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Early diagnosis of liver fibrosis with bacterial biomarkers

A literature study examining the possibility of bacterial biomarkers in the blood and faeces

Name:	Mirthe Stavast
S-number:	S3714756
Course:	Bachelor Research Project IIb
Faculty:	Science and Engineering
Department:	Nanomedicine & Drug Targeting
Supervisor:	Prof. dr. K. Poelstra
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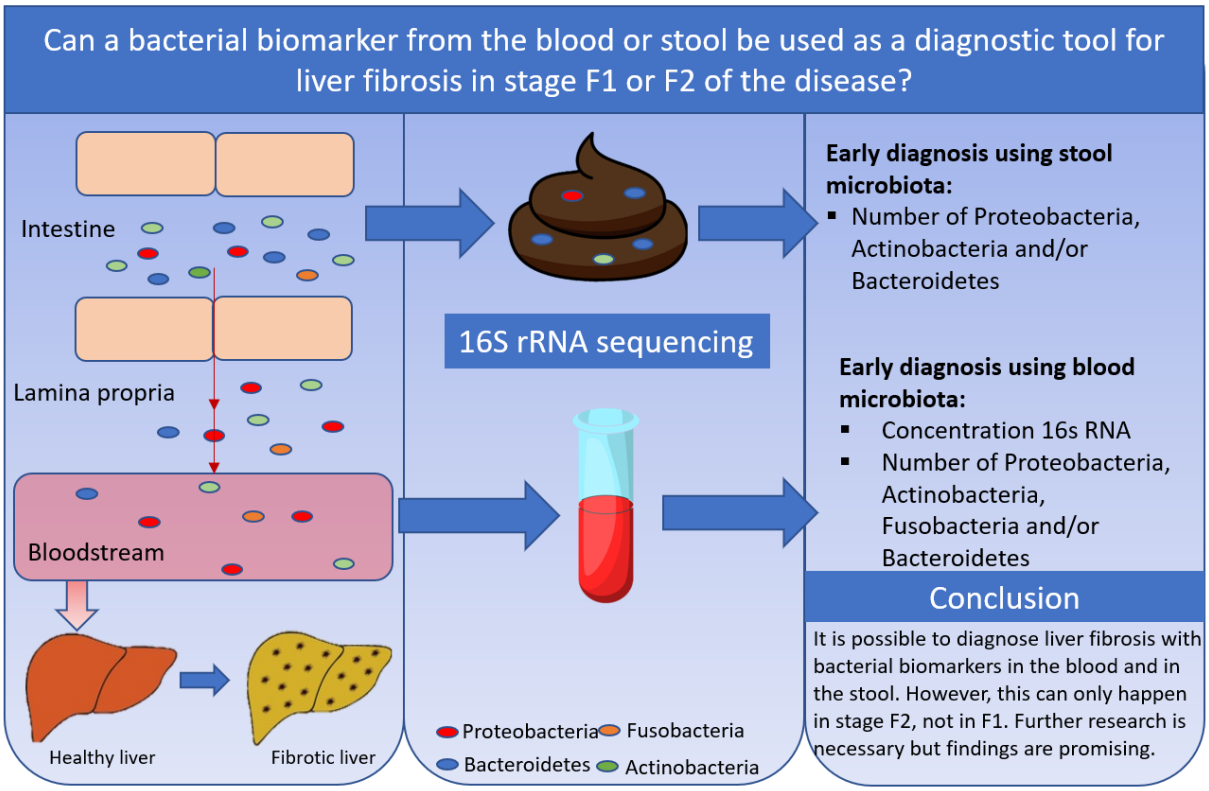
Content

Abstract

Introduction: Diagnosis of liver fibrosis often comes too late, resulting in only one treatment option: liver transplantation. As liver fibrosis causes (almost) no symptoms in early stages *and* most diagnostic tools focus on advanced liver fibrosis, there is a high need for early diagnostic tools. The interaction between the liver and the intestine in liver diseases becomes of more interest in the latest research. However, is it also possible to use this interaction for the diagnosis of liver fibrosis? Moreover, can a bacterial biomarker from the blood or stool be used as a diagnostic tool for liver fibrosis in stage F1 or F2 of the disease?

Results: It is more likely that a biomarker for liver fibrosis can be found in stage F2 of the disease, instead of stage F1. There are specific changes in phyla (*Proteobacteria, Actinobacteria, Fusobacteria and Bacteroidetes*) that might be of interest in this research. Also, the concentration of 16S rDNA in the blood might be used for the early diagnosis.

Conclusion: It can be concluded that it might be possible to diagnose liver fibrosis in stage F2 of the disease, using a bacterial biomarker in the blood and in the stool of patients. Further research is necessary, but the findings are extremely promising.



Introduction

Liver fibrosis

The liver is an extremely important organ in the human body and has many functions. It is crucial for metabolism, stores glycogen, regulates the blood volume, secretes bile acids, and breaks down toxic compounds (for example drugs).[1] With liver fibrosis, a chronic liver disease, scars are formed in the liver tissue.[2] As a result, the liver cannot function properly anymore. This can be problematic as it has many important functions. Around 2 million people worldwide die every year from chronic liver diseases, where fibrosis plays an important role.[3] The high number of deaths are caused by the lack of diagnosis of the disease at early stages. Early diagnosis is difficult because symptoms are only expressed when liver fibrosis has progressed into cirrhosis. These symptoms are caused by portal hypertension and hepatocellular failure.[4] The patients enter the clinic too late and the disease has progressed into an irreversible state, leading eventually to death. Also, the recovery capacity of the liver is diminished when the diagnosis of liver fibrosis comes too late.[5] Therefore, it is extremely important to detect liver fibrosis at an early stage, as this leads to less liver damage and results in more capacity to recover.

Liver fibrosis is caused by repeated injury to the liver, resulting in the accumulation of extracellular matrix proteins (ECM) and scar formation.[2] These injuries can be induced by excessive alcohol use, obesity, hepatitis B and C, specific genes, and other toxic compounds. A distinction can be made between liver diseases that are induced by alcohol and those that are induced by other factors. Alcohol use may lead to alcoholic liver disease (ALD), while other factors may lead to non-alcoholic fatty liver diseases (NAFLD) which can be progressed into non-alcoholic steatohepatitis (NASH).[4] Furthermore, hepatitis B and C viruses may lead to viral hepatitis and impaired bile acid flow may lead to cholestatic liver diseases.[6]

After an acute injury, hepatocytes die and go into apoptosis or necrosis. With necrosis, the intracellular components of the hepatocytes are released into the extracellular space. These intracellular components and the apoptotic cells are seen by Kupffer cells; the macrophages of the liver.[7] When the Kupffer cells detect these components and cells, they release chemokines and cytokines, and therefore cause an inflammatory response. The inflammatory response results in the removal of the injured hepatocytes. The removed hepatocytes can be replaced by regenerated ones. Cytokines, such as IL-6 and TNF-α, play an important role in this.[7]

However, when the injury persists, regenerated hepatocytes do not have the chance to replace all injured cells. Cytokines that are released by Kupffer cells and other factors activate hepatic stellate cells and they transform into myofibroblast-like cells.[7] Especially TGF-α, TGF-β and platelet-derived growth factors (PDGF) are important in this process. Myofibroblast-like cells produce ECM proteins (collagen, elastin, laminin, etc.) to replace the injured cells.[2] This is a wound-healing process that also happens with an acute injury, but this can be reversed. The myofibroblast-like cells go into apoptosis and the Kupffer cells produce factors to resolve the ECM. With a chronic injury, there is less chance for resolution because the number of myofibroblast-like cells increases.[7] This results in an accumulation of ECM proteins and leads to scar formation of the liver tissue.[2]

There are five stages, classified by the META-VIR scale, that describe the process of liver fibrosis and progression into cirrhosis.[8] A healthy liver is in the first stage (F0) and no fibrosis is occurring. When the fibrotic process starts due to chronic injury, ECM is produced and there is minimal scarring (F1). If the injury persists, more scarring occurs, and this can expand outside the liver (F2). The fibrosis can spread and cross with other fibrotic parts of the liver, which is called severe fibrosis (F3). Finally, the fibrotic process has progressed even further into cirrhosis (F4).[9] This might develop into hepatocellular carcinoma after several years and in the end, the liver ceases to function (liver failure).[8][10] In figure 1, the progression of a healthy

liver to hepatocellular carcinoma can be seen.[11] As the liver comes into higher stages, there is less chance of reversing the process.

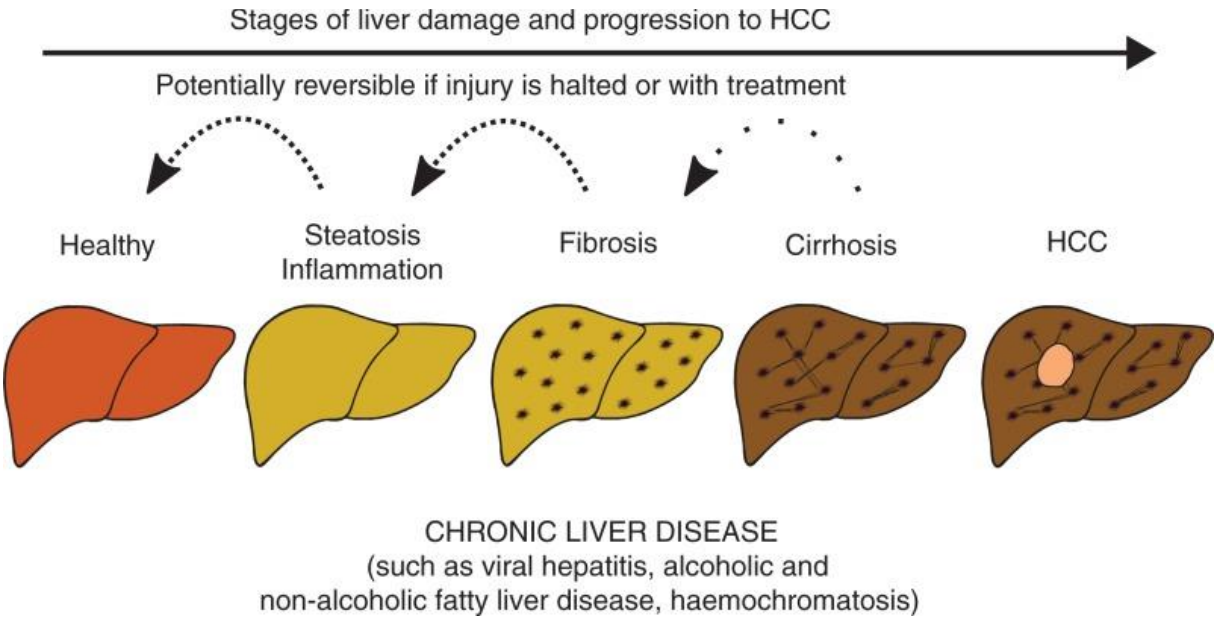


Figure 1: The stages of liver disease; from a healthy liver to hepatocellular carcinoma.[11]

