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Table of contents

List of tables

Figure index

List of abbreviations

AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ADRB3	Adrenergic receptor ß3
ALA	Alpha-linolenic acid
BFM	Body fat mass
BMI	Body mass index
CAD	Cardiovascular disease
CpG	Cytosine-guanine dinucleotide
DHA	Docosahexaenoic acid
DM2	Diabetes mellitus 2
EPA	Eicosapentaenoic acid
FADS1	Fatty acid desaturase1
FFQ	Food frequency questionnaire
FTO	Fat mass and obesity associated gene
GIT	Gastro intestinal tract
GHQ	General health questionnaire
GWAS	Genome wide association study
HDL	High density lipid
HRM	High resolution melting
IL6	Interleukin 6
LA	Linoleic acid
LEPR	Leptin receptor

LINE1	Long interspersed element 1
LPS	Lipopolysaccharide
Mets	Metabolic syndrome
MC4R	Melanocortin 4 receptor
MUFAs	Monounsaturated fatty acids
NFW	Nuclease free water
NPY	Neuropeptide Y
PA	Physical activity
PCR	Polymerase chain reaction
POMC	Proopiomelanocortin
PPARG2	Peroxisome proliferator-activated receptor gamma 2
PUFAs	Polyunsaturated fatty acids
SCFA	Short chain fatty acids
SCL6A14	Solute carrier family 6 member 14
SFA	Saturated fatty acid
SNP	Single nucleotide polymorphisms
TCF7L2	Transcription factor 7-like 2
TFAP2B	Transcription factor AP-2ß
TG	Triglycerides
TNF- apha	Tumor necrosis factor alpha

1. Introduction

Humans vary in their need and reply to diet (7). Weight loss/ gain and metabolic rate but also nutrition related disorders like obesity or diabetes mellitus 2 (DM2) result from different factors like genetics, dietary inclusive gut microbiota, and epigenetic: environment and lifestyle (1). Overweight is characterized by an excessive accumulation of lipids in body cells. It is the result of an imbalance of energy intake and expenditure but also microbial. Total body fat, for example, can be inherited up to 75-80% (3)

Epigenetic is focusing on heritable changes in gene expression without changing the underlying DNA sequence (1), but can be used as predictors for weight loss and further body mass index (BMI). Epigenetic mechanisms alone seem to be insufficient to explain individual metabolisms, thus, polymorphisms have been indentified which regulate food intake and nutrient metabolism (3). These single nucleotide polymorphisms (SNPs) are appearing millions of times in the human genome and can be associated with different disorders. They are effecting gene expression and protein functions (1).

Further, it is suggested to look at interactions between SNPs, environmental factors, dietary and in addition the gastrointestinal microbiota, so called microbiom (Figure 1) (2). Many researches enforce the fact that the microbiom is involved in developing metabolic disorders (1). Excretion of nutrients can be determined by genetic makeup (5).

Putting all these tree factors together we tried to classify the participants into different metabolic types (metabotypes) and provide a personalized nutrition based on the genotype.

1.1 Personalized nutrition

Dietary preferences and habits are controlled by biological, socioeconomic, psychological and behavioral determinants. Hunger, satiety and sensory aspects, control the individual food choice and preferences (6).

Body weight and composition, as well as metabolic rate can be influenced by genetic

and nutrient intake. Biochemical pathways, controlled by genes regulate nutrient absorption, distribution, metabolism, excretions and other cellular energy processes (5). Weight loss treatments, based on dietary intervention can have different success. Several studies show that genetic and epigenetic predispositions forecast the response to a weight loss intervention (4).

The field nutrigenetics offers a new opportunity to evaluate the role of genes, which determine metabolism, disorders and further use it as a benefit for a personalized nutrition. The need of the different amounts of macronutrients is the reason why dietary interventions should be considered on an individual basis (5).

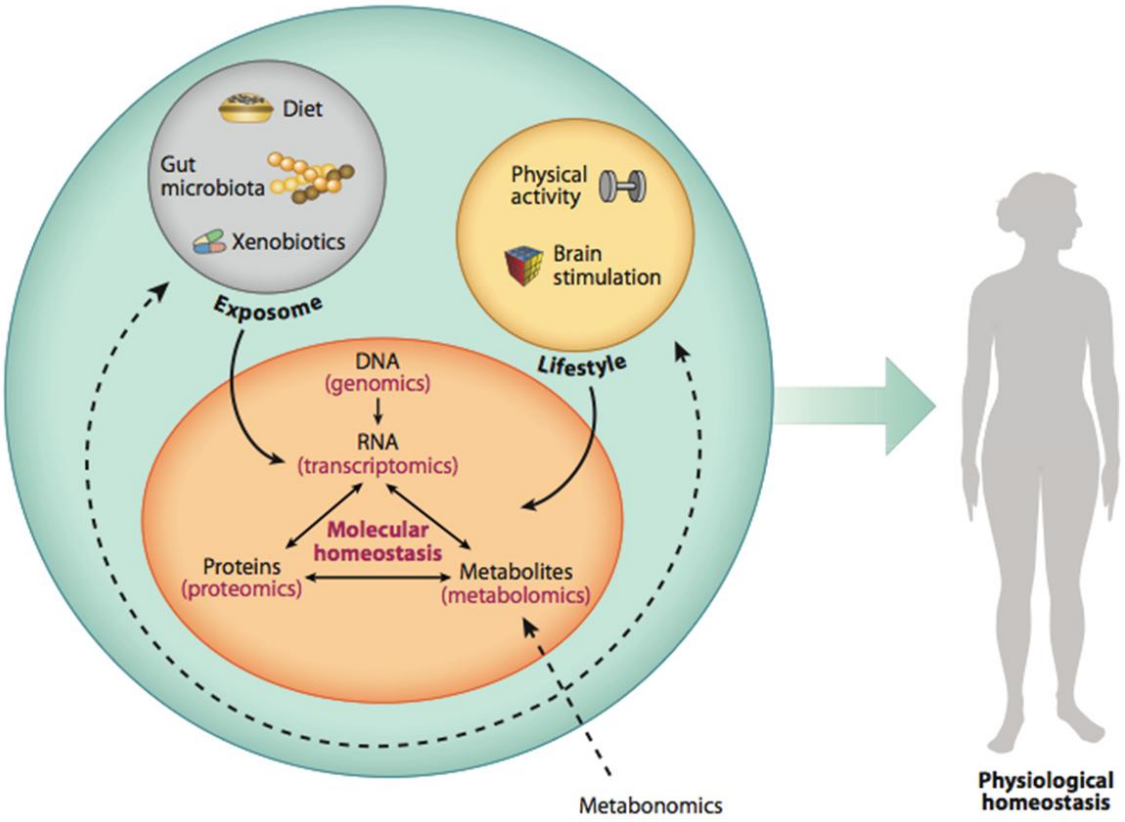

Figure 1: Interaction between DNA, RNA, gut microbiota and lifestyle (49)

1.2 Single nucleotide polymorphism

Single nucleotide polymorphism is a variation in a single nucleotide that occurs at a specific position in the genome. Polymorphisms are defined as the less common variant that has at least a frequency of 1% within the population. SNPs can occur in the coding, none coding or in the region between two genes. SNPs in the coding region are of two types, synonymous and nonsynonymous SNPs. Synonymous SNPs do not affect the protein sequence, but can still affect gene splicing, transcription factor binding, messenger RNA degradation, or the sequence of non-coding RNA, while nonsynonymous SNPs change the amino acid sequence of the protein (7,26, 47)

1.2.1. *FTO* rs9939609

Many *fat mass and obesity associated gene* (*FTO*) polymorphisms are related with obesity and excess body weight, by affecting adiposity (2, 19). The mechanism by how this mutation is influencing adiposity is unclear. Animal studies assume *FTO* regulates energy homeostasis, but it is not certain whether it influences energy intake or energy expenditure (20).

Genome wide association studies (GWAS) show that *FTO* SNP increase the BMI by 0.4kg/m²/allele, caused by an increased intake of fat and lower carbohydrate intake (18)

Goni et al. reported that individuals with the genotype AA variant have a higher BMI, percentage of body fat mass (BFM) and waist/height ratio, and greater waist circumference (2). Kilpeläinen et al. confirmed that the *FTO* SNP rs99396909 is associated with waist circumferences and additionally showed and relationship between *FTO*, physical activity (PA) and waist circumferences. Results show that waist circumferences were 33% smaller in the active individuals than in the one without physical activity comparing participants caring a risk allele. Phillip et al. showed carrier with AA and AT risk allele showed higher waist circumferences then TT carriers when high saturated fatty acids (SFAs) are consumed (4,18).

1.2.2. 1.2.2 *FTO* rs1121980

The *FTO* gene can affect appetite, satiety and eating behaviors (54). Vimaleswaran et al. reported that the risk allele of rs1121980 is significantly linked with a higher BMI and waist circumference. Furthermore researchers assume that this gene can also interact with physical activity. Vimaleswaran and colleagues did not find any significant association of *FTO* and PA. Nevertheless they could see that the BMI increased with lower PA in participants with the risk allele. The physical activity was clustered into 4 types: active BMI higher about 0.26; moderately active 0.28; moderately inactive 0.22; inactive 0.44 kg/m²/allele) (55).

1.2.3. *MC4R* rs17782313

Melanocortin 4 receptor (*MC4R*) is a hypothalamic regulator of the energy balance and is related to obesity (4). *MC4R* is expressed in the hypothalamus, the cerebral cortex, brain stem and spinal cord. Moreover it is a component of the leptin system, which is controlling food intake. This means, putting the body into a negative energy state, leads to a decreased level of leptin and further to a continuously repression of *MC4R*, thus results in an increased food intake, tendentially fat (21, 22). More than 100 variants in human *MC4R* have been found. A Chinese study confirmed the association between the SNP rs17782313 and obesity (4). Like the *FTO* gene, *MC4R* SNP increases the BMI with 0.25kg/m²/allele (18).

Children, who had the mutation in this gene, were less successful in weight maintenance. Moreover, *MC4R* rs17782313 is associated with increased intake of protein (4.4g/day) in woman, but several studies did not show associations between the gene and macronutrient intake (18).

MC4R variants can be linked with lifestyle, food intake and eating habits and as well with stress. This gene activates stress neuropeptides, like pro- opiomelanocortin and alpha- melanocyte stimulating hormone, which further increase the level of *MC4R* and thus changing eating behavior. Perk et al. investigated the interaction between *MC4R* rs17782313 and mental stress as well as nutrient intake in overweight and obesity development. Results show, that carrier of the risk allele had a significant higher intake

of processed food and increased fruit intake. There was a positive interaction between *MC4R,* stress and the risk of obesity. Participants with the risk allele had high stress and higher BMI and further greater energy intake. To conclude, *MC4R* interferes with nutrition and mental stress which is promoting obesity (21). Although, the outcomes are still controversy in different studies and more researches are needed (22).

1.2.4. *TCF7L2* rs7903146

The *transcription factor 7-like 2* (*TCF7L2*) is a key regulator of glucose homeostasis and metabolism (23). The gene is expressed in pancreatic islet and contributes to the insulin release regulation (24). The risk T allele results in a greater *TCF7L2* expression and decreased insulin content and secretion (23). *TCF7L2* has been most consistently associated with diabetes type 2, whereas rs7903146 has been reported with the highest effect of DM2 (18). The polymorphism is located between exon 4 and 5 on chromosome 10. It affects the splicing pattern of the gene, which is a transcription factor of the Wnt signalling pathway. Furthermore it influences beta and alpha cell metabolisms and proglucagon gene expression (24, 25)

Martinez et al. described that humans with this SNP had more weight loss in response to a low fat and high fibre intake (45). Moreover, it has been investigated with weight maintenance, showing the result that *TCF7L2* interacts with protein and attenuates weight changes. This means, carrier for the minor risk allele have more trouble to maintain their weight (18).

1.2.5. *PPARG2* rs1801282

Peroxisome proliferator-activated receptor gamma (*PPARG)* encodes a regulator of adipocyte differentiation. The gene is most related with developing obesity, lipid storage and insulin sensitivity. Robitaille et al. studied the relationship of the SNP rs1801282 Pro12Ala between waist circumference and the intake of dietary fat. They showed that the homozygous P12/P12 participants with a high total fat and SFA intake had the highest waist circumference. A12 carriers did not change with the same conditions. Dedoussis et al. demonstrated an interaction with monounsaturated fatty acids (MUFAs). P12/P12 had low waist circumferences when a high MUFA intake.

Looking at both studies it can be assumed that homozygous have a higher risk for obesity when consuming a high fat diet, but a low risk when MUFA intake is high (4). Galbete et al. showed subjects carrying the Ala allele of the *PPARG2* gene and consuming a high of carbohydrates (246 g/day) had an increased obesity risk compared to Pro12Pro subjects (28). In another study the authors investigated the relation between *PPARG2* and BMI. They showed a higher BMI with an overall estimation of 0.065 kg/m2 for homo-and heterozygous carriers (27).

1.2.6. *FADS1* rs174547

Fatty acid desaturase (*FADS*) are enzymes, like delta-5 desaturase, coded by *FADS* genes. Delta-5 desaturase is involved in the metabolism of n- 3 polyunsaturated fatty acids (PUFAs), by incorporating a double bound and result from a 20:4 n-3 to arachidonic acid (AA) (20:5 n-3) and from 20:3 n-6 to eicosapentaenoic acid (EPA) (20:5 n-6) (14). The precursors are linoleic acid (LA) for the n-6 family and alpha-linolenic acid (ALA) for the n-3 family (Figure 2) (15).

