Role of gut-liver axis in circadian exercise and dietary interventions to improve metabolic health

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Chapter 8

General discussion and future perspectives

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When I started my PhD approximately four years ago, the world was slowly recovering from the COVID-19 pandemic, but it was clear that we are facing another, potentially more dangerous, pandemic, the pandemic of obesity[1]. Obesity affects more than 15% of the population and contributes to the development and worse prognosis of diseases such as COVID-19 itself, ischemic heart disease as due to atherosclerosis, type 2 diabetes (T2D) and, the main focus of this thesis, metabolic dysfunction-associated steatotic liver disease (MASLD)[2]. However, while four years ago lifestyle interventions, the focus of this thesis, were the main prescribed treatment for obesity and MASLD, recently Semaglutide (Ozempic, GLP-1 receptor agonist)[3,4], Tirzepatide (a dual GIP and GLP-1 receptor agonist)[5] and a number of others, emerged as potent drugs for clinically relevant weight loss and T2D treatment. They also have promising effects in the treatment of MASLD[6]. Additionally, Resmetirom (a liver-targeted selective thyroid hormone receptor-β agonist) has been recently conditionally approved as a treatment for MASLD[7]. More drugs, such as other GIP/GLP-1 receptor agonists, triple agonists (additionally targeting the glucagon receptor)[8,9] and FGF21 based drugs[10], are currently in phase 2 and 3 trials. The first results promise potentially improved health outcomes. This would allow to finally decrease the number of individuals with obesity with GIP/GLP-1 receptor agonists, and specifically MASLD with FGF-21 based drugs, turning the tide of increasing numbers that have been observed for the last decades.

With the advent of these drugs, the question arises whether optimization of lifestyle interventions is still needed at all. For now, lifestyle interventions are still commonly used and needed, as these drugs are expensive and are only prescribed to patients if lifestyle interventions were not successful[11,12]. While these drugs will work for the majority of the population affected by obesity, the side effects of these drugs, as well as other causes, limit their effectiveness for some of the affected population[13,14]. For instance, 80% of people using the drugs experience at least some adverse effects, mainly gastrointestinal, and 7% and 15% of patients discontinue treatment with Tirzepatide and Semaglutide respectively[3,5]. Additionally, these drugs induce weight loss in individuals with obesity to a certain point (approximately 20%), but then the weight loss seems to plateau and does not decrease further[4,5]. Finally, and importantly, recent findings demonstrate that while

the drugs work well for weight loss, weight regain is likely upon cessation[15]. Hence, lifestyle changes are still the primary choice for prolonged and sustained metabolic health, and improving and optimizing lifestyle interventions remain crucial. In this chapter, I will discuss the findings of this thesis on which lifestyle intervention modifications worked best, as well as indicate which further research is warranted, including the combination of lifestyle interventions and treatment with newly emerging drugs.

Exercise training modes optimization and cardiometabolic health

Exercise training is an effective lifestyle intervention to improve metabolic health. Specifically, exercise training induces weight loss by increasing energy expenditure, where 10% weight loss or more leads to steatosis resolution if one has MASLD[16,17]. Exercise training also aids muscle growth, which improves (peripheral) insulin sensitivity and, over time, leads to an increased basic metabolic rate[18]. At the same time, starting exercise training may be a complex challenge for individuals with obesity. While the positive effects of exercise training are undeniable, there are also possibilities of injuries, fatigue, post-exercise overeating, overtraining, gut (microbiota) damage[19] and, in patients with T2D, dysregulation of glucose signalling[20,21]. Hence, there is a need for developing optimized exercise training lifestyle interventions, which would increase its positive effects and simultaneously lower the possibility of negative consequences.

Resistance versus endurance training

The first major choice that one should make when starting an exercise training lifestyle intervention is whether one chooses resistance (strength) training or endurance (cardio) training. While both positively affect cardiometabolic health, the way they do it differs. Specifically, endurance exercise training leads to a higher energy expenditure during the exercise bout, fat loss through fatty acid oxidation and higher cardiovascular system training. Resistance exercise training, meanwhile, better promotes muscle growth, resulting in a higher increase in basic metabolic rate over time[18,22]. Curiously, when comparing both interventions in similar cohorts, all endurance interventions, but 85% of resistance interventions, are successful in reducing body mass, fat mass and hepatic lipid accumulation[18]. Studies that did not find a difference between the exercise training modes, however, mostly recommend

resistance training, as it is more accessible for individuals with poorer cardiometabolic fitness[18]. However, the advice may also depend on additional treatment targets, such as insulin control or muscle mass gain[22].