As mentioned before, the diagnosis of liver fibrosis often comes too late and other treatment options than liver transplantation, antiviral agents for hepatitis B and C for example, are not possible anymore. The liver has a large overcapacity and therefore patients with fibrosis or cirrhosis usually do not have many symptoms. The liver function appears to be normal but is compensated for a long time.[7] However, when cirrhosis becomes too severe, there could be effects on metabolism, and portal hypertension can occur. Portal hypertension may lead to variceal bleeding and rebleeding, which can be dangerous.[12] For these patients, liver transplantation is the only treatment option left. Therefore, it is extremely important to diagnose liver fibrosis at an earlier stage of the disease (preferably at F1 or F2) and prevent progression. Unfortunately, most diagnostic tools focus on the diagnosis of advanced liver fibrosis. An example is the NFS (NAFLD fibrosis score). The NFS diagnoses advanced liver fibrosis with a receiver operating characteristic (ROC) of 0.85.[13] The ROC measures the probability to distinguish patients from healthy persons. When the maximum of ROC (1) is achieved, the model can distinguish patients from healthy persons perfectly.[14] In addition, the ELF (enhanced liver fibrosis) panel also diagnosis liver fibrosis in advanced stages with a ROC of 0.90.[13] So, new diagnostic tools are necessary to diagnose liver fibrosis in the early stages. To find a new way for the diagnosis, the bidirectional interaction between the intestinal microbiome and the diseased liver is of interest.[15] This interaction is also called: 'the gut-liver axis'.[16]

The effects of liver fibrosis on the intestine

The liver and the intestine are connected via the portal vein. Nutrients from the food can be absorbed through the intestinal membrane and enter the liver via the portal vein. In the liver, these nutrients are metabolized, set aside, and/or released into the circulatory system.[15] The intestine also contains a microbiome that is composed of bacteria, viruses, fungi, and archaea. These organisms live in mutualism with the host and can be influenced by the liver.[16] Bile acids and other mediators are produced in the liver and enter the intestine via the biliary tract. They mainly act as digestive surfactants, enhancing the digestion of lipids, but they also have antimicrobial functions. These antimicrobial activities help to keep the bacteria in balance and keep the intestine in a good health.[15]

The intestinal barrier prevents bacteria and bacterial products to enter the circulatory system and the liver.[16] This barrier is composed of several compartments that are important for intestinal permeability. Commensals are present in the lumen, they are part of our microbiome and produce antimicrobial products to keep the balance between our microbiome and pathogens. Additionally, the layers with mucous, unstirred water, and glycocalyx prevent the adhesion of bacteria to the intestinal wall. Furthermore, the epithelial cells of the intestinal membrane prevent the entering of bacteria into the circulatory system. These epithelial cells are closely packed together and are separated by tight junctions.[17]

However, fibrotic processes in the liver can influence the intestinal microbiome and permeability. As explained before, cytokines are released by Kupffer cells during fibrosis. These cytokines (for example IL-1, IL-6, IL-8, and TNF) are released from the liver, enter the circulatory system, and influence the membrane of the intestine. They increase the permeability, leading to an increased translocation of bacteria (and bacterial products) over the intestinal membrane into the circulatory system.[15] Also, the factors that cause fibrosis (for example alcohol use) can directly influence the microbiome of the intestine and thereby the permeability.[15] Fibrosis has many causes and each of these causes can influence the intestine differently.

Excessive alcohol abuse can result in alcoholic liver disease (ALD), which is connected to intestinal dysfunction.[16] It enhances/diminishes the growth of specific bacteria from our microbiome, and this leads to changes in composition. Also, inflammatory processes happen in the intestine due to alcohol abuse, which increases the number of monocytes and macrophages in the intestine. These cells produce cytokines such as TNF-α, which can bind to the TNF receptor-1. Upon binding, the tight junctions between epithelial cells are broken down, which makes the membrane leakier and increases intestinal permeability.[18] Especially Gram-negative bacteria, such as *Enterobacteriaceae* and *Proteobacteria*, are increased in the gastrointestinal tract due to chronic alcohol use.[15][16] These bacteria produce the endotoxin lipopolysaccharides (LPS), which can be translocated over the intestinal membrane due to the increased permeability. This means that LPS is present in the portal vein and enters the liver. Furthermore, the phyla Bacteroidetes and Firmicutes are diminished in patients with alcoholic liver disease.[16] The effects of alcohol on the microbiome can be seen long before the fibrotic process occurs.

Obesity can lead to NAFLD, which may progress into NASH and cirrhosis. This leads to bacterial overgrowth in the intestine of Gram-negative bacteria, especially *Proteobacteria*.[16] LPS is produced and can be translocated into the portal vein and the same effects can be seen as with ALD. Also, dysbiosis changes the effects of bile acids. It lowers the conversion of primary bile acids into secondary ones and therefore lowers the activation of nuclear bile acid receptor (FXR). This leads to increased permeability and therefore more translocation of bacteria from the intestine to the liver.[19] Dysbiosis also changes the intestinal metabolism of food, leading to increased production of endogenous ethanol and short-chain fatty acids, while choline will be reduced. Ethanol can be metabolized into acetaldehyde by alcohol-metabolizing enzymes that are present in bacteria of the microbiome. Acetaldehyde weakens the tight junctions and downregulates antimicrobial peptides in the intestine.[18]

Impaired bile flow from the liver to the intestine can result in cholestatic liver diseases. These liver diseases are characterized by the build-up of bile acids in the liver, leading to chronic injury and fibrosis.[15] As mentioned before, bile acids have antimicrobial properties and keep the balance between bacteria in the intestine. When fewer bile acids enter the intestine, some bacteria can overgrow and change the composition. This leads to an increased intestinal barrier and leakage of bacteria (and bacterial products) into the circulatory system.

Furthermore, hepatitis B and C viruses can lead to liver fibrosis due to chronic injury. Especially the hepatitis C virus influences the intestinal microbiome and permeability. There is an overgrowth of *Clostridiales*

bacteria, Enterobacteriaceae, Lachnospiraceae, and *Ruminococcaceae*.[15] Due to the change in the composition of these bacteria, the tight junctions between the epithelial cells are broken down and the permeability of the membrane increases.

The effects of the intestine on liver fibrosis

As explained before, the intestine contains a microbiome with bacteria (and other organisms) that live in mutualism with the host. The bacteria from the microbiome help with the digestion of food and prevent the overgrowth of other pathogenic bacteria by producing antimicrobial products. During liver fibrosis, the intestinal permeability increases because tight junctions are broken down (under the influence of TNF-α).[18] Also, the composition of the microbiome changes and there is bacterial overgrowth. The effects of the increased intestinal permeability and dysbiosis can be seen in figure 2.[16]

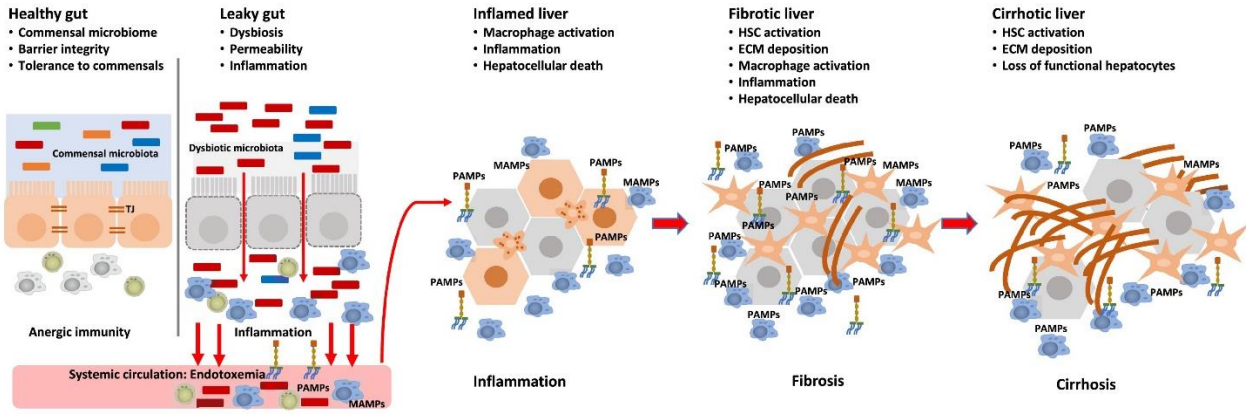


Figure 2: Effects of increased intestinal permeability and dysbiosis on the liver.[16]

The bacteria or their products (microbe-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs)) can translocate over the leaky intestinal wall and enter the lamina propria.[16][18] In the lamina propria, immune cells are present that can be activated by these bacteria/bacterial products. They reach the portal vein and enter the liver where they can activate Kupffer cells. This activation happens when they bind to pathogen recognition receptors on Kupffer cells. For example, the Toll-like receptor-2 can be activated by Gram-positive bacteria and the Toll-like receptor-4 can be activated by endotoxins (LPS).[18] Upon binding, they activate a downstream cascade, leading to the production of cytokines and chemokines. These cytokines activate hepatic stellate cells (HSC), and they transform into myofibroblast-like cells. These cells produce ECM and therefore cause fibrosis/cirrhosis.[2] In addition, the Toll-like receptor-4 downregulates Bambi, which is a decoy receptor for TGF-β. Decoy receptors bind cytokines (in this case TGF-β) and therefore inhibit their action and their binding to other receptors. Due to the decreased expression of Bambi, more TGF-β is available, leading to more inflammation and liver damage.[18]

Also, more intestinal bacteria may lead to increased production of trimethylamine (TMA) from choline in NAFLD. Choline is taken up via the diet and can be converted into phosphatidylcholine by the host or into TMA by bacteria.[18] Phosphatidylcholine is important for the prevention of the accumulation of fats in the liver and therefore prevents liver steatosis. However, when more bacteria are present in the intestine (bacterial overgrowth), more choline is converted into TMA. Therefore, less choline is available to be converted into phosphatidylcholine which leads to an accumulation of fats in the liver. Moreover, the amount of choline in the intestine is reduced due to dysbiosis, resulting in an even lower production of phosphatidylcholine and an increased accumulation of fat. This enhances the fibrotic process in the liver and results in liver damage.[18]

To summarize, liver fibrosis increases intestinal permeability, leading to an increased translocation of bacteria (and bacterial products) from the intestine to the portal vein.[15] The factors that cause liver fibrosis itself can also influence the intestinal permeability and the composition of the microbiome, leading to a leakier membrane and bacterial overgrowth.[16] Bacterial overgrowth does not only lead to more bacteria in the blood, but also to more bacteria in the stool of patients. When the bacteria (and bacterial products) translocate over the intestinal membrane, they can reach the liver and cause an immune response. Cytokines will be produced, and myofibroblast-like cells produce ECM proteins that form scars in the liver tissue.[2][18] Therefore, a vicious circle is happening; liver fibrosis induces bacterial translocation from the intestine to the blood, which again induces liver fibrosis.

Research Question

As mentioned before, new diagnostic tools are necessary to diagnose liver fibrosis in the early stages. Because the intestinal microbiome and the liver are closely related, it might be possible to use this interaction for the prediction of early liver fibrosis. This leads to the following research question: '*Can a bacterial biomarker from the blood or stool be used as a diagnostic tool for liver fibrosis in stage F1 or F2 of the disease?*'.

