (5 ng/µL)
- Bisulfite converted DNA solutions of control DNA (5 ng/µL)
- RNase free water

(The 25 mM $MgCl_2$ and the Q-Solution contained in the *PyroMark* PCR kit were not used.)

First, the master mix was prepared for DNA extracts, a no template control (NTC) and two tolerance samples containing the components mentioned above, except the DNA solution. Table 23 shows the pipetting scheme for the master mix.

Table 23: Pipetting scheme of a master mix for one sample

Component	**Volume [µL]**
PyroMark PCR master mix	12.5
CoralLoad concentrate	2.5
Forward primer	1 (0.4 µM)
Reverse primer	1 (0.4 µM)
RNase free water	6
∑	23

The master mix was pipetted in 200 µL reaction tubes, which were inserted into a cooled loading block. Afterwards, 2 µL sample DNA solution or control DNA solution were added. In each run, control DNA (0% and 100% methylated) and a NTC were included.

6.4.2 PCR settings

The thermal cyclers *Rotor-Gene Q* (Qiagen) and *iCycler* (BioRad) were programmed with their corresponding software (iQ5 Optical System 2.1, Rotor-Gene Q Series 2.1.0) according to Table 24. The PCR tubes were placed in the thermal cycler and the program was started.

Table 24 shows the temperature program for the *MAOB* method.

Table 24: Temperature program for the MAOB method

		Time	Temperature
Initialization		15 min	95°C
3 step cycling (50 cycles)	Denaturation	30 s	94°C
	Annealing	30 s	56.4°C
	Extension	30 s	72°C
Final extension		10 min	72°C

The PCR products were stored at -20°C after the run.

6.5 Agarose gel electrophoresis

Gel electrophoresis was performed by using the BioRad Mini Sub Cell GT and the power supply PowerPac HV.

6.5.1 Sample preparation

2 µL DNA ladder, 8 µL RNase free water and 2 µL 5x loading dye were mixed together. The DNA ladder (*Invitrogen™ 25 bp DNA ladder*) was supplied by life technologies. The loading dye (*5x Nucleic Acid Sample Loading Buffer*) was supplied by BioRad.

6.5.2 Procedure of agarose gel electrophoresis

First the 1x tris-acetate-EDTA (TAE) buffer was prepared, by mixing 20 mL 50x TAE buffer with 1980 mL distilled water. Next 2 g of agarose and 100 mL 1x TAE buffer were boiled until the solution was clear. 10 µL GelRed were added and the melted agarose was poured in the gel tray. A comb was placed in the tray to create wells for loading samples. After 30 min the solidified agarose gel was transferred in the electrophoresis cell. The cell was filled with 1x TAE buffer to submerge the agarose gel and the comb was removed. Then the samples (5 µL) and the DNA ladder (10 µL) were loaded in the wells. The electrophoresis cell was closed and the electrodes were connected. Then the power supply was started at 80 V.

6.6 Pyrosequencing

The experiments were performed using the *PyroMark Q24* from Qiagen with the corresponding *PyroMark Q24 Advanced 3.0.0* software. The working steps were performed with *PyroMark*

Q24 Advanced CpG Reagents from Qiagen according to the manufacturer's protocol (72). The streptavidin sepharose beads (*Streptavidin Sepharose™ High Performance*) for immobilization of PCR products were supplied by GE Healthcare.

6.6.1 Preparation for sequencing

First the *PyroMark Q24 Cartridge* (Qiagen) was loaded with the appropriate volumes of nucleotides, enzyme mixture and substrate mixture. The cartridge was then inserted in the *PyroMark* instrument. Then the DNA immobilization mix was prepared according to Table 25. The volumes given in the table refer to one sample. The immobilization mix was prepared including two tolerance samples.

Table 25: Pipetting scheme for the DNA immobilization mix

Component	Volume [µL]
Streptavidin Sepharose™	1
PyroMark binding buffer	40
High purity water	24
∑	65

A sufficient volume of 0.357 µM sequencing primer was prepared. Afterwards, 15 µL of the PCR product and 65 µL of the immobilization mix were added in an *iCycler iQ PCR plate* from BioRad. The PCR plate was sealed with adhesive foil and agitated at room temperature for 10 min at 1400 rpm. Meanwhile 20 µL of the prepared sequencing primer were added to each well of the *PyroMark Q24 Plate*, which was placed on the vacuum workstation. The PCR plate was also placed on the vacuum station. With the vacuum tool the immobilized DNA was denaturized, washed and transferred to the sequencing plate. The sequencing plate was heated for 5 min at 80°C and within 30 seconds placed on the heating block of the sequencing instrument and the run was started.

6.7 Avoiding contamination

To avoid any contamination, the preparation steps for PCR and pyrosequencing were carried out in two PCR working stations (Peqlab), which were located in two different rooms. Before the preparation the working stations were decontaminated for 30 min with UV light. Additionally, the working surface and the pipettes were cleaned with *DNA Exitus Plus IF* spray. In the first working station diverse mixes were prepared. The second working station was used to add bisulfite converted DNA or control DNA.

The thermo cyclers for PCR amplification were placed in one laboratory and the amplification products were stored in a separate room and fridge where no other reagents were stored. *The PyroMark* instrument for PSQ was also placed in another laboratory.

6.8 Data analysis

6.8.1 Pyrosequencing

The raw data obtained with the software *PyroMark* Q24 Advanced 3.0.0 were exported to Microsoft Exel 2013.

The quality assessment of the PSQ analysis results (DNA methylation status) is shown by the background color (73):

- Blue: passed
- Yellow: check
- Red: failed

Results with a red background color were not used for evaluation.

6.8.2 Methylation levels below LOQ

According to Qiagen, the limit of quantification (LOQ) of PSQ methods run on the *PyroMark Q24* instrument is 5%. For data evaluation, values below LOQ were treated as LOQ/2 (74).

6.8.3 T-test

A t-test was used to determine if there were significant differences between two groups. The test was carried out with the software *IBM SPSS 20.0*.

