Role of intestinal microbiota in cardio-metabolic diseases

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GENERAL INTRODUCTION

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1.1. ROLE OF INTESTINAL MICROBIOTA IN CARDIO-METABOLIC DISEASES

THE obesity epidemic forms one of the major healthcare challenges of the current era and forms a global healthcare challenge [1]. Obesity is strongly associated with a disturbed glucose and lipoprotein metabolism and is also associated with a proinflammatory state [2, 3]. Obesity plays a causal role in a range of comorbidities like type 2 diabetes (T2D) and cardiovascular disease (CVD) [4]. One of the most prominent factors causing obesity is the modern lifestyle characterized by a disbalance between caloric consumption and energy expenditure due to an overall sedentary lifestyle. However, since not all obese individuals develop T2D or CVD, additional factors like genetic background and specific environmental exposures must contribute to the development of obesity and obesity associated diseases.

Within the past decade, our understanding of the intestinal microbiota, the microbes harbouring our gastrointestinal tract (GI-tract), has increased remarkably. It has been shown that intestinal microbiota are associated with different diseases, and that they play a role in our metabolism and immunity [5, 6].

Our intestinal microbiota are the cumulative result of (early and late) environmental exposure, life style and genetics and are strongly implicated in the development of obesity and associated diseases [7, 8, 9, 10]. As such, they may explain at least a portion of the interindividual variability in susceptibility to T2D and CVD. In this thesis, we focus on the role of the intestinal microbiota in cardiometabolic disease. This was conducted by studying the effects of *A. municipia* on plasma lipids, immunity and neointima formation in the hyperlipidemic E3L.CETP mouse model and by studying the effects of cohousing in mice on immunity in the setting of bone marrow transplantation (BMT). Because of the inherent limitations and biases of the DNA sequencing technologies. In addition, we investigated the effects of different DNA sequencing technologies on the interpretation of experiment outcomes.

1.1.1. RISK FACTORS FOR CARDIOMETABOLIC DISEASE

CVD is a leading cause of death responsible for 32% of deaths globally in 2019 and the contribution of CVD to mortality is expected to increase over the next decades [1]. The main underlying cause of CVD is atherosclerosis, which is chronic deterioration of the heart and blood vessels, promoted by multiple risk factors including, dyslipidemia, high blood pressure, disturbed glucose metabolism and inflammation. The co-occurrence of metabolic risk factors that specifically increase the risk of T2D and CVD is termed the Metabolic syndrome (MetS). MetS is defined by the presence of three out of the five following factors: abdominal obesity, hypertriglyceridemia, hypertension, fasting hyperglycemia and low High Density Lipoprotein (HDL)-cholesterol [11]. The prevalence of MetS is currently increasing rapidly in parallel with the obesity epidemic.

Atherosclerosis is initiated by damage to the endothelium, which can be caused by dyslipidaemia, smoking and/or high blood pressure. Atherogenic lipoproteins, predominantly LDL and very low-density lipoprotein (VLDL) particles, accumulate in the vessel wall and after oxidation are taken up by macrophages via endocytosis [12]. Excess uptake of oxidized lipids causes macrophages to differentiate into foam cells [13]. Macrophages

and foam cells secrete pro-inflammatory mediators such as cytokines like TNF-α and IL-6, which activate other immune cells and result in exacerbation of inflammation. Vascular inflammation subsequently induces smooth muscle cell activation. Foam cells, smooth muscle cells, and accumulated lipoproteins together form lesions or atherosclerotic plaques, narrowing the vascular lumen [14].

Since atherosclerosis is driven by dyslipidaemia and local inflammation, it is likely exacerbated by a systemic pro-inflammatory state [15, 14]. Obesity is clearly associated with systemic inflammation. The obesity associated systemic inflammation originates at least partly from white adipose tissue (WAT) [16, 17]. Expansion of WAT leads to infiltration of immune cells like macrophages and T-cells in this tissue. These infiltrated immune cells express pro-inflammatory cytokines such as TNF-α and IL-6, that spill over in the circulation, resulting in systemic inflammation, that subsequently may aggravate atherosclerosis [18].

