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

Kovynev, A.S.

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Chapter 1

General introduction and thesis outline

General introduction

While the world has successfully managed to tackle the COVID-19 pandemic, it is facing another dangerous, widespread, and costly healthcare challenge, the pandemic of obesity[1]. According to the World Health Organization, one in in eight people were obese in 2022, and 43% of all adults were overweight; an increasing trend from previous years[2]. Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD)[3], is a condition that is characterized by an accumulation of lipids in the liver[4]. This disease, affecting more than 30% of the population, may slowly progress into liver fibrosis and later cirrhosis[5]. Yet, there is only one conditionally-approved pharmaceutical treatment option[6], with the first-line treatment being lifestyle interventions aimed at weight loss. A loss of 10% of body weight results in clinical improvement of steatosis[7].

Achieving weight loss is not easy. Any social media feed is full of a plethora of videos and articles with contradictory weight loss advice: “aerobic exercise is best”, “never exercise in the evening”, “only do resistance training”, “exercise does not matter, it is only what you eat” and many more. Asking a clinician for advice is also challenging, as the current clinical guidelines are broad and recommend increased fiber intake and regular exercise training[8]. However it is not known, for instance, what timing or type of exercise training is best, which fiber sources to use or what is the best way of integrating fiber in the diet[8]. Fine-tuning this advice is crucial for the future of combating obesity and MASLD.

Even in the scenario where a patient manages to lose weight and thereby manages to successfully resolve hepatic steatosis, more challenges likely follow. Unfortunately, within a year, an average patient would regain 50% of the lost weight back, with some cases resulting in a higher weight regain than the actual weight loss[9,10]. The reasons for this are complex and multifaceted. The problem of lifestyle interventions and anti-obesity drug treatment is that they are considered exactly that, an intervention or treatment that would eventually stop[9,10]. However, if one does not change their lifestyle permanently by adapting food and exercise habits, relapse is likely. Hence, it is imperative to develop interventions that are sustainable in the long term. While body fat loss may be achieved rather quickly, it takes a much longer time for certain biological processes to reach homeostasis and adapt to a new, leaner norm. Some of the processes, like the epigenetic memory of the adipose tissue, the number of adipocytes in white adipose tissue or leptin dysregulation[11-13], are outside of the scope of this thesis.

An important factor that plays a role in the pathogenesis of metabolic disorders is the gut microbiota[14,15]. Diet is one of the crucial factors shaping the gut microbiota, and unhealthy diet leads to unfavorable microbiota changes and increased inflammation[16]. This, in turn, results in more harmful metabolites being produced and absorbed by the gut (see next section for details), entering the liver via the portal vein. This may result in chronic inflammation and progression of the disease. Shockingly, transplanting the gut microbiota of an obese adult to healthy mice on a chow diet leads these mice to develop obesity, supporting the causal relationships between gut dysbiosis and obesity[17]. Hence, developing interventions that positively impact the gut microbiota are likely important for long-term metabolic health[18].

Further in this introduction, I will go more deeply into the outlined topics, defining MASLD and its pathogenesis, the gut-liver axis, and will also go over the existing research about the various lifestyle interventions, such as exercise training and diet modifications.

MASLD pathogenesis

The field of fatty liver disease research went through many changes of defining the disease and its diagnosis in the recent years. Three years ago, it was known as NAFLD. For a brief period, it was known as metabolic dysfunction-associated fatty liver disease (MAFLD), while now the commonly used name is MASLD. This rapid change in definitions underlines the progress that the field has achieved in the last decade to understand the disease's pathogenesis. In the last century, ethanol was considered to be the main contributor to liver dysfunction, so for an average hepatologist the only cause of the liver steatosis would be alcohol intake[19]. In 1986, the term NAFLD was introduced, to highlight that liver steatosis can happen without alcohol intake[20]. However, the name itself does not clarify what the underlying causes are, as it only described that the disease was not related to alcohol intake, but did not refer to the actual cause[3]. Similarly, in clinical practice it was diagnosed as steatosis that was not caused by alcohol or other identifiable causes, such as hepatitis. Correspondingly, the main theory of NAFLD development was a "two-hit hypothesis"[21], with the first hit being the development of hepatic steatosis, an accumulation of lipid droplets in hepatocytes leading to lipotoxicity[21]. The second hit was considered an inflammatory response to stressed hepatocytes, resulting in an influx of immune cells and progressing fibrosis[22]. To address the problems with naming conventions and diagnosis, the disease definition was briefly changed to MAFLD[23], highlighting the cause being a metabolic dysfunction. This shifted the disease determination to steatosis in the liver and a presence of any metabolic dysfunction, such as obesity, type 2 diabetes (T2D), or other evidence of metabolic dysregulation. The name was promptly changed once again to MASLD to clarify that it is specifically liver steatosis that is the underlying problem, and also to get rid of "fatty", a stigmatizing word[24]. Notably, the new guidelines do not exclude the use of alcohol when reaching the disease diagnosis, provided that alcohol consumption is not the underlying cause.

