Maastricht University

Microbiota, obesity and insulin resistance

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MICROBIOTA, OBESITY AND INSULIN RESISTANCE

Unraveling the impact of the microbiome on metabolic health in humans

The studies presented in this thesis were performed within the framework of TI food and Nutrition and NUTRIM School of Nutrition and Translational Research in Metabolism. NUTRIM participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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MICROBIOTA, OBESITY AND INSULIN RESISTANCE

Unraveling the impact of the microbiome on metabolic health in humans

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Voor Opa

PREFACE

‘All diseases begin in the gut’. More than 2000 years after this quote from Hippocrates, we have been starting to realize how right he was. Research in the last decade demonstrated the importance of the numerous microbial gut inhabitants in health and disease. The symbiotic relationship between microbes and the human host was first described in 1683 by the pioneer in microbiology, Antoni van Leeuwenhoek. In that year, he wrote a letter to the Royal Society of London, describing “very little animalcules, very prettily a-moving,” which he had seen under a microscope in plaque scraped from his teeth. In the following century, *The Germ Theory of Disease* was developed, which stated that many diseases are caused by the presence and actions of specific micro-organisms within the body. The more formal experiments of Louis Pasteur in the 1860s, followed by Robert Koch in the consecutive decades, provided the scientific proof for the Germ Theory.

Already in 1907, Nobel prize winner Ilya Ilitch Metchnikov, described in his book, “Essais optimistes; la longevite dans la serie animale” (The prolongation of life; optimistic studies), the numberless microbes in the large intestine, the colonization of the intestine shortly after birth, and microbial variations depending on food intake. He was also the first to describe the concept of beneficial microbes. Metchnikov worked at the Pasteur Institute, where he and Dr. Cohendy discovered that *Lactobacillus bulgaricus* in yoghurt and sour milk could reside in the human intestinal microbiota and would prevent against ‘intestinal putrification’. They linked the lactobaccilli to longevity based on the observation of high numbers of cenetarians in Bulgarian peasant populations, who consumed large amounts of fermented milk.

For more than three centuries after van Leeuwenhoek’s observation, the trillions of synbiotic microbes, remained largely unstudied. Because of the anaerobic properties of most intestinal bacteria, it was difficult to extract and culture them in a laboratory. Or, as Metchnikov 100 years ago wrote: ‘unfortunately, our actual knowledge of the intestinal flora is still very imperfect because of the impossibility of finding artificial media in which it could be grown.’ The development of 16s RNA gene-based approaches in the late 20th century finally provided the possibility to obtain a complete identification of bacteria in the human gastrointestinal tract. Since these techniques are yet widely available, research about the role of these bacteria has increased dramatically in the last 10 years. And this is just the beginning…

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CHAPTER 1

General Introduction

1. Obesity

Over the last decades, the prevalence of obesity has risen to epidemic proportions, and affects all ages and socioeconomic groups in the developed world[1,2]. Yet, also in many low- and middle-income countries, obesity and its metabolic consequences such as type 2 diabetes mellitus (T2DM)[3], cardiovascular disease, mental disorders[4] and several types of cancer[5], are contributing to increased morbidity and mortality rates. The last update on the global age-standardized prevalence of overweight and obesity showed that in 2014, 13.1% of men and 19.9% of all women were overweight or obese. If post-2000 trends continue, by 2025, global obesity prevalence will reach 18% in men and surpass 21% in women. Severe obesity (body mass index > 40 kg/m^2) will surpass 6% in men and 9% in women in 2025. These numbers indicate that obesity-related (cardiometabolic) complications will increase, accompanied by a large social and financial burden for society[6].

The etiology of obesity involves the dysbalance between energy intake, energy expenditure and energy excretion, which are dependent on the interaction between environment, genetic background and other biological factors[7]. Moreover, the obese insulin resistant state is characterized by adipocyte hypertrophy and disturbances in adipose tissue function due to an increased adipose tissue mass[8]. Impairments in the adipose tissue lipid buffering capacity in obese individuals contribute to lipid overflow and deposition of fat in ectopic organs such as the liver and skeletal muscle[9], in particular since the insulin resistant muscle is accompanied by an impaired capacity to increase fat oxidation upon an increased fatty acid supply[10,11]. The ectopic fat deposition is associated with insulin resistance[12], which in concert with progressive β-cell failure results in an increased blood glucose concentration in the non-diabetic range, classified as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). IFG (fasting glucose >5.6 mmol/l) and IGT (2h oral glucose tolerance test (OGTT)-derived glucose concentration >7.8 mmol/l) are intermediate states of glucose intolerance, preceding the development of T2DM. According to the WHO Global Report on diabetes 2016, the total burden of deaths from high blood glucose has been estimated to amount 3.7 million. This number includes 1.5 million diabetes deaths, and an additional 2.2 million deaths from cardiovascular diseases, chronic kidney disease, and tuberculosis related to higher-than-optimal blood glucose[13].

2. Insulin resistance

The obesity-related ectopic deposition of lipids may reduce insulin-stimulated translocation of glucose transporters to the cell membranes, thereby disrupting glucose uptake and glycogen storage, either through direct interference of bioactive lipid-intermediates with insulin signaling or through a stimulation of inflammatory pathways[3,11,14-16]. Although both isolated IFG and isolated IGT are insulin resistant states, these conditions may differ in their (predominant) site of insulin resistance[17,18]. Subjects with isolated IFG predominantly have hepatic insulin resistance and normal or slightly lower peripheral insulin sensitivity. In contrast, individuals with isolated IGT have a normal to slightly reduced hepatic insulin sensitivity and moderate to severe peripheral insulin resistance. Consequently, combined IFG and IGT is reflected by both peripheral and hepatic insulin resistance[19].

3. Interorgan cross-talk in obesity-related insulin resistance

The balance between energy intake, energy expenditure and energy excretion is maintained by a large regulatory network between metabolic organs. There is close interaction between the adipose tissue, skeletal muscle, pancreas and the liver in the regulation of energy and substrate metabolism. As mentioned above, disturbances in lipid metabolism are involved the etiology of obesity and related insulin resistance, impaired glucose metabolism and β-cell dysfunction.

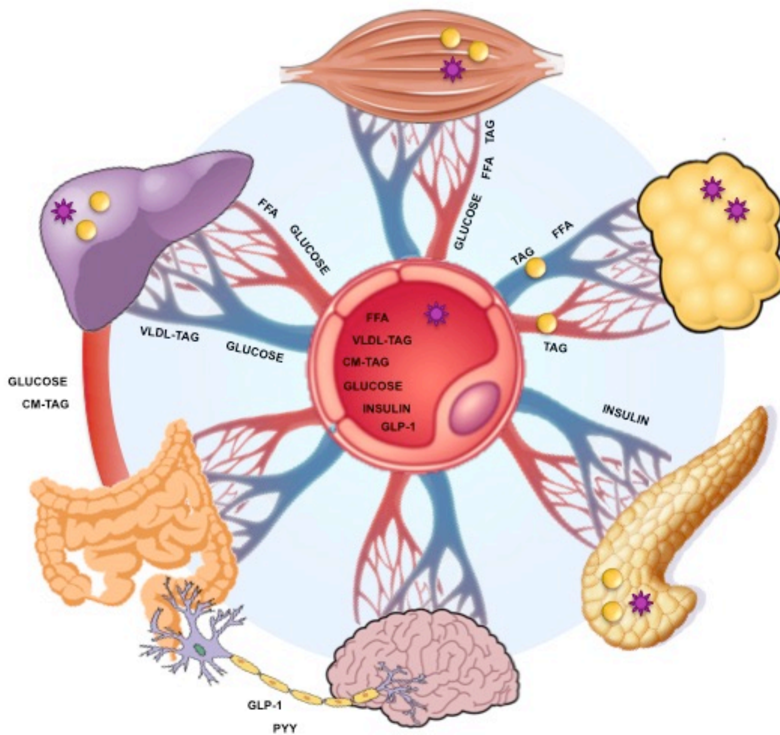
In healthy conditions, energy that is taken up from dietary fatty acids is stored as triacylglycerol in the adipose tissue. Under conditions of a chronic positive energy balance (i.e. obesity) the adipocytes become enlarged, resulting in an impaired lipid buffering of adipose tissue when the storage capacity of adipose tissue is exceeded. Furthermore, these hypertrophic adipocytes become less sensitive to the action of insulin. Insulin is the major anti-lipolytic hormone in human adipose tissue, and induces lipid uptake from the circulation through activation of lipoprotein lipase (LPL), while at the same time suppressing lipid breakdown through inhibition of hormone-sensitive lipase (HSL)[20]. Together, a reduced lipid uptake in combination with increased endogenous lipolysis causes overflow of lipids into the circulation[8,21]. The elevated lipid supply to non-adipose tissues such as the liver, skeletal muscle and pancreas may result in ectopic fat

storage in these tissues if lipid supply exceeds the fat oxidative capacity[8,11]. High intramuscular concentrations of lipids and lipid-intermediates, driven by an impaired ability to adjust fat oxidation to the increased lipid supply and altered intramuscular fatty acid handling, disrupt insulin-mediated glucose metabolism in skeletal muscle[14,22]. Also in the liver, hyperlipidemia is associated with insulin resistance and glucose intolerance[23]. In addition, an increased lipid supply results in an elevated hepatic triacylglycerol content and very low-density lipoprotein (VLDL-TAG) output[11,24,25]. Together with a higher hepatic glucose production and reduced insulin clearance[26], this results in increased plasma glucose and insulin concentrations, and a decreased glucose-stimulated insulin secretion by the pancreatic β-cells on the longer term[27,28].

Hypertrophic adipose tissue is furthermore characterized by a pro-inflammatory phenotype, which is the result of an imbalance between the production of pro-inflammatory and anti-inflammatory factors. The increased production and secretion of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 might contribute to the development of peripheral insulin resistance and disturbances in glucose homeostasis and, therefore, precede the development of T2DM.

Recent evidence also indicates that the gut, particularly the gut microbiota and its products, is part of the inter-organ crosstalk. Intriguingly, the gut microbiota has been implied in the development of insulin resistance and impaired glucose metabolism[29]. More specific, there is accumulating evidence that alterations in the gut microbiota composition and function affect adipose tissue, liver and skeletal muscle lipid and glucose metabolism, at least partly through altered production of bile acids (BA), incretins, inflammatory factors and short-chain fatty acids (SCFA)[30-33]. In the next paragraphs, these factors and the role of gut microbial imbalance (dysbiosis) in the metabolic interorgan cross-talk will be addressed in more detail.

Figure 1.1 Schematic overview of the inter-organ cross talk in obesity-induced insulin resistance. Products secreted by different tissues, including lipid species (FFA, TAG) yellow circles), proteins, hormones and inflammatory cytokines (purple stars) affect the functioning of other tissues via metabolic and endocrine cross-talk. Lipid spill-over from the adipose tissue contribute to ectopic lipid storage in the liver, skeletal muscle and pancreas, and might thereby induce insulin resistance. Also inflammatory cytokines may decrease insulin sensitivity. FFA: free fatty acids, TAG: triacylglycerol, VLDL very-low density lipoprotein, CM chylomicron.



4. The gastrointestinal tract and the microbiota within

Anatomically, the human gastrointestinal (GI) tract has a surface of approximately 400 m^2 and is composed of several compartments, each with varying metabolic functions. The digestion of food is initiated already in the mouth by chewing and the activity of amylase in saliva. After being exposed to the acidic environment ($pH<2$) in the stomach, food enters the duodenum where it is exposed to bile released from the gall bladder, and enzymes and hormones released from the pancreas. In the jejunum and ileum, food is further digested and nutrients are absorbed, whereas in the colon mainly water is resorbed. Throughout the intestine, a continuous simple columnar epithelial cell layer, which is interconnected by tight junctions and covered with a thin mucus layer, forms the physical and biochemical barrier between the host and the intestinal lumen[34].

The gastrointestinal tract is host to >10^{14} microbes of all three domains of life (Bacteria, Archaea and Eukaryota)[35,36]. The microbiome is dominated by five bacterial phyla (Bacteroidetes, Firmicutes, Verrucomicrobia, Actinobacteria and Proteobacteria) and Archaea. The most relevant genera are shown in Table 1.1, which provides an overview of the bacterial taxonomy. Microbial cells outnumber human cells by 10-fold, and the collective microbial genome contains an estimated 150 times more genes than the human genome[37,38]. There are significant differences in concentrations and populations of microbes within the gut itself, ranging from 10^1 to 10^3 microbes per gram of content in the stomach and duodenum, to 10^{12} to 10^{14} microbes per gram of content in the colon[31]. Colonization of the GI tract with bacteria is thought to begin in utero[39] and is strongly influenced by the first exposure to environmental micro-organisms. Microbial profiles in infants are highly divergent and change with the introduction of solid foods to gradually reach adult profiles that show less interpersonal diversity but are still unique to a given individual[40-42]. In adulthood, microbial populations are typically more stable, although these can rapidly be perturbed by diet, antibiotics or states of disease, and become less diverse and more unstable again in the elderly[27, 43].
Depending on the type of diet, various nutrients are provided to the host and the microbiota. The microbiome encodes proteins that are not independently coded for in the human genome, including enzymes (e.g. glycoside hydrolases and bile acid hydrolases) involved in the degradation of otherwise indigestible dietary elements (e.g. resistant starch, inulin and cellulose) and bile acids. An important step in the bacterial bile acid modification is 7α/7β-dehydroxylation, dehydrogenation or desulfation, performed by clostridia from the Firmicutes-phylum. These reactions convert the most abundant primary BA in humans (cholic acid (CA) and chenodeoxycholic acid (CDCA)) into their respective secondary BA (deoxycholic acid (DCA) and litocholic acid (LCA)). Furthermore, the microbiota are involved in the biosynthesis of essential amino acids, and perform unique catabolic biotransformations, thereby producing several bioactive metabolites. Although SCFA are mainly produced from indigestible carboydrates, microbiota-dependent amino acid metabolism also plays a role in the production of SCFA. More specific, propionate and butyrate are the predominant end products of the bacterial catabolism of threonine and lysine,

respectively[44]. Finally, microbiota are involved in the metabolism of xenobiotics[45] and the biosynthesis of certain vitamins[46].

5. The gastrointestinal microbiota

5.1 Factors shaping the gut microbiota composition

Studies in monozygotic and dizygotic twins showed that monozygotic twins have more highly correlated microbiotas, indicating a genetic component in a person's core gut microbiota composition[47]. During pregnancy, the developing fetal gut is 'primed' by the maternal gut microbiota and intestinal permeability, particularly towards the later stages of gestation[41,48]. A major determinant of the microbiota composition of newborns is the mode of delivery. Vaginally-delivered infants harbor bacterial communities resembling those of the maternal vagina, whereas gut microbiota of Caesarian section-delivered infants are enriched in maternal skin microbiota[49,50]. Following birth, the microbiota diversity develops throughout infancy and childhood, and reaches an adult-like stable composition at 2-year-old age[51]. Microbiota development is highly dependent on the nutritional regime (e.g. breast feeding versus formula milk[52]). Other factors such as incidental environmental exposures to antibiotics[53], country of birth[54] and type and duration of infection[55] play a major role in the distinctive characteristics of the microbial community in the infant's first year of life. Importantly, manipulating the dynamic gut microbiota of infants by the use of antibiotics may pose an increased risk of being overweight in later childhood[56].

5.2 The human microbiota composition

Although there is a high interindividual variability in microbial composition, the two most abundant of the 5 dominating phyla in the human gut are the Firmicutes and Bacteroidetes, comprising >90% of all bacteria[57].

The Gram-positive Firmicutes phylum is, among others, represented by the genera *Ruminiococcus, Clostridium* and *Lactobacillus,* which all comprise butyrate-producing bacterial species such as *Faecalibacterium prausnitzii*, *Roseburia intestinalis* and *Lactobaccilus plantarum*[58,59]. Lactobaccilli and clostridia are also involved in the modification of BA. A low abundance of butyrate-producing species has previously been associated with T2DM in humans[60,61].

The Gram-negative Bacteroidetes phylum is represented by *Bacteroides* and

Prevotella, and is able to degrade complex glycans. In the Actinobacteria-phylum, the most important commensal bacteria are from the genus *Bifidobacterium*, which provides the well-known probiotic species *Bifidobacterium bifidum.* Bifidobacteria are involved in the production of lactate and the SCFA acetate, and may thereby affect gastrointestinal immunity[62,63]. Proteobacteria that colonize the intestine include commensal, pathogenic and opportunistic species, such as *Salmonella*, *Helicobacter spp.*, and *Escherichia coli*. A high abundance of Proteobacteria has been related to inflammation in rodents[64]. Microbes responsible for mucosal degradation are included in the phylum Verrucomicrobia, in which for example *Akkermansia* is classified. The Archaea domain is primarily represented by the methanogen *Methanobrevibacter smithii*, which is involved in the removal of bacterial end products of fermentation, thereby affecting the specificity and efficiency of bacterial digestion of dietary polysaccharides[65].

Table 1.1. Taxonomy of the human gastrointestinal microbiota

<table>
<tr><th></th><th></th><th colspan="4">Gram-positive Bacteria</th></tr>
<tr><td>**Domain**</td><td>Archea</td><td colspan="4">Bacteria</td></tr>
<tr><td>**Kingdom**</td><td>Archea</td><td colspan="4">Bacteria</td></tr>
<tr><td>**Phylum**</td><td>Euryarchaeota</td><td colspan="4">Firmicutes</td></tr>
<tr><td>**Class**</td><td>Methanobacteria</td><td colspan="3">Clostridia</td><td>Bacilli</td></tr>
<tr><td>**Order**</td><td>Methanobacteriales</td><td colspan="3">Clostridiales</td><td>Lactobacillales</td></tr>
<tr><td>**Family**</td><td>*Methanobacteriaceae*</td><td>*Ruminococcaceae*</td><td>*Clostridiaceae*</td><td>*Lachnospiraceae*</td><td>*Lactobacillaceae*</td></tr>
<tr><td>**Genus**</td><td>*Methanobrevibacter*</td><td>*Ruminococcus*</td><td>*Faecalibacterium*</td><td>*Roseburia*</td><td>*Lactobaccillus*</td></tr>
<tr><td>**Species**</td><td>*Methanobrevibacter smithii*</td><td>*Ruminococcus bromii*</td><td>*Faecalibacterium prausnitzii*</td><td>*Roseburia intestinalis*</td><td>*Lactobaccilus plantarum*</td></tr>
</table>

<table>
<tr><th></th><th></th><th colspan="7">Gram-negative Bacteria</th></tr>
<tr><td>**Domain**</td><td></td><td colspan="7">Bacteria</td></tr>
<tr><td>**Kindom**</td><td></td><td colspan="7">Bacteria</td></tr>
<tr><td>**Phylum**</td><td></td><td colspan="2">Bacteriodetes</td><td colspan="3">Proteobacteria</td><td>Actinobacteria</td><td>Verrucomicrobia</td></tr>
<tr><td>**Class**</td><td></td><td colspan="2">Bacteroidia</td><td colspan="2">Gammaproteobacteria</td><td>Epsilonproteobacteria</td><td>Actinobacteria</td><td>Verrucomicrobiae</td></tr>
<tr><td>**Order**</td><td></td><td colspan="2">Bacteroidales</td><td colspan="2">Enterobacteriales</td><td>Campylobacterales</td><td>Bifidobacteriales</td><td>Verrucomicrobioales</td></tr>
<tr><td>**Family**</td><td></td><td>*Prevotellaceae*</td><td>*Bacteroidaceae*</td><td colspan="2">*Enterobacteriaceae*</td><td>*Helicobacteraceae*</td><td>*Bificobacteriaceae*</td><td>*Verrucomicrobiaceae*</td></tr>
<tr><td>**Genus**</td><td></td><td>*Prevotella*</td><td>*Bacteroides*</td><td>*Salmonella*</td><td>*Escherichia*</td><td>*Helicobacter*</td><td>*Bifidobacterium*</td><td>*Akkermansia*</td></tr>
<tr><td>**Species**</td><td></td><td>*Prevotella sp.*</td><td>*Bacteroides intesitinalis*</td><td>*Salmonella sp.*</td><td>*Escherichia coli*</td><td>*Helicobacter sp.*</td><td>*Bifidobacterium bifidum*</td><td>*Akkermansia muciniphila*</td></tr>
</table>

6 Gut microbiota dysbiosis, obesity and insulin resistance

6.1 The discovery of an obesity-associated microbiome

The first studies linking the gut microbiota to obesity were performed in germ-free mouse models. Bäckhed *et al.* [66] showed that 8- to 10-week-old conventionally-raised mice had 42% more total body fat than mice raised in the absence of microorganisms, even though germ-free mice consumed 29% more chow (57% carbohydrates, 5% fat). Furthermore, when germ-free mice were colonized with the microbiota of adult conventionally-raised mice, these animals gained significantly more weight than their germ-free counterparts when fed a Western diet[30]. In these studies, it was hypothesized that the microbiota stimulates hepatic lipogenesis through increased monosaccharide uptake from microbial fermentation of indigestible carbohydrates, and promotes LPL-directed fat storage into adipocytes through transcriptional suppression of intestinal angiopoietin-like 4 (ANGPTL4)[30,66].
Studies using genetically obese mice (*ob/ob*) indicated that these animals developed a distinct microbiome as compared to lean mice, with an increased capacity to harvest energy from the diet. This was further illustrated by showing that transferring the obese microbiota into germ-free lean mice resulted in a significantly greater increase in total body fat than colonization with a 'lean microbiota'[67]. Ley *et al.*[68] demonstrated that the cecal microbiota of these obese animals had a 50% reduction in Bacteroidetes and a significantly greater proportion of Firmicutes, relative to lean mice. An analogous increase in the Firmicutes/Bacteroidetes-ratio was found in the distal microbiota in obese humans[69]. Moreover, the dominant Firmicutes-phylum decreased after caloric restriction and Roux-en-Y gastric bypass[70]. Importantly, however, not all studies confirmed this correlation between obesity and the Firmicutes/Bacteroidetes-ratio in humans[71,72]. In a larger follow-up study in obese and lean twins, Turnbaugh *et al.*[73] reported that the fecal microbiome of obese subjects had significantly lower proportions of Bacteroidetes and higher levels of Actinobacteria compared to lean individuals, while there was no change in the proportion of Firmicutes. Other studies found that the abundance of bifidobacteria and *M. smithii*[74] and *Akkermansia muciniphila*[75] was decreased in obesity, whereas *Lactobacillus reuteri was* increased[74]. Taken together, these

data underscore that different gut microbial populations may have different effects on host adiposity, both in rodents and humans.

6.2 The discovery of an insulin resistant and diabetes-associated microbiome

In addition to a potential role of gut microbes in the development of obesity, alterations of gut microbial populations have been linked to altered glucose metabolism, changes in insulin sensitivity and the development of T2DM [76]. In the first study of Bäckhed *et al.*[66], conventionalization of germ-free mice significantly increased fasting glucose and insulin levels and induced insulin resistance. Later, Larsen *et al.* [61] demonstrated that T2DM is associated with gut compositional changes mostly apparent at phylum and class levels. The relative abundance of Firmicutes was significantly lower, while the proportion of Bacteroidetes and Proteobacteria was higher in patients with T2DM compared to healthy controls. Karlsson *et al*.[77] identified lower abundances of *Clostridium* species, similar to Larsen *et al.*[61], as well as higher abundances in four *Lactobacillus* species in T2DM. *Lactobacillus* species correlated positively, whereas *Clostridium* species were inversely associated with fasting glucose and HbA1c. In addition, Remely *et al.*[78] showed an increase in *Akkermansia*, *Enterobacteria*, and Archeae in T2DM patients. Furthermore, fecal levels of *Bifdobacterium* and *Clostridium coccoides* were found to be inversely related to fasting insulin and HOMA index in women[79].

Another large metagenome-wide association study indicated that T2DM patients had only a moderate degree of gut bacterial dysbiosis[60]. However, functional annotation analyses did indicate a decline in butyrate-producing bacteria and an increase in opportunistic pathogens. Importantly, not only patients with T2DM but also prediabetic subjects showed a lower abundance of the butyrate-producer *Faecalibacterium prausnitzii* and a higher abundance of *Akkermansia* than glucose tolerant subjects[80].

Taken together, rodent studies indicated a link between gut bacteria, insulin resistance and glucose homeostasis, which has been confirmed in human observational studies. Additional intervention studies should deepen our insight in the exact role of the microbiota composition and its functionality in human metabolism.

7. Gut microbiota-host interactions in obesity and insulin resistance

The dysbiosis of the gut microbiota as found in obesity, insulin resistance and T2DM provides the host with different concentrations of microbiota-derived metabolites as compared to lean, healthy individuals. As described above, the microbiota is involved in many metabolic processes in the gut, such as fermentation of complex carbohydrates and dehydroxylation of BA. *In vitro*, *ex vivo* and *in vivo* studies have suggested multiple mechanisms by which these and other microbial products can interfere with the gut-brain axis and the cross-talk between the adipose tissue, skeletal muscle, liver and pancreas. The following paragraphs provide an overview of important gut-derived factors, their receptors and signaling pathways, which might provide the link between gut microbiota and host physiology, thereby playing a role in the etiology of obesity and T2DM.

7.1 Microbiota-related effects on energy regulation

The presence of gut bacteria has been found to influence energy balance, since germ-free mice have significantly lower body weight and body fat percentage, despite a higher caloric intake than conventionalized mice[66]. The gut microbiota might affect both sides of the energy balance, as a factor that influences energy intake and energy harvesting, as well as energy expenditure[30,32].

Energy intake and satiety

First, the gut microbiota produces SCFA, which through a G-protein coupled receptor (GPR)41 dependent mechanism might modulate the secretion of incretin hormones like glucagon-like peptide-1 (GLP-1) and the expression of Peptide-YY (PYY)[81]. GLP-1 and PYY are satiety signals that can drive insulin secretion[82] and regulate food intake and blood glucose via complex overlapping endocrine and neuronal pathways[83]. Indeed, in humans with obesity, inulin propionate-ester intake versus inulin alone for 24 weeks prevented body weight gain through effects on appetite regulation[84]. Thus, by modulating SCFA production and subsequent satiety signals, the gut microbiota composition may affect energy intake and, therefore, energy balance.

Energy harvesting

An additional role for microbiota in energy balance is the capacity to increase energy harvest from the diet, mainly through fermentation of indigestible carbohydrates. The conversion of polysaccharides to acetate, butyrate or propionate accounts for approximately 5-10% of the daily energy uptake[85,86], but thus far evidence in humans is scarce. Although the concept of altered energy harvest as a mechanism that may contribute to differences in body weight or body composition is used frequently throughout the scientific literature[30,66], the significance of (altered) energy harvest from indigestible carbohydrates in humans is currently largely unknown.

Energy expenditure

Accompanied by an increased provision of energy substrates, the gut microbiota may also affect processes that favor energy storage over energy expenditure. Supportive for the role of microbiota-derived BA in energy regulation is a study in FXR-deficient mice, which had a reduced adipose tissue mass[87,88] possibly mediated via pronounced activation of the Wnt/β-catenin signaling pathway, which inhibits adipocyte differentiation and PPARγ function. Furthermore, the gut microbiota might enhance LPL-directed storage of (*de novo*) triglycerides in adipocytes. This was suggested upon the finding that conventionalization of germ-free mice promoted adipocyte hypertrophy by suppressing the intestinal expression of the circulating LPL inhibitor ANGPTL4[30,66.]

