Targeting inter-organ cross-talk in cardiometabolic diseases

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General discussion and future perspectives

GENERAL DISCUSSION

Over the past few decades we have witnessed advances in understanding of how tissues communicate with one another in health and disease. Now, it is well-established that cardiometabolic diseases are far more than simple dysfunction within local tissues and organs. Rather, cardiometabolic health is regulated by highly controlled coordination between various metabolic organ systems. By generating signaling molecules, such as peptide/protein hormones, bioactive lipids and small molecules, these functional metabolic organ systems are able to work together to maintain cardiometabolic health. However, dysregulation of the inter-organ cross-talk by disturbing the production of these signaling molecules, for example, contributes to the development of obesity and its associated cardiometabolic diseases, such as non-alcoholic fatty liver disease (NAFLD) and atherosclerotic cardiovascular disease (asCVD).

In recent years, signaling molecules involved in the inter-organ communication have been proposed as potential targets for combating cardiometabolic diseases. These molecules are often easier to manipulate than other determinants of cardiometabolic diseases, such as genetic makeup and environmental factors. Therefore, approaches aiming to regulate local and/or systemic levels of these signaling molecules (e.g. organokines and metabolites) are currently being explored to alleviate cardiometabolic diseases. Despite some progress made in the treatment of these diseases, more can be done to tackle the risks of cardiometabolic diseases. In particular, cardiovascular diseases (CVDs) remain the leading cause of death worldwide, taking an estimated 18 million lives each year [1]. Therefore, there is still an unmet need for additional therapeutic targets and approaches that can effectively combat cardiometabolic diseases.

In this thesis, by using dietary and pharmacological interventions, the potential of targeting inter-organ cross-talk to combat cardiometabolic diseases has been further explored. Two main therapeutic targets have been investigated to treat cardiometabolic diseases, namely **1)** gut microbiota-centered inter-organ cross-talk and **2)** liver-centered inter-organ crosstalk. The promise and future of these therapeutic targets and potential approaches will be discussed in this chapter.

1. Drugging the gut microbiota to improve inter-organ crosstalk for combating cardiometabolic diseases

The vast community of micro-organisms that inhabit the gut, i.e. the gut microbiota, has influence far beyond food digestion. Recent studies uncovered a potential contribution of the gut microbiota to the development of certain cardiometabolic

diseases. The mechanisms by which the gut microbiota modulates etiopathogenesis of cardiometabolic diseases are just beginning to be dissected. The gut microbiota forms a bioreactor that is fueled by exogenous (e.g. dietary nutrients) and endogenous (e.g. glycoproteins of the intestinal mucus layer) compounds. Upon breaking down these molecules, the gut microbiota can generate various metabolites, such as short chain fatty acids (SCFAs) and trimethylamine (TMA). These fermentation processes may also cause increased intestinal permeability, thereby causing translocation of microbiota-associated molecules e.g. lipopolysaccharide (LPS), into the circulation of the host. Upon entering to the circulation, gut microbial metabolites and gut microbiota-associated bioactive molecules can signal to metabolic organs, like adipose tissue and the liver, of the host to affect the host cardiometabolic health. Therefore, intervention studies aiming to manipulate the gut microbiota to influence local and/or systemic levels of its associated signaling molecules are crucial next steps. The gut microbiota-centered inter-organ cross-talk has thus been regarded as a novel therapeutic target for cardiometabolic diseases. The section below describes the therapeutic potential of such approaches that have been investigated in this thesis.

1.1 Regulating the production of gut microbiota-associated signaling molecules by dietary interventions

The diet and gut microbiota interact in a mutualistic manner. On the one hand, dietary factors are among the most potent modulators of the gut microbiota. On the other hand, microorganisms in the gut in turn affect the utilization and storage of ingested dietary nutrients, with potentially profound impact on host health. To understand how the diet affects the gut microbiota to influence cardiometabolic health, and to search for suitable dietary regimens that can effectively alleviate cardiometabolic diseases, various dietary intervention strategies are being tested in preclinical and clinical research. The main strategies include manipulating specific dietary nutrient(s) and optimizing dietary patterns (e.g. Ketogenic, Mediterranean and Paleolithic diet). In this thesis, to uncover the regulatory role of dietary components in the gut microbiota-host cross-talk and the consequences of this cross-talk for cardiometabolic health, I chose to modify the content of specific dietary nutrient(s) (**Chapter 2** and **Chapter 3**). One limitation of this approach is that dietary nutrients are rarely consumed in isolation. Nevertheless, manipulation of specific dietary components is required to gain mechanistical insights into their roles of in the microbiota-host communication, and is necessary to develop effective dietary regimens that can limit cardiometabolic dysfunction, which have been elaborated on below.

Reducing dietary simple sugar and saturated fat intake. Diet rich in simple sugars and/or saturated fat has been shown to cause a rapid remodeling of the gut microbiota

and trigger the development of cardiometabolic diseases in both animals and humans [2-5]. One mechanism by which the gut microbiota impacts cardiometabolic health upon high consumption of these dietary components is the damage of the gut barrier [5, 6]. Preclinical studies show that high simple sugars and/or saturated fat consumption increases the abundance of mucus-degrading bacteria that compromise intestinal barrier integrity [7, 8]. As a result, LPS, a cell wall component of Gram-negative bacteria, is released into the circulation, binds to Toll-like 4 receptors that are expressed in various immune cells (e.g. macrophages) and tissues (e.g. hepatocytes and adipocytes), and elicits pro-inflammatory responses [8-10]. In line with this, clinical studies observed that circulating LPS levels correlate with the development of cardiometabolic diseases, such as obesity [11] and NAFLD [12]. To investigate whether limiting the consumption of dietary sugar and fat is able to reduce the incidence of cardiometabolic diseases, several dietary intervention strategies have been evaluated in experimental animal models and in the clinic. The ketogenic diet, for example, which is characterized by low carbohydrates (CHOs; ~5-10% of total caloric intake) and was originally developed as a treatment for epilepsy, has been optimized for dietary fat and protein quantity and quality, and adopted for body weight management. Animal studies have shown that a ketogenic diet protects against obesity, while contradictory findings have been reported for e.g. type 2 diabetes (T2D) and asCVD [13, 14]. Some human studies reported a negative impact of ketogenic diets on the gut microbiota and cardiometabolic health [15, 16]. However, these studies were performed in small populations, limiting generalization to larger cohorts. Recently, a meta-analysis of 5 clinical trials with 447 participants [17] and recent clinical study involving 311 women with obesity [18] indicated that a low-fat diet is a feasible alternative to a low-CHOs diet for inducing weight loss with beneficial cardiometabolic effects, which were associated with decreased abundance of Gram-negative bacteria in the gut [19]. Likewise, a Mediterranean diet with a small proportion of complex CHOs (i.e. dietary fiber) and a high proportion of monounsaturated fat elicited favorable microbiota profiles (e.g. lower ratios of *Firmicutes*: *Bacteroidetes*) and metabolite production (high fecal SCFAs) and exhibited cardiovascular benefits [20, 21].

These findings thus suggest that limiting simple sugars and/or saturated fat consumption holds great promise in combating cardiometabolic diseases. However, future studies are needed to find the most suitable dietary fat and/or sugar content that can safely maintain cardiometabolic health in the long-term. Also, evaluating the impact of different types and sources of fat and CHO on the gut microbiota and cardiometabolic diseases is required, which would be helpful in improving current existing dietary patterns and developing novel dietary patterns that can effectively protect against cardiometabolic diseases. Besides, as modified versions of ketogenic

and Mediterranean diet protocols are rapidly growing in popularity, it is important to examine their long-term impact and safety on the gut microbial community and cardiometabolic health.

Increasing dietary fiber intake. In contrast to unfavorable effects of dietary saturated fat and simple sugars, dietary fiber (i.e. complex CHO) has been shown to beneficially modulate the gut microbiota and consequently improve cardiometabolic diseases. Dietary fibers are indigestible by the human gut as human cells do not have the enzymatic capacity to break down complex CHOs. However, unique taxa of gut microbes utilize dietary fiber as an important energy source. Fermentation of dietary fibers by gut microbes results in the production of large amounts of SCFAs, which benefit the host by serving as both recovered energy from otherwise in accessible dietary fiber and potent regulatory molecules with broad physiological effects. SCFAs were first identified as a principal energy source for intestinal epithelial cells, and most of the gut microbiota-derived SCFAs, in particular butyrate, have been estimated to be consumed by these cells, resulting in maintained intestinal barrier function and protection from intestinal inflammation [22]. Recent evidence revealed that butyrate can exerts functions beyond the intestine in the brain and peripheral tissues. SCFAs signal via the vagal nerve and via several G protein-coupled receptors in the intestine to regulate whole-body metabolism [23, 24]. By using *APOE*3-Leiden.CETP* mice, a well-established mouse model for human-like cardiometabolic diseases, our group reported that dietary butyrate regulates the gut-brain axis to improve energy metabolism through decreasing food intake and increasing fat oxidation by activating thermogenic tissues. As a result, dietary butyrate potently prevented the development of high-fat diet (HFD)-induced obesity and its associated metabolic disorders, including dyslipidemia, insulin resistance and hepatic steatosis [25]. Consistently, plant-rich and vegan/vegetarian diets rich in fiber, have been shown to promote weight loss through modulating the gut microbiota [26]. However, it should be noted that despite the observed protective effects of dietary fiber and SCFAs on obesity-associated disorders, humans with obesity were shown to have high fecal and cecal levels of SCFAs, indicating that SCFAs may also contribute to increased energy harvest [27]. Therefore, the individual's status of energy homeostasis may determine whether the beneficial effects of SCFAs on metabolism outweigh the extra calories obtained.