Another currently largely overlooked yet major difference is the impact of exercise training on the gut microbiota. Resistance training hardly impacts the gut microbiota, both in healthy populations and in individuals with metabolic disorders[23,24]. Overall changes in alpha- and beta-diversity (diversity metrics within and between samples respectively) are rarely reported[23], although certain bacteria, such as those of *Roseburia* genus, increase with resistance training[24,25]. Overall, changes in the gut microbiota are scarce and have a limited additional impact on the individual's long-term cardiometabolic health[23]. Endurance training, however, more strongly remodels the gut microbiota composition[26]. Running interventions change beta-diversity and sometimes lead to an increased alpha-diversity[27]. Additionally, endurance exercise training increases the relative abundance of short-chain fatty acid (SCFA) producers from *Akkermansia* and *Lachnospiriceae* genera, which has been associated with cardiometabolic benefits and may further improve fitness[28]. SCFA production may also increase VO_2max, another cardiometabolic health metric that predicts healthier outcomes[29]. Therefore, as already outlined in **Chapter 1**, gut microbiota remodelling towards a healthier state may be one of the ways to sustain weight loss and prevent weight regain.

If endurance exercise training is possible for the affected patient, then this should either be the preferred intervention, or at least be incorporated into the training. This thesis mostly investigated the effect of the lifestyle interventions on cardiometabolic health via gut microbiome remodelling, and resistance exercise training has scarce effects. Consequently, the remainder of this chapter will be devoted to endurance exercise training and referred to as just "exercise training", unless specified otherwise.

Timing of exercise training as an additional tool for the improvement of the metabolic health

Once the choice of the type of exercise training is made, the next question is *when* does one exercise. On the surface, this question does not seem that important – why would it matter, if it induces muscle damage and calorie usage regardless? Metabolism, like nearly any other process in the human body, is controlled by the circadian rhythm: an approximately 24 hours long cycle of changes in bodily

processes[30]. Circadian rhythm is mainly controlled by the central clock in the suprachiasmatic nucleus in the brain and is adjusted by *Zeitgebers*: environmental stimuli, with sun light being the most crucial[30]. Additionally, most tissues, such as gut, liver and muscles, have their own, peripheral circadian clocks, which are adjusted by the central clock, but also by additional *Zeitgebers*, such as food intake or exercise training [31,32]. Consequently, aligning exercise training with these peripheral clocks may allow to improve the exercise-exerted benefits.

For resistance exercise training, the consensus is that late active period exercise training (training in the second half of the active period, evening for humans, end of the dark phase for mice) is more beneficial for building muscles than early exercise training[33]. A consensus for endurance exercise, however, remains elusive. Theoretically, exercise training directly after waking up, without breaking the fast, allows for a faster switch to beta oxidation of lipids, as glycogen stores are depleted. The available clinical studies, however, show opposing outcomes. When measuring the period of the day with the highest physical activity, UK Biobank data demonstrated that those who had more of their physical activity in the afternoon had a lower risk of cardiometabolic events[34]. A Dutch NEO cohort study, though, concluded the opposite, despite using similar methods of determining a period with the highest physical activity[34,35]. Similarly, there are two studies with T2D patients that propose opposing results; one suggesting late exercise training and another early exercise training resulting in worse glucose regulation[20,21]. In patients with obesity, there is also discrepancy, though most studies suggest that late exercise training is more beneficial for weight loss than early exercise training[36]. These inconsistencies appear due to a number of factors that are difficult to control in clinical studies. For instance, chronotype (one's natural timing of the sleep-wake cycle) plays a big role in health, as people that naturally wake up earlier usually also have healthier outcomes[37]. These people with early chronotype may prefer to exercise in the morning, introducing bias in the outcomes. Hence, timing of exercise training studies should control (or at least account) for this factor. The timing of the last food intake also plays a big role[38]. By itself, earlier final food intake of the day is causally associated with better outcomes[38,39]. The combination of dinner and exercise training is more complex. Exercise training before and after dinner may lead to different results. A meta-analysis showed that light exercise (such as a walk) after dinner decreases the glucose spike, and the magnitude of the effect depends on the time after dinner when the exercise training was performed[40]. There was no effect

of light exercise training before dinner[40]. A number of mouse studies also show that post-dinner exercise has positive effects, but pre-dinner exercise does not, indicating that this is another factor to consider when investigating exercise training timing[41,42]. Sleep quality may also play a role, as exercise training generally improves sleep quality, but very strenuous exercise training or exercise training too close (1<h) to bedtime may result in poorer sleep[43,44].