Lastly, the intestinal microbiota has been found to be involved in the expression of the fuel sensor adenosine monophosphate–activated protein kinase (AMPK) in the liver and skeletal muscle, which plays a key role in fatty acid β-oxidation and energy expenditure[89]. Germ-free mice were resistant to a high-fat diet and showed a 40% increase of phosphorylated-AMPK in skeletal muscle as compared to conventionally-raised mice, which was reversible upon microbiota transplantation in these mice[30].

In summary, the gut microbiota has been suggested to play a role in energy harvesting, energy intake and energy expenditure in rodents, thereby affecting energy balance and the development of obesity. However, evidence in humans in largely lacking.

7.2 Microbiota and systemic low-grade inflammation

The microbiota residing in the colon plays an important role in the host immune system via interaction with the intestinal epithelium[90]. Peptide-glycans, such as the Gram-negative bacterial cell wall component lipopolysaccharides (LPS), can be recognized by stromal and epithelial cells, through pattern recognition receptors such as Toll-like receptor 4 (TLR4)[91-93]. Subsequent activation of pro-inflammatory kinases and transcription factors (e.g. nuclear factor kappa B, NFkB), causes an increased expression of inflammatory factors such as TNF-α, monocyte chemotactic protein (MCP)-1 and IL-6, which have been associated with the induction of insulin resistance in various tissues in rodents[94].

Additionally, LPS can reach the systemic circulation via uptake in chylomicrons or intestinal translocation. Indeed, high circulating levels of LPS have been associated with increased systemic inflammation[91,95] and lower intestinal integrity in subjects with obesity, the metabolic syndrome and T2DM[96]. Furthermore, human studies demonstrated that circulating levels of bacterial DNA, mainly derived from Proteobacteria, were enhanced in prediabetes, indicating a role for gut bacteria in metabolic inflammation[97].

Microbiota-produced SCFA might enhance the intestinal barrier and decrease gut permeability, e.g. by modulating the expression of tight junction protein and mucins, thereby decreasing LPS translocation and systemic inflammation[76,98]. Indeed, butyrate has been found to regulate the colonic size and function of the anti-inflammatory regulatory T-cell network[99-101] and to decrease the expression of pro-inflammatory cytokines. Also the expression of the SCFA receptor GPR43 in neutrophils, macrophages and monocytes[102,103] suggests that microbiota-dependent products such as SCFA may play a role in peripheral inflammation[99]. In fact, acetate and butyrate decreased LPS-stimulated release of TNF-α from human neutrophils *in vitro*[104] and inhibited NFkB-expression in murine macrophage cell lines[105].

Also the BA receptors FXR and G-protein coupled receptor TGR5 are highly expressed in the GI-tract, monocytes and macrophages as well as other immune cell types[106,107]. Therefore, BA are thought to be involved in the regulation of a normal GI inflammatory tone, the prevention of pathogen invasions and maintaining cell integrity[33]. Deconjugated and secondary BA within the GI tract counterregulate activities of innate immune cells, leading to the inhibition of inflammatory cytokine production in macrophages and monocytes, involving a

FXR-mediated inhibition of NFkB activity[93,108,109]. Abnormalities in BA metabolism have been linked to gut microbiota dysbiosis in the metabolic syndrome and inflammatory disorders such as inflammatory bowel disease or irritable bowel syndrome[110], but also in nonalcoholic steatohepatitis[92].
To summarize, the interactions between gut microbiota, epithelial cells and the immune system are the main framework underlying a possible altered intestinal permeability and low-grade inflammation in obesity, and might therefore also play an important role in insulin resistance.

7.3 Microbiota, glucose homeostasis and insulin sensitivity

As briefly described above, the microbiota composition may be related to insulin resistance[111]. In fact, transplantation of the microbiota from lean donors to recipients with the metabolic syndrome slightly improved insulin sensitivity in a subset of the study participants[112]. It has previously been found that butyrate-producing species are often decreased in T2DM patients[60,61,77], suggesting a role for SCFA in the pathogenesis of insulin resistance. In line, the improved insulin sensitivity after fecal transplantation was accompanied with higher levels of butyrate-producing bacteria. Depletion of certain butyrate-producing strains by the antibiotic vancomycin, on the other hand, was found to slightly but significantly decrease insulin sensitivity[113]. Additionally, inflammation-related Proteobacteria increased after vancomycin treatment, suggesting a role for inflammatory cytokines in insulin signaling. In addition, insulin sensitivity might be influenced by the metabolism of BA in the gut. The antibiotic-induced alteration of the gut microbiota of obese subjects slightly decreased peripheral insulin sensitivity, with a concomitant decrease of fecal secondary BA and a simultaneous increase in postprandial primary BA. In contrast, another study with short-term antibiotic exposure in healthy volunteers did not find effects on glucose tolerance[114], whereas broad-spectrum antibiotics in mice improved rather than impaired fasting glucose levels and glucose tolerance[115].
As an alternative approach, animal and human studies demonstrated that microbiota manipulations by probiotics improved glucose tolerance[116]. For example, *Akkermansia*-administration enhanced glucose tolerance in high-fat diet-fed mice[117]. A recent meta-regression analysis identified that probiotics can significanlty reduce glucose, HbA1c, insulin and HOMA-IR in participants with T2DM, but not in participants with other risk factors such as hypertension, hyperlipidaemia or obesity[118].

To conclude, although rodent studies have suggested several mechanisms that relate gut microbiota composition to insulin resistance and T2DM, human data is scarce and inconclusive. Therefore, it is important to determine which microbiota-related mechanisms regulate insulin resistance, how different organs are affected and which mechanisms are disturbed in the interorgan crosstalk in humans.

7.4 Microbiota-related effects on adipose tissue metabolism

Adipose tissue inflammation

The low-grade inflammatory state in obesity and T2DM is characterized by increased production and secretion of proinflammatory cytokines and adipokines by adipocytes[8,11,94]. The M1-polarization of adipose tissue macrophages increases the production of TNF-α and IL-6, which can directly inhibit insulin action in target cells (e.g. hepatocytes, myocytes and adipocytes) via autocrine, paracrine and endocrine mechanisms [94]. Moreover, the expression of SCFA and BA receptors on macrophages and adipocytes indicates a role for a gut-adipose tissue interaction in the adipose tissue inflammatory phenotype[119]. Acetate, propionate and butyrate might all prevent chronic low-grade inflammation by upregulating colonic anti-inflammatory T_{REG} cells dependent on GPR43, thereby decreasing metabolic endotoxemia and reducing production of proinflammatory adipocytokines and chemokines in mice. Overall, in addition to interacting with systemic inflammation, the gut microbiota composition might be involved in adipose tissue inflammation by effects on AT macrophages and monocytes. However, most data is derived from animal studies or cell models and therefore needs to be confirmed in humans.

Lipolysis

In the postprandial period, VLDL- and chylomicron-TAG are hydrolyzed in the process of extracellular lipolysis by LPL[120]. The FFAs that are liberated by this process can be taken up by the adipose tissue and can be directed towards oxidation or storage, or are spilled over and remain in the circulation[121]. The activity of LPL is regulated by nutrients and hormones such as insulin and ANGPTL4[122-124]. In obesity, insulin resistance and T2DM, the removal of TAG across adipose tissue is impaired due to a reduced insulin-mediated stimulation of LPL activity[8,125]. A role for the gut microbiota in LPL activation has been

suggested via the transcriptional suppression of ANGPTL4 by gut microbiota upon conventionalization in germ-free mice[66].

Moreover, ANGPTL4 might also reduce intracellular lipolysis under fasting conditions and during catecholamine stimulation in murine adipocytes[126,127]. Intracellular lipolysis describes the hydrolysation of TAG into glycerol and FFA by adipose tissue lipase (ATGL) and hormone-sensitive lipase (HSL), and involves a complex pathway that is dependent on the regulation of lipolytic and antilipolytic hormones and molecules. Catecholamines and TNF-α induce lipolysis, whereas insulin is the main antilipolytic hormone[128]. The production of SCFA in the gut, which relates to the microbiota composition and diet, might affect adipose tissue lipolysis. Several human studies have shown that oral or rectal administration of SCFA, in particular acetate, significantly decreased plasma free fatty acid (FFA) concentrations[129-131].

Treatment of differentiated murine *3T3-L1* adipocytes with acetate and propionate reduced intracellular fasting and catecholamine-mediated lipolysis via activation of GPR43, which might be explained by a decreased HSL phosphorylation[119]. In addition, binding of SCFA to GPR41 on adipocytes inhibited lipolysis, and increased adipogenesis and leptin synthesis in mice[132]. In the absence of the BA receptor FXR (FXR knock-out mouse model) basal intracellular lipolysis was increased, leading to elevated plasma FFA levels, indicating that BA signaling might also be involved in adipose tissue biology.

In summary, although several studies have indicated mechanisms via which the gut microbiota composition might affect adipose tissue lipolysis, the exact mechanisms in human physiology remain to be elucidated.

Adipogenesis

The differentiation of pre-adipocytes into mature adipocytes is known as adipogenesis. This process is highly dependent on peroxisome proliferator-activated receptor γ (PPARγ), which encodes a regulatory protein of adipocyte differentiation. During persistent positive energy balance, the adipose tissue is forced to expand to facilitate increased TAG storage[8,11] The inability of the adipose tissue to differentiate new adipocytes and store excess lipids in mature adipocytes results in adipocyte hypertrophy. Impaired adipose tissue expandability is associated with adipose tissue dysfunction, which is characterized by decreased insulin sensitivity, increased immune cell infiltration and increased intracellular and systemic FFA flux[8]. It has been shown in a FXR

knock-out model, that the BA receptor FXR controls adipocyte differentiation and function by promoting PPARγ activity and inhibiting the Wnt/β-catenin pathway[88,133]. In addition, a role for FXR activation has been suggested in adipogenesis and insulin signaling[133], which might be due to reduced lipogenesis[87,88]. Moreover, a role for SCFA in adipogenesis has been found, as propionate induced GPR43 expression during adipose differentiation of 3T3-L1 cells, with an up-regulation of PPARγ[134].
Taken together, several studies suggest a role for BA and SCFA on adipogenesis and PPARγ activity. However, the exact mechanisms by which the microbiota composition might be involved in adipogenesis, need to be further investigated in human *in vivo* models.

7.5 Microbiota-related effects on liver metabolism

Hepatic inflammation

Although the intestinal mucosa acts as an effective barrier against translocation of microbes and microbial products such as LPS and bacterial DNA from the gut to the circulation, small quantities do enter the portal vein. The liver is thereby the first filter for nutrients and microbial products that can alter glucose production and increase inflammation, steatosis, and insulin resistance. For example, the gut microbiota endotoxin LPS might set off the inflammatory cascade in the liver via binding to TLR-4 and consequent activation of Kupffer cells[135]. In addition, LPS has been found to play a role in the development of steatohepatitis in obese patients[92]. In line, bacterial DNA has been found in the liver[136] and NASH patients presented with a higher abundance of Proteobacteria in their intestines[137]. The increased inflammatory environment in the liver, together with hepatic lipid accumulation, plays a role in the development of hepatic insulin resistance.

Hepatic lipid regulation

Lipids derived from adipose tissue spillover and a high dietary fat intake increase the flux of lipids to the liver, which as a consequence enhances the production and secretion of VLDL particles, and thereby increases cardiovascular disease risk. The gut microbiota has been indicated to be involved in hepatic lipid regulation, via stimulation of hepatic triglyceride production mediated by transcription factors such as ChREBP[66], and the inhibition of ANGPTL4[66]. In addition,

microbiota-derived SCFA might play a role, as acetate and proprionate, taken up by the liver, may be used as substrates for lipogenesis and gluconeogenesis[138]. Furthermore, acetate has been found to induce the liver enzyme CYP7A1, responsible for BA synthesis[139]. A study in mice showed, that the increase of the bile acid pool size by overexpression of CYP7A1, protected from Western diet-induced obesity, hepatic steatosis and insulin resistance[140].
Taken together, the gut microbiota could influence signaling pathways involved in energy and lipid metabolism, leading to alterations in the production of hepatic fatty acids and triglyceride storage.

7.6 Microbiota-related effects on skeletal muscle metabolism

Insulin signaling

The skeletal muscle is the main site responsible for insulin-stimulated glucose disposal[141]. In postprandial conditions, insulin stimulates the transport of the glucose transporter GLUT4 to the cell membrane, thereby facilitating glucose uptake into the muscle[142]. In insulin resistant obesity, intramuscular toxic lipid-intermediates and inflammatory molecules inhibit the insulin signaling cascade, thereby contributing to elevated blood glucose concentrations, which in turn predispose towards T2DM[13,14,15]. As described above, T2DM and insulin resistance are associated with compositional changes of the microbiota[60,61,75,77]. These compositional changes might affect concentrations of endotoxin and inflammatory cytokines. In addition to the general assumption that cytokines inhibit insulin signaling, it has been found that also plasma LPS levels were negatively correlated with clamp-derived whole-body insulin sensitivity[143,144]. A recent study has shown that BA might affect skeletal muscle insulin sensitivity in mice, since the intestinal expression of FXR was required for the development of insulin resistance and obesity in response to a high-fat diet[145]. Moreover, BA can activate TGR5 in mice, which is expressed in skeletal muscle, thereby exerting protective effects against diet-induced obesity and insulin resistance[146].

Substrate metabolism

Impairments in fasting and postprandial fatty acid uptake, storage and oxidation may contribute to intramuscular accumulation of lipid-intermediates like diacylglycerol and ceramides[22,147]. It has been indicated that the metabolic profile of the insulin resistant muscle is more directed towards fat storage rather than

oxdation[148,149]. The lipid content in the muscle reflects a net balance between rates of fatty acid supply, uptake and mitochondrial fat oxidation[11]. As described above, the intestinal microbiota has been found to be related to the expression of AMPK in the muscle, which plays a key role in fatty acid β-oxidation[30]. Furthermore, SCFA may affect muscle insulin sensitivity and glucose metabolism through intestinal GLP-1 secretion. It has been demonstrated in rats that GLP-1 might modulate muscle microvascular function, thereby altering substrate supply and uptake in skeletal muscle[150]. SCFA-stimulated secretion of PYY might also improve insulin-mediated glucose uptake in skeletal muscle and increase whole-body fat oxidation, as shown in animal studies[151,152]. Finally, SCFA may alter peripheral substrate metabolism since their receptors, GPR41 and 43, are found to be expressed in skeletal muscle[153,154].

Taken together, several lines of evidence suggest that the gut microbiota-skeletal muscle crosstalk is involved in substrate metabolism and insulin sensitivity[32,155]. However, human studies that provide evidence for this have not been perfomed yet.

7.7 Microbiota-related effects on insulin secretion

Gut-derived factors might have an effect on insulin secretion by pancreatic β-cells. GLP-1 is the primary incretin hormone secreted from L-cells in the intestine, which may affect pancreatic β-cells by increasing insulin secretion[156]. At the same time, GLP-1 may decrease glucagon release from α-cells[157]. Indeed, elevated GLP-1 levels were correlated with improved glucose homeostasis in patients after bariatric surgery[158]. As discussed earlier, SCFA have been indicated to modulate the secretion of incretin hormones through a GPR41-dependent mechanism[81]. Moreover, a recent study has demonstrated a regulatory pathway for the SCFA-GPR43 axis in the modulation of β-cell function[159]. In addition, BA might be involved in the regulation of insulin release. The expression of BA receptor FXR has recently been reported to play a role in the pancreas, where it regulates glucose-induced insulin secretion[160,161] and protects against lipotoxicity[160]. Taken together, these findings highlight a novel relationship between the gut microbiota composition and glucose homeostasis. However, human studies that have investigated the effect of the gut microbiota on β-cell function and insulin secretion are lacking.

8. How to target the adult gut microbiota composition?

The resilient microbiota of adults can be subjected to minor and major disturbances such as infection, exposure to antibiotics and major dietary shifts[162]. Diet can specifically influence the microbiota via the diet composition and total energy content of the diet, or indirect via regulation of the gastrointestinal pH and transit time[163]. Long-term consumption of hunther-gatherer, plant-based diets have been associated with greater microbiome diversity[43], increased abundance of *Prevotella* and higher levels of SCFA production. The animal-based, high-fat Western diet, on the other hand, is associated with the *Bacteroides* enterotype[57]. The addition of prebiotic fibers to a diet, such as resistant starch, has been found to reduce mesenteric (visceral) fat accumulation, enhance insulin sensitivity, improve blood glucose levels and lipid metabolism[164]. Probiotics, consisting of one or more species of live bacteria such as lactobacilli and bifidobacteria, have been found not only to affect the intestinal flora directly but are also able to modulate immunological parameters and intestinal permeability, produce regulatory metabolites and improve glucose homeostasis[116,165-167]. The strong influence of dietary habits is underscored by the pronounced distinction of the gut microbial profiles of U.S. *vs.* Malawian and Amerindian populations across all ages[43]. In addition, recent evidence has indicated that even visits of short duration to other geographic locations can influence the gut microbial composition[168]. Lastly, short-term interventions with different diets varying in macronutrient composition have demonstrated that the gut microbiome can rapidly respond to altered diet, reflecting the diversity of human dietary lifestyles[169].

A more robust way to manipulate the microbiota composition is by administration of antibiotics, which can be selectively administered to target a specific pathogenic population. Antibiotic treatment typically induces a decrease in the diversity of the microbiota[170]. Antibiotics shift the composition of the microbiota in different ways, depending on their spectrum of activity, thereby specifically changing the metabolic profiles of intestinal contents and feces[171]. For example, models of antibiotic-treated mice show reduced fecal BA and decreased BA diversity, as a result of reduced microbial BA metabolism[33,172]. Another common feature of the metabolic profiles of antibiotic-treated mice and humans is a reduced production of SCFA[173-175]. Moreover, long-term antibiotic exposure has been found to promote weight gain[174,175] and, therefore, was among

the first growth promoters used in agriculture[176]. Importantly, when animals were exposed to sub-therapeutic dosages of antibiotics early in life, the effects on growth promotion were greater than if the exposure occured later in life, indicating that early life is a metabolically vulnerable stage[177]. Furthermore, it has been demonstrated that the risk of developing T2DM increases with the number of antibiotic prescriptions, together with a decline of intestinal integrity[178]. However, mouse studies have shown that antibiotic treatment improved metabolic impairments such as insulin resistance and glucose homeostasis[175,179,180].

Overall, it has been shown that antibiotic treatment can alter the microbiota composition, with consequences for the host metabolic phenotype. Therefore, antibiotic-treatment is a relative useful tool to investigate the role of the microbiota in human metabolism.

9. Outline of this thesis

Research performed to date has demonstrated that the gut microbiota may be related to host energy and substrate metabolism. Moreover, perturbations of microbial populations may contribute to an obesogenic phenotype, inflammation, an altered glucose metabolism, and insulin resistance. Importantly, however, mechanistic studies almost exclusively have been performed in rodent models. Therefore, these rodent studies need to be confirmed and/or extended to human clinical trials to better understand if and how specific microbial populations may contribute to the development of obesity and related metabolic and inflammatory perturbations, including the development of T2DM.

This thesis focuses on the effects of microbiota manipulation using broad-spectrum and small-spectrum antibiotics in obese, insulin resistant humans with an impaired glucose metabolism, taking into account multiple mechanisms that have been proposed to contribute to metabolic impairments based on rodent studies. **Chapter 2** describes the results of a double-blind, randomized placebo-controlled trial that was designed to investigate the effects of antibiotic treatment on tissue-specific insulin sensitivity, the gut microbiota composition, SCFA and BA concentrations. Moreover, this chapter also describes secondary outcomes of energy expenditure and substrate utilization, gut permeability, inflammation, adipocyte morphology and the adipose tissue transcriptome.

In the same clinical trial, the effect of antibiotics on forearm blood flow and

substrate fluxes across forearm muscle were investigated before and after consumption of a high-fat mixed-meal. Substrate fluxes of glucose, TAG, FFA and lactate across the forearm muscle were assessed by combining measurements of arterio-venous concentration differences across forearm muscle and forearm blood flow (venous occlusion plethysmography). The results of these measurements are described in **Chapter 3.** To obtain insight into the mechanisms that might affect lipid metabolism and insulin sensitivity in adipose tissue after antibiotic treatment, **Chapter 4** describes the effects of microbiota manipulation on *ex vivo* lipolysis in freshly isolated abdominal subcutaneous adipocytes. In this cell model, both basal and β-adrenergically-stimulated lipolysis were measured. Moreover, the effects of antibiotic treatment on adipose tissue expression of lipolytic genes and proteins were examined in human adipose tissue.

Observational studies have indicated that the gut microbiota composition is associated with T2DM and insulin resistance. However, most studies compared the microbiome between lean and obese or T2DM subjects, without having examined host metabolic characteristics in detail. Therefore, in **Chapter 5**, we aimed to obtain a better insight in the association between the microbiota composition and peripheral, hepatic and adipose tissue insulin sensitivity. This chapter describes a cross-sectional analysis of two independent cohorts of obese men (Maastricht and Amsterdam), in which the relationship between gut microbiota composition and tissue-specific insulin sensitivity was investigated in detail.

Finally, the findings of the studies described in this thesis are discussed in **Chapter 6,** in addition to implications for future studies

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CHAPTER 2

Effects of gut microbiota manipulation by antibiotics on host metabolism in obese humans: a randomized double-blind placebo-controlled trial

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Abstract

The gut microbiota has been implicated in obesity and cardiometabolic diseases, although evidence in humans is scarce. We investigated how gut microbiota manipulation by antibiotics (7-day administration of amoxicillin, vancomycin or placebo) affects host metabolism in 57 obese, prediabetic men. Vancomycin, but not amoxicillin, decreased bacterial diversity and reduced Firmicutes involved in short-chain fatty acid and bile acid metabolism, concomitant with altered plasma and/or fecal metabolite concentrations. Adipose tissue gene expression of oxidative pathways was upregulated by antibiotics, whereas immune-related pathways were downregulated by vancomycin. Antibiotics did not affect tissue-specific insulin sensitivity, energy/substrate metabolism, postprandial hormones and metabolites, systemic inflammation, gut permeability and adipocyte size. Importantly, energy harvest, adipocyte size and whole-body insulin sensitivity were not altered at 8-weeks follow-up, despite a still considerably altered microbial composition, indicating that interference with adult microbiota by 7-days antibiotic treatment has no clinically relevant impact on metabolic health in obese humans.

Introduction

Accumulating evidence indicates that the composition of the gut microbiota plays a prominent role in body weight regulation and the development of type 2 diabetes mellitus[1,2]. The gut microbiota regulates energy extraction from otherwise indigestible carbohydrates, determines the integrity of the intestinal epithelial layer, and influences the production and absorption of multiple signaling molecules involved in host metabolism. Several studies have demonstrated that germ-free mice are protected from diet-induced obesity, low-grade inflammation and glucose intolerance as compared to conventionally raised animals[3,4]. Furthermore, it has been shown that transferring microbiota via fecal transplantation evoked alterations in body weight and insulin sensitivity in both rodents[4] and humans[5,6] Taken together, these data indicate that modulation of the gut microbiota may provide a promising avenue to target obesity-related metabolic disorders[7].

The gut microbiota composition can be modulated by, amongst others, prebiotics, probiotics and antibiotics[8], thereby altering the presence and expression of microbial genes and derived metabolites such as bile acids (BA) and short-chain fatty acids (SCFA)[9,10]. Particularly, the use of antibiotics has been associated with increased metabolic impairments, mainly when exposure occurs in early life[11,12]. Of note, these findings are primarily based on animal studies, in which the animals have mostly been exposed to a combination of antibiotics for periods varying from two to twenty weeks[13-17]. It has recently been shown that antibiotics may improve peripheral insulin sensitivity in a small number of obese subjects[18]. Nevertheless, the effects observed in the latter study were relatively minor and, importantly, the study was not placebo-controlled. Thus, well-controlled, large human studies examining the effects and underlying mechanisms of microbiota modulation on host metabolism are currently lacking.

Here, we report on a randomized, double-blind, placebo-controlled trial that was designed to investigate the effects of broad and narrow-spectrum antibiotic treatment for seven days on gut microbiota composition, tissue-specific insulin sensitivity, energy expenditure, substrate oxidation, fecal and plasma BA and SCFA concentrations, gut permeability, abdominal subcutaneous adipocyte size and systemic low-grade inflammation in obese men with impaired glucose homeostasis. Moreover, eight weeks after cessation of antibiotic treatment, we

again determined microbiota composition, whole-body insulin sensitivity (HOMA-IR), fecal energy harvest and adipocyte size.

Methods

Study participants

57 low active (<3 h organized sports activities per week), weight-stable (<2 kg body weight change 3 months prior to inclusion) overweight/obese (BMI 25-35 kg/m^2), Caucasian men, between 35 and 70 years with impaired glucose metabolism (either fasting glucose >6.1 mmol/l, and/or 2h glucose between 7.8-11 mmol/l) and HOMA-IR>2.2 were included in this study (ClinicalTrials.gov, NCT02241421). Subjects were recruited via advertisements in local newspapers, and were all living in the area around Maastricht, The Netherlands. All subjects gave written informed consent for participation in this study, which was reviewed and approved by the local Medical Ethical Committee of Maastricht University Medical Center+. All procedures were according to the declaration of Helsinki (revised version, October 2008). Exclusion criteria were: the use of antibiotics for a period of three months before entering the study, known allergic reactions to any type of antibiotics, hearing disorders, cancer, liver malfunction, major illnesses with a life expectancy less than five years and pulmonary, hepatic, cardiovascular, kidney, and gastrointestinal disease. Subjects did not use β-blockers, lipid and glucose lowering-drugs, anti-oxidants or chronic corticosteroids.

Study design & randomization

This randomized, placebo-controlled, double-blind study had a three-armed parallel design. Participants were randomized to oral intake of amoxicillin (broad-spectrum antibiotic), vancomycin (directed against Gram-positive bacteria) or placebo (microcrystalline cellulose) for seven consecutive days (1500 mg/d). Antibiotics and placebo were equally capsulated to blind the content to subjects and investigators (BasicPharma, The Netherlands). The allocation sequence was established by computer-generated randomization (https://nl.tenalea.net). Block-randomization with stratification for BMI, age and 2h-glucose values was used to increase the homogeneity of the treatment arms (block size, n=6). After completion of the study, returned capsules were counted to assess compliance. Participants were asked to maintain their habitual physical activity pattern and

dietary habits (monitored by 3-day food diaries) throughout the study. The evening before an investigation day, a low fiber, low fat meal was consumed. Before and after intervention, study measurements were conducted following a 10-h overnight fast. To ensure complete systemic and gastrointestinal clearance of antibiotics, a 2-day wash-out period was taken into account before post-treatment measurements. Participants returned for a follow-up visit eight weeks after treatment cessation.

Hyperinsulinemic-euglycemic clamp

A two-step hyperinsulinemic–euglycemic clamp combined with a [6,6-$^{2}H_{2}$]-glucose tracer (Cambridge Isotope Laboratories, Andover, MA, USA) was performed to measure Rd, EGP[41] and the insulin-mediated suppression of FFA[42,43]. Blood samples were taken from a superficial dorsal hand vein, which was arterialized by using a hot-box (~50 °C). After a bolus-injection (2.4 mg/kg), tracer-infusion was started at 0.04 mg/kg/min, which was continued throughout the measurement. After 2 h, low-dose insulin was infused at 10 mU/m^2/min for 2 h[44], followed by high-dose insulin at 40 mU/m^2/min for 2 h[45]. By variable co-infusion of a 17.5%-glucose solution, enriched by 1.1% tracer, plasma glucose concentrations were maintained at 5.0 mmol/l. For calculation of steady-state-kinetics, additional blood samples were taken in the last 30 min of each step (0, 10 and 40 mU/m^2/min insulin).

