universität wien

MASTERARBEIT

Titel der Masterarbeit

„Epigenetic changes of FFAR3 and LINE1 in obese and diabetic patients“

verfasst von

Christine Merold, Bakk.

angestrebter akademischer Grad

Master of Science (Msc)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 066 838
Studienrichtung lt. Studienblatt:	Master Ernährungswissenschaften UG2002
Betreuerin / Betreuer:	Doz. Dr. Alexander Haslberger

Danksagung

Ganz besonders bedanken möchte ich mich bei meinen Eltern, die mir das Studium überhaupt erst ermöglicht haben und mich nicht nur finanziell, sondern auch moralisch immer unterstützt und mir den Rücken gestärkt haben.

Außerdem möchte ich mich auch ganz herzlich bei meinem Betreuer Doz. Dr. Alexander Haslberger und der ganzen Haslberger AG bedanken, die mir immer zur Seite standen und mir eine sehr große Hilfe bei der Erstellung meiner Masterarbeit waren.

Danke!

Contents

1 List of figures

2 List of tables

3 List of abbreviations

AMPK	…	AMP-activated protein kinase
BAK	…	BRI1-associated receptor kinase 1
Bax	…	Bcl-2-associated X protein
Bcl-2	…	B-cell lymphoma 2
BMI	…	body mas index
cAMP	…	cyclic-Adenosinmonophosphate
Cdk	…	cyclin-dependent kinase
DNMT	…	DNA-methyltransferases
ERK	…	extracellular signal-regulated kinases
FFAR3	…	free fatty acid receptor 3
GLP-1	…	glucagon like peptide-1
GPR	…	G-protein-receptor
GST	…	glutathion –S-transferase
HbA1c	…	glycol hemoglobin
HDAC	…	histone deacetylase
HDACi	…	histone deacetylase inhibitor
HIF-1a	…	hypoxia inducible factor-1a
HMT	…	histone methyltransferase
HNE	…	hydroxynenal
IBD	…	inflammatory bowel disease
IFN-γ	…	interferon-γ
IP3	…	inositol 1,4,5-trisphosphate
IRES	…	Internal ribosome entry site
LINE1	…	long interspersed nuclear element 1
miRNA	…	micro RNA

NAD	…	nicotinamide adenine dinucleotide
NF-κB	…	nuclear factor κB
PGC-1α	…	proliferator-activated receptor-γ coactivator
PPAR-γ	…	peroxisome proliferator-activated receptor-γ
PTP1B	…	protein tyrosine phosphatase 1B
PYY	…	Peptide YY
SAM	…	S-adenosylmethionine
SCFA	…	short chain fatty acid
SOCS3	…	suppressor of cytokine signaling 3
STAT3	…	signal transducer and activator of transcription 3
VEGF	…	vascular endothelial growth factor

4 Zusammenfassung

Es wird angenommen, dass kurzkettige Fettsäuren einen Einfluss auf die epigenetische Regulation von Nahrungsaufnahme, Sättigung und inflammatorische Prozesse haben. Dabei wird vermutet, dass unter anderem der Methylierungs-Level von G-Protein-Rezeptor-41 eine wesentliche Rolle spielt.

Während dieser Studie wurden drei Gruppen untersucht. Dazu zählten unter anderen zwei Kontrollgruppen welche aus adipösen und normal gewichtigen Personen bestanden. Außerdem eine Interventionsgruppe mit Diabetikern die eine drei monatige Ernährungsberatung sowie Medikation in Form von Victoza®, einem GLP-1 Antagonisten erhielten. Anhand von Blutproben wurden Methylierungs-Level von 5CpGs in der Promotor-region von FFAR3 und 3CpGs bei LINE1 mittels Pyrosequenzierung untersucht. LINE1 diente als globale Methylierungs-Kontrolle.

CpG-Analysen in FFAR3 zeigten eine signifikant, niedrigere Methylierung in normal Gewichtigen im Vergleich zu Adipösen und Diabetikern. Methylierung in LINE1 ergaben den niedrigsten Methylierungs-Level in adipösen Personen. Signifikant, negative Korrelationen zwischen Methylierung und BMI konnten in FFAR3 gezeigt werden. In LINE1 konnte hingegen ein leicht positiver Trend bezüglich Methylierung und BMI beobachtet werden. Alters bedingte Hypomethylierungen konnten durch heranziehen von LINE1 ausgeschlossen werden.

Unsere Ergebnisse vermuten einen Zusammenhang zwischen der von Microbiota, produzierten kurzkettigen Fettsäuren, der Aktivierung von FFAR3 und den daraus resultierenden veränderten epigenetischen Regulationen. Es wird angenommen, dass dies zu einer Beeinflussung der Leptinproduktion in Adipozyten führen kann und so Einfluss auf die Sättigungsregulation nimmt. Einfluss auf PYY Expression ist ebenfalls möglich. Desweitern wird eine Beeinträchtigung inflammatorischer Regulationen vermutet, wobei die Hemmung der Histonacetylierung, bedingt durch SCFA und GPR41 Aktivierung, als zugrunde liegender Mechanismus diskutiert wird.

5 Summary

Influence of SCFAs like butyrate are believed to play an important role in epigenetic regulation of food intake and satiety and even though in anti-inflammatory reactions. These affected regulations are supposed to be mediated by the G-protein-receptor 41.

During this study there have been analysed three groups. Two control groups which consist of obese subjects and lean controls. Third group are Diabetics whose intervention included three month medication with GLP-1 antagonist Victoza® and nutritional counselling. Blood samples were taken at the beginning, after one month and after three months. These samples were used for analysing promoter methylation of 5 CpGs in FFAR3 and 3 CpGs in LINE1. Analyses were done by pyrosequencing. LINE1 served as global methylation control.

CpG-analysis in FFAR3 showed significant lower methylation in obese and diabetic subjects compared to the lean control group. Methylation of LINE1 seems to be lowest in obese controls. Significant negative correlation was found in FFAR3 compared to methylation level and BMI. In contrary a trend for positive correlation between LINE1 methylation and BMI could be observed. Age specific hypomethylation could be excluded by analysis of LINE1 which did not show any age specific methylation distribution.

Our results suggest a possible link between SCFAs produced by microbiota and their activating function on FFAR3 resulting in a modified epigenetic regulation. This link may include modified leptin production in adipocytes followed by changed regulation of satiety. Also a possible influence on PYY production is discussed. Underlying mechanism might be inhibition of histone acetylation by SCFA and FFAR3.

6 Introduction

6.1 Epigenetic mechanisms

Epigenetic mechanisms consist of three interacting mechanisms: DNA-methylation, histone modification and non-coding microRNAs. These mechanisms influence gene expression throughout life (McKay und Mathers 2011). Against some presumptions epigenetic modifications can be influenced by several environmental triggers like nutrient intake, physical exercise and age. These mechanisms include inherited changes which include altered gene functions without changing the DNA sequence (Franks und Ling 2010).

6.1.1 DNA methylation

DNA methylation means the addition of a methyl group to a DNA molecule, which in most of all cases is the 5´position of a cytosine residue. Cytosine will be followed by a guanine residue (5´→3´) which then is called a CpG dinucleotide (McKay und Mathers 2011). “p” means the phosphodiester bond between cytosine and guanine. A conglomeration of these CpG dinucleotides is called CpG Island. (Vo und Millis 2012).CpG islands occur predominantly in promoter regions, exons, 5´flanking regions and 3´terminal areas (Jei Kim 2009). This kind of DNA modifications are heritable and reversible changes. The most important association to this epigenetic modification is the transcriptional repression. Thereby it can be distinguished between repression caused by inhibition the binding of different factors to their related DNA recognition sequence or repression through the recruitment of methylated-CpG-binding proteins with bounded co-repressor molecules (Guil und Esteller 2009). Generally DNA methylation is involved in several physiological procedures like X-chromosome inactivation, imprinting and silencing of germline-specific genes and repetitive elements (McKay und Mathers 2011).

DNA methylation is controlled by the DNA methyltransferase (DNMT) family. S-adenosylmethionine (SAM) serves as methyl donor. There are three existing DNA methyltransferases DNMT1, DNMT3A and DNMT3B. DNMT1 is responsible for the maintaining DNA methylation in hemi-methylated DNA after DNA replication in

cell division. DNMT3A and DNMT3B will form de novo DNA methylation. There is also a third enzyme in the DNMT3 family, DNMT3-like it controls the functions of DNMT3A and DNMT3B. It is also considered to activate DNMT3A or to lure DNMT3A to specific regions on the genome by binding to N-terminal tails of histone H3 (He et al. 2011).

Demethylation of CpGs plays also an important role in life and is not well understood yet. But it is already known that DNA demethylation can result in activation of specific genes and has influence on cellular regulatory mechanisms (Franchini Don-Marc 2012).

6.1.2 Histone modification

Histones are alkaline proteins in eukaryotic cell nuclei which are surrounded by DNA molecules and can be posttranslational modified at their N-terminal tails. Acetylation, Methylation, phosphorylation, sumoylation, ubiquitination and ADP ribosylation are possible modifications. The consequence of these modifications can alter DNA-histone interactions and further chromatin structure (Guil und Esteller 2009). This means changes in gene regulation. The histone code hypothesis claims that specific histone modifications effect specific biological functions in specific regions in the genome where they occur. This could result in a locus specific gene regulation (Jiménez-Chillarón et al. 2012).

The enzymatic regulation of histone methylation for example is conducted by histone methyltransferases (HMTs) and histone demethylases. Even though S-adenoyslmethionin (SAM) is necessary for the activation of HMTs. Histone acetylation is regulated by histone acetyl-transferase (HAT) and three classes of histone deacetylase I,II and III (HDAC). Class I and II can be inhibited by short-chain carboxylic acids and polyphenols. Class III needs nicotinamide adenine dinucleotide (NAD+) as cofactor (Jiménez-Chillarón et al. 2012).

6.1.3 microRNA

MicroRNAs are small non-coding RNAs with an average length of 20-22 nucleotides. It is suggested that miRNAs regulate about 30% of the genomic expression. These modifications can be induced through posttranscriptional or transcriptional regulation of chromatin structure (Jiménez-Chillarón et al. 2012).

The synthesis of miRNAs takes place in the nucleus and is regulated by RNA Polymerase II. This produced RNA is called primary-microRNA (pri-miRNA) it´s a long capped and polyadenylated transcript. The post-transcriptional way of miRNAs starts with cropping of pri-miRNAs and the release of hairpin-shaped precursors by the Drosha/DGCR8 complex. Further the export from the nucleus, trimming by the type III ribonuclease Dices and incorporation into the argonaute-containing RISC complex occurs. The RISC complex is responsible for the pairing of mature miRNA with mRNA through imperfect Watson-Crick base pairing. This will result in translational arrest or degradation of the transcript. In total miRNAs play a negative role in target gene expression (Guil und Esteller 2009).

All in all recent studies emphasize that DNA methylation and histone modifications are co-workers which are able to silence genes and repress transcription. It is supposed that histone deacetylation and H3K9 methylation are clearing the way for CpG methylation. Otherwise interaction of these two mechanisms are also discussed and supposed to result in long term transcriptional silencing (Fuks 2005).

6.2 G-protein receptor 41(GPR41) / Free fatty acid receptor 3 (FFAR3)

GPR41 belongs to a G-protein-coupled receptor cluster which includes FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41) and GPR42. These members are 30-40% identical. The human pseudo-gene GPR42 differs only in six amino acids positions from FFAR3 but without any function. It is indicated that hGPR42 be a result of gene duplication of hFFAR3 when human lineage separated from the rodent and bovine lineage (Ichimura et al. 2009). This GPCR-cluster is part of the rhodopsin-like family A, which contains most of the GPC-receptors (Frank Reimann 2012). GPCRs contain three different subgroups, α-,ß- and γ-subunit. There are 16 different α-subunits, GPR41 belongs to the Gαi/o subunit. On the one hand this α-subgroup inhibits adenylate cyclase (Frank Reimann 2012) which in turn is responsible for cAMP and phospholipase C production (Alberts Bruce 2010) which is decreased in this subunit (Frank Reimann 2012). On the other hand, inositol 1,4,5-trisphosphate (IP3) production inhibition occurs which can be

due to phospholipase C activation. IP3 production induces Ca2+ channel opening in the endoplasmatic reticulum. Intracellular Ca2+ concentration is increased and functions as signal for other proteins (Alberts Bruce 2010). ERK1/2 pathway is activated, too (Ichimura et al. 2009). In humans GPR41 is expressed in spleen, lymph node, bone marrow, peripheral blood mononuclear cells, colon, sympathetic ganglia and mainly in adipose tissue (Wu et al. 2012).

Another characteristic is the fact that Halpern et al. indicates that GPR41 does not have an own promoter region and is transcribed by the promoter region of GPR40. This means GPR41 transcription is induced by a promoter region 6619bp upstream and results in generating a bicistronic transcript of GPR41 and GPR40. Means both genes are determined on the same mRNA. But GPR40 does also have a monocistronic transcript. That means this one promoter produces two transcripts. Translation is controlled by an internal ribosome entry site (IRES) which is placed in the intergenic region. Further studies are necessary to ensure these results (Keren Bahar Halpern 2012).

Already proven is the hypophagic character of GPR41 which leads amongst others to leptin production (Ichimura et al. 2009). Leptin is a 167 long peptide which is exclusively produced in adipose tissue. Studies demonstrated positive correlation of leptin plasma levels and body fat mass (Schloegl et al. 2011). The hypophagic effect involves the regulation of energy expenditure and food intake. This characteristic is regulated by binding to specific receptors in the hypothalamus (Soliman et al. 2007). It was shown that SCFA stimulates GPR41 which in turn results in leptin production. This is strengthened by studies which indicate direct correlation of FFAR3

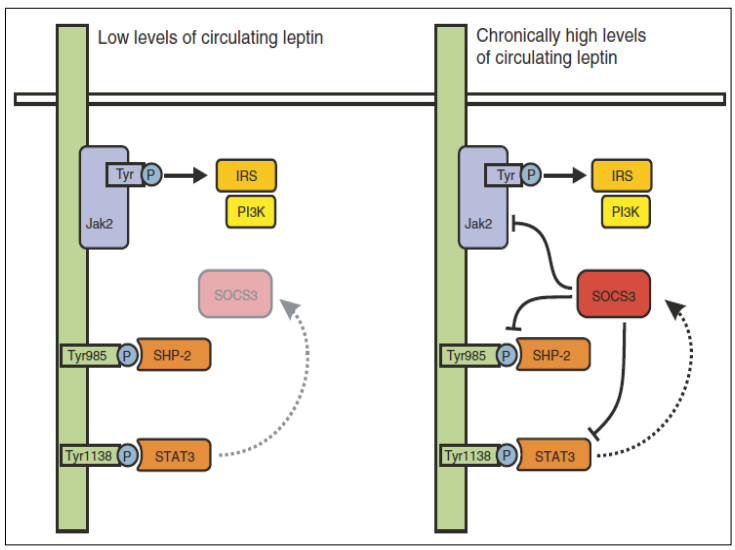

Figure 1 Leptin receptor: Leptin resistant state (Münzberg und Myers 2005)

expression and leptin production and was underlined by studies with knockout mice without FFAR3 which did not produce leptin (Ichimura et al. 2009). Additional it could be shown that oral induction of SCFA like propionate could increase leptin levels in mice by 80%. Same dose dependent effects were observed after butyrate and acetate induction (Arora et al. 2011). The correlation of fat mass and leptin production (Schloegl et al. 2011) plays an important role in obese patients because of the massive production of leptin resulting in a leptin resistant state. This means obese individuals are not able to respond to leptin with decreased food intake and consequently reduced body weight as normal weight subjects do. There are two discussed mechanisms for leptin resistance (Münzberg und Myers 2005).

On the one hand it is supposed that leptin is not able to reach its target in the brain (Münzberg und Myers 2005) whereby high levels of triglycerides which are elevated in obese subjects, may contribute amongst others to impaired blood-brain barrier transport (Banks A. William 2004). And on the other hand an inhibition of intracellular leptin receptor signalling cascade is debated. It is already proven that leptin induced hypothalamic signalling is reduced in obesity. In focus there are two inhibitory molecules, SOCS3 and the protein tyrosine phosphatase PTP1B responsible for leptin receptor regulated signalling. Overexpression of PTP1B and SOCS3 both result in decreased leptin signalling. Knockdown of PTP1B and SOCS3 results in increased leptin receptor signalling. Additional mutation of Tyr985 which marks the binding site of SOCS3 on the leptin receptor results also in increased leptin receptor signalling. It is supposed that high levels of leptin and simultaneously increased STAT3 activity in obese subjects result in elevated SOCS3 expression and consequently decreased leptin receptor signalling. This suggests a feedback inhibition during high leptin receptor activation. This mechanism involves STAT3 signalling pathway and SOCS3 expression itself and could be a possible explanation for the decreased function of increased leptin levels in obese individuals (Münzberg und Myers 2005).

Besides hypophagic effects of leptin studies from Samuel et al. show that deficiency in GPR41 results in decreased PYY expression (Buck S. Samuel 2008). Peptide YY is a 36 amino acid peptide produced from L cells especially in

the gastrointestinal tract (Schloegl et al. 2011). This peptide has the ability to signal to the brain and induce several pathways which are connected to food intake and satiety (Holzer et al. 2012). It´s main function is the ability of appetite and caloric intake reduction. Further PYY inhibits stomach emptying and increases the water and electrolyte absorption in the gastro intestinal tract. PYY release is higher in protein rich meals. Different PYY-production in obese and lean subjects is well-known. Obese patients show lower fasting PYY-levels compared to lean patients and also the postprandial release of the peptide is decreased which may lead to diminished satiety (Schloegl et al. 2011).