In addition to the beneficial effects of dietary fiber and SCFAs on body weight control, recent studies showed that butyrate can reduce atherosclerosis in inflammation-driven atherosclerotic mouse models (i.e. *Apoe* $^{-/-}$ and *Ldlr*$^{-/-}$ mice) partially via improving the gut barrier function [28]. Butyrate can inhibit the overgrowth of Gram-negative bacteria in the gut, thereby alleviating intestinal barrier permeability and systemic

inflammation. As a result, butyrate can block macrophage infiltration into plaques and protect against atherosclerosis progression. Given that hypercholesteremia is another key driver of atherosclerosis development, in **Chapter 2,** we examined whether butyrate confers its antiatherogenic effects in human-like *APOE*3-Leiden.CETP* mice. We demonstrated that while butyrate beneficially modulates the gut microbiota composition and function, we found no influence of butyrate on atherosclerotic plaque size, severity and composition, including the macrophage-positive areas within the lesions. Our data indicate that increased SCFA levels *per se* may not be sufficient to protect against asCVD. However, future studies are still needed to evaluate the therapeutic effects of dietary fibers themselves on asCVD in humanized animal models (e.g. *APOE*3-Leiden.CETP* mice) and in humans.

Optimizing dietary protein quantity and quality. Dietary protein provides gut microorganisms with essential nitrogen and carbon. Amino acid catabolism generates various functional molecules, most of which can influence cardiometabolic health [19]. For example, amines, phenols and indoles can combine with nitric oxide to form genotoxic N-nitroso compounds that increases the risk of carcinogenesis in humans populations [29]. In contrast, indole-propionic acid, a gut microbiota-associated metabolite of tryptophan, can protect against asCVD by promoting reverse cholesterol transport [30, 31]. The source of dietary protein also regulates gut microbiota-dependent metabolic output. This is best exemplified by the production of trimethylamine N-oxide (TMAO) from L-carnitine and its derivative choline, which are abundant in animal protein such as red meat, eggs and fish [32-34]. Studies in mice (e.g. *Apoe*$^{-/-}$, *Ldlr*$^{-/-}$, diet-induced obese (DIO) and *ob*/*ob* mice), have linked high dietary L-carnitine and choline consumption to the pathogenesis of cardiometabolic diseases, as caused by the generation of TMAO through the communication between the gut microbiota and liver [34]. The gut microbiota can convert dietary choline into trimethylamine (TMA) that is delivered via the portal vein to the liver where hepatocytes rapidly oxidize TMA into TMAO. TMAO was reported to aggravate atherosclerosis via several pathways, such as promoting foam cell formation and activating proinflammatory responses [34, 35]. However, in **Chapter 2**, by using humanized *APOE*3-Leiden.CETP* mice, we surprisingly demonstrated that diet rich in choline beneficially modulates the gut microbiota without affecting atherosclerosis. In **Chapter 3**, we further reported that dietary choline activates brown adipose tissue (BAT) to alleviate adiposity. In line with our findings, human studies showed a profound body weight reduction upon high dietary choline intervention [36]. Moreover, we demonstrated that increased plasma TMAO levels did not associate with the development of atherosclerosis (**Chapter 2**). The fact that TMAO induces atherosclerosis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice but not in humanized *APOE*3-Leiden.CETP* mice, may suggest that TMAO lacks atherogenic

properties in humans. Similarly, many clinical trials did not find any association between plasma TMAO and CVD risk [37-39]. Multiple systematic reviews and cohort studies have shown that the high intake of eggs, rich in TMAO precursors, is not associated with asCVD risk and mortality [40, 41]. Notably, a very recent study reported that TMAO even promotes anti-tumor immunity in triple negative breast cancer. This study reported that TMAO inhibits tumor growth by activating $CD8^+$ T cell-mediated anti-tumor immunity [42]. Also, the TMAO precursor choline enhances response to immunotherapy in triple negative breast cancer [42]. It would be interesting to investigate the role of dietary choline in other aspects of cardiometabolic diseases, such as obesity-associated cancers in the future. The current results highlight that vast effects of amino acid metabolites that are generated by the gut microbiota on host physiology are now just beginning to emerge and represent an area ripe for future research.

Optimizing dietary micronutrients. Besides macronutrients, dietary micronutrients also modulate the gut microbiota and affect cardiometabolic health. Vitamin D, for example, was shown to mitigate obesity-associated metabolic dysfunction in part by beneficially shaping the gut microbiota composition [43]. Like vitamins, metals can dramatically alter the composition and function of the gut microbiota, with profound impact on the host's cardiometabolic health. A recent study reported that high dietary iron consumption promotes NAFLD development in the context of obesity. By performing fecal microbiota transplantation, this study demonstrated that iron-induced liver damage is mediated by the gut microbiota [44]. Moreover, the high-salt diet can induce hypertension, as caused by decreased abundance of *Lactobacillus* and increased proportion of proinflammatory T helper 17 cells [45]. Overall, the interactions observed thus far between micronutrients and the gut microbiota, as well as a myriad of other interactions that undoubtedly still await discovery, represent a promising avenue for future research. These findings also emphasize the critical role of determining micronutrient composition in gut microbiota-based dietary intervention studies and warrant the need for clinical research in people at high risk of inadequate or excessive micronutrient intake.

Collectively, currently available data have shown that modulation of the dietary content of macro- and micro-nutrients has profound impact on the gut microbiota-centered inter-organ cross-talk and holds great promise for combating cardiometabolic diseases. Herein, we have further added novel insights into the role of dietary fiber and protein interventions in regulating cardiometabolic health. We have shown that while dietary supplementation with butyrate can effectively promote weight loss, it may not be able to protect against asCVD (**Chapter 2**). Future studies are required to examine

the protective effects of dietary fiber itself on asCVD in various animal models and in humans. Besides, we demonstrated that dietary choline, which is highly present in animal proteins (e.g. red meat, eggs, fish and sea food) has favorable effects on the gut microbiome and body composition, without affecting atherosclerosis (**Chapters 2** and **3**). Furthermore, plasma TMAO levels did not correlate with atherosclerosis (**Chapter 2**). Given that alternative voices regarding the effect of choline/TMAO on health and disease are getting louder, future studies are needed to examine whether high intake of (specific) animal proteins is in fact detrimental or not. Taken together, the plasticity of the gut microbiota makes their dietary modification an attractive approach for cardiometabolic disease prevention and treatment. However, one main factor that complicates the implementation of dietary interventions is the heterogeneity of gut microbiota composition among individuals which is caused by e.g. their different genetic backgrounds. In this regard, a main challenge for the future will be to design optimal dietary intervention strategies for populations with similar gut microbiota profiles, or even a personalized nutrient regimen for each individual.

1.2 Modulating the production of gut microbiota-associated signaling molecules by pharmacological interventions

In addition to dietary interventions, treatment with antibiotics, prebiotics and probiotics, and more recently, bacterial enzyme inhibitors and bacteriophages, are currently tested as intervention strategies aiming to modulate the gut microbiota profile and function in several diseases. The section below describes the potential of such pharmacological approaches specifically in the treatment of cardiometabolic diseases.

Antibiotics, prebiotics and probiotics. Although several studies reported an association between the development of atherosclerosis and the presence of pathogens such as *Helicobacter pylori* and *Cytomegalovirus* [46-48], the use of antibiotics to selectively eliminate potentially harmful microbial species has not paid off so far. In both animal and human studies, antibiotic treatment has been associated with various metabolic disorders, such as obesity and peripheral insulin resistance [49]. Aside from this, frequent exposure of the gut microbiota to antibiotics can induce rapid genetic modification of bacteria [50], which can accelerate the development of antibiotic resistance. These findings indicate that antibiotics will probably not be a viable option for the treatment of cardiometabolic diseases.

With technological advances, holistic examination of gut microbiota responses to dietary nutrients has led to a recent expansion of the prebiotic concept, and the use of prebiotics has been proposed as a therapeutic option for cardiometabolic diseases.

Prebiotics are defined as selectively fermented ingredients that induce specific changes in the composition and/or function of the gut microbiota, thus conferring health benefits for the host. Favorable effects of prebiotics on cardiometabolic health have been observed in both preclinical and clinical studies [51]. However, manipulating the gut microbiota non-specifically by prebiotics can be dangerous. For example, a compositionally defined diet combined with soluble inulin can induce the development of gut microbiota-dependent hepatocellular carcinoma [52]. Thus, future studies to provide mechanistic insights into the relationship between a prebiotic and a downstream metabolic phenotype are very much needed. However, this task is very challenging, since the gut microbial community and the gut microbiota-host cross-talk are very complex. Natural modulations of the gut microbiota linked to e.g. diet, lifestyle and drug interactions, also hinder the development of prebiotic therapeutics. Another study reported that interindividual differences in the post meal glucose response are associated with individual dissimilarities in the gut microbial composition [53]. These studies indicate that a personalized strategy may be necessary and should be systematically addressed to maximally exploit the benefits of prebiotic supplements.