Postprandial test

Blood was sampled from a superficial dorsal hand vein, which was arterialized by placing the hand into a hot-box (~50 °C). Blood samples were taken during the fasting state (t-30, t-15, t0 min) and postprandial (t=30, 60, 90, 120, 180 and 240 min) after ingestion of the test meal. The liquid test meal, that was consumed within 5 min, provided 2.6 MJ (61 E% fat, 33 E% carbohydrate, 6 E% protein), which was consumed within 5 min at t=0[46].

Indirect calorimetry

For indirect calorimetry during fasting (30 min) and the 4-h postprandial state, the open-circuit ventilated hood system was used (Omnical, Maastricht University, Maastricht, Netherlands)[47]. Calculations of energy expenditure and substrate oxidation were performed according to the formulas of Weir[48] and

Frayn[49]. Nitrogen excretion was based on the assumption that protein oxidation represents ~15% of total energy expenditure[50].

Gut Permeability test

After baseline urine collection, subjects drank a 150 ml multisaccharide test mix [1 g sucrose (Van Gilse, Dinteloord, the Netherlands), 1 g lactulose (Centrafarm, Etten-Leur, the Netherlands), 1 g sucralose (Brenntag, Sittard, the Netherlands), 1 g erythritol (Danisco Sweeteners, Copenhagen, Denmark), 0.5 g of l-rhamnose (Danisco)][29]. Urine was collected for determination of the urinary sucrose concentration in the 0-120 min urine collection, representing gastro-duodenal permeability, whereas in this collection small intestinal permeability is represented by the lactulose/rhamnose ratio. Proximal colon permeability is represented by the suclarose/erythritol ratio of the 120-300 min urine collection.

Biochemical analyses for plasma variables

Blood was collected into pre-chilled tubes, centrifuged at 1000*g*, and plasma was snap-frozen and stored at -80ºC until analyses. Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography–mass spectrometry and expressed as tracer-to-tracee ratio for steady-state calculations of Rd and EGP[41]. Plasma glucose, lactate, FFA and glycerol were determined with the Cobas Fara auto-analyzer (Roche, Switzerland). Plasma insulin was measured with a double antibody radioimmunoassay (Millipore, MA, USA). Plasma leptin concentrations were analyzed using commercially available radioimmunoassay kits (Human Leptin RIA, Millipore Corporation, Billerica, MA, USA). Plasma ANGPTL4 concentrations were measured by ELISA as described[51]. Plasma concentrations of IL-6, IL-8 and TNF-α were determined using a multiplex enzyme-linked immuno-sorbent assay (Human ProInflammatory II 4-Plex Ultra-Sensitive Kit, Meso Scale Diagnositics, Rockville, MD, USA). Isocratic ion-exchange HPLC (Model PU-1980 pump, Jasco Easton, MD) with mass spectrometry (Model LTQ XL, Thermo Fisher Scientific, Waltham, MA) was used to determine sugar concentrations in plasma and urine for gastrointestinal permeability assessment[29]. LPS-binding protein was measured using non-commercial ELISA as described before[52]. Plasma concentrations of GLP-1 were measured by radioimmunoassays as previously described[53]. Plasma BA profile was measured using liquid chromatography tandem mass spectrometry (LC-

MS/MS)[18]. The total amount of primary (cholic acid and chenodeoxycholic acid and their taurine and glycine conjugated forms) and secondary BA (deoxycholic acid, lithocholic acid and their conjugated forms) was calculated as the sum of the individually quantified BA. Plasma SCFA were determined by LC-MS/MS as reported before[54]. The detection limits for acetate, propionate and butyrate were 0.1, 0.05 and 0.05 µmol/L, respectively.

Laboratory analysis of adipose tissue

Abdominal subcutaneous AT biopsies were taken under local anesthesia under fasted conditions. One portion was embedded in paraffin. Sections were cut for staining, digital imaging and computerized morphometric measurement of individual adipocytes55. One portion (~500 mg) was snapfrozen in liquid nitrogen, from which RNA was extracted (Trizol chloroform extraction, Invitrogen, Cergy Pontoise, France) and used for microarray analysis. 100 ng total RNA was labeled by Whole-Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays, targeting 19793 unique genes (Affymetrix, Santa Clara, CA, USA). Quality control and data analysis pipeline have been described in detail previously56. Individual genes on the array were defined as changed when comparison of the normalized signal intensities showed a FDRq<0.05 in a two-tailed paired t-test with Bayesan correction (Limma)57. Further functional data analysis was performed on the filtered data set with Gene Set Enrichment Analysis (GSEA, http://www.broad.mit.edu/gsea). Gene sets were selected based upon FDRq <0.2. Array data have been submitted to the Gene Expression Omnibus: number GSE76003.

Laboratory analysis of feces

Feces was collected at home for two consecutive days at baseline, seven days and eight weeks after intervention using the BMP commode specimen collection system, and divided over sterile tubes at home. Subjects were provided with a box of dry ice to freeze their stool samples immediately after defecation at approximately -80°C and for transport to the university. Total fecal amount was weighed, and 24-h fecal samples were used to determine energy content using adiabatic bomb calorimetry (CBB 330, standard benzoic acid 6320 cal/g, BCS-CRMno90N). 24-h fecal BA composition was determined by using gas chromatography (GC) as described before[41]. Fecal SCFA were measured by gas

chromatography-mass spectrometry (GC-MS, Medical laboratory 'Dr. Stein & Collegae' Germany), according to the method described before[58].
For microbiota profiling, DNA was isolated from 24-h fecal samples as described before[18] and subsequently used for phylogenetic profiling using the HITchip phylogenetic microarray[19]. Standardized quality control was maintained through our library of a duplicated set of 3,631 probes targeting the 16S rRNA gene sequences of over 1,000 intestinal bacterial phylotypes. A more detailed description of microbiota profiling procedures can be found in the Supplemental Experimental Procedures.

Statistics

The calculated sample size (n=19 per treatment arm) was based on a 20% physiologically relevant change of insulin sensitivity ($\alpha = 0.05$, $\beta = 0.8$). All data were evaluated for normality. Univariate analysis (ANOVA) was applied to compare group characteristics at baseline. Differences between treatments were analyzed using repeated-measures ANOVA with time and treatment as factors. ANCOVA analysis of the delta (post-pre value) was used for parameters when significantly different at baseline, taking the baseline value into account as covariate. The postprandial response (energy expenditure, substrate oxidation and GLP-1) is given as incremental area under the curve (iAUC/min), which was calculated by the trapezoid method. For HITchip analysis, log10-transformed signals were used as a proxy for bacterial logarithmic abundance. To determine which bacterial groups were significantly different in relative abundance before and after treatment within each group, a paired Wilcoxon test was used. Between-treatment group effects were assessed with linear mixed models using the lme4 package [59]. Benjamini–Hochberg correction was applied for multiple testing. We used Random Forests, a supervised machine-learning technique, and the pre and post treatment classes to confirm these results[60]. To determine whether individuals could be grouped into classes of specific metabolic responses to the interventions, we used the lcmm R package[61] to perform Latent Class Analysis. Diversity of the microbiota was quantified based on non-logarithmized HITchip oligo-level signals by inverse Simpson's index using the Vegan package[62]. ANOVA with Tukey's Honest Significant post hoc analysis was applied to compare diversity between and within groups. Data are expressed as means ±

standard error of the mean (SEM), with a two-sided significance level of $P<0.05$. Statistical analysis was performed using SPSS 20.0 for Macintosh and R 3.03.

Results and Discussion

Subject characteristics

To study the role of the gut microbiota, we randomized 57 overweight and obese 35-70 year old Caucasian men to oral administration of the broad-spectrum antibiotic amoxicillin (AMOX), narrow-spectrum antibiotic vancomycin (VANCO, directed against Gram-positive bacteria), or placebo (PLA) for seven days. No significant differences in baseline characteristics were present between groups (Table 1). All subjects had impaired fasting glucose levels (plasma glucose≥5.6 mmol/l) and/or impaired glucose tolerance (2h plasma glucose during a 75g oral glucose tolerance test 7.8-11.1 mmol/l), and were insulin resistant (homeostasis model assessment for insulin resistance; HOMA-IR>2.2). One subject randomized to the AMOX intervention was considered a dropout due to use of other antibiotics during the study period. No serious adverse events and only a few cases of mild gastrointestinal discomfort were reported. There were no differences in daily energy and macronutrient intake, as monitored by a three-day food diary, between and within groups before and after intervention. Furthermore, body weight remained unchanged for all treatment groups throughout the study period and at follow-up (data not shown).

Table 2.1. Baseline characteristics of the study population

Variable	PLA (n=19)	AMOX (n=18)	VANCO (n=19)
Age (years)	60.9±1.7	55.7±1.5	60.6±1.5
Body weight (kg)	96.7±2.3	96.3±2.5	97.6±1.9
Body mass index (kg/m^2)	31.0±0.5	31.1±0.8	31.5±0.6
Waist/Hip ratio	1.04±0.01	1.04±0.01	1.07±0.01
Waist circumference (cm)	98.0±8.1	101.1±6.4	106.7±6.3
Fasting glucose (mM)	6.0±0.1	6.1±0.1	6.1±0.1
2h OGTT glucose (mmol/l)	7.7±0.4	7.0±0.5	7.2±0.4
Fasting insulin (mU/l)	15.7±1.5	17.9 ±1.6	16.8±1.1
HOMA-IR	4.2±0.4	4.9±0.5	4.6±0.3
HbA_{1c} (%)	5.5±0.1	5.6±0.1	5.6±0.1

Plus-minus values are means±SEM. HOMA-IR denotes homeostasis model assessment of insulin resistance, HbA_{1c} glycated haemoglobin

Efficacy of microbiota manipulation by antibiotic treatment

The fecal microbiota composition was determined by analyzing 16S ribosomal RNA (rRNA) gene amplicons, using the Human Intestinal Tract Chip Microarray (HITchip)[19], which showed that seven-day VANCO markedly decreased microbial diversity (P<0.001), whereas this was not affected by AMOX (P=0.42) as compared to PLA (Figure 2.1). VANCO decreased the relative abundance of mainly Gram-positive bacteria of the Firmicutes phylum. Among the most strongly affected groups were genus-like groups that contain known butyrate-producing species from *Clostridium* clusters IV and XIVa, such as *Coprococcus eutactus*, *Faecalibacterium prausnitzii* and *Anaerostipes caccae*, as well as species involved in BA dehydroxylation such as *Clostridium leptum*. Conversely, Gram-negative Proteobacteria, members of *Clostridium* cluster IX and VANCO-resistant Gram-positive *bacilli* such as *Lactobacillus plantarum* and *Enterococcus*, showed increased relative abundance after VANCO treatment (Figure 2.1 and Table S2.1), which is in line with previous studies[18,20]. This pattern was confirmed with a supervised machine-learning technique (Random Forests analysis, Table S2). Importantly, microbiota composition was still affected eight weeks after cessation of VANCO treatment. Microbial diversity was still lower (q=0.053), and overall similarity and composition were deviant from baseline (pre-treatment) as compared to PLA. Although the bacterial groups that increased in abundance due to VANCO treatment had in general returned to baseline levels, several members of *Clostridium* clusters IV and XIVa were still decreased as compared to PLA. Furthermore, observed dynamics with respect to gut microbiota composition and diversity were individual-specific (Figure S2.1). In contrast, AMOX treatment did not affect microbiota composition after seven days treatment or at eight weeks follow-up compared to PLA, which is in accordance with a previous study in obese humans[18].

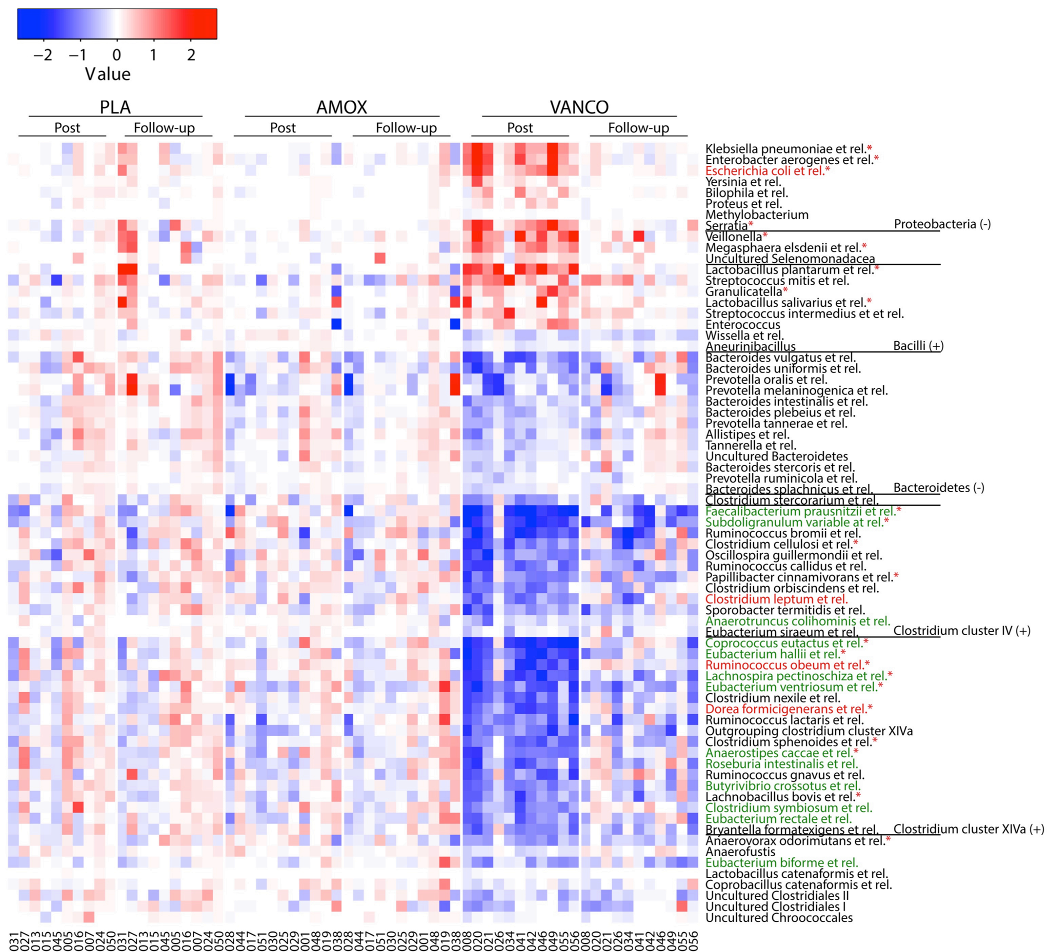


Figure 2.1. The effect of vancomycin and amoxicillin treatment on microbiota composition. Heatmap of bacterial groups (at genus and order like level with Gram staining between brackets) whose relative abundance was significantly different ($q<0.05$) post-treatment within the VANCO group. Color value shows $\log_{10}$ fold changes compared to baseline. Genus like groups containing known butyrate producing and BA dehydroxylating species are depicted in green and red, respectively. *Groups that exhibited a significant difference between VANCO and PLA treatments.

Antibiotic treatment does not affect tissue-specific insulin sensitivity

The primary outcome of this study was peripheral insulin sensitivity (insulin-stimulated glucose rate of disappearance, Rd), as determined by a two-step hyperinsulinemic-euglycemic clamp with [6,6-$^{2}H_{2}$]-glucose tracer infusion. Antibiotic treatment did not significantly alter Rd as compared to PLA (Figure 2.2). Additionally, no effects were found on hepatic and adipose tissue (AT) insulin sensitivity, as determined by the insulin-mediated suppression of endogenous glucose production (EGP) and plasma free fatty acid (FFA) concentrations, respectively. In accordance, antibiotic treatment did neither alter whole-body insulin sensitivity (HOMA-IR) immediately after cessation of treatment, nor at eight weeks follow-up (Figure S2). Our data are in contrast with several previous studies in rodents, which indicated that antibiotic treatment may improve glucose homeostasis and metabolic impairments[14-17,21-23]. Nevertheless, a more recent study showed that VANCO-treated mice had little weight change and no improvement in glycemic control[24]. Consistent with the present data, a four-day treatment with a broad-spectrum antibiotic cocktail did not affect postprandial glucose metabolism in lean healthy men[25]. Furthermore, it has recently been shown in a limited number of obese subjects with the metabolic syndrome that VANCO slightly but significantly reduced peripheral insulin sensitivity, despite comparable changes in microbial composition and BA metabolism as found in the present study[18]. Although the data of the latter study seems at odds with the present findings, it is important to emphasize that in the study by Vrieze and colleagues[18] the modest (~4%) VANCO-induced decrease in peripheral insulin sensitivity was based on a within-group comparison (post-treatment versus pre-treatment), since a placebo group was not included in the study design. Additionally, in the present study, follow-up measurements that were performed eight weeks after treatment cessation also did not show an effect on whole-body insulin sensitivity, despite a still considerably altered microbial composition as compared to pre-treatment as well as placebo.

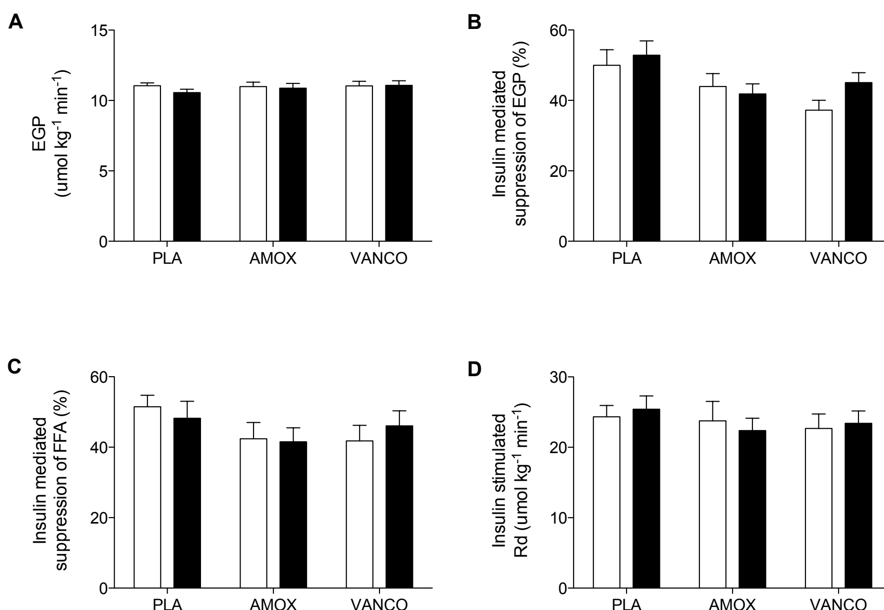


Figure 2.2. The effect of 7 days placebo, amoxicillin and vancomycin on hepatic, adipose tissue and peripheral insulin sensitivity. Bars represent means ± SEM (n=56). Tissue specific insulin sensitivity did not change by short-term antibiotics. **(A)** EGP: liver endogenous glucose production at baseline **(B)** steady state insulin-mediated EGP suppression (%) upon 10 mU/m^2/min insulin infusion **(C)** steady state 10 mU/m^2/min insulin-mediated suppression (%) of circulating free fatty acids (FFA) as measure for adipose tissue insulin sensitivity. **(D)** 40 mU/m^2/min insulin stimulated (oxidative) glucose disposal (Rd).

Antibiotic treatment does not affect energy and substrate metabolism

To examine the effect of gut microbiota modulation on postprandial metabolite concentrations, energy expenditure and substrate oxidation, we performed a high-fat mixed-meal test (2.6MJ [61E% fat, 33E% carbohydrates, 6E% protein]). We determined arterialized plasma metabolite concentrations and measured energy expenditure and substrate oxidation by whole-body indirect calorimetry. Neither VANCO nor AMOX significantly affected basal and postprandial plasma glucose, insulin, FFA, triacylglycerol (TAG) and lactate concentrations (Table 2.2 and Figure S2.3). Also, no significant effects on basal and postprandial energy expenditure, carbohydrate and fat oxidation were found (Figure 2.3). After

adjustment for fecal weight, intestinal energy harvest, which is reflected by daily fecal energy content, was neither changed immediately after treatment cessation, nor after eight weeks follow-up (Figure 2.3). Although previous studies in rodents have shown a prominent role of the gut microbiota in energy harvest and body weight[3,13], our findings suggest that antibiotics do not alter energy harvest in humans. Of note, in rodent studies, animals were exposed to antibiotics in their drinking water for two up to twenty weeks[13-17,21-23]. Similarly, more prolonged treatment (four to six weeks) with a higher dosage or a combination of different antibiotics increased body weight in endocarditis patients[26,27]. These studies may indicate that a long-term dysbalance in microbiota composition has more pronounced effects as compared to short-term manipulation. However, it is hard to differentiate between the role of the gut microbiota and systemic effects of antibiotics in the latter studies. Noteworthy, we have applied a two-day wash-out period before post-treatment measurements were performed to exclude that effects may be mediated via direct systemic effects of antibiotics. Additionally, VANCO does not pass the gastrointestinal barrier and, therefore, does not reach the circulation[28].

Antibiotic treatment does not alter gut permeability and systemic inflammatory markers

We investigated the effect of seven days of AMOX and VANCO treatment on gut permeability and the related translocation of bacterial lipopolysaccharide (LPS) from the intestinal lumen into the circulation. The pronounced VANCO-induced microbial alterations were not accompanied by changes in small intestine and proximal colon permeability (Figure S2.4), as assessed by a multi-saccharide test[29]. This is in accordance with unchanged LPS-binding protein (LBP) concentrations after VANCO and AMOX treatment as compared to PLA (Table 2.2). LPS, which is released by Gram-negative bacteria, may trigger the immune system by increasing inflammatory cytokine production in AT and is frequently used as an indicator of metabolic endotoxemia[30]. Therefore, we have additionally determined plasma interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α concentrations. In line with unchanged LBP concentrations, neither of these inflammatory factors was affected by seven-day VANCO or AMOX as compared to PLA. This was observed despite a substantial increase in relative abundance of potentially pro-inflammatory Gram-negative Proteobacteria.

Table 2.2. Effect of 7 days placebo, amoxicillin and vancomycin on metabolic, inflammatory, and hormonal parameters

Variable		PLA (N=14)	AMOX (N=12)	VANCO (N=12)	P value[a]
Fasting plasma metabolite and ANGPTL4 concentrations					
Glucose (mM)	Pre	6.31±1.12	6.48±0.25	6.25±0.19	0.177[b]
	Post	6.29±0.14	6.39±0.20	5.99±0.13	
TAG (mM)	Pre	1.40±0.17	1.08±0.15	1.03±0.09	0.511
	Post	1.47±0.21	1.04±0.15	1.06±0.10	
FFA (uM)	Pre	699±34	683±48	679±38.	0.423[b]
	Post	661±34	579±58	626±54	
Lactate (mM)	Pre	0.80±0.07	0.93±0.04	0.88±0.11	0.238
	Post	0.91±0.11	0.90±0.05	0.79±0.06	
ANGPTL4 (ng/ml)	Pre	5.1±0.7	4.3±0.5	4.9±0.5	0.137
	Post	5.5±0.7	3.8±0.5	4.3±0.3	
Postprandial (0-4h) plasma metabolite concentrations					
Glucose (iAUC/min)	Pre	0.60±0.10	0.50±0.12	0.41±0.19	0.633
	Post	0.54±0.13	0.55±0.10	0.45±0.09	
TAG (iAUC/min)	Pre	0.84±0.07	0.83±0.10	0.75±0.10	0.945[b]
	Post	0.92±0.07	0.94±0.01	0.81±0.07	
FFA (iAUC/min)	Pre	-364±27	-341±36	-339±31	0.547[b]
	Post	-332±28	-245±37	-300±40	
Lactate (iAUC/min)	Pre	0.65±0.05	0.45±0.07	0.40±0.08	0.154[b]
	Post	0.50±0.05	0.41±0.06	0.41±0.06	
Fasting plasma hormone concentrations					
GLP-1 (pmol/l)	Pre	8.7±0.7	8.5±0.7	9.7±1.1	0.670
	Post	9.3±1.1	8.7±0.8	10.2±1.2	
Insulin (mU/l)	Pre	11.5±1.3	12.6±1.3	14.3±1.8	0.504
	Post	12.7±1.6	13.4±1.8	13.9±1.5	
Leptin (ng/ml)	Pre	11.4±1.6	10.1±2.1	9.7±0.8	0.106[b]
	Post	12.9±2.3	10.0±1.8	8.8±0.8	
Postprandial (0-4h) plasma hormone concentrations					
GLP-1 (iAUC/min)	Pre	5.0±0.7	4.3±0.7	3.4±0.6	0.451
	Post	4.5±0.8	4.3±0.8	4.3±0.9	
Insulin (iAUC/min)	Pre	21.1±2.1	20.0±3.0	25.0±4.5	0.294
	Post	23.1±3.3	21.7±3.0	22.5±3.0	

Variable		PLA (N=19)	AMOX (N=18)	VANCO (N=19)	P value[a]
Fasting inflammatory marker concentrations					
LBP (pg/ml)	Pre	19.6±1.8	17.5±1.8	25.7±4.3	0.456
	Post	18.4±3.3	20.4±2.9	23.6±3.6	
IL-6 (pg/ml)	Pre	0.8±0.1	0.8±0.1	1.0±0.1	0.775
	Post	1.0±0.1	0.8±0.1	1.1±0.1	
IL-8 (pg/ml)	Pre	6.2±0.5	4.3±0.4[c]	5.2±0.4	0.444
	Post	5.9±0.5	4.8±0.4	5.9±0.4	
TNF-α (pg/ml)	Pre	2.6±0.1	2.3±0.1[c]	2.7±0.1	0.424
	Post	2.7±0.1	2.5±0.1	2.8±0.1	

Data are mean ± SEM. For determination of plasma hormones and metabolites, only a subgroup of n=38 was analyzed. There were no significant differences between the groups after intervention (Post) compared to baseline (Pre). Triacylglycerol (TAG), free fatty acids (FFA), glucagon-like peptide (GLP), angiopoietin-like 4 (ANGPTL4), lipopolysaccharide-binding protein (LBP), interleukin (IL), tumor necrosis factor (TNF). [a] P value represents the overall intervention effect between groups assessed by repeated measures ANOVA (time×treat P value) or ANCOVA when baseline concentrations were different between groups. [b] time effect ($P<0.05$). [c] baseline group difference ($P<0.05$)

Vancomycin inhibits bile acid conversion and short-chain fatty acid production

SCFA, notably butyrate, can be produced by several groups within the Firmicutes phylum (mainly *Clostridium* clusters XIVa and IV including *Coprococcus eutactus* and *F. prausnitzii*), some of which are also involved in BA dehydroxylation[9,31]. Indeed, we found a decreased relative abundance of these groups after VANCO, which was accompanied by a marked reduction in plasma (P=0.005) and fecal (P=0.001) concentrations of secondary BA as compared to PLA (Figure 2.4). This was accompanied by an increase of fecal primary BA (P=0.013). In addition, fecal SCFA concentrations (acetate (P=0.001), butyrate ($P<0.001$), caproate ($P<0.001$) and valerate (P=0.009)) were significantly decreased following VANCO, whilst in plasma only butyrate tended to decrease after VANCO (P=0.078) but not following AMOX treatment (Figure 2.5).

