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Role of intestinal microbiota in cardio-metabolic diseases

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6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

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6.1. THE ROLE OF INTESTINAL MICROBIOTA IN LIPID METABOLISM AND CARDIOVASCULAR DISEASE RISK FACTORS

Obesity is associated with a variety of comorbidities and predisposes an individual to type 2 diabetes and cardiovascular disease (CVD). The globally increasing prevalence of obesity challenges the medical scientific community to find new targets and strategies for the treatment of obesity and associated cardiometabolic diseases. Because of their role in host metabolism and immunity, intestinal microbiota form a new and interesting target in treating obesity and associated comorbidities such as the prevention and treatment of CVD.

6.1.1. *A. muciniphila*: A CANDIDATE SPECIES FOR LOWERING CIRCULATING TRIGLYCERIDES AND TOTAL CHOLESTEROL?

In Chapter 4 we showed that administration of *Akkermansia muciniphila* (*A. muciniphila*) decreased plasma TG and TC levels in the hyperlipidemic E3L.CETP mouse model remarkably. The E3L.CETP mouse model is characterized by a human-like lipoprotein metabolism and its circulating cholesterol is, unlike in wild-type (WT) mice, mainly present in the pro-atherogenic LDL and VLDL fraction. Since high circulating TG and TC levels are main risk factors for atherosclerosis, lowering these plasma lipids by *A. muciniphila* administration should help in prevention of atherosclerosis progression. Additional evidence for an anti-atherogenic potential of *A. muciniphila* has been described by Li *et al.* They have shown that administration of *A. muciniphila* decreased atherosclerotic lesion area in apolipoprotein E-deficient (apo$E^{-/-}$) mice by restoring gut barrier function and preventing LPS translocation, and thus preventing systemic inflammation [1]. Whether the beneficial effects of *A. muciniphila* administration are translatable to humans has recently been assessed by Depommier *et al.* [2]. They reported that administration of *A. muciniphila* to overweight and obese humans modestly improved plasma lipid levels. However, although *A. muciniphila* administration significantly decreased TC, it did not decrease LDL-C. The question thus remains whether this reduction in TC was truly anti-atherogenic, since it may well have occurred in the HDL-C fraction, which is not causally associated with CVD. In addition, *A. muciniphila* administration did not affect plasma TG nor inflammatory parameters. However, it should also be noted that the overweight and obese humans in this study were not overtly hyperlipidemic. In conclusion, whether *A. muciniphila* administration has anti-atherogenic potential in humans should be addressed in more appropriate study populations.

A major challenge in developing a novel treatment for hyperlipidemia to reduce CVD is the fact that effective and safe pharmacological treatments are already available. Statins are the most commonly used class of drugs to reduce LDL-C. Statins are relatively cheap and have demonstrated efficacy. Since these treatments cannot ethically be withheld from patients at risk, novel treatments have to be tested on top of statin therapy in prolonged and large outcome trials. The cost of these type of large scale trials is prohibitive. It seems likely that more convincing and clear evidence from both pre-clinical as well as (small scale) clinical trial needs to be generated before such trials for a microbial species or component could be started.

6.1.2. POTENTIAL MECHANISM FOR LOWERING CIRCULATING TRIGLYCERIDES AND CHOLESTEROL BY INTESTINAL MICROBIOTA

As previously described, administration of well-studied commensal bacteria like members of *Bifidobacterium* and *Lactobacillus* genera reduce circulating TG and TC levels [3, 4]. The exact mechanism of TG and TC reduction by these commensals has not been revealed yet. Most *Bifidobacterium* and *Lactobacillus* species that are known to be able lowering TC levels contain bile salt hydrolase (BSH) genes in their genomes and are thus able to deconjugate bile salts [5, 4]. However, the relationship between BSH and lowering TC is still not completely understood [4]. Several hypotheses have been proposed to explain the cholesterol-lowering effect of these intestinal bacteria [4], including the deconjugation of bile salts that promote the consumption of blood cholesterol to synthesize bile lost in feces [6, 5] and cholesterol assimilation in bacterial cells [7]. A study conducted by Wang *et al.* compared the *in vivo* cholesterol reducing effects of two BSH containg *Lactobacillus casei* strains with a *Lactobacillus casei* strain not containing the BSH gene, in high cholesterol diet fed mice. The BSH containing strains showed lowered serum TC levels compared to the control group, downregulated farnesoid X receptor (FXR) expression and upregulated expression of cholesterol liver X receptor (LXR) and low density lipoprotein receptor (LDLR) genes in the liver [4]. These findings indicate that BSH loweres cholesterol levels in vivo by reducing cholesterol absorption and increasing cholesterol catabolism [4].