Hypothesis

Nowadays, most diagnostic tools of liver diseases focus on the diagnosis of advanced liver fibrosis (F4).[13] Early liver fibrosis is hardly detected, due to the lack of symptoms and lack of early diagnostic tools. Symptoms are only expressed when the disease has progressed into cirrhosis.[4] Cirrhosis is an irreversible state of liver disease, meaning that diagnosis often comes too late and the only treatment possible is liver transplantation. For that reason, new diagnostic tools are necessary to improve the prognosis of chronic liver diseases.

As mentioned before, the liver and the intestine are closely related. Liver fibrosis increases the permeability of the intestinal wall and changes the composition of the microbiome.[15] This means that there will be bacterial overgrowth in the intestine, resulting in bacterial translocation over the intestinal barrier. Ideally, there is a bacterial biomarker in the blood of patients who are in the early stages of liver fibrosis, that can be used to diagnose the disease. However, there is also a changed bacterial composition in the stool of patients that might be used to diagnose liver fibrosis. In addition, too many similarities can be expected between patients in stage F1 and healthy individuals, meaning that a diagnostic tool in F2 might be more feasible. Therefore, it is expected that liver fibrosis can be diagnosed in stage F2 by either measuring a bacterial biomarker in the blood or by identifying bacteria in the stool.

Bacterial translocation from the intestine to the liver

As mentioned in the introduction, bacterial translocation from the intestine to the liver is extremely important for the progression of liver fibrosis. There is a vicious circle, where liver fibrosis leads to dysbiosis and increased intestinal permeability, which in turn leads to liver fibrosis. To elaborate on the effects of bacterial translocation on the disease progression, the bacterial influences on the progression of isolated steatosis into NASH and the progression of other liver diseases will be explained.

Bacterial influences on the progression of steatosis

Worldwide, 25% of the adults are suffering from non-alcoholic fatty liver disease (NAFLD) caused by obesity or metabolic diseases.[20] NAFLD includes multiple diseases; from isolated steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. Only a small amount of patients with isolated steatosis develops NASH. NASH can develop further into cirrhosis, which is an irreversible state of the disease and may lead to hepatocellular carcinoma. However, when NASH is diagnosed early, it is still possible to go back into isolated steatosis or even healthy states.[20] Therefore, it is extremely important to find a specific biomarker (preferably in the blood of patients) that indicates which patients are at high risk for developing NASH and which patients are only at low risk. To find this biomarker, the key factors that play a role in the progression from NAFLD to NASH need to be determined.

In a study by Qian et al[20], the changes of the intestinal microbiome and lipidome differentiates are investigated in the transition from steatosis to steatohepatitis. Ten mice with identical properties (genetic background, weight, etc.) received the same fatty diet to induce NAFLD. Half of the mice developed isolated steatosis and half developed NASH, which was not intended. This confirms the need for a specific biomarker to determine which mice (or humans) with isolated steatosis will develop NASH and which not. The mice were classified into two groups: one group with steatosis and one group with steatohepatitis. The transcriptome, gut microbiome, and lipidome of these mice were studied, and compared to humans. In this study, the plasma from 14 humans with NAFLD (9 with NASH and 5 with isolated steatosis) was used to analyse the lipidome.[20]

The lipidome in the plasma from mice and humans was examined, and it was seen that the overall amount of lipids in the plasma was similar in NASH and isolated steatosis. However, there were changes seen in the number of specific lipids in NASH cases compared to isolated steatosis and these patterns were the same in humans and mice. Some lipids increased in amount with NASH, while others decreased. From this study, it was concluded that the lipids free fatty acid(20:1), ceramide(d34:1), and phosphatidylcholine(18:1/18:2) were positively correlated to the process of hepatic ballooning.[20] This indicates that it might be possible to use these lipids in the diagnosis of patients with isolated steatosis that will progress into NASH.

The lipidome in the liver of mice was also determined. Here it was seen that most lipids were present in lower amounts in NASH compared to isolated steatosis. While many lipids were decreased, the LysoPC group was increased in NASH. The transcriptome in the liver of mice was determined to see the mechanism for the decreased/increased lipids in the liver. It was seen that triglyceride and ceramide synthesis and hydrolysis were decreased, while the LyscoPC and free fatty acid synthesis was increased.[20]

Furthermore, the gut microbiome of the mice with isolated steatosis was compared to mice with steatohepatitis. The phylum *Bacteroidetes* was decreased in NASH, leading to a lower *Bacteroidetes/Firmicutes* ratio, which was also seen in humans.[20][21]. Moreover, a decrease in the genus Bacteroides, a decrease in species *B. uniformis*, and an increase in species *M. schaedleri* were seen. They showed that these species were also connected to the lipidome, meaning that the gut microbiome influences the transition from steatosis to steatohepatitis. A decrease in *B. uniformis* leads to a decrease in triglycerides and an increase in free fatty acids, while an increase in *M. schaedleri* causes an increase in free fatty acids and an increase in groups of LyscoPC.[20] These changes in lipids were important for the transition from steatosis to steatohepatitis, and therefore the gut microbiome is also important in the disease progression.

Based on the study by Qian et al., it was concluded that the microbiome influences the progression from isolated steatosis to NASH. This study was based on mice, however, findings were confirmed using human plasma samples and other studies. Therefore, it might be possible to use specific bacterial biomarkers that

indicate whether a patient is at high risk of developing NASH. However, further research is necessary to demonstrate the full effect of the changes in the human microbiome on the transition from steatosis to steatohepatitis.

Bacterial influences on the progression of other liver diseases

Alcoholic liver disease

To see whether bacterial translocation does not only influence the transition of isolated steatosis to NASH but also the progression of other liver diseases, the development of alcoholic liver diseases was examined. Alcoholic liver disease is caused by excessive alcohol use and when the liver is inflamed it is called alcoholic hepatitis. However, the relationship between excessive alcohol use and alcoholic hepatitis is not straightforward. Not all individuals that chronically drink alcohol develop alcoholic hepatitis, meaning that there could be other factors involved in the development of the disease, for example the microbiome.[22] Therefore, it is important to find a biomarker (in the blood/stool of individuals) that indicates which alcoholics are at high risk for developing alcoholic hepatitis and which are at low risk.

In a study by Smirnova et al.[23], the relation between the faecal microbiome and the development/severity of the alcoholic liver disease is established. In total, 78 individuals were enrolled in the study and these individuals were placed in 4 different groups. One group contained the healthy controls (24 individuals), while another group contained heavy drinking controls (20 individuals). These controls did not have signs of liver fibrosis yet. Furthermore, the patients with alcoholic hepatitis were divided into two groups; one with moderate alcoholic hepatitis (10 individuals) and one group with severe alcoholic hepatitis (24 individuals).[23] Based on the results, they concluded that there are differences in the intestinal microbiome of patients with alcoholic hepatitis compared to the heavy drinking controls. However, there were no differences in the microbiome of patients with severe alcoholic hepatitis and moderate alcoholic hepatitis. The bacterial diversity of the healthy controls and the heavy drinking controls also did not differ from each other, while the bacterial diversity of alcoholic hepatitis (severe and moderate) was decreased compared to the controls. This might indicate that the bacterial composition can be a key factor in the development of alcoholic hepatitis. The bacterial microbiome of the heavy drinking controls and the patients with alcoholic hepatitis can be compared to each other to see the differences in bacteria. Especially the families *Ruminococcaceae* and *Lachnospiraceae* were important in the transition to alcoholic hepatitis. The differences in these families in the microbiome of patients with alcoholic hepatitis and the heavy drinking controls can be seen in figure 3.[23]

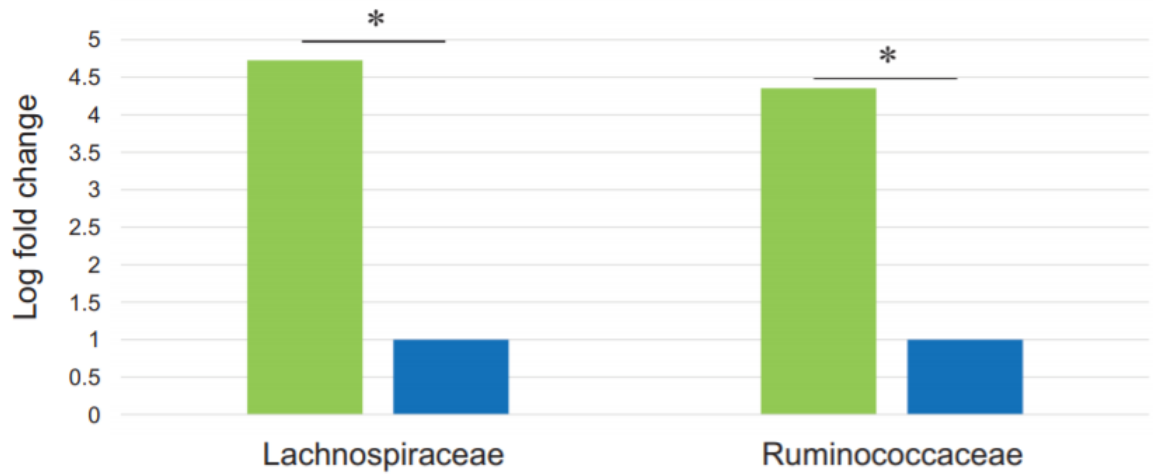


Figure 3: The differences in Lachnospiraceae and Ruminococcaceae in heavy drinking controls (green) and patients with alcoholic hepatitis (blue).[23]

In this figure, it can be seen that the families *Ruminococcaceae* and *Lachnospiraceae* were decreased in patients with alcoholic hepatitis. These families are the main producers of short-chain fatty acids in the intestine. Short-chain fatty acids have a protective role against liver diseases, and a decrease might therefore be a contributing factor in the development of alcoholic hepatitis. They play a role in the maintenance of the intestinal barrier, control inflammation, and influence hepatic metabolism.[23] Therefore, a decrease in the short-chain fatty acid-producing bacteria might be of importance for the development of alcoholic hepatitis.

These findings suggest that not only in NAFLD but also in ALD, the progression to hepatitis (non-alcoholic steatohepatitis and alcoholic hepatitis) is associated with changes in the microbial composition. However, there are more liver diseases where the intestinal microbiome might influence the progression of these diseases. One of these is cholestatic liver disease, caused by impaired bile flow or formation. Cholestatic liver diseases can be divided into primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC).[24]

Cholestatic liver diseases

A review article by Blesl et al.[24] combines existing information about the gut microbiome in cholestatic liver diseases. They mention several changes in the microbiome of patients with primary biliary cholangitis and primary sclerosing cholangitis, such as an increase in *Veilonella* species and a decrease in the genus *Faecalibacterium*. *Veilonella* species produce LPS, which can cross the intestinal barrier (due to increased permeability) and reach the liver. In the liver, LPS can induce inflammation and progress the disease. *Faecalibacterium prausnitzii* has anti-inflammatory effects, and when there is less of this bacterium present in the intestine, there will be more inflammation.[24] This means that the progression of PSC and PBC is associated with the changes in the microbiome of these patients.