Although BA and SCFA may control incretin release[10,32] and affect energy metabolism in rodents[33], no effects on postprandial energy and substrate metabolism, and fasting and postprandial glucagon-like peptide 1 (GLP-1) concentrations were found in the present study (Table 2.2).

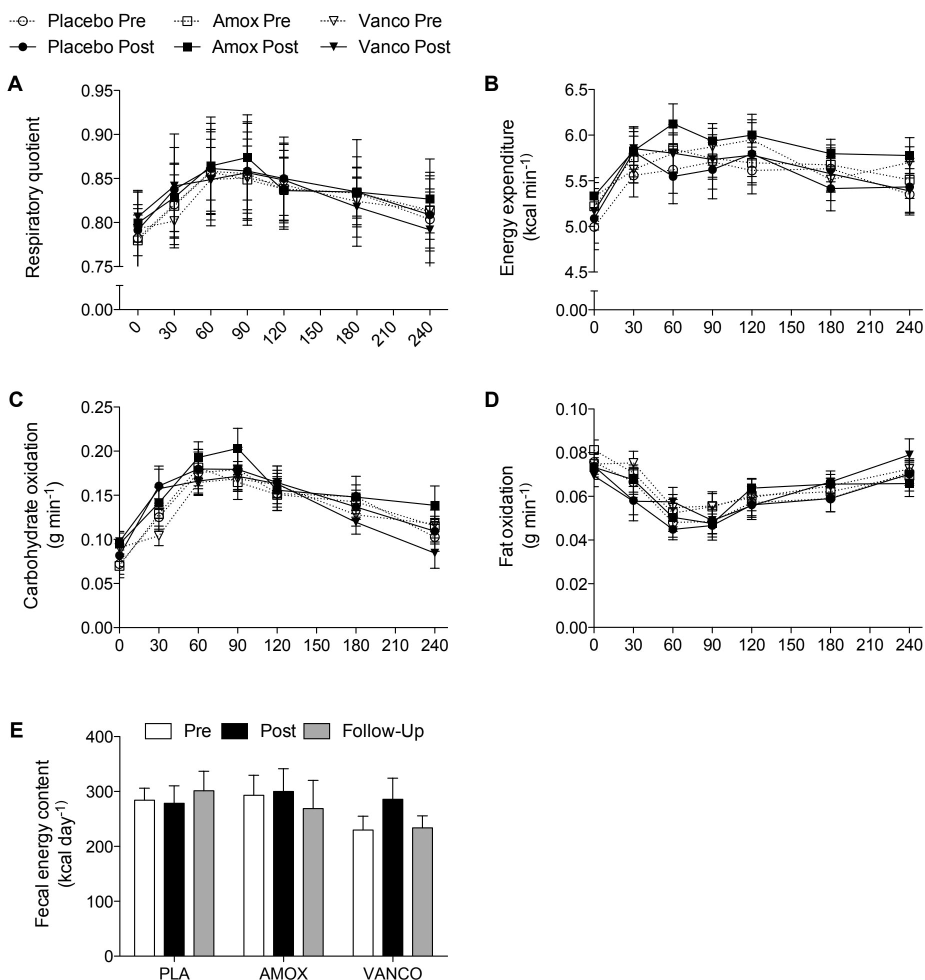


Figure 2.3. The effect of 7 days placebo, amoxicillin and vancomycin on energy expenditure, substrate metabolism and fecal energy excretion. Data are given as means ± SEM. Indirect calorimetry was performed during fasting conditions and for 4 hours after intake of a liquid high fat mixed meal (HFMM) in a subgroup of n=37. Mean O2-consumption and CO2-production over 20 minutes were used for calculations. (A-D) Fasting respiratory quotient (RQ), energy expenditure (EE), carbohydrate oxidation and fat oxidation did not differ after intervention (time×treat P value >0.05). Incremental AUC's after ingestion of HFMM were also not affected by AMOX or VANCO. (E) Fecal Energy excretion (kcal/day) did not significantly change after VANCO or AMOX compared to PLA (n=56).

Antibiotic treatment alters adipose tissue gene expression but not adipocyte morphology.

To determine the effect of an altered gut microbiota composition on AT, we collected abdominal subcutaneous AT biopsies to examine adipocyte size and gene expression profiles using Affymetrix microarray transcriptomic analysis. Antibiotic treatment had no significant effect on abdominal subcutaneous adipocyte size and the proportion of small and large adipocytes, neither directly after treatment cessation nor at eight weeks follow-up (Figure S2.5). Remarkably, when comparing the gene expression data with the Kyoto Encyclopedia of Genes and Genomes (KEGG), we found that VANCO and, to a lesser extent, AMOX increased AT expression of genes involved in pathways related to peroxisome-proliferator activated receptor (PPAR)-signaling and of genes encoding proteins involved in the mitochondrial Krebs cycle, fatty acid degradation and other components of the oxidative machinery, suggestive of increased oxidative metabolism in AT (Figure S2.6). In addition, VANCO decreased the expression of histone clustering genes. Although we found no differences in adipocyte morphology and circulating FFA, TAG, leptin and angiopoietin-like 4 (ANGPTL4) concentrations (Table 2.2), these alterations in the AT transcriptome may translate into changes in AT function over longer periods of time.

Finally, VANCO decreased the expression of gene sets involved in apoptosis and nuclear factor NFkB signaling as well as adaptive and innate immune responses, including genes of major histocompatibility complex-I, T-cell, B-cell and Natural Killer cell signaling. In contrast, genes related to lysosomal breakdown were upregulated as compared to PLA (Table S2.3). Lower NFkB-dependent gene expression and diminished NK and CD8+ T cell function in macrophages have been observed in germ-free and antibiotic-treated mice[34]. In the latter study, the effects were ascribed to a reduced activation of Farnesoid X receptors by a reduction of unconjugated and secondary BA[9], which seems in line with the present findings. In addition, although the exact role of SCFA in the systemic and AT immune cell responses is unknown, SCFA may be involved in the regulation of T-cells in the gut and peripheral tissues via the G-protein coupled receptor 43[10,35-37]. Despite the effects of antibiotic treatment on the KEGG-pathways described above, no significant associations (FDR<0.25) were found between individual bacterial groups and AT gene expression (data not shown).

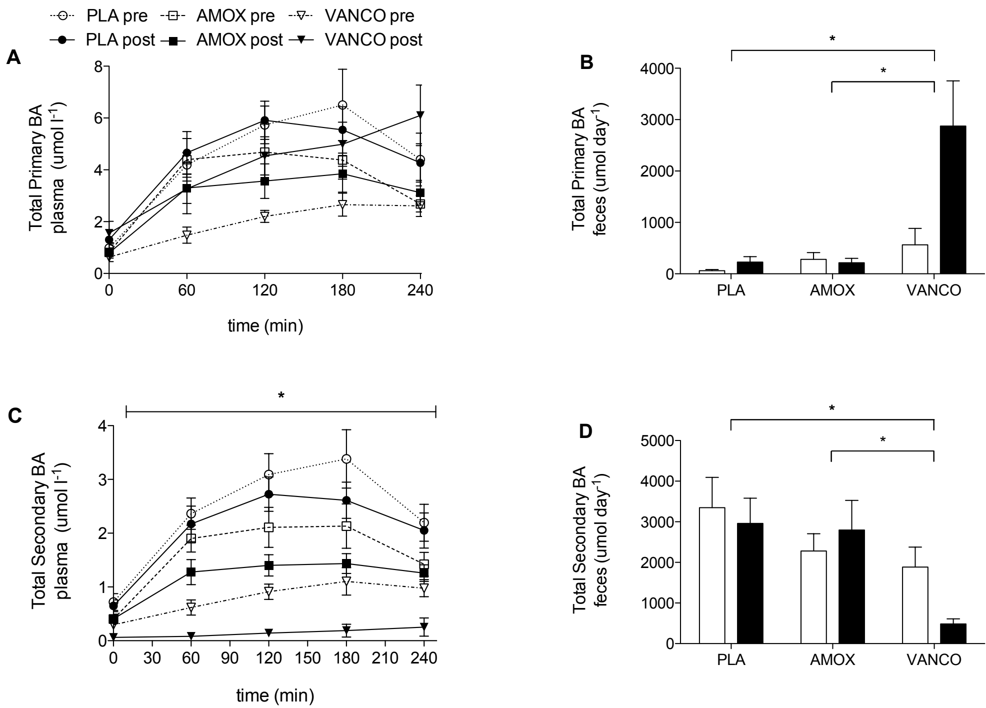


Figure 2.4. The effect of 7 days placebo, amoxicillin and vancomycin on bile acid concentrations in plasma and feces. Means ± SEM. In a subgroup of n=37, (A) plasma postprandial primary BA did not change significantly, (B) VANCO increased fecal primary BA, (C) decreased plasma secondary BA and (D) fecal secondary BA compared to PLA and AMOX. * time×treat P-value <0.05 for VANCO vs. PLA.

Microbial groups are not associated with host metabolic parameters

Although overall host metabolism did not change significantly following antibiotic treatment, we used univariate and multivariate statistics (redundancy analysis) to assess possible associations between specific characteristics of gut microbial profiles and host metabolic parameters. However, we did not find any significant and consistent associations when we evaluated the abundance and dynamics of individual bacterial taxa, combinations of taxa, the complete microbiota and bacterial diversity at baseline, as well as seven days and eight weeks post-intervention.

Furthermore, we investigated whether we could identify and connect patterns of specific metabolic and/or microbiological perturbations with the response to the intervention. First, we evaluated the stratification of subjects based on the extent of the microbial shift in diversity, as well as microbial composition. Secondly, based on the extent and direction of the metabolic response to the intervention, we used univariate and cluster analysis to discover microbial patterns. Lastly, we used latent class analysis[38], to define groups of subjects with certain metabolic patterns before and after treatment. Neither of these analyses showed groups of individuals with specific associations of the microbiota with host metabolic parameters (data not shown).

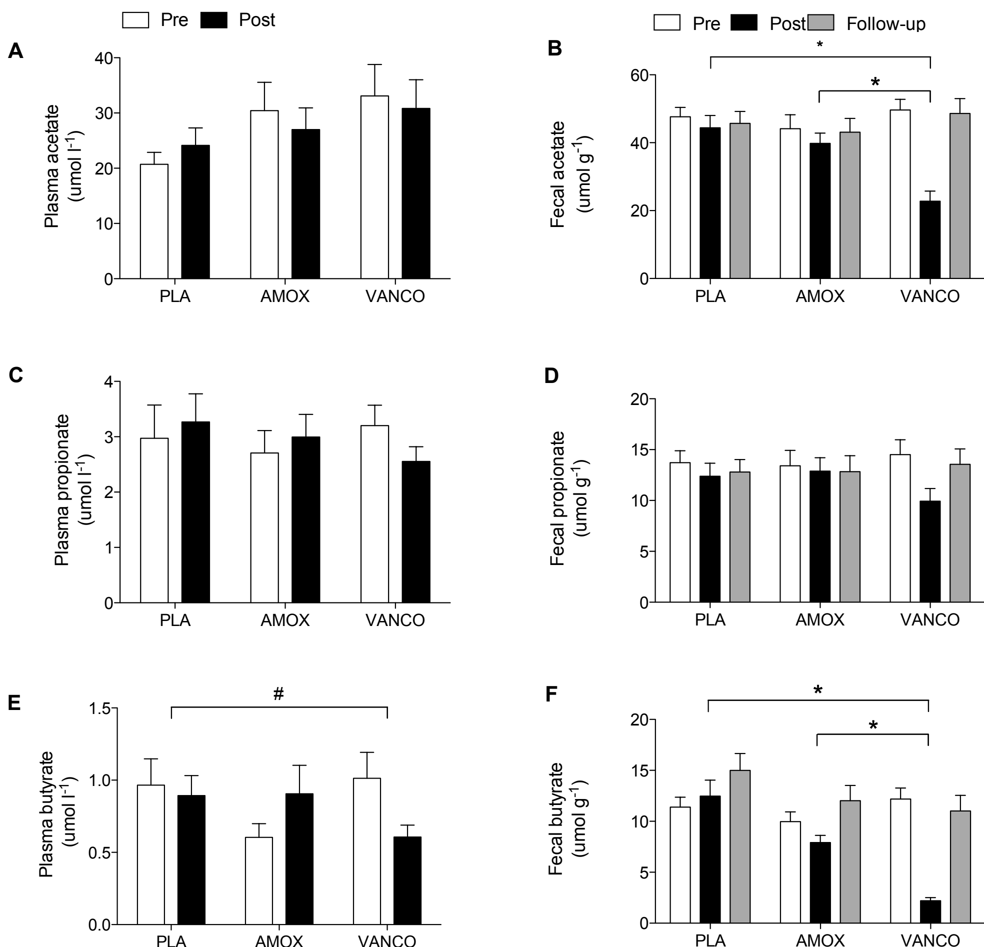


Figure 2.5: The effect of 7 days placebo, amoxicillin and vancomycin on plasma and fecal short chain fatty acid concentrations. Values are given as mean ± SEM (n=56). No significant effect was found for (A) plasma acetate, (C) plasma propionate, (D) fecal propionate, and a trend (#P=0.07) for (E) plasma butyrate. Fecal acetate and butyrate (B, F) decreased after VANCO treatment but not after AMOX. * time×treat P-value <0.05 for VANCO vs. PLA and vs. AMOX.

Perspectives

In the present study, we demonstrated that seven days VANCO treatment markedly affected microbial diversity and composition, which was accompanied by a reduced conversion of primary to secondary BA and a lower production of SCFA in the gut. Importantly, these alterations did not translate into significant effects on peripheral, hepatic and AT insulin sensitivity, energy and substrate metabolism and systemic low-grade inflammation immediately after treatment cessation. Moreover, no clinically relevant effects on energy harvest, abdominal subcutaneous adipocyte size and whole-body insulin sensitivity (HOMA-IR) were found at eight weeks follow-up. In contrast to VANCO, no effects of AMOX treatment on gut microbial composition, metabolic and inflammatory parameters were found. Taken together, the present study implies that interference with a resilient adult microbiota by antibiotics has no clinically relevant short-term (seven days) and long-term (eight weeks) effects on the metabolic parameters measured in this study. This contradicts many previous rodents studies and again highlights that rodent data cannot always be extrapolated to humans.

Noteworthy, several nuances have to be made with respect to the conclusions of the present study. First, since we studied obese, insulin resistant men with impaired glucose metabolism, we cannot exclude that microbiota manipulation by antibiotics may have more pronounced effects in women or less metabolically compromised individuals. Secondly, the duration of the intervention was relatively short, compared to rodent studies. Furthermore, it has been demonstrated that the risk of developing type 2 diabetes was increased when subjects were exposed to >5 antibiotic treatments[39], and that the number of prescriptions may accelerate the ageing-related decline of intestinal integrity[40]. Of note, the participants that were included in the present study had received on average 1.7 antibiotic treatments over the past 10 years, without any antibiotic

use 3 months prior to the start of the study. As mentioned above, several studies have indicated that a long-term or more frequent perturbation in microbiota composition may have more pronounced effects on metabolic health than short-term manipulation. For this reason, it is important to emphasize that the present study does not exclude an important role for the gut microbiota manipulations in changes of host metabolism. This should be further investigated in future prospective and long-term (dietary, prebiotic and/or probiotic) intervention studies in humans.

Supplementary Figures and Tables

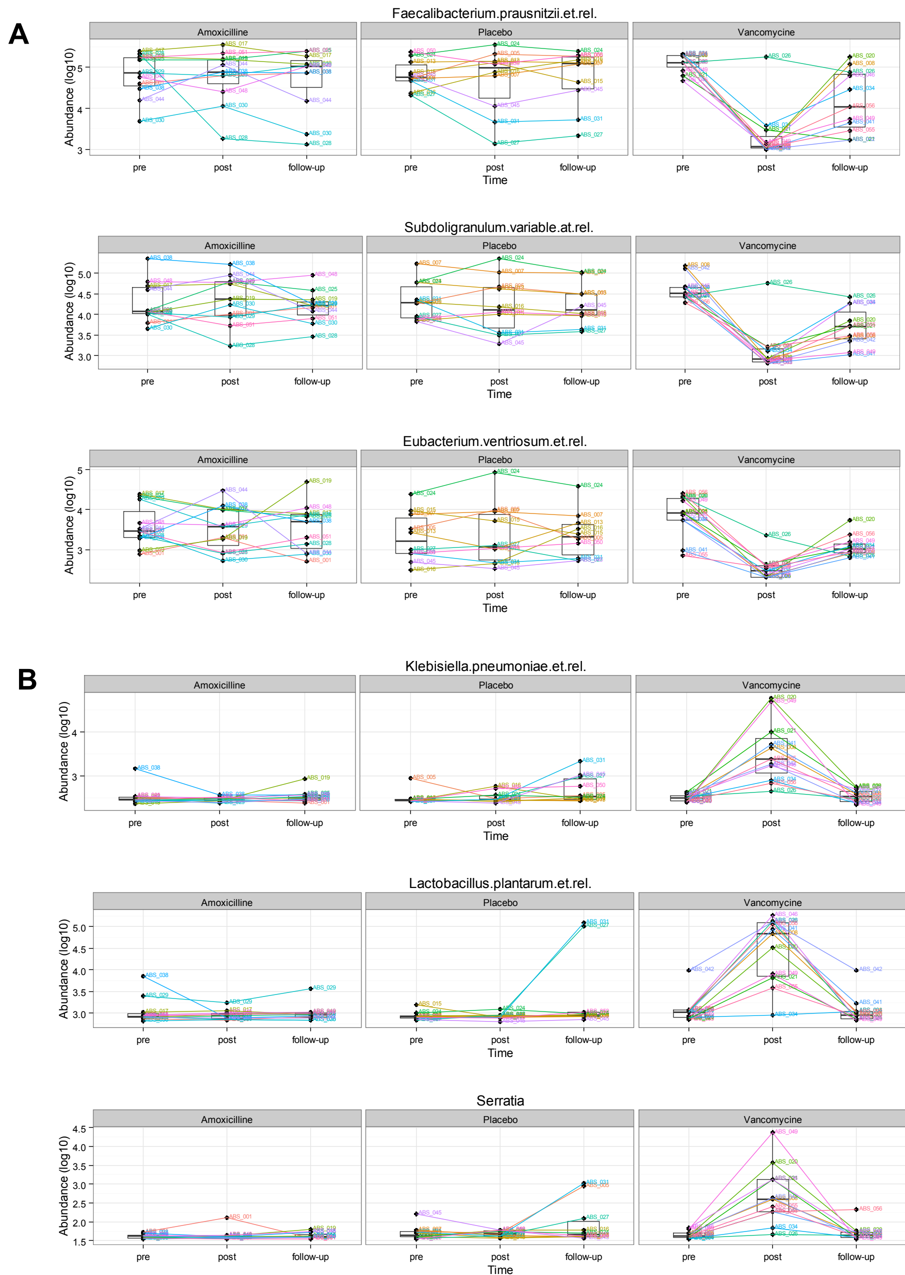
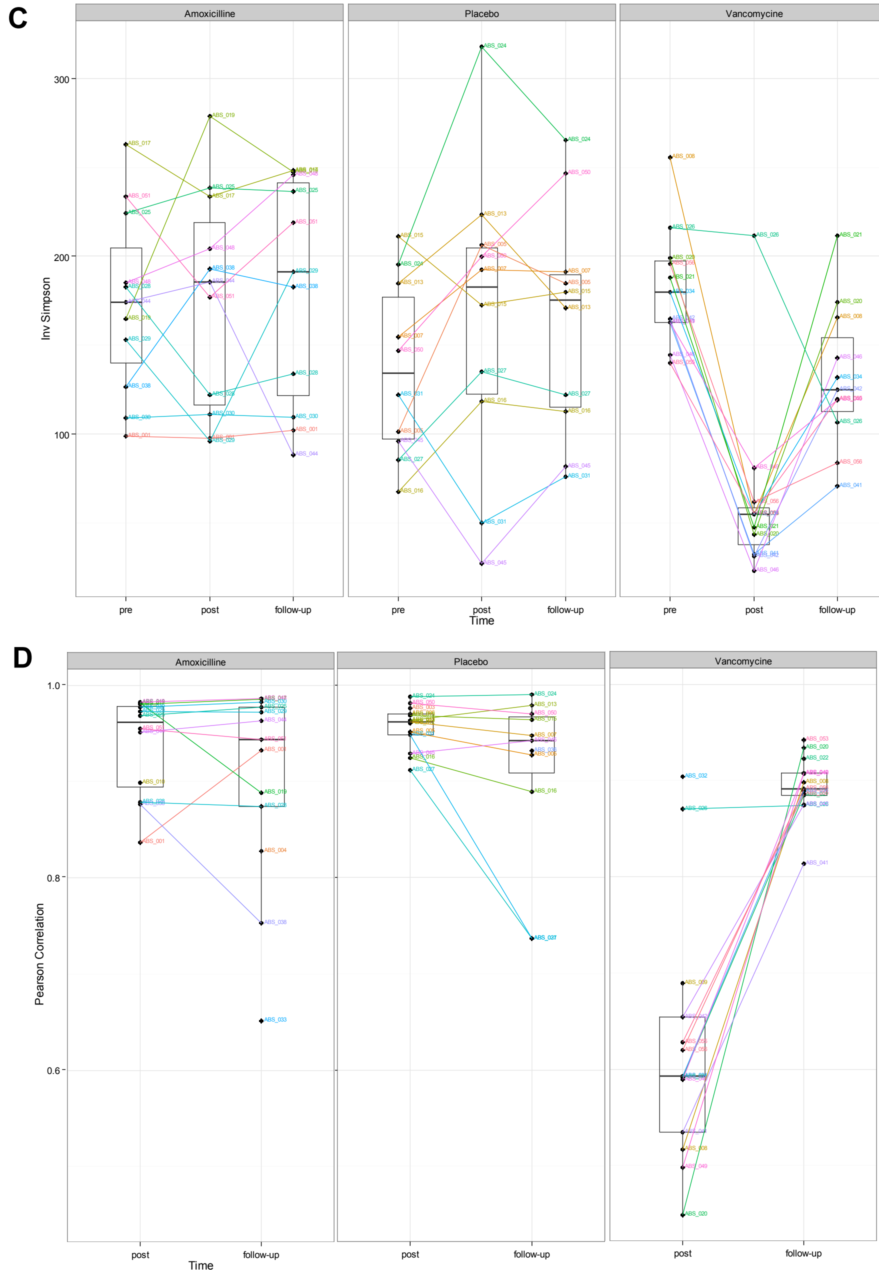


Figure S2.1. The effect of vancomycin, amoxicillin and placebo treatment on gut microbiota composition before, 7 days after and 8 weeks after intervention. Vancomycin treatment had highly pervasive effects on decreasing (A) and increasing (B) bacterial abundance, diversity (C) and overall similarity (D) as compared to baseline and placebo. Different individuals are indicated with different colours.



Although the abundance of most Gram-negative and vancomycin-resistant bacteria returned to near pre-treatment (baseline) levels at follow-up, some Gram-positive bacteria had not recovered in abundance in some individuals. A similar trend was observed for both diversity and compositional similarity at follow-up compared to baseline.

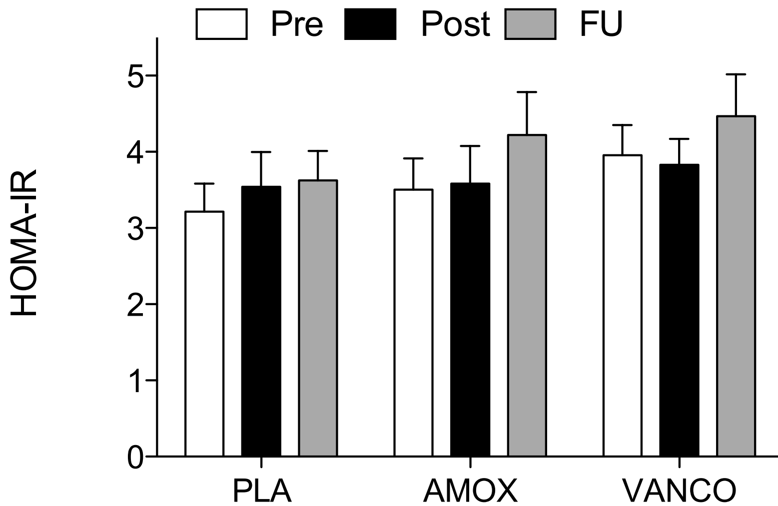


Figure S2.2. The effect of vancomycin, amoxicillin and placebo treatment for 7 days on whole-body insulin sensitivity directly after cessation of treatment and at 8-weeks follow-up. Data are mean ± SEM (*n*=37). AMOX and VANCO did not affect whole-body insulin sensitivity, reflected by the homeostasis model assessment of insulin resistance (HOMA-IR). There was a trend for a time-effect, but no time×treatment effect for FU compared to baseline (P=0.078). PRE, baseline values; POST, values directly after treatment cessation; FU, values at 8-weeks follow-up.

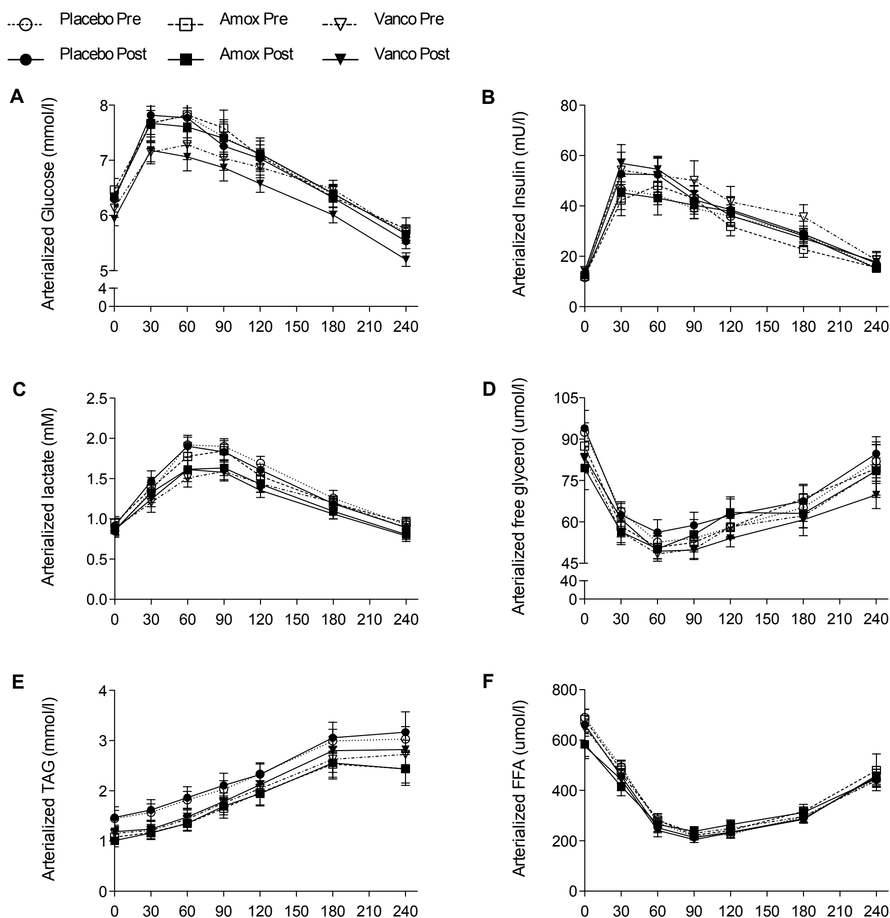


Figure S2.3. The effect of vancomycin, amoxicillin and placebo treatment for 7 days on plasma metabolite concentrations. Data are mean ± SEM (n=37). Fasting (t=0) and postprandial concentrations of (**A**) arterialized glucose, (**B**) lactate, (**C**) free glycerol, (**D**) triacylglycerol (TAG) and (**E**) free fatty acid (FFA) concentrations were not affected by AMOX or VANCO as assessed by repeated measures ANOVA.