6.3 Ligands of FFAR3: Butyrate

The most important SCFA produced from bacteria are acetate, propionate and butyrate which are produced in the ratio of 60:25:15 (Arora et al. 2011). Most attention will be given to butyrate which has several beneficial roles in human health (Berni Canani et al. 2012).

Butyrate has demonstrable histone deacetylase inhibitor (HDACi) activity already at very low concentrations in the millimolar range. Beneficial roles in chemoprevention may be the ability of butyrate to derepress silenced genes in cancer cells and to activate relevant genes in normal cells, like p21 and the proapoptotic protein Bcl-2 homologous antagonist/killer (BAK). HDACi is associated with antiangiogenic and antimetastatic effects by the repression of angiogenesis and reduced expression of proangiogenesis factors like hypoxia inducible factors (HIF-1a) and vascular endothelial growth factor (VEGF). Indirect effects of butyrate to prevent cancer will be the effect of slowed growth and the activation of apoptosis in colon cancer cells. The mechanism which is supposed behind these observations may be the transcriptional upregulation of detoxifying enzymes like glutathione-S-transferase (GST) which could be the protector against genotoxic carcinogens like H2O2 and 4-hydroxynonenal (HNE). These mentioned mechanisms are associated with the idea of dietary fiber induced prevention of colorectal cancer (Berni Canani et al. 2012).

Butyrate induced inhibition of histone deacetylase can also help to reduce inflammatory symptomes in patients with inflammatory bowel disease (IBD) (Berni Canani et al. 2012). Inflammatory bowel disease is an inflammation of the intestine. The two major forms of this disease are ulcerative colitis and crohn´s disease (Daniel K. Podolsky 1991). Several studies could show reduced abdominal symptoms and inflammation after increased consumption of dietary fibers and which results in increased butyrate production (Berni Canani et al. 2012). Inflammatory profile could be improved by reduction of macrophages, B-lymphocytes and T-lymphocytes (Vieira et al. 2012). These results were observed in patients with ulcerative colitis. The mechanism responsible for the improvement in inflammatory bowel disease patients might be the suppression of nuclear factor κB (NFκB), inhibition of interferon γ production and the upregulation of peroxisome proliferator-activated receptor γ (PPARγ) (Berni Canani et al. 2012).

These anti-inflammatory effects may be protective against low grade inflammation which occurs mostly in obesity, insulin resistance, type 2 diabetes and cardiovascular diseases by activating proliferator-activated receptor-γ coactivator 1α (PGC-1α). This activation may be due to the HDACi and the activated AMP-activated protein kinase (AMPK) (Berni Canani et al. 2012).

7 Objective

Involvement of epigenetic regulation in several physiological functions like satiety and food intake as well as, inflammation plays a crucial role in obese and diabetics patients. Methylation analysis of blood samples in obese diabetics, lean controls and obese controls should give information about the methylation levels in different individuals. Different methylation levels should help to understand the impaired regulation of satiety and food intake and especially the development of leptin resistant state which occurs in obese subjects due to increased leptin production in adipocytes. Further, an impact of bodyweight on methylation levels was supposed. To exclude age related methylation levels in FFAR3, LINE1 was analysed to rebut age related methylation distribution in FFAR3. Methylation levels were conducted by analysing 5 CpGs in the promoter region of FFAR3 and by analysing the global methylation of LINE1 in 3 CpGs.

8 Material and Methods

8.1 Study design

The study was directed by the department of nutritional science at the University of Vienna. Study period was from January 2011 to June 2013.

DNA methylation of LINE-1 and FFAR3 were analysed at three different time points. Time point 1 was before the Intervention, time point 2 was after one month of the intervention and time point 3 was after four months of the intervention.

Study participants of group one included obese diabetic people (n= 23), who received a formula diet to lose weight and a drug called Victoza®. Group two includes lean people, who serve as control group (n= 16). The third group also serves as control group but includes obese people (n=7). Both control groups were encouraged not to change their lifestyles, especially nutrition and sporting habits.

8.2 Inclusion criteria for the intervention and the obese control group

All patients with Diabetes mellitus type 2, impaired glucose tolerance, impaired fasting glucose or insulin resistance and with at least two of the following criteria were accepted for this study and were recruited to the hospital of Burgenland. These criteria include blood pressure higher than 140/90mmHg, dyslipidaemia with triglycerides higher than 1,695mmol/L and HLD-C under 0,9mmol/L in male and HLD-C under 1mmol/L in female patients and finally a BMI over 30.

8.3 Sample collection

Blood samples were taken at time point 1, before the intervention, at time point 2, after one month of the intervention and at time point 3, after four month of the intervention. Therefor PAXgene Blood DNA tubes (Qiagen, Hilden, Germany) were used. Samples were stored at -20°C.

8.4 DNA isolation and bisulfite conversion

DNA of collected samples was extracted using the PAXgene® Blood DNA Kit (Qiagen, Hilden, Germany) according to the manufactures' instructions.

For methylation analysis bisulfite-conversion of all samples was necessary. Therefore the EpiTect® Bisulfite Kit (Qiagen, Hilden Germany) was used. Principle of this kit is the sodium bisulfite induced conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged.

DNA concentration was measured by using the Pico100 (Picodrop Limited, Hinxton, UK). All reactions were carried out according to manufactures protocols.

Samples were stored at -20°C.

8.5 PCR conditions and gel electrophoresis

At the beginning primer design software (Qiagen, Hilden, Germany) was used to find adequate primers for the bisulfite converted DNA to guarantee a proper amplification of the FFAR3 region.

The PCR was carried out in a 25µl reaction mix which contains 12,5µl PyroMark PCR Master Mix (2x), 2,5µl CoralLoad Concentrate (10x) (Qiagen, Hilden, Germany), 12,5pmol of each primer (FFAR3) and 25pmol of each primer (LINE-1) and 5 ng of converted DNA for FFAR3 and 10ng of converted DNA for LINE-1.

The PCR conditions for FFAR3 were 95°C for 15 minutes, 45cycles of 94°C for 30 seconds, 52°C for 45 seconds and 72°C for 45 seconds, final elongation at 72°C for 10 minutes. The PCR conditions for LINE-1 were 95°C for 15 minutes, 45 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, final elongation at 72°C for 10 minutes. After the PCR reaction 3 µl of each product inclusive the control without sample, were checked by a 2% agarose gel electrophoresis.

The remaining 22µl of PCR products will be used for the Pyrosequencing. The samples were prepared as a single batch for the PCR reaction. After the PCR

reaction the samples were divided into two parts for the PyroMark reaction. Double batch of 11µl for each sample will be used for pyrosequencing.

8.6 Quantitative gene methylation analysis of FFAR3 by pyrosequencing

As mentioned above 11µl of PCR product were used for further investigation. Therefore a mixture was prepared containing 11µl of PCR product, 3µl streptavidin-coated Sepharose® beads (GE Healthcare, Vienna, Austria) 40µl PyroMark binding buffer (Qiagen, Hilden, Germany) and 26µl high purity water for a total volume of 80µl.

Pyrosequencing was done by using a PyroMark® Q24 System (Qiagen, Hilden, Germany). For this method biotinylated primer used for the PCR reaction is necessary. The biotinylated end of the amplicon can bind to the streptavidin-coated Sepharose® beads. This complex can be sucked by PyroMark® Vacuum Workstation. The PCR-products were purified and denaturized by using 70% ethanol, 0,2M NaOH solution and washing buffer. The purified single-stranded DNA was released into the annealing buffer (Qiagen, Hilden, Germany), which contains a corresponding pyrosequencing primer (0,4pmol/µl). This mixture was used for pyrosequencing.

The Pyrosequencing assay was designed with the PyroMark Q24 Software (Qiagen, Hilden, Germany). For quality control, each experiment included non-CpG cytosines as internal controls to guarantee efficient sodium bisulfite DNA conversion.

Table 1 Primers: PCR and Pyrosequencing

Primer name	Sequence 5`-3`	Size	Annealing Temperature
LINE1 (F)	TTTTGAGTTAGGTGTGGGATATA	23	50°C
LINE1 (R)	*Biotin*-AAAATCAAAAAATTCCCTTTC	21	50°C
LINE1 (S)	AGTTAGGTGTGGGATATAGT	20	
FFAR3-1 (F)	GTGATAGGGAAAATAGTAGTTGGTA	25	52°C
FFAR3-1 (R)	*Biotin*-CCCTACAAAACAAAACTCATCATAAT	26	52°C
FFAR3-1 (S)	GGGAAAATAGTAGTTGGTAT	20	

Table 2 Sequence to analyze: FFAR3 and LINE1

Assay	Sequence 5´-3´	Number of CpGs	Size
LINE1-P	TTCGTGGTGCGTCGTTT	3	17
FFAR3-P	CCG CTG CCT GCT CAC GAC CAC ACG CCA GGC TTC CGT CAA ACC ACT CAA CAT GTA TTA G	5	58

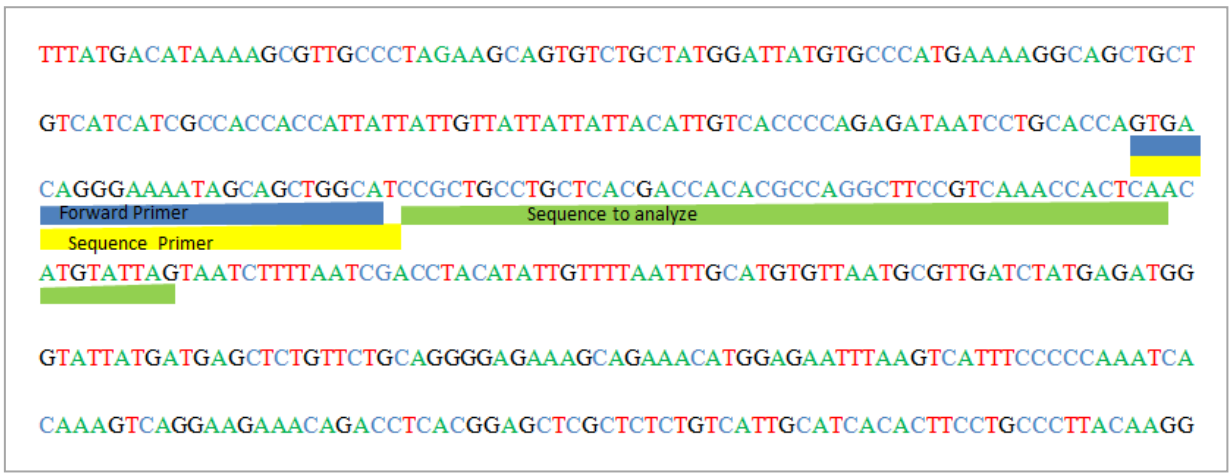

Figure 2 FFAR3 gene sequence region used for pyrosequencing

8.7 Statistical analysis

Quantitative methylation analysis of LINE-1 and FFAR-3 were analysed with IBM® SPSS® Statistics Version 20 (IBM, Armonk, NY). The Kolmogorov-Smirnov test was used to test for normality of the distributions. Also homogeneity of variance test was done. The student´s two tailed paired t-test and one-way analysis of variance (ANOVA) with the Tukey post hoc correction test were used to determine significant differences. To analyse correlations, the two-tailed Pearson test was used. A p-value <0,05 was considered to be statistically significant. All data shown are mean ± SD.

9 Results

9.1 FFAR3

During pyrosequencing of FFAR3 5 CpGs were analysed. Three bisulfite treatment controls (position 10, 20 and 37) were used to guarantee successful bisulfite conversion.

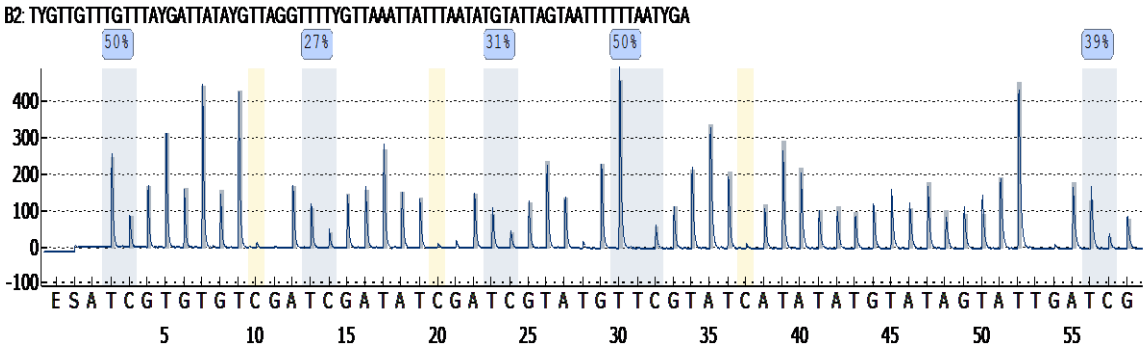

Figure 3 FFAR3-pyrogramm of diabetes patient at time point 3

9.1.1 General Methylation: all CpGs and single CpGs in each goup

In Table 3 mean methylation of all 5 CpGs in the three examined groups at each time point is shown. The results show that the lean control group is higher methylated compared to diabetics and obese controls. At time point 1 lean controls are significant (p=0,003) higher methylated than diabetics and also significant (p= 0,031) higher than obese controls. At time point 2 and 3 lean control is higher methylated than diabetics (T2: p=0,011; T3: p=0,000).

View at the mean methylation of all three time points underline the significant higher methylated lean controls than diabetics (p=0,008). The tendency of lean controls being higher methylated than obese controls can be observed (p=0,065).

During the intervention period no trend in methylation level was found in participating diabetics. Almost unchanged methylation levels during 4 months were also observed in lean control groups. Only the obese controls tend to have increasing methylation levels from time point one to time point three.

Table 3 FFAR3: mean methylation at each time point

		N	Mean [%]	Standarddeviation	Significance
CpG T1 mean	D	22	31,46	5,74	LC – D: p= 0,003
	OC	6	30,69	6,46	LC – OC: p= 0,031
	LC	17	38,19	6,19	
CpG T2 mean	D	22	32,53	7,17	LC – D: p= 0,011
	OC	7	32,34	7,13	
	LC	16	39,12	5,44	
CpG T3 mean	D	22	31,25	6,85	LC – D: p= 0,000
	OC	5	34,51	2,93	
	LC	13	40,39	5,68	
Mean all Cpgs T1_T2_T3	D	24	32,96	7,29	LC – D: p= 0,008
	OC	7	32,69	4,60	LC – OC: p= 0,065
	LC	18	39,11	5,10	

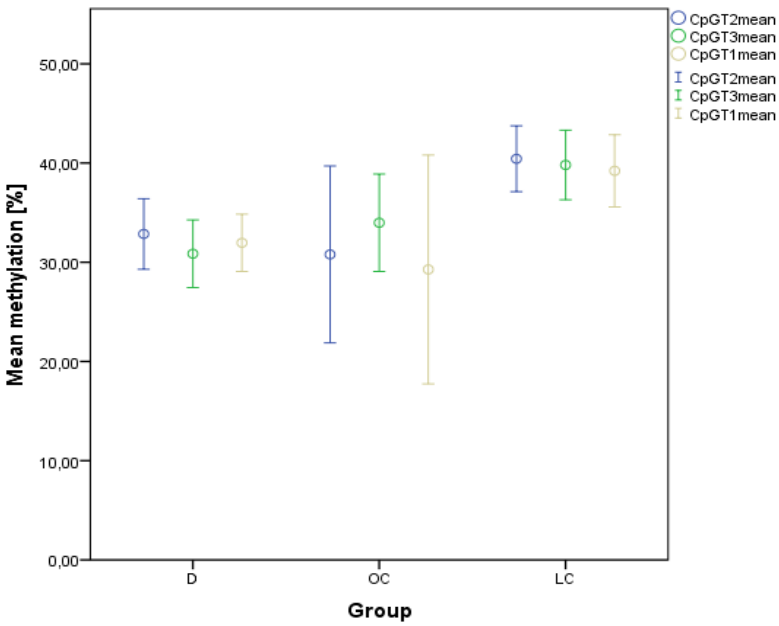

Figure 4 FFAR3: mean methylation of every group

In Table 4 to Table 6 the single CpGs are listed for each time point. It can be clearly seen that the CpGs are not equally methylated. CpG4 for example is highest methylated at all three time points. CpG2 is reduced in all three time points.

View at the significant differences between the different groups at single CpGs show that at time point 1 only CpG 4 and 5 have significant differences. At CpG4 lean controls are significant (p=0,000) higher than diabetics and obese controls (p=0,001). At CpG 5 lean control is also significant higher than diabetics (p=0,000) and obese controls (p=0,034).