Another cause for the induction of systemic inflammation in obesity is leakage of bacterial components like lipopolysaccharides (LPS) from the intestine into the circulation [19]. Consumption of a high fat diet has been associated with dysfunction of the mucin producing goblet cells and degradation of the mucin layer in the gastrointestinal tract, which forms a physical barrier between the intestinal content and the system [19]. Consumption of a high fat diet also decreases intestinal tight junction protein content [20]. Tight junctions are intercellular junctions in the intestinal epithelial cell monolayer, which seal neighbouring epithelial cells together and control paracellular permeability of the intestinal cell monolayer [20, 21, 22]. Dysfunction of the mucin layer and disrupted tight junctions results in a so called 'leaky gut' and translocation of bacterial components like LPS into the portal vein blood and circulation [23, 19, 20]. This LPS binds to Toll-like receptors (TLRs) on various immune cells. Binding of LPS to TLRs leads to activation of immune cells and results in an (innate) immune response [24]. Studies in obese and diabetic mice have shown that restoration of the mucin layer thickness by bacterial interventions ameliorates metabolic endotoxemia induced inflammation [25, 26, 27]. Thus, leakage of LPS from the intestinal lumen into the circulation results in systemic inflammation, which may also promote atherosclerosis [25, 28].

1.1.2. MOUSE MODELS TO STUDY ATHEROSCLEROSIS

Wild type mice are relatively resistant to the development of atherosclerosis. That is because in mice, unlike in humans, plasma cholesterol is mainly present in the HDL fraction and not in the pro-atherogenic LDL or VLDL fraction [29]. However, several mouse models have been developed to study the effects of experimental interventions on atherogenesis. The Apolipoprotein E*3Leiden. Cholesteryl Ester Transfer Protein (E3L.CETP) mouse, expressing a mutated human apolipoprotein E (APOE) and the human Cholesteryl Ester Transfer Protein (CETP) gene, is a hyperlipidemic, diet sensitive, atherosclerosis model characterized by a human-like lipid and lipoprotein metabolism [29, 30]. On a cholesterol containing Western type diet, E3L.CETP mice develop atherosclerosis in the aortic root area after ~8-16 weeks. This model is particularly responsive to human drugs that target lipid metabolism [30].

In addition, more rapid models of vascular damage and response have been developed. One such model entails placement of a non-constricting polyethylene cuff around

the femoral artery of E3L.CETP mice on a Western type diet. Vascular remodelling with signs of accelerated atherosclerosis takes place in a relatively short time frame of 2–3 weeks in the cuffed femoral artery [31]. This model of cuff-induced neointima formation resembles both restenosis as it occurs after balloon angioplasty in humans as well as the very early steps of atherosclerotic plaque formation, as the lesions formed after cuff placement contain both smooth cells and macrophages that might become foam cells [32]. This model is particularly responsive to immune modulation [33].

1.1.3. BONE MARROW TRANSPLANTATION, A TOOL TO STUDY GENE FUNCTION IN ATHEROGENESIS

A widely used tool to study the role of immune cells in many immune-associated disorders such as atherosclerosis, is experimental bone marrow transplantation (BMT) in mouse models. With this technique, host hematopoietic cells are depleted by lethal total body irradiation (TBI) and replaced by donor bone marrow cells harbouring genetic alterations in a relevant inflammatory pathway [34]. Both the more natural and cuff-induced models of atherosclerosis development have been used in conjunction with bone marrow transplantation (BMT) to determine the effects of deletion or addition of a gene to the bone marrow compartment on atherosclerosis development in mouse models [35, 36].

Experimental BMT in mice is relatively easy, effective and cost-efficient. However, a drawback is that BMT, and in particular the lethal TBI that is part of the procedure, may induce metabolic disturbances *per se* such as decrease in body weight, reduced adiposity, reduced organ weight, reduced insulin secretion and glucose intolerance [37, 38, 39]. While BMT is a relatively easy and effective method to assess gene function, the BMT procedure has multilevel effects on the organism and affects metabolically important organs and processes that are involved in development and progression of atherosclerosis. Therefore, BMT might affect atherosclerosis development *per se*, which should be taken in consideration in experimental design and interpretation of experimental outcome.