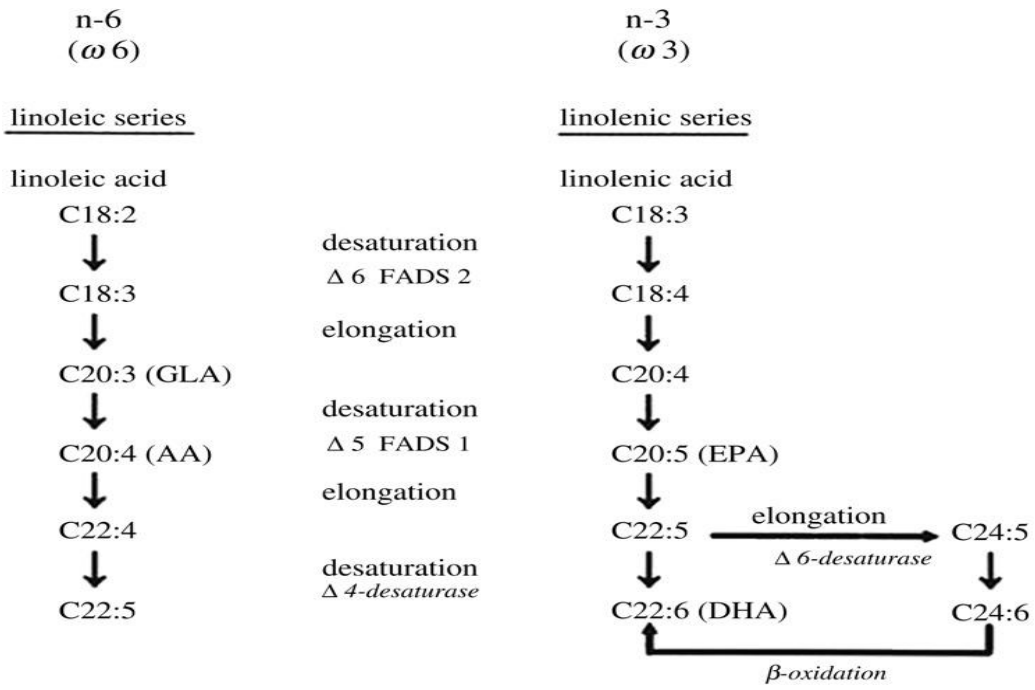

Figure 2: Transformation n-6 and n-3 fatty acids (15)

Both, LA and ALA are essential for normal growth and development and cannot be synthesized in the human body and must be obtained from the diet (15). AA and EPA are processed to eicosanoids, such as prostaglandins and leutokriens (Figure 3), which can be both harmful and beneficial for the human body, depending of the prostaglandin formation of n-6 or n-3. EPA and AA compete for prostaglandin and leutokriene synthesis at the cyclooxygenase and lipoxygenase level. Omega-3 fatty acids prostaglandins have antithrombotic and anti-inflammatory effects and decrease triglycerides and cholesterol (15)

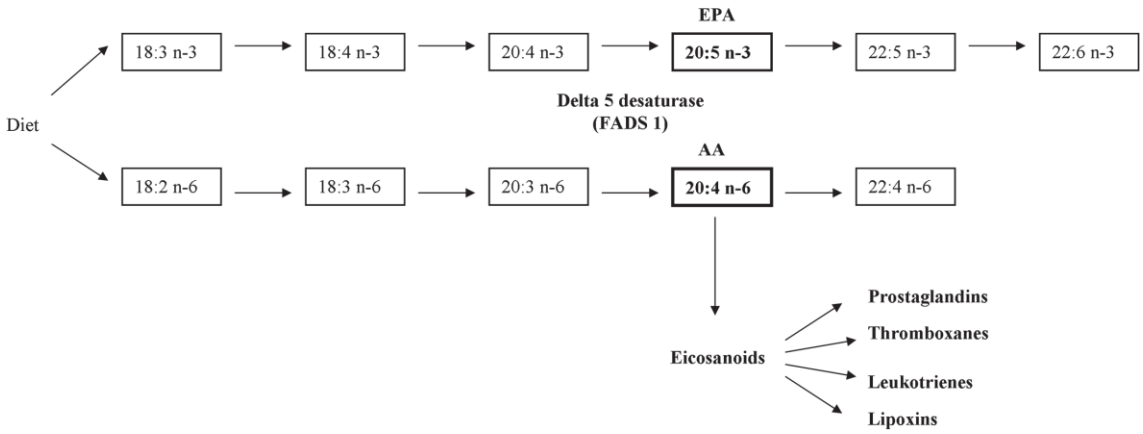

Figure 3: Eisosanoids from AA (14)

It has been reported that participants with a polymorphism in rs174547 in the *FADS1* gene have increased triglyceride (TG) levels, decreased high density lipoproteins (HDL) cholesterol and increased coronary artery disease risk. Controversial, Liu et al. showed, that carrying the T allele increases cardiovascular disease (CVD) risk, although this effect was only seen, if EPA was low (14). Rs174547 is shown to be associated with TG and HDL concentrations in nearly 40,000 European individuals. The C allele was associated with increased triglyceride levels and decreased high density lipoprotein HDL levels in the Japanese population (16).

1.2.7. *IL6* rs1800795

Interleukin 6 (*IL6*) encodes a cytokine that is involved in inflammatory and immunostimmulation. The IL6 rs1800797 GG phenotype had higher waist

circumferences compared with the ones without the risk genotype when PUFA and SFA concentration in plasma was low. Among those with risk genotype high PUFA /SFA concentration correlated with lower pro-inflammatory status, triglyceride levels and insulin resistance. This SNP is further associated with a high diabetes risk (4).

1.2.8. *SLC6A14* rs2011198

The *solute carrier family 6 member 14* (*SLC6A14*) gene plays an important role in the availability of tryptophan. This neurotransmitter is needed for the serotonin synthesis which can affect appetite and mood. Therefore, *SLC6A14* polymorphisms have been linked with obesity in adults (56), also verified by Suviolahti et al. (57).

1.2.9. *LEPR* rs9436740

Leptin is an adipocyte-secreted hormone and regulates energy homeostasis, blood pressure and food intake. Leptin is released in adipose tissue and correlates with the amount of adipose tissue. High leptin levels are suggested to reduce appetite and food intake and moreover increases energy metabolism. Controversy, high levels of leptin have been also observed in obese people. Polymorphisms at the *leptin receptor* (*LEPR*) lead to decreased signalling and further decrease the beneficial effects of leptin. Sport alone or in combination with dietary intervention result in weight loss, decreased adipose tissue and increases the levels of leptin, further decrease the positive effect of leptin (43, 44).

1.2.10. *ADRB3* rs4994

The *adrenergic receptor ß3* (*ARDB3*) is part of the adipocyte metabolism. It is mediating the rate of lipolysis in response to catecholamine. Their agonists decrease diabetes and obesity risk. This polymorphism is characterized by arginine replacing tryptophan in the position 64. The variation is associated with lower lipolysis and further increased accumulation of lipids in the adipose tissue (51). Arg64 allele carriers lost less weight than Try homozygote with a low –caloric diet and exercise. A low fat diet is recommended (48). Several studies reported resistance to weight loss for participants with the homozygous risk allele in comparison to heterozygous or wildtypes. Moreover losing visceral fat is more difficult for carries (51).

1.2.11. *ACE* rs4341

The human *angiotensin-converting enzyme* (*ACE*) gene is described as the insertion of I allele or the deletion of the D allele. Thus, these variants exist: II, ID and DD (10). *ACE* is a regulatory enzyme of the rennin- angiotensin- aldosterone system, which is connected with blood pressure. It converts angiotensin I to angiotensin II, which is a vasoconstrictor and inactivates bradykinin and kallidin, who are vasodilators (11, 17). High *ACE* activity (D allele), lowers bradykinin and the synthesis of angiotensin II. Both have an effect on cell growth. The stimulatory effect of angiotensin II in the DD subjects may result in a potential hypertrophic training that may have an advantage in power sport with greater training related strength gain and sprinting ability. The I or II genotype is associated with endurance performance, like swimming, cycling and running, based on lower *ACE* activity and increased bradykinin. This mutation results in more oxygenated blood, which is delivered to the working muscles (10, 11, 17).

1.2.12. *TFAP2B* rs987237

The encoding *transcription factor AP-2ß* (*TFAP2B)* is expressed in adipose tissue and is associated with BMI and insulin resistance. The gene could be involved in a positive energy balance. The SNP *TFAP2B* rs987237 has a significant association with waist to hip ratio (4). Martinez et al. showed a higher weight loss in wildtypes with a low fat and low caloric diet. The opposite response was recognized caring the risk allele (45). Stocks et al. investigated the association between rs987237 and a high protein diet, whereas a high protein diet was beneficial for weight maintenance in the AA genotype. No differences have been observed between homozygous and heterozygous types (46).

1.3 Epigenetic

Twins with the same genotype develop different phenotypes when they are separated and living in different environments, determined by epigenetic mechanism (1). The main epigenetic mechanisms are DNA methylation and histone modification. Histone modification includes methylation, acetylation, ubiquination and somoylation of lysine, phosphorylation of serine and threonine, and methylation of argentine (4).

DNA methylation of cytosines in CpG (cytosine-guanine dinucleotide) (methyl group to the pyrimidine ring in position 5 of cytosine) in the promoter region inhibit the transcription of a gene (1, 9). Influenced by dietary, lifestyle, toxins and endocrine disruptors DNA methylation at CpGs are specific and can vary over time within an individual and can be passed to the next generation (1, 3, 4). Methylation can be increased or decreased in response to environmental factors, including dietary components (50).

Weight loss can be influenced by genetic makeup and epigenetic mechanism. Epigenetic markers are used as predictors for metabolic risks and predictors for the success of a diet related treatment, like weight loss or weight maintenance (8). Different DNA methylation levels of *Interleukin 6* (*IL6*) were observed between obese and lean individuals (53).

Researches try to reveal how genetic and epigenetic mechanisms interact in their pathogenesis for different diseases. One major mechanism is CpG creating SNPs, which leads to genetically driven variation in DNA methylation and effects gene expression (8).

1.3.1 IL6 and LINE1

IL6 is a small glycoprotein and is produced in innate immune cells. Thus, it can represent a tissue of damage and stress. *IL6* concentrations are associated with several inflammatory diseases and can be used as an inflammatory marker. (10). Moreover, different studies showed that in obese individuals *IL6* is up to two or three fold higher compared to lean individuals. Aumüller et al. came to the result that low *IL6* methylation is associated with a better weight loss which results in a lower BMI (53).

Omega-3 polyunsaturated fatty acids (PUFAs) inhibit systematic inflammation through reduction of IL6 plasma concentration. High concentrations are associated with CVD. Supplementation with both EPA and DHA for several weeks reduced the plasma concentration of IL-6 but also its gene expression in adipose tissue (42)

The *long interspersed element 1 (LINE1)* is a retrotransposon, which is widely expressed in the human genome (4) and is associated with genetic instability and

chromosomal abnormalities (50). Usually assessed to estimate global DNA methylation, this marker is related to BMI, DM2, insulin resistance, cardiovascular disease, inflammatory response and cancer (4, 13). Some investigations have linked changes in DNA methylation to obesity and metabolic syndrome (MetS), before clinical symptoms are starting. LINE1 methylation has been associated with fasting glucose, blood lipids, and a greater risk for MetS in the presence of obesity and may be considered as a good biomarker in the response to weight loss treatment in obese. (9)

Nicoletti et al. assigned a study dividing woman into three different groups. First, control group (n=9) including normal weight test persons. Second group, (n=22) energy restricted group, where obese patients had to follow an energy restricted Mediterranean based dietary treatment and third group, the bariatric surgery group (n=14). Here the participants underwent a hypocaloric diet followed by a bariatric surgery. *LINE1* and *IL6* were two of the evaluated genetic markers. *LINE1* correlated positively with BMI at the baseline, but did not change after weight loss. Moreover hypermethylation of LINE1 is associated with high blood glucose which can be connected to higher risk of developing DM2 (13).

Nevertheless which strategy is used for losing weight; epigenetic markers can be used as early predictors for metabolic risks and the success of weight losing programs (13).

1.4 Microbiota

The microorganisms in the gut are a highly metabolic active community and are regarded as a regulator of its host homeostasis. The gut microbiota contains 100 times more genes than human cells. Therefore modifying the gut bacteria balance can affect the own metabolism. The composition of the microbiota varies over lifetime. Diet is the strongest factor that influences the composition (29). Changing from a high fat/low fiber diet to a low fat/high fiber can change the abundance of gut bacteria tremendously (36). The gut bacteria generate for example organic acids and alcohol to products which become available for the host. Indigestible complex carbohydrates are

a major source for carbon, the main source for the gut microbiota. After their fermentation short chain fatty acids (SFAs), like acetate, propionate and butyrate are produced. Those are absorbed via the colon mucosa and have an important role for human health. Furthermore they have beneficial effect in appetite regulation, lipid and glucose metabolism (29-31).

Lipids and proteins can as well influence the composition. Bacteria are able to form secondary and tertiary bile acids out of sterols, which are an important interplay between the microbiota and the host. This process regulates the absorption of dietary lipids during digestion (29). Evidence suggests that the trillions of bacteria that normally reside within the human gastrointestinal tract affect nutrient absorption and energy regulation and further help with weight maintenance (35).

However, an imbalanced gut microbiota and further their metabolites, like butyrate and lipopolysaccharide (LPS) affect epigenetic mechanisms (52). LPS binds on its receptor CD14 and thus triggers pro-inflammatory processes (61) (Figure 4).