Table 4 FFAR3: Methylation of each CpG at time point 1

Group T1	CpG1	CpG2	CpG3	CpG4	CpG5
D	37,29 ±12,10	20,47 ±5,22	24,48 ±5,71	41,27 ±7,85	33,82 ±7,95
OC	37,80 ±14,32	18,78 ±5,09	24,89 ±3,78	37,13 ±9,16	34,84 ±6,31
LC	42,86 ±13,25	23,66 ±3,95	27,21 ±3,61	51,70 ±6,46	46,13 ±11,13

At time point 2 there are more significant differences between the groups. At CpG2 lean controls are significant higher methylated than diabetics (p=0,001). At CpG3 lean controls are significant higher than diabetics (p=0,000) and obese controls (p=0,048). At CpG4 lean controls are significant higher than diabetics (p=0,000) and obese controls (p=0,005). At least at CpG5 lean controls are significant higher methylated than diabetics (p=0,005).

Table 5 FFAR3: Methylation of each CpG at time point 2

Group T2	CpG1	CpG2	CpG3	CpG4	CpG5
D	36,78 ±12,71	19,81 ±4,63	24,03 ±4,66	42,26 ±7,52	35,27 ±8,00
OC	42,05 ±13,40	21,61 ±4,61	25,82 ±4,12	43,03 ±6,20	35,61 ±6,60
LC	42,07 ±13,53	25,64 ±3,96	30,38 ±3,18	53,19 ±5,86	44,30 ±8,92

At time point 3 lean controls are significant higher in all 5 CpGs; CpG1: p=0,025; CpG2: p=0,009; CpG3: p=0,013; CpG4: p=0,000; CpG5: p=0,000. There is no significant difference between lean controls and obese controls.

Table 6 FFAR3: Methylation of each CpG at time point 3

Group T3	CpG1	CpG2	CpG3	CpG4	CpG5
D	36,53± 13,32	19,64 ±5,22	23,08 ±5,51	42,35 ±8,22	34,66 ±8,92
OC	41,83 ±9,85	22,49 ±3,38	26,83 ±4,55	45,02 ±3,81	36,39 ±4,21
LC	48,79 ±12,62	24,45 ±2,80	28,16 ±3,45	53,25 ±6,89	47,30 ±9,00

In total it can be observed that the number of significant differences between the groups at single CpGs is changing. On the one hand the significant difference between lean control and obese control is decreasing from time point 1 to time point 3. But on the other hand the significant differences between lean control and diabetics are increasing from time point 1 to time point 3.

9.1.2 Correlation: methylation and BMI

The correlation between BMI at different time points with the mean methylation of all groups at different time points shows following results.

In every time point a negative correlation can be observed. Like at time point one and time point three significant negative correlations between BMI and methylation can be seen. The comparison of mean BMI of all three time points and mean methylation of all groups at all three time points shows also significant negative correlation.

Table 7 FFAR3: Correlation of methylation and BMI

	Mean CpG T1 vs. BMI T1	Mean CpG T2 vs. BMI T2	Mean CpG T3 vs. BMI T3	Mean CpG T1T2T3 vs. Mean BMI T1T2T3
Correlation (Pearson)	-0,474**	-0,245	-0,544**	-0,451**
Significance	p=0,001	p=0,297	p=0,000	p=0,001

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

This means, the higher the BMI, the lower the methylation regardless in which group. In Figure 5 the significant negative correlation can be clearly seen.

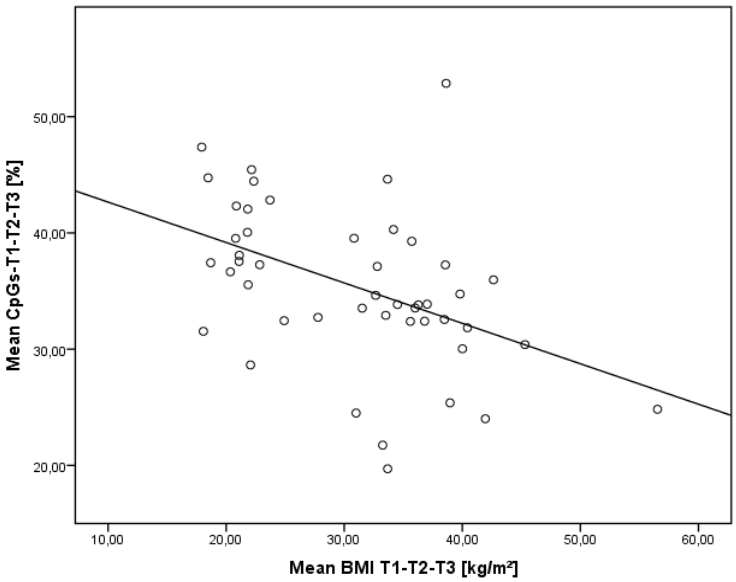

Figure 5 FFAR3: Mean Methylation against mean BMI at time point 1

The single groups showed the tendency of negative correlation with BMI but there were no significant correlations between the single groups and BMI at any time point.

9.1.3 Methylation and abdominal girth in diabetics

Abdominal girth (AG) was measured at all three time points only in the diabetic group. Correlations between abdominal girth and methylation were investigated. It could not be shown any significant correlation in this group. View at Table 8 shows a negative correlation but the correlation is very low and not significant. It could be possible to claim a trend to a negative correlation between abdominal girth and methylation level. This would mean that higher AG means a lower methylation. But in fact of very low correlation this fact should be neglected.

Table 8 FFAR3: Mean methylation against abdominal girth in diabetics

	CpG T1 vs. AG T1	CpG T2 vs. AG T2	CpG T3 vs. AG T3	Mean CpGs vs. Mean AG
Correlation(Pearson)	-0,057	-0,194	-0,046	-0,037
Significance	0,803	0,387	0,839	0,864

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

9.1.4 Methylation and age

Examination of FFAR3 gene showed an age specific methylation level. Unfortunately the age between the groups varies significantly (p=0,002). So lean control group with an average age of 25,67 years is the youngest group. Followed by obese group with an average age of 39,63 years. The oldest group is the diabetic intervention group with an average age of 58,36 years.

Table 9 FFAR3: Mean methylation vs. age

	Mean Methylation T1-T2-T3	Mean Methylation T1	Mean Methylation T2	Mean Methylation T3
Age:				
Correlation(Pearson)	-0,375*	-0,414**	-0,380*	-0,543**
Significance	0,012	0,005	0,010	0,000

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

This means that high age can be associated with lower methylation at FFAR3 gene and higher methylation in young persons. This can also be clearly seen in Figure 6.

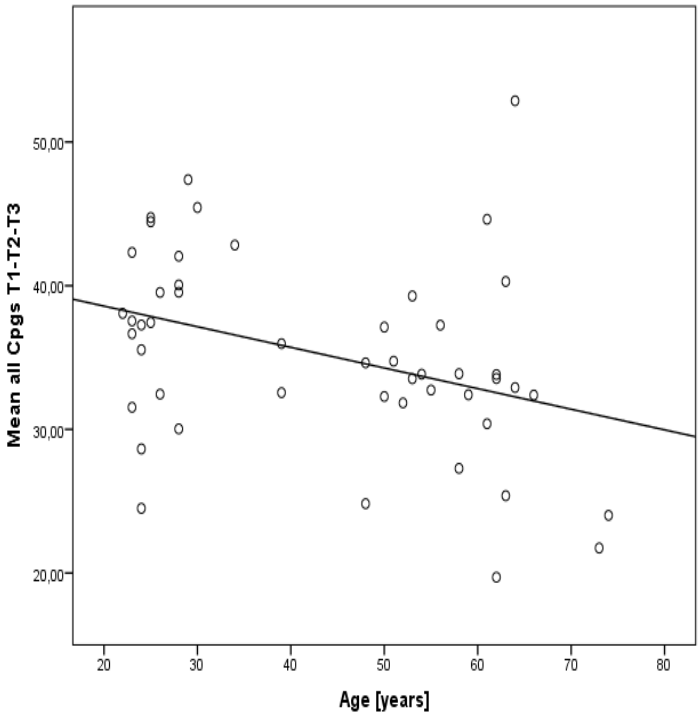

Figure 6 FFAR3: Mean methylation vs. age

9.1.5 Methylation and weight: weight loss

The weight distribution in the three groups shows that diabetics are the group with the highest weight (112,83kg ± 17,85kg). Followed by the obese group with an average weight of 101,38kg ± 10,46kg. The lean group has significant lower weight than the other two groups, with an average weight of 60,61kg ± 7,85kg.

Comparison of responders and non-responders, whereby responder means weight loss of minimum 5% of the documented bodyweight during intervention and non-responder means no weight loss or less than 5% of bodyweight during intervention. There was no significant correlation found between bodyweight loss and methylation. But looking at the weight of all patients and their methylation, significant, negative correlation of bodyweight and methylation can be observed. This means the higher the bodyweight the lower the methylation level and the other way round. This can be clearly seen in Figure 7 and Table 10.

Table 10 FFAR3: Methylation vs. bodyweight

	Methylation T1 [%] vs. weight T1 [kg]
Correlation (Pearson):	-0,409**
Significance:	0,005

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

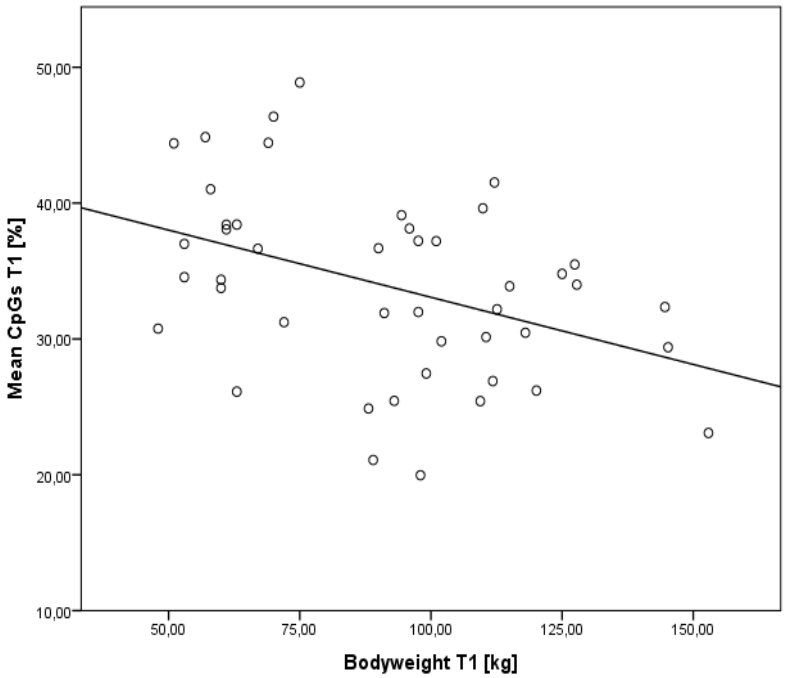

Figure 7 FFAR3: Mean methylation vs. weight at T1

9.1.6 Methylation and sex

The gender distribution is almost equally distributed with 54,9% women and 45,1 men. Looking at the sex specific methylation shows increased methylation in women (mean: 35,63%) compared to men (mean: 34,63%). But this might be due to the significant higher bodyweight of male (107,69kg) participants in comparison to female (79,00kg). As investigated above methylation correlates significantly, negative with bodyweight.

9.1.7 Methylation Hba1c

HbA1c was measured at all three time points in the diabetics intervention group. There is no correlation between changes in HbA1c and methylation during intervention. View at the single time points show a tendency of negative correlation of HbA1c with methylation. That means the higher the HbA1c level is, the lower the methylation level. During the intervention the patients received Victoza® which has an influence on HbA1c level.

Table 11 FFAR3: Mean methylation vs. HbA1c

	HbA1c T1 vs. Mean CpG T1	HbA1c T2 vs. Mean CpG T2	HbA1c T3 vs. Mean CpG T3
Correlation (Pearson)	-0,125	-0,242	-0,310
Significance	0,579	0,277	0,160

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

9.2 LINE1

During pyrosequencing of LINE1 three CpGs were analysed. One bisulfite treatment control (position 2) was used to guarantee successful bisulfite conversion.

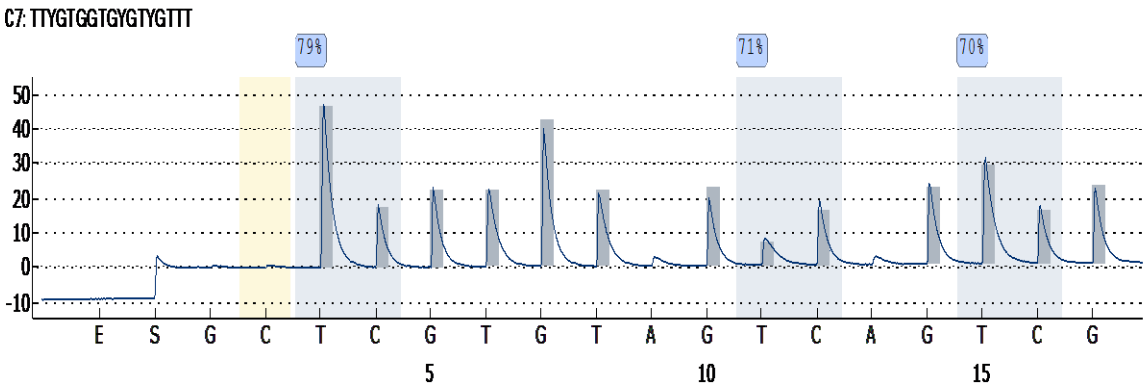

Figure 8 LINE1-pyrogramm of diabetes patient time point 1

9.2.1 General Methylation: all CpGs and single CpGs in each group

Table 12 shows mean methylation of LINE1 at each time point. Compared with FFAR3 there are no existing significant differences in methylation level between the different groups. No trend between the different time points in any group can be seen. Also there is no outranging group in the three groups with higher or lower methylation level. All in all Line1 shows steady methylation levels.

Table 12 LINE1: mean methylation

		N	Mean [%]	Standarddeviation
Line1_Mean_T1	D	20	66,09	5,32
	OC	7	64,68	3,27
	LC	16	67,33	5,55
	Gesamt	43	66,32	5,12
Line1_Mean_T2	D	21	69,17	5,22
	OC	5	63,60	3,92
	LC	14	68,83	5,85
	Gesamt	40	68,36	5,50
Line1_Mean_T3	D	18	67,64	6,39
	OC	5	63,41	3,61
	LC	14	67,02	5,78
	Gesamt	37	66,83	5,89
Mean all CpGs T1_T2_T3	D	23	67,57	0,99
	OC	7	64,36	2,92
	LC	18	68,06	5,01
	Gesamt	48	67,29	0,68

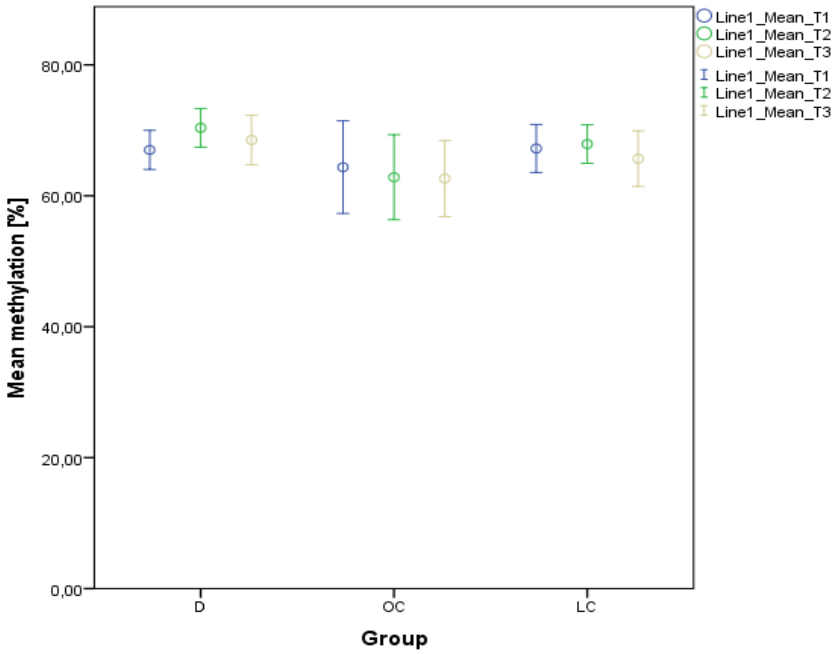

Figure 9 LINE1: Mean methylation of every group

At time point 1 CpG1 points out to be higher methylated than CpG2 and 3. This can be observed in all three groups. CpG2 and CpG3 show almost same methylation level.

Table 13 LINE1: Mean methylation at time point 1

Group T1	CpG1	CpG2	CpG3
OD	71,58 ± 6,04	63,85 ± 4,87	62,82 ± 6,24
OC	71,02 ± 4,04	61,36 ± 3,15	61,66 ± 3,06
LC	73,51 ± 5,48	63,69 ± 5,30	64,84 ± 6,34

Time point two shows same distribution as T1. CpG1 shows highest methylation level in every group. Also obese control group is lowest methylated in all three CpGs this trend is not well visible in T1 but in T2. Lean controls and diabetics are still almost at the same methylation level.

Table 14 LINE1: Mean methylation at time point 2

Group T2	CpG1	CpG2	CpG3
OD	74,45 ± 4,94	66,46 ± 5,23	66,60 ± 5,87
OC	67,92 ± 6,32	61,68 ± 1,84	61,18 ± 3,81
LC	74,85 ± 5,88	65,29 ± 5,24	66,34 ± 6,67

At time point three CpG3 still has the highest methylation level. Also the low methylated obese control group is still little less methylated (not significant) than the other groups.