1.2. MICROBIOTA

1.2.1. INTESTINAL MICROBIOTA

Over the past decade, our understanding of the mutual symbiotic relationship between mammals and microorganisms has evolved rapidly. The human microbiome project revealed that the human body is populated by hundreds of different bacterial species, collectively termed microbiota [40], harbouring our skin, body cavities and our internal organs like the gastrointestinal tract (GI tract) [41, 8]. The composition, complexity and abundance of microbiota are shaped by both the local micro-environment and environmental exposure and therefore differ at different body sites [42].

It is becoming clear that disruption of homeostasis of the intestinal microbiota (i.e. microbial dysbiosis) is associated with and is potentially causal in the development of various diseases in humans. Dysbiosis may not only play a role in intestinal diseases such as inflammatory bowel disease and ulcerative colitis, but also in diseases with a metabolic and an immune component such as type-2 diabetes [43, 44, 45].

Multiple factors like age, sex, lifestyle, medication use, host genetics, geography and

diet modulate and shape intestinal microbiota acutely and longitudinally. Diet is one of the most direct modulators and dietary intervention changes the activity and composition of intestinal microbiota already within 24 hours [46]. Individuals consuming a paleolithic diet like the Hadza hunter gatherers were shown to have a distinct GI tract microbiota composition as compared with European lean subjects [47]. It has also been shown that intestinal microbiota of lean and obese human individuals and mice differ. Obese individuals and obese mice have, in general, a less diverse microbiota and it has been shown that certain gut microbial patterns correlate with obesity [48, 7]. More recently, the first evidence for the causality of gastrointestinal microbiota in metabolic disease by direct interaction with the immune system, by either their metabolites or cell components, have been shown in animal studies [49, 50, 51]. For instance transplanting uncultured fecal microbiota of obese people to germ-free mice promoted obesity in these mice, showing the causal role of intestinal microbiota in obesity [52]. Although causality of intestinal microbiota in disease in different mouse studies has been shown, proving the causality of intestinal microbiota with disease in human subjects remains challenging.

1.2.2. BENEFICIAL INTESTINAL BACTERIA

Over the past decade, investigations of beneficial microorganisms that reside in the human gut have attracted much attention, in order to study the utilization of intestinal microbiota as a potential treatment agent for metabolic and intestinal inflammatory diseases. To this end, controversial approaches like Fecal Microbiota Transplantation (FMT) and less controversial approaches like oral administration of single bacterial specie have been used. The aim of FMT is to transplant healthy fecal microbiota to an individual with unhealthy fecal microbiota, while the aim of administration of single bacterial specie is to expose an individual to a beneficial bacterium.

Fecal Microbiota Transplantation is one of the oldest strategies for modulation of intestinal microbiota as it was applied in Traditional Chinese medicine back in the 4th century B.C. and described in western medicine in 1958 [53]. In humans, Fecal Microbiota Transplantation is achieved by blending donor fecal matter with saline solution to a homologous liquid solution. This liquid solution is delivered to the recipient either via a duodenal tube or via colonoscopy [54]. More recently, FMT studies have been conducted, using capsules containing fecal matter for oral administration [55]. FMT in rodents can be achieved by oral gavage of donor fecal material into the recipient or by co-housing. Since rodents are coprophagic, co-housing of a donor and a recipient rodent, results in fecal microbiota exchange [52]. Currently, FMT is applied as an effective therapy for the treatment of recurrent *Clostridium dificile* infection (CDI) in humans. Multiple studies have shown more than 90 percent efficacy in resolving recurrent CDI by FMT [56]. Animal studies have also demonstrated the ability of co-housing to alter the metabolic phenotype of the host [52].

Although FMT is an effective therapy for CDI and showed promising experimental outcomes in the onset of other inflammatory and metabolic diseases, it is a black box approach. FMT transplants several hundred or thousands bacterial species from the donor to the recipient and thus the procedure does not pinpoint which bacterial species are responsible for the beneficial outcome. Oral administration of a single bac-

terial specie rather than a complex bacterial population has also been extensively studied within the past decade. *Akkermansia muciniphila* (*A. muciniphila*) and *Faecalibacterium prausnitzii*(*F. prausnitzii*) are two potential beneficial bacterial species that have been subjected to multiple animal and human studies for their antiglycemic and anti-inflammatory properties [57, 58, 59, 60, 61].