Hepatitis B and C

Another factor that can cause liver disease is the hepatitis virus. There are two important hepatitis viruses related to liver diseases: HBV (hepatitis B virus) and HCV (hepatitis C virus). When someone is infected with the hepatitis B virus, acute-on-chronic liver failure might arise. However, not all individuals that are infected will develop hepatitis B virus-associated acute-on-liver failure (HBV-ACLF). This means that other factors (such as the microbiome) might play a role in the development of HBV-ACLF.[25]

In a study by Yao et al.[25], the relationship between the microbiome and the progression of HBV-ACLF was examined. Stool samples of 91 patients with HBV-ACLF were collected and these patients were placed in different groups according to their stage of the disease. Nine patients experienced each stage during their treatment, and therefore 103 faecal samples were collected in total. Patients in stage 1 (N=39) were in the rising stage, patients in stage 2 (N=34) were in the plateau stage and patients in stage 3 (N=36) were in the recovery stage. Furthermore, 30 control samples were used of individuals without hepatitis.[25]

When comparing all HBV-ACLF groups to the control groups, the phylum *Bacteroidetes* decreased while the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria* increased. When the liver disease progresses from stage 1, it ends up in stage 2. However, when the disease will be treated, it can end up in stage 3 (which is better than stage 1 or 2). So, to see if intestinal bacteria influence the progression of the disease, it is important to look at the bacteria in stage 1 that may lead to the progression into stage 2. Table 1 shows the changes in bacteria in the different stages, compared to the stage before.[25]

Table 1: Changes in the intestinal microbiome between different stages of HBV-ACLF.[25]

Stage 1 (compared to control) Rising stage		Stage 2 (compared to stage 1) Plateau stage		Stage 3 (compared to stage 2) Recovery Stage	
Increase	**Decrease**	**Increase**	**Decrease**	**Increase**	**Decrease**
Veillonella	Bacteroidetes	Veillonella	Bacteroidetes	Bacteroidetes	Enterococcus
Enterococcus	Prevotella	Enterococcus		Megamonas	
Streptococcus	Megamonas				
Klebsiella					
Lactobacillus					
Blautina					

In table 1, an increase in *Streptococcus*, *Klebsiella*, *Veillonella*, *Enterococcus*, *Lactobacillus*, and *Blautina* can be seen in stage 1. Especially *Enterococcus* and *Klebsiella* are pathogenic and may lead to damage to intestinal cells. Therefore, more bacteria/bacterial products can reach the liver and progress the disease further. Furthermore, the phylum *Bacteroidetes* is decreased in stage 1 and further decreased in stage 2. These bacteria are protective against liver diseases, and when these bacteria are decreased, the liver disease progresses further.[25] When the patient gets into the recovery phase, the phyla *Bacteroidetes* and *Megamonas* increases again, while *Enterococcus* decreases. Therefore, it is likely that these bacteria are involved in the development of HBC-ACLF.[25]

To conclude, it is likely that bacteria are involved in the development of HBV-ACLF. It might be possible to use bacterial biomarkers as an indication if an individual infected with HBV will develop HBV-ACLF. However, further research is necessary to confirm these findings and see which biomarkers might be used. Also, the progression of liver diseases induced by the hepatitis C virus is associated with changes in the gut microbiome. In the different stages, the gut microbiome changes, and this is related to further disease progression.[26]

This means that the progression of liver diseases is likely caused by changes in the intestinal microbiome and translocation of bacteria/bacterial products to the liver. Therefore, it might be of great importance to see whether these microbial changes (in the blood or stool of patients) can be used to diagnose early liver diseases. To do so, the identity and quantity of bacteria must be determined, which will be explained in the next section.

Identifying/quantifying bacteria

As mentioned before, the composition of the bacterial microbiome changes in chronic liver diseases and influences fibrosis.[18][20] Some bacteria are increased during the fibrotic process and some bacteria are decreased, depending on the cause of the disease.[15] In general, many bacteria will overgrow in the intestine and this causes increased intestinal permeability. There will be bacterial translocation from the intestine to the liver, which enhances the fibrotic process.[18] Also, there will be a different microbial composition in the stool and blood of these patients. To measure the alterations of the microbiome of patients with liver fibrosis compared to healthy patients, the bacterial composition must be identified and quantified.

To identify and quantify bacteria in the blood or stool of patients with liver fibrosis, it is important to distinguish the bacterial cells from the human cells. Bacteria are prokaryotic microorganisms, meaning that they do not have a membrane-bound nucleus and other membrane-bound structures in their cytoplasm (for example mitochondria). Other major differences between bacteria and human cells are the cell wall

and the ribosome. Bacterial cells contain a cell wall made of peptidoglycan, while human cells do not have a cell wall. Also, the bacterial ribosome differs from the human ribosome. In general, the ribosome contains proteins and ribosomal RNA (rRNA), that are present in two subunits. The human ribosome subunits are 40S and 60S, while the bacterial ribosome subunits are 30S and 50S. The differences between the human and bacterial ribosome can be used to distinguish bacterial from human cells. The 30S part of the bacterial ribosome contains 16S rRNA, which is specific for each bacterium and is used to identify and quantify bacteria.[27]

The 16S rRNA gene contains 9 hypervariable regions: V1 to V9.[27] In most studies, only specific variable regions (for example V4) from the 16S rRNA gene are used to identify and quantify bacteria. The PCR technique is used to amplify these regions, using a forward primer and a reverse primer.[28] The DNA from bacteria in the blood and stool can be extracted and isolated.[13][28] This DNA is only present in extremely low amounts and needs to be multiplied several times when you want to study it. This can be done by the PCR technique (Figure 4). The first step of the PCR technique is heating the sample. The double-stranded DNA is denatured, and two single-strand DNA chains are created.[29] The primers can bind to the single-strand DNA chains, leading to the process called 'annealing'.[28] After this, extension happens, where DNA-polymerase synthesizes a new DNA strand. This process can be repeated several times until enough DNA is made that can be studied.[29] The 16S rDNA is amplified and can be analysed quantitatively and qualitatively.

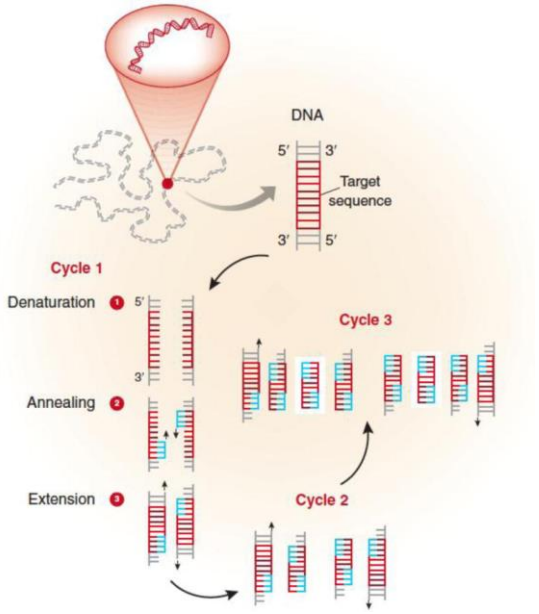


Figure 4: The PCR technique: denaturation, annealing, extension, and repeat.[18]

In a study by Johnson et al.[27], bacterial identification using 16S rRNA sequencing is evaluated. They showed that full 16S rRNA genome sequencing results in better taxonomic resolution compared to short-read sequencing (sequencing of only specific variable regions).[27] In many studies, short-read sequencing was used due to technological restrictions. They found that the V4 sub-region was the most inferior in matching their region to the original taxonomic level. Only 44% of the amplicons were able to match their sequence to the original taxonomic level. If the full length of the 16S rRNA was used to identify bacteria, it was possible to match all sequences with the correct species.[27] This can also be seen in figure 5, where the trees are presented based on the taxonomy of sequences. The redder colour is present in the trees, the higher the percentage of unclassified sequences. It can be seen that the full 16S rRNA gene sequencing (V1-V9) results in a white tree, and therefore no unclassified sequences. When amplifying the V4 region only, the reddest coloured tree can be seen and therefore the most unclassified sequences.[27] Therefore, it can be concluded that full 16S rRNA sequencing is better than sequencing sub-regions.

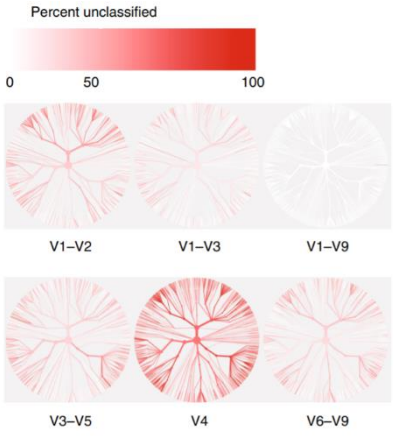


Figure 5: Percent unclassified sequences in trees.[17]

So, the hypervariable regions of the 16S rRNA gene are specific for bacterial species and can be used to quantify and qualify bacteria in the stool or blood of patients with early liver fibrosis. Preferably, the full

16S rRNA gene will be amplified and studied, in order to get the best results. The use of 16S rRNA as a diagnostic tool for early liver fibrosis will be mentioned in the next sections.

Bacterial biomarker in the blood

During liver fibrosis, the permeability of the intestinal wall increases and there is bacterial overgrowth in the intestine. Therefore, more bacteria (and their products) can cross the intestinal barrier and enter the bloodstream. As explained before, this process enhances the fibrotic processes even further. Because most diagnostic tools focus on the diagnosis of advanced liver fibrosis, new diagnostic tools are necessary to diagnose liver fibrosis in the early stages. Ideally, this could be possible by measuring a specific bacterial biomarker in the bloodstream because of the increased translocation. These bacterial patterns might then be used to diagnose liver fibrosis in patients that are suspected to develop early liver fibrosis (for example due to excessive alcohol use or obesity).

To understand the differences in bacteria, it is important to know the bacterial taxonomy. The kingdom of bacteria can be classified into 6 taxonomic ranks. Starting with phylum, then class, order, family, genus and finally species.[28] The main bacterial taxa that are associated with liver diseases are from the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria*.[15][30] They are related to liver fibrosis and the progression of liver fibrosis into cirrhosis.