Table 15 LINE1: Mean methylation at time point 3

Group T3	CpG1	CpG2	CpG3
OD	72,43 ± 7,19	65,47 ± 5,47	65,00 ± 6,89
OC	68,72 ± 5,45	60,88 ± 1,98	60,62 ± 3,83
LC	74,19 ± 4,82	63,17 ± 5,54	63,67 ± 7,26

All in all there is no trend visible in the different time points. Single CpGs show same trends as mean methylation levels.

9.2.2 Methylation and BMI

LINE1 does not show clear tendency between methylation level and measured BMI during three time points. View at T2 and T3 could assume very weak and not significant positive correlation. There cannot be made a clear definition of positive or negative correlation. But a positive tendency in T2 and T3 can be proposed.

Table 16 LINE1: Correlation of mean methylation vs. BMI

	BMI T1 vs. Mean T1	BMI T2 vs. Mean T2	BMI T3 vs. Mean T3	BMI T1T2T3 vs. Mean T1T2T3
Correlation (Pearson)	-0,044	0,277	0,140	-0,031
significance	0,784	0,250	0,429	0,836

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

Figure 10 shows correlation of mean methylation at T2 and BMI at T2. Positive correlation can be seen but not significant and mainly only at T2. Relevance of this correlation cannot be clearly figured out.

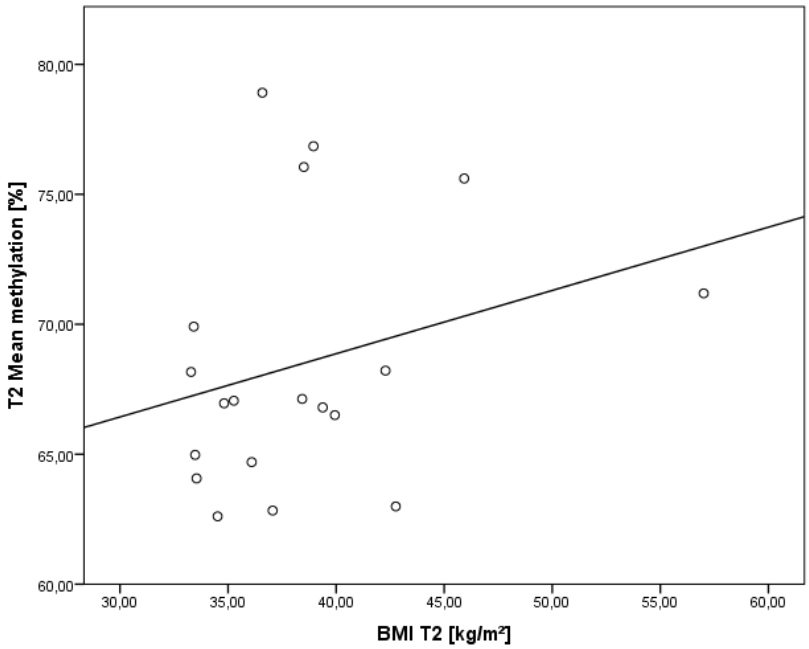

Figure 10 LINE1: Mean methylation vs. BMI at time point 2

9.2.3 Methylation and abdominal girth in ODs

Comparison of abdominal girth (AG) in diabetics and methylation level did not show any significant correlation or trend. Only time point 2 shows minimal trend for positive correlation similar as in Table 16. But this single correlation in one time point has no evidence.

Table 17 LINE1: Mean methylation vs. abdominal girth

	AG T1 vs. Mean T1	AG T2 vs. Mean T2	AG T3 vs. Mean T3	AG T1T2T3 vs. Mean T1T2T3
Correlation (Pearson)	-0,219	0,346	-0,012	-0,025
significance	0,354	0,125	0,964	0,910

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

9.2.4 Methylation and age

LINE1 does not show any correlation between age and methylation level. View at single CpG shows similar results as mean methylation levels.

Table 18 LINE1: Mean methylation vs. age

	Methylation T1 vs. Age	Methylation T2 vs. Age	Methylation T3 vs. Age	Mean Methylation T1T2T3 vs. Age
Correlation (Pearson)	-0,146	0,060	0,094	-0,022
Significance	0,350	0,715	0,582	0,882

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

Figure 11 clearly shows that age do not have any influence on methylation levels in every control and intervention group.

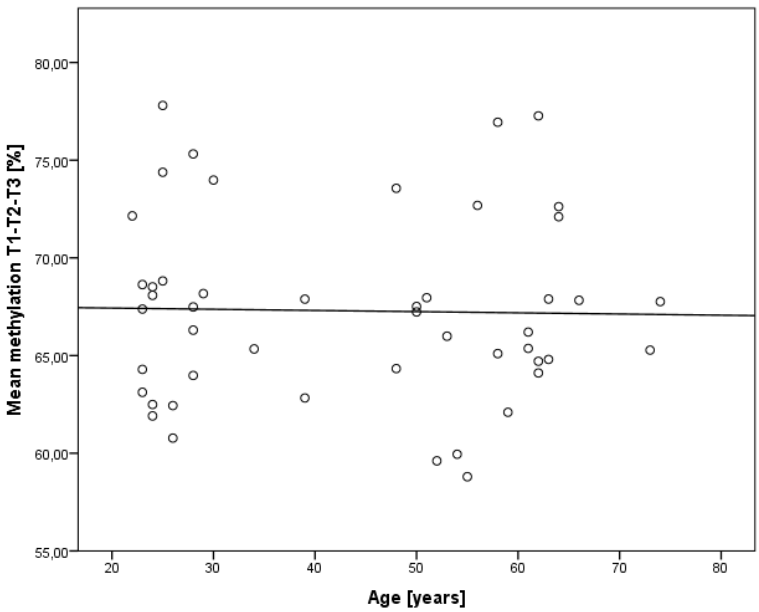

Figure 11 LINE1: Mean methylation vs. age

9.2.5 Methylation and sex

Methylation levels are equally separated in male and female. Average methylation in male is 67,33 ± 5,22 % and 67, 26 ± 4,39 % in female.

9.2.6 Methylation Hba1c

Hba1c was measured in diabetics in all three time points. Correlation analysis show positive correlation of Hba1c and mean methylation level at each time point. This means the higher the HbA1c value the higher the mean methylation level of LINE1. These results are contrary to FFAR3 where negative correlation can be observed.

Table 19 LINE1: Mean methylation vs. HbA1c

	HbA1c T1 vs. Mean CpG T1	HbA1c T2 vs. Mean CpG T2	HbA1c T3 vs. Mean CpG T3
Correlation (Pearson)	0,197	0,341	0,189
Significance	0,406	0,130	0,453

**Correlation is significant on the level of 0,01.
*Correlation is significant on the level of 0,05.

Generally Hba1c values were decreasing from time point one to time point three, this can be due to the medication of Victoza® which was given throughout the intervention period.

Table 20 LINE1: Mean HbA1c value

	Mean [%]	Standarddeviation
HbA1c T1	8,45	1,31
HbA1c T2	8,19	1,61
HbA1c T3	7,44	0,84

10 Discussion

Methylation of five CpGs in the promoter region of FFAR3 and three CpGs of LINE1 which used as global methylation control were analysed by pyrosequencing. Blood samples from three groups at three time points were investigated. The role of methylation levels in the different groups and the influence on different epigenetic regulations will be discussed.

Investigations of FFAR3 showed different methylation levels in the three analysed groups. Thereby lean controls showed significant higher methylation than obese controls and diabetics. LINE1 which was used as general methylation control showed no significant differences in methylation levels between the three groups. No correlation could be found in diabetics during intervention period. Significant results were found for a negative correlation of increasing BMI and decreasing FFAR3 methylation. Interestingly LINE1 shows a positive trend of increasing BMI and increasing LINE1 methylation.

Several studies demonstrated dose dependant SCFA activation of GPR41 and consequently regulated leptin production in adipocytes (Ichimura et al. 2009). Studies on animals and humans could confirm the hypophagic effect of SCFA induced leptin production (Arora et al. 2011). The difficulty in obese patients is the higher fat content which leads to increased leptin production which provokes leptin resistant state (Münzberg und Myers 2005). Leptin resistant state is hypothesised to develop because of defect signalling to the brain whereby also triglycerides which are well known to be increased in adipose subjects are able to inhibit blood-brain barrier transfer. Or defect leptin receptor signalling is debated as cause for leptin resistant states (Banks A. William 2004). Our findings show hypermethylated lean controls and low methylation levels in obese and diabetics. This is supposed to result in higher expression of FFAR3 in obese and diabetics compared to lean subjects. That means higher FFAR3 expression leads to higher leptin production which is consistent with studies on mouse adipocyte cell line (Ichimura et al. 2009). It is supposed that leptin overexpression and thereby promoting a leptin resistant state might be amongst others avoided through hypermethylation resulting in lower expression levels of FFAR3 and less leptin

production in lean controls. These results are consistent with our results of significant negative correlation of BMI and methylation levels found in FFAR3 gene. It means the higher the BMI the lower the methylation level in the study participants. This also indicates higher leptin production in patients with high BMI.

Besides hypophagic effects of leptin, studies from Samuel et al. show that deficiency in GPR41 results in decreased PYY expression (Buck S. Samuel 2008). Main function of PYY is the ability of appetite and caloric intake reduction. Further it inhibits stomach emptying and increases the water and electrolyte absorption in the gastro intestinal tract. General obese patients show lower fasting PYY-levels compared to lean patients and also the postprandial release of the peptide is decreased which may lead to diminished satiety (Schloegl et al. 2011). Until now underlying mechanisms are not cleared yet and maybe studies on expression levels might help to understand the role of PYY and consequently findings of our study could be explained. Because if decreased methylation levels in obese subjects as found in our study, are indicated to result in higher expression than in lean controls this would result in contrary outcomes as reported by Schloegl et al. 2011 (Schloegl et al. 2011).

Another important aspect includes butyrate being an important mediator in inflammation. It´s ability to induce inhibition of histone deacetylase helps to reduce inflammatory symptoms (Berni Canani et al. 2012) by reduction of macrophages, B-lymphocytes and T-lymphocytes (Vieira et al. 2012). The mechanism behind might be suppression of nuclear factor-κB (NFκB), inhibition of interferon γ production, the upregulation of peroxisome proliferator-activated receptor γ (PPARγ) and activation of proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Berni Canani et al. 2012). PPARγ occurs mostly in adipose tissue and is able to regulate genes involved in fatty acid uptake, inflammation and glucose homeostasis (Staels Bart and Fruchart Jean-Charles 2005). These activations may be due to the HDACi. Further these anti-inflammatory effects may be protective against low grade inflammation which occurs mostly in obesity, insulin resistance, type 2 diabetes and cardiovascular diseases. Further studies could show that increased consumption of dietary fibers results in reduced inflammation and increased butyrate production which might result in increased FFAR3

activation (Berni Canani et al. 2012). Recently findings of Wu et al. 2012 suggest possible inclusion of GPR41 in histone acetylation, proliferation, apoptosis and cell cycle. As already mentioned butyrate induction causes inhibition of histone acetylation, but only in cells with GPR41 and not in cells without GPR41. Furthermore anti-apoptotic function could be demonstrated after 500µmol/l of butyrate induction followed by tripled apoptotic cell number without GPR41 whereby in cells with GPR41 there was no increase in apoptotic cells detected. Involvement of Bcl-2 and Bax proteins was investigated by western blot. Results showed dose dependant increase of pro-apoptotic Bax proteins and decreased anti-apoptotic Bcl-2 proteins after butyrate induction. In GPR41 cells expression of anti-apoptotic Bcl-2 proteins was increased. Consequently it is supposed GPR41 prevents butyrate induced pro-apoptotic impacts. Finally, also inclusion of GPR41 in cell cycle alteration could be shown. Therefore flow cytometry analysis of relative DNA content was used to establish the inclusion of butyrate in cell cycle alterations. So it could be shown that GPR41 is able to transfer more cells from G1- (post mitotic) phase to S- (DNA synthesis) phase checkpoint. Almost no effects could be observed in cells without GPR41. These results may explain the lowered anti-proliferative effect of GPR41 induced by butyrate. Interestingly GPR41 is able to inhibit an increase of p21, a cyclin-dependent kinase (Cdk) inhibitor, which is able to inhibit Cdk 2 and Cdk 4, after butyrate induction (Wu et al. 2012). View at our results especially lean controls show significant higher methylation than obese patients. Assuming this would lead to decreased expression of GPR41, this would mean that the butyrate induced pro-apoptotic, anti-proliferative effect and inhibition of HDAC will be less blocked by GPR41 in lean subjects. Further studies are necessary to clearly understand the role of GPR41 at this topic.

Halpern et al. indicate that GPR41 does not have an own promoter region and is transcribed by the promoter region of GPR40. This would mean GPR41 transcription is induced by a promoter region 6619bp upstream and results in generating a bicistronic transcript of GPR41 and GPR40. So both genes are determined on the same mRNA. Translation is controlled by an internal ribosome entry site (IRES) which is placed in the intergenic region. The importance of these

findings is not clear yet and also the consequences of promoter methylation in GPR41 will have to be reviewed. According to Halpern et al., changes in methylation levels of GPR41 promoter region will have no influence on epigenetic regulation. Otherwise it is supposed that changes in methylation could affect transcription of both genes. Further studies are necessary to ensure these results and also their influences on transcriptional and translational levels have to be investigated (Keren Bahar Halpern 2012).

Long interspersed element-1 (LINE1) was used as control for global methylation and includes 17% of the whole genome (Turcot et al. 2012). General hypomethylation occurs in many forms of cancer and even environmental risk factors like benzene, tobacco smoke and persistent organic pollutants can be reasons for LINE1 hypomethylation. (Di et al. 2011) Beside this also ageing is a possible characteristic for global low methylation levels (Richardson 2003). According to Turcot et al. LINE1-hypomethylation should be also considered as potential marker for metabolic syndrome like plasma fasting glucose, plasma lipid levels but also ischemic heart disease and stroke (Turcot et al. 2012). In our study LINE1 methylation did not show any age dependant correlation which could exclude age specific influence on measured methylation levels in the three observed groups. There could not be observed lower methylation level of LINE1 in female compared to male which was observed in visceral adipose tissue in the study from Turcot et al (Turcot et al. 2012). Our findings indicate a positive trend of increasing BMI and increasing methylation levels, which was contrary in FFAR3 methylation. Overall methylation level was lower in obese controls compared to diabetics. Maybe this goes back to medication of diabetics with GLP-1 antagonist, Victoza® which results in improved HbA1c levels.

11 Conclusion

Taken together our results indicate involvement of GPR41 in several physiological functions like satiety and food intake as well as, inflammation. The responsible mediators for these physiological functions are on the one hand the microbiota which mainly produces SCFA like butyrate and on the other hand methylation levels which are supposed to regulate GPR41 expression. For future studies this link from microbiota over GPR41 activation and the affected epigenetic regulation of several physiological functions will be a very interesting challenge. We suppose that GPR41 would not only serve as valuable biomarker but also as therapeutic target for metabolic diseases. Several future studies will be necessary to evaluate GPR41 as possible target.

12 Paper, abstracts and poster

12.1 Poster: Third Clinical Epigenetics International Meeting, Clinical Epigenetics in Solingen, Germany

Do changes in GI-microbiota influence epigenetic methylation of inflammatory genes in type 2 diabetes and obesity?

Marlene Remely[1], Christine Merold[1], Ya Chen[1], Eva Aumüller[1], Daniela Jahn[1], Simone Dworzak[1], Berit Hippe[1], Helmut Brath[1], Alexander G. Haslberger[1*]

[1]: University of Vienna, Department of Nutritional Sciences, Althanstrasse 14/UZA2/2D541, 1090 Vienna, Austria

[*]: Corresponding and presenting author,
e-mail: alexander.haslberger@univie.ac.at
Tel: 0043/1/4277 54996

Abstract

Obesity and Diabetes mellitus type 2 (DM2) are associated with a systemic low grade inflammation. Changes of the gastro-intestinal (GI) microbiota as well as epigenetic regulation are discussed to trigger this inflammatory response. Different interactions between GI-microbiota and epigenetic mechanisms are discussed. Possible mechanisms are the NF-κB signalling mediated from bacterial cell wall components DNA or the signalling via short chain fatty acids produced by the GI-microbiota.

We enrolled 3 groups of study participants: individuals with (OD) and without (OC) DM2 and lean controls (LC). The OD-participants underwent three months of intervention consisting of nutritional counselling and the glucagon-like peptide-1 agonist Liraglutide. Blood and stool samples were collected at the beginning of the study, after one and after three months. Stool samples were analysed for the abundance and diversity of GI-microbiota using PCR-DGGE, qPCR and 454 Pyrosequencing. Epigenetic methylation of CpG sites in the promoter regions of TLR2, TLR4, FFAR3, TNF-α and Line1 was investigated with bisulfite-pyrosequencing of the DNA extracted from the blood samples.

The diversity and the band pattern of the GI-microbiota were reduced in the OD- and OC-group compared to LC-individuals. The ratio of *Firmicutes*/*Bacteroidetes*, the abundance of lactic acid bacteria subgroups and of *Enterobacteria* increased during the intervention period in OD-participants. In contrast, in OC-individuals with weight loss the ratio of *Firmicutes*/*Bacteroidetes* was decreased.