These factors are individually difficult to control in a clinical study and together pose an even larger challenge. Hence, to investigate the biology and the exact mechanisms between specifically the difference in the exercise training timing, we decided to conduct these studies in APOE*3-Leiden.CETP mice, a humanized mouse model for MASLD. In **chapter 3**, young male mice fed MASLD-promoting high-fat high-cholesterol (HFHC) diet were exercise-trained at either ZT13, one hour after the start of their active period, or ZT22, two hours before the end of their active period, for 8 weeks. This study set up allowed us to investigate the very early stage of MASLD, with development of steatosis without fibrosis. In this model, we saw that while the exercise training did not have a clear effect on liver fat loss (as the steatosis was only developing), only early exercise training significantly increased hepatic inflammation and the influx of monocytes into the liver. That can be interpreted as an early alarm system of activating the immune system to protect the liver, though it is also possible that early exercise accelerates inflammation development in MASLD. Our study in female APOE*3-Leiden.CETP mice, an atherosclerosis model, also showed a trend for an increased inflammation in early exercising group[45]. This coincided with an increased atherosclerotic lesions in early exercise training group, while the opposite was observed in the late exercising group[45]. Additionally, to better investigate the inflammatory changes, in **chapter 2** we reviewed human studies on the impact of timing of exercise training on inflammation. We concluded that late exercise training leads to a higher release of exercise-mediated IL-6, which, after exercise, exerts anti-inflammatory influence on the body. Together, these findings suggest that late exercise training may be more beneficial for inflammation prevention than early exercise training.

To investigate the role of circadian exercise training further, in **chapter 4** we extended the timed exercise experiment conducted in **chapter 3**, using older male APOE*3-Leiden.CETP mice, up to twelve weeks instead of eight. Mice in this experiment developed advanced hepatic steatosis with some fibrosis. Strikingly, in this experiment only mice that were exercise-trained in the late active period had a

significant reduction in body weight gain and liver steatosis. This is the first proof in animal models that timing of exercise training has a differential impact on MASLD amelioration. This protective effect of specifically late exercise coincided with an altered gut microbiome composition, i.e., an increase in the SCFA-producing genera and specifically in *Akkermansia muciniphila*. SCFA production is generally beneficial for the amelioration of cardiometabolic health and MASLD[46-49], but combined with exercise training, it may also increase energy expenditure during the exercise[50]. We then further demonstrated that fecal microbiota transplantation (FMT) from late exercising mice to sedentary mice partially replicated the effects on MASLD amelioration, demonstrating a causal role of gut microbiota remodelling in amelioration of MASLD by late exercise.

A question that remains unanswered is how exactly does late exercise training initiate a shift in gut bacteria. It has been shown that high fat diet and obesity result in disruption of central and peripheral circadian functions[51]. Specifically, the loss of circadian rhythmicity in the gut leads to microbial dysregulation and obesity[52]. Consequently, adding exercise training as an additional *Zeitgeber* may allow for some restoration of the circadian rhythm. One mechanism via which this may happen is lactate production in the muscles during exercise training[53]. Later in the active period, muscle oxidative capacity is increased[54], possibly causing higher lactate production. As in the gut certain bacteria may utilize lactate for production of SCFA[53,55], that may explain the observed SCFA increase. To test this, in an unpublished experiment, we have repeated the set up of the study in **chapter 4**, but with eight weeks of training, and then intravenously injected the mice with radiolabelled lactate, 24 hours after the last training bout. In line with expectations, the amount of lactate in the plasma of mice that exercised late was decreased, while the amount of SCFA in portal vein blood was increased. Lactate and SCFA concentrations were unchanged in other groups. This experiment indicates that exercising late mice have both a possibly higher leakage of lactate into the gut and a higher capability to convert lactate into SCFA. Some lactate-utilizing bacteria are known to oscillate and increase in their abundance in the late active period[56,57]. Together, this positive reinforcement cycle of higher lactate production and its utilization into SCFA may explain why late exercise training is more effective than early exercise training. This and future studies, however, still need to describe whether it is higher production, or higher leakage of lactate into the gut (or both) that lead to higher SCFA production. Overall, when applying these findings in clinical

conditions, timing can be used as medicine, by utilizing the natural daily fluctuations for higher exercise training benefits. While more research is definitely needed, overall our findings in mice suggest that late active period exercise training may be the preferable time window.