6.9 Chemicals and kits

Acetic Acid	Sigma Aldrich
Agarose	Sigma Aldrich
DNA Exitus Plus™ IF	AppliChem
DNA Ladder 25 bp Invitrogen™	ThermoFisher Scientific
EDTA	
EpiTect Fast DNA bisulfite kit	Qiagen

Ethanol absolute (EtOH)	VWR
GelRed™	VWR
Loading dye (5x)	BioRad
MilliQ water	
Primers	Sigma Aldrich
PyroMark® denaturation solution	Qiagen
PyroMark® PCR Kit	Qiagen
PyroMark® Q24 Advanced CpG reagents	Qiagen
PyroMark® wash buffer	Qiagen
RNase free water (ultra-filtered and autoclaved)	Sigma Aldrich
Streptavidin Sepharose™	GE Healthcare
Tris base	Sigma-Aldrich

6.10 Sample material and control DNA

CpGenome universal methylated DNA	Millipore
DNA extracts from whole blood samples	Provided by Mag. Dr. Ellen Umlauf and Ass. Prof. Dipl. Ing. Dr. Maria Zellner, Institute of Physiology, Department of Physiology and Pharmacology, Medical University of Vienna
EpiTect control DNA (1000)	Qiagen

6.11 Consumable material

Biosphere filter tips 0.5-20 µL	Sarstedt
Flat cap strips	BioRad
iCycler iQ PCR plates	BioRad
Microseal® adhesive seals	BioRad
PCR tubes 200 µL	VWR
Pipette Tips, BIO-CERT® 5-200 µL and 50-1000 µL	VWR
PyroMark Q24 cartridge	Qiagen
PyroMark Q24 plate	Qiagen
Reaction tubes 1.5 mL	VWR

6.12 Equipment

Analytical balance	TE2144S	Sartorius
Centrifuge	Centrifuge 5424	Eppendorf
Electrophoresis cell	Mini Sub Cell GT	BioRad
PCR working station		Peqlab
Pipettes		Eppendorf, BioRad
Power supply	Power Pac HV	BioRad
Pyrosequencing device	PyroMark Q24	Qiagen
Thermo cycler	Rotor-Gene Q	Qiagen
Thermo cycler	iCycler iQ5	BioRad
Vortex mixer	Classic Advanced	Velp scientifica
Vortex mixer		Janke & Kunkel

6.13 Software

BioRad iQ5 Optical System Software 2.1

IBM SPSS 20

Microsoft Exel 2013

Rotor-Gene Q Series Software 2.1.0

Sigma Plot 13.0

6.14 Webservers

Oligo Analyzer 3.1	http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/
Oligo Calc	http://www.basic.northwestern.edu/biotools/OligoCalc.html
RNAfold	http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi

6.15 Databases

Eukaryotic Promoter Database	http://epd.vital-it.ch/
National Centre for Biotechnology (NCBI)	http://www.ncbi.nlm.nih.gov/nucleotide/

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List of abbreviations

%	Percent
°C	Degrees Celcius
µg	Microgram
µL	Microliter
A	Adenine
AD	Alzheimer's disease
APH-1	Anterior pharynx defective 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid β protein
BACE	β-secretase
BC	Bisulfite conversion
bcDNA	Bisulfite converted DNA
bp	Base pairs
C	Cytosine
CpG	Cytosine-phosphate-guanine dinucleotide
CV	Coefficient of variation
dATP	Deoxyadenosine triphosphate
dATPαS	Deoxyadenosine alpha-thiotriphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Desoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleoside triphosphate
dsDNA	Double stranded DNA
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
EOAD	Early onset Alzheimer's disease
EPD	Eukaryotic promoter database

et al.	Et alii/aliae/alia (English: and others)
G	Guanine
gDNA	Genomic DNA
H_2O	Water
Hcy	Homocysteine
L	Liter
LOAD	Late onset Alzheimer' disease
LOQ	Limit of quantification
MAOB	Monoamine oxidase B
Mg	Magnesium
MgCl2	Magnesium chloride
min	Minute
mL	Mililiter
mM	Millimolar
mRNA	Messenger RNA
MTHF	Methylenetetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
MTRR	Methyltransferase reductase
NCBI	National Center for Biotechnology Information
NCT	Nicastrin
NTC	No template control
NFT	Neurofibrillary tangles
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PEN-2	Presenilin enhancer 2
PHF	Paired helical filaments
PP2A	Protein phosphatase 2A
PPi	Pyrophosphate
PS1	Presenilin 1
RBC	Red blood cell
RNA	Ribonucleic acid
SAH	S-Adenoxyl homocysteine
SAM	S-Adenosyl methionine
SNP	Single nucleotide polymorphism
SP	Senile plaques
ssDNA	Single stranded DNA
T	Thymine

Ta	Annealing temperature
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
THF	Tetrahydrofolate
Tm	Melting temperature
U	Uracil
UV	Ultra violet
WB	Whole blood
WBC	White blood cell

Literature

1. Krstic D, Knuesel I. Deciphering the mechanism underlying late-onset Alzheimer disease. Nature Reviews Neurology. 2013;9(1):25-34.
2. Hardy J. A hundred years of Alzheimer's disease research. Neuron. 2006;52(1):3-13.
3. Mattson MP. Pathways towards and away from Alzheimer's disease. Nature. 2004;430(7000):631-9.
4. Burns A, Iliffe S. Alzheimer's disease. British Medical Journal. 2009;338.
5. Bollati V, Galimberti D, Pergoli L, Dalla Valle E, Barretta F, Cortini F, et al. DNA methylation in repetitive elements and Alzheimer disease. Brain, Behavior, and Immunity. 2011;25(6):1078-83.
6. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. Nature Reviews Genetics. 2007;8(4):253-62.
7. Portela A, Esteller M. Epigenetic modifications and human disease. Nature Biotechnology. 2010;28(10):1057-68.
8. Haluskova J. Epigenetic studies in human diseases. Folia Biologica. 2010;56(3):83-96.
9. Bird A. Perceptions of epigenetics. Nature. 2007;447(7143):396-8.
10. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nature Reviews Genetics. 2012;13(2):97-109.
11. Kaminsky Z, Wang SC, Petronis A. Complex disease, gender and epigenetics. Annals of Medicine. 2006;38(8):530-44.
12. Ambros V. microRNAs: tiny regulators with great potential. Cell. 2001;107(7):823-6.
13. Coppedè F. Advances in the genetics and epigenetics of neurodegenerative diseases. Epigenetics of Degenerative Diseases. 2013;1(1).
14. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nature Reviews Genetics. 2012;13(7):484-92.
15. Singal R, Ginder GD. DNA methylation. Blood. 1999;93(12):4059-70.
16. Ling C, Groop L. Epigenetics: A molecular link between environmental factors and type 2 diabetes. Diabetes. 2009;58(12):2718-25.
17. Wang J, Yu JT, Tan MS, Jiang T, Tan L. Epigenetic mechanisms in Alzheimer's disease: implications for pathogenesis and therapy. Ageing Research Reviews. 2013;12(4):1024-41.
18. Trasler J, Deng L, Melnyk S, Pogribny I, Hiou-Tim F, Sibani S, et al. Impact of Dnmt1 deficiency, with and without low folate diets, on tumor numbers and DNA methylation in Min mice. Carcinogenesis. 2003;24(1):39-45.
19. Mastroeni D, Grover A, Delvaux E, Whiteside C, Coleman PD, Rogers J. Epigenetic mechanisms in Alzheimer's disease. Neurobiology of Aging. 2011;32(7):1161-80.
20. Wang SC, Oelze B, Schumacher A. Age-specific epigenetic drift in late-onset Alzheimer's disease. PloS One. 2008;3(7):e2698.