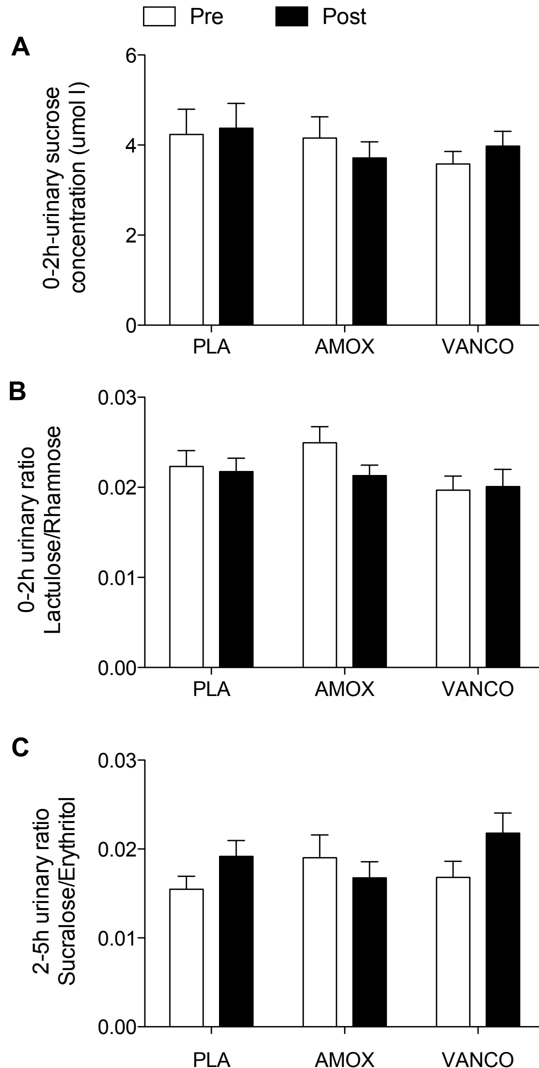


Figure S2.4. The effect of vancomycin, amoxicillin and placebo treatment for 7 days on gut permeability. Bars represent means ± SEM (n=56). To determine gut permability, urine was collected immediately before drinking a multisaccharide-mix (to), at t=120 and t=300 minutes. AMOX and VANCO did not affect (**A**) gastroduodenal permeability as expressed by the urinary sucrose concentration at t=120 min, (**B**) small interstine permeability expressed by the lactulose/rhamnose ratio (urine collection 0-120 min), (**C**) proximal colon permeability: suclarose/erythritol ratio (urine collection 120-300 min).

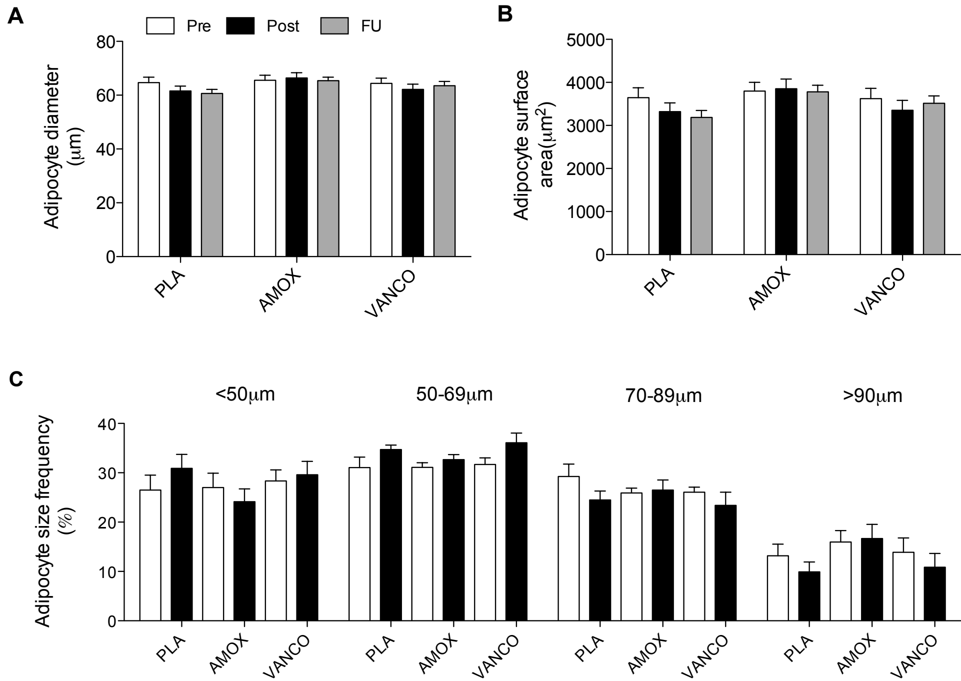


Figure S2.5. Abdominal subcutaneous adipocyte morphology before and immediately after placebo, amoxicillin and vancomycin treatment, as well as after 8 weeks follow-up. Representative sections of stained adipose tissue were used for adipocyte size determination in a subgroup of subjects (*n*=18). AMOX and VANCO did not affect **(A)** mean adipocyte diameter, **(B)** mean adipocyte surface area and **(C)** adipocyte size distribution.

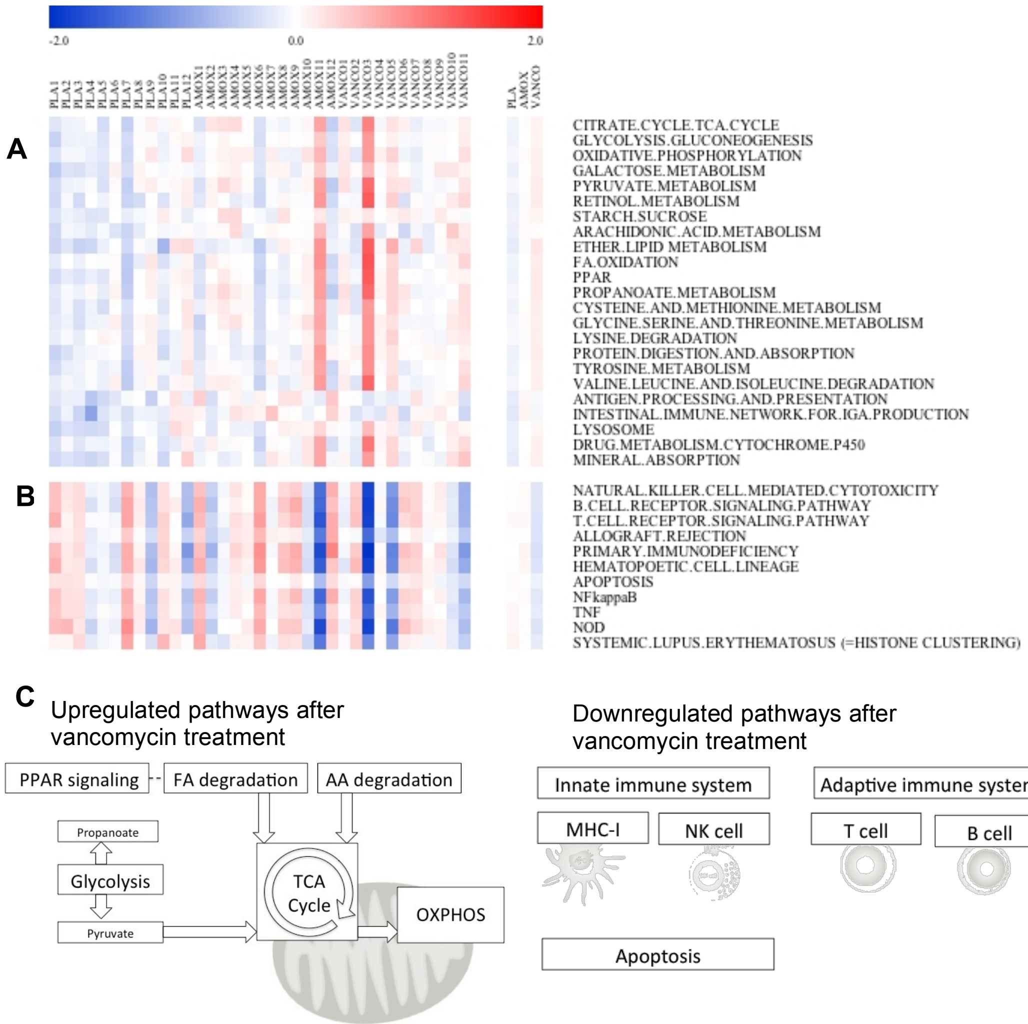


Figure S2.6. Heatmap of 7 days placebo, amoxicillin and vancomycin-induced effects on abdominal subcutaneous adipose tissue gene expression. **(A)** Upregulated genes, **(B)** downregulated genes determined in adipose tissue biopsies (*n*=30). The heatmap shows pathways related to metabolic function (Kyoto Encyclopedia of Genes and Genomes (KEGG) database) derived from Gene Set Enrichment Analysis. Signal log-ratio's (SLR) of genes in the heatmap represent genes that significantly contribute to the described pathways (FDR q-value <0.2). Color in the heatmap reflects the SLR per subject and group, with blue color being downregulated and red color being upregulated genes following treatment. (**C**) Schematic illustration of pathways that are upregulated and downregulated after vancomycin treatment as compared to placebo.

Table S2.1. Significantly different microbial taxa after intervention with vancomycin and placebo in feces using linear mixed models.

	BH_	P	log fold change within	log fold change within
Coprococcus.eutactus.et.rel	1.46E-	1.91E-	162.262.964	-0.036098988
Ruminococcus.bromii.et.rel.	4.57E-	1.09E-	152.679.587	-0.338380905
Faecalibacterium.prausnitzii	1.52E-	2.35E-	14.508.797	0.075849958
Lactobacillus.plantarum.et.	6.98E-	5.37E-	-139.338.187	-0.026993041
Eubacterium.hallii.et.rel.	2.96E-	1.25E-	133.243.286	-0.114103865
Ruminococcus.obeum.et.rel	1.20E-	1.29E-	125.172.537	-0.051553
Lachnospira.pectinoschiza.	1.07E-	1.07E-	119.017.372	-0.144882415
Clostridium.cellulosi.et.rel.	9.21E-	8.50E-	114.016.546	-0.267960043
Clostridium.nexile.et.rel.	1.51E-	2.21E-	1.103.251	-0.158269081
Anaerostipes.caccae.et.rel.	2.73E-	4.83E-	108.875.593	-0.225845465
Dorea.formicigenerans.et.re	4.03E-	9.31E-	105.973.413	-0.123118158
Veillonella	9.45E-	2.91E-	-0.99977749	0.008042942
Clostridium.sphenoides.et.r	1.44E-	1.77E-	0.91030989	-0.036075241
Serratia	3.30E-	7.11E-	-0.85859596	-0.132395735
Papillibacter.cinnamivorans	3.21E-	6.66E-	0.82835965	-0.156871014
Klebisiella.pneumoniae.et.r	1.92E-	3.15E-	-0.8215981	-0.144113787
Megasphaera.elsdenii.et.rel	7.84E-	1.81E-	-0.8001288	-0.016009582
Escherichia.coli.et.rel.	6.70E-	4.64E-	-0.79077723	-0.086406762
Lachnobacillus.bovis.et.rel.	3.21E-	6.43E-	0.78609501	-0.130157333
Enterobacter.aerogenes.et.r	3.21E-	6.48E-	-0.76953625	-0.145197198
Anaerovorax.odorimutans.e	1.47E-	2.04E-	0.66699492	-0.054689642
Sutterella.wadsworthia.et.r	3.21E-	6.35E-	-0.54038979	-0.096457386
Granulicatella	2.96E-	1.37E-	-0.49661041	-0.135840177
Lactobacillus.salivarius.et.r	4.99E-	1.23E-	-0.3206781	0.003000548
Collinsella	7.84E-	1.72E-	0.30211314	-0.045063251
Eubacterium.cylindroides.et	3.56E-	7.93E-	-0.28758184	-0.020381399
Clostridium.difficile.et.rel.	5.00E-	3.07E-	-0.26460033	-0.082852343
Bifidobacterium	1.27E-	1.47E-	0.18320543	-0.092371693
Eggerthella.lenta.et.rel.	7.26E-	6.14E-	0.15800988	-0.079697689
Aerococcus	1.92E-	3.25E-	-0.06422419	-0.034350517
Atopobium	3.79E-	2.04E-	0.02943863	0.005546336

This table shows bacteria taxa that were significantly different after placebo or vancomycin treatment using linear mixed models in addition to between-group analysis as determined by paired Wilxocon test. BH-adjustment: Benjamini–Hochberg corrected

Table S2.2. The effect of 7 days vancomycin treatment confirmed by Random Forests analysis.

Bacteria	MDA	Bacteria	MDA
Klebsiella pneumoniae et rel.	*2,15E-02*	*Bacteroides stercoris et rel.*	*1,94E-03*
Clostridium leptum et rel.	*1,33E-02*	*Outgrouping Clostridium cluster XIVa*	*1,89E-03*
Clostridium nexile et rel.	*1,05E-02*	*Streptococcus mitis et rel.*	*1,68E-03*
Lachnobacillus bovis et rel.	*9,86E-03*	*Yersinia et rel.*	*1,62E-03*
Megasphaera elsdenii et rel.	*9,51E-03*	*Bryantella formatexigens et rel.*	*1,58E-03*
Serratia	*9,40E-03*	*Subdoligranulum variable at rel.*	*1,49E-03*
Veillonella	*9,20E-03*	*Clostridium sphenoides et rel.*	*1,48E-03*
Eubacterium hallii et rel.	*8,77E-03*	*Papillibacter cinnamivorans et rel.*	*1,33E-03*
Clostridium symbiosum et rel.	*8,58E-03*	*Granulicatella*	*1,21E-03*
Prevotella tannerae et rel.	*8,40E-03*	*Streptococcus intermedius et rel.*	*1,15E-03*
Uncultured Clostridiales I	*8,34E-03*	*Eubacterium biforme et rel.*	*1,13E-03*
Lachnospira pectinoschiza et rel.	*7,44E-03*	*Clostridium stercorarium et rel.*	*1,11E-03*
Ruminococcus gnavus et rel.	*7,22E-03*	*Coprococcus eutactus et rel.*	*8,78E-04*
Ruminococcus lactaris et rel.	*6,77E-03*	*Faecalibacterium prausnitzii et rel.*	*6,84E-04*
Enterobacter aerogenes et rel.	*5,39E-03*	*Eubacterium rectale et rel.*	*6,58E-04*
Dorea formicigenerans et rel.	*5,34E-03*	*Actinomycetaceae*	*6,53E-04*
Uncultured Clostridiales II	*4,61E-03*	*Streptococcus bovis et rel.*	*6,21E-04*
Sporobacter termitidis et rel.	*4,45E-03*	*Coprobacillus catenaformis et rel.*	*6,10E-04*
Lactobacillus plantarum et rel.	*4,37E-03*	*Bacteroides splachnicus et rel.*	*5,73E-04*
Bacteroides vulgatus et rel.	*3,98E-03*	*Uncultured Mollicutes*	*5,58E-04*
Eubacterium ventriosum et rel.	*3,74E-03*	*Anaerofustis*	*5,53E-04*
Bacteroides plebeius et rel.	*3,64E-03*	*Eubacterium cylindroides et rel.*	*5,34E-04*
Butyrivibrio crossotus et rel.	*3,46E-03*	*Aneurinibacillus*	*5,03E-04*
Anaerostipes caccae et rel.	*3,41E-03*	*Bacteroides ovatus et rel.*	*4,95E-04*
Anaerovorax odorimutans et rel.	*3,08E-03*	*Methylobacterium*	*3,98E-04*
Clostridium orbiscindens et rel.	*3,02E-03*	*Eubacterium siraeum et rel.*	*3,58E-04*
Escherichia coli et rel.	*3,01E-03*	*Peptococcus niger et rel.*	*2,72E-04*
Anaerotruncus colihominis et rel.	*2,98E-03*	*Collinsella*	*2,48E-04*
Clostridium cellulosi et rel.	*2,87E-03*	*Prevotella melaninogenica et rel.*	*2,38E-04*
Bacteroides uniformis et rel.	*2,71E-03*	*Eggerthella lenta et rel.*	*2,37E-04*
Ruminococcus obeum et rel.	*2,68E-03*	*Catenibacterium mitsuokai et rel.*	*2,27E-04*
Ruminococcus bromii et rel.	*2,47E-03*	*Burkholderia*	*1,87E-04*
Oscillospira guillermondii et rel.	*2,40E-03*	*Vibrio*	*1,74E-04*
Roseburia intestinalis et rel.	*2,39E-03*	*Uncultured Chroococcales*	*1,38E-04*
Bacteroides intestinalis et rel.	*2,12E-03*	*Brachyspira*	*1,35E-04*
Ruminococcus callidus et rel.	*1,99E-03*		

This tables shows the mean decrease accuracy of bacterial taxa that were also found to be altered after 7 days vancomycin treatment as determined by paired Wilcoxon test. MDA (mean decrease accuracy) represents the impact of bacterial taxa on the accuracy of the model.

Table S2.3. Effects of vancomycin and amoxicillin vs. placebo on gene set enrichment of the adipose tissue

CLASS	UPREGULATED GENE SETS	VANCO-PLA		AMOX-PLA	
KEGG BRITE	KEGG PATHWAYS	NES	FDR-q	NES	FDR-q
Energy metabolism	OXIDATIVE.PHOSPHORYLATION	2.24 *	0.000	1.75*	0.081
Carbohydrate metabolism	GLYCOLYSIS.GLUCONEOGENESIS	1.95 *	0.006	1.81*	0.062
	GALACTOSE.METABOLISM	1.76 *	0.023	2.02*	0.029
	PYRUVATE.METABOLISM	1.99 *	0.006	1.38	0.263
	CITRATE.CYCLE.TCA.CYCLE.	1.83 *	0.016	1.86*	0.049
	PROPANOATE.METABOLISM	1.89 *	0.010	1.39	0.258
	STARCH.AND.SUCROSE.METABOLISM	1.51	0.113	1.74*	0.079
Lipid metabolism	ARACHIDONIC.ACID.METABOLISM	0.97	0.719	1.74*	0.081
	FATTY.ACID.DEGRADATION	1.86 *	0.014	1.14	0.522
	ETHER.LIPID.METABOLISM	1.84 *	0.015	0.94	0.804
	PPAR.SIGNALING.PATHWAY	1.89 *	0.010	0.85	0.889
Amino acid metabolism	LYSINE.DEGRADATION	1.87 *	0.012	0.89	0.852
	CYSTEINE.AND.METHIONINE.METABOLISM	1.89 *	0.010	1.76*	0.078
	TYROSINE.METABOLISM	1.83 *	0.016	1.72*	0.083
	VALINE.LEUCINE.AND.ISOLEUCINE.DEGRADATION	1.79 *	0.018	1.59	0.144
	GLYCINE.SERINE.AND.THREONINE.METABOLISM	1.73*	0.030	1.6	0.137
	LYSOSOME	1.99 *	0.006	2.22*	0.000
	COMPLEMENT.AND.COAGULATION.CASCADES	2.21 *	0.001	1.6	0.133
Digestive system	PROTEIN.DIGESTION.AND.ABSORPTION	2.01 *	0.006	n.a.	n.a.
	MINERAL.ABSORPTION	1.86 *	0.013	1.79*	0.068
Immune system (MHC-II)	INTESTINAL.IMMUNE.NETWORK.FOR.IGA.PRODUCTION	n.a.	n.a.	2*	0.027
	ANTIGEN.PROCESSING.AND.PRESENTATION	n.a.	n.a.	1.8*	0.059

Xenobiotics metabolism	DRUG.METABOLISM.CYTOCHROME.P450	2.09 *	0.003	1.61	0.131
	METABOLISM.OF.XENOBIOTICS.BY.CYTOCHROME.P450	1.98 *	0.006	1.42	0.237
	DRUG.METABOLISM.OTHER.ENZYMES	1.07	0.621	1.68*	0.100
Cofactor and vitamin metabolism	RETINOL.METABOLISM	1.99 *	0.006	1.12	0.560
Signaling molecules	ECM.RECEPTOR.INTERACTION	1.77*	0.021	0.82	0.920

CLASS	**DOWNREGULATED GENE SETS**	**VANCO-PLA**		**AMOX-PLA**	
KEGG BRITE	**KEGG PATHWAYS**	**NES**	**FDR-q**	**NES**	**FDR-q**
Immune system	NATURAL.KILLER.CELL.MEDIATED.CYTOTOXICITY	-2.4*	0.001	-0.74	1.000
	B.CELL.RECEPTOR.SIGNALING.PATHWAY	-1.8*	0.026	-0.76	1.000
	T.CELL.RECEPTOR.SIGNALING.PATHWAY	-2.23*	0.002	-0.77	1.000
	NOD.LIKE.RECEPTOR.SIGNALING.PATHWAY	-2.24*	0.003	-1	0.831
	HEMATOPOIETIC.CELL.LINEAGE	-1.72*	0.039	n.a.	n.a.
Immune diseases (MHC-I)	ALLOGRAFT.REJECTION	-1.55*	0.078	n.a.	n.a.
	GRAFT.VERSUS.HOST.DISEASE	-1.66*	0.053	n.a.	n.a.
	PRIMARY.IMMUNODEFICIENCY	-2.08*	0.006	n.a.	n.a.
	INFLAMMATORY.BOWEL.DISEASE.IBD.	-1.55*	0.079	n.a.	n.a.
	SYST.L.ERYTHEM. (GENES OF HISTONE CLUSTER)	-1.71*	0.040	-1.35	0.489
Substance dependence	ALCOHOLISM (GENES OF HISTONE CLUSTER)	-1.85*	0.023	-1.88*	0.085
Replication and repair	DNA.REPLICATION	-1.57*	0.072	n.a.	n.a.
Cell growth and death	APOPTOSIS	-1.99*	0.010	n.a.	n.a.
Signal transduction	NF.KAPPA.B.SIGNALING.PATHWAY	-1.56*	0.076	n.a.	n.a.
	TNF.SIGNALING.PATHWAY	-1.63*	0.061	n.a.	n.a.

Gene set enrichment analysis found enriched pathways of the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. KEGG BRITE denotes clustering of KEGG-pathways in functional hierarchies, NES normalized enrichment score, FDR-q false discovery rate adjusted p-value, not appliccable means no significant upregulation, respectively downregulation after vancomycin or amoxicillin of the selected pathway. Data of n=10 per group was used for microarray analysis. * FDR-q<0.1, significant enrichment of the selected pathway.

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CHAPTER 3

Antibiotic treatment does not affect skeletal muscle substrate metabolism in obese men: a randomized, double-blind, placebo-controlled trial

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To be submitted

Abstract

Aim To investigate the impact of gut microbiota manipulation on fasting and postprandial skeletal muscle metabolism in obese humans.

Methods 40 obese, insulin resistant males were randomized to amoxicillin (broad-spectrum antibiotic), vancomycin (targeting gram-positive bacteria) or placebo treatment (7 d, 1500 mg/d). Before and after treatment, forearm blood flow and metabolite fluxes across forearm muscle were measured.

Results Neither vancomycin nor amoxicillin treatment significantly affected fasting and postprandial plasma glucose, free fatty acids (FFA), triacylglycerol (TAG), glycerol, lactate and insulin concentrations, and forearm blood flow. Fasting and postprandial net forearm muscle glucose uptake and the release of lactate across forearm were not significantly altered by antibiotic treatment as compared to placebo. Finally, antibiotic treatment did not change fasting and postprandial glycerol, FFA and TAG fluxes across forearm muscle.

Conclusion The present study demonstrated that skeletal muscle substrate metabolism, forearm blood flow and postprandial glucose metabolism were not affected following short-term antibiotic treatment in obese men with impaired glucose metabolism.

Introduction

Accumulating evidence indicates that the composition of the gut microbiome is involved in host energy and substrate metabolism, thereby contributing to the etiology of obesity and type 2 diabetes mellitus (T2DM)[1]. The intestinal microbiota is involved in the secretion of various gut hormones and substrates, which in turn influence metabolic processes in peripheral organs such as the liver, adipose tissue and skeletal muscle. The skeletal muscle accounts for approximately 80% of insulin-stimulated glucose disposal and, therefore, plays a key role in development and progression of insulin resistance and T2DM[2]. In the obese state, a reduced adipose tissue lipid buffering capacity contributes to an increased supply of lipids to non-adipose tissues such as the skeletal muscle, causing ectopic lipid accumulation when oxidative capacity is insufficient[3]. In addition, impairments in postprandial fatty acid uptake, storage and oxidation may contribute to intramuscular accumulation of lipid-intermediates like diacylglycerol and ceramides[4,5]. Accumulation of these bioactive metabolites has been associated with insulin resistance.

Interestingly, the gut microbiota may affect host metabolic health through effects on skeletal muscle metabolism, at least in rodents. It has been shown that the persistent lean phenotype of germ-free mice may be associated with increased skeletal muscle levels of phosphorylated AMPK and its downstream targets involved in fatty acid oxidation[6]. Additionally, the gut hormones glucagon-like peptide 1 (GLP-1) and Peptide YY have been reported to affect muscle metabolism and insulin sensitivity in rats through effects on microvascular blood flow and fat oxidation, respectively[7].

Finally, SCFA, derived from microbial fermentation of indigestible carbohydrates, may have effects on peripheral substrate metabolism since their receptors are expressed in skeletal muscle[8,9]. Taken together, several lines of evidence suggest that the gut microbiota-skeletal muscle cross-talk is involved in substrate metabolism and insulin sensitivity in rodents[1,10]. However, human studies that have investigated the effects of microbiota manipulation on skeletal muscle substrate metabolism and insulin sensitivity under physiological conditions are lacking. To unravel the impact of gut microbiota manipulation on fasting and postprandial skeletal muscle metabolism in humans, we investigated the effect of broad (amoxicillin; AMOX) and narrow-sprectrum (vancomycin; VANCO)

antibiotic treatment on skeletal muscle blood flow and metabolite fluxes across forearm muscle in obese men with impaired glucose metabolism.

Methods

This study was performed in a sub-cohort of a larger clinical trial in which we investigated the impact of antibiotic treatment on microbiota composition and tissue-specific insulin sensitivity. In this randomized, placebo-controlled, double-blind study with a 3-armed parallel design, participants received either AMOX, VANCO (1500 mg/d) or placebo (PLA) for 7 consecutive days. The inclusion criteria are presented in detail before[11]. Here, the effects of gut microbiota manipulation on skeletal muscle substrate metabolism and postprandial insulin sensitivity were investigated in 40 obese men with impaired impaired fasting glucose and/or impaired glucose tolerance, who all gave written informed consent (reviewed and approved by the local Medical Ethical Committee). All procedures were according to the declaration of Helsinki (revised version, 2008)

Study procedures

Before and after the intervention, forearm muscle metabolism and blood flow were studied in the fasting state and for 4 hours after consumption of a high-fat mixed meal (HFMM; 2.6 MJ, [61 E% fat, 33 E% carbohydrate, 6 E% protein])[11]. The evening before an investigation day, participants were asked to consume the same evening meal (low in fiber and fat)[12]. Skeletal muscle metabolism was studied in the forearm muscle, using arterio-venous concentration differences corrected for forearm blood flow. Therefore, catheters were inserted into the superficial dorsal hand vein, which was heated in a hot box (55°C) to allow arterialized venous blood sampling, and retrogradely in a deep anticubital vein of the contralateral forearm to sample venous blood[13]. Blood samples were taken simultaneously from these catheters during fasting and postprandially (t30, t60, t90, t120, t180, t240 min). During this timeframe, total forearm blood flow (FBF) was measured before each blood sampling by venous occlusion plethysmography with a mercury strain gauge (Periflow 0699; Janssen Scientific Instruments), as described previously[14]. To prevent contamination of the blood from the forearm vein with blood from the hand, a wrist cuff was inflated to 200 mm Hg for 3 min before the samples were taken[15].