Meanwhile, probiotics (i.e. live microorganisms) have also been shown to exert health benefits on the host when administered in adequate amounts. For example, *Akkermansia muciniphila* (*A. muciniphila*), a SCFA producer, has been approved, in pasteurized form, as a food supplement and categorized as a novel food by the European Food Safety Authority [54]. Both live and pasteurized *A. muciniphila* were shown to improve insulin resistance and dyslipidemia both in mouse models [55] and in humans [56]. The beneficial effects of pasteurized *A. muciniphila* on cardiometabolic health was likely caused by a thermostable outer-membrane protein of *A. muciniphila* [57]. Similarly, administration of multi-strain probiotics, which contain 3 strains of *Lactobacillus*, and 3 strains of *Bifidobacterium*, was shown to reduce fasting plasma insulin levels in people with T2D [58]. Although probiotic interventions are clinically feasible and could be valuable in the treatment of cardiometabolic diseases [59], there is still no consensus on the intervention period, optimal dose and mechanistic pathways underlying the efficacy of probiotics to improve cardiometabolic diseases. It is important to point out that the current selection of probiotics is primarily in accordance to abundance-based analyses of gut microbial composition, where microorganisms whose proportions closely correlate with beneficial phenotypes are the main focus of interest. However, the keystone commensal, which organizes the gut microbial community or provides an crucial gain of function, can be a low-abundant component and is often not easily detected by current sequencing depths of analyses.

Bacterial enzyme inhibitors and bacteriophage therapy. Given the very recent insight that metabolites produced by gut bacteria may be harmful for cardiometabolic health of the host, one of the most recent lines of research focuses on developing small molecules capable of selectively targeting microbial enzymes involved in the production of those metabolites. However, data are very limited at the moment. Thus, due to high therapeutic potential, such intervention strategies represent an important avenue of future research. Bacteriophage therapy was initially described in the 1910's but has seen a recent renewal in interest with regard to the treatment of cardiometabolic diseases [60]. It was recently reported that phage therapy can be utilized to deplete susceptible gut bacteria and affect the metabolism of gut microbiota [60]. Bacteriophage therapy has been examined in a mouse model of liver diseases, showing that that bacteriophages can reduces the abundance of *Enterococcus faecalis* in the gut to improve liver damage [61]. However, using this therapy to improve cardiometabolic diseases has not been investigated outside of rodents and thus needs further investigation for its therapeutic efficacy and clinical safety.

Taken together, the gut microbiota-targeted pharmacotherapy requires a progressive and experimental pipeline approach. The first step towards developing novel therapeutics for cardiometabolic diseases would be understanding the global relationships between the gut microbiota and the host. Next, studies should focus on classifying cardiometabolic diseases based on disease phenotypes and gut microbiota profiles, so that different therapeutic strategies can be applied to different gut microbial communities. Investigating molecular mechanisms of microbiota-host interactions during pathological processes should be pursued, which would assist in developing the gut microbiota-oriented therapy. Meanwhile, bioanalytical profiling technologies, such as metagenomics and metabolomics, on human fecal samples, can be utilized to provide holistic and dynamic biochemical information, which can help researchers establish personalized gut microbiota-targeted therapeutic strategies (Gut microbiota-oriented therapies for cardiometabolic diseases are shown in **Figure 1**).

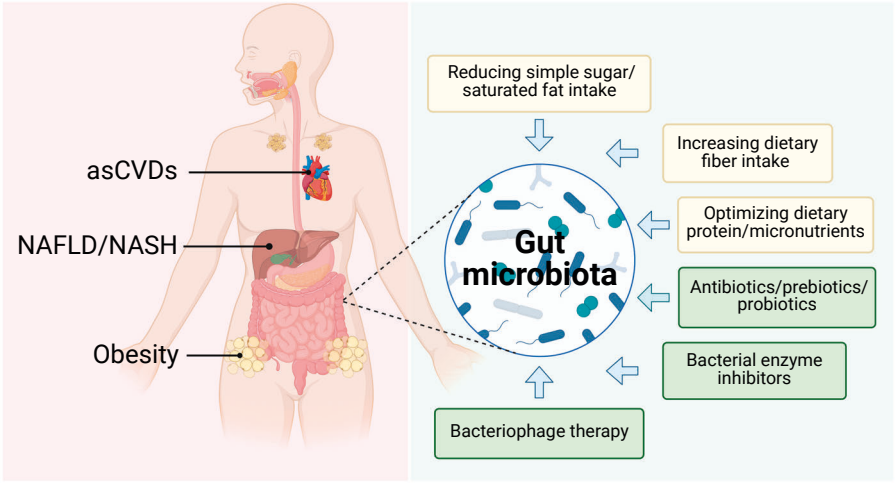

Figure 1. Drugging the gut microbiota to improve cardiometabolic diseases. The currently available gut-microbiota-oriented therapeutic strategies for cardiometabolic diseases, i.e. obesity, non- alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH) and atherosclerotic cardiovascular disease (asCVD), are mainly dietary and pharmacological interventions. Dietary interventions, such as optimizing dietary macro- and micro-nutrient quantity and quality, can beneficially modulate gut microbiota-centered inter-organ cross-talk to combat cardiometabolic diseases. Likewise, gut microbiota-oriented pharmacological interventions, such as the use of antibiotics, prebiotics and probiotics, and more recently, bacterial enzyme inhibitors and bacteriophage therapy, have also shown therapeutic potential in the treatment of cardiometabolic diseases.

2. Targeting the liver to improve inter-organ cross-talk for combating cardiometabolic diseases

Besides the gut, the liver is another key organ that can impact cardiometabolic health and disease. Through fine-tuning circulating levels of metabolic substrates (e.g. glucose and lipids) and through producing bioactive molecules (e.g. hepatokines), the liver can communicate with various metabolic organ systems to regulate whole-body metabolism. As such, liver-centered inter-organ cross-talk is an important target for combating cardiometabolic diseases. The section below describes the therapeutic potential of targeting this cross-talk that has been investigated in this thesis.

2.1. Improving liver metabolic function by dietary and pharmacological interventions

The liver is the hub of many metabolic pathways, including those of lipids and glucose. In health, the liver is a well-controlled machinery, and it can coordinate the whole-body metabolic flexibility that is characterized by the ability to dynamically

adapt to fluctuations in energy needs and supplies. However, when the disposal of metabolic substrates of e.g. fatty acids (FAs) within the liver is overwhelmed, lipids may accumulate in the liver, thereby impairing liver metabolic function and carving the path towards to the development of NAFLD. Given that liver steatosis, an early stage of NAFLD, is an early indicator of hepatic and systemic insulin resistance, it is not surprising that NAFLD is associated with increased risk of cardiometabolic diseases. Indeed, feeding C57BL/6J mice with a HFD for 8 weeks induces liver steatosis, and mice develop more severe liver steatosis accompanied with adiposity and insulin resistance by prolonging the HFD feeding for another 8 weeks (**Chapter 4**). Likewise, in most epidemiological studies, liver fat content correlates with high body mass index (BMI) and high incidence of CVDs [62]. Moreover, by feeding *APOE*3-Leiden.CETP* mice with a high fat and high cholesterol diet (HFCD) for 23 weeks, a humanized progressive NAFLD model could be established (**Chapter 6**). Of note, compared to the liver steatosis model (**Chapter 4**), this model exhibits more severe cardiometabolic complications, such as hyperlipidemia, hyperglycemia, insulin resistance and obesity (**Chapter 6**). This is in line with recent findings demonstrating that NAFLD severity correlates positively with the severity of other cardiometabolic diseases [63]. As such, the metabolic function of the liver has become an indicator of cardiometabolic heath, and a barometer that allows the identification of individuals who are insulin resistant, who are in a metabolic state of severe adipose tissue dysfunction, and who are at a high risk of CVD development [62]. To improve liver metabolic function and cardiometabolic health, two main liver-oriented strategies are being examined in both preclinical and clinical research (i.e. dietary interventions and hepatocyte mitochondria-targeted therapy), which will be elaborated on below.