A. muciniphila is a strictly anaerobic, gram-negative commensal bacterium of the human and mouse GI-tract and has been isolated from the human GI-tract. *A. muciniphila* degrades mucin and produces propionate and acetate [62]. Treatment with viable *A. muciniphila* decreased fat mass, restored mucus layer thickness, decreased endotoxemia and improved glycaemia in obese mice [26]. More recently, a clinical trial showed beneficial metabolic effects of *A. muciniphila* administration in overweight/obese insulin-resistant humans [63]. Administration of pasteurised *A. muciniphila* tended to reduce body weight and fat mass. Plasma total cholesterol, insulinemia were reduced and insulin sensitivity was improved compared to the placebo group [63]. Numerous in vivo and in vitro studies have addressed the anti-inflammatory properties of *F. prausnitzii*. *F. prausnitzii* exerts its anti-inflammatory properties via different mechanisms [64]. *F. prausnitzii* produces a 15kDa peptide named microbial anti-inflammatory molecule (MAM), which enhances the anti-inflammatory milieu in the gut by inhibition of pro-inflammatory cytokines such as IL-8, Th1 and Th17 [65, 66]. Furthermore, *F. prausnitzii* exerts its anti-inflammatory effects by inducing secretion of anti-inflammatory IL-10 and enhancing the intestinal barrier function via expression of tight junction proteins claudin-1 and claudin-2 [60, 67]. *A. muciniphila* and *F. prausnitzii* are examples of beneficial bacteria, which potentially can be used as a single bacterial species with therapeutic properties.

1.3. INTESTINAL MICROBIOTA AND THE IMMUNE SYSTEM

1.3.1. PHYSICAL AND IMMUNOLOGICAL BARRIERS OF THE INTESTINE

The gastrointestinal tract organ contains the largest number of microorganisms of all body sites. These microorganisms are comprised of viruses, fungi, parasites and, predominantly, bacteria [68]. The main purpose of intestinal bacteria is degradation of otherwise indigestible compounds and thus supply additional nutrients to the host [69]. Their concentration per gram luminal content increases from the upper intestine towards the colon [70]. To prevent exposure and migration of the intestinal luminal bacteria to the circulation, the lumen is confined by both physical and immunological barriers [71].

The first physical barrier or the extrinsic barrier is the mucus layer consisting of mucin glycoproteins, produced by goblet cells. The mucus layer prevents exposure of bacteria to the epithelium layer which forms the second physical barrier or the intrinsic barrier. The epithelial cells are circumferentially linked together by tight junctions making them impermeable for whole bacteria [72].

In addition to physical barriers, the intestinal lumen is also guarded by immunological barriers. The immune system has a variety of tools like anti-microbial peptides and immune cells belonging both to innate and adaptive immunity which can recognize and respond to microbiota and their components. The first immunological barrier is via secretion of anti-microbial peptides such as defensins and lysozyme by Paneth cells and

immunoglobulin alpha (IgA) by B cells into the mucus layer [73].

On top of secretion of antimicrobial compounds, different immune cells are able to recognize and eliminate microorganisms that have penetrated the intestinal epithelial layer and entered the underlying connective tissue called lamina propria, before they enter the circulation. Neutrophils which can phagocytose these intruders along with monocytes and T-cells are patrolling the lamina propria. Upon infection, inflammatory monocytes and T-cells can be recruited to the lamina propria [74]. It seems likely that the immune system can distinguish between symbiotic microorganism which are beneficial for the host and the pathogenic bacteria which might have a negative effect on the host health [71].