While here we showed a clear effect of exercise training timing on MASLD amelioration in mice, as mentioned earlier, clinical studies are more complex due to other lifestyle factors. These factors may also be utilized to improve the effect of the exercise training further. Specifically, diet and meal timing, which I will discuss in more detail in the next sections, may be adjusted to increase the abundance of SCFA producing bacteria within the gut and also better restore gut and gut microbiome function and rhythmicity.

Gut barrier, exercise training intensity and (cardiometabolic) health

Another important factor that should be considered when designing an effective exercise training intervention is the intensity and frequency of the intervention, related to both beneficial and adverse effects. Organisms require time to recover from exercise, and rest is crucial for injury prevention[58]. While sports medicine has guidelines for general injury prevention, gut health and the integrity of the gut epithelial lining should also be taken into account.

Gut epithelial health is crucial for a healthy gut and body[59]. If its integrity is disturbed, the gut becomes “leaky”, with poorer control over which molecules enter the circulation[60]. Specifically, lipopolysaccharides (LPS), which are already increased in blood plasma of individuals with obesity or MASLD, may more easily pass the epithelial lining, possibly resulting in increased inflammation[60]. The main fuel for colonocytes is butyrate, one of the SCFA produced by gut microbiota[61], and a healthy microbiome that produces sufficient butyrate ensures a healthy epithelial lining[62]. However, depending on its intensity and/or duration, exercise training can induce hypoxia and local ischemia, which leads to higher production of reactive oxygen species (ROS), higher temperature in the gut and lower blood supply towards the gut[63-65]. Since all of these may damage the gut epithelial lining, it is crucial to leave enough time for recovery[63,64]. To better understand the interaction between exercise intensity, frequency and recovery, in **chapter 5** we investigated the effects of frequent moderate exercise training and less frequent vigorous exercise training on the gut microbiome and gut permeability of male C57BL/6J mice. Five days a week of moderate exercise training resulted in damage to

epithelial lining, as confirmed by histological stainings, while vigorous exercise training every second day resulted in less damage. Curiously, the relative abundance of SCFA producers was increased more in vigorous exercise training. As vigorous exercise training leads to a higher production of lactate, it is plausible that with more intense exercise training, more lactate passes via the more temporarily damaged epithelial lining, resulting in higher production of butyrate to accelerate recovery. The addition of a rest day after the exercise bout also allows for longer time to repair the lining. If these findings can be translated to humans, current interventions that usually involve only three days of exercise training a week, with a day of rest in between, may be adequate for protecting the gut epithelial lining.

Taken together, our animal experiments show for the first time that exercise training optimization can play a marked role in improving body weight, body fat and liver steatosis in MASLD. Specifically, we showed that late exercise training is more beneficial than early exercise training due to the impact on the gut microbiome and metabolome. Similarly, exercise intensity and frequency should be investigated for healthier systemic and intestinal outcomes. However, while our animal studies clearly show the impact of late exercise training on MASLD amelioration and also partially elucidate the mechanism via which the additional benefits on the weight loss are exerted, clinical studies are obviously still needed to confirm the beneficial effects of late exercise and underling mechanisms in humans. With such studies, guidelines for patients to facilitate successful lifestyle interventions may be improved. Additionally, while here we attempt optimizing exercise training interventions, it has to be noted that generally any exercise training is better than no exercise training, and this is something that should also be emphasized to those who want to lose weight and/or improve metabolic health.

Optimization of dietary regiments modes and cardiometabolic health

Besides exercise, dietary intervention is another popular lifestyle intervention for the management of obesity, T2D and/or MASLD. However, “dietary intervention” is an umbrella term that includes many different approaches to changes dietary habits. Ultimately, the main aim of such interventions is calorie restriction, though ways to achieve that differ widely. The most straightforward way is calorie counting, determination of one’s approximate calories consumed and burned throughout the day[66]. While the method sounds simple and is helpful for some, it is not very