Reducing hepatic metabolite substrate influx by dietary interventions. Dietary factors can affect the metabolic function of the liver in addition to modulation of the gut microbiota-centered inter-organ cross-talk (as described in the section 1.1). Dietary interventions for NAFLD rely on the central hypothesis that optimizing the quality and quantity of dietary nutrients can reduce the influx of exogenous metabolic substrates (e.g. dietary CHOs and lipids) and endogenous metabolic substrates (e.g. white adipose tissue [WAT]-derived FAs) into the liver. Currently, diets used for improving liver metabolic function are mainly those that contain small amounts of saturated fat, free sugars and/or refined CHOs [64]. High saturated fat consumption can increase saturated free FAs in the circulation, which generally results in elevated influx of these FAs into the liver. On the other hand, excess consumption of saturated fat can cause endotoxemia, which can result in WAT inflammation and lipolysis, thereby further elevating the supply of WAT-derived FAs to the liver. In the liver, excess saturated fatty acetyl coenzyme A (acyl-CoA) can be metabolized into diacylglycerol (DAG). DAGs

are precursors of TGs which can be stored in the liver in the form of lipid droplets. Of note, DAGs can also activate protein kinase Cε, resulting in impaired hepatic insulin signaling [65]. Besides, saturated fatty acyl-CoAs, particularly palmitoyl-CoA, can enter to the *de novo* ceramide synthetic pathway. Ceramides can impair hepatocytic mitochondrial function, and reduce hepatic and systemic insulin signaling, thereby promoting the development of NAFLD and its associated cardiometabolic diseases [66]. Thus, reducing saturated fat intake may hold great promise in alleviation of NAFLD-associated cardiometabolic diseases. Indeed, the Mediterranean diet that contains high ratios of monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) relative to saturated FAs, has been shown to reduce liver fat content and obesity in both preclinical and clinical studies [67]. Currently, the Mediterranean diet is also recommended for people with NAFLD by guidelines of the European Associations for the Study of both Obesity, Diabetes and the Liver (EASO, EASD and EASL, respectively) [68]. However, thus far limited data are available to support the use of a Mediterranean diet as a strategy for preventing and treating NAFLD. Thus, future studies are required to examine its therapeutic efficacy, preferably at various stages of NAFLD in both experimental animal models and ultimately in humans.

Reduction of dietary sugar and refined CHOs intake can reduce hepatic *de novo* lipogenesis (DNL), which is a key contributor to NAFLD. DNL is a biochemical process in which FAs are synthesized from acetyl coenzyme A (CoA) subunits which can be derived from various sources, mainly dietary CHOs. High consumption of dietary sugars can enhance DNL, which exclusively synthesized saturated FAs [69]. An intermediate in the DNL pathway, malonyl-CoA, can inhibit mitochondrial FA uptake, thereby limiting β-oxidation. Hence, reducing dietary CHOs and saturated fat may be an effective strategy for combating NAFLD and its associated cardiometabolic diseases. Consistently, a recent study has reported that short-term intervention with an isocaloric low-CHO diet induces a dramatic reduction of liver fat and other cardiometabolic risk factors paralleled by a markedly decreased DNL and largely increased mitochondrial β-oxidation [70]. These results highlight the potential of low-CHO interactions for treating NAFLD. Despite this, future studies are still needed to examine the influence of low-CHO on cardiometabolic health and disease in the long-term.

In summary, current guidelines recommend dietary modifications, in particular reducing dietary content of saturated fat, free sugars and/or refined CHOs, in the treatment of NAFLD and its associated cardiometabolic diseases. However, it remains unclear whether optimizing diet composition or following a particular dietary pattern provides greater benefit. The Mediterranean diet has received the most attention with the most promising results, and is recommended by the EASO, EASD and EASL for

people with NAFLD. Other dietary strategies, such as ketogenic, plant-based and high-protein diets, have all shown promise in beneficially affecting cardiometabolic diseases, including NAFLD. However, prospective, long-term and randomized clinical studies with liver histopathological endpoints are still required before recommendations should be developed regarding most of these dietary interventions in the specific NAFLD treatment.

Improving hepatocyte mitochondrial function by pharmacological interventions. As mentioned above, unhealthy diets lead to liver metabolic dysfunction, and dietary interventions are still the mainstay in the management of patients with NAFLD. Nevertheless, lifestyle interventions are insufficient to fully prevent/reverse NAFLD [71]. As such, pharmacological interventions that can effectively improve liver metabolic function and cardiometabolic diseases are urgently needed. Over the past decades, important advances have been made in the understanding of pathogenesis of liver damage, fibrogenesis, and carcinogenesis in relation to mitochondrial dysfunction of hepatocytes. Although liver-targeted, especially hepatocyte mitochondria-targeted medicine, is still largely in the developmental stage, we will discuss here the therapeutic potential of such a strategy in the alleviation of NAFLD and its associated cardiometabolic diseases.

The liver is composed of several cell types, among which hepatocytes, making up 70-85% of the liver mass [72]. Hepatocytes play a critical role in modulating CHO and lipid metabolism, and thus are most susceptible to cellular damage. Within these cells (i.e. hepatocytes), mitochondria are important organelles that are considered a metabolic hub for controlling hepatocyte function. Hepatocytic mitochondria orchestrate energy metabolism by substrate oxidation via a combination of β-oxidation of FAs and glycolysis of glucose, coupled to the tricarboxylic acid cycle (TCA), adenosine triphosphate (ATP) synthesis through oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) formation [72]. Aberrant alterations in these processes can contribute to liver injury [73], indicating that improving hepatocyte mitochondrial function is a potential strategy for the alleviation of NAFLD and its associated cardiometabolic diseases. Indeed, in **Chapter 4**, we demonstrated that in developing obesity, improved hepatocyte mitochondrial function was linked to decreased sphingolipid accumulation and reduced inflammation in the liver. It is well-studied that the early onset of obesity is characterized by aberrant lipid accumulation and impaired mitochondrial function in the liver. Under obesogenic conditions, FA disposal through β-oxidation and TG formation is overwhelmed in the liver, so that excess FAs can form lipotoxic species, such as sphingolipids, that dampen mitochondrial function and induce local and systemic inflammation. With disease progression, obesity-driven

NAFLD is associated with impaired mitochondrial ATP synthesis in hepatocytes [74-76]. Indeed, we observed that upregulation of hepatic expression of mitochondrially encoded ATP synthase membrane subunit 6 (*mt-ATP6*), a gene encoding ATP synthase, was associated with a profound reduction of hepatic steatosis and inflammation. During progressive NAFLD, hepatic inflammation leads to hepatic stellate cell (HSC) activation, and activated HSCs are characterized by the release of retinoids, which can induce production of excessive amounts of extracellular matrix proteins and consequently promotion of liver fibrogenesis [77, 78]. In **Chapter 4**, we further reported the protective effects of improved mitochondrial function against HSC activation, as related to increased mitochondrial ATP synthesis. In agreement, depriving HSCs from retinol reduces mitochondrial ATP synthesis, while energy output increased by restoring retinol [78, 79]. Consistently, previous studies did show that within the liver, the transcription level of the *mt-ATP6* gene correlates with retinol levels [80], indicating that improvement of mitochondrial function can alleviate NASH. Notably, improved mitochondrial function of hepatocytes also contributed to reduced body fat mass accompanied by improved adipose tissue function and insulin sensitivity (**Chapter 4**). Our findings further provide convincing evidence that hepatocyte mitochondria function closely links to cardiometabolic health, and mitochondria-directed therapy holds great promise in preventing and treating NAFLD and other cardiometabolic diseases such as T2D and obesity.

In line with our findings, several drugs that are still used for treating obesity and/or T2D in the clinic have shown profound impact on hepatocyte mitochondrial function, and therefore have been proposed as potential therapeutics for NAFLD, such as metformin. Metformin can directly act on hepatocytes to decrease gluconeogenesis partially via modulation of mitochondrial complex I activity, AMP-activated protein kinase (AMPK) activation, and the AMP concentration [81-83]. It can also enhance the hepatic cytosolic redox state by inhibiting glycerol-3-phosohate dehydrogenase activity to decrease glucogenesis [84, 85]. Consistently, several clinical trials observed that metformin treatment improved hepatic steatosis and inflammation in people with NAFLD. Likewise, liraglutide, an acylated glucagon-like peptide-1 (GLP-1) agonist that is also used as anti-diabetic drug, has also been shown to improve NAFLD in HFD-fed mice by improving mitochondrial function and reducing ROS production [86]. In line with this, a randomized placebo-controlled study showed that compared with the placebo group, liraglutide treatment promoted histological resolution of NASH [87]. Although these anti-diabetic drugs thus show some promise in alleviating NAFLD, their efficacy in the treatment of more severe NAFLD, i.e. fibrotic NASH, requires further investigation. Moreover, given that these therapeutic agents do not specifically target hepatocyte mitochondria, these aforementioned findings only show an association

between improved mitochondrial function and improved progressive NAFLD. Thus, it is still unclear whether direct hepatocyte mitochondria-targeted therapy is able to combat NAFLD and its associated cardiometabolic diseases. Future studies are thus needed to evaluate the therapeutic potential of hepatocyte mitochondria-targeted therapy in the treatment of NAFLD, and in particular fibrotic NASH.

Taken together, hepatocyte mitochondria are appealing targets for treatment of liver pathologies. The development of drugs targeted to hepatocyte mitochondria is strongly encouraged, as such drugs may be able to effectively combat NAFLD, and even fibrotic NASH. This hypothesis is supported by our findings showing that improving hepatocyte mitochondrial function can inhibit HSC activation (**Chapter 4**), indicating the anti-fibrotic potential of mitochondria-targeted therapies. Currently, mitochondrial medicine is largely in the developmental stage, and future pre-clinical and clinical studies should pay more attention and efforts to explore its application in the treatment of cardiometabolic diseases.