21. Coppede F, Tannorella P, Pezzini I, Migheli F, Ricci G, Caldarazzo lenco E, et al. Folate, homocysteine, vitamin B12, and polymorphisms of genes participating in one-carbon metabolism in late-onset Alzheimer's disease patients and healthy controls. Antioxidants & Redox Signal. 2012;17(2):195-204.
22. Matsui T, Ingelsson M, Fukumoto H, Ramasamy K, Kowa H, Frosch MP, et al. Expression of APP pathway mRNAs and proteins in Alzheimer's disease. Brain Research. 2007;1161:116-23.
23. Huang Y, Mucke L. Alzheimer mechanisms and therapeutic strategies. Cell. 2012;148(6):1204-22.
24. O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. Annual Review of Neuroscience. 2011;34:185-204.
25. Kaether C, Haass C, Steiner H. Assembly, trafficking and function of gamma-secretase. Neuro-degenerative Diseases. 2006;3(4-5):275-83.
26. Wang B, Yang W, Wen W, Sun J, Su B, Liu B, et al. Gamma-secretase gene mutations in familial acne inversa. Science. 2010;330(6007):1065.
27. Fuso A, Seminara L, Cavallaro RA, D'Anselmi F, Scarpa S. S-adenosylmethionine/homocysteine cycle alterations modify DNA methylation status with consequent deregulation of PS1 and BACE and beta-amyloid production. Molecular and Cellular Neuroscience. 2005;28(1):195-204.
28. Fuso A, Nicolia V, Cavallaro RA, Ricceri L, D'Anselmi F, Coluccia P, et al. B-vitamin deprivation induces hyperhomocysteinemia and brain S-adenosylhomocysteine, depletes brain S-adenosylmethionine, and enhances PS1 and BACE expression and amyloid-beta deposition in mice. Molecular and Cellular Neurosciences. 2008;37(4):731-46.
29. Duyckaerts C, Brion JP, Hauw JJ, Flament-Durand J. Quantitative assessment of the density of neurofibrillary tangles and senile plaques in senile dementia of the Alzheimer type. Comparison of immunocytochemistry with a specific antibody and Bodian's protargol method. Acta Neuropathologia. 1987;73(2):167-70.
30. Lovestone S, Reynolds CH. The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. Neuroscience. 1997;78(2):309-24.
31. Brion JP. Neurofibrillary tangles and Alzheimer's disease. European Neurologoy. 1998;40(3):130-40.
32. Morris M, Maeda S, Vossel K, Mucke L. The many faces of tau. Neuron. 2011;70(3):410-26.
33. Zellner M, Baureder M, Rappold E, Bugert P, Kotzailias N, Babeluk R, et al. Comparative platelet proteome analysis reveals an increase of monoamine oxidase-B protein expression in Alzheimer's disease but not in non-demented Parkinson's disease patients. Journal of Proteomics. 2012;75(7):2080-92.
34. Bortolato M, Chen K, Shih JC. Monoamine oxidase inactivation: from pathophysiology to therapeutics. Advanced Drug Delivery Reviews. 2008;60(13-14):1527-33.
35. Parnetti L, Reboldi GP, Santucci C, Santucci A, Gaiti A, Brunetti M, et al. Platelet MAO-B activity as a marker of behavioural characteristics in dementia disorders. Aging 1994;6(3):201-7.
36. Da Prada M, Cesura AM, Launay JM, Richards JG. Platelets as a model for neurones? Experientia. 1988;44(2):115-26.

37. Bock C. Epigenetic biomarker development. Epigenomics. 2009;1(1):99-110.
38. Strimbu K, Tavel JA. What are biomarkers? Current Opinion in HIV and AIDS. 2010;5(6):463-6.
39. Tost J. DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. Molecular Biotechnology. 2010;44(1):71-81.
40. Müller HJ, Prange DR. PCR - Polymerase-Kettenreaktion. Springer Spektrum. 2016;2.Auflage:2-7.
41. Jansohn M. Gentechnische Methoden. Eine Sammlung von Arbeitsanleitungen für das molekularbiologische Labor. Elsevier Spektrum Akademischer Verlag. 2006;4. Auflage:135-71.
42. Available from: https://www.abmgood.com/marketing/knowledge_base/polymerase_chain_reaction_introduction.php 2016
43. Glick B, Pasternak, J. J. Molecular biotechnology: Principles and applications of recombinant DNA. American Society for Microbiology. 2002;3. edition.
44. McPherson M, Moller S. PCR second edition. Taylor & Francis Group. 2006:15-8, 222.
45. Chawla HS. Basic techniques. Introduction to plant biotechnology. Science Publishers. 2004;2nd edition.
46. Stellwagen NC. DNA gel electrophoresis. In: Tietz D, editor. Nucleic Acid Electrophoresis. Berlin, Heidelberg: Springer Berlin Heidelberg; 1998. p. 1-53.
47. Matissek R, Steiner G, Fischer M. Lebensmittelanalytik. 5. Auflage: Springer Berlin Heidelberg; 2014. p. 415-21.
48. Lottspeich F, Engels JW. Bioanalytik. Springer Spektrum. 2012;2. Auflage:750.
49. Robyt JF, White BJ. Biochemical techniques theory and practice. Waveland Press. 1990.
50. "Agarose gel electrophoresis" Available from: http://ocw.mit.edu/courses/biological-engineering/20-109-laboratory-fundamentals-in-biological-engineering-fall-2007/labs/mod1_2/.
51. Structural formula of ethidium bromide. Available from: https://wwwthermofishercom/order/catalog/product/15585011. 2016.
52. Huang Q, Fu WL. Comparative analysis of the DNA staining efficiencies of different fluorescent dyes in preparative agarose gel electrophoresis. Clinical Chemistry and Laboratory Medicine. 2005;43(8):841-2.
53. Waring MJ. Complex formation between ethidium bromide and nucleic acids. Journal of Molecular Biology. 1965;13(1):269-82.
54. Genereux DP, Johnson WC, Burden AF, Stöger R, Laird CD. Errors in the bisulfite conversion of DNA: modulating inappropriate- and failed-conversion frequencies. Nucleic Acids Research. 2008;36(22):e150-e.
55. Hayatsu H. Discovery of bisulfite-mediated cytosine conversion to uracil, the key reaction for DNA methylation analysis - a personal account. Proceedings of the Japan Academy Series B, Physical and Biological Sciences. 2008;84(8):321-30.
56. Krueger F, Kreck B, Franke A, Andrews SR. DNA methylome analysis using short bisulfite sequencing data. Nature Methods. 2012;9(2):145-51.