reliable, as precise calorie counting is difficult[67]. Additionally, it is not a convenient method to sustain long-term, and it may also contribute to the development of eating disorders[68]. Consequently, various fasting modes, such as intermittent fasting[69] or 5:2 fasting, gained popularity[70]. These methods allow for somewhat prolonged fasting, which leads to activation of ketogenesis, the use of lipids as a primary energy source. However, while it was theorized that these methods may yield additional benefits for weight loss, via ketogenesis and metabolic remodelling, compared to simple calorie restriction, recent studies showed that the weight loss resulting from these alternative fasting modes is only due to the calorie restriction itself[71]. That, however, is not a negative thing, as these methods make calorie restriction more sustainable than simple calorie counting, and all of these methods lead to a loss of 6-8kg after a year-long intervention[71]. Recently fasting-mimicking diets (FMD), which restrict calorie intake to 600-700 kcal per day for 5 days a month, emerged as a new promising dietary intervention[72]. Fasting-mimicking diets activate prolonged fasting state, which may have more beneficial effects, such as prolonged longevity and improved mitochondrial health in mice, and improvement of insulin sensitivity in humans, in addition to sustained weight loss of 3kg already after a 3 months long intervention[73-76]. Together, these calorie restriction methods are an effective tool for weight loss if they are manageable to sustain for a participant, though, importantly, they are interventions and should be followed up by persistent lifestyle changes.

Hence, a different approach to dietary interventions is improving the quality of consumed food, and not the quantity, although improving food quality can also be used in combination with calorie-restriction methods. Once again, a number of different diets exist, such as Mediterranean diet[77] or diets that include/exclude certain foods[78]. The aim of these diets is to achieve healthier food consumption and also improve satiety/reduce appetite. This way, the achieved calorie restriction is more long-term and sustainable, plus consumption of diets high in vegetables and low in processed foods and red meat also leads to an improved gut and gut microbiota health[79]. These effects are mostly achieved via the increase in the fiber intake in the diet and, as a result fiber fermentation by gut bacteria into SCFA[49]. Increase of fiber by itself leads to better gut motility and faster passage of food, potentially also resulting in less calorie consumption[80]. Additionally, production of butyrate leads to a healthier epithelial lining, next to a decrease in appetite and activation of browning of white adipose tissue, resulting in higher energy

expenditure[46,47,81]. Other SCFA, such as acetate and propionate, also contribute to lower inflammation and higher muscle oxidative capacity[48,50]. Together, this makes increased fiber intake one of the most potent treatments for weight loss, though questions still remain on how much and which fiber should be consumed.

Based on the notion of fiber having beneficial effects, and late exercise training increasing bacteria that can ferment fiber, the question arises whether combining late exercise with high dietary fiber would lead to synergistic beneficial metabolic effects. We have tested this combined intervention in APOE*3-Leiden.CETP mice in **chapter 6**. The results of this chapter were probably the most surprising out of the whole thesis. As expected, both exercise training and high dietary fiber separately led to a decrease in fat mass gain and an improvement in glucose and triglyceride levels in plasma. The combination was extra effective, with body fat mass even actually decreasing compared to the start of the experiment. However, no additional positive effects of the combination treatment were observed in the liver. Only exercise training, regardless of fiber intake, had a positive impact on liver lipids reduction. Additionally, only exercise training *without* fiber resulted in decreased inflammation in the liver. The explanation for why that was happening is, once again, in the gut. Fiber was the main determinant of the gut microbiome compositional changes. However, right after exercise bout, only exercise training groups had a higher abundance of SCFA in portal vein, and not the fiber group without exercise training. This finding indicates that exercise training is a major contributor to the production of SCFA in the body, with our unpublished findings suggesting the increase happening via lactate metabolism. Consequently, it may be important to have at least some exercise training when losing weight with help of fiber-rich diets. However, as mentioned earlier, here the addition of fiber and these extra produced SCFA, while beneficial for the whole body, had no effect on the liver steatosis. This is potentially due to the fact that while SCFA are generally beneficial for metabolic health, acetate, which constituted more than 90% of all SCFA in portal vein and had the highest relative increase after treatment, may also be harmful for for the liver in MASLD context. Excess acetate may be easily converted into acetyl-CoA and integrated into the *de novo* lipogenesis[82,83]. Admittedly, as we have only measured SCFAs in the portal vein, butyrate concentrations and activity in the gut could be different in our set up. Yet, in regards to liver health, it seems to be a matter of balancing the amount of fiber intake and the types of fiber that is most beneficial. Developing fiber supplements, dietary guidelines or probiotics (foods promoting

healthy bacteria growth) that would result in the increase of butyrate, and not acetate, can then be a beneficial way forward.