Interestingly, the genome of *A. muciniphila* has recently been sequenced and it seems not to harbour a bile salt hydrolase gene. Therefore, *A. muciniphla* probably lowers plasma TC by another mechanism of action [8]. *A. muciniphila* mainly produces SCFAs acetate and propionate upon mucin degradation [9, 10]. A previous study by Lukovac *et al.* explored the transcriptional response of mouse intestinal organoids to *A. muciniphila* by incubating the organoids with *A. muciniphila* culture supernatants [11]. This study revealed that *A. muciniphila* metabolites affect various transcription factors and genes involved in fatty acid, cholesterol and bile acid metabolism. Some of these effects may be attributable to the short chain fatty acids (SCFAs) produced by *A. muciniphila*, as a previous study in rats showed that SCFAs down regulate cholesterol synthesis in both liver and intestinal tissue [12]. Alternatively, acetate can be converted to butyrate by buterogenic bacteria and affect host lipid metabolim by another mode of action [13]. Li *et al.* recently suggested that butyrate increases brown adipose tissue activity by neural activation, leading to increased oxidation of intracellular fatty acids resulting in a compensatory influx of TG-derived fatty acids and lowering of plasma TG [14]. These findings show that bacterial species have multiple mechanisms that could explain the reduction in TG and TC after administration.

6.1.3. BOOSTING BACTERIAL LIPID LOWERING POTENTIAL BY GENETIC MODIFICATION

In order to increase the lipid lowering ability of intestinal bacteria in humans, it is an interesting thought to enhance these particular properties by genetic manipulation. Genetically modified bacteria with enhanced lipid lowering ability can serve as a future treatment to reduce cardiometabolic risk factors by lowering host peripheral lipid levels. Well studied commensal bacteria like members of *Bifidobacterium* and *Lactobacil-*

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lus genera might be appropriate candidates for a genetically engineered enhanced lipid lowering capability. For instance, bile salt hydrolase overexpressing strains with potential cholesterol lowering ability can be constructed by creating a genetic construct with the bile salt hydrolase gene downstream of a strong overexpression promoter and transforming this construct into the desired strains. With use of *in vitro* tests one can assess whether the overexpression of this gene results in increased bile salt deconjugation and *in vivo* experiments can provide the evidence for its potential supra-physiological cholesterol lowering properties.

The idea of a genetically modified microorganism treatment might not be as far-fetched as it sounds, since similar approaches have been exploited in different contexts. Póhlmann *et al.* engineered the probiotic yeast strain *Saccharomyces boulardii* (*S. boulardii*) for secretion of biologically active viral IL-10 homologs for treatment of inflammatory bowel disease. The *in situ* data of this particular study were promising but *in vivo* data about the secretion of IL-10 are lacking [15]. Another group showed that an engineered mutant of *S. boulardii* could survive the mouse gastrointestinal tract and they could detect viable *S. boulardii* cells in the mouse Peyer's patches (PP) [16]. In particular, the idea of cytokine expression by micoorganisms in peripheral immune active sites like the PP might be interesting to modulate the local or systemic immune response to bacterial triggers from the intestine.

Another example showing the therapeutic potential of genetically engineered microorganisms is a study of Chen *et al.* They designed and constructed a N-acylphosphatidylethanolamines (NAPEs) overexpressing *E. coli* Nissle 1917 strain and administered it for 8 weeks to obese mice, via drinking water. NAPEs are precursors to the N-acylethanolamide (NAE) family of lipids, which are synthesized in the small intestine in response to feeding and they reduce food intake and obesity. Mice that received engineered bacteria had lower food intake, adiposity, insulin resistance and hepatosteatosis compared to control mice [17]. This particular study showed the therapeutic potential of such a strategy in the treatment of obesity related comorbidities. Although design and creation of such a genetically modified therapeutic bacterial strains nowadays is not a technological breakthrough anymore, the public acceptance of such a treatment is still very challenging.

6.2. Strategies for manipulating intestinal microbiota

6.2.1. Controlled and uncontrolled strategies for modulation of intestinal microbiota composition

Intestinal microbiota interact with host immunity and metabolism as shown in Chapter 3 and Chapter 4 of this thesis. There are different strategies for modulation of intestinal microbial communities such as Fecal Microbiota Transplantation (FMT) or oral administration of defined bacterial cultures.