57. Harrington CT, Lin EI, Olson MT, Eshleman JR. Fundamentals of pyrosequencing. Archives of Pathology & Laboratory Medicine. 2013;137(9):1296-303.
58. Schock G, Traeger T. Techniques to overcome bottlenecks in epigenetic research. Qiagen GmbH.
59. Ronaghi M. Pyrosequencing sheds light on DNA sequencing. Genome Research. 2001;11(1):3-11.
60. Lehmann U, Albat C, Kreipe H. High-resolution quantitative methylation analysis of microRNA genes using Pyrosequencing. Methods in Molecular Biology. 2012;878:229-40.
61. Ronaghi M, Uhlén M, Nyrén P. A sequencing method based on real-time pyrophosphate. Science. 1998;281(5375):363-5.
62. Griffiths AJF. Introduction to genetic analysis. New York: W.H. Freeman; 2012.
63. Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P. Real-time DNA sequencing using detection of pyrophosphate release. Analytical Biochemistry. 1996;242(1):84-9.
64. National Center for Biotechnology Information (NCBI). Available from: https://wwwncbinlmnihgov/.
65. Eucariotic Promoter Database (EPD). Available from: http://epdvital-itch/.
66. RNAfold Available from: http://rnatbiunivieacat/cgi-bin/RNAfoldcgi.
67. Oligo Calc: Oligonucleotide Properties Calculator. Available from: http://biotoolsnubicnorthwesternedu/OligoCalchtml.
68. Launay JM, Del Pino M, Chironi G, Callebert J, Peoc'h K, Megnien JL, et al. Smoking induces long-lasting effects through a monoamine-oxidase epigenetic regulation. PloS One. 2009;4(11):e7959.
69. Qiagen. EpiTect, fast bisulfite conversion handbook 2012.
70. OligoAnalyzer 3.1. Available from: http://euidtdnacom/calc/analyzer.
71. Qiagen. *EpiTect, PyroMark* PCR handbook. 2009.
72. Qiagen. PyroMark Q24 Advanced CpG reagents handbook. 2012.
73. Qiagen. PyroMark Q24 Advanced software user guide. 2012:59.
74. Programs OoP. Assigning values to non-detected, non-quantified pesticide residues in human health. Food Exposure Assessments. 2000.

APPENDIX A

Table A 1: DNA methylation status of MAOB in AD patients

Methylation status [%]											
	CpG										
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A031	31	33	51	35	54	20	20	48	24	11	12
	22	38	44	40	38	33	32	39	28	22	38
	43	32	63	49	41	25	36	45	36	28	34
	59	53	63	45	58	53	49	68	35	61	44
A032	36	31	53	41	32	34	29	35	17	21	11
	41	42	43	31	34	27	20	67	37	36	18
	28	40	61	22	43	5	27	43	<LOQ	25	26
	24	40	44	38	32	36	34	36	45	41	13
A033	24	28	43	19	20	16	23	29	19	33	31
	18	27	36	27	18	24	19	34	16	14	20
	49	42	60	34	47	22	12	37	33	26	31
A034	29	13	23	10	13	12	15	29	21	8	13
	43	26	54	51	33	17	25	38	28	21	15
	32	30	40	20	20	26	34	33	21	19	13
A038	17	18	46	27	15	20	32	25	18	24	19
	9	15	39	17	24	11	26	19	17	16	5
	17	9	39	10	45	13	26	49	32	41	34
A041	43	29	57	18	35	39	36	40	26	22	26
	40	29	34	25	35	23	40	30	31	17	27
	14	30	44	28	34	23	26	40	20	19	31
A042	27	47	27	7	6	13	16	41	9	7	5
	25	51	52	37	22	26	23	28	18	27	9
	41	35	53	36	39	33	49	35	22	17	18
	12	21	26	17	20	32	35	25	29	11	15
A043	18	33	43	25	31	30	25	38	28	14	13
	17	24	55	21	25	24	24	44	25	25	21
	33	39	51	56	32	32	23	40	33	<LOQ	14
	27	26	34	25	24	27	25	41	29	20	18
A044	9	38	29	26	13	17	20	21	16	12	12
	28	26	48	24	23	28	38	21	24	24	23
	23	33	46	26	16	8	24	30	10	11	7
	28	60	40	21	34	32	24	35	26	14	18
A055	32	21	42	41	36	23	47	39	20	<LOQ	12
	55	14	40	43	48	31	38	20	27	16	11
	23	11	36	27	42	20	25	17	10	14	11
A060	34	*	46	11	<LOQ	33	25	18	12	18	21
	46	17	51	13	11	34	20	23	12	14	13
	47	22	55	19	21	53	28	28	11	18	27
A064	38	37	49	9	27	8	23	33	12	22	11
	21	38	34	10	27	16	11	15	11	15	12
	25	32	47	14	33	25	24	45	15	24	15