Biochemical analyses

Blood was collected in pre-chilled EDTA-tubes and immediately centrifuged (1,000 *g*, 10 min, 4 °C). Plasma was frozen in liquid nitrogen and stored at –80°C until analysis. Plasma free fatty acids (FFA) were analyzed using standard enzymatic techniques automated on a Cobas Fara centrifugal spectrophotometer (Roche Diagnostics, Basel, Switzerland). Plasma triacylglycerol (TAG), glycerol, glucose, and lactate were analyzed enzymatically on a Cobas Mira automated spectrophotometer. Plasma insulin was measured with a radioimmunoassay (Millipore). Hematocrit was determined in heparinized blood using a microcapillary system.

Calculations and statistics

The power calculation for the total inclusion can be found elsewhere[11]. To be able to detect a 5% difference in skeletal muscle lipid handling, n=12 per intervention group were needed[16]. To take into account block-randomization (n=6 per block) and possible drop-outs, we included 40 participants for this sub-study.

The postprandial response is given as incremental area under the curve per minute, which was calculated by the trapezoid rule from the start of the meal to 240 min ($iAUC_{0–240}$).

The FBF (ml $100ml^{-1}$ min^{-1}) was calculated as the slope of the plethysmograph-graph muliplied by 6. The blood flow rate is FBF*(100-hematocrit/100). The forearm muscle fluxes of metabolites (i.e. glucose uptake, lactate release) were calculated by the arteriovenous difference of glucose multiplied by the blood flow rate. A positive flux indicates net uptake across forearm muscle whereas a negative flux indicates net release into the circulation.

Analysis of variance (ANOVA) was applied to compare group characteristics at baseline. Differences between treatments were analyzed using repeated-measures ANOVA with time and treatment as factors. Data are expressed as mean ± SEM. $P<0.05$ (two-sided P-value) was considered to be statistically significant. Statistical analysis was performed using SPSS for Mac version 20.0 (Chicago, IL, USA).

Results

No serious adverse events of the antibiotic treatment were reported. At baseline, body weight, BMI, and body fat distribution did not differ between groups (Table 3.1). Furthermore, baseline fasting plasma glucose, insulin, lactate, glycerol, TAG and FFA concentrations were comparable between groups (Figure 3.1). As expected, plasma glucose, lactate, insulin and TAG concentrations increased compared to baseline levels after meal intake in all groups (Figure 3.1). Furthermore, meal intake decreased postprandial FFA and free glycerol concentrations, which returned to baseline values after 240 minutes.

VANCO and AMOX treatment did not significantly affect fasting arterialized concentrations and the postprandial response (iAUC/min) of circulating metabolites and insulin as compared to PLA (all $P > 0.05$).

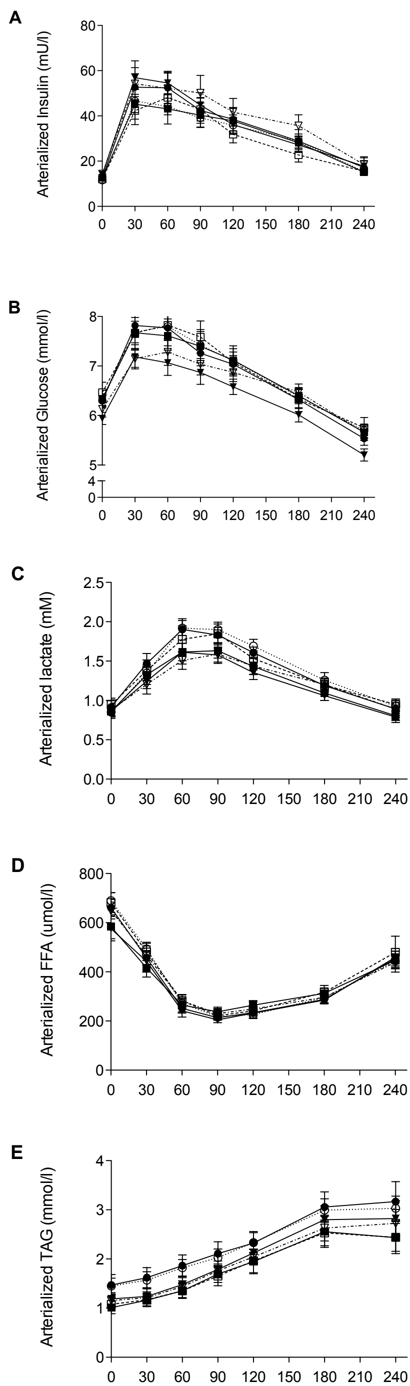
Baseline fasting FBF was comparable between groups (mean FBF, 4.48 ± 0.23 ml $100ml^{-1}$ min^{-1}), and postprandial FBF did not significantly increase after meal intake. Antibiotic treatment had no significant effect on baseline and postprandial FBF as compared to PLA (Figure 3.1).

We found neither significant effects of VANCO or AMOX on fasting and postprandial net forearm muscle glucose uptake nor the release of lactate across forearm muscle as compared to PLA. Similarly, antibiotic treatment did not alter fasting and postprandial glycerol, FFA and TAG fluxes across forearm muscle (Figure 3.1).

Table 3.1. Subject characteristics

	PLA (n=13)	AMOX (n=12)	VANCO (n=13)
Age (years)	62.6±1.3	56.3±2.0	60.0±1.9
Body Weight (kg)	92.0±2.4	96.7±2.9	96.5±2.5
BMI (kg/m2)	30.0±0.6	31.3±1.1	31.2±0.8
WHR	1.05±0.01	1.04±0.02	1.07±0.02
Waist circumference (cm)	108.3±2.1	107.7±3.4	111.5±3.0
Fasting glucose (mmol/l)	6.3±0.1	6.5±0.2	6.3±0.2
Fasting insulin (mU/l)	11.5±1.3	12.6±1.3	14.3±1.8
HOMA-IR	3.3±0.3	3.1±0.4	3.9±0.3

Data are expressed as mean ± SEM. No significant differences were found between groups (one-way ANOVA, $P > 0.05$)


A
Arterialized Insulin (mU/l)
80
60
40
20
0
0 30 60 90 120 150 180 210 240
B
Arterialized Glucose (mmol/l)
8
7
6
5
4
0
C
Arterialized lactate (mM)
2.5
2.0
1.5
1.0
0.5
0.0
D
Arterialized FFA (umol/l)
800
600
400
200
E
Arterialized TAG (mmol/l)
4
3
2
1

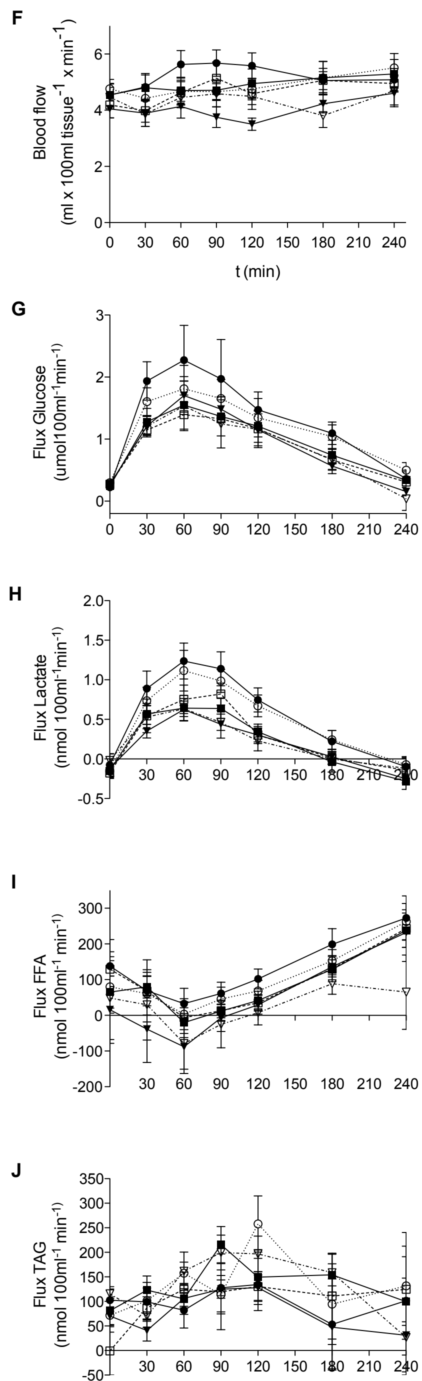

F
Blood flow
(ml x 100ml tissue-1 x min-1)
t (min)
G
Flux Glucose
(umol100ml-1min-1)
H
Flux Lactate
(nmol 100ml-1min-1)
I
Flux FFA
(nmol 100ml-1 min-1)
J
Flux TAG
(nmol 100ml-1 min-1)

Figure 3.1. Effect of 7 days PLA, AMOX or VANCO treatment on plasma concentrations and fluxes of glucose, lactate, FFA, TAG and insulin across forearm muscle. Fasting (t=0) and postprandial concentrations of (A) arterialized insulin, (B) glucose, (C) lactate, (D) free fatty acids (FFA) and (E) triacylglycerol (TAG) concentrations were not affected by AMOX or VANCO as assessed by repeated measures ANOVA. AMOX and VANCO did not affect forearm blood flow (F) and net fluxes of (G) glucose, (H) lactate, (I) FFA, and (J) TAG across forearm muscle are shown. A positive flux indicates net uptake across the forearm muscle whereas a negative flux indicates net release. Values are given as mean ± SEM (n=37).

Discussion

The present study demonstrated that gut microbiota manipulation using antibiotics had no significant effects on fasting and postprandial forearm muscle substrate metabolism and postprandial forearm muscle insulin sensitivity in obese men with impaired glucose metabolism.

VANCO reduced microbiota diversity after 7 days treatment, as we have previously reported[11]. The abundance of butyrate-producing species and species involved in bile acid dehydroxylation decreased following VANCO, whilst there was a compensatory upregulation of gram-negative Proteobacteria and VANCO-resistant bacilli. AMOX did not induce any significant changes in microbiota diversity or composition, and did not affect related metabolites[11].

Here, we found that antibiotic treatment did neither influence systemic lipid overflow, i.e. fasting and postprandial TAG and FFA concentrations, nor postprandial glucose and insulin concentrations. Together with an unchanged postprandial forearm muscle glucose flux following VANCO or AMOX, this indicates that postprandial insulin sensitivity did not change by antibiotic treatment. In contrast, rodents studies have indicated that the gut microbiota may exert important effects on substrate and energy metabolism, thereby contributing to the etiology of obesity and type 2 diabetes through various mechanisms. Alterations in gut hormones like GLP-1 and microbial products such as SCFA[17,18] have been suggested to underlie changes in skeletal muscle fat oxidation and substrate metabolism following gut microbiota manipulation. More specific, it has been suggested that the stimulation of fat oxidation in skeletal muscle might be under influence of gut-derived hormones and substrates through metabolic pathways involving the phosporylation of AMPK[6].

Also, AMPK is able to regulate the transcription of the glucose transporter GLUT4[19], thereby influencing skeletal muscle glucose uptake. However, it is important to emphasize that most data and putative mechanisms are obtained from animal studies.
Previously, we have reported that this 7-day VANCO treatment significantly reduced fecal SCFA (butyrate and acetate) and, to a lesser extent, plasma butyrate concentrations. Based on these findings, we hypothesized that this may induce alterations in skeletal muscle substrate metabolism. However, the present data clearly show that these changes in SCFA and BA concentrations neither affect skeletal muscle substrate fluxes nor postprandial insulin sensitivity. Another group of gut derived-signals, the incretins peptide YY and GLP-1, might affect microvascular blood flow[17,20], thereby altering substrate supply and uptake in skeletal muscle. In line with previously reported unchanged GLP-1 concentrations following antibiotic treatment[11], the present data indicate that short-term antibiotic treatment did not alter fasting and postprandial forearm muscle blood flow.

Conclusion

The present study demonstrated that, despite marked changes in gut microbiota composition following VANCO treatment, skeletal muscle substrate metabolism and postprandial glucose metabolism were not altered by 7-days antibiotic treatment in obese men with impaired glucose metabolism. Further research should establish whether more prolonged manipulation of the gut microbiota may induce changes in skeletal muscle substrate metabolism.

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CHAPTER 4

Gut microbiota manipulation and lipolysis in human adipose tissue

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Abstract

Background Recent evidence indicates that the intestinal microbiota and its products may contribute to the development of obesity and related cardiometabolic complications by affecting lipid metabolism and insulin sensitivity. Here, we investigated the effects of microbiota manipulation on *ex vivo* basal and β-adrenergically-stimulated lipolysis in human adipocytes, and adipose tissue gene and protein expression.

Methods Thirty-six obese men were randomly assigned to amoxicillin (AMOX; broad-spectrum antibiotic n=12), vancomycin (VANCO; targeting gram-positive bacteria n=9) or placebo (n=15) treatment (7d, 500 mg t.i.d). Before and after treatment, *ex vivo* adipose tissue lipolysis was assessed under basal conditions and during stimulation with the non-selective β-agonist isoprenaline (ISO) using freshly isolated mature adipocytes. Glycerol release into the medium was measured as an indicator of lipolysis. In addition, gene (targeted microarray) and protein expression analyses were performed to provide insight into underlying pathways. **Results** Antibiotic treatment had no significant effects on basal and maximal ISO-mediated glycerol release from adipocytes. However, β-adrenergic sensitivity was slightly improved after VANCO (EC_{50}: $2.4 \times 10^{-6} \pm 5.7 \times 10^{-7}$M *vs* $1.0 \times 10^{-6} \pm 5.9 \times 10^{-7}$M, $P<0.05$). Surprisingly, this was not accompanied by alterations in β-adrenoceptor expression or post-receptor signaling (i.e. hormone-sensitive lipase-phosphorylation).

Conclusion We demonstrate that short-term antibiotic treatment by VANCO slightly increased adipose tissue β-adrenergic sensitivity. Whether this translates in an improved adipose tissue function in the long term remains to be determined.

Introduction

The intestinal microbiota seems to contribute to the development of obesity and related cardiometabolic complications, at least in rodents[1-3]. Gut microbial products and their stimulation of gut hormonal secretion may influence metabolic processes in the skeletal muscle, liver and adipose tissue, thereby affecting lipid storage[4,5] and lipolysis[6]. The release of lipids from adipose tissue of obese insulin resistant individuals is characterized by a blunted catecholamine-induced lipolysis, which is related to reduced β-adrenoceptor expression and decreased lipase activation[7,8]. This impaired lipolytic response might contribute to adipose tissue dysfunction and, as a consequence, the development of insulin resistance[9].

The gut microbiota might affect adipose tissue lipid turnover through multiple mechanisms, including altered production of angiopoietin-like protein 4 (ANGPTL4), short-chain fatty acids (SCFA), bile acids (BA), lipopolysaccharides (LPS), as well as gut-derived incretins[3]. First, the gut microbiota regulates the production of ANGPTL4, also known as Fasting-Induced Adipose Factor, which is a circulating antagonist of lipoprotein lipase (LPL) that is present in adipose tissue, liver and the intestinal tract. Additionally, ANGPTL4 promotes the intracellular *lipolytic* response to fasting and catecholamines in murine adipocytes[10]. In line, human in vivo studies have shown that plasma ANGPTL4 concentrations were positively associated with fasting levels of free fatty acids (FFA) and adipose tissue lipolysis [11]. Another potential mechanism through which our gut can affect host lipid metabolism is fermentation of nondigestable carbohydrates to SCFA in the distal ileum, cecum and colon. These SCFA, of which acetate, butyrate and propionate are the most abundant, are ligands for the G-protein-coupled receptors (GPCRs), GPCR41 and GPCR43, which are expressed on several peripheral tissues including adipose tissue[12]. Several human studies have shown that oral or rectal administration of SCFA, in particular acetate, significantly decreased plasma FFA concentrations[13-15]. In addition, treatment of differentiated murine 3T3-L1 adipocytes with SCFA reduced fasting and catecholamine-mediated lipolysis via activation of GPR43[16]. A decreased hormone-sensitive lipase (HSL) phosphorylation may underlie this antilipolytic effect of SCFA, as has recently been shown in murine 3T3-L1 adipocytes[17]. Finally, also gut-derived BA[18,19] and incretins, including glucagon-like peptide 1 (GLP-1)[20], might affect adipose tissue lipid metabolism. Taken together, modulation of the

gut microbiota and its products may provide a promising strategy to target adipose tissue lipolysis, thereby improving obesity-related metabolic impairments. Importantly, however, most data are based on *in vivo* and *in vitro* murine studies, whilst well-controlled human studies investigating the effect of microbiota modulation on adipose tissue lipolysis are currently lacking.
Here, we investigated the effects of microbiota manipulation, achieved by oral broad or small-spectrum antibiotic treatment, in obese men with impaired glucose homeostasis on *ex vivo* basal and β-adrenergically stimulated lipolysis in human adipocytes. In addition, targeted gene and protein expression analyses in adipose tissue biopsies were performed to investigate potential underlying mechanisms.

Methods

Study participants

The current research was conducted as part of larger clinical trial on the effect of gut microbiota manipulation on host metabolism[21]. In addition, we included data from 12 subjects from a recently conducted study using the same antibiotics-intervention[22]. In total, 36 overweight and obese (BMI 25-35 kg×m^2) Caucasian men between 35-70 years were included in the present study. Detailed inclusion and exclusion criteria have previously been described[21,22]. All subjects gave written informed consents for participation in both studies, which were reviewed and approved by the local Medical Ethical Committee of Maastricht University Medical Centre+. All procedures were according to the declaration of Helsinki (revised version, October 2008, Seoul, South Korea).

Study design

Study participants were randomized (double-blind) to the oral intake of amoxicillin (AMOX, broad-spectrum antibiotic), vancomycin (VANCO, directed against gram-positive bacteria) or placebo (PLA) for 7 consecutive days (500 mg t.i.d.). Participants were asked to maintain their habitual physical activity pattern and dietary habits throughout the study. An abdominal subcutaneous adipose tissue biopsy was taken under local anaesthesia under fasted conditions before and after 7 days antibiotics treatment for *ex vivo* characterization of adipocyte lipolysis, targeted microarray and Western Blot analyses, as described in more detail below.

To ensure proper systemic and gastrointestinal clearance of antibiotics, a 2-day wash-out period was taken into account before the adipose tissue biopsies were collected.

Ex vivo adipocyte lipolysis

A part of the adipose tissue biopsy (~500 mg) was used for the isolation of mature adipocytes following collagenase digestion in Dulbecco's Modified Eagle's Medium (DMEM)-Ham's F12 at 37°C[23]. The resulting suspension was filtered through a 200 µm filter and adipocytes were washed once with DMEM-Ham's F12 to eliminate collagenase. Isolated mature adipocytes were diluted in DMEM-Ham's F12 supplemented with 3% bovine serum albumin for lipolysis assays and incubated with increasing concentrations of isoprenaline (ISO, a non-selective β-adrenergic agonist; $10^{-10} – 10^{-3}$ M) in duplicate at a finale volume of 100 µl for 3 h at 37°C. Following incubation, 60 µl cell-free aliquots of the infranatant were collected for glycerol determination (lipolysis index) using the EnzyChrome™ Adipolysis assay kit (Gentaur, Eersel, The Netherlands). Glycerol release was expressed per cell number and relative to baseline, as lipolysis index.

Adipose tissue gene expression analyses

RNA was extracted from frozen AT (~500 mg) using Trizol chloroform extraction (Invitrogen, Cergy Pontoise, France). Next, total RNA (100 ng per sample) was labeled by Whole-Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays targeting 19793 unique genes (Affymetrix, Santa Clara, CA, USA). Quality control and data analysis have been described in detail previously[24]. Individual genes on the array were defined as changed when comparison of the normalized signal intensities showed a FDRq (false-discovery adjusted p-value) < 0.05 in a 2-tailed paired t-test with Bayesan correction[24].

Adipose tissue protein expression

Adipose tissue biopsies (100-300 mg) were ground to a fine powder under liquid nitrogen and homogenized in 200-600 µL radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease/phosphatase inhibitor cocktail (Cell Signalling Technology Europe, Leiden, The Netherlands). Lysates were vortexed for 5 min at room temperature and centrifuged at 14.000 rpm for 30 min at 10°C. Infranatant was carefully transferred to new tubes. Protein concentrations were

determined using the Pierce® BCA protein assay kit (Santa Cruz Biotechnology Inc., Heidelberg, Germany). Samples were stored at -80°C prior to analysis. 25 µg protein was separated on Any kD SDS-PAGE gels (Bio-Rad Laboratories, Veenendaal, The Netherlands) and subsequently blotted semi-dry onto nitrocellulose membrane using Trans-Blot® Turbo Transfer System (Bio-Rad Laboratories, Veenendaal, The Netherlands). Following transfer, membranes were blocked for 1 h in blocking buffer (Tris-buffered saline with 0.1% Tween 20 (TBS-T), 5% nonfat dry milk). The membranes were incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Next, membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. The primary antibodies used were adipose triglyderide lipase (ATGL) (#2138), total HSL (#4107) and Phospho-HSL Ser563 (*equivalent to Ser552 of human HSL*), a major protein kinase A (PKA) target (#4139), all from Cell Signalling. β-actin was used as loading control (Santa Cruz, #sc-47778, 1:1000 dilution). Secondary antibodies were horse-radish peroxidase-conjugated-IgG α-swine or α-rabbit (DAKO, Heverlee, Belgium). Antigen-antibody complexes were visualized by chemiluminescence using SuperSignalTM West Femto extended Duration Substrate (Life Technologies, Gent, Belgium). Visualization and analysis was performed using a Chemidoc XRS system (Bio-Rad Laboratories, CA, USA) and Quantity One software.

Calculations and statistical analysis

The concentration of agonist producing the half-maximum effect (EC50) was determined using logistic conversion of each dose-response curve as described previously[25]. The negative logarithm of the EC50 value (pD2) was defined as the β-adrenergic sensitivity. Analysis of variance (ANOVA) was applied to compare subject characteristics at baseline. Treatment effects within and between groups were tested using repeated-measures ANOVA. A dependent or independent t-test was used

to assess differences before and after treatment and to assess differences between PLA, AMOX or VANCO, respectively. Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS version 20.0 (Chicago, IL, USA). $P<0.05$ was considered to be statistically significant.

Results

Subject characteristics

Baseline subject characteristics are shown in Table 4.1. No significant differences were present between the PLA and VANCO group and the AMOX and VANCO group, except for a higher insulin sensitivity (homeostasis model assessment for insulin resistance (HOMA-IR) 3.3±0.4 vs. 5.0±0.6, $P<0.05$) and lower fasting insulin concentrations (12.3±1.4 vs. 18.4±2.0 mU/l, $P<0.05$) in the PLA versus AMOX group.

Table 4.1. Baseline characteristics

	PLA (n=15)	AMOX (n=12)	VANCO (n=9)	P-value
Age (years)	62.7±1.6	54.3±3.4	58.7±2.8	0,068
Body Weight (kg)	90.5±3.3	93.7±2.7	97.8±3.2	0,429
BMI (kg/m^2)	29.5±0.9	29.6±1.0	31.7±0.8	0,238
Fasting glucose (mmol/l)	5.9±0.1	6.0±0.2	6.0±0.2	0,919
Fasting Insulin (mU/l)	12.5±1.5*	18.4±2.0	18.2±1.6	0,022
HOMA-IR	3.3±0.4*	5.0±0.6	4.8±0.4	0,028
TAG (mmol/l)	1.13±0.11	1.40±0.22	1.04±0.17	0.347
FFA (umol/l)	587.6±29.2	579.2±53.8	580.9±48.5	0.990

Characteristics given as mean ± SEM. The P-value represents the difference between the 3 groups (one-way ANOVA). *Significant difference between PLA–AMOX (Bonferroni-post hoc testing, $P<0.05$); BMI: Body mass index; HOMA-IR: Homeostasis model assessment for insulin resistance.

Ex vivo lipolysis

We investigated the effect of antibiotics treatment on basal and β-adrenergically mediated lipolysis in freshly isolated mature adipocytes. Basal glycerol release, expressed per number of cells, was not altered following treatment (ANOVA $P=0.314$, Table 4.2). In addition, maximal ISO-mediated lipolytic response (adjusted for cell number) was comparable between groups ($P=0.671$, Table 4.2). The half-maximal effective concentration (EC50) and pD2 (-logEC50) values for ISO, which were calculated based on the dose-response curves (Supplementary Figure S4.1), were slightly but significantly reduced following VANCO (Table 2, $P<0.05$), indicative of increased β-adrenergic sensitivity. In line, VANCO induced a modest leftwards shift of the dose-response curve of ISO (Supplementary Figure S4.1).

Table 4.2. Basal and maximal ISO-mediated lipolytic response in SCAT adipocytes before and after intervention, compared to placebo

		PLA	AMOX	VANCO	P-value
Basal (μM/10^{-6}cells/3h)	pre	19169±4114	17879±4144	19156±4743	0.314
	post	18734±3328	14280±2542	15275±2734	
Max (μM/10^{-6}cells/3h)	pre	60274±9391	88075±19080	67341±14669	0.671
	post	40029±5182	73856±21591	49254±8018	
EC50	pre	$2.1x10^{-7}$±$1.6x10^{-7}$	$9.1x10^{-7}$±$3.2x10^{-7}$	$2.4x10^{-6}$±$5.7x10^{-7}$	0.012
	post	$6.5x10^{-8}$±$9.5x10^{-8}$	$6.1x10^{-7}$±$1.5x10^{-5}$	$1.0x10^{-6}$±$5.9x10^{-7}$*	
pD2	pre	6.5±0.3	6.2±0.3	6.0±0.3	0.012
	post	7.1±0.3	5.8±0.5	6.7±0.4 *	

Values are given as mean ± standard error of the mean (SEM). P-value represents the overall time×treat P-value of the repeated measures ANOVA. Post-hoc testing was performed using a dependent t-test.* P<0.05 pre vs.post.

β-adrenoceptor expression and lipase activation

Next, targeted microarray and Western Blot analyses on adipose tissue biopsies were performed to determine whether the improved β-adrenoceptor responsiveness following VANCO was related to changes in receptor expression and activation of major lipolytic proteins. The microarray data revealed no significant changes in adrenoceptor expression or post-receptor signaling after VANCO treatment (Table 4.3). In accordance with these findings, total protein content of ATGL, HSL and the phosphorylation status (on Ser563) of HSL were not significantly altered following VANCO treatment (Figure 4.1A-C and Supplementary Figure S4.2).

Discussion

The present study investigated whether short-term antibiotic treatment alters adipose tissue lipolysis in humans. Here, we demonstrated that oral administration of VANCO but not AMOX for 7 days slightly but significantly increased *ex vivo* β-adrenergic sensitivity in adipocytes derived from obese insulin resistant men. Surprisingly, however, this was not accompanied by

changes in adipose tissue β-adrenoceptor expression or post-receptor events (i.e. HSL phosphorylation). Together, the present data suggest that short-term microbiota manipulation by antibiotics treatment has no pronounced effects on adipose tissue lipolysis in obese men.