With FMT procedure, the recipient is (re)populated with the fecal microbiota of a donor, sometimes after ablation of the endogenous microbiota [18]. This seems to be an effective strategy for modulation of intestinal microbiota in humans and mice. Currently, FMT is used as a treatment for patients suffering from intestinal diseases like Clostridium difficile-associated diarrhoea (CDAD) and inflammatory bowel disease (IBD) for

restoration of the intestinal microbiota [19, 20]. Despite its effectiveness, FMT is a poorly controlled tool for modulation of the intestinal microbiota. Although the FMT procedure is relatively easy to perform, there is wide inter-institutional variability in methodology, for example in preparation of FMT and or in processing and mode of delivery of the fecal matter [21]. In most cases, prior to FMT, the recipient is treated with antibiotics in order to reduce the native, dysbiotic population [21, 22]. The total fecal composition of the donor is transplanted to the receiver, and the receiver is repopulated with a wide range of bacterial, fungal and viral species of the donor. As a consequence, it is impossible to pinpoint which donor species was responsible for the beneficial (clinical) outcome.

Another uncontrolled strategy for modulation of intestinal microbiota composition is oral administration of prebiotic compounds. Prebiotics are indigestible dietary fibres, which are degraded by intestinal microbiota [23]. A prebiotic compound, such as e.g. inulin, can be degraded by several different species like members of the genera *Bifidobacterium* and *Allobaculum* which as a consequence increase their abundance [24]. To our knowledge, there are no prebiotics known that modulate the abundance of only one specific species. Upon fibre degradation, the bacteria secrete metabolites in the lumen, as side or waste products of their metabolism. These metabolites can serve as precursors for metabolism of the host and/or other bacteria and in this way there is a cross feeding between different species. By using labelled [^{13}C] lactate, Belenguer *et al.* showed that lactate produced by *Bifidobacterium adolescentis* L2-32 in culture is used by *Eubacterium hallii* L2-7 for butyrate production and confirmed the cross feeding between these two human intestinal commensal species [25]. In this way, administration of one single prebiotic compound can up and down regulate the abundance of several additional species, genera or even bacterial families.

Modulation of the intestinal microbial composition can be somewhat more directly and targeted by administration of a single bacterial culture, compared to FMT or oral administration of prebiotic compounds. In this way, a defined strain with a defined concentration can be administered to the host and its effects on host physiological parameters can be described, as applied in Chapter 4 of this thesis. This modulation strategy has been well described in humans and mice for aerobic, facultative aerobic and anaerobic bacteria [26, 27, 28]. However, because of the intricate interplay and cross feeding between different bacterial taxa and species, administration of one single strain can also up or down regulate the abundance of it cross feeding partners or competitors. It seems reasonable to conclude, that even a controlled strategy for manipulation of intestinal microbiota is still only partly controlled. In Chapter 4, we cannot formally exclude the possibility that some of the observed effects are not mediated by *A. muciniphila* but mediated by other bacteria that were affected by administration of *A. muciniphila*.

6.2.2. MODULATION OF INTESTINAL MICROBIOTA BY ELIMINATION OF SINGLE BACTERIAL SPECIES; A FUTURE TOOL

We (Chapter 4) and others have shown that the potentially beneficial effects of bacterial species on host lipid metabolism and immunity can be studied by introducing this bacterial species into the intestinal microbiota population of the host by oral administration. However, it might also be interesting to study the absence of potentially detrimental bacterial species by eliminating these from the intestinal microbiota population

of the host. Elimination of bacterial species in a complex bacterial population can be achieved by bacteriophages. Bacteriophages are viruses which infect bacterial cells and not eukaryotic cells [29, 30]. Like other viruses, they inject their viral DNA into the host cell and use the host cell machinery for the replication and assembly for new bacteriophages until the host cell is eliminated by eruption. This idea has inspired many scientists and even the industry. One of the commercial pioneers in this field is the in France based start-up company Eligo Biosciences. By using CRISPR-Cas technology, Eligo Biosciences aims to design bacteriophage based antimicrobials. By using RNA-guided nucleases (RGNs) targeting specific DNA sequences or polymorphisms, including antibiotic resistance and virulence determinants, a specific bacterium can be targeted and eliminated from the community without affecting other bacterial members of that community [31, 32, 33]. In this way, the effects of presence or absence of a single species within a complex community can be studied and assessed for its contribution or causality in host (patho)physiology. However, as mentioned in the previous paragraph, eliminating one species from a complex bacterial population might have further downstream effects on the whole bacterial population because of the intricate interplay and cross feeding between different bacterial taxa and species in the population.