Methylation status [%]											
	CpG										
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A066	20	18	36	22	16	9	20	27	20	18	12
	20	25	33	14	14	18	13	29	21	12	15
	22	12	34	21	17	8	23	25	27	9	15
A069	43	67	62	21	50	24	15	46	20	16	11
	23	69	46	29	37	17	10	38	18	12	14
	15	50	59	18	44	10	18	43	13	<LOQ	16
A070	28	28	31	25	11	8	<LOQ	20	9	26	5
	44	42	48	33	11	13	14	25	6	36	17
	33	32	43	37	9	23	20	18	11	28	11
A072	34	22	48	37	20	13	21	37	21	8	19
	28	19	44	32	30	21	27	30	14	20	21
	32	31	47	28	11	16	25	8	26	15	24
A081	26	33	37	21	23	37	26	40	15	25	12
	29	24	40	33	22	30	30	37	9	21	11
	23	25	25	18	22	26	29	22	18	8	16
A111	17	17	29	21	19	18	24	29	19	15	21
	20	12	30	13	10	10	10	21	12	<LOQ	17
	17	26	32	5	6	<LOQ	11	40	7	23	23
	20	20	28	21	21	20	27	29	20	17	18
A114	25	19	33	18	38	24	22	33	12	9	17
	19	27	25	13	34	15	15	22	8	13	8
	17	14	14	18	32	17	19	16	11	7	7
A117	38	31	51	28	33	21	32	44	22	26	18
	26	24	36	25	36	26	30	33	14	22	20
	29	21	58	33	20	26	20	28	<LOQ	<LOQ	6
	35	40	58	26	28	21	27	48	8	18	15
A134	23	30	48	30	22	31	40	34	20	30	13
	20	13	32	25	23	23	27	39	24	20	28
	24	12	60	28	35	19	28	55	33	23	17
A148	42	32	89	24	28	53	58	40	9	14	32
	46	26	70	21	25	61	54	29	6	11	11
	29	25	47	23	37	43	54	45	14	18	15
	43	24	53	18	24	47	46	33	19	16	23
A155	37	59	36	*	5	39	29	53	<LOQ	35	5
	33	39	42	38	35	33	40	51	16	26	17
	43	61	66	50	44	35	57	53	39	49	36
A159	34	25	73	36	41	38	36	76	16	28	38
	29	14	35	16	13	29	28	22	18	12	5
	24	13	37	30	18	11	26	19	16	11	13
	19	27	41	30	24	30	25	26	11	7	14
A161	41	21	66	8	15	<LOQ	9	17	5	12	<LOQ
	27	39	58	<LOQ	14	<LOQ	<LOQ	34	12	18	13
	18	40	42	8	23	17	16	23	12	11	28
	14	39	31	14	26	13	17	21	16	13	10

Methylation status [%]											
	CpG										
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A163	30	25	43	20	34	20	31	38	24	17	15
	28	48	55	41	42	20	37	36	29	15	16
	26	51	60	45	35	33	31	51	37	20	28
	28	41	49	31	33	23	28	25	14	19	24
	29	35	44	27	29	21	26	32	21	23	18
A172	34	16	47	22	28	22	28	32	25	34	20
	7	19	39	12	12	19	7	21	5	20	16
	37	35	42	24	21	39	36	45	23	34	28
A174	17	13	86	20	14	14	17	16	*	*	*
	65	74	89	77	75	73	38	98	13	9	61
	41	45	79	41	21	45	33	58	11	39	19
	8	58	47	54	53	18	21	55	10	44	19
A175	33	35	50	27	29	20	27	33	20	20	13
	32	36	27	27	30	13	16	26	30	22	12
	30	31	33	34	30	18	39	35	22	17	22
A179	27	42	67	40	11	13	9	20	18	27	24
	22	34	51	36	18	19	29	40	23	33	34
	23	23	61	28	23	19	15	30	21	22	25
A184	48	66	49	48	19	18	18	13	10	29	26
	55	91	55	60	20	5	21	18	<LOQ	40	13
	64	64	59	60	12	34	16	19	29	33	22
A187	26	20	25	5	10	26	22	21	9	10	12
	28	23	55	33	21	27	29	32	23	28	22
	32	39	55	36	27	26	29	46	24	14	13
A192	22	43	62	26	30	25	28	46	25	23	13
	25	39	43	29	35	11	24	36	23	37	24
	15	31	36	22	28	18	22	28	15	25	15
A199	28	23	44	12	28	22	17	30	13	13	5
	20	23	37	21	15	16	28	27	17	17	11
	14	20	25	19	17	15	21	23	7	15	20
A214	40	56	59	44	30	29	31	43	18	36	19
	16	28	53	24	37	26	31	29	6	19	18
	21	8	48	<LOQ	20	33	8	42	6	32	18
A249	25	27	39	25	13	17	30	27	7	24	14
	21	13	37	22	18	14	22	28	15	15	9
	39	41	63	42	28	25	28	37	32	29	15

Table A 2: DNA methylation status of MAOB of the control group

Methylation status [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
K146	51	19	52	10	15	28	8	13	14	28	23
	40	6	54	23	18	12	10	31	18	26	8
	36	44	69	28	49	31	35	37	24	28	16
	39	19	73	49	48	25	42	38	53	37	45
K148	21	25	45	13	29	34	37	36	20	23	30
	36	22	38	24	34	24	30	35	25	23	13
	8	20	80	6	8	<LOQ	87	91	19	<LOQ	15
	76	83	33	35	39	<LOQ	32	53	<LOQ	17	13
K149	32	38	53	29	27	31	26	39	25	29	17
	21	19	40	36	20	20	11	26	14	12	21
	39	42	45	45	19	29	29	14	10	27	8
	24	17	39	31	47	6	8	53	20	26	12
K150	32	54	41	23	43	46	34	44	29	26	31
	33	34	39	29	31	26	32	25	39	25	27
	23	37	25	51	72	41	35	48	50	16	33
K152	27	32	46	18	19	18	25	36	17	18	20
	27	28	47	28	25	23	28	41	22	24	14
	13	20	37	6	8	8	15	24	17	24	3
K153	29	32	47	21	35	32	37	37	28	32	23
	39	24	42	20	35	21	33	30	32	27	14
	28	32	61	22	52	34	40	37	30	26	17
K155	39	45	50	43	53	40	40	31	15	16	22
	46	51	51	42	36	38	36	27	15	12	11
	31	39	60	41	50	35	37	36	25	18	20
K157	34	22	39	29	17	19	25	30	17	19	11
	32	40	43	30	13	21	25	35	21	19	19
	30	32	47	30	21	15	21	44	19	5	16
K158	31	36	60	24	41	26	26	39	9	29	12
	25	41	48	26	30	28	41	34	25	20	14
	<LOQ	24	53	26	26	25	25	40	18	23	24
K159	46	52	43	29	30	21	20	29	18	31	24
	38	48	57	52	47	46	43	47	38	35	33
	53	20	75	40	27	24	44	32	44	13	40
K177	55	<LOQ	52	54	<LOQ	40	30	41	6	25	9
	26	16	48	20	19	25	31	33	9	22	12
	<LOQ	35	28	13	34	<LOQ	<LOQ	27	32	9	11
	46	39	61	25	57	27	52	57	44	45	35
K184	14	25	30	22	27	24	24	32	<LOQ	<LOQ	13
	28	38	32	15	31	11	23	27	6	15	6
	14	30	60	62	57	16	13	39	13	24	<LOQ
	12	17	57	<LOQ	8	18	32	30	12	7	<LOQ