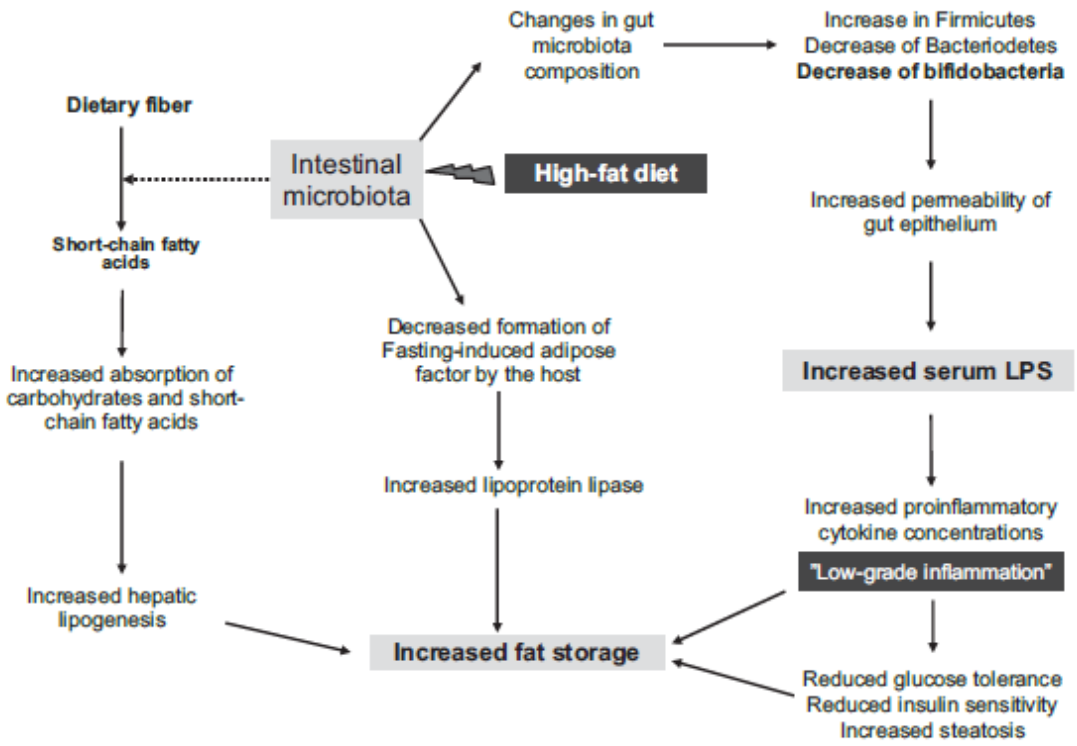

Figure 4: Microbiota and inflammatory status (61)

TABLE. **Major Bacteria and Archaea Phyla and Genera Found in the Human Gut Microbiota**[a]

Phyla	Representative genera
Bacteria	
Firmicutes	*Ruminococcus*
	Clostridium
	Peptostreptococcus
	Lactobacillus
	Enterococcus
Bacteroidetes	*Bacteroides*
Proteobacteria	*Desulfovibrio*
	Escherichia
	Helicobacter
Verrucomicrobia[b]	
Actinobacteria	*Bifidobacterium*
Cyanobacteria[b]	
Synergistes[b]	
Archaea	
Euryarchaeota	*Methanobrevibacter*

Table 1: Genera from Bacteroidetes and Firmicutes (35)

1.4.1 Bacteroidetes

Bacteroidetes are gram-negative bacteria and the one off the most common in the gastro intestinal tract (GIT). The phylum includes two genera *Bacteroides* and *Prevotella*. In this class there are many SCFAs producers and therefore important against pathogens (Table 1). They are associated with high protein, animal fat or carbohydrate intake (31, 34).

Lean people have a higher proportion of Bacteroidetes comparison with obese people. (32)

1.4.2 Firmicutes

Two groups of beneficial bacteria are dominant in the human gut, the *Bacteroidetes* and the *Firmicutes* (33). Firmicutes is the largest phylum of bacteria and is separated into *Clostridium cluster XIVa*, *Clostridium cluster IV* and *Lactobacillus* group. *Lactobacillus*, acetate producer, are a sub-dominant group, whereas *Clostridium cluster XIVa* and *Clostridium cluster IV*, both butyrate producers, make 10-40% of the gut microbiota (38*). Lactobacilli* tend to have a beneficial effect and are popular used as probiotic bacteria (39). Several researches showed an increased *Lactobacilli* amount

in association with weight gain and inflammatory processes, but more studies are needed (40,41).

1.4.3 Firmicutes/ Bacteroidetes ratio

The *Firmicutes* and *Bacteroidetes* ratio has often been mentioned as a marker for human health, whereas the ratio is higher in obese human compared to lean individuals. Several studies show a shift in the ratio during dietary intervention and weight loss. This means the lean phenotype results in a decrease of *Firmicutes* and a high *Bacteroidetes* abundance (36-37). A negative shift can lead to chronic inflammation by increasing plasma LPS and results in obesity (31,34). Comparing diabetics with healthy persons, diabetics have an decreased amount of *Firmicutes* but higher amount of *Bacteroidetes* (40)

1.4.4 Microbiom and epigenetic

The dominant groups, either *Firmicutes* or *Bacteroidetes*, correlate with different methylation status, which can be linked with obesity and other diseases (59). These bacteria are producing SCFAs by fermenting indigestible fiber into sugar, protein and peptides. SCFAs like butyrate, acetate and propionate are thought to influence epigenetic mechanisms and gene expression (58).

Butyrate and propionate regulate gene expression as histone deacetylase inhibitors and methyl CpG binding proteins which results in a different gene expression, like the downregulation of pro-inflammatory cytokine IL6 (58, 62). Histone acetylation by SCFAs is seen to reduce obesity through anti-inflammatory effects. Though, LPS has an important role in the epigenetic regulation of immune and intestinal cells and is known as an inflammatory molecule (59, 58) Line 1 is usually highly methylated and can be associated with stroke, DM2, obesity, BMI and other diseases (58).

2. Objectives

The Metabotype-Study at the Department for Nutritional Science of the University of Vienna, supervised by Univ.Doz. Dr. Haslberger, was initiated to define a clustering of participants into four different metabotypes based on differences in genetics: polymorphisms, epigenetics: DNA methylation and in the gut microbiota composition.

Lots of research has been done in the field nutrigenetics, knowing that the gut bacteria have a major impact on the host´s health, metabolism and methylation. Moreover studies show the importance of individual genetic makeup for their weight maintenance and the different need of macro- and micronutrients to serve humans individual health. Epigenetic impact and their mechanisms can predict human's response to weight maintenance and weight loss; but methylation levels alone are insufficient to recommend personalized nutrition.

For genetic analysis we decided to genotype 12 different SNPs, knowing there influence on diverse metabolism and the preference for the macronutrient. What is more, some SNPs can predict the risk for DM2, obesity. With ACE we characterize different sport types. To include environmental and lifestyle impact of each individual, we analysed LINE 1 and IL6, two epigenetic markers, whereas the different methylation levels predict the efficiency of losing weight and moreover can be used as inflammatory markers and global methylation marker.

Thus, we created this pilot study, where we combined genetic, epigenetic and the microbiom analysis to divide 37 study participants into different metabotypes, like glycol, fat, protein and balanced type, which so far no study has done before. Moreover we want to show the importance of combining these three factors for human health statements and to assure a personalized nutrition.

3. Material and Methods

3.1 Study design

The population included healthy men and women at the age from 30 to 60 years. Exclusion criteria were chronic diseases, colitis ulcerosa, supplementation of pre- or probiotics, antibiotic intake and body mass index (BMI) over 30. Genotype information of 40 individuals was available. Two had to be excluded with the reason of DM2. Another one had to be excluded with the reason of BMI 33 and antibiotic intake. BMI was calculated dividing weight (kg) by the square of height. After ensuring that participants understood the information and signed the consent, those were enrolled.

3.1.1 Procedure and sample collection

The group of volunteers could choose between tree different dates, where we met in the practice of Christina Schnitzler (Cooperation partner). Diet information was collected by a food frequency questionnaire in which basic food were classified into 16 food groups: dairy products, eggs, meat and sausage, fish, vegetable, fruits, grain, snacks, oil, grains, sugar, convenience food, water, alcohol and tee. Moreover they were asked to report how often they are consuming each food group. Environmental factors, like smoking, physical activity, stress, allergies and intolerances were questioned. Such factors can interfere with the genetic data. With the general health questionnaire we collected information to the health status. For molecular analysis we collected blood, mucus and stool. For the blood samples we used blood spots, for mucus swaps and for stool we provided collection tubes, which the proband delivered back to us.

3.2 DNA Extraction (blood spots)

For the molecular analysis, blood spots were used for genotyping after DNA extraction (total 100µl) which was done with the QIAamp DNA Mini Kit.

3.2.1 Procedure

We used the protocol from QiAmp, which was as followed:

1. Place 1-2 dried blood spot into a 1.5 ml microcentrifuge tube and add 180 µl of Buffer ATL.. Incubate at 85°C for 10 min. Centrifuge shortly
2. Add 20 µl Proteinase K, vortex, and incubate at 56°C for 1 h. centrifuge shortly
3. Add 200 µl Buffer AL to the sample, vortex, and incubate at
70°C for 10 min. centrifuge shortly
4. Add 200 µl ethanol (96–100%) to the sample, vortex and centrifuge shortly
5. Carefully put the mixture to the QIAamp Spin Column. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the Spin Column in a clean 2 ml collection tube and discard the tube containing the filtrate. Do this step twice.
6. Carefully add 500 µl Buffer AW1. Close the cap and centrifuge at 8000 rpm for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube, and toss the collection tube with the filtrate.
7. Carefully add 500 µl Buffer AW2. Close the cap and centrifuge at full speed 14,000 rpm for 3 min.
8. Place the QIAamp Spin Column in a new 2 ml collection tube and trash the collection tube with the filtrate. Centrifuge at 14,000 rpm for 1 min.
9. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge, and discard the collection tube with the filtrate. Carefully add 100 µl Buffer AE. Incubate at room temperature for 1 min, and then centrifuge at 8000 rpm for 1 min.

3.3 Genotyping

The SNP analysis was made with the StepOne Plus (Thermo Fisher, Massachusetts, USA) using TaqMan Mastermix and TaqMan SNP Genotyping Assays from Thermo Fisher (Massachusetts, USA).

The TaqMan SNP Genotyping assay contained 2 primers, to amplify the sequence of interest and 2 probes, VIC and FAM, to detect the allele. The assay is delivered with a concentration of 40X. It is recommended to dilute each SNP Genotyping Assay with the TE buffer to a 20X concentration. The *PPARG2* SNP is characterized as a Drug Metabolism Enzyme Genotyping Assay and contained already the required concentration of 20X.

Each well of the 96 Fast Plate contained 5µl TaqMan Mastermix, 0.5µl TaqMan SNP Genotyping Assay and 4.5µl DNA template, which was diluted with the TE Puffer to 5ng/ µl. The temperature programme and cycles are different using TaqMan SNP Genotyping Assay or TaqMan Drug Metabolism Enzyme Assay.

Step	**SNP Genotyping Assay**			**Drug Metabolism Enzyme Assay**		
	Temp.	**Duration**	**Cycles**	**Temp.**	**Duration**	**Cycles**
Enzyme Activation	95°C	10 min	Hold	95°C	10 min	Hold
Denaturation	95°C	15 sec.	40	95°C	15 sec.	50
Annealing/ Extension	60°C	1 min		60°C	90 sec	

Our SNPs of interest were as followed

MC4R	rs17782313	GTTTAAAGCAGGAGAGATTGTATCC**[C/T]**GATGGAAATGACAAGAAAAGCTTCA	Risk allele **C**
TCF7L2	rs7903146	TAGAGAGCTAAGCACTTTTTAGATA**[C/T]**TATATAATTTAATTGCCGTATGAGG	Risk allele

			T
FADS1	rs174547	TGTTTTTGCTGTTTTCACCTACGCA**[C/T]**CCTTTTCA ATAGTTGTGTTATGCTC	Risk allele **C**
IL6	rs1800795	ACTTTTCCCCCTAGTTGTGTCTTGC**[C/G]**ATGCTAA AGGACGTCACATTGCACA	Risk allele **G**
SLC6A14	rs201198	AATATACTTCTATTAACAACTAGCT**[C/A]**CATTTGA TTATACCTTTTTCTTGGT	Risk allele **C**
LEPR	rs9436740	AGCACTTTAGGCACCAGGAAAGCTG**[A/T]**ATGCTA AGGCCCGGAGACATGAAAG	Risk allele **T**
ARDB3	rs4994	GTCATGGTCTGGAGTCTCGGAGTCC**[A/G]**GGCGA TGGCCACGATGACCAGCAGG	Risk allele **G**
FTO	rs1121980	TCCTAGTCACGTGTCTTGGTACTAT**[A/G]**TGAGATT TCAGATCCACCTGCCTAC	Risk allele **A**
FTO	rs9939609	GGTTCCTTGCGACTGCTGTGAATTT**[A/T]**GTGATG CACTTGGATAGTCTCTGTT	Risk allele **A**
PPAG2	rs1801282	AACTCTGGGAGATTCTCCTATTGAC**[C/G]**CAGAAA GCGATTCCTTCACTGATAC	Risk allele G
ACE	rs4341	GGTGAGCTAAGGGCTGGAGCTCAAG**[C/G]**CATTC AAACCCCTACCAGATCTGAC	
TFAP2B	rs987237	GTGCTTTAATTTGTAAACAAAATTC**[A/G]**TGTCACT GCAGAATTGCTTTCACTG	Risk allele **G**

Using a real time PCR, the runs had be post read with the TaqMan Genotyping Software. Considering the brackets in the target DNA sequence, the VIC dye probe of the TaqMan SNPs Genotyping Assay is detecting the first allele in the bracket. FAM dye probe detects the second risk allele in the bracket. A heterozygous type of for example *MC4R* would be detected as VIC and FAM. The C/C genotype for the same gene would be detected only from the VIC probe. In contrast to MC4R the homozygous form of *TCF7L2* would be detected of FAM/FAM probe, the wildtype, logically with the VIC/VIC probe (Figure 5).