2.2 Hepatokine FGF21-based pharmacotherapy

As an endocrine organ, the liver secretes various peptide and protein hormones, namely hepatokines, that can influence cardiometabolic health through cross-talk with multiple metabolic organ systems via autocrine, paracrine and endocrine signaling. As such, these bioactive molecules are of great interest as potential targets for the treatment of cardiometabolic diseases, especially as many of these are much easier to manipulate than other factors contributing to cardiometabolic diseases. Among these hepatokines, fibroblast growth factor (FGF) 21 has been brought to the foreground as a promising potential therapeutic for cardiometabolic diseases.

FGF21 is an atypical member of the endocrine FGF family that lacks mitogenic activity. Although *Fgf21* mRNA can be detected in numerous tissues (e.g. the liver and adipose tissue), circulating FGF21 is mainly derived from the liver [88]. Indeed, we (**Chapter 5**) and others [48, 89] observed that circulating FGF21 levels correlate well with hepatic *Fgf21* mRNA expression levels. FGF21 elicits its biological effects by binding and activating a receptor complex comprised of FGF receptors (FGFRs) and its co-receptor-klotho (KLB) [90]. Whereas FGFRs exhibit a ubiquitous expression pattern, the expression of KLB is primarily restricted to specific metabolic organs (e.g. the liver and adipose tissue) [91]. Physiologically, FGF21 is a stress-induced hormone, whose levels rise in metabolically compromised states (e.g. obesity, T2D and NAFLD) [88]. Hepatocyte lipid overload is an important signal that triggers FGF21 production and release [92]. Induction of FGF21 is thought to mediate a compensatory response to limit metabolic dysregulation, although such a physiological response is insufficient

to actually compensate [88]. Indeed, administration of (long-acting) FGF21 has shown beneficial effects on obesity and T2D, as reproducibly observed in many preclinical studies [93-95]. For instance, FGF21 administration in DIO mice reduces fat mass and improves insulin sensitivity and lipid profiles (that is, decreases in TGs and low-density lipoprotein cholesterol, and increases in high-density lipoprotein cholesterol) [95-97]. Given that native FGF21 is unsuitable for clinical use owing to poor pharmacokinetic and biophysical properties [91], a large number of long-acting FGF21 analogues have been developed. Several FGF21 analogues have even progressed to early phases of clinical trials in patients with e.g. obesity and T2D. In these trials, substantial improvements were observed in dyslipidemia and hepatic fat fractions in individuals with obesity and T2D [48, 94]. In this section, we will further discuss the therapeutic efficacy of FGF21-based pharmacotherapy on fibrotic NASH and atherosclerotic CVD.

FGF21-based pharmacotherapy in the treatment of NASH. Very recently, two phase IIa clinical trials reported that pharmacological treatment with FGF21 analogues reduce liver fat content in people with NASH [98, 99]. In **Chapter 5**, by using *APOE*3-Leiden.CETP* mice, we further demonstrated that FGF21 treatment limits all features of fibrotic NASH, including liver lipotoxicity, inflammation and fibrogenesis. We proposed that the protective effects of FGF21 on liver lipotoxicity result from the combined effects of FGF21 on adipose tissue and the liver, resulting in decreased lipid influx from adipose tissue into the liver coupled with the activation of FA oxidation and cholesterol elimination pathways in the liver [90, 91]. Our findings are in agreement with observations observed in humans showing that administration with FGF21 analogues in people with NASH not only decreased liver lipid content [100, 101], but also increased cholesterol removal, reducing the risk for further hepatocyte lipotoxicity [98]. While NASH is initiated by liver lipotoxicity, NASH progression is primarily triggered by inflammation [102]. We demonstrated that FGF21 prevented HFCD-induced inflammatory responses, as proved by improved lobular inflammation and hepatocyte ballooning and reduced numbers of inflammatory foci and crown-like structures. Notably, FGF21 reduced pro-inflammatory activation of various subsets of Kupffer cells (KCs; i.e. the resident macrophages in the liver). Furthermore, we reported that FGF21 treatment prevented liver fibrosis, which was associated with reduced numbers of lipid- and scar-associated KCs [103, 104]. Previous studies have shown that scar-associated macrophages are enriched in fibrotic liver [104-107], and these cells are able to prime quiescent primary HSCs to upregulate the expression of fibrillar collagen [104], thereby promoting liver fibrosis. As such, our findings likely indicate that FGF21 inhibits liver fibrogenesis by preventing lipid- and scar-associated macrophage accumulation, thereby inhibiting HSC activation to produce collagen. Collectively, our data further strengthen the therapeutic potential of FGF21 for treatment of NASH and provide

mechanistic insight supporting the currently ongoing clinical trials evaluating the impact of long-acting FGF21 on fibrotic NASH.

FGF21-based pharmacotherapy in the treatment of atherosclerotic CVD. While clinical trials are underway with long-acting FGF21 analogues to combat e.g. NAFLD [108], the pharmacological effects of FGF21 on atherosclerosis are far from being elucidated. Current studies evaluating the therapeutic effects of FGF21 on atherosclerosis are mainly derived from genetic studies, demonstrating that *Fgf21* deficiency in *Apoe*$^{-/-}$ mice promotes atherosclerosis [109]. Of note, FGF21 treatment reduces hypercholesterolemia in obese non-human primates [110] and humans [111], indicating that (long-acting) FGF21 may have the ability to attenuate atherosclerosis. A study has shown reduced atherosclerotic lesion size upon FGF21 administration in *Fgf21*$^{-/-}$*Apoe*$^{-/-}$ mice [109]. However, considering that APOE plays crucial role in mediating hepatic uptake of TG-rich lipoprotein remnants, *Apoe*$^{-/-}$ mice may not be the best model to investigate potential therapeutic effects of FGF21 on atherosclerosis by modulating lipid metabolism. Therefore, in **Chapter 6**, we explored the effects of FGF21 treatment on cardiovascular risk factors, particularly on lipoprotein metabolism in relation to atherogenesis using *APOE*3-Leiden.CETP mice*. We showed that FGF21 reduces hypercholesterolemia by accelerating TG-rich lipoprotein turnover as a result of brown fat activation and white fat browning, thereby reducing atherosclerotic lesion severity and increasing atherosclerotic lesion stability index. Mechanistic studies revealed that FGF21 treatment enhances lipolytic conversion of VLDL by brown fat and by white fat. In line with this, a clinical study was reported that FGF21 administration increased thermogenesis-related gene/protein expression in human adipocytes [112]. The avid uptake of generated VLDL remnants that result from lipolysis by BAT and beige WAT by the liver is likely mediated via the APOE-LDLR pathway, as we previously found that clearance of VLDL remnants is impaired in *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice [113]. Moreover, we showed that decreased atherosclerotic lesion area was mainly predicted by the reduction in non-high-density lipoprotein cholesterol (non-HDL-C). Indeed, clinical studies showed that early interventions to lower non-HDL-C levels in the circulation can fully block and even reverse earlier stages of atherosclerosis [114]. FGF21 also increased atherosclerotic plaque stability index by reducing the plaque macrophage content relative to the collagen and smooth muscle cell content. This suggests that in addition to lowering atherogenic cholesterol levels to reduce atherosclerosis initiation, FGF21 also suppresses inflammation. Consistently, FGF21 was shown to block foam cell formation and inhibit inflammatory responses in oxidized low-density lipoprotein-induced macrophages *in vitro* [115]. Therefore, our present data, together with available clinical data, suggest that FGF21 is a promising therapeutic for asCVD.

Taken together, clinical studies on FGF21 analogues conducted thus far have yielded mixed results. While the effects of FGF21 on glycemic control are disappointing, it has shown promising results in terms of liver function and lipid metabolism, making it a potential treatment for NASH and CVD comorbidities rather than T2D. It is worth noting that while many therapeutics for T2D effectively lower glucose levels, they do not have the same therapeutic effects on dyslipidemia as FGF21. Therefore, further research is needed to explore the potential benefits of combining FGF21 with these anti-diabetic drugs to treat a group of obesity-associated metabolic disorders. Additionally, large-scale clinical studies with long-term treatment periods are needed to assess whether improved lipid metabolism upon FGF21 treatment is sufficient to effectively treat NASH and asCVDs. Furthermore, to translate the therapeutic potential of FGF21-based therapeis from the laboratory to the clinic, some crucial questions need to be answered [91]. Considering that the current findings highlight the complexity and interspecies variations of FGF21 biology, future research should focus on identifying the specific tissues and cellular pathways that mediate the diverse pharmacological effects of FGF21 in humans. This would assist in developing tissue-specific FGF21 agonists with improved specificity and safety. Besides, clinical trials have observed