Methylation status [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
K185	42	34	49	33	36	15	20	36	23	26	17
	50	21	62	28	39	28	16	55	12	16	17
	43	26	65	42	31	32	35	61	33	11	<LOQ
	60	16	64	19	50	45	45	30	36	<LOQ	27
K200	27	49	40	21	27	12	24	26	12	5	24
	28	52	33	28	20	16	19	25	22	11	14
	23	48	43	15	10	6	20	20	8	14	20
K203	46	68	62	12	31	19	20	12	17	12	32
	14	18	45	24	30	28	21	15	29	20	15
	22	66	26	31	<LOQ	20	25	50	7	14	<LOQ
	7	10	35	<LOQ	7	12	31	17	7	<LOQ	<LOQ
K206	20	19	53	29	49	23	23	40	20	13	12
	19	25	40	38	37	19	26	32	31	9	23
	35	38	42	20	32	26	38	33	27	37	19
K210	24	37	49	18	17	19	36	24	13	<LOQ	13
	28	50	46	18	24	12	27	22	9	5	44
	34	28	42	43	36	33	37	33	30	15	22
K214	27	30	47	22	30	19	37	38	21	20	7
	39	51	73	33	31	25	37	26	8	38	23
	43	32	56	32	25	34	49	28	8	29	21
	32	43	50	45	33	39	34	48	29	17	15
K217	25	8	37	19	51	35	57	48	23	<LOQ	<LOQ
	9	22	20	15	10	8	39	6	<LOQ	10	7
	19	14	14	13	6	11	31	20	16	15	9
	8	12	19	<LOQ	9	10	10	10	<LOQ	9	11
K221	29	16	28	20	19	21	30	42	14	19	22
	32	13	35	17	14	20	36	39	13	12	21
	37	28	41	20	12	24	28	40	23	19	28
K230	40	24	40	21	32	25	25	26	23	17	23
	36	22	35	22	29	19	29	22	25	28	19
	24	9	22	18	19	17	15	26	19	20	17
K257	31	35	36	19	18	17	23	32	13	8	14
	21	37	71	36	21	40	24	58	12	<LOQ	35
	33	34	45	20	6	15	8	14	20	9	12
K263	36	19	54	19	15	29	31	26	32	9	16
	38	28	52	19	14	19	33	18	27	14	20
	42	20	35	26	28	22	32	29	30	12	11
K268	26	14	39	17	23	37	39	49	27	28	38
	26	27	53	25	33	29	41	34	18	22	15
	33	17	30	25	18	39	41	39	16	26	24
K293	22	29	46	17	23	13	29	29	24	<LOQ	<LOQ
	21	27	42	18	27	11	21	24	17	11	16
	28	46	51	21	40	22	41	47	27	18	18

Methylation status [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
K305	40	42	52	15	11	45	41	44	36	24	19
	31	31	88	31	14	38	43	49	47	29	20
	3	8	46	8	16	38	37	40	35	9	7
K307	40	47	75	38	10	24	42	37	35	32	9
	36	51	60	35	32	29	26	28	20	26	29
	17	42	60	16	27	6	25	38	14	31	23
K317	21	35	33	18	20	21	19	42	18	21	28
	21	37	40	15	25	19	26	47	13	27	32
	27	36	31	13	17	14	15	51	25	26	25
	18	29	31	19	25	18	24	35	26	25	26
K319	50	71	31	12	27	35	37	41	33	26	28
	34	34	39	31	43	33	22	38	22	13	11
	20	11	12	21	29	15	32	15	<LOQ	8	<LOQ
K320	54	34	67	49	32	30	24	43	25	26	19
	54	35	56	17	23	21	19	39	7	20	31
	32	57	35	28	30	<LOQ	23	28	26	22	47
	32	30	46	47	28	16	27	30	18	18	25
K324	19	35	64	37	19	19	22	21	17	15	15
	24	35	47	24	26	5	20	42	35	12	17
	34	*	48	27	25	5	18	38	24	14	19
K329	23	42	44	17	7	9	15	20	9	7	5
	21	43	63	16	15	10	18	14	6	6	12
	13	30	46	24	18	20	27	16	10	18	17
K336	31	34	42	29	30	22	19	40	17	15	30
	24	25	44	20	25	24	17	34	16	27	20
	27	24	44	21	29	26	32	39	16	29	22
	21	31	50	18	31	29	22	28	21	26	28
K337	17	29	41	36	23	19	23	37	17	20	23
	31	32	52	40	26	19	29	56	28	23	27
	47	44	44	29	33	38	42	68	36	22	40
	19	19	30	26	21	15	16	39	10	15	15
K348	22	25	38	18	21	23	16	32	20	20	13
	20	15	34	22	19	22	22	25	12	18	14
	24	13	49	11	17	14	22	37	15	10	9
K357	36	32	51	20	13	21	34	41	16	21	18
	33	17	38	22	20	22	25	34	18	12	9
	16	45	23	30	14	13	20	34	15	<LOQ	<LOQ
	35	32	59	26	25	38	39	45	19	24	22
K361	35	24	52	32	31	27	38	47	34	37	22
	29	29	55	30	30	35	33	42	38	32	21
	38	23	50	31	26	29	25	35	22	23	20
K374	33	15	36	29	30	17	15	34	14	11	13
	31	26	32	22	42	20	36	39	17	16	26
	43	32	43	23	36	22	37	51	19	20	19