We have previously reported that VANCO but not AMOX treatment markedly reduced microbiota diversity and altered its composition, predominantly by decreasing gram-positive Firmicutes[21]. The present findings indicated that VANCO slightly improved β-adrenoceptor sensitivity as compared to PLA.

This was, however, not accompanied by changes in gene expression of β-adrenoceptors or lipolytic genes. In line, we did neither observe any changes in downstream signaling, including ATGL and HSL protein content, nor HSLphosphorylation at Ser563 (*equivalent to Ser552 of human HSL*). However, we cannot exclude effects of VANCO on β-adrenoceptor responsiveness via catecholamine-mediated phosphorylation of HSL at other serine residues, although this seems unlikely based on the unchanged basal and maximal stimulated lipolysis that we observed in these human adipocytes.

Table 4.3. Fold change of lipolysis-related genes in adipose tissue before and after intervention, compared to placebo

Gene	Description	AMOX vs. PLA	VANCO vs. PLA
PNPLA2	patatin-like phospholipase domain-containing protein 2	1,051	1,142
LIPE	hormone sensitive lipase	1,015	1,215
PLIN1	perilipin 1	1,079	1,185
ABHD5	abhydrolase domain containing 5	-1,125	-1,181
G0S2	G0/G1 switch 2	1,075	1,164
ADRB1	adrenoceptor beta 1	1,091	1,096
ADRB2	adrenoceptor beta 2	-1,035	-1,016
ADRA2A	adrenoceptor alpha 2A	1,028	1,290
ADRA2B	adrenoceptor alpha 2B	1,077	1,051
ADRA2C	adrenoceptor alpha 2C	-1,022	1,138
AQP7	aquaporin 7	1,120	1,244

Micro-array analysis-derived adipose tissue gene expression for lypolysis-related genes. The mean fold change per gene is expressed as differential fold change compared to placebo (Limma FC for (VANCO_post-VANCO_pre)-(PLA_post-PLA) and: (AMOX_post-AMOX_pre)-(PLA_post-PLA_pre).

Orally administered VANCO is not absorbed into the bloodstream to an appreciable extent as compared to oral intake of AMOX. This suggests that the improved β-adrenergic responsiveness of adipocyte lipolysis is mainly due to changes in gut microbial-induced signaling rather than an effect of VANCO *per se*[26]. We have previously demonstrated that VANCO treatment for 7 days decreased the abundance of *Clostridium* clusters XIVa and IV, which was accompanied by a reduced conversion of primary to secondary BAs, and a lower concentration of fecal SCFA. Interestingly, SCFA and BA receptors are expressed in adipose tissue, and both SCFA and BA can modulate fasting and β-adrenoceptor-mediated lipolysis in murine and human adipocytes[16,27], indicating a possible role for BA and SCFA signaling in catecholamine-induced lipolysis. In addition, ANGPTL4, of which the intestinal expression can be influenced by the gut microbiota, has been suggested to be involved in the lipolytic response to catecholamines in murine adipocytes [10]. However, we have previously shown that VANCO or AMOX did not affect circulating ANGPTL4 concentrations[21]. LPS, derived from Gram-negative bacteria, may also be involved in gut-host signaling[28]. Indeed, LPS has been shown to induce lipolysis in human adipocytes, and stimulated the formation of adrenaline and noradrenaline by macrophages[29]. However, previous data from our group demonstrated that VANCO or AMOX treatment did not affect LPS-binding-protein concentrations. Taken together, gut microbial-induced changes in gut-derived signaling molecules, such as BA and SCFA, might be involved in the improved adrenergic responsiveness of the human *ex vivo* adipose tissue lipolysis following VANCO treatment.
The slight improvement in VANCO-induced β-adrenergic sensitivity did not translate in effects on *in vivo* insulin sensitivity of adipose tissue lipolysis. As previously reported[21], we found no significant differences in the antibiotic treatment-induced suppression of plasma FFA concentrations during a hyperinsulinemic-euglycemic clamp compared with PLA in these subjects. A limitation of this study is that the gene and protein expression data reflect the total adipose tissue profile rather than cell membrane or lipid droplet-specific expression. Moreover, biopsies were taken after an overnight fast. Therefore, we cannot draw any conclusions regarding changes in adipose tissue lipase activation and translocation of lipid droplet-associated proteins following β-adrenergic stimulation. Future studies should address underlying mechanisms of microbiota effects on β-adrenergic sensitivity of lipolysis, in more detail.

In summary, we demonstrated that short-term manipulation of the gut microbiota by VANCO treatment slightly increased *ex vivo* β-adrenergic sensitivity in adipose tissue of obese patients. Future research is needed to establish whether this translates into improved adipose tissue function in the long-term.

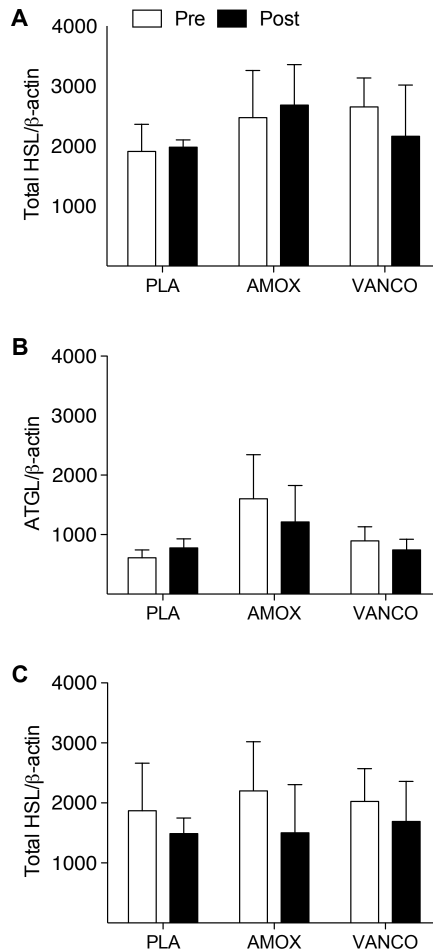

Figure 4.1. Quantitative analysis of the Western blots of HSL (A), ATGL (B) and phosphorylated HSL on Ser563 *(*equivalent to Ser563 of human HSL) (C). Pre (white bars) and post (black bars) intervention data are normalized for the loading control β-actin. Values are given as mean ± SEM (n=5 for PLA, n=5 for VANCO and n=6 for AMOX).

Supplemental Figures

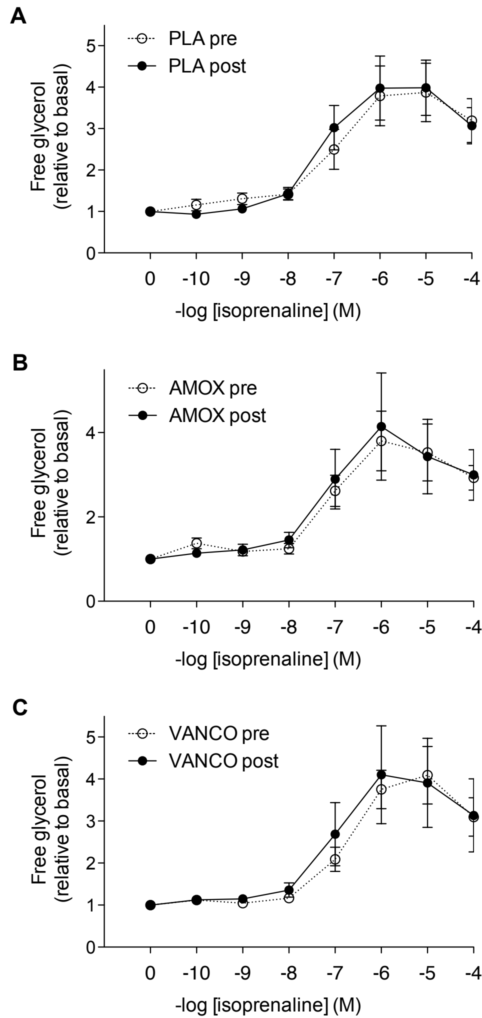

Figure S4.1. Dose-response curves for ISO-mediated lipolytic response in human mature adipocytes derived from the SCAT before and after intervention. Lipolysis (glycerol release in the medium) is expressed compared to baseline, following incubation with increasing concentrations ISO (10^{-10} to 10^{-4} mol/l) before (circles) and after (triangles) 7d treatment with placebo (**A**), amoxicillin (**B**) or vancomycin (**C**).

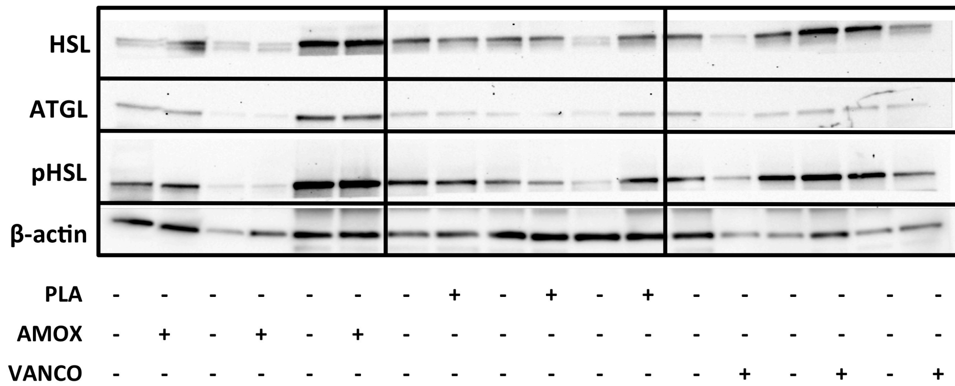

Figure S4.2. Representative Western Blot for lipolytic markers in human adipose tissue. Membranes were probed with antibodies directed against total ATGL, total HSL, phosphorylated HSL (pHSL) on Ser563 (*equivalent to Ser563 of human HSL*) and β-actin was used as a loading control. A subset of 3 subjects per group is shown.

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CHAPTER 5

Microbiota composition is not correlated to insulin sensitivity, but represents distinct alternative dysbiotic states in two independent cohorts of overweight and obese males

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CHAPTER 6

General Discussion

Obesity and its metabolic consequences are major contributors to morbidity and mortality rates worldwide[1,2]. Recent studies suggest that alterations in the gut microbiota composition may be one of the underlying mechanisms. Several years ago, the lack of anaerobic culturing systems hampered detailed insight into the bacterial composition. Due to technical advances, the bacterial abundance can now be determined at species level, and the field of microbiota research has since then rapidly gained interest. It were the early studies by Backhed[3,4], Cani[5] and Turnbaugh[6], which showed for the first time that, in mice, the microbiota composition was associated with adiposity. Additional studies in rodents[7-10], horses[11], piglets[12] and even zebrafish[13] and fruit flies[14] provided several hypotheses related to the importance of microbial populations on obesity outcomes. More recently, large metagenomic studies in humans have also indicated that different gut microbial populations may have differential effects on host adiposity and insulin sensitivity[15-17]. The gut microbiota may be of importance in the metabolism of nutrients beyond host-dependent metabolism[18,19], for the uptake of micronutrients[20] and in the host metabolic and inflammatory phenotype[5,21-24]. Therefore, interference with a resilient microbiome might alter concentrations of bacterial products such as short chain fatty acids (SCFA), bile acids (BA), amino acids (AA), lipopolysaccharides (LPS) as well as gut hormones (e.g. incretins), inflammatory markers and cytokines. These molecules might affect the gut-brain axis and the cross-talk between the gut, adipose tissue, skeletal muscle, liver and pancreas[25,26], affecting thereby substrate and energy metabolism. However, human studies that have investigated the contribution of the microbiome to body weight control and insulin resistance are scarce, and underlying mechanisms have not been elucidated yet.

Therefore, the present thesis aimed to address the role of the human gut microbiota in host metabolism. More specific, we determined the effects of gut microbial manipulations using antibiotics on tissue-specific insulin sensitivity and metabolic profile (**Chapter 2**) and on host adipose tissue (**Chapters 2** and **4**), liver (**Chapter 2**) and skeletal muscle metabolism (**Chapters 2** and **3**). In addition, in a cross-sectional analysis using two cohorts of obese men we have determined the association between gut microbial species and tissue specific insulin sensitivity and glucose homeostasis (**Chapter 5**).

Manipulation of the gut microbiota - an antibiotics approach

The resilient bacterial populations in the gut are prone to environmental pertubations. Although the microbiota composition is generally stable over a long period during adulthood, fluctuations may occur due to changes in diet, particularly the amount of fat and fiber, medication, the intake of probiotics and disease state[27]. To investigate whether the microbiota is involved in metabolic processes of the host, we intended to temporarily modulate the gut microbiota composition in a robust way and examined the effects on tissue-specific insulin sensitivity, energy and substrate metabolism and the inflammatory profile of the host after the intervention. As a proof-of-concept, we used antibiotics to induce pronounced changes in the bacterial composition. Based on pharmacokinetic and pharmacodynamic properties, and taking into account medical-ethical aspects (e.g. chance of side-effects and antibiotics resistance), we decided to use a regular used dose of vancomycin (VANCO) and amoxicillin (AMOX) in our study (seven days, 1500 mg/day). VANCO, which is not absorbed in the gut and as such does not exert direct systemic effects, is directed against Gram-postive bacteria[28]. Indeed, in **Chapter 2**, we found that VANCO treatment decreased microbial diversity, with a decrease of the relative abundance of Gram-positive bacteria of the Firmicutes phylum. Among the most strongly affected groups were butyrate-producing species (*e.g. Faecalibacterium prausnitzii)* as well as species involved in BA dehydroxylation, such as *Clostridium leptum*. Indeed, fecal and plasma concentrations of butyrate and secondary BA decreased after VANCO. Furthermore, VANCO decreased acetate, caproate and valerate concentrations in feces, whereas primary BA were increased. Noteworthy, we found a compensatory increase of the abundance of some Gram-negative Proteobacteria, members of *Clostridium* cluster IX and VANCO-resistant Gram-positive bacilli.

AMOX is a broad-spectum antibiotic, targeting both Gram-positive and Gram-negative bacteria. AMOX is distributed systemically and, therefore, may have direct effects on peripheral organs. To exclude these systemic effects, we applied a two day wash-out period. Strikingly, in our study neither microbial diversity nor microbial metabolites were significantly affected by AMOX. In line, a previous study also showed no marked microbial changes after AMOX treatment[29]. Nevertheless, mild to moderate effects of AMOX with or without clavulanic acid on the normal microbiota has previously been shown[30]. Our findings, however,

do not exclude that total bacterial numbers decreased, since HIT-chip methodology provides insight in relative but not absolute changes of specific species. In order to obtain a more complete overview of the bacterial changes in the gut, PCR techniques should be additionally used to determine the amount of bacteria present in the intestine after intervention.

Microbiota manipulation and effects on host metabolism

Effects on insulin sensitivity

In **Chapter 2**, we investigated whether antibiotic-induced modification of the gut microbiota may affect tissue-specific insulin sensitivity. However, we did not find effects of antibiotics-treatment on peripheral insulin sensitivity, hepatic insulin sensitivity, and adipose tissue insulin sensitivity. Our data are in contrast with rodent studies, and again highlights that rodent data cannot always be extrapolated to humans. In these animal studies, the manipulation of the microbiota composition has been indicated to alter insulin sensitivity[7-9,31-34]. For example, Backhed *et al.*[3] showed a 40% decrease of insulin sensitivity after only two weeks colonization of germ-free mice with gut microbiota. However, a more recent study showed that VANCO-treated mice had little weight change and no improvement in glycemic control[35].

Furthermore, our data are in contrast to a previous relatively small study in humans (n=8 per group), showing that VANCO induced a slight but significant reduction in peripheral insulin sensitivity[29]. The reason for this apparent discrepancy may be explained by the fact that in the latter study, results were based on a within group comparison (post-treatment versus pre-treatment) since no placebo-group was included in the study design. Moreover, the VANCO-induced decrease in peripheral insulin sensitivity was very modest (~4%) and might not be clinically relevant. Additionally, a more recent trial showed that four days of treatment with a broad-spectrum antibiotic cocktail did not affect postprandial glucose metabolism in lean healthy men[36]. Taken together, these and our data clearly demonstrate that in a physiological setting, pronounced microbiota alterations and alterations in SCFA and BA metabolism evoked by antibiotics, do not affect insulin sensitivity in humans.

In line with this, in a cross-sectional analysis of two seperate cohorts of obese, insulin resistant men, we were not able to demonstrate a microbial profile that was associated with tissue-specific sensitivity (**Chapter 5)**. This is in contrast with

large metagenome-wide association studies, which have shown that obese individuals and patients with T2DM or insulin resistance had decreased intestinal microbiota diversity[15-17,37]. More specific, lower Firmicutes and higher Proteobacteria abundances in T2DM[6,15,16,38] and prediabetic humans[37] as compared to healthy controls have been described. Importantly, these associations have mostly been based on fasting values, whereas the relationship between gut microbiota and insulin action, as determined by a hyperinsulinemic-euglycemic clamp as we have employed, has never been established in obesity before. Notably, additional analysis of our cohorts regarding fasting parameters also did not show a significant correlation between the gut microbiota composition and glucose homeostasis. Therefore, our data do not support a major role of the gut microbiota in glucose homeostasis and insulin sensitivity when the obese state has already developed.

Effects on energy regulation, inflammation, hormones and metabolites

In addition to effects on insulin sensitivity, we investigated the effect of the antibiotics-intervention on mechanisms that have been suggested to underly the microbiota-insulin sensitivity relationship. As mentioned above, we found that VANCO treatment for seven days markedly changed SCFA and BA profiles, whereas no effects of AMOX on these parameters were found. The alterations of SCFA and BA profiles did not affect fasting plasma glucose or insulin levels, postprandial glucose tolerance, and plasma concentrations of (gut) hormones and metabolites. Strikingly, we did not find any significant impact of antibiotic treatment on energy harvesting, whole-body energy expenditure and substrate oxidation. In contrast to animal studies[6,38], we found a lower energy density (kcal/g feces) in fecal samples after VANCO treatment, suggesting increased energy harvest. Importantly, however, VANCO treatment yielded a higher fecal weight, resulting in a similar total amount of calories excreted via the feces per day between groups.

It has been indicated that the reduction of SCFA, deconjugated BA and secondary BA concentrations may interfere with the host immune response through a reduced activation of the GPR43 on macrophages and monocytes[39,40], reduced activation of the anti-inflammatory T_{reg} network[21,41,42] and increased innate immune cell activity[23,43]. The decrease in secondary BA and butyrate after VANCO did, however, not result in increased systemic inflammation in our study.

In line, no effects of AMOX and VANCO on gut permeability, circulating LPS-binding protein and pro-inflammatory cytokine concentrations were found. Our results are in contrast with rodent studies, showing that (prebiotic) microbial manipulation improved[44], whereas antibiotics reduced gut epithelial integrity[5]. Proteobacteria have been suggested to have properties that can directly exacerbate inflammation and (GI)-tissue damage[45-47], whereas butyrate may conserve the intestinal barrier and decrease gut permeability by modulating the expression of tight junction protein and mucins[48,49]. Our results demonstrated that the abundance of Proteobacteria increased and butyrate concentations decreased by VANCO treatment. We cannot exclude that the apparent opposite effects of Proteobacteria and butyrate on inflammation may have counteracted each other to some extent, and might therefore underlie the absence of any effects on gut permeability and systemic concentrations of inflammatory markers. Moreover, since obese subjects may already have an impaired GI barrier function[50,51], it might be that the antibiotic-related dysbiosis has no substantial additive effect on gut permeability in our study population.

Microbiota manipulation - effects on the long term?

Even though antibiotics are widely used in the clinical setting, the exact long-term effects on commensal bacterial populations and microbial community resilience are largely unknown[27,52]. In agreement with observations that some populations of bacteria do not immediately or completely recover after antibiotic treatment[52,53], we found some microbiota populations in the VANCO-treated group to be still deviant from baseline after eight weeks follow-up. Most bacterial groups that increased in abundance immediately after seven days antibiotic treatment (e.g. Proteobacteria*)* had returned to baseline levels, but several members of *Clostridium* clusters IV and XIVa were still decreased in some individuals at the end of follow-up.

Also in animal studies, VANCO evoked a pronounced effect on *Clostridium leptum* and *Clostridium coccoides* clusters, which had recovered to a large extent two-three days post-treatment[10]. However, in the latter study, levels of some metabolites did not recover immediately after the cessation of antibiotic treatment. Therefore, in our study, we have determined fasting insulin and glucose concentrations (to calculate HOMA-IR), as well as adipocyte size and fecal energy harvest eight weeks after treatment cessation. Strikingly, we

demonstrated that whole-body insulin sensitivity (HOMA-IR), adipocyte size and fecal energy did neither alter immediately following seven days antibiotic treatment, nor after eight weeks follow-up, even though the microbiota composition in the VANCO-treated group had not completely recovered at the end of follow-up. In agreement with our findings, it has recently been shown a four-day treatment with an antibiotic cocktail in lean healthy humans did not cause short (four days) or long-term (180 days) changes in glucose tolerance[36]. It is, however, tempting to postulate that it takes a longer and more persistent manipulation of the microbiome before alterations in host metabolism become evident. For example, the expression of SCFA and BA receptors, tight-junction proteins in the gut and the inflammatory response might need some time to adapt to different bacterial concentrations and their metabolites. Most animal studies that have demonstrated a large impact of antibiotic use on metabolic health outcomes, exposed these animals to antibiotics in their drinking water for at least two-four weeks[5,7,8]. In addition, studies in humans that have shown a relation between antibiotic use and adiposity also found this effect when subjects were exposed to a more prolonged treatment (four-six weeks) with a higher dosage of antibiotics, or a combination of antibiotics.[54,55] Although day-to-day variations in microbiota composition have been reported, the adult microbiota is in general stable over time, with a certain resilience that decreases during the ageing proces[27]. The fact that we do not find any metabolic effects, except for adipose tissue (AT) gene expression, shows that human metabolism is not affected following short-term alterations in the microbiome, nor after a prolonged time period in which the microbiome has not returned to the baseline composition (eight wk follow-up).

Nevertheless, it is important to emphasize that the present study does not fully exclude a role for the gut microbiota in host metabolism. Since we studied obese, insulin resistant men with impaired glucose metabolism, we cannot exclude that microbiota manipulation by antibiotics may have more pronounced effects in women or less metabolically compromised individuals.

Furthermore, the extent to which antibiotics affect the gut microbiota composition and host metabolism might be largely dependent on the subjects' long-term dietary habits. Dietary composition (especially fat and fiber content) has been found to be extremely important in shaping the microbiome[56,57]. Therefore, enriching a diet with prebiotics, such as resistant starch or

oligosaccharides for a long period of time might be an effective way to alter host metabolism in obese insulin resistant subjects[58,59]. Moreover, this might be a relatively easy and physiologically relevant way to investigate long-term manipulation of the microbiome. Supplementing the diet with probiotics, which have been linked to positive health outcomes, is an alternative to study long-term effects on metabolism.

Microbiota manipulation - effects on adipose tissue metabolism.

In order to obtain a more detailed insight into mechanims that may provide a link between gut microbiota and metabolism, we investigated the effects of antibiotics specific for AT function. First, in **Chapter 2**, we investigated the effect of seven days antibiotics on AT gene expression (microarray analysis). VANCO, but not AMOX treatment, significantly decreased immunity-related gene sets in AT. The fact that we did not find this downregulation after AMOX treatment, suggests that the VANCO-induced alterations in microbiota metabolites (e.g. SCFA, BA) might have played a role. However, these gene expression alterations did not translate into changes in systemic low-grade inflammatory markers, including TNF-α, IL-6 and IL-8) Both VANCO and AMOX increased AT gene expression of pathways related to oxidative metabolism (TCA cycle, oxidative phosphorylation) and pathways feeding into these cycles (glycolysis, beta-oxidation) as compared to placebo. Noteworthy, the VANCO-treated subjects showed this upregulation of genes related to oxidative metabolism pathways independently of changes in the placebo group, whereas AMOX-induced effects on these genes were due to a decrease observed in the placebo-group. Mitochondrial ATP synthesis in mature white adipocytes is essential for major metabolic pathways such as lipolysis, *de novo* fatty acid synthesis, triacylglycerol (TAG) synthesis, glyceroneogenesis and the re-esterification of fatty acids[60]. However, in **Chapter 4** we demonstrated that antibiotic treatment had no significant effects on *ex vivo* basal lipolysis and β-adenergic stimulation of lipolysis in mature adipocytes. In agreement with these functional lipolysis data, β-adrenoceptor expression and post-receptor signaling pathways were unaltered following antibiotics treatment. This is in line with unchanged adipose tissue insulin sensitivity, as determined *in vivo* by the insulin-mediated suppression of free fatty acid concentrations during the hyperinsulinemic-euglycemic clamp

(Chapter 2). Interestingly, it has previously been found that fasting and β-adrenoceptor-mediated lipolysis in murine and human adipocytes is modulated by SCFA and BA[61,62]. Moreover, human studies have shown that oral or rectal administration of SCFA, in particular acetate, significantly decreased plasma FFA concentrations[63-65]. The reduction in concentrations of SCFA and BA metabolites in our study might not have been large enough to induce alterations in the lipolytic response in AT. Multiple other mechanisms have been suggested to affect adipose tissue lipid turnover, including an altered production of angiopoeitin-like 4 (ANGPTL4), lipopolysaccharide (LPS) and incretins[66]. However, plasma concentrations of these factors were not altered by antibiotics in our study. Interestingly, the adipocyte β-adrenergic sensitivity was slightly but significantly improved after VANCO treatment, although the physiological importance of this slight effect remains to be determined.

Finally, as described in **Chapter 2**, we investigated AT morphology since abdominal subcutaneous adipocyte size is tightly associated with AT function and insulin resistance[67,68]. Adipocyte hypertrophy, as frequently observed in obese individuals, is correlated with AT inflammation and impairments in lipid metabolism[67,68]. The gut microbiota imbalance that may be present in obesity is considered as an important trigger of white adipose tissue (WAT) plasticity via the regulation of energy harvesting[69] and fat storage[3] in animals. We did not find any changes in adipose tissue morphology, neither in mean adipocyte size nor in the proportion of small and large adipocytes. In agreement with unchanged adipocyte morphology, we did not find changes in circulating concentrations of leptin, an adipokine that is secreted from subcutaneous adipose tissue in proportion to adipocyte size[70]. Interestingly, this indicates that the VANCO-induced effects on AT gene expression of pathways related to oxidative metabolism and inflammation, as discussed above, are independent of changes in adipocyte size.

Microbiota manipulation - effects on skeletal muscle metabolism

The skeletal muscle accounts for approximately 80% of insulin-stimulated glucose disposal and, therefore, plays a prominent role in development and progression of insulin resistance and type 2 diabetes mellitus[71]. In **Chapter 3**, we investigated the effects of modulation of the microbiota composition by antibiotics on postprandial insulin sensitivity and skeletal muscle substrate metabolism under fasting and postprandial conditions. Interestingly, postprandial glucose and insulin concentrations and forearm glucose uptake after the intake of a high-fat mixed-meal did not change following VANCO and AMOX treatment. Additionally, we found that antibiotics treatment did neither change fasting and postprandial TAG and FFA concentrations, nor the extraction of TAG and FFA across the forearm muscle. Finally, no changes in lactate flux across forearm muscle were observed. These findings clearly indicate that antibiotics treatment did not significantly affect human forearm muscle metabolism.