We saw a decreased CpG methylation in the promoter regions of TLR2, TLR4 and FFAR3 in the OC- and OD-groups compared to LC-individuals. CpG methylation of TNF-α and Line1 did not show significant changes between the groups or during the time course. Further, we observed correlations between anthropometric measurements and methylation of TLR4.

Changes in the GI-microbiota and the DNA-methylation may not only be useful biomarkers but might also act as starting points for prevention and therapy of obesity and DM2.

Conflict of interests

All authors declare no conflict of interests

Funding

The project was supported by FFG – The Austrian Research Promotion Agency.

12.2 Poster: FEMS Kongress in Leipzig, Germany

Relation of *Faecalibacterium prausnitzii* phylotypes and epigenetic regulation in obese, diabetic patients and controls

Berit Hippe, Simone Dvorzak, Marlene Remely, Eva Aumüller, Christine Merold, Alexander G Haslberger

Background: *Faecalibacterium prausnitzii* is one of the most abundant commensal bacteria and one of the main butyrate producers in the healthy human gut. Information on their genetic diversity and role in the inflammatory genesis is limited. Epigenetic regulation via FFAR or NFκB signaling are discussed

Objectives: We analyze *F.prausnitzii* phylotypes, butyrate production and epigenetic rgulation in obese, diabetic 2- and lean controls under a four month nutrition intervention.

Methods: We compare fecal and blood samples of the 3 groups at three time points with qPCR and high resolution melt curve analysis (HRMA) and automated ribosomal intergenic spacer analysis (ARISA). *F. prausnitzii* depending butyrate gene levels are detected by qPCR. Epigenetic methylation of CpG sites in promoter regions of FFAR3, TLR2, TLR4, TNF-α and Line1 are investigated with bisulfite-pyrosequencing of the DNA extracted from the blood samples.

Results: Obese- and diabetic groups differ significantly at all-time points compared to controls with fewer *F. prausnitzii* copies. *F. prausnitzii* butyrate levels do not correlate with the total *F. prausnitzii* amount. Analysis of CpGs in the promoter regions of FFAR3, TLR2, and TLR4 showe lower methylation% in obesity and type 2 diabetes. CpG methylation of LINE-1and TNF- show no significant changes between the groups or during the time course. Negative correlation between BMI and methylation of FFAR3 was observed.

Conclusion: Different phylotypes of *F. prausnitzii* and their various functions may lead to differences in the inflammatory genesis in the host. Butyrate producers may have an influence in developing obesity and might also act as starting points for prevention and therapy of obesity.

12.3 Poster: IUNS 20th International Congress of Nutrition in Granada, Spain

Changes in microbiota and epigenetic methylation of inflammation relevant genes in type 2 diabetes and obesity

Marlene Remely, Christine Merold, Ya Chen, Eva Aumüller, Daniela Jahn, Simone Dworzak, Berit Hippe, Alexander G. Haslberger

Metabolic diseases are believed to involve changes in the structure of microbiota causing low grade inflammation.

We studied obese and type 2 diabetes patients in a four month intervention period in comparison to a lean control group. Intervention involved Victoza for type 2 diabetics and nutritional counselling for both intervention groups. Microbiota were analysed for abundance and diversity by PCR-DGGE, qPCR and 454 Pyrosequencing. Epigenetic methylation of 3-7 CpG sites in promoter regions of TLR2, TLR4, FFAR3, TNF-α and Line1 as a marker for global methylation was analysed.

In type 2 diabetes and obesity the diversity of band patterns was decreased compared to healthy controls. *Firmicutes*/*Bacteroidetes* ratio, abundance of lactic acid subgroups and *Enterobacteria* increased during intervention period in type 2 diabetes. In contrast the ratio of *Firmicutes*/*Bacteroidetes* was decreasing in obese patients with weight loss.

We generally found a significant decreased methylation in TLR2, TLR4 and FFAR3 in obesity but also decreased methylation in diabetics, (TLR2: obese: 2,96%, TLR4: obese: 4,30%, FFAR3: diabetes: 31,75%; obese: 32,51%).CpG methylation of TNF-α and Line1 did not show significant changes between the groups. In general we found a significant correlation between an increased BMI/WHR and decreased methylation of TLR2, TLR4 and FFAR3 but increased methylation of Line1.

Our results suggest that a different composition in microbiota in obesity and type 2 diabetes effect the epigenetic regulation of inflammatory molecules, possibly also underlying a progression of the metabolic disease. Interactions between microbiota and epigenetic regulation may involve NF-κB signalling from bacterial cell wall components or DNA as well as SCFA binding to SCFA-receptors or TLRs. Significant correlations of anthropometric measurements and TLR2, TLR4, FFAR3 or in contrast to global methylation assessed by Line1 could develop into useful markers.

12.4 Paper: Applied and Translational Genomics, 2013

Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity

Marlene Remely [1], Christine Merold [1], Simone Dworzak [1], Eva Aumüller[1], Berit Hippe [1], Julia Zanner [1], Helmut Brath [2], Alexander G. Haslberger [1]

[1] Department of Nutritional Sciences, Vienna, Austria
Tel 0043 6991 2211212, alexander.haslberger@univie.ac.at

[2] Diabetes Outpatient Clinic, Health Center South, Vienna, Austria

Abstract

Gut microbiota and microbial metabolic activities of the lipid and glucose metabolism, satiety and chronic low-grade inflammation are known to be involved in the metabolic syndrome. Especially fermentation end products, SCFAs are believed to engage the epigenetic regulation of inflammatory reactions via FFARs and other SCFA-receptors.

We studied a potential interaction of microbiota with epigenetic regulation in obese and type 2 diabetes patients compared to a lean control group over a four month intervention period. Microbiota was analyzed for abundance, b*utyryl-CoA: acetate CoA-transferase* gene, and for diversity by qPCR, DGGE and 454 high-throughput sequencing. Epigenetic methylation of the promoter region of FFAR3 and Line-1 was analyzed using bisulfite conversion and pyrosequencing.

The diversity of the microbiota as well as *Faecalibacterium prausnitzii* were significantly lower in obese and type 2 diabetic patients compared to lean individuals. Results from *Clostridium cluster IV* and *Clostridium cluster XIVa* showed a decreasing trend in type 2 diabetics in comparison to the *butyryl-CoA: acetate CoA-transferase* gene and according melt curve analysis.

The analysis of the FFAR3 promoter region showed a significant lower methylation% in obese and type 2 diabetics with an increase in obese patients over intervention period. LINE-1, a marker of methylation, indicated no significant differences between the three groups or the time points, although methylation of

type 2 diabetics tended to increase over time. In addition we found a significant correlation between a higher BMI and lower methylation% of FFAR3.

Our results give evidence that a different composition of gut microbiota in obesity and type 2 diabetes affect the epigenetic regulation of inflammatory molecules. Interactions between microbiota and epigenetic regulation may involve SCFAs binding to FFARs but also NF-κB signaling from bacterial cell wall components or DNA.

Keywords

butyryl-CoA: acetate CoA-transferase gene – *Faecalibacterium prausnitzii* – FFAR3 – Line-1- GLP-1 Agonist

Abbreviations

BMI	body mass index
DGGE	denaturing gradient gel electrophoresis
FFAR	free fatty acid receptor
FFG	Austrian Research Promotion Agency
FFQ	food frequency questionnaire
FWF	Austrian Science Fund
GLP-1	glucagon-like peptide 1
Gpr	G-protein coupled receptor
HbA_{1c}	glycol hemoglobin
HDAC	histon deacetylase
IFN-γ	interferon-γ
IL	Interleukin
LCFA	long chain fatty acid
Line-1	long interspersed nuclear element 1
NF-κB	nuclear factor κB
PCA	principal component analysis

PGB	Porphobilinogen-Deaminase
PPAR-γ	peroxisome proliferator-activated receptor-γ
PYY	Peptide YY
SCFA	short chain fatty acid

Introduction

The gut microbiota plays a critical role in the establishment and maintenance of health. A wide range of inflammatory and metabolic diseases is associated with microbial imbalance [1,2]. Obesity has been associated with changes in abundance, diversity and metabolic function of the gut microbiota [2]. These compositional changes are indicated by a higher abundance of *Firmicutes* and a decreased abundance of *Bacteroidetes* [1,2,3]. However, controversial data [4] or no link of obesity to the ratio of *Firmicutes /Bacteroidetes* are also described [5]. A higher *Firmicutes* versus *Bacteroidetes* ratio is hypothesized to increase the fermentation end-products butyrate and acetate, as many *Firmicutes* are butyrate producers [6]. *Faecalibacterium prausnitzii* is well described for butyrate synthesis, anti-inflammatory activity, decreasing the rates of NF-κB (nuclear factor-κB) activation and IL-8 (interleukin) secretion, increasing IL-12, IL-10 and IFN-γ (interferon-γ), and also as an indicator of microbial diversity [7]. Evidence is accumulating that a higher diversity is related to health, whereas a lower diversity is observed in relation to various diseases [8]. The gut microbiota is also involved in obesity-associated metabolic disorders, type 2 diabetes, metabolic endotoxemia and low-grade inflammation [9]. Type 2 diabetes has often been associated with lower abundance of *Firmicutes*, while *Bacteroidetes* and *Proteobacteria* tended to be more abundant [1]. Controversial data showed lower abundance of *Bacteroidetes vulgatus* and *Bifidobacterium* spp. and a higher of *Clostridium leptum cluster* in diabetics compared to healthy volunteers [10]. In addition, the relative abundance of Bacilli is increased in diabetic mice models [1]. Our recently published data support these findings of a higher *Firmicutes /Bacteroidetes* ratio with the highest contribution coming from lactic acid bacteria. *Lactobacilli* together

with *Enterobacteria* may contribute to the associated systemic low-grade inflammation of obesity and type 2 diabetes. Both are mentioned to induce inflammation via their immune modulating properties and to provoke the macrophagen-mediated adipose tissue inflammation [11].

A part of the microbiota, SCFA-producing bacteria are known to be involved in the fermentation of dietary fibers by converting sugars partially to short-chain fatty acids (SCFAs), including acetate, propionate and butyrate [12]. These SCFAs may help explain some mechanisms by which gut microbiota affect obesity and chronic diseases. They have a role in the regulation of energy metabolism, immunity and adipose tissue expansion [13]. On the other hand all three SCFAs are proposed to protect against diet-induced obesity, with butyrate and propionate being more efficient than acetate [14]. They are ligands of two different G-protein coupled receptors, GPR41 (FFAR3; free fatty acid receptor) and GPR43 (FFAR2). The affinity of SCFAs to FFAR3 and FFAR2 differs with the length of the fatty acids. FFAR3 prefers the ligands butyrate and propionate, whereas FFAR2 prefers acetate and propionate [14,15]. An additional receptor, GPR40, does not bind SCFAs but LCFAs (long chain fatty acid) and is located upstream very close to GPR41 gene. Halpern et al. mentions that GPR41 may has no promoter region and thus is transcribed by the promoter of GPR40 [16]. Biological functions of FFARs are the modulation of lipid metabolism, expression of the leptin gene and the control of food intake. Effects of SCFAs are also believed to include epigenetic control of gene expression [17]. Butyrate is well-known for its epigenetic activity as histone deacetylase inhibitor (HDACi). Modulation of histone acetylation by SCFAs may prevent obesity through anti-inflammatory and anti-carcinogenic effects. Activities of histone deacetylases and methyl CpG binding proteins may influence DNA-methylation [18]. Folat is the best known methyl group donor, however other substances such as methionine, choline and butyrate are also able to influence the DNA methylation status. Changes in the CpG methylation of genes or promoter regions of inflammatory mediators or receptors regulate the expression. Highly methylated promoter regions are proposed to decrease transcriptional activity of the downstream gene [19]. In contrast, a global DNA

methylation, often analyzed by the methylation of long interspersed nuclear element 1 (LINE-1) is discussed for DNA stability. LINE-1 elements are usually high methylated and their variability can be associated with cancer, age, ischemic heart disease and stroke as well as with plasma fasting glucose and plasma lipid levels [20].

We investigate differences of FFAR3, LINE-1 in human blood samples, *butyryl-CoA: acetate CoA-transferase* gene and compositional changes in *Firmicutes* of microbial DNA from feces of obese patients without established insulin resistance, type 2 diabetics under GLP-1 Agonist therapy (glucagon like peptide-1 Agonists) and a lean control group. The GLP-1 Agonist increases the glucose-dependent insulin secretion in beta cells and suppresses the glucagon secretion in the Pancreas. It also slows the gut passage in order to decrease the postprandial spike in blood glucose [21]. In addition, we investigated the response of gut microbiota and according epigenetic consequences to intervention and its potential role in controlling metabolic syndrome.

Materials and methods

Study participants and study design

The study “Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity” was approved by the Viennese Human Ethics committee. All study participants gave written consent for using blood and stool samples and data obtained from food frequency questionnaires (FFQ) for the analyses presented here. The FFQ asked participants to report the frequency of consumption and portion size over a defined period (last three months). Additional to dietary habits, the FFQ included questions about lifestyle (i.e. smoking, alcohol consumption, physical activity), medical relevant influences (i.e. vitamin supplements and additives), BMI (body mass index) and age at enrollment. To ensure comparable data, patients were

interviewed for their history of gastrointestinal diseases, use of antibiotics, probiotics or prebiotics.

We studied fourteen obese (OC) with no established insulin resistance (aged 38 +/- 14 years; BMI 33,71 ± 3,97 kg/m^2) and twenty-four insulin-dependent type 2 diabetes (D) patients (aged 56 +/- 9 years; BMI 38,01 ± 5,81 kg/m^2) over a four month intervention period and compared to a lean control (LC) group (n = 19; aged 30 +/- 8 years; BMI 21,78 ± 2,35 kg/m^2). Intervention comprises GLP-1 Agonist for type 2 diabetics and nutritional counseling for both intervention groups. Blood and fecal samples were collected at three occasions; before treatment, during and after treatment, with a distance of 1 month between the first two time points and 3 months between the second and third time point. HbA_{1C} (glycol hemoglobin) blood level was determined at laboratory Health Center South in Vienna. The levels were between 7-8 mmol/mol hemoglobin in our type 2 diabetes group, a value that is according to American Diabetes Association protective against long-term consequences.

Fecal sample collection, processing, and analysis

Stool samples were stored at -70 C after collection. Bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (Qiagen, Germany) according to the manufacturer's protocol. Additional, samples were treated in FastPrep™ Lysing Matrix E tubes (MP Biomedicals, USA) twice for 45 sec in a bead-beater (Mini-Beadbeater 8 Bio-Spec Products, USA) with an intervening minute on ice. DNA concentration and quality was determined with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis.

The total bacterial diversity was measured by DGGE (denaturing gradient gel electrophoresis) using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' [22] and 518r 5'-ATT ACC GCG GCT GCT GG-3' [23] according to Remely et al. (2013) [11].

For further analysis in qPCR, we created clone libraries from dominant members of *Faecalibacterium prausnitzii* in two stool samples of healthy volunteers. Cloning was done following Remely et al. 2012 [11]. We quantified bacterial abundance with TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science, Australia) using 16S rDNA group specific primers (Table 1). *Butyryl-*

CoA:acetate CoA-transferase gene was amplified and thermal denatured with SYBR Green qPCR analysis. The amplicons were divided into three areas, as described by Louis & Flint (2009). These peaks are assigned to represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0 1C), *Roseburia/E. rectale spp.* (85.5–89.0 1C) and *F. prausnitzii* (89.5–92.5 1C).

The specificity of primer and probes was checked with the ProbeMatch function of the ribosomal database project 10 (http://rdp.cme.msu.edu/). The PCR reactions mixture and serial DNA dilution of typically strains were prepared according to Pirker et al. 2012 [24].

Sixteen samples were analyzed with Roche GS FLX + Titanium next generation sequencing (LGC sequencing GmbH, Germany). For alignment and further analyses we used the pyro pipeline of the ribosomal database project 10 (http://rdp.cme.mus.edu/) and qiime. We then performed significance tests and principal coordinate analysis in qiime.

Blood sample collection, processing, and analysis

Blood samples were collected in PAXgene Blood DNA Tubes (Qiagen, Germany) and stored at -20°C. For extraction of DNA, they were treated with the PAXgene Blood DNA Kit (Qiagen, Germany). For epigenetic analysis the DNA was converted with the EpiTect Bisulfite Kit (Qiagen, Germany) according to manufacturer's protocol. The DNA was measured and approved for purity with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis. Suitable primers for the selected 5 CpGs in the promoter regions of FFAR3 (Table 1-2) were designed using the PyroMark Assay Design Software 2.0 (Qiagen, Germany). The PCR was carried out in a total reaction volume of 25 µL, it contains 12,5 µL Pyromark 2X PCR master mix, 5 pmol (FFAR3) or 1 pmol (LINE-1) of each primer, 2,5 µL Coralload Concentrate 10X (Qiagen,, Germany), and 5 ng (FFAR3) or 10 ng (LINE-1) bisulfite converted DNA. PCR product quality was investigated with agarose gel-electrophoresis. Analysis of CpG methylation was performed with a PyroMAark Q24 MDx (Qiagen, Germany).

Statistical analysis

The normality of distribution was approved with the Kolmogorov-Smirnov test. The comparison of gut microbiota was performed using the non-parametric Mann-Whitney U test and for three unpaired groups the non-parametric Kruskal Wallis ANOVA. Differences in the percent of methylation of LINE-1 and FFAR3 were determined using the student's two tailed T-test, one-way analysis of variance (ANOVA) and with the Tukey post hoc correction test. For correlation analysis we used the two-tailed Pearson test. P values < 0.05 were determined as statistically significant. All data shown are mean ± sd.