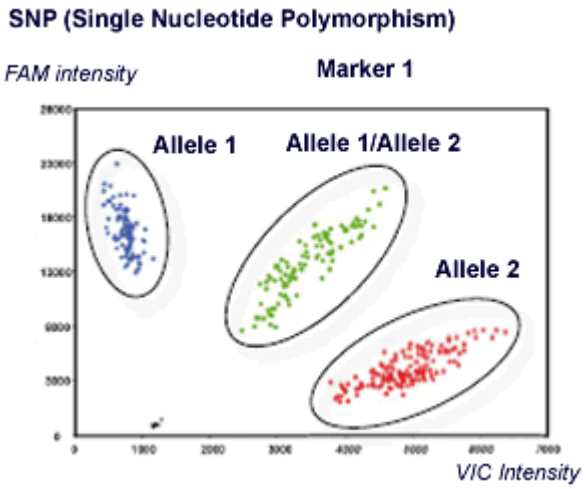

Figure 5: Cluster of genotyping analysis (TagMan Genotyping protocol)

The *ACE* needed to be considered differently. According to Eisenmann et al. the DD genotype was coequal with rs4341 G/G polymorphisms, ID and C/G genotype and II with the C/C phenotype. Thus, the G/G SNP is associated with affinity for power sport and the C/C phenotype is associated with endurance sport (11).

3.4 Bisulfit conversion and high resolution melting analysis

For epigenetic analysis 20µl DNA was bisulfite converted, using the EpiTec bisulfite kit (Qiagen, Hilden, Germany), from the total 100µl extracted DNA from the blood spots.

We used the High resolution melt method to measure *LINE1* and *IL6* as epigenetic markers. High resolution melting (HRM) analysis is a technique that is based on DNA melting analysis. The method characterizes DNA samples according to their dissociation behaviour and moreover the transition from double stranded DNA to single stranded DNA according to increasing temperature. Previously the target DNA sequence needed to be amplified. EvaGreen is used as a fluorescent dye. The changes in the fluorescence measure the increase of the DNA concentration during the PCR and afterwards measure DNA melting, induced by temperature, by the HRM.

Following Primers and temperature were used (12).

IL-6 HRM fw	DNA	taagtgggttgaagtaggtgaaga
IL-6 HRM rev	DNA	actacctaaccatcctcaaattt
LINE-1 HRM fw	DNA	ttttgagttaggtgtgggatata
LINE-1 HRM rev	DNA	aaaatcaaaaaattccctttc

temperature programme

5min	95°C	
25s	95°C	45 cycles
40s	54°C	
25s	72°C	
1min	95°C	
1min	45°C	
HRM	65-95°C	

The temperature programme and cycle numbers for *LINE1* and *IL6* were the same.

In each well 5µl EpiTech HRM KIT Mastermix, 0.05µl Primer forward, 0.05µl Primer reverse, 3.9µl nuclease free water (NFW) and 1µl DNA were added. For each run 5 standards were applied, made out of CaCo cells. Standard 1 to 5 was methylated with 8.7%, 26.5%, 44.4%, 62.2% and 80%.

3.5 DNA extraction (stool) and real time PCR

The real-time polymerase chain reaction (PCR) is used for absolute and relative quantification of microbial DNA. For specific amplification of one species sensitive and specific primers have to be designed.

For the PCRs of the microbiota we extracted the DNA from the stool samples using QIAamp Fast DNA Mini Stool Kit and diluted with NFW to a 5ng/ µl concentration. The quantity of bacteria was measured using TaqMan qPCR and SYBER Green qPCR in a Rotergene 3000.

Each well contained half of the total 10µl KAPA™ SYBR® FAST qPCR Mastermix or KAPA™ PROBE® FAST qPCR Mastermix, each 1µl Primer forward and Primer reverse, 2µl DNA template and 1µl sonde if using a probe (see table beneath), otherwise 1µl NFW, like for *Lactobacilli*.

Bakterien	**Primer**	**Sequenz 5´-3´**	**PCR Programm**	**Standard**
Lactobacilli	Lac1	AGCAGTAGGGAATCTTCCA	95° 10´ (95° 30", 61° 60", 72° 50") x 40	*L. casei*
	Lac2	ATTYCACCGCTACACATG		
Bacteroidetes	allBacteroid296f	GAGAGGAAGGTCCCCAC	95° 10´ (95° 30", 60° 30", 72° 50") x40	DSM 2079
	allbacteroid412r	CGCTACTTGGCTGGTTCAG		
	allbacteroid375P	CCATTGACCAATATTCCTCACTGCTGCCT		
Cluster IV	C.lept F	GCACAAGCAGTGGAG T	95° 10´ (95° 30", 55° 30", 72° 50") x40	*C.leptum*

	C.lept R	CTTCCTCCGTTTTGTCAA		
	C.lept PE	(FAM)-AGGGTTGCGCTCGTT-(BHQ-1)		
Cluster XIVa	195 F	GCAGTGGGGAATATTGCA	95° 10´ (95° 15", 56° 15", 72° 45") x40	*C. blautia ger.*
	C.cocc R	CTTTGAGTTTCATTCTTGCGAA		
	C.cocc PE	AAATGACGGTACCTGACTAA		

The procedure of the DNA extraction from the stool is enclosed in the appendix.

3.6 Metabolic Types

The aim of this study was, putting genetic epigenetic and the human microbiom together and to classify different metabolic types, identify the individual obesity and diabetes risk and moreover give advices for the personal nutrition and sport type.

For our study, in total we chose 12 SNPs. Seven of them are associated with obesity and DM2. *MC4R* rs17782313, *TCF7L2* rs7903146, *IL6* rs1800795, *SLC6A14* rs2011198, *FTO* rs9939609, *PPARG2* rs1801282 have been associated either with BMI and obesity or DM2 (2). Moreover *MC4R* rs17782313 and *LEPR* rs9436740 are linked with satiety, *IL6* rs1800795 with weight regain and *SLC6A14* rs2011198 with eating disorder development. Others like *TFAP2B* rs987237, *FADS1* rs174547, and *ADRB3* rs4994 but also *FTO* rs993609 and *TCF7L2* rs7903146 are connected with affinities to different metabolisms. The *ACE* gene can divide our participants into different sport types (43,44,56,55,57).

For the classification of the different Metabotypes we focused only at the SNPs, which are correlated to nutrition and metabolism.

According to literature humans with a risk allele at the *FADS1* rs174547 have a decreased HDL and high cholesterol (42). Therefore a low fat diet is recommended. Carriers of the risk allele at the *ARDB3* gene have a low lipolysis and a low fat diet would be accurate. For risk allele carriers of *FTO* rs9939609 and *PPARG2* rs1801282 a high protein diet is recommended. Heterozygous and homozygous forms of *TFAP2B* rs987237 should consume a high fat diet. The same recommendation exists for the wild type form of *TCF7L2* rs7903146 (19,24,27).

how u should eat				
	SNP	**Ponits**	**No SNP**	**Points**
Glyco Type	FADS1		PPARG2	
	ARDB3		FTO rs99	
			TFAP2B	
Protein Type	FTO rs99		TFAP2B	
	PPARG2			
	TCF7L2			
	ARDB3			
Fat Type	TFAP2B		ARDB3	
			FTO rs99	
			TCF7L2	
			FADS1	
Balanced Type			TFAP2B	
			FADS1	
			FTO rs99	
			ADRB3	
			PPARG2	

Table 2: Classification of the metabotypes

This table shows how we divided the SNPs according the different Metabotypes. In each category it is possible to have either a SNP or no SNP. Like Martinez et al. we gave points from zero to two for each SNP (2).

In the column SNP, for the wild type form the participant collects zero points, one point for the heterozygous and two for the homozygous form of a SNP. The other way

around would be like for the no SNP column. Here, the test person collects zero points if a proband has a homozygous SNP, one point for a heterozygous form and two points for the wild type form. For each category it is possible to collect up to ten points. After collecting points and calculating them together, the test person can be divided in a metabotype according the category with the highest score. In case a participant has the same scores in different Metabotypes, a closer look at the different SNPs is required for a reasonable decision.

	SNP	Points	No SNP	Points
obesity risk	**MC4R**			
	TCF7L2			
	SLC6A14			
	ARDB3			
	FTO rs99			
DM2 risk	ARDB3			
	TCF7L2			
	IL6			
Sport type and general informations	ACE			
	FTO rs11			
	LEPR			
	SLC6A14			

Table 3: classification obesity risk, DM2 risk and health information

For scoring the obesity and diabetes risk we used the same procedure as for the metabotypes. The genes *MC4R*, *TCF7L2*, *SLC6A14*, *ARDB3* and *FTO* are associated with obesity. In total ten points could have been collected. *ARDB3*, *TCF7L2* and *IL6* are linked with a higher risk of DM2 for risk allele carriers. Here, in total six points could have been collected. In these two categories the collected points in each category were converted into percentages. 0-20% is seen as a low risk for obesity and scored for the statistics as zero. 21-40%, scored with 1, is seen as a mediate risk and results above 41%, scored with 2, demonstrate a high risk for developing obesity. Exactly the same procedure was used for Diabetes risk score.

Participants with the CC allele in rs4341 polymorphism of the *ACE* gene should focus on sport variants based on endurance. GG allele carrier can be recommended sport

connected to speed and strength. Heterozygous test persons can be seen as balanced. The *LEPR* gene in this case gives information about the individual satiety of the test persons. Participants with SNPs are likely to eat more, because of a decreased satiety. *SLC6A14* gene is not only correlated to diabetes. It can predict the affinity of developing eating disorders (11,43,44,56,57).

The field nutrigenetics offers a new opportunity to evaluate the role of metabolism determined genes and disorders related genes and further use it as a benefit for a personalized nutrition. The unique genetic predisposition determines the need of the amount of macronutrients, which is the reason why dietary interventions should be considered on an individual basis (5).

3.7 Statistics

For statistical analysis we used SPSS. Correlation between SNPs and epigenetic markers were made with non-parametric tests using Kruskal-Wallis-Test or Mann-Whitney-U, if looking at a variable with only 2 factors.

4. Results

Figure 6 shows the contribution of the different metabotypes using the SNP analysis. In total we found 23 balanced types, 7 glyco, 2 protein and 5 fat types.

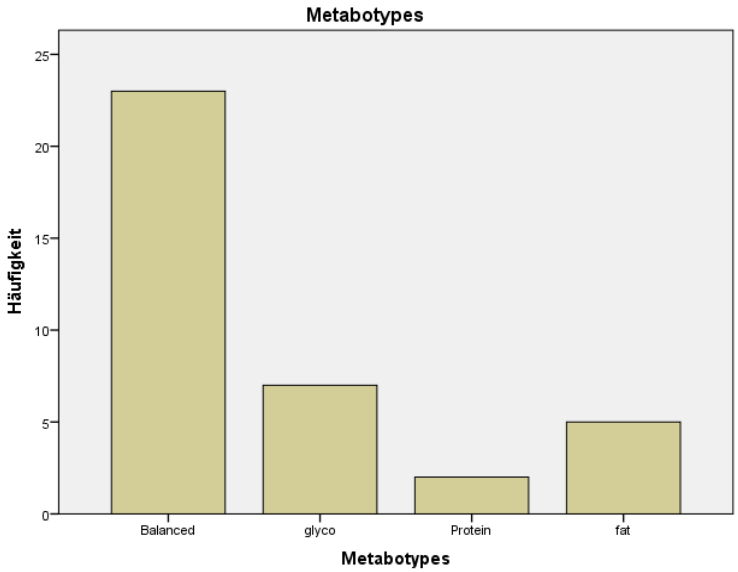

Figure 6: Different Metabotypes

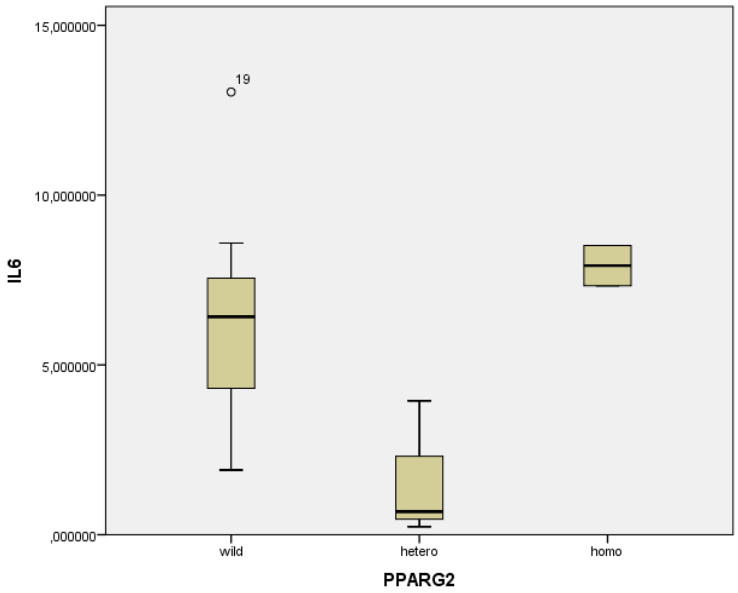

Figure 7: Boxplot *IL6* methylation and *PPARG2*

Ränge

	PPARG2	N	Mittlerer Rang
IL6	wild	16	11,75
	hetero	3	3,00
	homo	2	17,00
	Gesamt	21	

Statistik für Test[a,b]

	IL6
Chi-Quadrat	7,091
df	2
Asymptotische Signifikanz	,029

a. Kruskal-Wallis-Test

b. Gruppenvariable: PPARG2

Ränge

	PPARG2	N	Mittlerer Rang	Rangsumme
IL6	wild	16	11,31	181,00
	hetero	3	3,00	9,00
	Gesamt	19		

Statistik für Test[a]

	IL6
Mann-Whitney-U	3,000
Wilcoxon-W	9,000
Z	-2,348
Asymptotische Signifikanz (2-seitig)	,019
Exakte Signifikanz [2*(1-seitig Sig.)]	,014[b]

a. Gruppenvariable: PPARG2

b. Nicht für Bindungen korrigiert.