Alterations in glucagon-like peptide-1 (GLP-1) and SCFA concentrations have been suggested to underlie changes in skeletal muscle fat oxidation and substrate metabolism[72,73] through metabolic pathways involving the phosporylation of AMPK[4] or effects on microvascular blood flow[18]. Furthermore, SCFA have been suggested to affect peripheral substrate metabolism, since their receptors (GPR41 and 43) are expressed in skeletal muscle[74,75]. Although circulating butyrate concentrations were altered after VANCO treatment, this did not alter fasting and postprandial whole-body energy expenditure, fat and carbohydrate oxidation, as described in **Chapter 2**. Thus, although several lines of evidence suggest that the gut microbiota-skeletal muscle cross-talk is involved in substrate metabolism and insulin sensitvity in rodents[25,76], the contribution of AMKP, GLP-1, SCFA and other factors in the microbiota-muscle interaction is not supported by our antibiotics study, and requires more investigation.

Conclusion

Our data, showing pronounced modulation of microbial composition but no effects on host metabolism, are not in line with many data derived from animal experiments and a few other human studies. VANCO treatment for seven days

markedly changed the composition of the gut microbiota, with consequent alterations of SCFA and BA profiles. However, these alterations did not affect tissue-specific insulin sensitivity, energy harvest, energy expenditure and substrate metabolism, gut permeability, adipocyte morphology and plasma concentrations of hormones, metabolites and low-grade inflammatory markers. Even after eight weeks follow-up, when the microbiota compostion was still considerably altered, no differences in energy harvesting, adipocyte size and whole-body insulin sensitivity (HOMA-IR) were found.
The fact that we were not able to confirm the clear effects observed in animal studies shows, once again, that the human organism and its interaction with the environment is extremely complex, and mice and man are not alike. Inter-individual differences in lifestyle, diet, country of origin, mode of delivery at birth, medical history, travel habits and cultural habits make it hard to determine the exact role of the microbiome in obesity and comorbities, and hamper comparisons of outcomes between studies. Nevertheless, human data thus far does not provide robust evidence that the gut microbiota has a pronounced impact on host metabolism. Long term, moderate alterations of the microbiota composition (e.g. by prebiotics or probiotics or altering diet composition), might be needed to obtain health benefits. Whether this 'slow and steady' approach wins the race needs to be confirmed in large, well-controlled human trials.

Future perspectives

Based on the outcomes of the present work, as extensively described in this thesis, together with available evidence in literature, several suggestions for future studies can be made:

1. It is important that future studies include both men and women in a study protocol that aims to investigate metabolism. Gender differences in physiology and sex hormones alter the susceptibility to intervention-effects. Moreover, recent studies have shown that fecal microbiota composition might differ between men and women. The gut microbiome in women was characterized by a lower abundance of Bacteroidetes than men[77,78], and it has been shown that the response to dietary fibers differ between males and females[77,79]. Therapies to treat microbiota dysbiosis by for example prebiotics, might therfore not work equally well for both sexes. In addition, the relationship between microbiota and BMI has been

found in women but not in men[77], whereas the relationship between peripheral insulin sensitivity and the microbiome was only found in men (unpublished data from our group).

2. The large inter-individual differences in the response to food might be explained by differences in the gut microbiota composition and metabolic phenotype, suggesting that dietary interventions may need to be personalized[80]. Screening of the microbiota profile in combination with the metabolic phenotype before designing a weight-loss program or dietary intervention would be an important strategy to follow in the future. In that way, a better insight in beneficial bacteria and targeted supplements with probiotics or prebiotics can be obtained.
3. It has recently been shown that stool consistency is negatively correlated with species richness, positively associated with the Bacteroidetes/Firmicutes ratio, and linked to *Akkermansia* and *Methanobrevibacter abundance*[81]. Stool consistency reflects differences in fecal water content and colon activity, and is an indication for intestinal colon transit time. The associations between stool consistency and species richness and community composition emphasizes the importance of stool consistency and transit time assessment in future studies, since this may influence study outcomes.
4. Although we could not detect an association between the microbiome and insulin sensitivity in the present studies, large metagenome-wide association studies indicated differences in the microbiota compostion between lean, obese and diabetic subjects. Based on the present study, we cannot exclude that antibiotic treatment may have had more impact on host physiology in lean, metabolically healthy individuals. It is, therefore, important for future microbiota-manipulation studies to include a lean healthy control group.
5. Thus far, only animal studies have demonstrated the effects of specific bacterial colonies on epithelial integrity. Although animal data are convincing, it is important to investigate the 'leaky gut' hypothesis in a more extensive manner in humans. In order to target LPS-leakage and colonocyte health in future treatment strategies for obesity and metabolic complications, collection of colon biopsies should be considered to investigate the expression of tight-junction proteins.

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ADDENDUM

Valorization

The prevalence of obesity has reached epidemic proportions, and affects all ages and socioeconomic groups in the Western world[1,2]. In 2014, 13.1% of men and 19.9% of all women were overweight or obese. This worldwide obesity prevalence is expected to reach 18% in men and to surpass 21% in women in 2025. The excessive accumulation of body fat in the overweight and obese state increases the risk for the development of type 2 diabetes mellitus[3], cardiovascular disease, mental disorders[4] and several types of cancer[5]. The WHO Global Report on Diabetes 2016 showed that the total burden of deaths from high blood glucose has been estimated 3.7 million, including 1.5 million deaths due to diabetes alone. Yet, the rising obesity prevalence is also observed in many low- and middle-income countries. For example, Africa, which is home to 54 low- and middle-income countries, is expected to have the world's largest increase in the death rate caused by chronic diseases over the next decade[6].

The increasing worldwide prevalence of obesity and related cardiometabolic complications has a considerable economic and social impact. Health care costs include an expensive array of treatments. To delay disease progression, millions of individuals depend on medication and surgery to treat obesity-related complications. For example, medication to lower cholesterol, blood pressure and blood glucose, gastric bypass surgery or cardiovascular surgery. Despite improved medical and technological advances, a downward trend in longevity has been observed that is related to the increased incidence of obesity[7]. Moreover, this decrease in life expectancy might only accelerate in the future, since the current generation of children has a higher BMI and prevalence of related complications than ever before[1].

It is therefore important to implement new strategies to improve worldwide health and awareness of the problems that come along with being overweight and obese. For years, the public health approach to obesity was the advice to consume less and exercise more. However, attempts to lower body mass for a longer time period without the implementation of a healthy lifestyle in daily life and without addressing biological drivers of weight gain, inevitably fails for the majority of people. Therefore, better knowledge on weight loss strategies, healthy dietary components, and parameters of human metabolism as well as the triggers that may give lead to implementation of a healthy lifestyle are needed and warrant a close collaboration between research, industry and the government.

Research

The balance between energy intake, energy expenditure and energy excretion is maintained by a large regulatory network between metabolic organs. There is close interaction between the adipose tissue, skeletal muscle, pancreas and the liver in the regulation of energy and substrate metabolism. Studies performed over the last decade have indicated that the bacteria residing in the intestinal tract are part of this inter-organ crosstalk. Perturbations of microbial populations may contribute to an obesogenic phenotype, inflammation, alterations in glucose metabolism and insulin resistance. Importantly, however, mechanistic studies almost exclusively have been performed in rodents, and results from these studies cannot directly be used to optimize public health guidelines. This thesis describes a clinical trial in overweight and obese humans to better understand if and how specific microbial populations may contribute to the development of obesity and related metabolic and inflammatory perturbations, thereby making scientific knowledge suitable and available for societal or economic utilization. Our findings demonstrate that pronounced modulation of the gut microbiota composition did not significantly affect host metabolism. Even after eight weeks follow-up, when the microbiota composition was still considerably altered as compared to baseline, no significant differences in host metabolism were found. The extrapolation of findings from animal studies to relevance for humans is an important step in the process of valorization. Knowledge on the effect of microbiota manipulation in humans may provide useful information to develop and/or optimize (nutritional or lifestyle-related) strategies to prevent obesity and obesity-related metabolic disturbances. Moreover, the studies described in this thesis provide new perspectives for future research and suggestions for improvement of intervention protocols to further investigate the link between microbiota and human metabolism. For example, since we found large inter-individual differences in the gut microbiota composition and the relationship with metabolic phenotype, this suggests that dietary interventions may need to be more personalized. In addition, future studies may consider inclusion of a lean, metabolically healthy control group. Finally, it is important that future studies include both men and women, since increasing evidence indicates gender differences in physiology, microbiota composition and sex hormones regarding the susceptibility to intervention effects.

Industry

The research as described in the present thesis was part of an interdisciplinary collaboration between academia, industry, and government within the framework of the Top Institute Food and Nutrition (TIFN). The ambition of this private-public partnership is to provide the knowledge base that is needed for high-impact innovations in food and nutrition. The motto 'scientific excellence, industrial relevance' indicates the importance of valorization within TIFN. The close collaboration between industry and academia leads to demand-driven research, which might provide long-term business strategies. Although the industrial partners had no leading role in the design of this study, their critical questions and suggestions were taken into account to optimize the study design.
The goal of the study as described in this thesis was to provide the nutritional industry with insights in the importance of the intestinal microbiome in obesity and related metabolic impairments. The fact that we were not able to confirm a clear relationship between the gut microbiota and metabolic health parameters in an overweight/obese population shows that the human organism and its interaction with the environment is extremely complex. Inter-individual differences in lifestyle, diet, country of origin, mode of delivery at birth, medical history, travel habits and cultural habits make it hard to determine the exact role of the microbiome in obesity and comorbidities. Long term, moderate alterations of the microbiota composition (e.g. by prebiotics or probiotics) might be needed to obtain health benefits, if the gut microbiota appears to contribute to metabolic health in humans.

Government

The government may take measures to make the implementation of a healthy lifestyle more feasible, such as taxes on unhealthy products, creating an environment that stimulates physical activity, and the implementation of a healthy lifestyle into health care prevention. The studies described in this thesis are an important first step in elucidating the importance of the gut microbiota in host metabolism in humans, but do not provide the government with direct advice to change policy on guidelines for healthy nutrition. However, these studies show that we are still in an early phase regarding microbiota research, and governmental funding in this area of research remains important to answer a broad range of new questions.

Health care professionals

Although the results of the studies as described in this thesis show that microbiota manipulation by seven days antibiotics treatment does not affect human metabolism, the use of antibiotics should be decreased in common practice because of rising antibiotics resistance worldwide. In addition to the increasing problems regarding antbiotics resistance, it has been demonstrated that the risk of developing type 2 diabetes was increased when subjects have been exposed to >5 antibiotic treatments[8]. Moreover, the number of prescriptions and duration of antibiotic treatment may accelerate the ageing-related decline of intestinal integrity[9]. Health care professionals play an important role in encouraging healthy behavior and should provide dietary guidelines to obese patients, involving improved quality of food. The results of the studies described in this thesis do not provide direct guidelines for health care, but indicate that the promising animal data in the field of the gut microbiome generated and published over the last decade, including fecal transplantation data, should be critically evaluated and requires more studies in humans before developing or implementing treatment strategies for common practice.

General public

When it comes to trust the information that is provided to the general public, scientists appear to be the most trusted, whereas the least trusted group consists of politicians[10]. It is for the scientific community therefore extremely important to provide the general public with clear and understandable information regarding study results. The overwhelming load of information about 'healthy' and 'dangerous' food in the news and social media, often provides people with the wrong information and lifestyle advice. Moreover, the interpretation of scientific results by journalists often needs more consideration.

This thesis shows for the first time that care should be taken with all information available that considers the gut microbiota as the holy grail and, related to that, probiotic drinks as the holy wine. We are far from knowing what the exact role of the microbiota composition and functionality is. However, the large amount of information available within scientific literature indicates that the gut microbiota, as part of an important digestive organ, might differently affect metabolism dependent on metabolic phenotype, age and gender. It is the task of the

scientific community to elucidate these differences, and investigate whether long term, but moderate dietary or lifestyle manipulations can positively affect body weight and metabolic profile in overweight and obese humans, and may beneficially alter related cardio-metabolic complications.

Concluding remarks

The study results as described in this thesis have been incorporated in original articles that have been published or submitted to scientific journals in the field of metabolism, diabetes and gastroenterology. These research articles are accessible to scientists who are interested in this topic. In addition, results have been presented at several (inter)national conferences to colleagues inside and outside the specific field, and have been discussed within the TIFN-framework with partners from industry. This study shows once again that care should be taken to extrapolate animal data to guidelines for humans, and that the human organism and its interaction with the environment is extremely complex.

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Summary

Over the last decades, the prevalence of obesity has reached epidemic proportions, with an increasing impact on health care. Several recent studies have indicated that the composition of the gut microbiota might play a prominent role in body weight regulation and impairments in metabolic health. Moreover, perturbations in microbial populations may contribute to changes in body weight, inflammation, glucose metabolism and insulin sensitivity. Importantly, however, mechanistic studies almost exclusively have been performed in rodent models, and most human evidence comes from large metagenomic studies. Therefore, the studies described in this thesis investigated the impact of manipulation of the gut microbiota composition on human physiology in subjects at increased risk for developing type 2 diabetes mellitus. In addition, we examined the relationship between the microbiota composition at the genus level and tissue-specific insulin sensitivity, as determined by the gold-standard hyperinsulinemic-euglycemic clamp.

In **Chapter 2**, we demonstrated that a seven-day intervention with amoxicillin (AMOX; broad-spectrum) or vancomycin (VANCO; narrow-spectrum antibiotic) did not affect host metabolism as compared to placebo in 57 obese, insulin resistant males. In this double-blind, randomized placebo-controlled trial, we found a pronounced decrease in bacterial diversity and a reduction of Firmicutes-abundance after VANCO, but not after AMOX. The reduction of Firmicutes resulted in decreased plasma and/or fecal concentrations of short-chain fatty acids and bile acid metabolites. However, this did not affect hepatic, peripheral and adipose tissue insulin sensitivity, as determined by a two-step hyperinsulinemic-euglycemic clamp with [6,6-$^2H_2$]-glucose tracer infusion. In addition, gut permeability, circulating lipopolysaccharide binding protein (LPB) concentrations and fecal energy harvest were not affected by either VANCO or AMOX.
Despite a still considerably altered microbial composition, also at eight-weeks follow-up, we did not find effects on several aspects of human metabolism such as energy harvest, adipocyte size and whole-body insulin sensitivity.
Additionally, antibiotics intervention did not affect fasting and/or postprandial energy expenditure, substrate utilization and circulating insulin, GLP-1, ANGPTL4 and leptin. In **Chapter 3**, we studied the effect of a seven-day antibiotics intervention on forearm skeletal muscle metabolism in more detail by

combining measurements of arterio-venous concentration differences across forearm muscle and forearm blood flow. In line with Chapter 2, antibiotic treatment did neither affect fasting and postprandial plasma glucose, triacylglycerol (TAG) and free fatty acid (FFA) concentrations, nor the extraction of glucose, TAG and FFA across the forearm muscle. In addition, no changes in lactate flux across forearm muscle were observed.
As described in **Chapter 2**, microarray analysis showed that VANCO induced a significant decrease of immunity-related pathways in adipose tissue. However, these gene expression alterations did not translate into changes in systemic low-grade inflammatory markers. Both VANCO and AMOX increased adipose tissue gene expression of pathways related to oxidative metabolism and pathways feeding into these cycles, although adipocyte morphology remained unaltered. In addition, in **Chapter 4**, we performed functional measurements at the level of adipose tissue. Here, we found that seven-day VANCO treatment slightly but significantly improved ex vivoβ-adrenergic sensitivity. However, the physiological importance of this slight effect remains to be determined since no significant effects on ex vivo basal lipolysis and maximal β-adenergic receptor-mediated lipolysis in mature human adipocytes were observed. In line, β-adrenoceptor expression and post-receptor signaling pathways in adipose tissue were unaltered following antibiotics treatment.
Finally, in **Chapter 5**, we have investigated the relationship between gut microbiota composition and tissue-specific insulin sensitivity in detail in two independent cohorts of obese men (recruited in Maastricht and Amsterdam). Except for the association between centrain bacterial species and HbA1c in the Maastricht cohort, we did not find any significant associations between fasting and clamp-derived measures of tissue-specific insulin sensitivity in both cohorts.

In conclusion, although previous animal studies have suggested that the gut microbiota may be a promising target to improve metabolic health, our data demonstrated that short-term manipulation of the human gut microbiota by antibiotics does not have any metabolic consequences. A seven-day treatment with VANCO but not AMOX significantly decreased the microbiota diversity and reduced the abundance of Firmicutes, which was accompanied reduced bile acid metabolites and short-chain fatty acids in plasma and/or feces. This did not translate into significant effects on host metabolism directly after cessation of

the intervention, nor at eight-weeks follow-up, despite the fact that microbiota composition was not recovered completely to baseline at follow-up. In addition, we did not find a significant association between the gut microbiota and the host metabolic phenotype in humans in a cross-sectional analysis in two independent cohorts. Taken together, this thesis does not provide evidence that altering the gut microbiota in a robust, short-term manner has a pronounced impact on host metabolism in obese subjects with impaired glucose metabolism. Interference with the complex relation between the human being and the environment might need other strategies in order to obtain health benefits.

Samenvatting

De prevalentie van obesitas is de afgelopen decennia enorm toegenomen, wat geleid heeft tot een toegenomen druk op de gezondheidszorg en gerelateerde kosten. Recent onderzoek heeft aangetoond dat de samenstelling van de populatie darmbacteriën een prominente rol kan spelen in de regulatie van het lichaamsgewicht en het metabolisme. Verstoringen van de bacterie-samenstelling, ofwel microbiota-compositie, kunnen leiden tot veranderingen van het lichaamsgewicht, ontstekingsparameters, de suikerstofwisseling en insulinegevoeligheid. Studies die de onderliggende mechanismen in de mens hebben onderzocht zijn schaars, omdat het merendeel van de studies is uitgevoerd in dieren.
In de onderzoeken die zijn beschreven in dit proefschrift onderzochten we wat het effect was van het manipuleren van de microbiota-compositie op de stofwisseling in mensen met overgewicht en obesitas. Daarnaast onderzochten we de relatie tussen de microbiota en insulinegevoeligheid van de lever, spier en het vetweefsel, organen die een belangrijke rol spelen bij verstoringen in de stofwisseling die aanwezig zijn bij mensen met obesitas en type 2 diabetes mellitus. De insulinegevoeligheid hebben we gemeten met behulp van de hyperinsulinemische-euglycemische clamp, wat de gouden-standaard techniek is.

In **Hoofdstuk 2** hebben we aangetoond dat een interventie met amoxicilline (AMOX; breed-spectrum antibioticum) en vancomycine (VANCO; smal-spectrum antibioticum, gericht tegen Gram-positieve bacteriën) geen effect heeft op het metabolisme bij mensen met overgewicht of obesitas. Aan dit onderzoek namen 57 insulineresistente mannen met overgewicht of obesitas deel, die allen gedurende 7 dagen één van de antibiotica of een placebo gebruikten. Dit dubbelblinde, gerandomiseerde onderzoek toonde aan dat de diversiteit van darmbacteriën na VANCO-behandeling was afgenomen, net als de aanwezigheid van de bacteriestam 'Firmicuten'. De AMOX-behandelde groep toonde deze effecten niet.
De afname van Firmicuten resulteerde in een verlaging van plasma en/of feces concentraties van korte-keten vetzuren en galzoutmetabolieten. Dit leidde echter niet tot veranderingen van de insulinegevoeligheid van de lever, spier of vetweefsel (gemeten met de twee-stap hyperinsulinemische-euglycemische clamp met een [6,6-$^2H_2$]-glucose-tracer). Ook de doorlaatbaarheid van de

darmwand en het inflammatoir profiel, zoals gereflecteerd door circulerende concentraties van LBP en inflammatoire cytokines, bleven onveranderd.
Ondanks dat de microbiota compositie na acht weken follow-up nog niet volledig hersteld was, vonden we ook aan het einde van deze periode geen significante effecten op het metabolisme (energie-opname in de darm, de grootte van vetcellen en de insulinegevoeligheid (HOMA-IR) bij deze mensen met overgewicht en obesitas.

Verder onderzochten we of de interventie met AMOX of VANCO een effect had op het energiegebruik en de vet- en koolhydraatverbranding na een nacht vasten en na een vetrijke maaltijd in de vorm van een milkshake. Ook werden veranderingen in circulerende hormonen zoals insuline, GLP-1, ANGPTL4 en leptine onderzocht. We vonden geen veranderingen in deze hormonen na de interventie. De resultaten beschreven in **Hoofdstuk 3** zijn hiermee in overeenstemming. In dit hoofdstuk werd gekeken naar de stofwisseling van de spier in de onderarm met behulp van metingen van arteriële-veneuze concentratieverschillen en doorbloedingsmetingen van de onderarm. Dit onderzoek toonde aan dat antibiotica-behandeling geen significant effect had op de plasma glucose-, vrije-vetzuren- en lactaatconcentraties en de opname van glucose en lipiden door de spier, zowel na een nacht vasten als na het eten van een vetrijke maaltijd.

Zoals beschreven in **Hoofdstuk 2**, verlaagde behandeling met VANCO de expressie van genen gerelateerd aan het immunsysteem in het vetweefsel. Deze genexpressie veranderingen vertaalden zich echter niet in veranderingen van inflammatoire parameters in het bloed. Zowel VANCO als AMOX verhoogden de expressie van genen die betrokken zijn bij een verhoogde verbranding van koolhydraten en vetten, maar hadden beide geen effect op de vetcelgrootte.
In **Hoofdstuk 4** hebben we functionele metingen verricht op het niveau van het vetweefsel (*ex vivo*). We vonden dat behandeling met VANCO gedurende 7 dagen de β-adrenerge gevoeligheid voor stimulatie van de lipolyse (vetafbraak) in vetcellen enigszins verhoogde. De fysiologische relevantie van dit effect vereist echter verder onderzoek, mede omdat er geen effect gevonden werd op de basale en β-adenerge recptor-gemedieerde stimulatie van de lipolyse.

Bovendien bleken de expressie van de β-adrenoceptor alsmede post-receptor effecten onveranderd na de interventie.

Tot slot hebben we in **Hoofdstuk 5** de relatie tussen de microbiota samenstelling en de weefselspecifieke insulinegevoeligheid onderzocht in twee onafhankelijke cohorten van mannen met obesitas. De cohorten waren gerekruteerd in Maastricht en Amsterdam. Met uitzondering van de associatie tussen bepaalde bacteriën en HbA1c, een maat voor de regulatie van de glucose spiegel over een langere periode, in het Maastricht-cohort, vonden we geen enkele significante associatie tussen de microbiota en insulinegevoeligheid in beide cohorten, bepaald aan de hand van zowel gevaste parameters in het bloed en de insulinegevoeligheid zoals gemeten met behulp van de gouden-standaard techniek.

Concluderend kunnen we zeggen dat, in tegenstelling tot dierstudies die suggereerden dat de microbiota een grote rol speelt bij het metabolisme, wij geen relatie vonden tussen de bacteriesamenstelling in de darm en de stofwisseling bij mannem et overgewicht en obesitas. Behandeling met VANCO, maar niet AMOX, gedurende zeven dagen verlaagde de diversiteit van de microbiota en de aanwezigheid van de bacteriestam Firmicuten. Dit ging gepaard met een afname van galzoutmetabolieten en korte-keten vetzuren in de plasma en/of feces, maar vertaalde zich niet in een significant effect op het metabolisme van de gastheer. Ook twee maanden na het stoppen van de antibiotica vonden we geen aantoonbare effecten op de insulinegevoeligheid, ondanks het feit dat de microbiota nog steeds niet volledig hersteld was naar de samenstelling zoals aan het begin van het onderzoek.
Kortom, de onderzoeken beschreven in dit proefschrift tonen aan dat het veranderen van de darmbacterie-samenstelling met antibiotica op korte termijn geen significante effecten heeft op de stofwisseling in mensen met overgewicht en obesitas. Andere strategieën lijken noodzakelijk om de complexe relatie tussen de mens en zijn omgeving te manipuleren om de metabole gezondheid te bevorderen.

Curriculum Vitae

Dorien Reijnders was born on July 10th 1988 in Heerlen, The Netherlands. She completed secondary school at the Sintermeerten College in Heerlen in 2006. Consecutively, she studied Biomedical Sciences at the Radboud University in Nijmegen, The Netherlands. She combined the Masters Toxicology and Pathobiology. As part of the Toxicology Master, she visited the Toxicology lab at the Karolinska Institute in Stockholm, Sweden, for a 6-month internship to perform experiments related to cell cycle mechanisms in 2010. During the Pathology Master in 2011, she investigated epithelial-mesenchymal transition of kidney cells during a 6-month interniship at the Department of Toxicology and Pharmacology in Nijmegen.
In September 2011, she started her PhD research at the Department of Human Biology at Maastricht University (NUTRIM School of Nutrition and Translational Research in Metabolism), under supervision of Prof. Ellen E. Blaak and Dr. Gijs H. Goossens. Her research, which is described in this thesis, entiteld *'Microbiota, Obesity and Insulin Resistance - Unraveling the impact of the microbiome on metabolic health in humans'*, was performed within the framework of the Top Institute Food and Nutrition (TIFN). Dorien has presented these research findings at several national conferences, the 22th European Congress on Obesity in Prague, Czech Republic (2015), and the European Molecular Biology Laboratory (EMBL) conference in Heidelberg, Germany, in 2015.

List of Publications

Effects of Gut Microbiota Manipulation by Antibiotics on Host Metabolism in Obese Humans: A Randomized Double-Blind Placebo-Controlled Trial.
Reijnders D, Goossens GH, Hermes GD, Neis EP, van der Beek CM, Most J, Holst JJ, Lenaerts K, Kootte RS, Nieuwdorp M, Groen AK, Olde Damink SW, Boekschoten MV, Smidt H, Zoetendal EG, Dejong CH, Blaak EE.
Cell Metabolism. 2016 Jul 12;24(1):63-74.

Effects of amoxicillin and vancomycin on skeletal muscle substrate metabolism in obese men: a randomized controlled trial
Reijnders D, Goossens GH, Blaak EE
Submitted

Gut microbiota composition strongly correlates to peripheral insulin sensitivity in obese men but not in women
Most J, Goossens GH, **Reijnders D**, Canfora EE, Penders J, Blaak EE
Submitted

Gut microbiota manipulation and lipolysis in human adipose tissue
Reijnders D, Jocken JWE, Canfora EE, Plat J, Goossens GH, Blaak EE
To be submitted

Microbiota composition is not correlated to insulin sensitivity, but represents distinct alternative dysbiotic states in two independent cohorts of overweight and obese males
Reijnders D, Hermes GD, Kootte RS, Goossens GH, Smidt H, Nieuwdorp M, Zoetendal EG, Blaak EE
To be submitted

Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity.
Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, van Nood E, Holleman F, Knaapen M, Romijn JA, Soeters MR, Blaak EE, Dallinga-Thie GM, **Reijnders D**, Ackermans MT, Serlie MJ, Knop FK, Holst JJ, van der Ley C, Kema IP, Zoetendal EG, de Vos WM, Hoekstra JB, Stroes ES, Groen AK, Nieuwdorp M.
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Peeters K, Wilmer MJ, Schoeber JP, **Reijnders D**, Heuvel LP, Masereeuw R, Levtchenko E. *Pharmaceutics. 2011 Oct 27;3(4):782-92.*

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