Results

Analysis of the FFQ revealed differences in life style patterns and in the consumption of sugary products. At all three time points the total sweet consumption was the lowest in type 2 diabetics. In contrast obese patients showed the highest intake, 50 % of participants consumed sweets one to three times per week. No differences were observed in the consumption of grain, vegetables, fruits, meat, dietary products and fish. In life style patterns type 2 diabetics showed the lowest physical activity (64.0 % T1, 52.0 % T3 never do sports), followed by obese participants (37.5 % T1, 12.5% T3 never do sports), whereas 44.0 % of lean controls exercised 1-3 times per week. 19 out of 24 type 2 diabetes patients showed an average weight reduction of 3.1 ± 2.1 kg, obese patients lost about 4.9 ± 3.7 kg (Table 3).

DGGE fingerprinting analysis of total bacteria revealed significant differences between type 2 diabetics and lean controls at time point 2 ($p = 0.03$) and time point 3 ($p = 0.01$). Lean controls showed the highest diversity, followed by obese participants and by type 2 diabetics showing the lowest diversity. Although the first two principal components (PCs) explained 15.6 % and 12.0 % of variance (data not shown), principal component analysis (PCA) showed grouping of band patterns according to the three groups of study participants.

Lean controls had a higher Shannon's diversity index (6.48 ± 0.47) of 454 sequencing results and a higher number of sequences (683.3 ± 59.6) compared to

obese controls (4.51 ± 2.60; 571.5 ± 48.2) and type 2 diabetics (5.19 ± 2.62; 645.5 ± 22.6). A clustering of the participants in the PCA revealed grouping of type 2 diabetes subjects. The first three calculated PCs of 454 sequencing results explained 44.6 %, 17.2 % and 8.3 % of the variance in the data. *Firmicutes* and *Verrumicrobia* contributed most to the separation along PC1 and *Proteobacteria, Actinobactera* and *Bacteroides* contributed to the clustering along PC2.

We focused on groups of *Firmicutes*, known for their high number of SCFA-producing bacteria. *Clostridium Cluster IV* and *Clostridium Cluster XIVa* abundances were not significantly different between the groups or intervention period but tended to decrease with intervention in type 2 diabetics. High throughput sequencing confirmed these findings as type 2 diabetics were enriched in *Firmicutes* especially *Clostridiales* and *Bacilli*, in particular *Lactobacillales* as described before. Quantitative PCR analyses of *Faecalibacterium prausnitzii* go in line with high throughput sequencing analysis. Type 2 diabetics showed a significantly lower abundance at all three time points with an improvement at the third time point. The lean control group showed the highest abundance. These findings were significant at all three time points between lean control group, type 2 diabetics and obese patients (T1 $p < 0{,}001$; T2 $p < 0{,}001$; T3 $p < 0{,}001$). In addition, obese participants showed a higher abundance compared to type 2 diabetics. No significant changes within a group were observed over the study period (Figure 1).

The b*utyryl-CoA: acetate CoA-transferase* gene was used as a marker for butyrate production as the direct analysis of SCFAs in feces is difficult by rapid binding and degradation of SCFAs in the gut. *Butyryl-CoA: acetate CoA-transferase* gene tended to increase in type 2 diabetics over the intervention period from an already lower level compared to the lean control group. Dominant butyrate producers differ in their DNA G+C content which was shown by the analysis of the melt curves obtained from PCR amplicons of the *butyryl-CoA:acetate CoA-transferase* gene. Detailed analysis allowed to differentiate between *Faecalibacterium prausnitzii, Eubacterium hallii* and *Roseburia.* We observed no significant differences in between our study population, although *Faecalibacterium prausnitzii* showed a trend to increase in type 2 diabetics.

Analysis of 5 CpGs in the promoter region of FFAR3 gene showed a significant higher methylation in the lean control group compared to type 2 diabetics at all three time points (T1 p =0.003; T2 p= 0.011; T3 p= 0.0001) and to obese participants at the first time point (p= 0.031; Table 4, Figure 3). In type 2 diabetics, we observed no significant changes in the methylation during the intervention period (T1: 31.46 ± 5.74 %; T2: 32.53 ±7.17 %; T3: 31.25 ± 6.85 %). In comparison, obese patients showed an increase of methylation with nutritional counseling (T1: 30.69 ± 6.46 %; T2: 32.34 ± 7.13 %; T3: 34.51 ± 2.93 %), although they do not reach the methylation status of the lean control group (39.23 ± 0.9 %). The mean methylation of LINE-1 indicates no significant differences between the groups or the time points but obese individuals had the lowest methylation. We found a significant negative correlation between the BMI and methylation of FFAR3 (R= -0,474; p=0,001; Figure 4), whereas LINE-1 showed a tendency of a positive correlation. We observed no correlation between methylation of FFAR3 or Line-1 and age.

Discussion

We analyzed subgroups of the phyla *Firmicutes*, with a focus on the diversity, *Faecalibacterium prausnitzii* as a marker of inflammation, and the *butyryl-CoA: acetate CoA-transferase gene* as a proxy for SCFA-producers. Epigenetic methylation analysis included the CpGs in the promoter region of FFAR3 and LINE-1 as an indicator of the overall methylation. The results are discussed for the influence of SCFAs on epigenetic control of gene expression, especially of FFARs.

DGGE fingerprinting showed a lower diversity in type 2 diabetics compared to obese patients and lean controls. These findings were significant between lean controls and type 2 diabetics. However, we did not observe significant differences in Shannon's diversity index of our selected subgroup in 454 sequencing results. Diversity as an indicator of health and disease is still under discussion as other

research groups do not observe significant differences between type 2 diabetics and non-diabetics [10] or even higher diversity and richness in diabetics with higher BMI (BMI > 31) compared to lean diabetics. In agreement with our observed PCs, PC1 *Firmicutes* and *Verrumicrobia*, PC2 *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*, Larsen et al., 2010 also shows a separation between the groups attributed to *Bacteroides*, *Firmicutes* and *Verrumicrobia* (45 %) and in the second direction the combination of *Proteobacteria* and *Actinobacteria* (28 %) [1].

The analysis of one of the highest butyrate producing bacteria showed a lower abundance of *Faecalibacterium prausnitzii* in type 2 diabetics followed by obese patients (figure 1). However, our analyses of butyrate producing bacteria indicate no significant differences between type 2 diabetics and obese patients or the lean control group. In addition, analysis of *Clostridium cluster IV* and *Clostridium cluster XIVa* are in line with analysis of *butyryl-CoA: acetate CoA-transferase* gene. Thus we might have changes of species or even genus without a detectable outcome in the family itself. *Faecalibacterium prausnitzii* is identified as a dominant and conserved species in healthy human gut microbiota and is described to play a role during low-grade inflammation pathology [25]. A relationship between *Faecalibacterium prausnitzii* and inflammatory markers was observed in obese and diabetic patients and remained after adjustment for BMI. The proportions of *Faecalibacterium prausnitzii* were lower in type 2 diabetic subjects indicating a higher incidence of low-grade inflammation [7].

Melting curve analysis of *butyryl-CoA: acetate CoA transferase* gene and qPCR analysis of *Faecalibacterium prausnitzii* showed no clear correlation. One cause might be the described two pathways of butyrate production. The most important pathway is the *butyryl-CoA: acetate CoA-transferase*, using acetate as co-substrate. A minority of bacteria is able to use the *butyrate kinase* or *phosphotransbutyrylase* as an alternatively final step [26]. The used approach only detects bacteria which use the *butyryl-CoA: acetate CoA-transferase* pathway. Additional, butyrate producers do not form a homogenous phylogenetic group as butyrate producers and non-butyrate producers are in the same phylogenetic

cluster and although they are carrier of the gen there is no evidence for expression. Turnbaugh et al. (2006) estimated the role of gut microbiota profiles in the production of SCFAs at two levels: the capacity to produce SCFAs and the type of SCFAs produced [3]. Dependent on the initial SCFAs concentration status, there is evidence that inter-individual differences in polysaccharide fermentation are able to affect intervention with complex carbohydrates. Significant correlations are observed with gender, dietary factors, especially resistant starch and BMI. Dietary components and prebiotics affect also the growth of butyrate producers, for example the consumption of inulin stimulates the growth of *F. prausnitzii* and *E. rectale* [27]. Hippe et al. (2011) showed the important influence of diet or age on the butyrate production in the GI tract. The microbiota of the elderly is characterized by decreased butyrate production capacity, reflecting increased risk of degenerative diseases [26]. The absorption of SCFAs together with their conversion to fat in the liver and the regulation of host genes that promote deposition of fat in lipocytes, cause an increase in adipose tissue [28]. Butyrate in particular has an important impact on promoting body energy expenditure via a modulation of mitochondria function. The results are consistent with the role of SCFAs as an additional energy source promoting adiposity. Research of human SCFA content in feces indicated a higher proportion of SCFAs in overweight and obese participants compared to lean controls although the ratio of *Firmicutes /Bacteroidetes* is lower in overweight and obese [4]. A higher concentration of butyrate and acetate was measured in the caeca of obese mice, while propionate was not significantly different between the lean or obese group [3]. A higher ratio of *Firmicutes /Bacteroidetes* increases the energy harvest and the content of SCFAs and as such the lipogenesis and accumulation in adipocytes [29]. In contradiction, high-fat diets supplemented with butyrate prevent and also reverse insulin resistance in obese mice [30,31]. However, insufficient data currently prevent an estimation of optimal levels of fecal SCFAs for the promotion of colonic health. These observations support the hypothesized engagement of SCFAs on host inflammatory signaling processes via epigenetic control of gene expression [13]. However, the precise role of SCFAs in signaling pathways is still unclear [30].

We observed a higher methylation in the promoter of the FFAR3 gene in the lean control group. Both intervention groups had a lower methylation with the lowest in type 2 diabetics (table 4, figure 3). These differences of methylation between the groups result in a negative correlation between methylation and BMI (figure 4). Type 2 diabetics showed no significant differences over the intervention period, whereas obese patients showed an increase of methylation after nutritional counseling. Thus a lower expression of FFAR3 in the lean control group can be expected according to Canani et al. (2011). In mice an increase of leptin production caused by an over expression of FFAR3 after oral doses of propionate was observed [32]. A knockdown of FFAR3 by siRNA inhibits the ability of propionate to induce leptin expression mainly in adipocytes [33]. Leptin itself takes influence on the hunger-satiety-cycle by suppression of the appetite and contribution to the fat metabolism. Although obese patients harbor many fat cells with according higher leptin levels, leptin is no longer able to contribute to satiety. It was discussed that high leptin levels over time make the satiety cells insensitive to leptin, whereas a supplementation with SCFAs induces sensitivity or the production of leptin. Only few obese patients are effected from leptin deficiency, so therapies using recombinant leptin have not been successful [34] and might affect therapies with SCFAs. As we observed a negative correlation of FFAR3 methylation and BMI, a lower methylation of the promoter region in obese and type 2 diabetics may indicate the proposed interaction of FFAR3 expression and higher leptin levels. On the other hand FFARs are discussed to stimulate the secretion of satiety hormone PYY (Peptide YY), which inhibits gut motility, slows intestinal transit time and enhances nutrient absorption. These results are underlined by a study between Gpr41-deficient mice and conventional raised mice. A deficiency of the receptor is associated with a lower expression of PYY and as a consequence with a higher energy harvest from diet [35]. However, current researches indicate a FFAR3 independent influence of butyrate and propionate on body weight and food intake. In addition FFAR3 is mentioned to play a minor role in butyrate stimulation of GLP-1 secretion. SCFAs increase the reduced GLP-1 secretion of obese and type 2 diabetics and thus promote insulin secretion [14]. Therefore, a different microbial composition may contribute to

reduced GLP-1 release. The main production of GLP-1 is located in the mucosal L cells, in the ileum and colon but also in pancreatic α-cells and in neurons [2]. Prebiotic treatment induced GLP-1 secretion in ob/ob mice, whereas a deletion of GLP-1 prevented the beneficial effects of prebiotics on weight gain, glucose metabolism and inflammatory pathway activation [35]. However, there is no evidence, that an intervention with GLP-1 Agonists for adjustment of blood glucose levels influences epigenetic methylation.

Changes in global methylation can affect gene expression, genomic stability, and chromosomal structure. In the case of LINE-1 methylation of our type 2 diabetics and lean controls showed the same methylation status, whereas obese patients had the lowest methylation. Baccarelli et al. (2010) associated blood DNA hypomethylation with higher risk of cardiovascular disease caused by older age, smoking, exposure to air pollution, folate deficiency, hyperhomocysteinanemia and end-stage renal disease [36]. Turcot et al. (2012) revealed lower LINE-1 methylation levels in visceral adipose tissue especially in obese subjects without established insulin resistance with an association to higher plasma glucose level, lower HDL-cholesterol level and greater risk to be affected by metabolic syndrome [20]. The incidence, that our obese study population has a lower global methylation than our type 2 diabetics might be related to the well-adjusted HbA_{1c} value as a result of GLP-1 Agonist, whereas a higher blood glucose level in obese participants could not be excluded.

Conclusion

Our results suggest that a different composition of gut microbiota in obesity and type 2 diabetes effect the epigenetic regulation of inflammatory molecules, possibly also underlying a progression of the metabolic disease. Interactions between microbiota and epigenetic regulation may influence satiety and hunger cycle. Especially butyrate may help to develop strategies for regenerative medicine by promoting epigenetic remodeling and the expression of associated

genes. Our results indicate the necessity to concentrate on gene-specific methylation to better understand the involvement of epigenetic regulation in the development of obesity and type 2 diabetes.

Acknowledgment

The authors thank all the staff of the diabetes clinic at the Health Centre South and the head of the laboratory at the health center, Dr. Ernst Forster. Special thanks to all our participants without whom this study would not have been possible.

References

1. Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, et al. (2010) Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS One 5: e9085.
2. Vrieze A, Holleman F, Zoetendal EG, de Vos WM, Hoekstra JB, et al. (2010) The environment within: how gut microbiota may influence metabolism and body composition. Diabetologia 53: 606-613.
3. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, et al. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444: 1027-1031.
4. Schwiertz A, Taras D, Schafer K, Beijer S, Bos NA, et al. (2010) Microbiota and SCFA in lean and overweight healthy subjects. Obesity (Silver Spring) 18: 190-195.
5. Duncan SH, Lobley GE, Holtrop G, Ince J, Johnstone AM, et al. (2008) Human colonic microbiota associated with diet, obesity and weight loss. Int J Obes (Lond) 32: 1720-1724.
6. Turnbaugh PJ, Gordon JI (2009) The core gut microbiome, energy balance and obesity. J Physiol 587: 4153-4158.
7. Furet JP, Kong LC, Tap J, Poitou C, Basdevant A, et al. (2010) Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes 59: 3049-3057.
8. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, et al. (2008) Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A 105: 16731-16736.

9. Gerritsen J, Smidt H, Rijkers GT, de Vos WM (2011) Intestinal microbiota in human health and disease: the impact of probiotics. Genes Nutr 6: 209-240.
10. Wu X, Ma C, Han L, Nawaz M, Gao F, et al. (2010) Molecular characterisation of the faecal microbiota in patients with type II diabetes. Curr Microbiol 61: 69-78.
11. Remely M, Dworzak S, Hippe B, Zwielehner J, Aumüller E, et al. (2013) Abundance and diversity of microbiota in type 2 diabetes and obesity. diabetes Metab Res Rev 4.
12. Louis P, Flint HJ (2007) Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. Appl Environ Microbiol 73: 2009-2012.
13. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59-65.
14. Lin HV, Frassetto A, Kowalik EJ, Jr., Nawrocki AR, Lu MM, et al. (2012) Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLoS One 7: e35240.
15. Layden BT, Angueira AR, Brodsky M, Durai V, Lowe WL, Jr. (2012) Short chain fatty acids and their receptors: new metabolic targets. Transl Res.
16. Halpern KB, Veprik A, Rubins N, Naaman O, Walker MD (2012) PR41 Gene Expression Is Mediated by Internal Ribosome Entry Site (IRES)-dependent Translation of Bicistronic mRNA Encoding GPR40 and GPR41 Proteins. Journal of Biological Chemistry 287 20154-20174.
17. Wilson ME (2005) Diarrhea in nontravelers: risk and etiology. Clin Infect Dis 41 Suppl 8: S541-546.
18. Duranton B, Keith G, Goss, Bergmann C, Schleiffer R, et al. (1998) Concomitant changes in polyamine pools and DNA methylation during growth inhibition of human colonic cancer cells. Exp Cell Res 243: 319-325.
19. Canani RB, Costanzo MD, Leone L, Bedogni G, Brambilla P, et al. (2011) Epigenetic mechanisms elicited by nutrition in early life. Nutr Res Rev 24: 198-205.
20. Turcot V, Tchernof A, Deshaies Y, Perusse L, Belisle A, et al. (2012) LINE-1 methylation in visceral adipose tissue of severely obese individuals is associated with metabolic syndrome status and related phenotypes. Clin Epigenetics 4: 10.
21. Nauck M, Frid A, Hermansen K, Shah NS, Tankova T, et al. (2009) Efficacy and safety comparison of liraglutide, glimepiride, and placebo, all in combination with metformin, in type 2 diabetes: the LEAD (liraglutide effect and action in diabetes)-2 study. Diabetes Care 32: 84-90.
22. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695-700.