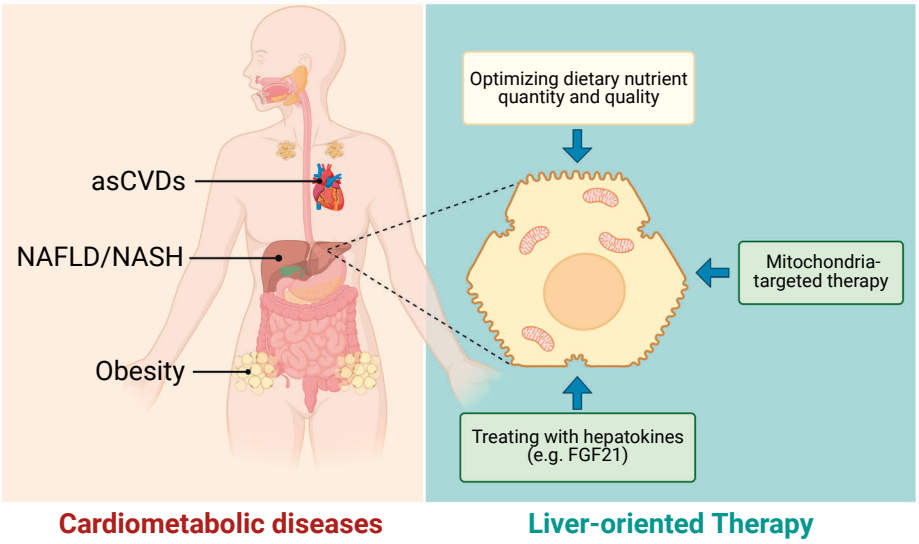

Figure 2. Improving the liver metabolic function to improve cardiometabolic diseases. Currently available liver-oriented therapeutic strategies for cardiometabolic diseases, e.g. obesity, non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH) and atherosclerotic cardiovascular disease (asCVD), are mainly dietary and pharmacological interventions. Dietary interventions, such as optimizing dietary macro- and micro-nutrient quantity and quality can reduce liver fat which improves cardiometabolic health. Likewise, liver-oriented pharmacological interventions, such as mitochondrial-targeted therapy, and more recently, administration of hepatokines have also shown therapeutic potential in the treatment of cardiometabolic diseases, including NASH and asCVD.

large variability in plasma levels of FGF21 among individuals, indicating that people may respond differentially to (long-acting) FGF21. As such, understanding genetic and metabolic factors that influence FGF21 responsiveness is necessary for the development of personalized FGF21-based pharmacotherapies that target populations with optimal FGF21 sensitivity (Liver-oriented therapies for cardiometabolic diseases are shown in **Figure 2**).

CONCLUDING REMARKS

In multicellular organisms, maintenance of systemic homeostasis and response to nutritional and environmental challenges require the coordination of multiple organs and tissues. To adapt to changing metabolic demands, higher organisms have developed a system of inter-organ communication by which one tissue can affect metabolic processes in a distant tissue. Dysregulation of these lines of communication contributes to the development of cardiometabolic diseases (e.g. obesity, T2D, NAFLD/NASH and asCVD), which are pressing public-health concerns worldwide. Therefore, there is an urgent need to identify novel strategies to limit cardiometabolic health risks associated with the disruption of inter-organ cross-talk.

On one hand, strategies can focus on regulating the gut microbiota-centered inter-organ cross-talk, which can be achieved by dietary and pharmacological interventions. The gut microbiota forms a bioreactor which is fueled by exogenous dietary components and endogenous compounds generated from microorganisms and the host to produce various bioactive compounds. These gut microbiota-derived metabolites signal to various metabolic organs in the body, which adds to cross-talk between the gut and the host. The gut microbiota-host interaction contains different layers, including dietary precursors, gut microbial communities and meta-organismal pathways, all of which are potential therapeutic targets for cardiometabolic diseases. The gut microbiota is most sensitive to the diet, and diet-induced changes of the gut microbiota can influence the production of the gut microbial metabolites (e.g. SCFAs). Administration of antibiotics, prebiotics or probiotics can also modulate the gut microbiota composition to affect the gut microbiota-centered inter-organ cross-talk. Moreover, bacterial enzyme inhibitors and bacteriophage therapy can also influence the production of the gut microbial metabolites by modifying the gut microbiota profile. This thesis focused on dietary intervention, as this strategy induce little discomfort and side effects. We have demonstrated that dietary interventions are efficient to modulate the gut microbiota composition and function, thereby regulating the gut microbial metabolite production. In particularly, we showed that dietary butyrate and choline

supplementation can beneficially modulate the gut microbiota to alleviate adiposity. Moreover, we showed that plasma levels of choline metabolite TMAO are not associated with the development of cardiometabolic diseases. Future studies are still required to evaluate the therapeutic potential of dietary butyrate and choline supplementation in the treatment of various stages of NAFLD and asCVD in both various experimental animal models and in humans.

On the other hand, therapies can also focus on liver-centered inter-organ cross-talk. Dietary and pharmacological strategies to improve hepatocyte mitochondrial function hold great promise for combating cardiometabolic diseases. Herein, we showed that improving hepatocyte mitochondrial function by γ-hydroxybutyric acid not only improves liver metabolic function, but also reverses obesity and its associated metabolic diseases including such as insulin resistance. In addition, cardiometabolic health can be improved by regulating systemic levels of hepatokines (e.g. FGF21). In this thesis, we showed that FGF21-based pharmacotherapies can regulate the cross-talk between the liver and adipose tissue to improve cardiometabolic diseases, especially fibrotic NASH and atherosclerotic CVD. Mechanistically, FGF21 upregulates FA oxidation in thermogenic tissues and in the liver, thereby improving lipid metabolism, and as a consequence largely attenuates all features of NASH and atherosclerosis development. Our data provide a strong experimental basis for the clinical development of FGF21 to treat NASH and asCVD. It will thus be very interesting to learn whether this approach is able to effectively (and safely) improve cardiometabolic health in humans in the future.

REFERENCES

1. Roth, G.A., et al., *Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study.* J Am Coll Cardiol, 2020. **76**(25): p. 2982-3021.
2. Collins, K.H., et al., *A High-Fat High-Sucrose Diet Rapidly Alters Muscle Integrity, Inflammation and Gut Microbiota in Male Rats.* Sci Rep, 2016. **6**: p. 37278.
3. Vors, C., et al., *Postprandial Endotoxemia Linked With Chylomicrons and Lipopolysaccharides Handling in Obese Versus Lean Men: A Lipid Dose-Effect Trial.* J Clin Endocrinol Metab, 2015. **100**(9): p. 3427-35.
4. Herman, M.A. and M.J. Birnbaum, *Molecular aspects of fructose metabolism and metabolic disease.* Cell Metab, 2021. **33**(12): p. 2329-2354.
5. Febbraio, M.A. and M. Karin, *"Sweet death": Fructose as a metabolic toxin that targets the gut-liver axis.* Cell Metabolism, 2021. **33**(12): p. 2316-2328.
6. Binienda, A., et al., *Dietary Carbohydrates and Lipids in the Pathogenesis of Leaky Gut Syndrome: An Overview.* Int J Mol Sci, 2020. **21**(21).
7. Desai, M.S., et al., *A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility.* Cell, 2016. **167**(5): p. 1339-1353 e21.
8. Cani, P.D., et al., *Metabolic endotoxemia initiates obesity and insulin resistance.* Diabetes, 2007. **56**(7): p. 1761-72.
9. Ferro, D., et al., *New Insights into the Pathogenesis of Non-Alcoholic Fatty Liver Disease: Gut-Derived Lipopolysaccharides and Oxidative Stress.* Nutrients, 2020. **12**(9).
10. Kallio, K.A., et al., *Endotoxemia, nutrition, and cardiometabolic disorders.* Acta Diabetol, 2015. **52**(2): p. 395-404.
11. Lassenius, M.I., et al., *Bacterial Endotoxin Activity in Human Serum Is Associated With Dyslipidemia, Insulin Resistance, Obesity, and Chronic Inflammation.* Diabetes Care, 2011. **34**(8): p. 1809-1815.
12. Marti, A., et al., *Higher Lipopolysaccharide Binding Protein and Chemerin Concentrations Were Associated with Metabolic Syndrome Features in Pediatric Subjects with Abdominal Obesity during a Lifestyle Intervention.* Nutrients, 2021. **13**(2).
13. Roberts, M.N., et al., *A Ketogenic Diet Extends Longevity and Healthspan in Adult Mice.* Cell Metab, 2017. **26**(3): p. 539-546 e5.
14. Paoli, A., et al., *Ketogenic Diet and Microbiota: Friends or Enemies?* Genes (Basel), 2019. **10**(7).
15. Swidsinski, A., et al., *Reduced Mass and Diversity of the Colonic Microbiome in Patients with Multiple Sclerosis and Their Improvement with Ketogenic Diet.* Frontiers in Microbiology, 2017. **8**.
16. Tagliabue, A., et al., *Short-term impact of a classical ketogenic diet on gut microbiota in GLUT1 Deficiency Syndrome: A 3-month prospective observational study.* Clin Nutr ESPEN, 2017. **17**: p. 33-37.
17. Nordmann, A.J., et al., *Effects of low-carbohydrate vs low-fat diets on weight loss and cardiovascular risk factors: a meta-analysis of randomized controlled trials.* Arch Intern Med, 2006. **166**(3): p. 285-93.
18. *Comparison of the Atkins, Zone, Ornish, and LEARN diets for change in weight and related risk factors among overweight premenopausal women: The A TO Z weight loss study: A randomized trial (vol 297, pg 969, 2007).* Jama-Journal of the American Medical Association, 2007. **298**(2): p. 178-178.
19. Gentile, C.L. and T.L. Weir, *The gut microbiota at the intersection of diet and human health.* Science, 2018. **362**(6416): p. 776-780.

20. Covas, M.I., et al., *The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial.* Ann Intern Med, 2006. **145**(5): p. 333-41.