Now that we have defined the main features of the disease and its diagnostics, let us have a closer look at its development (Figure 1). The disease development begins with a lack of physical activity and a caloric intake that is approximately 10% higher, or more, than the recommended amount for an individual[3,25]. Current research hypothesizes that this leads to a higher lipid accumulation in white adipose tissue and, over time, deteriorating insulin sensitivity of that tissue. With a lack of insulin-mediated lipolysis inhibition in white adipose tissue, more free fatty acids are released in the blood stream, and are then taken up by hepatocytes within the liver[26]. Simultaneously, increased calorie intake results in increased *de novo* lipogenesis in the liver itself. Together, these lead to an increased accumulation of triglycerides in the hepatocytes. This accumulation, in turn, leads to excessive utilization of the lipids via β-oxidation, causing lipotoxicity[26,27]. Lipotoxicity is manifested in a number of ways. Toxic intermediate components of lipid metabolism, such as saturated free fatty acids (FFAs), lysophosphatidyl choline or ceramides accumulate in mitochondria, damaging them[27]. Meanwhile, excessive β-oxidation leads to an increased formation of reactive oxidative species (ROS), damaging mitochondria and other hepatocytes' organelles[28]. The mitochondrial damage results in mitochondrial dysfunction and, despite sufficient energy availability, impaired ATP production[26]. Accumulation of toxic lipid metabolites in cytoplasm and ROS from mitochondria lead to endoplasmic reticulum stress and incorrect protein folding, furthering the damage[26,28].