23. Neefs JM, Van de Peer Y, De Rijk P, Goris A, De Wachter R (1991) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res 19 Suppl: 1987-2015.
24. Pirker A, Stockenhuber A, Remely M, Harrant A, Hippe B, et al. (2012) Effects of antibiotic therapy on the gastrointestinal microbiota and the influence of Lactobacillus casei. Food and Agricultural Immunology.
25. Tremaroli V, Backhed F (2012) Functional interactions between the gut microbiota and host metabolism. Nature 489: 242-249.
26. Hippe B, Zwielehner J, Liszt K, Lassl C, Unger F, et al. (2011) Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age. FEMS Microbiol Lett 316: 130-135.
27. Louis P, Flint HJ (2009) Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett 294: 1-8.
28. Tsukumo DM, Carvalho BM, Carvalho-Filho MA, Saad MJ (2009) Translational research into gut microbiota: new horizons in obesity treatment. Arq Bras Endocrinol Metabol 53: 139-144.
29. Diamant M, Blaak EE, de Vos WM (2011) Do nutrient-gut-microbiota interactions play a role in human obesity, insulin resistance and type 2 diabetes? Obes Rev 12: 272-281.
30. Kootte RS, Vrieze A, Holleman F, Dallinga-Thie GM, Zoetendal EG, et al. (2012) The therapeutic potential of manipulating gut microbiota in obesity and type 2 diabetes mellitus. Diabetes Obes Metab 14: 112-120.
31. Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, et al. (2009) Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes 58: 1509-1517.
32. Ichimura A, Hirasawa A, Hara T, Tsujimoto G (2009) Free fatty acid receptors act as nutrient sensors to regulate energy homeostasis. Prostaglandins Other Lipid Mediat 89: 82-88.
33. Wu J, Zhou Z, Hu Y, Dong S (2012) Butyrate-induced GPR41 activation inhibits histone acetylation and cell growth. J Genet Genomics 39: 375-384.
34. Schloegl H, Percik R, Horstmann A, Villringer A, Stumvoll M (2010) Peptide hormones regulating appetite--focus on neuroimaging studies in humans. Diabetes Metab Res Rev 27: 104-112.
35. Musso G, Gambino R, Cassader M (2010) Obesity, diabetes, and gut microbiota: the hygiene hypothesis expanded? Diabetes Care 33: 2277-2284.
36. Baccarelli A, Tarantini L, Wright RO, Bollati V, Litonjua AA, et al. (2010) Repetitive element DNA methylation and circulating endothelial and inflammation markers in the VA normative aging study. Epigenetics 5.
37. Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, et al. (2004) Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Appl Environ Microbiol 70: 167-173.
38. Matsuki T, Watanabe K, Fujimoto J, Takada T, Tanaka R (2004) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of

predominant bacteria in human feces. Appl Environ Microbiol 70: 7220-7228.
39. Walter J, Hertel C, Tannock GW, Lis CM, Munro K, et al. (2001) Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67: 2578-2585.
40. Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, et al. (2009) Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. Br J Nutr 101: 541-550.
41. Di JZ, Han XD, Gu WY, Wang Y, Zheng Q, et al. (2011) Association of hypomethylation of LINE-1 repetitive element in blood leukocyte DNA with an increased risk of hepatocellular carcinoma. J Zhejiang Univ Sci B 12: 805-811.

Tables

Table 1: Primers and TaqMan®-probes targeting 16S rDNA coding regions of bacteria, BCoAT-gene and for CpG-Methylation analysis

Target	**Primer /Probe**	**Sequence (5' - 3')**	**Conc. [pmol/µL]**	**Reference**
Clostridium cluster IV (Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	4	[37]
	Rev primer	CTT CCT CCG TTT TGT CAA	4	
	Probe	(6-FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	2	
Clostridium cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	5	[38]
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A	5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)	1.5	

Lactic Acid Bacteria (LAB)	Fwd primer	AGC AGT SGG GAA TCT TCC A	4	[39]
	Rev primer	ATT YCA CCG CTA CAC ATG	4	
***BCoAT* gene**	Fwd primer	GCI GAI CAT TTC ACI TGG AAY WSI TGG CAY ATG	27	[12]
	Rev primer	CCT GCC TTT GCA ATR TCI ACR AAN GC	27	
Faecalibacterium prausnitzii	Fwd primer	GGA GGA AGA AGG TCT TCG G	0.25	[40]
	Rev primer	AAT TCC GCC TAC CTC TGC ACT	0.25	
FFAR3	Fwd primer	GTG ATA GGG AAA ATA GTA GTT GGT A	0.5	
	Rev primer	*Biotin*-CCC TAC AAA ACA AAA CTC ATC ATA AT		
	Seq primer	GGG AAA ATA GTA GTT GGT AT	0.4	
LINE-1	Fwd primer	TTT TGA GTT AGG TGT GGG ATA TA	1	[41]
	Rev primer	*Biotin*-AAA ATC AAA AAA TTC CCT TTC		
	Seq primer	AGT TAG GTG TGG GAT ATA GT	0.4	

Table 2: Sequence to analyze: FFAR3 (geneID (NCBI) 2865) and LINE-1

Assay	Sequence 5´-3´	Number of CpGs	Size
LINE1	TTC GTG GTG CGT CGT TT	3	17
FFAR3	CCG CTG CCT GCT CAC GAC CAC ACG CCA GGC TTC CGT CAA ACC ACT CAA CAT GTA TTA G	5	58

Table 3: Characterization of study participants

Group	Age ± SD (years)	Time point	BMI ± SD (kg/m^2)
D	58,36 ± 9,35	1	38,44 ± 5,14
		3	37,63 ± 5,58
OC	39,63 ± 15,18	1	33,71 ± 4,24
		3	32,66 ± 3,75
LC	25,67 ± 3,09	1	21,19 ± 1,93
		3	20,63 ± 1,87

Tabble 4: Methylation of 5 CpGs in each group

GroupT1	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	Σ: CpGs
D	37,29 ± 12,10	20,47 ± 5,22	24,48 ± 5,71	41,27 ± 7,85	33,82 ± 7,95	31,46 ± 5,74
OC	37,80 ± 14,32	18,78 ± 5,09	24,89 ± 3,78	37,13 ± 9,16	34,84 ± 6,31	30,69 ± 6.46
LC	42,86 ± 13,25	23,66 ± 3,95	27,21 ± 3,61	51,70 ± 6,46	46,13 ± 11,13	38,19 ± 6,19

Table 4: Methylation of 5 CpGs in the promoter region of FFAR3. At the first time-point (T1) lean controls (LC) showed highest methylation over all 5 CpGs, the lowest methylation has been observed in type 2 diabetics (D) and obese patients.

Figures

Figure 1: 16S rDNA qPCR quantification with primer pair for *Faecalibacterium prausnitzii.* A significant ($p < 0.01$) lower abundance of *Faecalibacterium prausnitzii* was shown in type 2 diabetics (D) compared to obese patients (OC) and lean controls (LC) at all three time points

T1, samples taken before intervention; T2, samples taken after 1 month of intervention; T3, samples taken after 4 month of intervention

Figure 2: Bar Charts of 454 sequencing results reflect qPCR analysis. The abundance of *Firmicutes* especially *Clostridiales* and *Bacilli* in type 2 diabetics are increasing (D) between the time points. *Faecalibacterium prausnitzii* is significant higher abundant in lean controls (LC). In addition obese patients (OC) have a higher abundance compared to type 2 diabetics.

T1, samples taken before intervention; T3, samples taken after 4 month of intervention

Bacteroidales

Clostridiales, Faeclibacterium

Clostridiales, Blautia

Erysipelotrichales

Bacilli, Streptococcus

Figure 3: Mean methylation of FFAR3 promoter region between the three groups at the first time point. Lean controls (LC) showed highest methylation compared to both intervention groups. These differences were significant between lean controls and type 2 diabetics (D, $p = 0.003$) and between obese participants (OC) and lean ($p= 0.031$).

Figure 4: Correlation between BMI and FFAR3methylation%. Significant correlation between a higher BMI of all study participants and lower FFAR3 methylation% at time point 1 (T1), before intervention ($R = -0,474$; $p = 0,001$).

13 Appendix

13.1 DNA extraction of human whole blood

For the isolation of genomic DNA from 8,5ml human whole blood, PAXgene® Blood DNA Kit and PAXgene Blood DNA Tubes were used. All reactions were conducted according to manufactures protocol (Qiagen, Hilden, Germany).

All centrifugation steps necessary for the purification should be carried out at room temperature (15-25°C) in a swing-out rotor. Before purification can be started some things have to be prepared:

- Thaw frozen PAXgene DNA Tubes in a rack at room temperature for 2 hours or at 37°C in a water bath for 15 minutes. PAXgene Blood DNA Tubes should be inverted about 10 times before starting the procedure
- Heating block or water bath should be heated to 65°C
- Add 1,4ml Buffer BG4 (resuspension buffer) to lyophilized PreAnalytiX® Protease. Solved Protease should be stored at 2-8°C or in aliquots at -20°C.
- Mix 5ml Buffer BG3 (digestion buffer) and 50µl reconstituted PreAnalytiX® Protease, for each sample. This mixture should be prepared immediately before the start of the procedure.

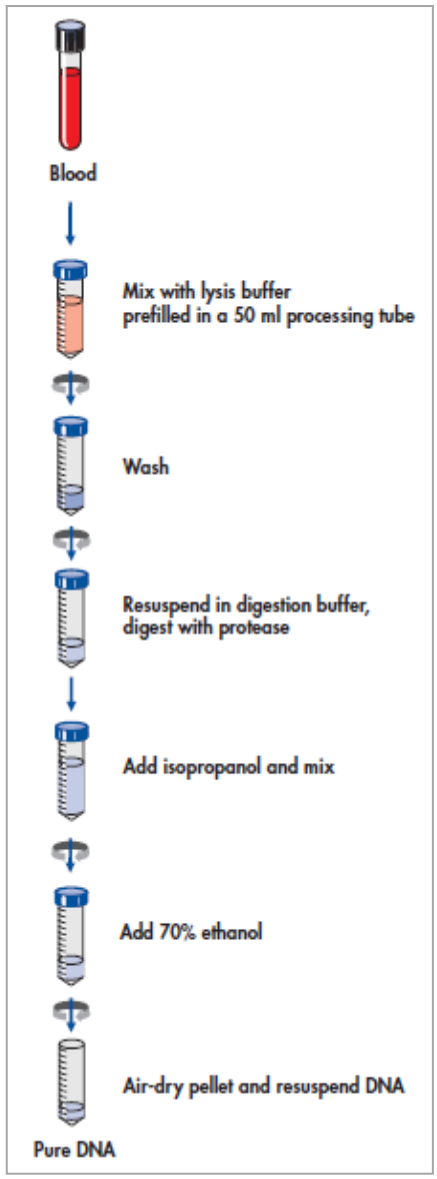

Figure 12 Scheme of blood extraction (Qiagen, Hilden, Germany)

Procedure:

1. Transfer all the blood from one PAXgene Blood DNA Tube into a Processing Tube containing 25 ml Buffer BG1. Close the tube. Mix by inverting the tube 5 times. Be sure the sample is homogenized, otherwise invert again 5 times.
2. Centrifuge for 5 minutes at 2500 x g in a swing-out rotor.
3. Carefully discard the supernatant and place the tube in a rack
4. Add 5 ml Buffer BG2, close the tube and wash the pellet by vortexing for 5 seconds.
5. Centrifuge for 3 minutes at 2500 x g in a swing-out rotor.
6. Carefully discard the supernatant and place the tube back in the rack.
7. Add 5ml Buffer BG3/PreAnalytiX® Protease, close the tube and vortex for 20 seconds at high speed until the pellet is dissolved completely
8. Place the tube in a heating block or water bath and incubate at 65°C for 10 minutes. Change of sample colour can be observed from light red to light green, indicating that protein digestion has occurred.
9. Vortex again for 5 seconds at high speed
10. Add 5 ml isopropanol (100%) and mix by inverting the tube at least 20 times until the white DNA strands clumb visibly together.
11. Centrifuge for 3 minutes at 2500 x g.
12. Disacard the supernatant and leave the tube inverted on a clean piece of absorbant paper for 1 minute.
13. Add 5 ml 70% (v/v) ethanol and vortex for 1 second at high speed.
14. Centrifuge for 3 minutes at 2500 x g.
15. Discard the supernatant and leave the tube inverted on a piece of absorbent paper for at least 5 minutes.
16. To remove ethanol from the rim, carefully dab the tube onto absorbent paper and leave it inverted for 5 minutes to dry the DNA pellet.
17. Add 1 ml Buffer BG4 and dissolve the DNA by incubating for 1 h at 65°C in a heating block or water bath. Incubate at room temperature overnight to guarantee the samples redissolve completely.

13.2 Complete bisulfite conversion and cleanup of DNA for methylation analysis

For bisulfite conversion EpiTect® Bisulfite kit was used (Qiagen, Hilden, Germany). With this kit DNA amounts of 1ng-2µg in a volume of up to 20µl can be processed. All reactions were carried out according to manufactures protocol (Qiagen, Hilden, Germany).

The incubation of the target DNA with sodium bisulfite results in the conversion of umethylated cytosine residues into uracil, while methylated cytosines remain unchanged. The complete conversion of unmethylated cytosines is the most critical step. Consequently very harsh conditions (low pH, high temperature and high bisulfite salt concentration) are chosen. But this often results in DNA fragmentation and loss of DNA during purification. Purification is necessary to remove bisulfite salts and chemicals used, which would inhibit sequencing reactions. DNA Protect Buffer will prevent DNA fragmentation during the conversion.

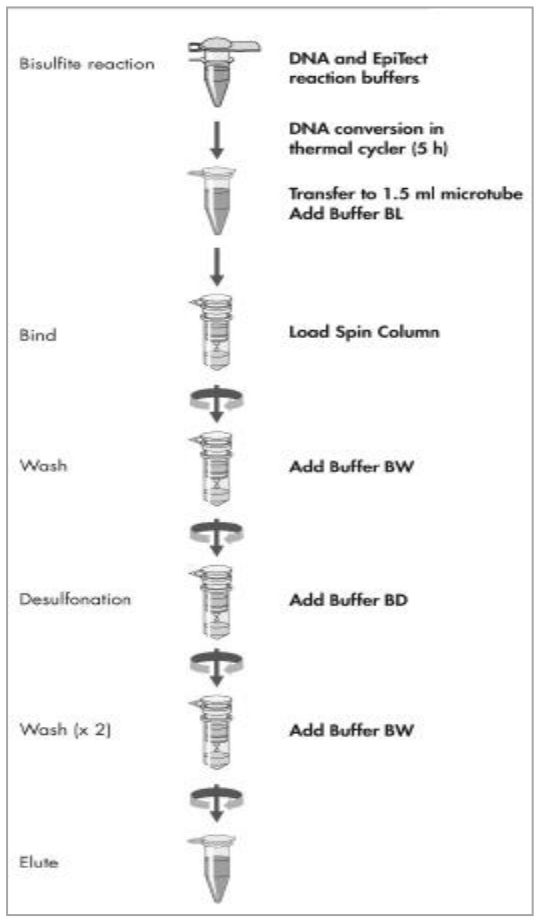

Figure 13 Scheme of bisulfite conversion (Qiagen, Hilden, Germany)

Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions. If converting fewer than 8 DNA samples, this Mix can be stored at -20°C, up to four weeks. Notice that DNA Protect Buffer should turn from green to blue after addition to DNA-Bisulfite Mix. Also well mixing and correct pH are necessary for the bisulfite conversion reaction. All centrifugation steps should be carried out at room temperature (15-25°C).

Before starting with the procedure some things have to be done:

- Add 30 ml ethanol (96-100%) to Buffer BW and store at room temperature (15-25°C). before starting the procedure invert the bottle several times.
- Add 27 ml ethanol (96-100%) to Buffer BD and store at 2-8°C. Invert the bottle before usage and close immediately after use.
- Equilibrate samples and buffers to room temperature

Procedure:

Bisulfite DNA conversion

1. Thaw DNA to be used in the bisulfite reactions. dissolve the Bisulfite Mix by adding 800µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 minutes. If the Bisulfite Mix will not dissolve properly, heat the Bisulfite Mix-RNase-free water solution to 60°C and vortex again. Do not place dissolved Bisulfite Mix on ice.
2. Prepare the bisulfite reactions in 200µl PCR tubes. Add each component in the order listed. The combined volume of DNA solution and RNase-free water must total 20µl.

Table 21 Bislufite conversion mix

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

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

3. Close the PCR tubes and mix the bisulfite reactions. Store the tubes at romm temperature (15-25°C). DNA Protect Buffer should turn from green to blue after addition to DNA-Bisulfite Mix.

4. DNA bisulfite conversion is performed in a thermal cycler. The complete cycle should about 5 hours.

Table 22 Bisulfite conversion PCR condition

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

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

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

Cleanup of bisulfite converted DNA

6. When the bisulfite conversion is complete, shortly centrifuge the PCR tubes containing the bisulfite reactions. Then transfer the complete bisulfite reactions to clean 1,5ml microcentrifuge tubes.
7. Add 560µl freshly prepared Buffer BL containing 10µl/ml carrier RNA to each sample. Vortex the solution and shortly centrifuge
8. Place the necessary number of EpiTect spin columns and collection tubes in a rack. The entire mixture from each tube is transferred into a corresponding EpiTect spin column.
9. Centrifuge the spin columns at maximum speed for 1 minute. Discard the flow through and place the spin columns back into the collection tubes.