Patients with NAFLD

A study by Champion et al.[31] identified and quantified the bacteria that are present in the blood in patients with early liver fibrosis (stage F1 or F2) and in healthy individuals (stage F0). Microbiota can be present in the liver if there is bacterial translocation possible from the intestine to the portal vein. As mentioned in the introduction, the intestinal permeability increases during liver fibrosis and therefore more bacteria can translocate over the intestinal membrane. In this study, patients with obesity from different European countries were included that are in the early stages of liver fibrosis (F1 or F2) or have no liver fibrosis (yet) (F0). Therefore, only the microbiota of NAFLD is examined, not of other liver diseases (such as ALD, cholestasis and hepatitis B/C). In total, there were 34 people with stage F0, 37 patients with F1 and 11 patients with F2 included.[31]

The identity and quantity of bacteria were determined using 16S rDNA sequencing (V3-V4 hypervariable regions). In all participants, the phyla *Proteobacteria* and *Actinobacteria* were most present in the liver. From the phylum *Proteobacteria*, the families *Pseudomonadaceae* and *Enterobacteriaceae* were dominating the most. This can also be seen in figure 6 and 7.[31] In figure 6, it can be seen that the percentage of *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* differs between people without liver fibrosis and patients with stage F1 of liver fibrosis. The increase in *Proteobacteria* and the decrease in *Firmicutes*, *Bacteroidetes* and *Actinobacteria* are significant.[31] In figure 7, it can be seen that the percentage of the families *Flavobacteriaceae*, *Caulobacteraceae* and *Propionibacteriaceae* differs significantly between F0 and F1. In both figures, the liver microbiota in F0 differed from the liver microbiota in F1 and F2.[31]

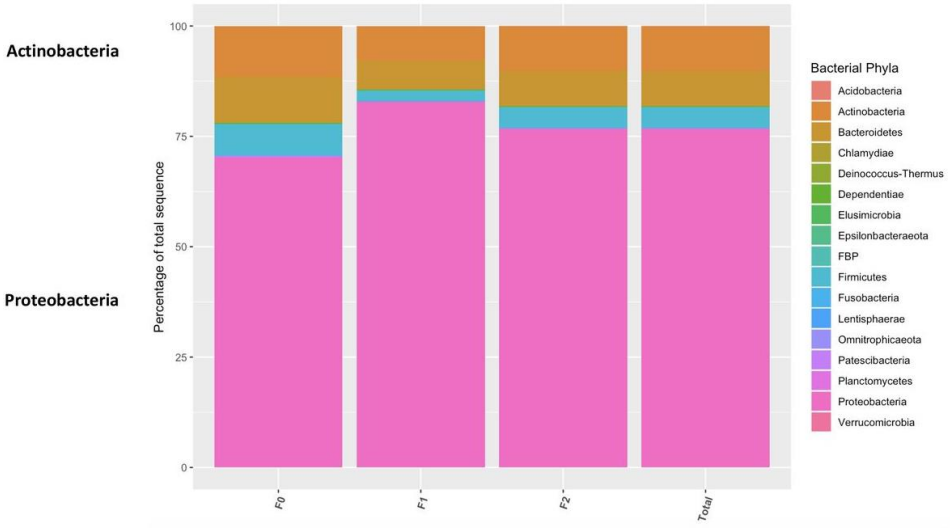


Figure 6: The percentage of bacterial phyla in different stages of liver fibrosis.[31]

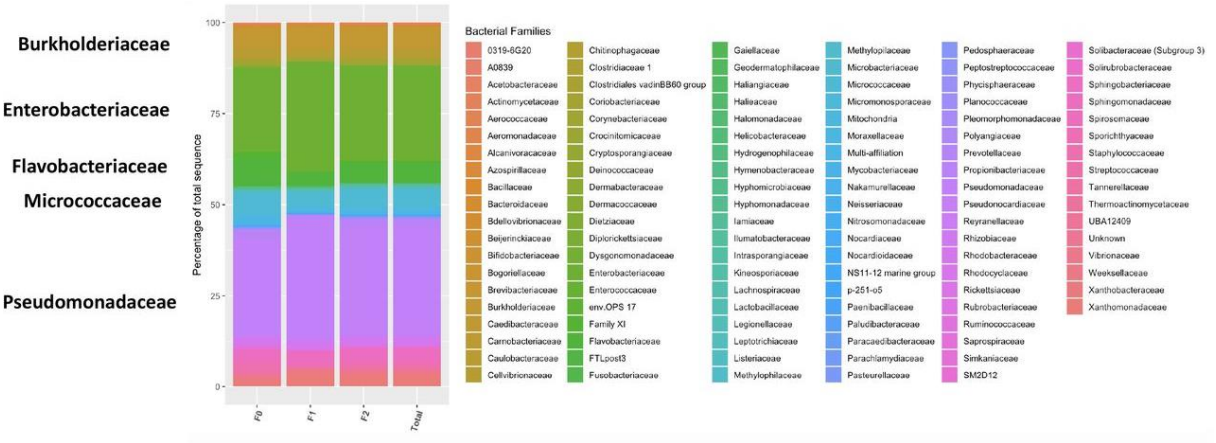


Figure 7: The percentage of bacterial families in different stages of liver fibrosis.[31]

When performing LEFSe, they discovered that there were almost no differences in the bacteria between F1 and F2. Therefore, these stages cannot be discriminated from each other when using liver microbiota as a diagnosis of liver fibrosis. However, they did find that early stages of fibrosis could be identified by biomarkers from the families of the *Proteobacteria* phylum. Especially the family *Enterobacteriaceae* from this phylum seems to be important in the progression of liver fibrosis, as it is present in high amounts in the liver. This is a gram-negative bacterium, meaning that LPS is produced which induces inflammation.[31]

A study by Lelouvier et al.[13] investigated if blood microbiota could be a possible biomarker of liver fibrosis in patients with NAFLD. They measured the concentration of 16S rDNA in the blood of patients (Spanish cohort) with the stage F1, F2 or F3 of the disease. They also measured the concentration of 16S rDNA of healthy individuals. These results can be seen in figure 8. It was concluded that patients with liver fibrosis had a significantly higher concentration of 16S rDNA in their blood compared to healthy individuals.[13] This was also expected, as there is increased bacterial translocation from the intestine to the blood

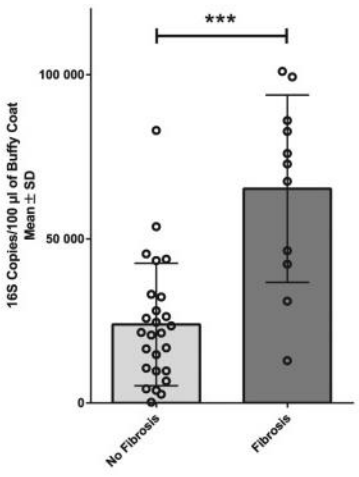


Figure 8: the concentration of 16S rDNA in the blood of healthy individuals versus patients with liver fibrosis.[13]

in patients with liver fibrosis. However, the variance of the results in the fibrotic group is relatively large. More research is necessary to see whether fibrotic patients could actually be distinguished from healthy individuals, using the 16S rDNA concentration. Furthermore, they examined another cohort (Italian cohort), where patients in stage F1 and F2 are present together with healthy controls. Again, the concentration of 16S rDNA in the blood of patients with liver fibrosis (stage F1 and F2) is higher compared to healthy individuals.[13]

When identifying and quantifying the bacteria in the blood of the patients from the Spanish cohort, they found that the phylum *Proteobacteria* dominated the most. Other phyla that were present in the blood of these patients and their proportions can be seen in figure 9. Although the phyla *Proteobacteria* and *Actinobacteria* have the highest proportions in the blood samples, this was not measured in faecal samples. In these faecal samples, they found much higher proportions of *Bacteroidetes* (34.5%) and *Firmicutes* (56.8%).[13] This suggests that there is no straightforward relationship between the bacteria in stool samples and bacteria in blood samples. In addition, the study by Lelouvier et al. measured the changes of bacteria in the stool of the patients and healthy controls. In the Spanish cohort, they found an increase in *Bacteroidetes* and *Fusobacteria* and a decrease in *Firmicutes* and *Actinobacteria*. However, these changes were not found in the Italian cohort.[13]

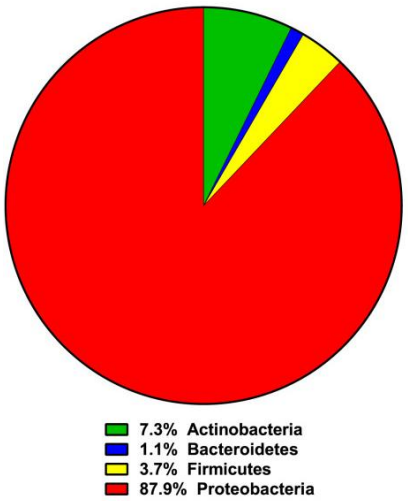


Figure 9: The proportion of phyla present in the blood of patients with liver fibrosis in the Spanish cohort.[13]

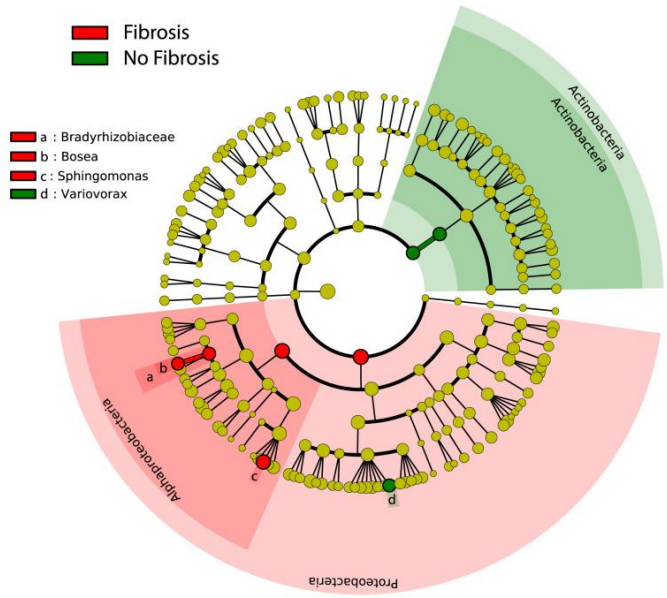


Figure 10: LEfSe Dendrogram comparing the bacteria in the blood of patients with liver fibrosis to healthy individuals.[13]

Using LEfSe analysis, they measured the differences in blood bacteria between patients in the Spanish cohort and the healthy controls. These results can be seen in figure 10.[13]

In figure 10, it can be seen that the *Proteobacteria* (in red) were increased in liver fibrosis, while the *Actinobacteria* (in green) were decreased. Furthermore, significant changes were seen in the family *Bradyrhizobiaceae* and in the genera *Variovorax*, *Bosea* and *Sphingmonas*.[13] These phyla, families and genera might be important in the early diagnosis of liver fibrosis.

When comparing the bacterial differences between the stages of liver fibrosis to the healthy controls in each cohort, the following results could be observed: in stage F1 of the Spanish cohort, an increase in the families *Bradyrhizobiaceae* and *Sphingomondaceae* from the phylum *Proteobacteria* was observed compared to healthy controls. Also, an increase in the genera *Spingomonas* and *Boseae* was seen. However, these findings were not seen in stage F1 of the Italian cohort. Furthermore, a decrease in the genera *Variovorax* from the phylum *Proteobacteria* can be seen in the Spanish cohort, which did not correspond to the data from the Italian cohort. Although stage F1 did not match between the two cohorts, stage F2 (together and without F3) did. These results can be seen in figure 11, where the different stages of liver fibrosis are present on the X-axis and the percentage of bacteria on the Y-axis.[13]