6.3. LOCAL AND SYSTEMIC IMMUNE MODULATION BY INTESTINAL MICROBIOTA

6.3.1. INTESTINAL MICROBIOTA MODULATE THE HOST IMMUNE RESPONSE LOCALLY AT THE INTESTINAL IMMUNE ACTIVE SITES

In Chapter 4 we showed that oral administration of *A. muciniphila* modulated the immune cell composition locally in the mesenteric lymph nodes (mLNs) by changing the abundance of different immune cell populations and their activation markers. There is a very fine bidirectional interaction between the host GI immune system and its microbiota. The immune system is constantly surveying the luminal environment and secretes anti-microbial compounds into the mucus layer to control bacterial outgrowth and control overexposure of the epithelial cells to bacteria [34]. Dendritic cells (DCs) survey the lumen and lamina propria constantly and capture bacteria that have crossed the epithelial layer. These antigen loaded DCs migrate to the mLNs for antigen presentation. This antigen presentation leads to the differentiation of commensal specific regulatory cells (Treg) Th17 cells and IgA producing B cells [35]. In Chapter 4, oral administration of *A. muciniphila* decreased total T cell population in the mLNs. Simultaneously, the total B cell population was increased and the expression of the T cell co-stimulatory molecule CD86 on both the follicular and mucosal B cell populations was reduced. *A. muciniphila* also tended to decrease the antigen-presenting molecule MHCII on different DCs subpopulation, indicating reduced activity of these DC subpopulations. Furthermore, the total amount of neutrophils in the mLNs was also decreased upon oral administration of *A. muciniphila*. These findings clearly indicate that *A. muciniphila* has immune modulating properties.

The immune modulation by *A. muciniphila* could be explained by the SCFA producing capability of *A. muciniphila* [11]. SCFAs modulate the host's immune system in different manners. SCFAs increase the expression of antimicrobial peptides secreted

into the lumen, modulate the production of cytokines and chemokines and regulate the differentiation, recruitment and activation of immune cells including neutrophils, macrophages and DCs [36]. A recent study showed that oral administration of butyrate reduced neutrophil associated inflammation in a DSS induced colitis mouse mode [37]. These findings are in line with the hypothesis that the decreased total number of neutrophils upon oral administration of *A. muciniphila* is due to its SCFA production. Thus, oral administration of *A. muciniphila* seems to excert a local anti-inflammatory response in the mesenteric lymph nodes, which may be both mediated via presentation of *A. muciniphila* antigens by the DCs in the mLNs and SCFA production and secretion of *A. muciniphila* in the lumen.

6.3.2. INTESTINAL MICROBIOTA MODULATE HOST PERIPHERAL IMMUNITY

Several human commensals, e.g. members of *Bifidobacterium* are known to affect the circulating immune cells and signaling molecules. Bernini *et al.* showed that daily intake of *B. lactis* HN109 over a period of 45 days decreased the pro-inflammatory cytokines TNF-α and IL-6 in blood samples of obese subjects [26]. Martin *et al.* showed that daily oral gavage for a period of 10 days with *Bifidobacterium animalis ssp. lactis* CNCM-I2494 increased splenic secretion of anti-inflammatory cytokines IL-4, IL-5 and IL-10 in a low-grade inflammation mouse model [38]. In Chapter 4, we showed that administration of *A. muciniphila* affected the host IL-10 response modestly and reduced portal vein LPS levels tremendously. Therefore, intestinal microbiota form an interesting and new target for modulation of the host peripheral immune system and might be useful in prevention or treatment of immunometabolic diseases. To this end, longitudinal oral administration of known beneficial single or mixed bacterial cultures can be used for diminishing the immunological effects of a dysbiosed microbiome.