Table 4: SPSS output *PPARG2* and *IL6* methylation

The Kruskal-Wallis- test comes to a significant correlation between *PPARG* and the methylation of *IL6.* Heterozygous test persons have a decreased methylation of *IL6* (Figure 7). The result has to be considered with caution, looking at the participant contribution. The same result can be seen for the *LEPR* with a p-value < 0.05 between wildtype and heterozygous type.

Considering *LINE1* we see as well a significant correlation with *PPARG* in the opposite direction. Test persons with a risk allele have a higher methylation in *LINE1* (Figure 8).

In figure 10 we show the sport frequency between the different sporttypes: In comparison of the endurance sporttype the strenght type are more keen to pratice sport daily or more times a day. Looking at the methylation there is a significant differnce between this two sport types and the *IL6* methylation (Figure 9). The bacterial amount of *Cluster IV* is significantly decreased in stenght sporttypes (Figure 11).

Thus, our participants have a significant increase in *Cluster IV* abundance, if practicing sport daily or 4-6 times a week compared to 1-3 times a week or 1-3 times a month.

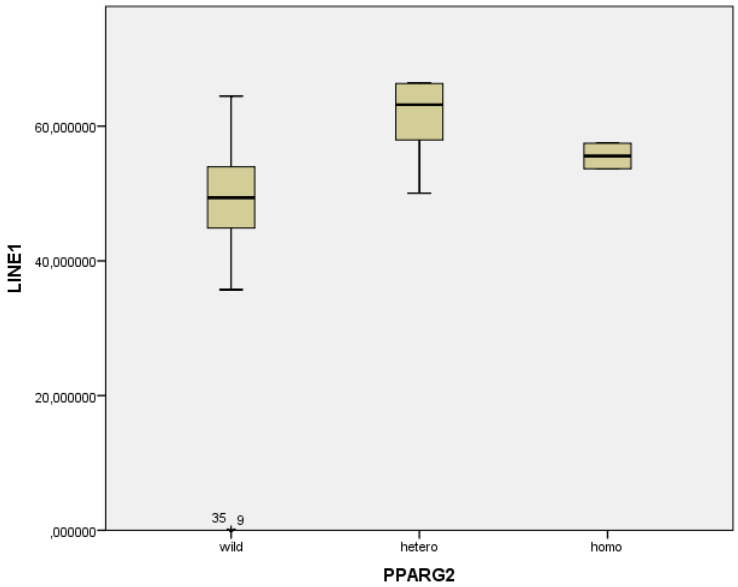

Figure 8: Boxplot *PPARG* and *LINE1* methylation

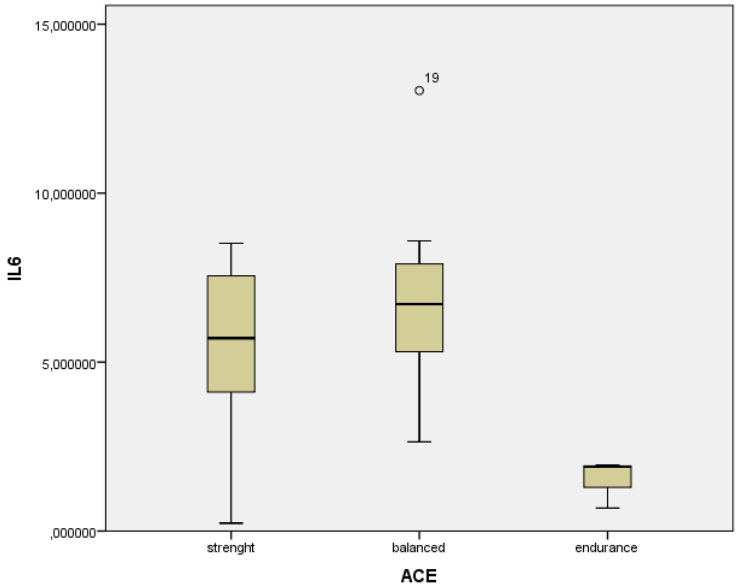

Figure 9: Boxplot *ACE* and *IL6* methylation

Verarbeitete Fälle

	Fälle					
	Gültig		Fehlend		Gesamt	
	N	Prozent	N	Prozent	N	Prozent
ACE * Körperliche_Bewegung_t0	37	100,0%	0	0,0%	37	100,0%

ACE * Körperliche_Bewegung_t0 Kreuztabelle

Anzahl

		Körperliche_Bewegung_t0					
		mehrmals täglich	täglich	4-6x/W	1-3x/W	1-3x/M	Gesamt
ACE	strenght	6	6	2	2	0	16
	balanced	4	3	2	2	2	13
	endurance	0	3	0	5	0	8
Gesamt		10	12	4	9	2	37

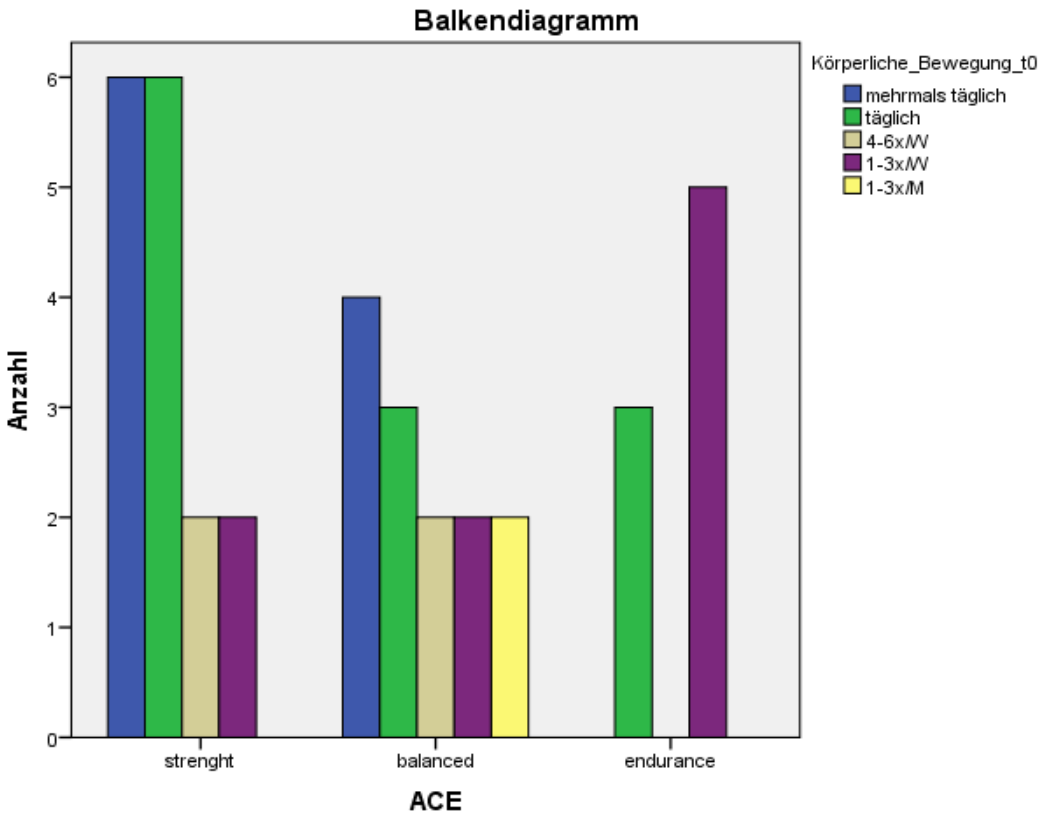

Figure 10: Sport types and physical activity

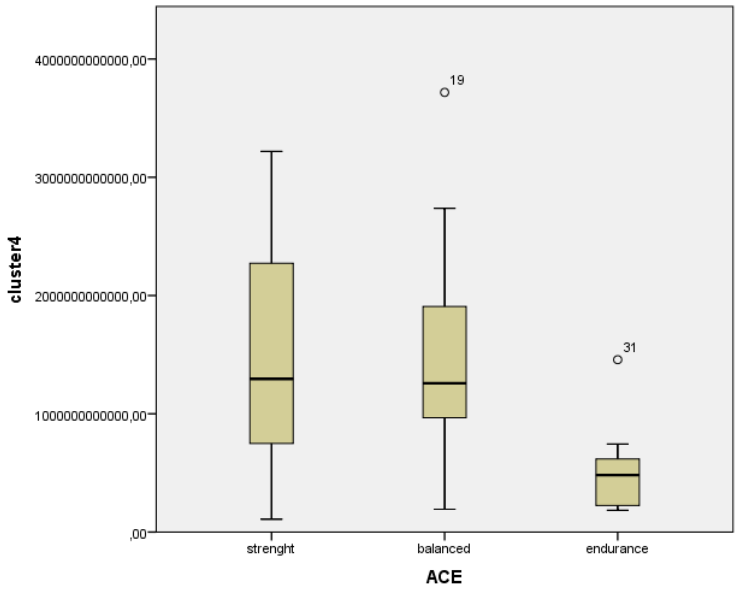

Figure 11: *Cluster IV* and different sport types

Ränge

	ACE	N	Mittlerer Rang
cluster4	strenght	16	21,50
	balanced	13	21,62
	endurance	8	9,75
	Gesamt	37	

Statistik für Test[a,b]

	cluster4
Chi-Quadrat	7,455
df	2
Asymptotische Signifikanz	,024

a. Kruskal-Wallis-Test

b. Gruppenvariable: ACE

Ränge

	ACE	N	Mittlerer Rang	Rangsumme
cluster4	strenght	16	15,13	242,00
	endurance	8	7,25	58,00
	Gesamt	24		

Statistik für Test[a]

	cluster4
Mann-Whitney-U	22,000
Wilcoxon-W	58,000
Z	-2,572
Asymptotische Signifikanz (2-seitig)	,010
Exakte Signifikanz [2*(1-seitig Sig.)]	,009[b]

a. Gruppenvariable: ACE

b. Nicht für Bindungen korrigiert.

Table 5: SPSS output sport types and *Cluster IV*

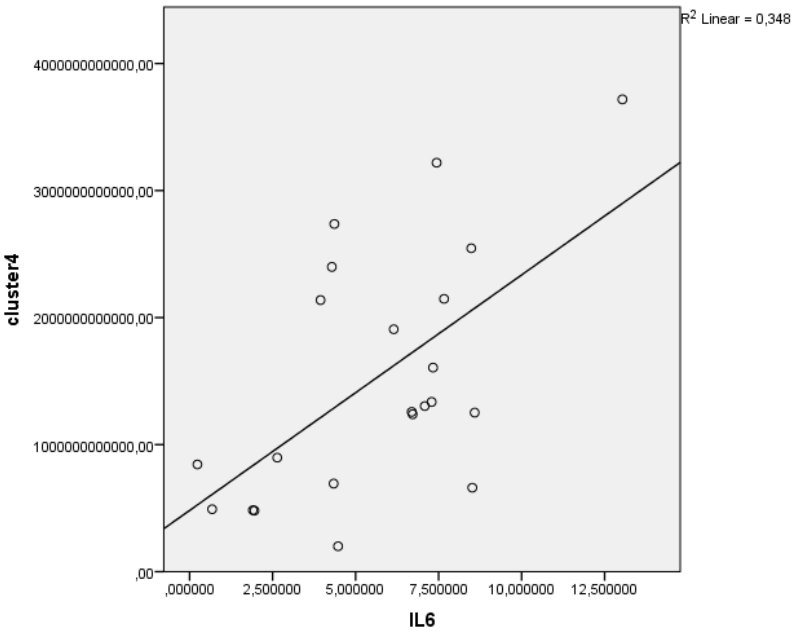

Figure 12: IL6 Methylation and amount of *Cluster IV* bacteria

Comparing the two outcomes the logistic regression shows a trend for the *IL6* methylation and *Cluster IV*. The amount of bacteria in this family increases with methylation percentage of IL-6 (Figure 7).

Thus, our participants have a significant increase in *Cluster IV*, if practicing sport daily or 4-6 times a week compared to 1-3 times a week or 1-3 times a month.
The same results can be regarded with the *Firmicutes*. Subjects with higher amount of *Firmicutes* show an increased IL6 methylation and were found more in the strength and speed sport type.