10. Add 500µl Buffer BW to each spin column. Centrifuge at maximum speed for 1 minute. Discard the flow-through and place the spin columns back into the collection tubes.
11. Add 500µl Buffer BD to each spin column. Close the lids and incubate for 15 minutes at room temperature (15-25°C). Do not transfer precipitates from the Buffer BD to the spin column.
12. Centrifuge the spin columns at maximum speed for 1 minute. Discard the flow-through and place the spin columns back into the collection tubes.
13. Add 500µl Buffer BW to each spin column and centrifuge at maximum speed for 1 minute. Discard the flow-through and place the spin columns back into the collection tubes.
14. Repeat step 13 once
15. Place the spin columns into new 2 ml collection tubes and centrifuge the spin columns at maximum speed for 1 minute to remove any liquid.
16. To remove/dry any remaining liquid place the spin columns with open lids into clean 1,5 ml microcentrifuge tubes and incubate the spin columns for 5 minutes at 56°C in a heating block.
17. Place the spin columns into clean 1,5 ml microcentrifuge tubes. Then dispense 20µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 1 minute at 15,000 x g.
18. If you want to increase the yield of DNA in the eluate, dispense an additional 20µl Buffer EB to the center of each membrane and centrifuge for 1 minute at maximum speed.
19. Store the DNA at -20°C.

13.3 PCR amplification of template DNA optimized for Pyrosequencing® analysis

For the PCR amplification of template DNA PyroMark PCR kit and the according protocol was used (Qiagen, Hilden, Germany).

Before starting its important to check several points. One primer must be biotinylated at its 5´end in order to prepare a single stranded PCR which is necessary for Pyrosequencing. The primer design should be considered by using PyroMark Assay Design Software 2.0. Even though the optimal PCR amplicon length for Pyrosequencing should between 80 and 200bp. HotStar Taq DNA Polymerase requires an activation of 15 minutes at 95°C. All reaction mixtures should be performed in an area separated from that used for DNA preparation or PCR product analysis. Even though, disposable tips containing hydrophobic filters are recommended to minimize cross-contamination.

Procedure:

1. Thaw PyroMark PCR Master Mix, CoralLoad Concentrate and primer solutions at room temperature or on ice. Make sure that all solutions are mixed proberly to avoid localized concentrations of salt.
2. Set up the reaction according to the following table. It is not necessary to keep reaction vessels on ice, because HotStar Taq DNA Polymerase is inactive at room temperature

Table 23 PCR-master mix

Component	Volume/reaction	Final concentration
Reaction mix **PyroMark PCR Master Mix, 2x**	12,5µl	Contains HotStar Taq DNA Polymerase, 1x PyroMark PCR Buffer, and dNTPs
CoralLoad Concentrate, 10x	2,5µl	1x
Primer A	Variable	0,2µM

Primer B	Variable	0,2µM
RNase-free water	Variable	-
Template DNA **Template DNA, added at step 4**	Variable	≤500ng/reaction or 10-20 ng bisulfite converted DNA
Total volume	**25µl**	

3. Gently pipet the master mix up and down to guarantee well mixing and dispense appropriate volumes into PCR tubes.
4. Add template DNA (≤500ng/reaction) to the individual PCR tubes. 10ng human genomic DNA or 10-20 ng bisulfite converted DNA are recommended.
5. When a thermal cycler with a heated lid is used, do not use mineral oil. Proceed with step 6. Otherwise , overlay with about 100µl mineral oil
6. Program the thermal cycler according to manufacturer's instruction.

Table 24 PCR conditions

			Additional comments
Initial PCR Activation step	15 min	95°C	HotStar DNA Polymerase is activated by this heating step
3-step cycling Denaturation	30 s	94°C	
Annealing*	30 s	56°C 60°C	For bisulfite converted DNA For genomic DNA
Extension	30 s	72°C	
Nuber of cycles	45		
Final extension	10 min	72°C	

*An annealing temperature that gives the highest specifity for the desired PCR product should be used.

7. Place the PCR tubes in the thermal cycler and start the cycling program. After amplification samples can be stored overnight at 2-8°C or at -20°C for longer storage.
8. Use 5-20µl of a 25µl PCR for subsequent Pyrosequencing analysis.

PCR products were checked by 2% agarose gel electrophoresis. The PCR products can be directly loaded onto an agarose gel without addition of loading dye. CoralLoad Concentrate contains a gel loading reagent and gel tracking dyes.

13.4 Reagents

PAXgene® Blood DNA Kit (25): PreAnalytiX™, A Qiagen/ BD Conpany

- Processing Tubes containing 25ml Buffer BG1 (Qiagen, Hilden, Germany)
- Buffer BG2 (wash buffer) (Qiagen, Hilden, Germany)
- Buffer BG3 (digestion buffer) (Qiagen, Hilden, Germany)
- Buffer BG4 (resuspension buffer) (Qiagen, Hilden, Germany)
- PreAnalytiX® Protease (Qiagen, Hilden, Germany)
- 100% isopropanol
- 70% ethanol

EpiTect Bisulfite Kit (48): (Qiagen, Hilden, Germany)

- Bisulfite Mix (Qiagen, Hilden, Germany)
- DNA Protect Buffer (Qiagen, Hilden, Germany)
- RNase-free water (Qiagen, Hilden, Germany)
- Buffer BL (Qiagen, Hilden, Germany)
- Buffer BW (Qiagen, Hilden, Germany)
- Buffer BD (Qiagen, Hilden, Germany)
- Buffer EB (Qiagen, Hilden, Germany)
- Ethanol 96%

PCR: (Qiagen, Hilden, Germany)

- PyroMark PCR Master Mix, 2x (Qiagen, Hilden, Germany)
- CoralLoad® Concetrate, 10x (Qiagen, Hilden, Germany)
- RNase-Free Water (Qiagen, Hilden, Germany)

Pyromark: (Qiagen, Hilden, Germany)

- Streptavidin-coated Sepharose® beads (GE Healthcare, Vienna, Austria)
- Pyromark binding buffer (Qiagen, Hilden, Germany)
- PyroMark Annealing Buffer (Qiagen, Hilden, Germany)
- 70% Ethanol
- Denaturation solution (Qiagen, Hilden, Germany)
- Wash Buffer (Qiagen, Hilden, Germany)

- PyroMark Gold Q24 Reagents (Qiagen, Hilden, Germany)

Gelelectrophoreses:

- TAE buffer (50x) (genXpress Service & Vertrieb GmbH)
- Biozym LE Agarose (Biozym, Wien, Austria)

Primer:

FFAR3: designed with primer design software (Qiagen, Hilden, Germany)

- FFAR3-1 (fw) (©Biomers.net GmbH)
- FFAR3-1 (R*) (©Biomers.net GmbH)
- FFAR3-1 (S) (©Biomers.net GmbH)

LINE1: was chosen from a paper of Di et al. 2011 (Di et al. 2011).

- LINE1 (fw) (©Biomers.net GmbH)
- LINE1 (R*) (©Biomers.net GmbH)
- LINE1 (S) (©Biomers.net GmbH)

13.5 Analysis of estrogen receptor-1

During pyrosequencing of ESR1 13 CpGs were analysed. Three bisulfite treatment controls (position 22, 46 and 72) were used to guarantee successful bisulfite conversion. ESR1- was designed by primer design software (Qiagen, Hilden, Germany). ESR1-7 (fw), ESR1-7 (R*) and ESR1-7 (S) were the exact names of the used primers, which were purchased from ©Biomers.net GmbH.

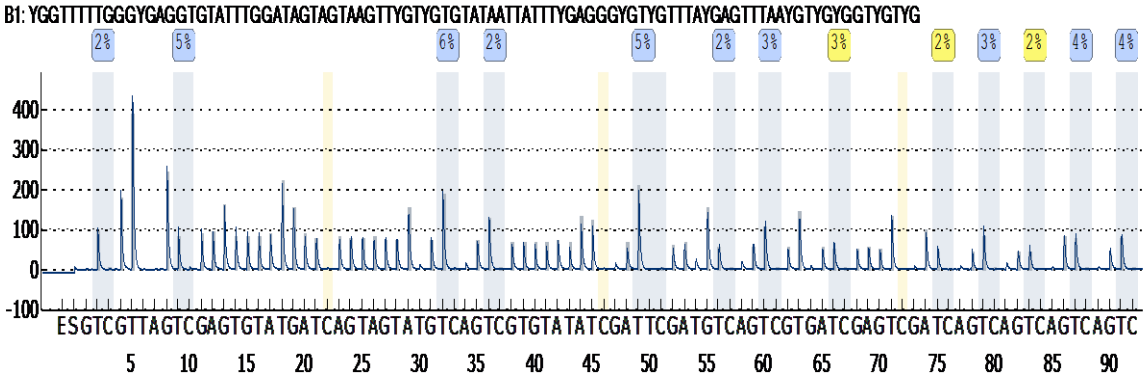

Figure 14 ESR1-pyrogram of obese diabetic patient at time point 2

Table 25 ESR1: Mean methylation of each time point

		N	Mean	Standarddeviation
ESR1_T1_Mean	OD	20	4,54	,42
	OC	3	4,60	,59
	LC	5	5,02	,78
	Total	28	4,64	,52
ESR1_T2_Mean	OD	21	4,44	,50
	OC	3	4,77	,56
	LC	5	5,38	1,02
	Total	29	4,64	,69
ESR1_T3_Mean	OD	19	4,64	,70
	OC	1	4,11	.
	LC	4	4,81	,98
	Total	24	4,64	,73

Not all samples were analysed this is why no exact results are available. General, methylation levels in ESR1 are very low compared to FFAR3 and LINE1. Significant differences can possibly showed at time point two between OD and LC (p=0,013).

14 Curriculum Vitae

CHRISTINE MEROLD

Persönliche Informationen

Geburtsdatum:	29.03.1988
Staatsangehörigkeit:	deutsch
Familienstand:	ledig
Religion:	römisch-katholisch

Schulische Ausbildung

10/2010 bis 06/2013	**Ernährungswissenschaften** an der Universität Wien *Abschluss:* • Master of Science (Molekulare Ernährung)
10/2007 bis 10/2010	**Ernährungswissenschaften** An der Universität Wien *Abschluss*: • Bachelor of Science
1998 bis 2007	Gymnasium Oberhaching *Abschluss:* • Allgemeine Hochschulreife
1994 bis 1998	Grundschule, Straßlach

15 References

Alberts Bruce, Bray Dennis Hopkin Karen Johnson Alexander Lewis Julian Raff Martin Roberts Keith Walter Peter (2010): Essential Cell Biology. third edition. New York: Garland Science Taylor & Francis Group.

Arora, Tulika; Sharma, Rajkumar; Frost, Gary (2011): Propionate. Anti-obesity and satiety enhancing factor? In: *Appetite* 56 (2), S. 511–515.

Banks A. William, Coon B. Alan Robinson M. Sandra Moinuddin Asif Schultz M. Jessica Nakaoke Ryota Morley E. John (2004): Triglycerides induce leptin reistance at the blood-brain barrier. In: *Diabetes Metab. Res. Rev.* (53), S. 1253–1260.

Berni Canani, Roberto; Di Costanzo, Margherita; Leone, Ludovica (2012): The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. In: *Clin Epigenetics* 4 (1), S. 4.

Buck S. Samuel, Abdullah Shalto Toshiyuki Motoike Federico E. Rey Fredrik Backhed Jill K. Manchester Robert E. Hammer S. Clay Williams Jan Crowley Masashi Yanagisawa Jeffrey I. Gordon (2008): Effects of the gut microbiota on host adiposity are modulated by short-chain fatty-acid binding G protein-coupled receptor, Gpr41. In: *The National Acadamy of sciences of the USA* 105 (43), S. 16767–16772.

Daniel K. Podolsky (1991): Inflammatory Bowel Disease. In: *The new England Journal of Medicine* 328, S. 928–937.

Di, Jian-zhong; Han, Xiao-dong; Gu, Wen-ye; Wang, Yu; Zheng, Qi; Zhang, Pin et al. (2011): Association of hypomethylation of LINE-1 repetitive element in blood leukocyte DNA with an increased risk of hepatocellular carcinoma. In: *J. Zhejiang Univ. Sci. B* 12 (10), S. 805–811.

Franchini Don-Marc, Schmitz Kerstin-Maike Petersen-Mahrt Svend K. (2012): 5-Methylcytosine DNA Demethylation: More than losing a methyl group. In: *Annual Review of Genetics* (46), S. 419–441.

Frank Reimann, Gwen Tolhurst Fiona M. Gribble (2012): G-Protein-Coupled Receptors in Intestinal Chemonsensation. In: *Cell Metabolism* 15, S. 421–432.

Franks, Paul W.; Ling, Charlotte (2010): Epigenetics and obesity: the devil is in the details. In: *BMC Med* 8 (1), S. 88.

Fuks, François (2005): DNA methylation and histone modifications: teaming up to silence genes. In: *Current Opinion in Genetics & Development* 15 (5), S. 490–495.

Guil, Sònia; Esteller, Manel (2009): DNA methylomes, histone codes and miRNAs: Tying it all together. In: *The International Journal of Biochemistry & Cell Biology* 41 (1), S. 87–95.

He, Xin-Jian; Chen, Taiping; Zhu, Jian-Kang (2011): Regulation and function of DNA methylation in plants and animals. In: *Cell Res* 21 (3), S. 442–465.

Holzer, Peter; Reichmann, Florian; Farzi, Aitak (2012): Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut–brain axis. In: *Neuropeptides* 46 (6), S. 261–274.

Ichimura, Atsuhiko; Hirasawa, Akira; Hara, Takafumi; Tsujimoto, Gozoh (2009): Free fatty acid receptors act as nutrient sensors to regulate energy homeostasis. In: *Prostaglandins & Other Lipid Mediators* 89 (3-4), S. 82–88.

Jei Kim, Jee-Yeon Kim Jean-Pierre J. Issa (2009): Aigng and DNA Methylation. In: *Current Chemical Biology* (3), S. 321–329.

Jiménez-Chillarón, Josep C.; Díaz, Rubén; Martínez, Débora; Pentinat, Thais; Ramón-Krauel, Marta; Ribó, Sílvia; Plösch, Torsten (2012): The role of nutrition on epigenetic modifications and their implications on health. In: *Biochimie* 94 (11), S. 2242–2263.

Keren Bahar Halpern, Anna Veprik Nir Rubins Oryl Naaman Michael D. Walker (2012): GPR41 Gene Expression Is Mediated by Internal Ribosome Entry Site (IRES)-dependent Translation of Bicistronic mRNA Encoding GPR40 and GPR41 Proteins. In: *Journal of Biological Chemistry* 287 (24), S. 20154–20174.

McKay, J. A.; Mathers, J. C. (2011): Diet induced epigenetic changes and their implications for health. In: *Acta Physiologica* 202 (2), S. 103–118.

Münzberg, Heike; Myers, Martin G. (2005): Molecular and anatomical determinants of central leptin resistance. In: *Nat Neurosci* 8 (5), S. 566–570.

Richardson, Bruce (2003): Impact of aging on DNA methylation. In: *Ageing Research Reviews* 2 (3), S. 245–261.

Schloegl, Haiko; Percik, Ruth; Horstmann, Annette; Villringer, Arno; Stumvoll, Michael (2011): Peptide hormones regulating appetite-focus on neuroimaging studies in humans. In: *Diabetes Metab. Res. Rev.* 27 (2), S. 104–112.

Soliman, Mohamed; Kimura, Kazuhiro; Ahmed, Mohamed; Yamaji, Daisuke; Matsushita, Yukiko; Okamatsu-Ogura, Yuko et al. (2007): Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in cultured bovine adipocytes. In: *Domestic Animal Endocrinology* 33 (4), S. 400–409.

Staels Bart and Fruchart Jean-Charles (2005): Therapeutic Roles of Peroxisome Proliferator-Activated Receptor Agonists. In: *American Diabetes Association* (54), S. 2460–2470.

Turcot, Valérie; Tchernof, André; Deshaies, Yves; Pérusse, Louis; Bélisle, Alexandre; Marceau, Simon et al. (2012): LINE-1 methylation in visceral adipose tissue of severely obese individuals is associated with metabolic syndrome status and related phenotypes. In: *Clin Epigenetics* 4 (1), S. 10.

Vieira, Erica L.M; Leonel, Alda J.; Sad, Alexandre P.; Beltrão, Nathália R.M; Costa, Thaís F.; Ferreira, Talita M.R et al. (2012): Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis. In: *The Journal of Nutritional Biochemistry* 23 (5), S. 430–436.

Vo, An T.; Millis, Richard M. (2012): Epigenetics and Breast Cancers. In: *Obstetrics and Gynecology International* 2012 (7261), S. 1–10.

Wu, Jin; Zhou, Zongli; Hu, Yinghe; Dong, Suzhen (2012): Butyrate-induced GPR41 Activation Inhibits Histone Acetylation and Cell Growth. In: *Journal of Genetics and Genomics* 39 (8), S. 375–384.