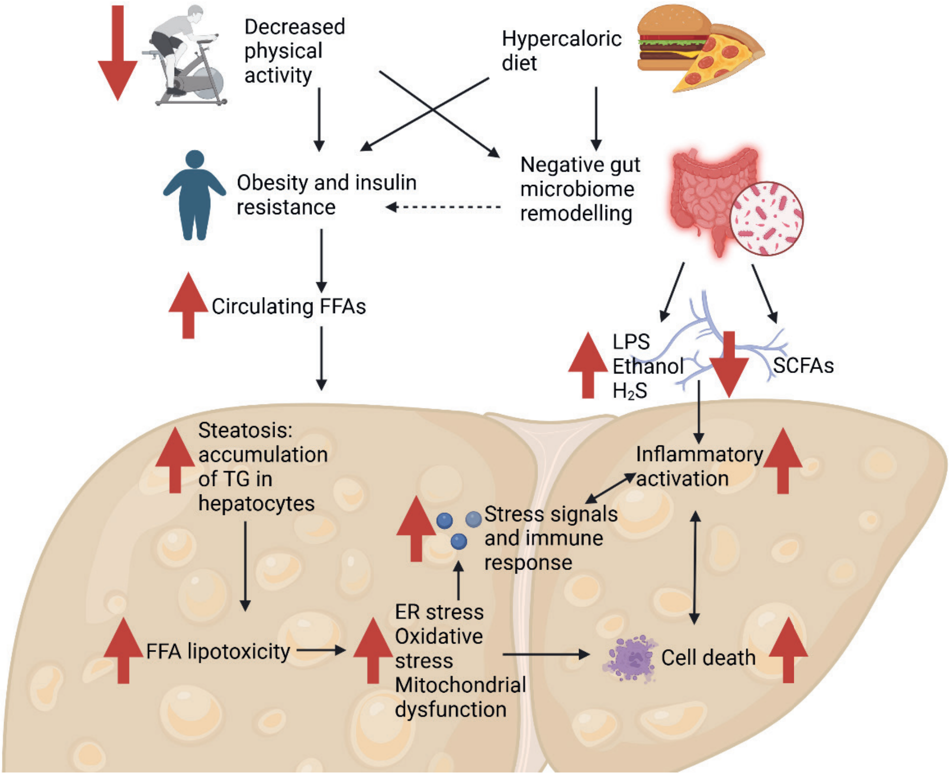

Figure 1. Mechanisms of MASLD development. Adapted from Li et al (2024)[26]. *Please see text for the explanation. Red arrows indicate possible negative impact on metabolic health, while black arrows show causality. Abbreviations: ER, endoplasmic reticulum; FFA, free fatty acids; LPS, lipopolysaccharides; SCFA, short-chain fatty acid; TG, triglyceride.*

These mechanisms lead to the hepatocytes’ stress and damage, causing them to release pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6. Consequently, Kupffer cells (i.e., resident liver macrophages) are activated, and more monocytes enter the liver from the blood stream[4,5]. This inflamed state leads to the activation of hepatic stellate cells and their transformation into myofibroblast-like cells, producing extracellular matrix components, such as collagen[29]. Together, hepatic inflammation and collagen production lead to the development of metabolic dysfunction-associated steatohepatitis (MASH) with pronounced fibrosis[4,5]. If not addressed, this fibrosis may later progress to cirrhosis, hepatocellular carcinoma, and, in rare cases, liver failure[4,5].

However, while lipid accumulation in hepatocytes is the main cause of liver damage, it is not the only one. Bile acid imbalance[30], lipopolysaccharides (LPS)[31], ethanol[32] and a lack of short-chain fatty acid (SCFA) influx[33,34] all contribute and are regulated by gut-liver axis, with an unhealthy gut having a large contribution to MASLD beyond altered calorie uptake.

Gut-liver axis and its role in MASLD development

Gut microbiota stability is instrumental for the maintenance of health. A healthy gut helps maintaining a strong gut epithelial lining, preventing pathogens and LPS from entering the circulation[14,18,35]. It also protects against pathogens themselves via colonization resistance and supplies the body with high levels of SCFAs[14,18,35]. The SCFAs are mainly acetate, propionate and butyrate, fermented from dietary fiber, which promote beneficial cardiometabolic health outcomes[14,18,35,36].

However, the recent western dietary trends, with increased portion size, calorie-dense snacks and processed foods, rich in (trans-)fats and low in fiber, may lead to a drastic remodeling of the gut microbial community[14]. While the gut microbiota composition of most healthy individuals can be classified into one of three enterotypes, patients with obesity/MASLD diffuse into an intermediate state of unfavorable gut microbial composition, with a lack of stable beneficial bacteria[37]. Correspondingly, this diseased state has recently emerged as one of the most crucial contributors to MASLD[14] (Figure 2). This state is characterized by low α-diversity (the amount of different bacterial species in a sample)[16], with less bacteria being able to produce required metabolites and protect against opportunistic pathogens. With ~70-80% of the immune system already allocated to the gut, this increases the burden on the immune system further[38]. Additionally, lack of fiber in the diet reduces the abundance of many bacterial producers of SCFAs, especially butyrate[14]. Since butyrate functions as the main energy source for enterocytes[39], reduction of butyrate in the gut worsens the function of the gut epithelial lining[36], resulting in the tight junctions becoming “leaky” and allowing the passing of more particles into the circulation[36]. A high-fat diet also increases the abundance of bacteria producing inflammatory LPS[40]. Consequently, with increased LPS production and leakage of the gut, more LPS ends up in portal vein and the liver, leading to an increase in inflammation via its recognition by the immune cells and subsequent immune system response[41]. Red processed meat is another potential contributor to a worsened gut health. Red meat is rich in sulfur-containing compounds, choline and carnitine. Upon reaching the gut, they are converted into H_2S, trimethylamine and trimethylamine N-oxide respectively. These compounds damage the epithelial lining and worsen inflammation further[42]. Change in bile acid composition is another gut-mediated factor that may contribute to MASLD. Bile acid metabolism is outside of the scope of this thesis, but the contribution of bile acids to the disease is excellently discussed by Arab et al (2017)[43] and expanded on by Lai et al (2023)[44].