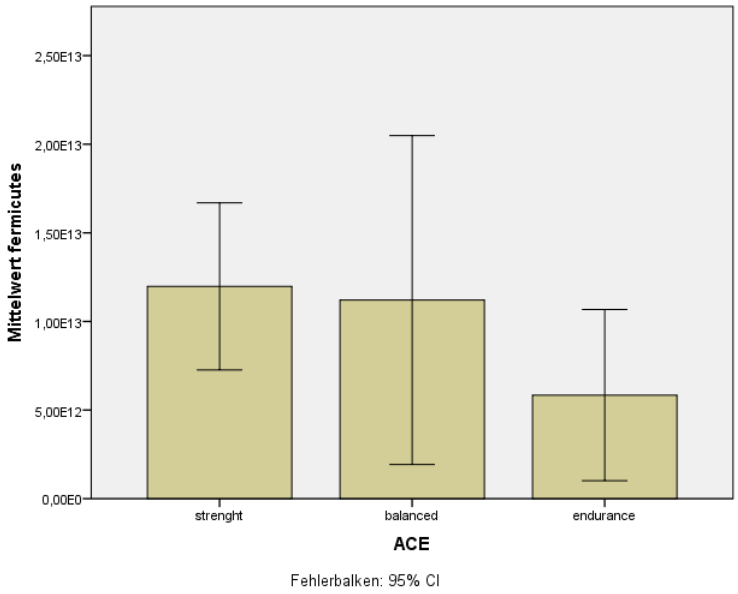

Figure 13: Sport types and amount of *Bacteroidetes*

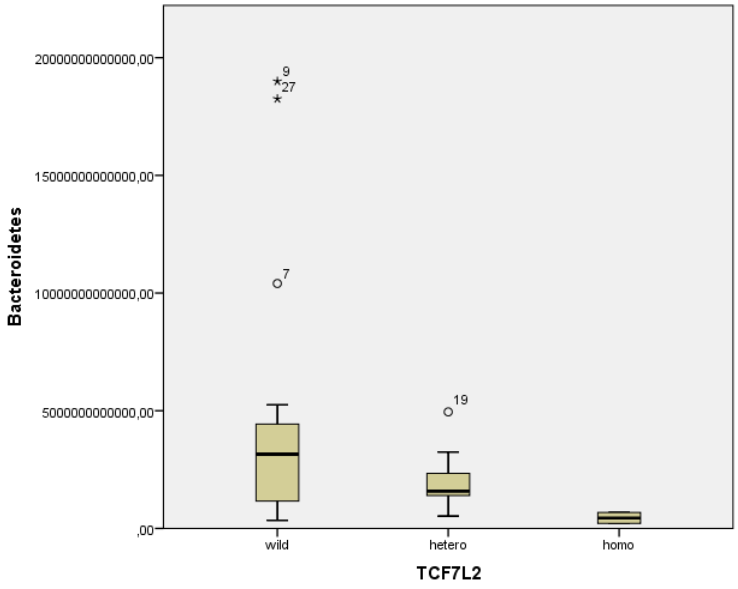

Figure 14: *TCF7L2* and the amount of *Bacteroidetes*

Ränge

	TCF7L2	N	Mittlerer Rang
Bacteroidetes	wild	19	22,21
	hetero	16	17,19
	homo	2	3,00
	Gesamt	37	

Statistik für Test[a,b]

	Bacteroidetes
Chi-Quadrat	6,490
df	2
Asymptotische Signifikanz	,039

a. Kruskal-Wallis-Test

b. Gruppenvariable: TCF7L2

Table 6: SPSS output *TCF7L2* and *Bacteroidetes*

Considering SNPs and gut bacteria, a significant correlation between *Bacteroidetes* and *TCF7L2* which is associated with the risk for diabetes, can be demonstrated with the figure 14. Looking at *LEPR*, there is as well a trend in the amount of *Bacteroidetes.* None carriers of the risk allele have higher amount of *Bacteroidetes* than compared with carrier of the risk allele. Both SNPs can be put in association with obesity further diabetes. Testing *Bacteroidetes* and the obesity risk, shows a significant outcome with a p-value < 0.049 but can be regarded as a trend. Participants with a low risk for diabetes have the highest abundance of this species.

Korrelationen

			bread_noodles_rice_potatoes_kat	Bacteroidetes
Kendall-Tau-b	bread_noodles_rice_potatoes_kat	Korrelationskoeffizient	1,000	-,312*
		Sig. (2-seitig)	.	,022
		N	40	37
	Bacteroidetes	Korrelationskoeffizient	-,312*	1,000
		Sig. (2-seitig)	,022	.
		N	37	37

*. Die Korrelation ist auf dem 0,05 Niveau signifikant (zweiseitig).

Table 7: SPSS output *Bacteroidetes* and FFQ

Recent studies show that a higher consumption of foods with carbohydrates result in higher numbers of *Bacteroidetes*. This fact can underline and strengthen our result in the correlation of *Bacteroidetes* and bread, noodle, rice, potatoes. Some other correlations could be seen with bacteria and the FFQ, like *Prevotella* and vegetable, legumes, fruits and *Bacteroidetes* and eggs (58).

Korrelationen

			obesityrisk	IL6
Kendall-Tau-b	obesityrisk	Korrelationskoeffizient	1,000	-,235
		Sig. (2-seitig)	.	,075
		N	37	37
	IL6	Korrelationskoeffizient	-,235	1,000
		Sig. (2-seitig)	,075	.
		N	37	37

Table 8: No correlation between obesity risk and *IL6* methylation

Korrelationen

			DM2risk	IL6
Kendall-Tau-b	DM2risk	Korrelationskoeffizient	1,000	-,289*
		Sig. (2-seitig)	.	,027
		N	37	37
	IL6	Korrelationskoeffizient	-,289*	1,000
		Sig. (2-seitig)	,027	.
		N	37	37

*. Die Korrelation ist auf dem 0,05 Niveau signifikant (zweiseitig).

Table 9: DM2 risk and *IL6* methylation

Korrelationen

			obesityrisk	Bacteroidetes
Kendall-Tau-b	obesityrisk	Korrelationskoeffizient	1,000	-,260*
		Sig. (2-seitig)	.	,049
		N	37	37
	Bacteroidetes	Korrelationskoeffizient	-,260*	1,000
		Sig. (2-seitig)	,049	.
		N	37	37

*. Die Korrelation ist auf dem 0,05 Niveau signifikant (zweiseitig).

Table 10: Correlation of obesity risk and *Bacteroidetes*

Korrelationen

			DM2risk	Bacteroidetes
Kendall-Tau-b	DM2risk	Korrelationskoeffizient	1,000	-,278*
		Sig. (2-seitig)	.	,033
		N	37	37
	Bacteroidetes	Korrelationskoeffizient	-,278*	1,000
		Sig. (2-seitig)	,033	.
		N	37	37

*. Die Korrelation ist auf dem 0,05 Niveau signifikant (zweiseitig).

Table 11: Correlation between DM2 risk and *Bacteroidetes*

Korrelationen

			DM2risk	TCF7L2
Kendall-Tau-b	DM2risk	Korrelationskoeffizient	1,000	,513**
		Sig. (2-seitig)	.	,001
		N	37	37
	TCF7L2	Korrelationskoeffizient	,513**	1,000
		Sig. (2-seitig)	,001	.
		N	37	37

**. Die Korrelation ist auf dem 0,01 Niveau signifikant (zweiseitig).

Table 12: Correlation between SNP and DM2 risk

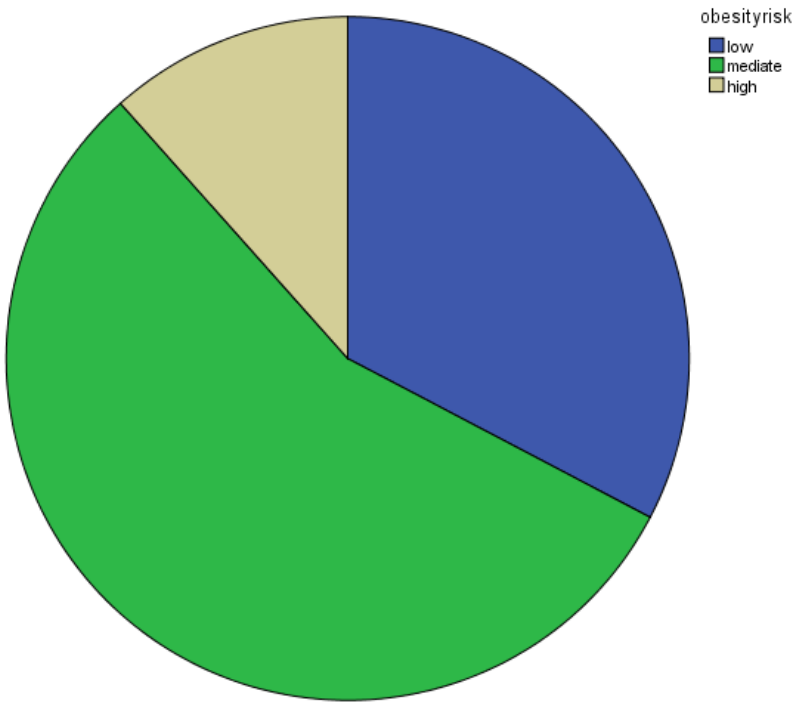

Figure 15: Amount of *Bacteroidetes* in association with obesity risk

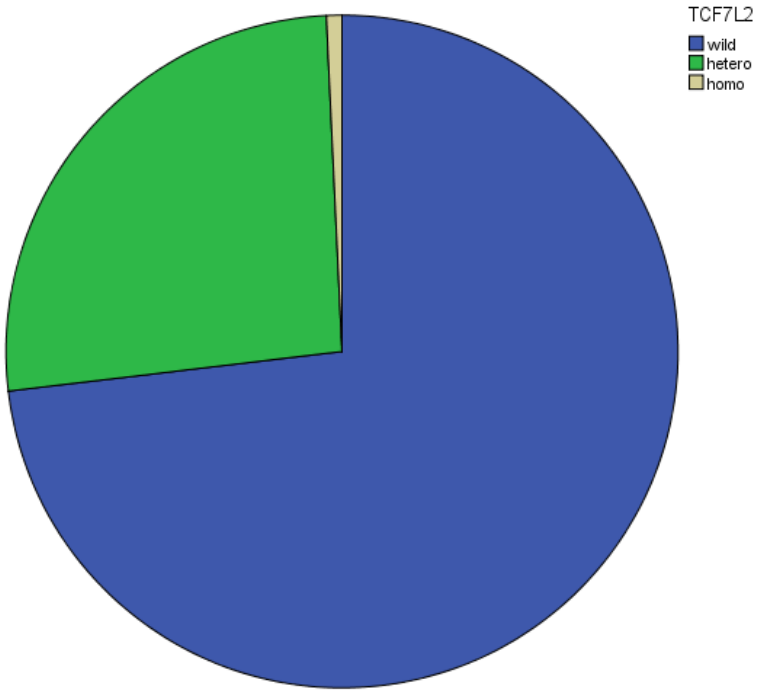

Figure 16: Amount of *Bacteroidetes* in association with TCF7L2 SNP

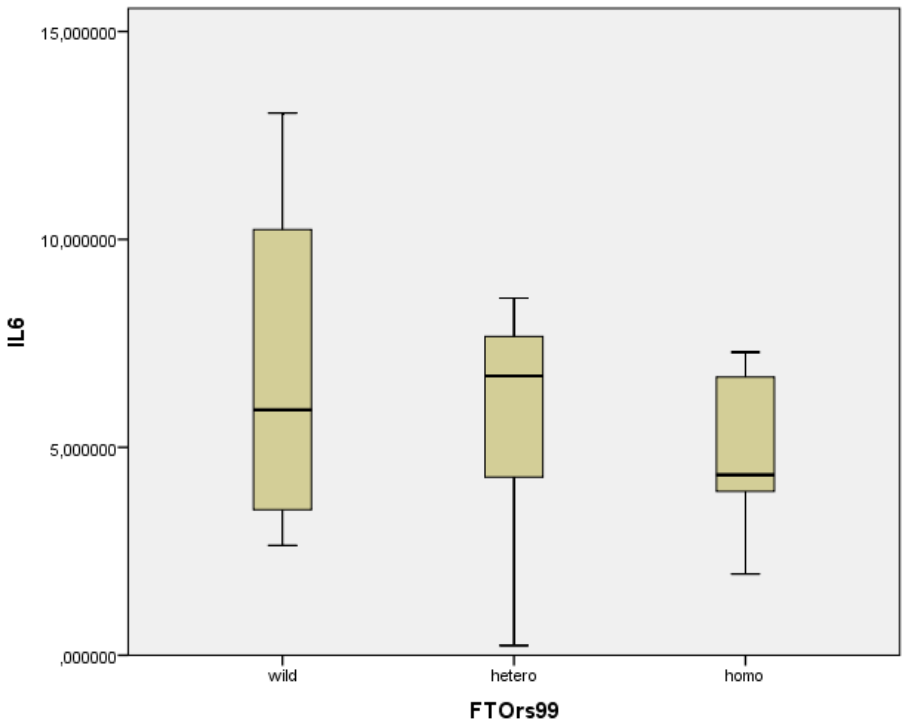

Figure 17: No significant correlation between IL6 methylation and FTO SNP

5. Discussion

SNPs, copy number variation, insertion and deletion and telomere length are genetic variants that affect the different response to diet. Moreover epigenetic markers can be used as predictors for adult onset diseases and to individual response to diet (48). Proteins, carbohydrates and lipids have a different metabolic role in energy homeostasis. Thus, a diet with similar energy content but with different macronutrient division can influence metabolism, appetite and thermogenesis differently (45). However, not only the quantity but also the quality plays an important role. Types of fatty acids, amino acids and carbohydrates have different pathways further effecting nutrient metabolism according to genotype (48). All these nutritional components are as well important sources for the gut microbiome. The gut bacteria are affecting metabolic functionality and further defining the host´s health (49). To our knowledge our study is the first that investigated all these aspects to provide a personalized nutrition.

Our main findings identify an association between genetic, epigenetic and gut microbiota variations. Considering the outcome for the strength sport type; they have a higher amount in *Firmicutes* in general and in *Cluster IV* and a higher methylation of *IL6*, which could be verified with testing *Firmicutes* or *Cluster IV* against *IL6*. This means, a high amount of *Cluster IV* or in general *Firmicutes*, which is mostly seen in more likely obese humans or human with higher BMI (58), is resulting in a low weight loss which is also demonstrated with the high methylation of *IL6*. *Firmicutes* and *Cluster IV* are also correlating with inflammatory and stress level according to FFQ. Remely et al. mentions that *Cluster IV* and *Cluster XIVa* is decreasing in people with DM2 after weight loss, which means people with higher BMI have an higher abundance of these bacteria, which we also could show with *Cluster IV* (58; 60).