21. Garcia-Mantrana, I., et al., *Shifts on Gut Microbiota Associated to Mediterranean Diet Adherence and Specific Dietary Intakes on General Adult Population.* Front Microbiol, 2018. **9**: p. 890.

22. Zhang, L., et al., *Butyrate in Energy Metabolism: There Is Still More to Learn.* Trends in Endocrinology and Metabolism, 2021. **32**(3): p. 159-169.

23. Samuel, B.S., et al., *Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41.* Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16767-72.

24. De Vadder, F., et al., *Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits.* Cell, 2014. **156**(1-2): p. 84-96.

25. Li, Z., et al., *Butyrate reduces appetite and activates brown adipose tissue via the gut-brain neural circuit.* Gut, 2018. **67**(7): p. 1269-1279.

26. Salas-Salvado, J., et al., *Prevention of diabetes with Mediterranean diets: a subgroup analysis of a randomized trial.* Ann Intern Med, 2014. **160**(1): p. 1-10.

27. Turnbaugh, P.J., et al., *An obesity-associated gut microbiome with increased capacity for energy harvest.* Nature, 2006. **444**(7122): p. 1027-31.

28. Kasahara, K., et al., *Interactions between Roseburia intestinalis and diet modulate atherogenesis in a murine model.* Nature Microbiology, 2018. **3**(12): p. 1461-1471.

29. Zheng, J., et al., *Dietary N-Nitroso Compounds and Risk of Hepatocellular Carcinoma: A USA-Based Study.* Hepatology, 2021. **74**(6): p. 3161-3173.

30. Zhang, B., et al., *The Mechanism Underlying the Influence of Indole-3-Propionic Acid: A Relevance to Metabolic Disorders.* Front Endocrinol (Lausanne), 2022. **13**: p. 841703.

31. Xue, H.L., et al., *Gut Microbially Produced Indole-3-Propionic Acid Inhibits Atherosclerosis by Promoting Reverse Cholesterol Transport and Its Deficiency Is Causally Related to Atherosclerotic Cardiovascular Disease.* Circulation Research, 2022. **131**(5): p. 404-420.

32. Koeth, R.A., et al., *Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis.* Nat Med, 2013. **19**(5): p. 576-85.

33. Zhu, W., et al., *Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk.* Cell, 2016. **165**(1): p. 111-124.

34. Wang, Z., et al., *Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease.* Nature, 2011. **472**(7341): p. 57-63.

35. Tang, W.H.W. and S.L. Hazen, *The contributory role of gut microbiota in cardiovascular disease.* Journal of Clinical Investigation, 2014. **124**(10): p. 4204-4211.

36. Chang, T.Y., et al., *Optimal Dietary Intake Composition of Choline and Betaine Is Associated with Minimized Visceral Obesity-Related Hepatic Steatosis in a Case-Control Study.* Nutrients, 2022. **14**(2).

37. Bordoni, L., et al., *Trimethylamine N-oxide and the reverse cholesterol transport in cardiovascular disease: a cross-sectional study.* Sci Rep, 2020. **10**(1): p. 18675.

38. Richard, C., et al., *Impact of Egg Consumption on Cardiovascular Risk Factors in Individuals with Type 2 Diabetes and at Risk for Developing Diabetes: A Systematic Review of Randomized Nutritional Intervention Studies.* Can J Diabetes, 2017. **41**(4): p. 453-463.

39. Shin, J.Y., et al., *Egg consumption in relation to risk of cardiovascular disease and diabetes: a systematic review and meta-analysis.* Am J Clin Nutr, 2013. **98**(1): p. 146-59.

40. Meyer, K.A. and J.W. Shea, *Dietary Choline and Betaine and Risk of CVD: A Systematic Review and Meta-Analysis of Prospective Studies.* Nutrients, 2017. **9**(7).

41. Nagata, C., et al., *Choline and Betaine Intakes Are Not Associated with Cardiovascular Disease Mortality Risk in Japanese Men and Women.* J Nutr, 2015. **145**(8): p. 1787-92.

42. Wang, H., et al., *The microbial metabolite trimethylamine N-oxide promotes antitumor immunity in triple-negative breast cancer.* Cell Metab, 2022. **34**(4): p. 581-594 e8.

43. Hussein, H.M., et al., *Vitamin D mitigates diabetes-associated metabolic and cognitive dysfunction by modulating gut microbiota and colonic cannabinoid receptor 1.* Eur J Pharm Sci, 2022. **170**: p. 106105.

44. Mayneris-Perxachs, J., et al., *Iron status influences non-alcoholic fatty liver disease in obesity through the gut microbiome.* Microbiome, 2021. **9**(1).

45. Wilck, N., et al., *Salt-responsive gut commensal modulates T(H)17 axis and disease.* Nature, 2017. **551**(7682): p. 585-589.

46. Epstein, S.E., et al., *The role of infection in restenosis and atherosclerosis: focus on cytomegalovirus.* Lancet, 1996. **348 Suppl 1**: p. s13-7.

47. Patel, P., et al., *Association of Helicobacter pylori and Chlamydia pneumoniae infections with coronary heart disease and cardiovascular risk factors.* BMJ, 1995. **311**(7007): p. 711-4.

48. Saikku, P., et al., *Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction.* Lancet, 1988. **2**(8618): p. 983-6.

49. Vrieze, A., et al., *Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity.* J Hepatol, 2014. **60**(4): p. 824-31.

50. Huemer, M., et al., *Antibiotic resistance and persistence-Implications for human health and treatment perspectives.* EMBO Rep, 2020. **21**(12): p. e51034.

51. Green, M., K. Arora, and S. Prakash, *Microbial Medicine: Prebiotic and Probiotic Functional Foods to Target Obesity and Metabolic Syndrome.* Int J Mol Sci, 2020. **21**(8).

52. Singh, V., et al., *Dysregulated Microbial Fermentation of Soluble Fiber Induces Cholestatic Liver Cancer.* Cell, 2018. **175**(3): p. 679-694 e22.

53. Zeevi, D., et al., *Personalized Nutrition by Prediction of Glycemic Responses.* Cell, 2015. **163**(5): p. 1079-1094.

54. Turck, D., et al., *Safety of pasteurised Akkermansia muciniphila as a novel food pursuant to Regulation (EU) 2015/2283.* Efsa Journal, 2021. **19**(9).

55. He, X., et al., *Akkermansia muciniphila Alters Gut Microbiota and Immune System to Improve Cardiovascular Diseases in Murine Model.* Front Microbiol, 2022. **13**: p. 906920.

56. Depommier, C., et al., *Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study.* Nat Med, 2019. **25**(7): p. 1096-1103.

57. Anhe, F.F. and A. Marette, *A microbial protein that alleviates metabolic syndrome.* Nat Med, 2017. **23**(1): p. 11-12.

58. Firouzi, S., et al., *Effect of multi-strain probiotics (multi-strain microbial cell preparation) on glycemic control and other diabetes-related outcomes in people with type 2 diabetes: a randomized controlled trial.* European Journal of Nutrition, 2017. **56**(4): p. 1535-1550.

59. Witkowski, M., T.L. Weeks, and S.L. Hazen, *Gut Microbiota and Cardiovascular Disease.* Circ Res, 2020. **127**(4): p. 553-570.

60. de Jonge, P.A., et al., *Gut virome profiling identifies a widespread bacteriophage family associated with metabolic syndrome.* Nat Commun, 2022. **13**(1): p. 3594.

61. Duan, Y., et al., *Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease.* Nature, 2019. **575**(7783): p. 505-511.

62. Stefan, N., H.U. Haring, and K. Cusi, *Non-alcoholic fatty liver disease: causes, diagnosis, cardiometabolic consequences, and treatment strategies.* Lancet Diabetes Endocrinol, 2019. **7**(4): p. 313-324.

63. Duell, P.B., et al., *Nonalcoholic Fatty Liver Disease and Cardiovascular Risk: A Scientific Statement From the American Heart Association.* Arterioscler Thromb Vasc Biol, 2022. **42**(6): p. e168-e185.

64. Yki-Jarvinen, H., et al., *Dietary carbohydrates and fats in nonalcoholic fatty liver disease.* Nat Rev Gastroenterol Hepatol, 2021. **18**(11): p. 770-786.

65. Kotronen, A., et al., *Hepatic stearoyl-CoA desaturase (SCD)-1 activity and diacylglycerol but not ceramide concentrations are increased in the nonalcoholic human fatty liver.* Diabetes, 2009. **58**(1): p. 203-8.

66. Chaurasia, B., et al., *Targeting a ceramide double bond improves insulin resistance and hepatic steatosis.* Science, 2019. **365**(6451): p. 386-392.

67. Moore, M.P., et al., *A Fad too Far? Dietary Strategies for the Prevention and Treatment of NAFLD.* Obesity (Silver Spring), 2020. **28**(10): p. 1843-1852.

68. Sberna, A.L., et al., *European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD) and European Association for the Study of Obesity (EASO) clinical practice recommendations for the management of non-alcoholic fatty liver disease: evaluation of their application in people with Type 2 diabetes.* Diabetic Medicine, 2018. **35**(3): p. 368-375.