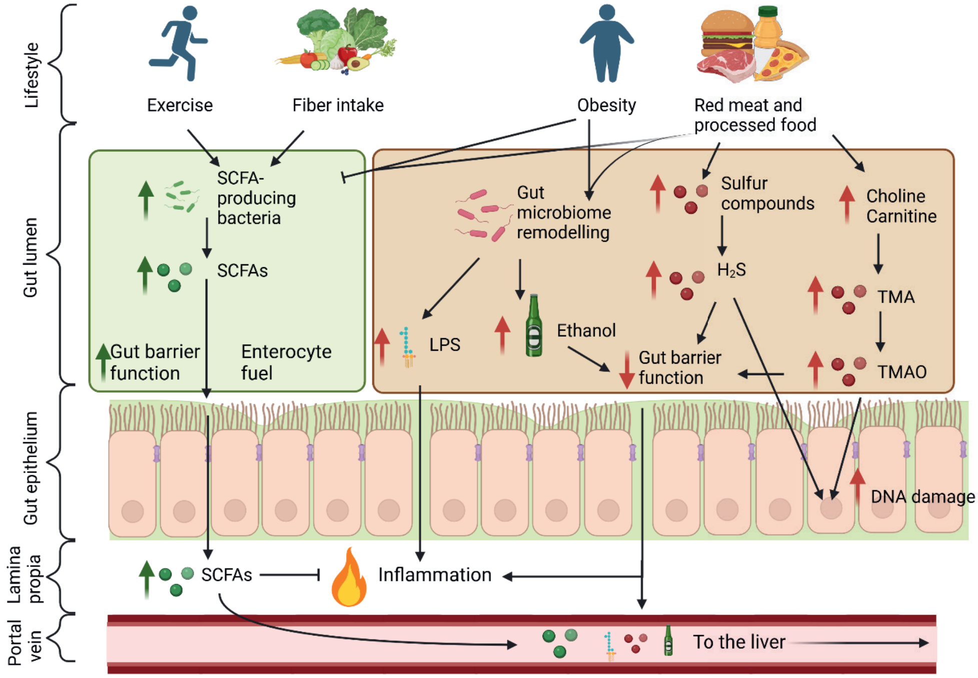

Figure 2. Influence of lifestyle on gut-produced metabolites. Adapted from Song et al (2020)[42]. Please see text for the explanation. Red arrows indicate possible negative impact on metabolic health, green arrows indicate possible positive effect, while black arrows show causality. Abbreviations LPS, lipopolysaccharides; SCFA, short chain fatty acid; TMA, trimethylamine; TMAO, trimethylamine N-oxide;

Surprisingly, another peculiar contributor to MASLD via the gut-liver axis, without alcohol intake, is ethanol. Humans may produce large amounts of ethanol in a rare condition, known as the auto-brewery syndrome, which even makes them intoxicated[45]. However, gut ethanol production was not explored beyond this condition until recently. Recent findings show that, while the original name of MASLD contained “non-alcoholic”, ethanol is produced in clinically relevant quantities in the gut of MASLD patients, and the elimination of a MASLD-associated microbiota with antibiotics also eliminates bacterial ethanol production[32,46].

Taken together, developing treatment strategies for MASLD that improve gut health in addition to steatosis resolution are crucial for successful long-term disease treatment.

Exercise training as MASLD treatment

Physical activity is considered one of the best preventive and curative treatments of cardiometabolic diseases[47]. While sedentary behavior is a massive risk factor, an increase in physical activity correlates with better clinical outcomes[48]. Physical activity refers to any non-sedentary behavior, while exercise training is a planned, more intense and repetitive activity.