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6.3.3. IMPLICATIONS OF INTESTINAL MICROBIOTA FOR HOST PATHOPHYSIOLOGY

In Chapter 2 and Chapter 3 we applied co-housing, a rodent analogue of FMT, for modulation of intestinal microbiota after BMT. Experimental BMT in mice is relatively easy, effective and cost-efficient tool to study the role of immune cells in many immune-associated disorders [39]. However, the procedure of BMT, which combines use of broad range antibiotics and ionizing radiation induces acute and chronic side effects like decreased body weight gain [40, 41], intestinal damage [42] and , disturbs the intestinal microbiota and causes dysbiosis [43, 44]. Similar findings have been recently reported in humans [35]. We hypothesized that co-housing could restore the dysbiosed microbiome to a eubiotic microbiome and as a consequence ameliorate the pathophysiological side effects of BMT in mice. Chapter 2 described that the decreased total body weight gain after BMT is a consequence of decreased weight gain of multiple organs and alterations in the adipose tissue cell pool composition as well as to a decreased pancreatic secretion of the anabolic hormone insulin. Co-housing with healthy, non-BMT mice, did not prevent nor restore this decreased total body weight gain (Body weight data of co-housed groups is not shown in this thesis). A mouse study examing the effect of FMT post BMT, showed that FMT preserved the intestinal bacterial composition, improved gastrointestinal tract function, intestinal epithelial integrity and prevented decreased body weight gain after

BMT [45]. A clinical study showed that oral supplementation of glutamine, fibre and oligosaccharides (GFO) from seven days prior and up to twenty-eight days post stem cell transplantation, decreased the severity of mucosal injury and prevented weight loss in humans compared with the control group [46]. Another clinical study reported beneficial clinical outcomes like mitigated mucosal injury and decreased incidence of acute Graft Versus Host Disease after oral administration of dietary fibres during the procedure of BMT [47]. These animal and clinical studies indicate that intestinal microbiota might serve at least partially as a target in prevention of decreased body weight gain after BMT and restoration of intestinal ephitelial intergity might play a role in this.

In Chapter 3 we show that splenocytes of BMT treated mice show higher cytokine release as compared to healthy controls, when stimulated with various pathogenic stimuli. This hyper-responsive phenotype of splenocytes is transferred by FMT from BMT treated mice to healthy control mice. By inoculation of germ free mice with cecum content of BMT treated mice, we showed the causal role of intestinal microbiota in hyperresponsiveness of splenocytes post BMT, as a measure of peripheral immunity. Interestingly, the FMT experiment showed the dominance of a dysbiosed microbiome over a healthy one and showed that the balance of a healthy microbiome can be overruled. The observation of a dominant dysbiosed microbiome has previously been described in different immune models. Elinav *et al.* showed that NLRP6 inflammasome deficient mice have altered intestinal microbiota composition, showed spontaneous intestinal inflammatory cell recruitment and were more susceptible for chemically induced colitis. Co-housing experiments revealed that the colitogenic intestinal microbiota of these NLRP6 inflammasome deficient mice were transferable by FMT [48]. It seems that the homeostasis of a healthy microbiome is based on the balance between the many different members of that community, the host and its environment. However, this balance might be more fragile and easier to perturb by endogenous or exogenous effectors, than was previously thought. In our specific experimental design, FMT perturbed this balance and drove the intestinal microbiota community of the recipient mice into a dysbiosis and affected host immunity as we have shown in Chapter 3.

Regarding the recent findings in literature, we can conclude that intestinal microbiota are able to ameliorate both the physiological and immunological side effects of BMT. However, it seems extremely important to choose the right strategy for modulation of intestinal microbiota in order to modulate host metabolism and immunity in a favourable way.

6.4. STUDYING MICROBIAL COMMUNITIES BY 16S SEQUENCING.

The emergence of the microbiome field became possible because of rapid evolution of Next Generation Sequencing technology (NGS). NGS developments have made it possible to sequence billions of nucleotides from multiple samples in a single sequencing run with reduction of time and cost [49]. Currently, a frequently used method for studying microbial community composition and dynamics is 16s sequencing. Despite the fact that the cost of sequencing per nucleotide rapidly decreased over the past decade, down to several cents for each base pair, using NGS as an experimental read-out is still expen-

sive. The commercial price of 16s sequencing for a single stool sample ranges from € 16-50, depending on a.o. sample size, sequencing depth requirements and sequencing platform [50, 51]. This price mostly excludes DNA isolation and NGS data analysis costs, so the actual cost per sample is higher. For a sizeable mouse experiment with an intervention and multiple stool samples over the study period, NGS sequencing costs can still be prohibitive.