Methylation status [%]											
	CpG										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
	42	28	62	36	35	28	41	37	27	34	28
K378	53	32	61	51	37	40	25	28	17	31	30
	33	45	51	36	32	36	37	39	25	13	26
	24	16	27	18	18	25	21	24	20	18	12
K447	34	41	44	35	21	24	31	58	22	31	13
	9	19	35	24	18	26	23	43	10	25	26

APPENDIX B

Table B 1: Characteristics of the patients/samples; "A" stands for AD patients, "K" stands for healthy control

AD patient/ healthy control	Gender	Age [year]	MAOB[1] [standardized abundance]	WBC[2] [10^3/µL]	RBC[3] [10^6/µL]	Age of sample [month]
A032	f	80	1.30	n.a.	n.a.	134
A033	f	65	1.37	n.a.	n.a.	134
A034	f	91	1.17	n.a.	n.a.	134
A038	f	80	1.58	n.a.	n.a.	131
A041	f	71	1.15	n.a.	n.a.	133
A042	f	83	1.27	n.a.	n.a.	133
A043	f	84	1.81	n.a.	n.a.	131
A044	f	77	1.48	n.a.	n.a.	131
A055	f	69	1.14	n.a.	n.a.	131
A060	f	87	1.10	5.2	3.55	96
A064	f	80	1.06	8.3	4.7	96
A066	f	94	1.31	5.5	4.18	96
A069	f	86	1.47	5.5	4.38	95
A070	f	74	1.10	7.6	4.53	95
A072	f	91	1.39	6.3	4.61	94
A081	f	81	1.15	9.6	4.85	93
A111	f	78	1.50	7.9	5.04	55
A114	f	89	0.82	7.7	3.32	57
A117	f	87	1.84	7	3.1	55
A134	f	87	1.29	12.5	5.15	56
A148	f	80	1.25	4.3	3.56	54
A155	f	84	1.26	8.2	6.36	54
A159	f	84	0.90	7.2	4.73	53
A161	f	79	1.02	6.1	3.83	53
A163	f	76	1.34	4.8	4.33	41
A172	f	68	2.15	n.a.	n.a.	39
A174	f	79	1.11	n.a.	n.a.	41
A175	f	87	1.45	5.1	4.43	39
A179	f	87	1.14	9.7	4.21	40
A184	f	68	1.66	4.9	4.75	34
A187	f	81	0.99	4.9	5.15	35
A192	f	85	0.78	8.8	4.97	34
A199	f	82	0.75	6.4	4.42	32
A214	f	70	1.35	5	4.4	26
A249	f	82	1.13	5.7	4.5	5
K146	f	87	0.94	n.a.	n.a.	132
K148	f	88	0.96	n.a.	n.a.	132
K149	f	87	0.97	n.a.	n.a.	132
K150	f	88	0.75	n.a.	n.a.	132
K152	f	80	0.93	n.a.	n.a.	132
K153	f	84	1.05	n.a.	n.a.	132
K155	f	87	0.79	n.a.	n.a.	132
K157	f	85	0.84	n.a.	n.a.	131
K158	f	66	0.84	n.a.	n.a.	131

AD patient/ healthy control	Gender	Age [year]	MAOB[1] [standardized abundance]	WBC[2] [10^3/µL]	RBC[3] [10^6/µL]	Age of sample [month]
K159	f	81	1.14	n.a.	n.a.	131
K177	f	75	1.02	n.a.	n.a.	125
K184	f	90	0.65	n.a.	n.a.	122
K185	f	72	0.67	n.a.	n.a.	119
K200	f	73	1.10	n.a.	n.a.	95
K203	f	85	1.05	4.8	4.03	94
K206	f	86	0.41	8.7	4.38	94
K210	f	74	0.51	7.5	4.6	91
K214	f	81	0.71	8.1	4.26	93
K217	f	90	0.96	6.6	4.53	93
K221	f	82	0.83	6.8	4.34	93
K230	f	79	0.81	5.7	4.86	55
K257	f	83	0.60	6.7	6.3	54
K263	f	66	0.85	3.9	4.41	56
K268	f	80	1.17	8	4.87	56
K293	f	87	0.94	5.5	4.73	52
K305	f	82	0.94	6.8	5.21	43
K307	f	77	0.76	3.9	3.77	43
K317	f	88	0.64	8.1	4.02	41
K319	f	80	1.09	6.6	4.94	43
K320	f	86	0.46	6.3	3.83	41
K324	f	89	0.71	n.a.	n.a.	42
K329	f	86	0.61	4.3	4.66	42
K336	f	70	0.78	6	4.5	40
K337	f	79	0.77	6.4	4.81	40
K348	f	69	0.66	6.3	4.41	34
K357	f	75	0.51	6.3	4.74	32
K361	f	82	0.96	6.1	5.17	34
K374	f	83	0.76	5.1	4.25	27
K378	f	68	0.85	6.4	4.37	27
K447	f	80	0.88	6.1	4.94	16

1 monoamine oxidase B

2 white blood cells

3 red blood cells

APPENDIX C

Table C 1: Composition of the pools, age of respective individuals and age of samples. "K" stands for healthy control. "A" stands for AD patient

Pool	AD patient	Age [y]	Age of sample [months]	Pool	Healthy control	Age [y]	Age of sample [months]
P1	A033	65	122	P11	K158	66	121
	A172	68	51		K263	66	62
	A184	68	46		K378	68	38
	A055	69	122		K348	69	46
	A214	70	38		K336	70	50
	A041	71	122		K185	72	121
	A070	74	101		K200	73	100
	A163	76	51		K210	74	99
	A044	77	121		K177	75	121
	A111	78	63		K357	75	45
P2	A161	79	63	P12	K307	77	51
	A174	79	51		K230	79	63
	A032	80	122		K337	79	50
	A038	80	118		K152	80	118
	A064	80	101		K268	80	62
	A148	80	63		K319	80	51
	A081	81	99		K447	80	22
	A187	81	46		K159	81	118
	A199	82	43		K214	81	99
	A249	82	18		K221	82	101
P3	A042	83	118	P13	K305	82	51
	A043	84	118		K361	82	45
	A155	84	63		K257	83	62
	A159	84	63		K374	83	38
	A192	85	45		K153	84	118
	A069	86	101		K157	85	118
	A060	87	101		K203	85	100
	A117	87	63		K206	86	100
	A134	87	63		K320	86	51
	A175	87	51		K329	86	51
P4	A179	87	51	P14	K146	87	118
	A114	89	63		K149	87	118
	A031	90	118		K155	87	118
	A034	91	118		K293	87	62
	A072	91	100		K148	88	122
	A066	94	101		K150	88	118
					K317	88	51
					K324	89	51
					K184	90	120
					K217	90	101