Interestingly, we could find an association between high methylation of *IL6* and a lower risk of diabetes and obesity. Aumueller et al. mentioned a higher methylation of IL6 deteriorates weight loss during intervention (53). The opposite was shown in the study of Remely and Haslberger, where subjects were high methylated in *IL6* after

weight loss, thus leaner and lower obesity risk (60), what we also could demonstrate with our study. The correlation between *IL6* SNP and DM2 risk results in a p value of 0.004, which can be interpreted as heterozygote carrier have a higher risk for DM2 than wildtype and homozygote have a higher risk than heterozygote.

We could also see that the wildtype form of *TCF7L2* and *LEPR* have a higher amount of *Bacteroidetes*, which were shown to be higher abundant in lean person. Both SNPs can be associated with obesity and further diabetes. Carrying no risk allele means participants are more likely lean, thus have a less risk for obesity and DM2. This outcome could be underlined by showing a trend of the correlation of a high amount of *Bacteroidetes* with a low risk for obesity (32).

LINE 1 is considered to be high methylated in participants with high BMI (50). Our study demonstrates a trend in this direction. Moreover we observe the interaction of *LINE1* with *PPARG2*, whereas heterozygotes was higher methylated, thus having a higher BMI. We come to the same result which is significant with a p value < 0.05. Controversially, the wildtype for *PPARG2* shows a positive correlation with *IL6,* which would mean that wildtype carriers have a less efficiency to lose weight but a lower risk to develop DM2.

We cannot demonstrate any differences in methylation or gut microbiom in the different metabotypes, beside *Cluster IV* is different in balanced and protein- typ, but this outcome has to be interpreted with caution, because of the population size in the protein metabotype.

BMI does not correlate with diabetes or obesity risk in our study, although it is known that a higher BMI is indicator for obesity and further can develop diabetes (62). The reason could be the small sample size.

Nevertheless this study demonstrated that the interactions between genetic, epigenetic variations, the gut microbial composition and their influences through diet and lifestyle but also physical activity are relevant for genotype based interventions.

Further there is an enormous potential in developing personalized diets based on the genotype (45).

5.1 Limitations

Sample size for this type of study must be much higher in order to identify reliable associations. In addition, dietary habits and genetic background differ between populations. Thus, drawing conclusions for other populations should be done carefully.

Dietary consumption is self reported. Inaccuracies in memory bias the result.

DNA methylation markers can predict the vain of weight regain. For this other epigenetic markers should have been investigated too, such as **tumor necrosis factor** (TNF) alpha**,** proopiomelanocortin (POMC) **or** neuropeptide **Y** (NPY). TNF- alpha correlates with a hypermethylation in increasing body fat. POMC is higher methylated in people with a great response to weight regain. NPY predicts weight regain if hypomethylated (4).

Thus, methylation is changing over time within an individual and further narrow diet interventions. In this case it would have been interesting to investigate methylation from two different time points and investigate possible changes.

6. Conclusion

Humans differ in height, weight, activity, cognition, strength, endurance and their preference for food, due to a wide range of biological variables. These variables can be allelic polymorphisms and due to environmental influences (7). DNA methylation is connected with the response to dietary interventions, weight loss and regain and developing diseases e.g. metabolic disorders (4).

With increasing knowledge of gene- diet interactions for macronutrient and micronutrient it will be possible to give recommendations based on the genetic make-up (18).

Dietary recommendations are for general population, only a few subgroups are considered for additional dietary advice (18). We know that all individuals within a population are not similar healthy when consuming the same food. Consumption of too many calories, imbalance of the different macronutrients and micronutrients and lifestyle result in metabolic imbalances and even disorders. Thus, medical care is increasing massively as atherosclerosis, diabetes, obesity; hypertension and allergy are increasing more and more worldwide. Still it is not yet clear which industrial processes and economic models are needed to finance the personalization of diet (7) The use of SNP technologies is already been practiced. To provide individuals with the knowledge of their gene predisposition for diet, lifestyle and even drugs will become more commercial reality (7). However testing alone is not enough. Participants should not draw their own conclusion from the results. This could lead to suboptimal self-administration of dietary advices. Involving health professionals can ensure the optimum of patient's benefit (45).

7. Summary

Metabolic diseases are a central burden for public health and heath care. Mechanisms contributing to metabolic diseases such as obesity and DM2 include genetic risk factors, epigenetic dysregulation, dysbalance of microbiom, lifestyle and nutrition. There is increasing evidence that genetic, epigenetic and microbiota aspects contribute to individual mechanisms, which result in individual pathways for metabolism and energy extraction from food. Genetic dispositions, such as SNPs are under scientific investigation but as well already in commercial use for defining metabolic types (metabotypes). These metabotypes define risk for metabolic diseases, preferences for energy extraction from food and individualized concepts for weight management or weight loss. However, until know metabotypes are mainly based on genetic disposition and do not consider environmental and nutritional effects on gene regulation. The objective of this study was to substantiate the need to integrate epigenetic and microbial analysis in the genetic analysis of metabotypes.

Genetic disposition based on analysis of 12 SNPS, further 2 epigenetic markers and 4 main groups of gut microbiota were analyzed in 37 subjects. Additionally food frequency questionnaires were assessed.

Results show that SNPS can be clearly attributed to metabotypes. Analysis of DNA methylation strengthens the outcome. Either gut microbiota composition shows significant correlation with SNP and methylation according to metabotype clustering.

Thus epigenetic and microbiota analysis significantly substantiate the classification of metabotypes and should be used as an important tool for improved individual nutritional counseling.

8. Zusammenfassung

Metabolische Krankheiten sind eine wesentliche Belastung für das Gesundheitswesen und Gesundheitsfürsorge. Zu den Mechanismen welche zur Metabolischen Krankheiten, wie Diabetes und Übergewicht beitragen, sind genetische Risikofaktoren, epigenetische Fehlregulation, Fehlbalance des Mikrobioms, Lebensstil und Ernährung inkludiert. Es gibt zunehmend Beweise, dass sich genetische, epigenetische und mikrobiotische Aspekte an individuelle Mechanismen beteiligen, welches in individuelle Stoffwechselwege des Metabolismus und Energieextraktion aus dem Essen resultiert. Genetische Disposition, unter anderem SNPs, sind in der wissenschaftlichen Forschung aber auch schon in kommerziellen Gebrauch um verschiedene metabolische Typen zu definieren. Diese Metabotypes definieren das Risiko für metabolische Krankheiten, Präferenz der Energieextraktion aus dem Essen und individuelle Konzepte für Gewichtsmanagement oder Gewichtsverlust. Bis heute basieren Metabotypes hauptsächlich anhand genetischer Disposition und umfassen nicht Umwelt- und Ernährungseinflüsse auf die Genregulation. Die Ziele dieser Studie war es die Nutwendigkeit des Miteinbeziehenden epigenetischer und mikrobieller Analysen in die genetische Analyse für Metabotypes zu untermauern.

Genetische Disposition wurde anhand von 12 SNPs analysiert, weiter wurden zwei epigenetische Marker und 4 Bakterienstämme 37 Probanden untersucht. Zusätzlich wurde ein Ernährungsfragebogen durchgeführt. Die Ergebnisse zeigen, dass sich Metabotxpes durch SNPs einteilen lassen. Epigenetische Untersuchungen verstärken dieses Ergebnis. Aber auch mikrobielle Untersuchungen zeigen einen signifikanten Einfluss auf SNPs und Methylierungen.

Deshalb untermauern die Epigentik und Mikrobiota signifikant die Einteilung der Metabotypes und sollten als eine wichtige Methode zur verbesserten individuellen Ernährungsberatung verwendet werden.

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10. Appendix

11.1 Food frequency questionnaire

Name:	
Geschlecht:	
Gewicht in kg:	
Größe in cm:	
Datum:	

Sind Sie mit Ihrem Gewicht zufrieden?			
ja			
nein			
weiß nicht			

Leiden Sie oft an einer dieser Entzündungen und/oder Infektionen?	
Grippale Infekte	
Entzündungen der Haut	
Entzündungen im Mundraum	
Entzündungen am Auge, Bindehautentzündungen	
Sonstiges: _____	
Entzündliche Erkrankungen im Magen-/Darmbereich	
Muskel-/Gelenksentzündungen	
Blasenentzündung	
Pilzinfektionen im Genitalbereich	
Pilzinfektionen von Haut oder Füssen	

Nehmen Sie derzeit Medikamente zu sich?	
nein	

ja, folgende: **_____** **_____**	

Rauchen Sie?	
ja	
nein	

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

6. Versuchen Sie den Stresspegel in den folgenden Bereichen grob zu schätzen. (z.B. Arbeit 45%, Freizeit 20%, Familie 35%, insg. 100 %)	
Arbeit	……%
Freizeit	……%
Familie/Partner	……%

Haben Sie eine der folgenden Stoffwechselkrankheiten?	
Diabetes mellitus Typ 1	
Diabetes mellitus Typ 2	
Schilddrüsenüberfunktion	
Schilddrüsenunterfunktion	

Gicht	
Pankreasleiden	
nein	

Fragen zu Ihrem Bewegungsverhalten						
	Mehrmals Täglich	Täglich	4-6x/ Woche	1-3x/ Woche	1-3x/ Monat	nie
Machen Sie regelmäßig körperliche Bewegung? (Einkäufe zu Fuß, Spazieren,...)						
Betreiben Sie regelmäßig Sport im Freien (< 30min mit Schwitzen)? z.B. Wandern, Skifahren, Laufen, Radfahren...						
Betreiben Sie regelmäßig Sport im Fitness-Studio (< 30 min mit Schwitzen)?						

Fragen zu Ihrem Essverhalten						
	Selten/ nie	1-3x/ Woche	3-5x/ Woche	1x/ Tag	2-3x/ Tag	>3x/ Tag
Milch- und Milchprodukte						

Weich- und Hartkäse						
Milch, Joghurt, Molke, Buttermilch						
Rahmprodukte: Süß- und Sauerrahm, Sahne/Obers, Creme Fraiche, Schmand						
Topfen, Frischkäse, Hüttenkäse						
Eier						
Fleisch						
Rind, Schwein, Lamm, Kalb,						
Pute, Huhn						
Fleisch- und Wurstprodukte						
Wurstaufschnitt, Salami						
Grill-, Brat-, Kochwürste						
	Selten/ nie	1-3x/ Woche	3-5x/ Woche	1x/ Tag	2-3x/ Tag	>3x/ Tag
Gemüse						
Kartoffel						
Hülsenfrüchte: Erbsen, Bohnen, Sojabohnen, Kichererbsen						
Zwiebelgemüse: Lauch, Zwiebel, Knoblauch, …						
grünes Blattgemüse, Kohlgemüse und Salat						

Sonstiges: Tomaten, Paprika, Zucchini, Karotten, etc.						
Obst						
Frischobst						
Trockenobst						
Fisch						
Fischfilets frisch oder tiefgefroren, ganz oder Block						
Verarbeiteter Fisch: Panierter Fisch, Dosenfisch						
Getreideprodukte Brot, Teigwaren, etc.						
Voll- oder Mehrkornprodukte						
Weißmehlprodukte						
Süßigkeiten						
Süßigkeiten (Schokolade, Keks, Fruchtgummi…)						
Mehlspeisen						
Süße Brotaufstriche: Nutella, Marmelade, etc.						
Knabbereien						
Salzgebäck (Chips, Soletti…)						
Nüsse und Samen						
Öle und Fette						
Tierische Fette: Schmalz,						

Butter						
Pflanzliche Fette: Olivenöl Kernöl, Rapsöl, Maiskeimöl, Nussöle, …						
Fertiggerichte						

Wurde bei Ihnen Laktoseintoleranz, Fruktoseintoleranz, HistaminIntoleranz oder Glutenunverträglichkeit (Zöliakie) festgestellt?
Was?