Another limitation of 16s sequencing is the resolution of 16s sequencing, which is limited to the amplicon sequence and availability of reference data in 16s databases. In 16s sequencing, variation in specific regions of the bacterial 16s rRNA gene is used as a marker gene for taxonomic analysis and distinguishing different taxa within the sample [52, 53]. A short amplicon of the 16s rRNA gene or its entire 16s rRNA gene can be sequenced and used for taxonomic analysis. However, in most cases a short amplicon of the 16s rRNA gene for e.g. the V4 region or a combination of two or more amplicons like the V3 and V4 regions is sequenced, rather than sequencing the full-length 16s RNA gene. Sequencing a short amplicon of 16s rRNA gene results a higher number of reads per sample and a higher sequencing depth in comparison with full-length 16s sequencing. However, it should be noted that the maximal resolution of 16s sequencing is predominantly at the genus level [54].

As described in Chapter 5, sequencing the full-length 16s rRNA gene provided a different view regarding bacterial relative abundance, in-sample diversity and in in-between-sample diversity, as compared to V4 sequencing regardless of sequence analysis platform. Thereby, the improvement of resolution in terms of taxonomic analysis was in our particular experimental setting marginal. In a recent study, bacterial communities in wastewater were characterized using PacBio full-length 16s rRNA gene sequencing and compared to *in silico* extracted V3-V4 short sequences from the same PacBio data [55].The authors claimed that analysing full-length 16s rRNA gene provided more refined and reliable taxonomic assignment, even to the species level. Detailed examination of their findings revealed that they only made this comparison for the genus *Acinetobacter* and not for their entire dataset. In this particular study, using full-length 16 rRNA gene only assigned six additional OTUs to species level compared to short V3-V4 reads. In our study, described in Chapter 5, we see comparable results on species level OTU assignment by full-length 16s rRNA gene (data not shown) and refer to these differences as a marginal improvement in resolution of taxonomic analysis. Another study using mouse gut samples, compared full-length 16s rRNA gene sequenced by Oxford Nanopore sequencing platform with short V3-V4 region reads sequenced by Illumina platform. The authors reported that full-length 16s rRNA gene data had better resolution than the short-read sequencing data, at the species level [56]. The results of these two studies are in line with our findings described in Chapter 5.

It has to be mentioned that both studies [55, 56] mainly focused on the differences in taxonomic assignment of OTUs on species level between full-length 16s rRNA gene and a short amplicon of the 16s rRNA gene. In contrast, in Chapter 5 we also took into account in-sample and in-between-sample diversity analysis to show the difference between using full-length 16s rRNA gene and a short amplicon of this marker gene. We used UniFrac distance as a measure for in-between-sample diversity. Full-length 16s rRNA sequenced samples had a different UniFrac distance compared to V4 sequenced

sample, showing that the full-length 16s rRNA data resembled a different bacterial phylogeny. Furthermore, using full-length 16s rRNA resulted in a more robust UniFrac distance, as the jack-knifing variance was smaller compared to the V4 data. These observations are not revealed by studying taxonomic assignment only. Therefore, focusing on taxonomic assignment of OTUs only and not taking into account differences in-sample and in–between-sample diversity does not utilise the full potential of using full-length 16s rRNA gene sequencing.

Another drawback of focusing on taxonomic assignment only is based on the fact that currently available 16s reference databases like the Greengenes and SILVA database [57, 58] mainly rely on 16s rRNA gene sequences of cultured taxa. Therefore, a certain proportion of the sequences in a typical 16s sequencing dataset can not be assigned, because of the lack of a reference sequence in these databases. Although, recently, uncultured taxa are being included in the databases, we can not yet understand the full potential of an uncultured taxon, because of the lack of information about the in vitro and in vivo properties of these uncultured bacteria.

Taken together, 16s sequencing is a useful tool for studying microbial community changes and dynamics globally, but less useful for studying individual members of the community. The scientific community, using this technology as a read-out tool for experimental outcome, should be aware of the inherent limitations and biases of NGS technology and take these limitation and biases in account for the interpretation of their experimental results.

6.5. Conclusion

The studies described in this thesis addressed the causal role of intestinal microbiota in host metabolism, immunity and pathophysiology of atherosclerosis. We showed that oral administration of the bacterial species *A. muciniphila* affected host lipid metabolism. In addition, our co-housing experiments after BMT indicated that intestinal bacteria play a crucial role in systemic immune responses. Furthermore, we provided critical insight in two generally accepted and widely applied research tools in cardiometabolic research and microbiome research, BMT and 16s rRNA sequencing. After BMT mice clearly suffer from a multitude of abnormalities that should be taken into account when designing and interpreting results from BMT experiments. Similarly, the choice of amplicon for 16S rRNA sequencing affects interpretation of the results and should be taken into account when designing and interpreting results from experiments which affect the microbiota.

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