1.3.2. INTESTINAL MICROBIOTA INTERACT DIRECT AND INDIRECTLY WITH HOST IMMUNE SYSTEM

Intestinal microbiota interact directly with antigen presenting immune cells (APC), such as dendritic cells (DCs). Pattern recognition receptors (PRR) like the TLRs recognize a variety of microbial components and allow (innate) immune cells to sense pathogen associated molecular patterns (PAMPs). These receptors are expressed on the intestinal epithelial cells (IEC) and the APCs [74]. Dendritic cells are also able to sense the intestinal lumen directly by protruding dendrites and take up epitopes from the intestinal lumen. The APCs migrate to secondary lymphoid organs like the Peyer's patches or mesenteric lymph nodes (MLNs) for antigen presentation to T cells, which results in T cell differentiation. T cells can differentiate into anti-inflammatory regulatory T cells (TReg) or pro-inflammatory T helper 1 (TH1) and TH17 cells [73].

Intestinal microbiota not only directly interact with the immune system, but also indirectly via the metabolites they produce. Intestinal microbiota convert indigestible carbohydrates to Short Chain Fatty Acids (SCFAs) which have been shown to interact with immune cells. Kim et all. demonstrated SCFA production in the intestine of mice after feeding them with a mixture of dietary fibres pectin and inulin. This was associated by antibody production in B cells [75]. SCFAs also seem to control gene expression to express molecules necessary for plasma B cell differentiation [75]. They also showed that increased SCFA levels not only affected local immunity in the intestine but also systemic immunity, because of increased numbers of IgA+ cells in mesenteric lymph nodes and the spleen.

Thus, the interaction of intestinal bacteria with the host does not only affect local immunity but also affects systemic immunity. Experiments in germ-free mice, born and raised in sterile conditions and therefore lacking intestinal bacteria, showed that the splenic lymphocyte population had decreased CD4+ T cell proportions compared to conventionally raised mice [76]. Mono-colonization of these germ-free mice with *Bacteroides fragilis* restored the splenic CD4+ T cell proportions. Therefore it can be concluded that intestinal microbiota play a crucial role in regulation of the systemic immune system. CD4+ T cells also play a role in atherogenesis as TH1 cells seem to be pro-atherogenic and Treg cells athero-protective [77].

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1.4. NEXT GENERATION SEQUENCING

1.4.1. NEXT GENERATION SEQUENCING AS A TOOL TO PROFILE THE INTESTINAL MICROBIOTA AND MICROBIOME

Microbiota are defined as an ecological community of commensal, symbiotic and pathogenic microorganisms whereas the microbiome is defined as the collective genomes of these microorganisms [78, 40]. Studying the microbiome has been made possible with the rapid evolution of Next Generation Sequencing (NGS). Due to NGS technology, it is now possible to sequence billions of nucleotides in multiple samples in limited time and at limited cost. Roche 454 pyrosequencing was one of the first commercially available NGS platforms. Pyrosequencing technology yielded an approximately 100-fold increase in throughput over conventional Sanger sequencing, reducing sequencing time and cost significantly [79]. This technological improvement made the fast rise of the microbiome field possible. Currently, there are multiple NGS platforms available, with different specific properties like for e.g. differences in amplicon sequence length, sequencing time per run and sequencing depth (number of sequence copies per run), from different companies like Oxford Nanopore, Pacific Bioscience and Illumina.

Every gram of stool sample contains billions of bacteria belonging to hundreds of different taxa. In order to distinguish these bacterial taxa the 16s ribosomal RNA gene (16s rRNA) serves as a marker gene [80]. 16s rRNA gene consists approximately of 1500 base pairs with nine hypervariable regions. The hypervariable regions are 70 up to 250 base pairs long and each of them is flanked by highly conserved regions [81]. Due to the hypervariability of these regions, bacterial taxa within a sample can be distinguished by sequencing one or multiple hypervariable regions and aligning the sequences to a reference sequence.

1.4.2. 16S SEQUENCING AND METAGENOMIC SEQUENCING

For studying the bacterial composition of a sample, sequencing one or multiple V-regions of the 16s gene is sufficient to distinguish different bacteria. This strategy is called 16s V-region sequencing and is currently the most commonly applied strategy for studying bacterial communities [82]. However, 16s sequencing provides only information about the bacterial taxa, not about eukaryotes, viruses or their gene content. Furthermore, 16s sequencing has a limited taxonomic resolution (~genus level) because of the relatively short sequenced amplicon and overlap in these sequences between different species.