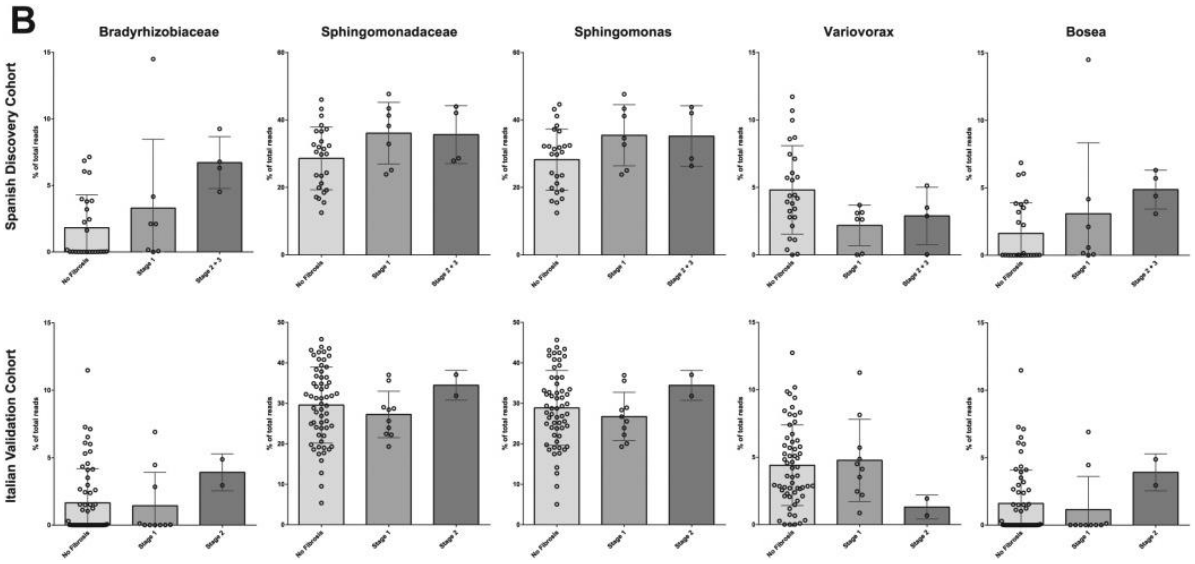


Figure 11: Changes in blood microbiota in the Spanish cohort and the Italian cohort.[13]

The data in the study by Lelouvier et al. suggests that there is a relationship between the concentration of 16S rDNA in the blood and early liver fibrosis in patients with NAFLD. They also suggest that it is not possible to diagnose liver fibrosis in stage F1, as the composition of bacteria in the blood differs a lot between the cohorts. However, it might be possible to diagnose liver fibrosis in stage F2 using bacterial concentration and the composition of the bacteria in the blood. The blood microbiota signature is similar between the two cohorts regarding stage F2 (or F3) of liver fibrosis.[13]

When combining the results from the study by Champion et al.[31] and Lelouvier et al.[13], it can be suggested that bacterial families from the phylum *Proteobacteria* are the most interesting in diagnosing early liver fibrosis in patients suspected to have NAFLD. However, more research is necessary to confirm the findings of these studies. Furthermore, there are also other liver diseases that need to be diagnosed in the early stages. The next section will elaborate on the possibility of using bacterial biomarkers in the blood to diagnose early liver fibrosis in alcoholic liver disease.

Patients with other liver diseases

As mentioned before, the *Proteobacteria* are the most interesting phylum that might be used to diagnose early liver fibrosis in NAFLD. However, the intestinal microbiome of individuals with obesity is different from individuals that consume a lot of alcohol. Therefore, the bacterial translocation is different and the bacteria/bacterial products that are present in the blood will also be different. These circulating bacteria can be measured using 16S rDNA sequencing and possibly be used as biomarkers for the diagnosis of early ALD.

In a study by Puri et al.[32], the blood microbiota is determined in different stages of alcoholic liver disease. They included 76 individuals and these individuals were placed in 4 different groups. There were two control groups without signs of liver fibrosis. These control groups were healthy controls (20 individuals) and heavy drinking controls (19 individuals). The healthy controls did not drink alcohol and the heavy drinking controls chronically used alcohol. Furthermore, there were two patient groups with signs of fibrosis. These were patients with moderate alcoholic hepatitis (18 individuals) and patients with severe alcoholic hepatitis (19 individuals).[32]

They measured the concentration of 16S rDNA in the blood of each individual, these results can be seen in figure 12.[32] In this figure, it can be seen that the concentration of 16S rDNA increases with the severity of the disease. Therefore, it can be assumed that alcohol consumption increases bacterial translocation as well as disease progression. However, the variance of the SAH-group is relatively large.[32]

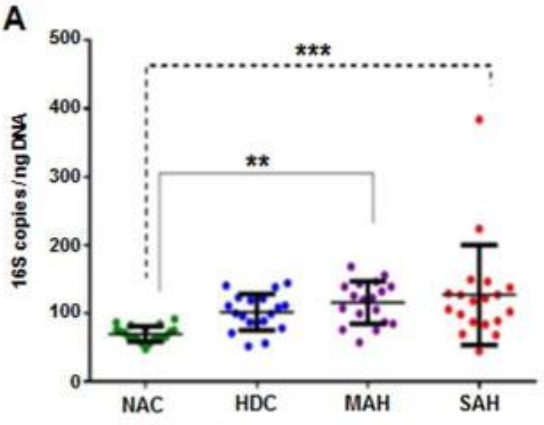


Figure 12: Concentration of circulating 16S rDNA in each group. NAC = not alcoholic controls; HDC = heavy drinking controls; MAH = moderate alcoholic hepatitis; SAH = severe alcoholic hepatitis.[32]

For the early detection of liver fibrosis, the composition of blood microbiota in patients with early liver fibrosis compared to patients with no liver fibrosis is important. Therefore, the transition from individuals that are drinking heavily but do not show signs of liver fibrosis to individuals that have moderate alcoholic hepatitis patients is of most interest. The differences in bacterial composition between these two groups can be seen in figure 13.[32]

It can be seen that the families *Coriobacteriaceae*, *Burkholderriales incertae sedis* and *Streptococcaceae* were enriched in patients with moderate alcoholic hepatitis compared to heavy drinking controls.[26] The genera that were increased in moderate alcoholic hepatitis patients (compared to heavy drinking controls) were *Turicella*, *Cloacibacterium*, *Streptococcus*, *Curvibacter* and *Actinobacter*. The families that were decreased in moderate alcoholic hepatitis patients were *Microbacteriaceae*, *Acetobacteriaceae* and *Oxalobacteraceae*. When comparing all alcoholics (so, heavy drinking controls, moderate alcoholic hepatitis patients and severe alcoholic hepatitis patients) to the healthy controls, they showed a general increase in the phylum Fusobacterium and a decrease in the phylum Bacteroidetes.[32]

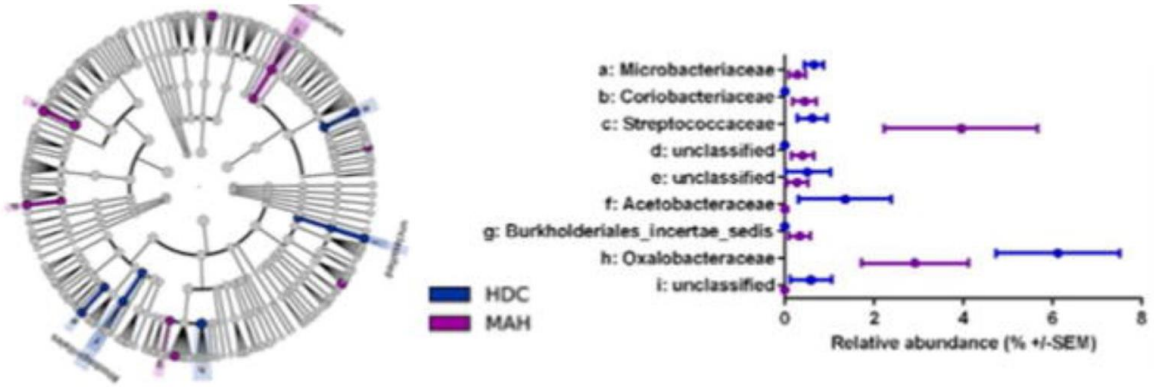


Figure 13: The differences in the bacterial composition of healthy drinking controls (HDC) compared to moderate alcoholic hepatitis (MAH) patients.[32]

However, this was the only study measuring the circulating microbiome in alcoholic liver diseases. So, to confirm these findings and increase the knowledge, more research should be done. Nevertheless, these findings about the blood microbiome in ALD are promising in the process of finding a bacterial biomarker to detect the early stages of the disease.

Furthermore, some studies suggested using bacterial biomarkers in the stool of patients to diagnose early liver fibrosis.[28][30] They quantified and qualified the bacterial composition in the stool, and measured the changes compared to healthy controls. These studies will be mentioned in the next section.

Bacterial biomarker in the stool

As mentioned before, the bacterial composition in the intestine changes during fibrotic processes. Specific bacteria can overgrow in the intestine of patients with liver diseases, while other bacteria might decrease in amounts. This observation is caused by liver fibrosis but also depends on the kind of liver disease. For example, the bacteria in the intestine from patients with alcoholic liver disease differs from the bacteria in the intestine from patients with non-alcoholic liver diseases.[30] Several studies showed the changes in the microbial composition of the stool in patients with advanced liver fibrosis. However, detecting advanced liver fibrosis might be too late and liver transplantation is the only treatment possible for these patients. Only a few studies investigated a microbiota biomarker in the stool for the detection of early liver fibrosis, these studies will be explained further.

In a study by Li et al.[28], the possibility of using a bacterial biomarker in the stool for the detection and staging of liver fibrosis is investigated in rats. They used 131 rats and induced liver fibrosis in 66 of them, using the compound carbon tetrachloride. The rats were placed into 5 groups according to their stages of liver fibrosis: F0, F1, F2, F3 and F4. There were 65 rats in the control group (F0), 15 rats with stage F1 of liver fibrosis, 22 rats with stage F2, 11 rats with stage F3 and 18 rats with stage F4. The bacteria in the stool of these rats were identified and quantified using 16S rRNA sequencing (V4 region).[28]

In the stool of rats with liver fibrosis (F1, F2, F3 and F4), the bacterial community diversity and the community richness was measured and compared to the controls. These results can be seen in figure 14[28], where the bacterial community richness is measured with Chao index and the bacterial community diversity is measured with the Simpson index. The Simpson index in rats with liver fibrosis is higher, meaning that there is less bacterial community diversity. The Chao1 index is lower in rats with liver fibrosis, meaning that there is less bacterial community richness.[28]

However, the community diversity in rats with stage F4 of fibrosis was higher than the community diversity in rats with lower stages of fibrosis. The study also confirmed these findings in humans using results from other studies. In humans with liver diseases (NAFLD, ALD, PBC, PSC, HCV, or HBV), the bacterial community diversity and richness is

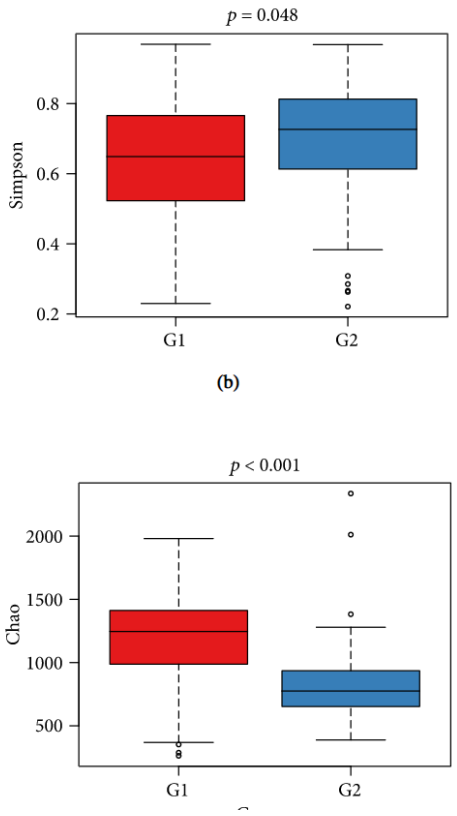


Figure 14: Bacterial community richness and bacterial community diversity in rats. G1 = control group; G2 = liver fibrosis.[28]

decreased compared to healthy persons. However, these human studies did not mention the relationship between the microbiome and liver fibrosis further.[28]

The study by Li et al.[28] identified the bacteria that were present in the stool of rats and quantified these bacteria. They showed that several bacteria (phyla/families/genera) were increased in liver fibrosis, while others were decreased. These different bacterial compositions could be used for diagnostic purposes with an accuracy of 0.99. However, when looking at the different stages of liver fibrosis (F1, F2, F3 and F4), it was not possible to distinguish the stages F1, F2 and F3 from each other. There is too much overlap between these stages and therefore it might only be possible to diagnose liver fibrosis in all these stages but not discriminate them from each other.[28]

Although the study was performed in rats and the differences in stage F1, F2 and F3 could not be distinguished from each other, the study showed promising results for a biomarker in the stool of patients with liver fibrosis. Further research is necessary to confirm these findings in humans and find out if the staging of liver fibrosis is also possible.