Exercise training has long been recommended as an MASLD treatment[47]. Exercise training is an effective tool for weight loss[49,50] and, as mentioned above, a 10% body weight reduction leads to improved clinical symptoms, such as a reduction in hepatic steatosis and circulating alanine transaminase (ALT) levels, another marker of liver dysfunction. However, weight loss is not the only benefit of exercise training, as exercise reduces liver lipid levels even without an overall body weight change after a 12 weeks intervention[51]. Here, a distinction has to be made between different modes of exercise training. First, all programs that were successful in reducing body weight had at least 3 days of exercise training per week, and, second, there is a difference between aerobic exercise training and resistance training[52]. All aerobic protocols, but only 85% of resistance training protocols, were effective in reducing body weight. Notably, studies, which show both mode of exercise as equally beneficial, recommend resistance training as a preferable mode of exercise as it is more accessible to patients with poor cardiorespiratory system[53].

Both exercise training modes exert positive effects via a number of different pathways (Figure 3). They by themselves use energy that allows to burn extra fat. Prolonged exercise training also builds muscle mass and increases the basic metabolic rate, allowing for higher overall energy expenditure[53]. Skeletal muscle mass increases with training, which leads to an increased glucose uptake, over time bypassing insulin resistance[52]. To support this, most exercise training studies show an increase in peripheral, but not hepatic, insulin sensitivity, which still contributes to improved MASLD outcome[52]. Finally, endurance exercise training has been observed to increase the abundance of SCFA-producing gut bacteria, though the mechanisms are not well-studied[54].

Additionally, exercise training also has an effect on the immune system and inflammation. Specifically, after an exercise bout, skeletal muscles release IL-6[55,56]. IL-6 has both pro- and anti-inflammatory functions. When released, it binds to IL-6 receptor (IL-6R), which is present on membranes of only some cell types (hepatocytes, leukocytes, endothelial and epithelial cells)[55,56]. This complex can dimerize with gp130, introducing an anti-inflammatory cascade. However, IL-6R can also be cleaved from the membrane, and bind IL-6 in plasma, forming a pro-inflammatory soluble IL-6/IL-6R complex[55,56]. Notably, muscle-released IL-6 only induces the anti-inflammatory cascade via binding to the membrane bound IL-6R[55,56]. Additionally, exercise increases levels of soluble gp130, which inactivates soluble IL-6/IL-6R, further blocking pro-inflammatory activity of IL-6[57]. Consequently, the immune response is partially suppressed and resources are used for muscle maintenance instead. As most of the cardiometabolic disorders are inflammatory, prolonged exercise training may have positive anti-inflammatory effects[58].

Overall, exercise is a great life-style intervention for improving cardiometabolic health, addressing both weight loss and general cardiometabolic improvement.

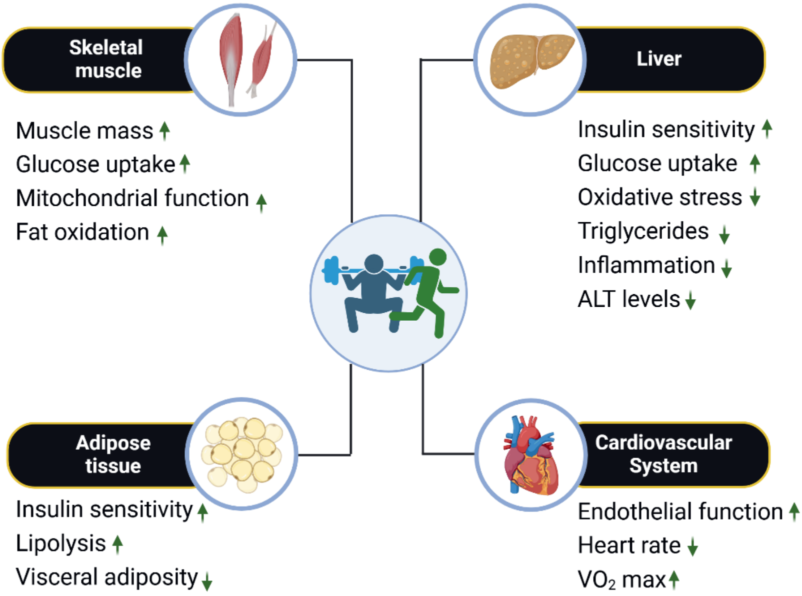

Figure 3. The effects of exercise on MASLD amelioration. Adapted from Razzak et al (2025)[59]. Please see text for the explanation. Green arrows indicate positive effects of the exercise on the organs. Abbreviation: ALT, alanine aminotransferase.