Wie gut vertragen Sie Milch?	
(Ohne Joghurt oder Käse. Haben Sie nach dem Konsum von Milch Beschwerden wie Bauchschmerzen, übermäßige Blähungen, Durchfall?)	
sehr gut	
leichte Bauchschmerzen	
Durchfall	
Bauchkrämpfe	
Blähungen	
weiß nicht	

Wie gut vertragen Sie Fructose?	
(Haben Sie nach dem Verzehr von Obst oder Fruchtsäften Beschwerden wie Bauchschmerzen, übermäßige Blähungen, Völlegefühl oder andere Beschwerden?)	
keine Beschwerden	
Völlegefühl	
Blähungen im oberen Bauch	
Bauchkrämpfe	

Bauchschmerzen	
Übelkeit	
weiß nicht	

Nehmen Sie Nahrungsergänzungsmittel zu sich?	
Bitte auch ankreuzen, wenn keine regelmäßige Einnahme (Nährstoffe in konzentrierter Form, Bsp. Vitamintabletten, Knoblauchkapseln, ...) Wenn ja, welche	
Ja	
nein	

Fragen zu Ihrem Trinkverhalten								
Wie viel Flüssigkeit nehmen Sie täglich zu sich?	< 1 Liter		1-2 Liter		2-3 Liter		>3 Liter	
Wie viele Tassen Kaffee trinken Sie täglich?	< 1 Tasse		1-2 Tassen		3-5 Tassen		> 5 Tassen	
Wieviele Tassen grünen Tee trinken Sie täglich?	< 1 Tasse		1-2 Tassen		3-5 Tassen		> 5 Tassen	
Wie oft trinken Sie Alkohol?	nie		nur zu Anlässen		1-2/ Monat		2-5/ Monat	
	1-2/ Woche		täglich		mehrmals/ Tag			
Wie viele Portionen Alkohol trinken Sie?	1-2/ Monat		3-4/ Monat		1-2/ Woche		3-5/ Woch	

1 Portion entspricht hierbei 1/3 l Bier, 1/8l Wein/Schaumweine, 1 Schnapsglas (2cl) >15% Alkohol							e	
	1-2 / Tag		≥3/ Tag					

Welche Getränke nehmen Sie hauptsächlich zu sich?			
Mehrfachnennungen möglich			
Kaffee		Verdünnte Säfte	
Wasser still		Tee (schwarz, grün,..)	
Wasser spritzig		Energiegetränke	
Limonade		Tee (Kräuter)	
Cola		Nektar	
Dicksaft		Saft (100% Frucht)	
Sonstiges:			

Haben Sie oft Blähungen?	
ja, den ganzen Tag über	
nur manchmal nach dem Essen	
hin und wieder (2 bis 3 mal in der Woche)	
nie	
bei Reisen	
weiß nicht	

Welche Konsistenz hat Ihr Stuhl?	
flüssig, ohne feste Bestandteile	
einzelne weiche Klümpchen mit unregelmäßigem Rand	
wurstartig mit glatter Oberfläche	
wurstartig mit rissiger Oberfläche	
wurstartig, klumpig	
einzelne, feste Kügelchen, schwer auszuscheiden	

Haben Sie Probleme bei oder mit der Darmentleerung?	
Nein, mein Stuhlgang ist regelmäßig und unproblematisch	
Schmerzen vor und/oder während der Entleerung	
mühsame, portionsweise Darmentleerung	
abwechselnd Durchfall und Verstopfung	
zu selten	

Nehmen Sie regelmäßig Abführmittel zu sich?	
Ja	
Nein	

Gab es im letzten Jahr einen oder mehrere Stoffe auf die Sie allergisch reagiert haben?	
Ja , nämlich	
Nein	

Wie reagierten Sie allergisch? Welche Symptome traten auf?	

Geben Sie bitte an, welche der folgenden Beschwerden bei Ihnen häufiger als gelegentlich auftreten (Anzahl im Monat).		
Symptom	Ja, wie oft	Nach welchen Nahrungsmitteln
Nasenjucken, Niesattcken		
Behinderte Nasenatmung, „Stockschnupfen“		
Heuschnupfen, wässriger Niesschnupfen		
Augentränen, Augenjucken		
Schwellung oder Brennen von Lippen oder Zunge		
Rauhe Mundschleimhaut oder Zunge		
Gaumen-, Hals-, Ohrenjucken		
Häufiges Räuspern, „beschlagene Stimme“		
Atembeklemmungen		
Übelkeit, Erbrechen, Durchfall		
Blähungen, starke Darmgeräusche		
Ekzeme, Hautausschläge, Hautrötungen		
Nesselsucht, Urtikaria, Hautjuken		
Kopfschmerzen, Migräne		
Andere Beschwerden:		

Nehmen Sie Cholesterinsenkende Medikamente zu sich? Wenn ja, welche?	

11.2 General health questionnaire

Ihr allgemeiner Gesundheitszustand der letzten Wochen

Liebe/r Teilnehmer/in,

im Zuge der Studie möchten wir Ihren Gesundheitszustand erfassen und bitten Sie daher, sich Zeit zu nehmen um die folgenden Fragen zu beantworten. Bitte beantworten Sie ALLE Fragen indem Sie **zu jeder Frage immer nur eine Box ankreuzen**

Haben Sie in letzter Zeit:		**1 = überhaupt nicht**	**2 = wie üblich**	**3 = mehr als durchschnittlich**	**4 = sehr oft/viel**
F1	die Fähigkeit gehabt sich auf alle Ihre Tätigkeiten zu konzentrieren?	☐ 1	☐ 2	☐ 3	☐ 4
F2	Schlafprobleme* gehabt a.G. von Sorgen?	☐ 1	☐ 2	☐ 3	☐ 4
F3	das Gefühl gehabt ein wichtiger Teil von etwas zu sein?	☐ 1	☐ 2	☐ 3	☐ 4
F4	das Gefühl gehabt, Sie sind fähig Entscheidungen zu treffen?	☐ 1	☐ 2	☐ 3	☐ 4
F5	das Gefühl gehabt, konstant angespannt zu sein?	☐ 1	☐ 2	☐ 3	☐ 4
F6	das Gefühl gehabt, Sie können Schwierigkeiten nicht bewältigen?	☐ 1	☐ 2	☐ 3	☐ 4

		1 = **überhaupt nicht**	2 = **wie üblich**	3 = **mehr als durchschnittlich**	4 = **sehr oft/viel**
F7	die Fähigkeit gehabt normale Alltagsaktivitäten zu genießen?	1	2	3	4
F8	die Fähigkeit gehabt, Ihren Problemen ins Gesicht zu sehen?	1	2	3	4
F9	das Gefühl von Unzufriedenheit und Niedergeschlagenheit empfunden?	1	2	3	4
F10	das Vertrauen in Sich selbst verloren?	1	2	3	4
F11	das Gefühl gehabt kein wichtiger Mensch zu sein?	1	2	3	4
F12	sich im Großen und Ganzen zufrieden gefühlt?	1	2	3	4

* Schlafprobleme: Schwierigkeiten beim einschlafen, Störungen des Durchschlafens, schlechte Schlafqualität

11.3 DNA extraction blood spots

• This protocol is suitable for blood, both untreated and treated with anticoagulants, which has been spotted and dried on filter paper (Schleicher and Schuell 903).

• Prepare an 85°C water bath for use in step 2, a 56°C water bath for use in step 3, and a 70°C water bath for use in step 4.

• Ensure that Buffers AW1 and AW2 have been prepared according to the instructions on page 24.

• If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 56°C.

• All centrifugation steps are carried out at room temperature (15–25°C).

1. Place 3 punched-out circles from a dried blood spot into a 1.5 ml microcentrifuge tube and add 180 µl of Buffer ATL.

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

2. Incubate at 85°C for 10 min. Briefly centrifuge to remove drops from inside the lid.

3. Add 20 µl Proteinase K stock solution, mix by vortexing, and incubate at 56°C for 1 h. Briefly centrifuge to remove drops from inside the lid.

Note: The addition of Proteinase K is essential.

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

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

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

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

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

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

6. Carefully apply the mixture from step 5 to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g*

(8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Close each spin column in order to avoid aerosol formation during centrifugation.

7. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

8. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 9, or to eliminate any chance of possible Buffer AW2 carryover, perform step 8a, and then continue with step 9.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, coming into contact with the QIAamp Spin Column. Removing the QIAamp Spin Column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Spin Column. In these cases, the optional step 8a should be performed.

8a. (Optional): Place the QIAamp Spin Column in a new 2 ml collection tube (not provided)

and discard the collection tube with the filtrate. Centrifuge at 20,000 x *g*

(14,000 rpm) for 1 min.

9. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 150 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

Note: Do not elute the DNA with volumes of less than 100 µl.

Three punched-out circles (3 mm diameter) typically yield 150 ng and 75 ng of DNA from anticoagulated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3.

The volume of the DNA eluate used in a PCR assay should not exceed 10%, e.g.,

for a 50 µl PCR, add no more than 5 µl of eluate.

11.4 Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA

DNA amounts of 1 ng – 2 µg in a volume of up to 20 µl can be processed using this standard protocol.

Things to do before starting

1. Add 30 ml ethanol (96–100%) to Buffer BW and store at room temperature (15–25°C). Invert the bottle several times before starting the procedure.
2. Add 27 ml ethanol (96–100%) to Buffer BD and store at 2–8°C. Invert the bottle several times before starting the procedure and make sure to close the bottle immediately after use. White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the EpiTect spin column.

3. Add 310 µl RNase-free water to the lyophilized carrier RNA (310 µg) to obtain a 1 µg/µl solution. Dissolve the carrier RNA thoroughly by vortexing. When processing 48 samples at once, add the complete volume of dissolved carrier RNA to the bottle of Buffer BL, and check the box on the bottle lid label. If processing fewer samples, split the dissolved carrier RNA into conveniently sized aliquots (e.g., 50 µl) and store at –15 to –30°C. Aliquots can be stored for up to 1 year. If fewer than 48 conversions will be performed in a 2-week period, then only make up enough Buffer BL–carrier RNA solution as required (see Table 1, page 17, for example volumes). Carrier RNA enhances binding of DNA to the EpiTect spin-column membrane, especially if there are very few target molecules in the sample. Carrier RNA is not necessary if >100 ng DNA is used.
4. Add dissolved carrier RNA to Buffer BL. Calculate the volume of Buffer BL and dissolved carrier RNA required for the number of samples to be processed (see Table 1 for example volumes). If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.
5. Equilibrate samples and buffers to room temperature.

6. Optional: Set a thermomixer, heating block, or heated orbital incubator to 60°C for use in step 1.

Table 1. Carrier RNA and Buffer BL volumes

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

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

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

Procedure

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

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

Note: Do not place dissolved Bisulfite Mix on ice.

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

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

Table 2. Bisulfite reaction components

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

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

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

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

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

The complete cycle should take approximately 5 h.

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

Table 3. Bisulfite conversion thermal cycler conditions

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

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

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

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

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

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

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

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

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

8. Place the necessary number of EpiTect spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube in step 7 into the corresponding EpiTect spin column.

9. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

10. Add 500 µl Buffer BW to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

11. Add 500 µl Buffer BD to each spin column, and incubate for 15 min at room temperature (15–25°C).

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

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

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

12. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

13. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

14. Repeat step 13 once.

15. Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

16. Recommended: Place the spin columns with open lids into clean 1.5 ml microcentrifuge tubes (not provided) and incubate the spin columns for 5 min at 56°C in a heating block.

This step enables evaporation of any remaining liquid.

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

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

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

1.5 Isolation of DNA from Stool for Pathogen Detection

1. Weigh 180–220 mg stool in a 2 ml microcentrifuge tube (not provided) and place tube on ice.
2. Add 1 ml InhibitEX Buffer to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized.
3. Heat the suspension for 5 min at 70°C. Vortex for 15 s.
4. Centrifuge sample at full speed for 1 min to pellet stool particles.
5. Pipet 15 µl proteinase K into a new 1.5 ml microcentrifuge tube (not provided).
6. Pipet 200 µl supernatant from step 4 into the 1.5 ml microcentrifuge tube containing proteinase K.
7. Add 200 µl Buffer AL and vortex for 15 s.
8. Incubate at 70°C for 10 min.

Centrifuge briefly to remove drops from the inside of the tube lid (optional).

9. Add 200 µl of ethanol (96–100%) to the lysate, and mix by vortexing.

Centrifuge briefly to remove drops from the inside of the tube lid (optional).

10. Carefully apply 600 µl lysate from step 9 to the QIAamp spin column.

Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate. Do this step twice

11. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.

12. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate.

13. Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 3 min.

14. Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube (not provided) and pipet 200 µl Buffer ATE directly onto the QIAamp membrane. Incubate for 1 min at room temperature, then centrifuge at full speed for 1 min to elute DNA. Do this step twice

1.6 Genotyping

For the PCR:

- Vic dye probe
- Fam dye probe

SNP genotyping assay

=First tree dots are in the tubes which are coming with the delivery

- 2 target specific primer
- 1-20ng DNA per well
- 2X TaqMan genotyping Master mix or Universal Master MIX II per well
- For each run it is recommended negative controls- NFW

It's recommended to dilute the SNP Genotyping assay 40X 80X to a 20X working stock with a 1X TE buffer→ vortex and centrifuge the mixture→ make aliquots.

NOTE: Drug Metabolism Enzyme (DME) Genotyping assay are supplied at 20X concentration.

(In our case PPARy= DME Gen. Assay)

As real-time PCR system, we are using the StepOne/ StepOnePlus System with a StepOne 96 well Fast, final reaction volume 10µl

Workflow

Preparation for the Master Master Mix:

Component	96-Well Fast plate (10µl reaction)
2X TaqMan Master Mix	5.00µl
20X Assay working stock	0.50µl
volume MMM/well	**5.50µl**
+ DNA	**4.50**µl
Totall volume	**10µl**

- swirl the bottle of "X TagMan Genotping Master Mix gently to mix the contents
- vortex and centrifuge the 20X Assay Working Stock, then mix briefly
- pipette the required volumes of 2X TagMan Genotyping Master Mix , 20X genotyping Assay Mix into steril tube-> cap the tube-> vortex briefly ->centrifuge briefly (Air bubbles→ wrong clusters)

prepare the reaction plate with the wet DNA-method

- Dilute each DNA in NFW in order to deliver 1-20ng per well. A final concentration of at least 0,2ng/µl is recommended
- Pipette the Master Master Mix into each well of the reaction plate. Use the appropriate volumes listed above.
- Pipette samples into the plate

- Cover the plate with MicroAmp Optical Adhesive Film or MicroAmp Optical caps if usung a MicroAmp Optical 96-well plate. Seal the plate with a MicroAmp Adhesive Film Applicator.
- Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions
- .

Step	Predesigned SNP and Custom SNP			DME		
	Temp.	Duration	Cycles	Temp.	Duration	Cycles
AmpliTaq Gold®, UP, Enzyme Activation	95°C	10 minutes	HOLD	95°C	10 minutes	HOLD
Denaturation	95°C	15 seconds	40	95°C	15 seconds	50
Annealing/ Extension	60°C	1 minute		60°C	90 seconds	

Note: TaqMan® DME Genotyping Assays use a 90 second extension time and 50 cycles. These conditions are for optimal performance due to the longer average amplicon size of TaqMan® DME Assays compared to that of TaqMan® SNP Genotyping Assays.

DME for PPARy Gene!