In a study by Liu et al.[30], the combination of gut microbiome sequencing and conventional risk factors were examined for the use in early diagnosis of liver diseases. The study used data from 7115 patients that had more than 15 years of electronic health records, and therefore this is a longitudinal study. From these data, 41 patients were included that had incident alcoholic liver disease and 103 patients were included that had other incident liver diseases. The stool samples of these patients were collected, and the bacterial composition was measured using shallow shotgun metagenomics.[30]

They made an overview of the bacteria from the stool that showed predictive signals for early-liver diseases, which can be seen in figure 15 on the next page. In this overview, bacteria are present from phylum to family level, and it might be possible to use them in the diagnosis of liver fibrosis in the early stages.[30] Some of the bacteria are present in larger amounts in liver fibrosis (compared to healthy individuals), but also some are present in smaller amounts.[15]

With alcoholic liver disease, an increase in the families *Streptococcaceae*, *Enterobacteriaceae* and *Chitinophagaceae* was seen in the stool of patients. Also, the genera *Rikenella*, *Blautia*, *Neisseria*, *Actinomyces* and *Dorea* were enriched. With non-alcoholic liver disease, the families *Streptococcaceae*, *Enterobacteriaceae* and *Erysipelotrichaceae* were increased in the stool of patients. Furthermore, the genera *Lactobacillus*, *Veilonella*, *Actinomyces* and *Prevotella* were also increased. It should be noticed that *Streptococcaceae*, *Enterobacteriaceae* and *Actinomyces* increases in both NAFLD and ALD. While many bacteria are enriched in liver diseases, the *Faecalibacterium*, *Akkermansia* and *Coprococcus* were decreased. These bacteria might have protective effects to prevent liver diseases. All these findings were also mentioned in previous studies.[30]

When they combined the microbiota from the stool together with conventional risk factors (obesity, alcoholism etc.), they could predict an early non-alcoholic fatty liver disease with an AUROC of 0.834 and early alcoholic liver disease with an AUROC of 0.956.[30]

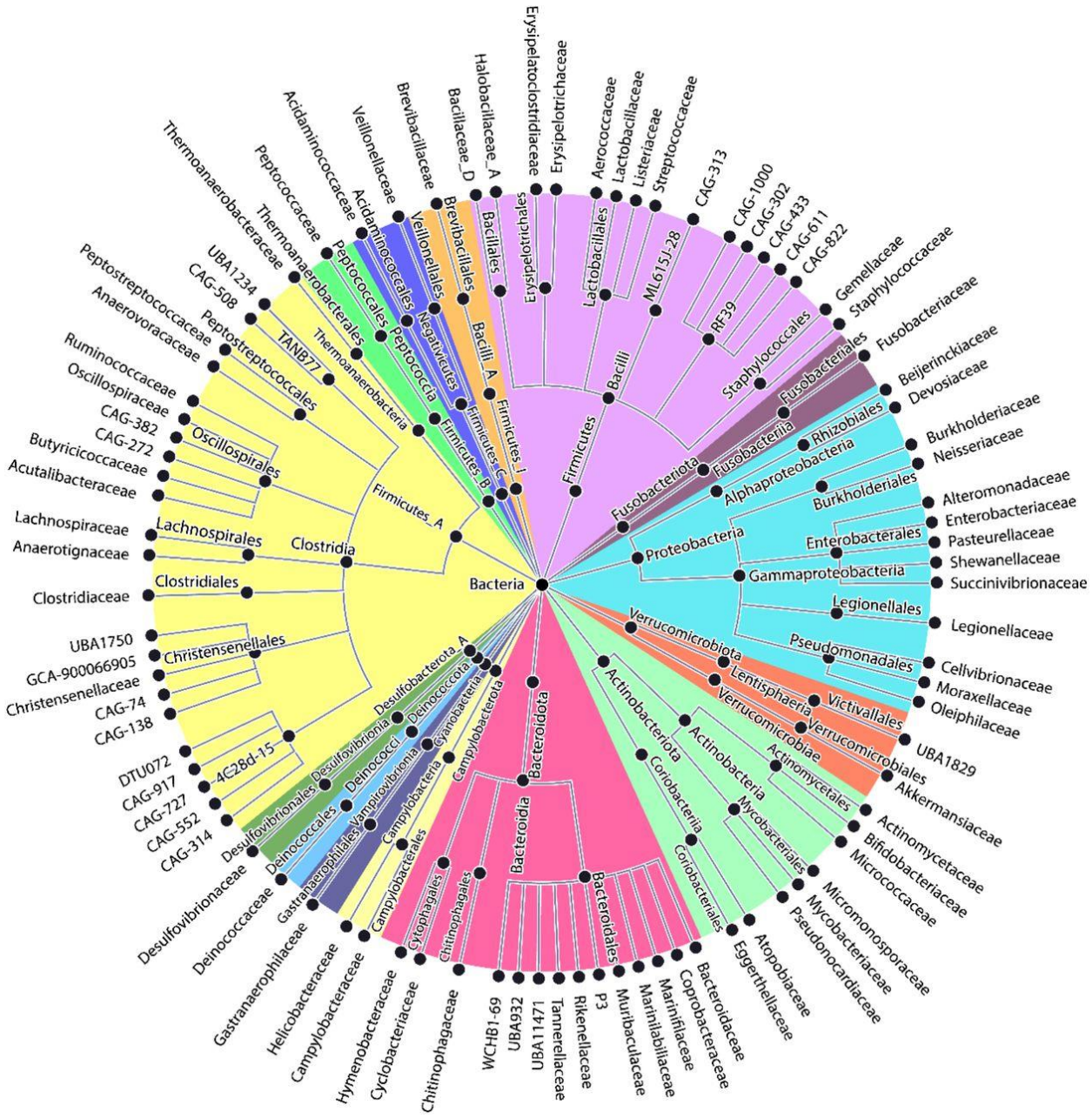


Figure 15: Bacteria in the stool that can be used to predict liver fibrosis.[30]

Discussion

As taking liver biopsies for the diagnosis of liver fibrosis is difficult, invasive, and often comes too late, other methods were examined that can replace the biopsies.[13] In this literature study, the possibility of using a bacterial biomarker in the blood or in the stool of patients to diagnose liver fibrosis in stage F1 or F2 is examined. The studies from Champion et al.[31], Lelouvier et al.[13] and Puri et al.[32] examined if blood microbiota can be used for the detection of early liver fibrosis. They measured the concentration of 16S rDNA in the blood of patients and/or identified and quantified these bacteria. The studies from Li et al.[28] and Liu et al.[30] determined if stool microbiota could be used to predict early liver fibrosis. They looked at the identity and quantity of these bacteria in the faeces. All studies that were mentioned previously concluded that it was possible to use a bacterial biomarker in the blood or in the stool of patients for the detection of early liver fibrosis. However, these findings were only based on a few studies (3 for in the blood; 2 for in the stool) and might therefore not be extremely reliable. Furthermore, many studies only measured the microbiota in a few patients (or animals) and therefore more research is necessary. Most studies included patients with ALD and/or NAFLD, while no study used patients with cholestatic liver diseases or hepatitis B/C induced liver diseases. To see if it is also possible to use a bacterial biomarker for the detection of these liver diseases, it is necessary to investigate the changes in stool microbiota, changes in intestinal permeability and changes in blood microbiota in these patients.

As all studies lead to the same conclusion, it can be advised to progress the research in both approaches (blood and stool). Both approaches may lead to a bacterial biomarker for the detection of liver fibrosis in early stages, however further research is necessary. To see which method has the most potential, it is important to know the advantages and disadvantages of measuring blood microbiota and stool microbiota. This will be mentioned in the next section of the discussion.

Pros and cons of measuring bacteria in blood and faeces

A couple of disadvantages can be noticed when looking at bacteria in the stool, compared to bacteria in the blood. There are a lot of different bacteria in the stool and these bacteria are present in extremely high amounts (around $0.9 * 10^{11}$ bacteria/gram stool).[33] Therefore, measuring specific changes in the microbiome of the stool might be difficult and it can be hard to find a specific pattern that matches each patient. Also, the composition of the bacteria will be different for each individual, as it can be influenced in different ways. The factors that influence the gut microbiome can be seen in figure 16.[22] In this figure, it can be seen that the genotype of the individual can influence the microbiome. However, this genetic influence on the microbiome is minor. Less than 2% of the bacterial diversity in the microbiome is influenced by the genotype of a person.[34] Other factors, for example, food intake or lifestyle, play a more important role in the composition of the microbiome.

One factor that influences the bacteria in the stool is the diet. A plant-rich diet leads to an increase in the bacteria *Prevotella*; while a high-fat diet leads to an increase in *Bacteroidetes*.[35] Also, alcohol use, smoking and medication intake influence the intestinal microbiome, leading to a different faecal microbiome.[34] Therefore, the faecal microbiome of patients with non-alcoholic fatty liver disease (caused by obesity) will be different from the faecal microbiome of patients with alcoholic liver disease.[30][34] Furthermore, the faecal microbiome among patients with for example a non-alcoholic fatty liver disease can differ from each other. Each person has a different environment, lifestyle and eating habit, which creates a specific faecal microbiome.[35] So, the microbiome of the stool can be influenced enormously by many different factors, and it might be difficult to detect specific changes that indicate liver fibrosis.