Another approach to lifestyle interventions is to combine dietary interventions targeting calorie restriction with a higher fiber intake, which we investigated in a clinical study in **chapter 7**. In that study, individuals with T2D followed a FMD protocol 5 days a month for a year. The applied FMD exceeded fiber intake according to Dutch guidelines, and that is probably why we observed an increase in the abundance of SCFA producers, especially *Akkermansia muciniphilla* and *Anaerostipes hadrus*, even after one fasting bout. Notably, this increase did not happen in other fasting studies, containing less fiber[84]. Specifically *Akkermansia muciniphila* has been demonstrated to play a role in the improvement of insulin sensitivity in patients with T2D, signifying the importance of fiber in the diet[47,85,86]. One of the possible dangers of prolonged, more intense fasting diets is remodelling of the gut microbiome with a decrease in bacteria that rely on continuous food intake, and an increase of mucin-digesting bacteria. They may use mucin as an energy source and disturb the mucin layer, while other bacteria die out due to the lack of available energy sources. However, in our study the ratio of mucin to dietary fiber digesting enzymes did not significantly increase after one fasting bout, unlike in other studies that investigated fasting impact on the gut microbiome[87]. This suggests that the presence of foods rich in fiber preserved bacterial energy sources. In addition to changes induced by one fasting bout, we also observed long-lasting changes in fecal microbiome after the whole year of intervention, even after a 3 weeks wash-out. Specifically, in addition to change in overall microbiome remodelling in patients on FMD, we saw an increase in fecal genes of the butyrate-producing pathway and a decrease in genes of the lipid degradation pathway. While we did not measure fecal SCFA directly in this study, this provides additional evidence that lifestyle interventions that target gut microbiome and increase SCFA producers result in positive cardiometabolic outcomes.

Overall, while there is a lot of evidence that any way of calorie restriction is an effective strategy for improving cardiometabolic health, we need to keep in mind the possibility of the weight regain after the end of the intervention. Hence, we need to prioritize interventions that lead to long lasting habitual and gut microbiome changes, as healthy eating habits and healthy gut microbiome serve as an additional protection against weight regain. Fiber supplementation or long-

lasting FMD seem to be such interventions that may induce long term metabolic benefits.

The possible pitfalls of microbiome research and how to avoid them

In this thesis, we show that the lifestyle interventions, be it manipulations of exercise training or different diet modes, have an impact on the remodelling of the gut microbiome. We also show that changes in the gut microbiome induced by exercise and dietary intervention correlate with more positive cardiometabolic health outcomes. However, only in one study, in **chapter 4**, we have proven that the microbiome has a causal role in the amelioration of MASLD with fecal microbiota transplantation, while the other findings obtained are still correlational. Additionally, even with the FMT study, while we do show a causal impact of fecal content, we still do not completely elucidate the mechanism via which this positive impact is conducted, and do not identify bacteria, or even other microbes, that have a causal positive impact on metabolic health. That is because microbiome research has many pitfalls that plague a number of recently published articles, which we have tried to avoid in this thesis to achieve more valid, even if less strong, conclusions. One big issue is that gut microbiomes between mice and humans are quite different from each other, and we cannot expect to find same bacteria changes in both, as certain species simply do not inhabit guts of both species[88]. This makes our findings of *Akkermansia muciniphila* and *Anaerostipes hadrus* being increased with fiber supplementation in both our mouse and human studies even stronger. Another potential issue is the methods used. Many of the studies, also in this thesis, rely on cheaper 16S sequencing, which results in low-resolution data and relatively poor characterization of observed changes, especially in mice, whose gut microbiome is less characterized[88]. An increasing number of studies now utilize metagenomics sequencing, as we have done in **chapters 4** and **7**. Compared to 16S sequencing, this provides better resolution and allows insights into the functional changes and better understanding of produced metabolites, but is more complex and expensive. In the same fashion, the actual methods for analysis changed a lot even in the 4 years that I have been doing my PhD, and it is extremely important to stay up to date. Re-analysing data from 4 years ago may lead to different outcomes, as quality control, the size and amount of databases and available statistical tools have progressed a lot, which may result in a differential annotation of sequences. Finally,

with some exceptions we, as a scientific community, need to move away from looking for one 'miracle' bacterium that is changed with an intervention and has a positive outcome. There are very few bacteria that can do that, and even then their impact is probably only marginal[86]. Instead, we need to move towards a better understanding of how treatments or interventions affect the general community structures and produced metabolites, to maximize the growth of beneficial bacteria and the production of SCFA and other beneficial metabolites.