Circadian biology and exercise training

While it is not a surprise that exercise training is generally beneficial, many questions remain on what frequency, mode and timing of exercise training are optimal for fat loss.

Our body is controlled by circadian rhythms, approximately 24 hour-long cycles of hormonal, physical and behavioral changes. The main circadian regulator is the suprachiasmatic nucleus in the brain, which is in charge of daily body activity. Its activity is regulated by *Zeitgebers* (environmental stimuli), primarily via the sunlight[60]. Disruption of circadian activity is a known contributor to the development of obesity and metabolic disease[61], with nightshift workers having an increased risk to develop obesity and T2D[62,63].

Circadian activity of the body is even more complicated by the fact that tissues have their own, peripheral, circadian clocks[64], affected by more different *Zeitgebers*, such as food intake or exercise. These clocks ensure the stable activity of organs, such as the gut, muscle, or liver, by regulating which processes happen when. A diet rich in fat may disrupt these peripheral clocks, contributing to the disease development[65]. Hence, aligning daily processes, such as exercise training, to the optimal function of the clock has potential for additional exercise benefits[66]. If utilized right, a good choice of exercise training timing could both reap higher benefits from skeletal muscles or the immune system, and help the body re-synchronize peripheral clocks in metabolic diseases[67]. For building muscle through resistance training, most of the research agrees that exercise training during evening is best[68]. However, for aerobic exercise training there is some conflicting evidence. For instance, studies that measured the period of the highest physical activity arrived at opposing conclusions[69,70]. One study in patients with obesity and T2D reported that early exercise has actually been unfavorable for patients' glycemic levels, while late exercise exerted benefits[71]. Another study with T2D patients showed the opposite results, with late exercise training having no benefits while early exercise training decreased blood glucose levels[72]. A number of other studies also show opposing results, as excellently summarized by Martinez-Montoro et al (2023)[73], with slightly more evidence towards late exercise training being more beneficial. Notably, no studies investigating the effect of timing of exercise training in context of MASH have been described as yet.

Exercise and gut microbiota

In addition to the benefits that exercise training exerts on the body, it also has a direct effect on the gut microbiota. Professional athletes, especially runners, have a different microbiota composition compared to healthy individuals with a sedentary lifestyle[74]. Prolonged bouts of exercise, such as running a (ultra-) marathon, can have a significant impact on the gut microbiota composition[75]. Importantly, observed changes, such as an increase in the *Firmicutes/Bacteroidetes* ratio, or an increased abundance of SCFA producers, is not just a by-product of exercise training, but an important adaptation[76]. SCFA influx into muscles increases oxidative capacity of muscles and is beneficial for prolonged endurance exercise[77]. Specifically, SCFA producers in the gut have been described to be able to convert lactate, produced by the muscles, into acetate, butyrate and propionate, serving as fuel for both the gut and muscles[74,78].

The gut's response to exercise training also differs between lean and obese exercising individuals. Some studies report that leaner individuals have a bigger shift towards SCFA-producing bacterial taxa and also have higher fecal SCFA levels than obese individuals[79], though other studies demonstrate a shift towards SCFA producers despite a lack of overall changes in beta-diversity (i.e., a metric of similarities between samples)[80]. Exercise training may also increase the alpha-diversity in exercising obese patients, a general marker of a more stable and diverse microbiota[81], though only some forms of exercise training appear to induce this change[82]. On a taxonomic level, exercise training increases the abundance of *Akkermansia* genus[83], implicated in improved cardiometabolic outcomes[84].

However, exercise training does not only have a positive effect on the gut. Prolonged or vigorous exercise training exerts a tremendous stress on the body[85]. For instance, vigorous exercise training can cause heat shock and hypoxia (from reduced perfusion) in the gut, which may lead to damage of the gut epithelial lining[86,87] and to suppression of a number of beneficial bacteria[88]. Hence, production of SCFAs that support the integrity of the intestinal lining, is crucial to counteract these changes. Consequently, it is crucial to study the effects of exercise training on gut health and develop sustainable approaches that do not cause harm to the gut and benefit the body.