For studying microbiomes, metagenomic sequencing has a substantially higher taxonomic resolution. With metagenomic sequencing, all the DNA present in the sample is sequenced. When using Illumina technology this will result in short reads. Subsequently, genomes are reassembled from these short reads and aligned against reference databases. Reads from the same genome are merged into a single contiguous sequence or so called contig [83]. After assembly, genes are predicted and functionally annotated using a sequence database such as for example KEGG [83]. Since metagenomic sequences deal with all the DNA present in the sample and genomes are reassembled from the sequence DNA it is also possible to identify eukaryotes, viruses and their gene content. Metagenomic sequencing provides far more information about the microbiota and their genomic potential compared to 16s sequencing. However it is a more compu-

tational and cost expensive strategy.

1.5. OUTLINE OF THE THESIS

As introduced above, atherogenesis and CVD are promoted by multiple risk factors including dyslipidaemia, high blood pressure, disturbed glucose metabolism and inflammation. Intestinal microbiota have been associated with many of these CVD risk factors. In this thesis, we set out to investigate the role of intestinal microbiota in cardiometabolic disease and its underlying risk factors.

We used different strategies for manipulation of intestinal microbiota and studied the effects of modified intestinal microbiota on cardiometabolic risk factors. In chapter 2 and chapter 3, we investigated the role of intestinal microbiota in bone marrow transplantation (BMT) studies, since the microbiota are more than likely severely affected by the drastic BMT procedure. We exploited natural coprophagy while co-housing BMT-treated and control mice, as a tool to cross-transfer fecal microbiota. Chapter 2 describes the effects of autologous BMT on metabolic parameters, since the BMT procedure itself was found to have pleiotropic organ-specific effects. In chapter 3, we show that the autologous BMT procedure itself triggers a pro-inflammatory immune response which is mediated and transferred via the intestinal microbiota from a BMT-treated mouse to its co-housed partner. These data are proof for the causality of intestinal microbiota in the modulation of the systemic immune response.

Specific intestinal microbiota have been proposed to play beneficial roles in the modulation of inflammation. Since fecal transplantation is not a selective manipulation strategy and potentially transfers multiple bacterial and eukaryotic strains, viruses and host material, we aimed to introduce a single bacterial strain as a selective manipulation strategy. In chapter 4, we modulated the intestinal microbiota directly by oral administration of *A. muciniphila* and studied its local and systemic immune modulating properties and determined whether *A. muciniphila* administration would prevent neointima formation. Although lipid metabolism and the immune system showed signs of improvement by *A. muciniphila* administration, this was insufficient to ameliorate neo-intima formation. In chapter 5, we focussed on strategies to analyse the bacterial composition of the microbiota. Using two different sequencing platforms, we studied whether full length versus V4-region sequencing of the 16S rRNA gene affected the interpretation of the bacterial composition. Our results do indicate that the sequencing platform and strategy indeed affect the results and interpretation of the bacterial composition of the microbiota before and after an intervention. The implications of the findings described in this thesis and the future perspectives of these findings are discussed in chapter 6.

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1.6. ABBREVIATIONS

T2D	Type 2 diabetes
CVD	Cardiovascular disease
MetS	Metabolic syndrome
BMT	Bone marrow transplantation
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
LPS	Lipopolysaccharides
E3L.CETP	APOE*3Leiden.humanCholesteryl Ester Transfer Protein
APOE	Apolipoprotein E
CETP	human Cholesteryl Esther Transfer Protein
IgA	Immunoglobulin alpha
SCFAs	Short Chain Fatty Acids
APC	Antigen presenting cells
DC	Dendritic cell
MLN	Mesenteric lymph node
PRR	Pattern recognition receptors
PAMPs	Pathogen associated molecular patterns
IEC	Intestinal epithelial cells
TReg	Regulatory T cells
TH1	T helper 1
TLR	Toll-like receptor
GI tract	Gastrointestinal tract
A. muciniphila	*Akkermansia muciniphila*
NGS	Next Generation Sequencing
16s rRNA	16s ribosomal RNA gene

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