However, even with these considerations taken into account, the question of causality of gut microbiota changes still remains. It is difficult to differentiate between a lifestyle intervention having an impact on both the gut microbiota and the body, a lifestyle intervention having an impact on the body via the gut microbiota, and also the body having an effect on the gut microbiota and leading to changes there. Germ-free mice, which are often used in the studies to show that elimination of the microbiome plays a role in certain processes, while useful, are not always the best model for cardiometabolic diseases. These diseases are inflammatory in nature, while germ-free mice have an entirely different phenotype compared to physiologically normal mice, especially in relation to inflammation and weight gain[89]. Hence, FMT studies, both mouse to mouse, like in **chapter 5**, human to mouse, and in some rare cases human to human, would probably be the future of causal microbial research.

Overall, future studies investigating the impact of lifestyle interventions on cardiometabolic health via modulation of the gut microbiome should preferably be done in humans, with metagenomics sequencing to investigate the observed changes, and be verified across different cohorts, as geographical location[90], medication[91] and sex[92] may play large roles in observed outcomes, while additional causal verification has to be conducted with FMT studies in either mice or humans.

New emerging pharmacological options and their interaction with lifestyle interventions

While lifestyle interventions are generally an effective way to lose weight, even though they have downsides, the recent emergence of pharmaceutical options, such as Semaglutide[4], Tirzepatide[5], and a number of other emerging drugs including Retatutride, will soon change our approach towards obesity. The most recent drugs, such as Tirzepatide, are already nearly as effective as bariatric surgery[5,93], which has

been a gold standard for complex obesity cases. However, a more intriguing question is how can lifestyle interventions be used *together* with the drugs to improve weight loss further or assist in other beneficial outcomes.

Firstly, one downside of Semaglutide and Tirzepatide is that they seem to plateau after a certain point and, while 15-25% of weight loss induced by Tirzepatide is already extremely promising, in certain cases additional weight loss is required[4,5]. Surprisingly, a recent study in mice showed that a brief seven days caloric restriction right before the start of the Semaglutide and Tirzepatide treatment resulted in an additional weight loss compared to groups that started the treatment without a prior calorie restriction[94]. While it is less studied in humans, one study showed that an introduction of a 12 weeks long lifestyle intervention, which resulted in a 5% weight loss, prior to the Tirzepatide treatment, resulted in a higher cumulative weight loss, compared to other Tirzepatide studies[95]. However, as this study did not have a control group with Tirzepatide but no lifestyle intervention, more studies on a combination of lifestyle interventions and these drugs are warranted.

(Late) exercise training can be another lifestyle intervention that can be used together with these drugs. One of the largest concerns about the side effects of the weight loss drugs is eventual muscle mass loss[96]. While overall muscle mass loss is natural, a particular concern is the loss of the myocardium, heart muscles. Specifically endurance exercise training can then prevent that muscle loss, together with an improved cardiometabolic fitness.

Another issue that exists for these drugs, as they are intervention treatments, and not life-long treatments, is weight regain[15]. That is an issue that of course exists for all interventions, but as the use of the drugs does not necessarily require any lifestyle adjustments, it makes it easier to return to previous habits and regain the lost weight quicker. As discussed throughout this thesis, gut microbiome changes towards a healthier, SCFA-producing profile may be crucial for the prevention of weight regain and general microbiome stability. Mouse studies show that these drugs actually lead to microbiome changes very similar to the ones we discuss in this thesis, such as an increase in *Akkermansia muciniphila*[97,98] and *Muribaculaceae*[99]. However, the for only available for now study on the impact of semaglutide on gut microbiome in humans did not register any particular taxonomic changes[100]. Promoting these changes further, with interventions, fiber

supplementation or other probiotics may be an additional boost to prevent weight regain and ensure the long-term success of combined treatment.

To sum up, while new pharmaceutical solutions open an opportunity for exciting and successful treatment of obesity and obesity-related diseases, combining them with the optimized lifestyle modifications, such as the ones we investigated, with promotion of endurance late exercise training, fiber supplementation and possible fasting, may lead to even better benefits and circumvent the remaining disadvantages of these emerging pharmaceutical options.

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