Finally, the gut and its microbiome, similarly to many other tissues and processes in the human body, are known to oscillate according to circadian rhythms[89]. Aligning the intestinal clock with other processes, for example, training when SCFA-producing bacteria are increased, can be yet another method to optimize exercise performance or exercise benefits.

Dietary fiber as lifestyle intervention treatment

While effective, exercise training also demands for patients to adhere to the intervention program. Another potentially more simple lifestyle change, is a change in diet. A plethora of different diets exist, though most of them are just different modes of calorie restriction that make it more manageable for patients to limit food intake. Nonetheless, a dietary modification that undoubtedly has a positive long-term impact on cardiometabolic health is the increase of fiber intake[90]. Even in healthy individuals, meeting the recommended levels of fiber intake reduces the chance of cardiometabolic events by 20-30%[90]. Fiber intake is also known to improve insulin sensitivity and accelerate fat loss in patients with T2D[91,92].

Fiber intake achieves these effects via a number of mechanisms. First, fiber generally improves digestion and food passage time[93]. Secondly, fiber serves as fuel for gut bacteria that can convert these complex carbohydrates via fermentation into SCFAs. SCFAs serve as energy for gut epithelial cells, reducing gut permeability[94,95], help to reduce appetite[96], reduce inflammation[36] and improve muscle oxidative capacity[97] (Figure 4). SCFAs act via G protein coupled receptors (GPRs), mainly GPR41, GPR43 and GPR109A. In plasma, SCFAs lead to a decrease in the levels of total cholesterol, LDL-cholesterol and fasting glucose[90].

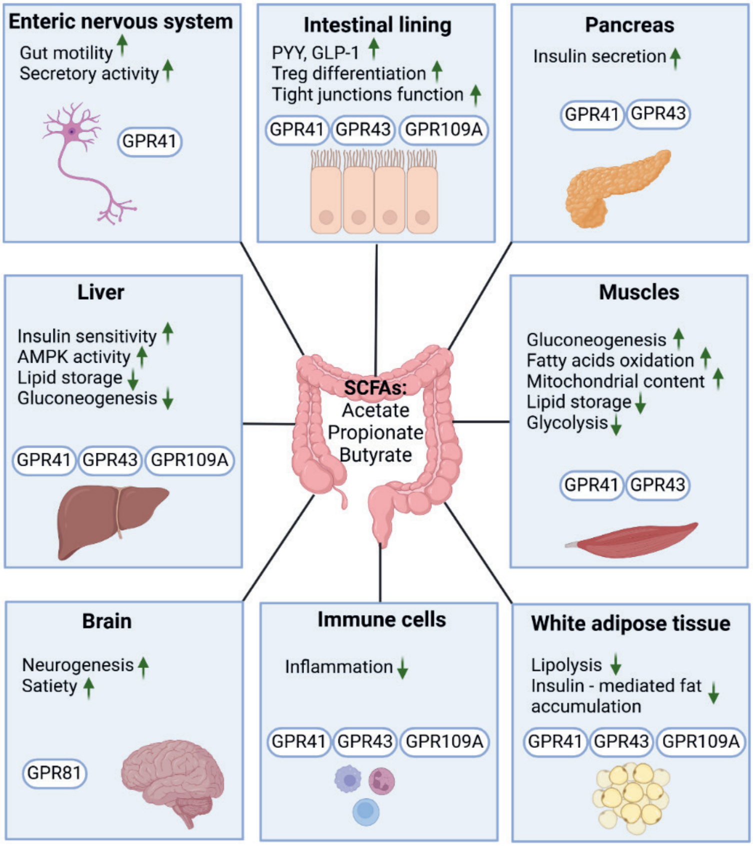

Figure 4. Effects of the most common short-chain fatty acids on cardiometabolic health. Adapted from Drevon (2021)[98]. Please see text for the explanation. Arrows indicate positive effect on the function of several cells and organs. Abbreviations: AMPK, AMP-activated protein kinase; GLP-1; glucagon-like peptide-1; GPR, G protein-coupled receptor; PYY, peptide YY; SCFA, short-chain fatty acid.

Incorporation of higher amounts of fiber into the diet also results in a remodeling of gut microbiota composition[14]. Specifically, fiber leads to an additional increase of SCFA producers potentially crucial for long-term positive metabolic outcomes[99].