69. Mardinoglu, A., et al., *An Integrated Understanding of the Rapid Metabolic Benefits of a Carbohydrate-Restricted Diet on Hepatic Steatosis in Humans.* Cell Metab, 2018. **27**(3): p. 559-571 e5.

70. Mardinoglu, A., et al., *An Integrated Understanding of the Rapid Metabolic Benefits of a Carbohydrate-Restricted Diet on Hepatic Steatosis in Humans.* Cell Metabolism, 2018. **27**(3): p. 559-+.

71. Romero-Gomez, M., S. Zelber-Sagi, and M. Trenell, *Treatment of NAFLD with diet, physical activity and exercise.* J Hepatol, 2017. **67**(4): p. 829-846.

72. Morio, B., et al., *Role of mitochondria in liver metabolic health and diseases.* Cell Calcium, 2021. **94**: p. 102336.

73. Serviddio, G., et al., *Targeting mitochondria: a new promising approach for the treatment of liver diseases.* Curr Med Chem, 2010. **17**(22): p. 2325-37.

74. Longo, M., et al., *Mitochondrial dynamics and nonalcoholic fatty liver disease (NAFLD): new perspectives for a fairy-tale ending?* Metabolism, 2021. **117**: p. 154708.

75. Verbeek, J., et al., *Roux-en-y gastric bypass attenuates hepatic mitochondrial dysfunction in mice with non-alcoholic steatohepatitis.* Gut, 2015. **64**(4): p. 673-83.

76. Lee, K., et al., *Hepatic Mitochondrial Defects in a Nonalcoholic Fatty Liver Disease Mouse Model Are Associated with Increased Degradation of Oxidative Phosphorylation Subunits.* Mol Cell Proteomics, 2018. **17**(12): p. 2371-2386.

77. Okuno, M., et al., *Retinoids exacerbate rat liver fibrosis by inducing the activation of latent TGF-beta in liver stellate cells.* Hepatology, 1997. **26**(4): p. 913-21.

78. Trivedi, P., S. Wang, and S.L. Friedman, *The Power of Plasticity-Metabolic Regulation of Hepatic Stellate Cells.* Cell Metab, 2021. **33**(2): p. 242-257.

79. Chiu, H.J., D.A. Fischman, and U. Hammerling, *Vitamin A depletion causes oxidative stress, mitochondrial dysfunction, and PARP-1-dependent energy deprivation.* FASEB J, 2008. **22**(11): p. 3878-87.

80. Berdanier, C.D., et al., *Role of vitamin A in mitochondrial gene expression.* Diabetes Res Clin Pract, 2001. **54 Suppl 2**: p. S11-27.

81. Zhou, G., et al., *Role of AMP-activated protein kinase in mechanism of metformin action.* J Clin Invest, 2001. **108**(8): p. 1167-74.

82. Rena, G., D.G. Hardie, and E.R. Pearson, *The mechanisms of action of metformin.* Diabetologia, 2017. **60**(9): p. 1577-1585.

83. Lien, F., et al., *Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk.* Journal of Clinical Investigation, 2014. **124**(3): p. 1037-1051.

84. Madiraju, A.K., et al., *Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase.* Nature, 2014. **510**(7506): p. 542-6.

85. Madiraju, A.K., et al., *Metformin inhibits gluconeogenesis via a redox-dependent mechanism in vivo.* Nat Med, 2018. **24**(9): p. 1384-1394.

86. Tong, W.X., et al., *Liraglutide ameliorates non-alcoholic fatty liver disease by enhancing mitochondrial architecture and promoting autophagy through the SIRT1/SIRT3-FOXO3a pathway.* Hepatology Research, 2016. **46**(9): p. 933-943.

87. Armstrong, M.J., et al., *Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study.* Lancet, 2016. **387**(10019): p. 679-690.

88. Zarei, M., et al., *Targeting FGF21 for the Treatment of Nonalcoholic Steatohepatitis.* Trends Pharmacol Sci, 2020. **41**(3): p. 199-208.

89. Nishimura, T., et al., *Identification of a novel FGF, FGF-21, preferentially expressed in the liver.* Biochim Biophys Acta, 2000. **1492**(1): p. 203-6.

90. Fisher, F.M. and E. Maratos-Flier, *Understanding the Physiology of FGF21.* Annu Rev Physiol, 2016. **78**: p. 223-41.

91. Geng, L., K.S.L. Lam, and A. Xu, *The therapeutic potential of FGF21 in metabolic diseases: from bench to clinic.* Nat Rev Endocrinol, 2020. **16**(11): p. 654-667.

92. Priest, C. and P. Tontonoz, *Inter-organ cross-talk in metabolic syndrome.* Nat Metab, 2019. **1**(12): p. 1177-1188.

93. Kharitonenkov, A., et al., *FGF-21 as a novel metabolic regulator.* Journal of Clinical Investigation, 2005. **115**(6): p. 1627-1635.

94. Talukdar, S., et al., *A Long-Acting FGF21 Molecule, PF-05231023, Decreases Body Weight and Improves Lipid Profile in Non-human Primates and Type 2 Diabetic Subjects.* Cell Metab, 2016. **23**(3): p. 427-40.

95. Xu, J., et al., *Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice.* Diabetes, 2009. **58**(1): p. 250-9.

96. Coskun, T., et al., *Fibroblast growth factor 21 corrects obesity in mice.* Endocrinology, 2008. **149**(12): p. 6018-27.

97. Berglund, E.D., et al., *Fibroblast growth factor 21 controls glycemia via regulation of hepatic glucose flux and insulin sensitivity.* Endocrinology, 2009. **150**(9): p. 4084-93.

98. Luo, Y., et al., *Pegbelfermin selectively reduces secondary bile acid concentrations in patients with non-alcoholic steatohepatitis.* JHEP Rep, 2022. **4**(1): p. 100392.

99. Harrison, S.A., et al., *A randomized, double-blind, placebo-controlled phase IIa trial of efruxifermin for patients with compensated NASH cirrhosis.* JHEP Rep, 2023. **5**(1): p. 100563.

100. Sanyal, A., et al., *Pegbelfermin (BMS-986036), a PEGylated fibroblast growth factor 21 analogue, in patients with non-alcoholic steatohepatitis: a randomised, double-blind, placebo-controlled, phase 2a trial.* Lancet, 2019. **392**(10165): p. 2705-2717.

101. Harrison, S.A., et al., *Efruxifermin in non-alcoholic steatohepatitis: a randomized, double-blind, placebo-controlled, phase 2a trial.* Nat Med, 2021. **27**(7): p. 1262-1271.

102. Cai, J., X.J. Zhang, and H. Li, *The Role of Innate Immune Cells in Nonalcoholic Steatohepatitis.* Hepatology, 2019. **70**(3): p. 1026-1037.

103. Bleriot, C., et al., *A subset of Kupffer cells regulates metabolism through the expression of CD36.* Immunity, 2021. **54**(9): p. 2101-+.

104. Ramachandran, P., et al., *Resolving the fibrotic niche of human liver cirrhosis at single-cell level.* Nature, 2019. **575**(7783): p. 512-+.

105. Remmerie, A., et al., *Osteopontin Expression Identifies a Subset of Recruited Macrophages Distinct from Kupffer Cells in the Fatty Liver.* Immunity, 2020. **53**(3): p. 641-+.

106. Daemen, S., et al., *Dynamic Shifts in the Composition of Resident and Recruited Macrophages Influence Tissue Remodeling in NASH.* Cell Rep, 2021. **34**(2): p. 108626.

107. Seidman, J.S., et al., *Niche-Specific Reprogramming of Epigenetic Landscapes Drives Myeloid Cell Diversity in Nonalcoholic Steatohepatitis.* Immunity, 2020. **52**(6): p. 1057-1074 e7.

108. Sonoda, J., M.Z. Chen, and A. Baruch, *FGF21-receptor agonists: an emerging therapeutic class for obesity-related diseases.* Horm Mol Biol Clin Investig, 2017. **30**(2).

109. Lin, Z., et al., *Fibroblast growth factor 21 prevents atherosclerosis by suppression of hepatic sterol regulatory element-binding protein-2 and induction of adiponectin in mice.* Circulation, 2015. **131**(21): p. 1861-71.

110. Kharitonenkov, A., et al., *The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21.* Endocrinology, 2007. **148**(2): p. 774-781.

111. Talukdar, S., et al., *A Long-Acting FGF21 Molecule, PF-05231023, Decreases Body Weight and Improves Lipid Profile in Non-human Primates and Type 2 Diabetic Subjects.* Cell Metabolism, 2016. **23**(3): p. 427-440.

112. Lee, P., et al., *Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans.* Cell Metab, 2014. **19**(2): p. 302-9.

113. Berbee, J.F., et al., *Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development.* Nat Commun, 2015. **6**: p. 6356.

114. Robinson, J.G., et al., *Eradicating the Burden of Atherosclerotic Cardiovascular Disease by Lowering Apolipoprotein B Lipoproteins Earlier in Life.* Journal of the American Heart Association, 2018. **7**(20).

115. Wang, N., et al., *Fibroblast growth factor 21 regulates foam cells formation and inflammatory response in Ox-LDL-induced THP-1 macrophages.* Biomed Pharmacother, 2018. **108**: p. 1825-1834.