Pool	AD patient	Age [y]	Age of sample [months]	Pool	Healthy control	Age [y]	Age of sample [months]
P5	A268	67	15	P15	K371	67	39
	A138	70	63		K493	74	15
	A276	70	11		K344	75	50
	A167	74	51		K464	75	22
	A091	79	68		K500	76	11
	A245	79	18		K312	77	51
	A135	80	63		K402	77	32
	A256	80	16		K509	77	11
	A258	80	16		K323	78	51
	A101	81	63		K366	78	39
P6	A129	81	63	P16	K219	79	101
	A197	81	43		K351	79	46
	A133	82	63		K454	79	22
	A124	83	63		K284	80	62
	A132	84	63		K376	81	38
	A173	84	51		K242	82	63
	A190	84	45		K367	82	39
	A202	84	39		K373	82	38
	A203	84	39		K259	83	62
	K343*	84	50		K261	83	62
P7	A141	85	63	P17	K277	83	62
	K246*	85	63		K291	83	62
	K264*	85	62		K283	84	62
	A106	86	63		K227	85	64
	A145	86	63		K309	85	51
	A195	86	43		K345	85	50
	A198	86	43		K453	85	22
	A205	86	39		K235	86	63
	A131	87	63		K244	86	63
	A176	87	51		K273	86	62
P8	A103	88	63	P18	K299	86	62
	A105	88	63		K353	86	46
	A119	88	63		K386	86	36
	A125	88	63		K397	86	34
	A126	88	63		K205	87	100
	A193	88	45		K365	87	39
	K287*	88	62		K387	87	36
	A152	89	63		K404	87	32
	A165	89	51		K431	87	29
	A169	89	51		K321	88	51
P9	A201	89	43	P19	K201	86	100
	A104	90	63		K303	89	62
	A266	90	16		K340	89	50
	A118	91	63		K245	90	63
	A186	91	46		K318	90	51
	A241	94	22		K308	91	51
	A248	94	18		K310	91	51
	K331*	94	50		K384	91	36
	A098	95	63		K278	92	62
	A157	95	63		K372	95	39

*samples of AD patients (although labeled with “K”)

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder and the most commonly diagnosed form of age-related dementia. Pathological hallmarks of AD are neurofibrillary tangles, senile plaques and neuronal cell loss. Currently, AD can only be diagnosed post mortem and therefore there is a great interest to develop biomarkers for an early diagnosis of AD.

In contrast to early onset AD (EOAD), which is caused by gene mutations, late onset AD (LOAD), the most prevalent type of AD, seems to be mediated by aberrant epigenetic modifications. High homocysteine and low folate levels in plasma of AD patients have been associated with hypomethylation of the genome. Aberrant DNA methylation patterns could therefore be suitable biomarkers for the early diagnosis of AD.

In the master thesis, the suitability of the DNA methylation status of *monoamine oxidase B* (*MAOB*) as biomarker for the diagnosis of AD was investigated. In previous studies, the MOAB protein has been found to be overexpressed in AD patients.

At the beginning, a pyrosequencing method was developed, allowing to determine the methylation status of 11 CpGs in the MAOB promoter. The pyrosequencing method was then applied to the analysis of DNA extracts from whole blood samples of 40 AD patients and 40 healthy controls. DNA extracts were provided by our cooperation partners from the Institute of Physiology, Medical University of Vienna. In addition, pools of DNA extracts were obtained, prepared by mixing DNA extracts from individual persons.

Statistical analyses of the methylation levels determined showed that the methylation status of the CpGs in DNA from whole blood of AD patients was not significantly different from that found in healthy controls. Significant differences were neither found for DNA extracts from individual persons nor for DNA extract pools.

Zusammenfassung

Alzheimer ist eine neurodegenerative Erkrankung und die häufigste Form von Demenz. Kennzeichnend für die Erkrankung sind die Bildung von neurofibrillären Bündeln und senilen Plaques und ein langsam fortschreitender Verlust der Nervenzellen. Da Alzheimer bislang nur post mortem sicher diagnostiziert werden kann, ist die Entwicklung eines Biomarkers für eine frühe Diagnose von großer Bedeutung.

Im Gegensatz zu der früheren Form von Alzheimer (EOAD), welche durch Mutation hervorgerufen wird, wird vermutet, dass die spät beginnende Form von Alzheimer (LOAD) durch epigenetische Modifikationen entsteht. Alzheimerpatienten zeigen oft eine niedrige Folat- und Homozysteinplasmakonzentration, welche zu einer Hypomethylierung des Genoms führen kann. Daher könnte der DNA Methylierungsgrad für eine frühe Diagnose von Alzheimer herangezogen werden.

In der Masterarbeit wurde untersucht, ob der DNA Methylierungsgrad der *Monoamino oxidase B* (*MAOB*) als Biomarker für eine frühe Diagnose von Alzheimer geeignet ist, da vorherige Studien eine Überexpression des MAOB Proteins bei Alzheimerpatienten gezeigt hatten.

Zunächst wurde eine Pyrosequenziermethode entwickelt und optimiert, um den Methylierungsgrad von 11 CpGs in der Promotorregion von *MAOB* bestimmen zu können. Anschließend wurden mit Hilfe der Pyrosequenziermethode DNA Extrakte aus Vollblutproben von 40 Alzheimerpatienten und 40 gesunden Kontrollen analysiert. Die DNA Extrakte wurden von unseren Kooperationspartnern Dr. Maria Zellner und Dr. Ellen Umlauf (Institut für Physiologie, Medizinische Universität Wien) zur Verfügung gestellt. Zusätzlich wurden Poolproben, welche durch das Mischen von DNA Extrakten unterschiedlicher Personen hergestellt wurden, analysiert.

Mit statistischen Tests wurden keine signifikanten Unterschiede im DNA Methylierungsgrad der CpGs zwischen Alzheimerpatienten und gesunden Kontrollen gefunden. Signifikante Unterschiede wurden weder für DNA Extrakte von individuellen Personen noch für DNA Extrakte von Poolproben erhalten