While the use of fiber itself is known to be beneficial, the question arises of what would happen if fiber is combined with exercise training, which also increases the abundance of SCFA producers.

Fasting as an emerging alternative lifestyle modification

Finally, different modes of fasting, such as intermittent fasting, alternating day fasting or 5:2 diet (i.e., 2 consecutive fasting days a week) have recently became a popular diet mode that allows for easier management of calorie intake. However, while these fasting modes indeed allow to decrease calorie intake by up to ~30% and hence is a successful weight loss strategy, less is known about the impact of fasting on the gut microbiota. Specifically, the longer and harsher the fast (meaning very low calorie intake), the less energy the gut microbiota has access to.

This impact of fasting on the microbiota is only beginning to be studied. Intermittent fasting has been shown to increase microbial richness and in most studies also changes microbial composition[100]. However, prolonged extreme fasting (5+ days with more than 90% calorie reduction), despite positive metabolic effects, results in a reduction of bacterial diversity, a negative factor that may hurt colonization resistance and heighten the risk for developing infections[101], and in an increase in the mucus-degrading bacteria that may damage epithelial lining[102]. Recently, fasting-mimicking diets, containing a low amount of calories, emerged as a new fasting alternative[103,104]. Individuals on these diets fast for a week per month, mimicking the effects of the fasting, but avoiding the aforementioned negative side effects. This approach may also possibly maintain the desirable microbes in the gut. More research into these fasting diets is warranted to optimize positive effects of the fasting while protecting and improving the gut microbiota and maximize fasting benefits.

Thesis outline

As outlined in **chapter 1**, there is a clear need for further optimization of lifestyle interventions to target MASLD, obesity and related cardiometabolic complications, mainly aimed at maximizing the effect of the intervention benefits, in part by improving the gut microbiota composition. While exercise training is a popular and accessible intervention, there are still many unanswered questions about its influence on organs and their cross-talk in health and disease. Consequently, most studies described in this thesis are devoted to modifying exercise training interventions to improve their cardiometabolic and anti-inflammatory benefits.

In **chapter 2**, we reviewed the existing knowledge on the impact of circadian exercise training on the immune system and inflammation. We summarized the findings on different modes of morning and evening exercise training in healthy adults and discussed the implications of these findings for the use of the exercise training as a therapeutic tool.

Next, in **chapter 3,** we aimed to investigate the impact of early and late active period exercise training on inflammatory changes in the early stages of MASLD. To do that, we used young male APOE*3-Leiden.CETP mice with developing liver steatosis, by being fed a high-fat diet. These mice were subjected to moderate exercise training on a treadmill either one hour after the start of their active period, or one hour before the end of their active period.

To investigate the effects of timed exercise training further, in **chapter 4**, we conducted a similar experiment in older mice that stayed on the high-fat diet longer. That allowed us to investigate the effects of timed exercise training on advanced liver steatosis. Since we found that exercise training in the late active period modified the gut microbiota composition, we also conducted a fecal microbiota transplantation to investigate the causal effect of this late exercise-modulated gut microbiota on MASLD development.

Exercise clearly has a pronounced effect on the gut microbiota composition. In **chapter 5**, we investigated the effect of exercise on the gut further by studying the impact of moderate and vigorous exercise training on the gut state in male C57BL/6J mice. In addition to gut microbiota composition itself, we also aimed to investigate changes in the gut epithelial lining, which can be damaged with intense training.

In most of the aforementioned studies, exercise changed the SCFA production in the gut. As the main source of SCFA is the fermentation of fiber, in **chapter 6,** we aimed to elucidate whether the addition of inulin, a dietary fiber, aids in ameliorating MASLD further, alone or combined with late active period exercise. To study this, we once again used male APOE*3-Leiden.CETP mice with fully developed steatosis.

To further understand the impact of lifestyle interventions on gut microbiome in the context of metabolic health, in **chapter 7**, we investigated the impact of prolonged treatment with repeated fasting, and its longitudinal impact, on the gut microbiota composition and functionality in patients with T2D.

Finally, in **chapter 8**, we discuss the findings in context of the currently available literature, and propose a framework for the future studies, and **chapter 9** summarizes the findings of this thesis.

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