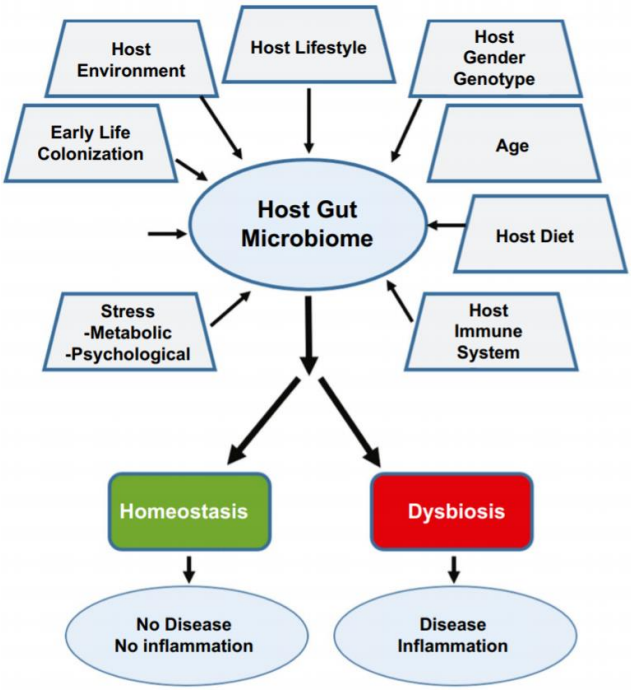


Figure 16: Factors that influence the microbiome.[22]

Also, many studies showed that liver fibrosis causes bacterial overgrowth in the (small) intestine. Many bacteria will be enhanced, while only a few will be decreased. However, the microbiota in the stool differs from the microbiota in the small intestine. The bacterial composition can change, and not all bacteria that are present in the intestine might end up in the stool. Therefore, the stool microbiota does not fully reflect the microbiota in the intestine. The bacteria in the stool can be seen as a 'photograph' from the changes in the microbiome and do not encompass the whole process.[22]

However, the advantage of measuring the stool microbiome is that no blood samples are necessary. Therefore, no needles are used, which might be more convenient for some patients. Although, it might also be possible that some patients prefer to give a blood sample over a faecal sample. Furthermore, no doctor's appointment is necessary when collecting stool, the patients can just hand in their faeces during open consultation hours.

The advantage of measuring the blood microbiome over the stool microbiome is related to the quantity of the bacteria. As mentioned before, there are many bacteria in the stool/intestine of individuals.[33] However, only a few of these bacteria pass the intestinal membrane and enter the bloodstream. Therefore, there are way fewer bacteria present in the blood and fluctuations can be seen more easily. In this way, not only the changed bacterial composition is measured but also the increased intestinal permeability. As an increased intestinal permeability is related to liver fibrosis, it might be more beneficial to examine the blood microbiota.

For these reasons, it might be more ideal to quantify and qualify bacteria in the blood of people who are suspected to have early liver fibrosis (instead of in the stool). This method will potentially have the highest success rates, as small fluctuations in the concentration of 16S rDNA and changes in the bacterial microbiome can be seen more clearly. Furthermore, blood sampling is easy and inexpensive (compared to liver biopsies).[19] Nevertheless, the research dealing with stool microbiota should also be progressed, as it did lead to promising results according to the study by Li et al.[28] and Liu et al.[30]

More research is necessary to find out which bacterial biomarkers are the most useful. In the next section, the information from the most promising studies dealing with the blood microbiome are compared to each other and the results are combined to find a biomarker for the early diagnosis of liver fibrosis in all liver diseases.

16s rDNA sequencing as a diagnostic tool for early liver fibrosis

Based on the advantages and disadvantages of measuring bacteria in the blood versus the stool, it can be advised to progress the research dealing with the blood microbiome. The studies from Champion et al.[31], Lelouvier et al.[13] and Puri et al.[32] examined if blood microbiota could be used to diagnose specific liver diseases. However, these studies did not combine information from all liver diseases and did not investigate a bacterial biomarker for liver fibrosis in general. The study by Champion et al. measured the possibility of using a bacterial biomarker in the blood to diagnose stage F1 or F2 liver fibrosis in patients with NAFLD.[31] The study by Lelouvier et al. also measured this but combined F2 with F3 in one cohort.[13] Both studies showed promising results for the early diagnosis of liver fibrosis using blood microbiota, however, it is not likely that this already can happen in stage F1. The study by Champion et al. mentioned that stage F1 and stage F2 could not be distinguished from each other, while the study by Lelouvier et al. showed that the bacterial composition in the blood of patients with stage F1 differed too much between cohorts.[13][31] Therefore, it can be suggested to focus more on early diagnosis in stage F2 of the disease (or stage F1 and F2 combined), and not on stage F1 of the disease alone. Furthermore, the study by Puri et al. investigated if the circulating microbiota could be used to diagnose early ALD. This study also showed promising results, as the bacterial microbiome in the blood is changed in patients with early liver diseases compared to alcoholics without liver disease.[32]

The studies from Lelouvier et al. and Puri et al. both measured the concentration of 16S rDNA in the blood. They discovered that this concentration is increased in the patients with early liver fibrosis, compared to healthy controls.[13][32] The study by Champion et al. did not measure this, as they focused more on bacterial changes (instead of a general increase/decrease in the blood).[31] Therefore, it can be suggested to perform further research on the concentration of 16S rDNA in the blood of patients with early liver fibrosis, especially because the variance of the fibrotic group in figure 9 is relatively large. These findings need to be confirmed in each kind of liver disease that leads to cirrhosis, so in NAFLD, ALD, cholestasis and hepatitis B/C induced liver diseases. The study by Yao et al. investigated bacterial changes in the stool of patients with HBV-ACLF, they showed that most phyla are enhanced in the intestinal microbiome while only Bacteroidetes are reduced in patients with HBV-ACLF.[25] Also, the intestinal permeability was increased in this disease. Therefore, it can be expected that the concentration of bacteria (and therefore of 16S rDNA) in the blood will also be increased.

When looking at specific bacterial changes in the blood that might predict if there is a liver disease present, it can be seen that many different bacteria are measured in each study.[13][31][32] Therefore, it might be difficult to find a specific bacterium in these studies that changes similarly in each disease. It would be ideal if one specific phylum could be used to diagnose each liver disease, leading to a general bacterial biomarker. However, this might not be possible, as differences in bacterial composition were seen between NAFLD and ALD patients.[13][31][32] Therefore, disease-specific bacterial biomarkers might also be an option, and should be examined further. To see if a general biomarker can be found, or if a disease-specific biomarker has more potential, the most important phyla in the blood will be discussed.

The phyla *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Bacteroidetes* are mentioned frequently in different studies.[13][15][31][32] The study by Champion et al. measured an increase in *Proteobacteria*, which

was confirmed in the study by Lelouvier et al.[13][32] An increase in *Proteobacteria* did not happen in patients with early ALD, according to the study by Puri et al.[32] However, the study by Zhou et al.[15] did mention an increase in the phylum *Proteobacteria* in the intestinal microbiome of patients with early ALD, confirming the suggestion that there is no straightforward relationship between the intestinal microbiome and the blood microbiome. Further research is necessary to confirm the findings from Puri et al. and see if an increase in *Proteobacteria* in the intestine does (not) lead to an increase in *Proteobacteria* in the blood of patients with early ALD. Furthermore, the study by Lelouvier et al. mentioned a decrease in *Actinobacteria* in the blood of patients with early NAFLD.[13] This was also confirmed in the study by Champion et al.[31] but did not happen in ALD according to Puri et al.[32] Furthermore, the study by Puri et al. measured an increase in *Fusobacteria*, which was also measured in the faeces according to Lelouvier et al., but not measured in the blood.[13][31] Therefore, it is not likely that an increase in Proteobacteria, a decrease in Actinobacteria and/or an increase in Fusobacteria can function as a general biomarker for liver fibrosis. However, these changes might be used as a disease-specific bacterial biomarker.

Furthermore, the phylum *Bacteroidetes* is decreased in the blood of patients with liver fibrosis according to different studies.[31][32] This was not expected, as *Bacteroidetes* are gram-negative bacteria, produce LPS and can activate Kupffer cells in the liver. This leads to further progression of liver fibrosis and more scar formation.[18] However, only a small part (1.1%) of the bacteria in the blood belongs to the phylum *Bacteroidetes*. The largest part of the bacteria in the blood belongs to the *Proteobacteria* (87.9%), which are also gram-negative bacteria.[13] These bacteria produce LPS, and probably have the largest interaction with liver fibrosis. In addition, the phylum *Bacteroidetes* is called a 'beneficial bacteria', according to the study by Yao et al.[25] and Lv et al.[36] This might indicate that they have a protective role against liver diseases that lead to cirrhosis. Furthermore, a decrease in *Bacteroidetes* in the blood was seen in patients with early ALD, measured in the study by Puri et al.[32] This decrease was seen in all alcohol-consuming groups (also in the group that did not develop liver fibrosis yet), which might indicate a relationship between alcohol consumption and a decrease in *Bacteroidetes* in blood samples. The study by Yao et al. measured a decrease in *Bacteroidetes* in the stool of patients with HBV-ACLF, which might suggest that this phylum is also decreased in the blood.[25] In addition, the study by Lv et al. measured the stool microbiome of patients with early-stage primary biliary cirrhosis. They found a decrease in beneficial bacteria, for example *Bacteroidetes*, which might indicate a decrease in *Bacteroidetes* in the blood as well.[36] However, this relation is not straightforward and should be examined in future studies. Unfortunately, a decrease in *Bacteroidetes* in the blood was not mentioned in NAFLD patients in the study by Lelouvier et al. They did not mention the changes of this phylum in their blood results and mostly focussed on the phylum *Proteobacteria*. However, they did find an increase in faecal *Bacteroidetes* in the Spanish cohort, but this was not confirmed in the Italian cohort.[13] In addition, it should be noticed that a decrease in bacteria might be more difficult to measure, compared to an increase. For this reason, and because not all studies show the same results, the changes in *Bacteroidetes* in the blood (and stool) and their use as a bacterial biomarker should be investigated further.

To summarize, it might be possible to diagnose early liver fibrosis (stage F2) in all kinds of liver diseases using the concentration of 16S rDNA in the blood. As the amount of *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* in the blood differs between patients with early ALD and early NAFLD, it can be expected that these bacteria cannot function as a general biomarker but more as a disease-specific biomarker. However, the connection between the number of these bacteria in the blood and liver fibrosis in all kind of liver diseases is still relatively unknown. Especially in cholestatic liver diseases and hepatitis B/C induced liver diseases more research is necessary. Furthermore, it seems promising to investigate the relationship between *Bacteroidetes* in the blood and the presence of liver fibrosis further, as most studies show a decrease in *Bacteroidetes*, which might lead to a general biomarker. These findings are based on only a few

studies and patients, meaning that the reliability of the biomarkers is not extremely high. Previous studies examined the possibility of a bacterial biomarker in a specific liver disease, while a bacterial biomarker for liver fibrosis in general is not studied (yet). So, to expand this literature research, to confirm the findings and to combine all information, a new study has to be developed. For this new study, it is advised to examine the concentration of 16S rDNA in the blood of patients with all kinds of liver diseases, to investigate the changes in *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* in the blood of these patients, and to combine this information (hopefully) leading to a general biomarker. An example of the next step in this research can be seen in the next section.

A follow-up study

As for the next step in the research towards a general bacterial biomarker for early liver fibrosis, the results from previous studies need to be extended, combined and confirmed. In this new study, the 16S rDNA concentration and the quantity of *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* in the blood of patients with early liver fibrosis will be examined and compared to healthy individuals. Not only patients with ALD or NAFLD will be examined, but also patients with cholestatic liver diseases and hepatitis B/C induced liver diseases will be included. The set-up of this study is as followed:

Research question

Can liver fibrosis in stage F2 be diagnosed using the 16S rDNA concentration and the quantity of *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and/or *Actinobacteria* in the blood?

Hypothesis

In previous studies, it was shown that the 16S rDNA concentration in the blood of patients with early ALD and early NAFLD was increased compared to healthy controls.[13][32] As an increase in intestinal bacteria and an increase in intestinal permeability was measured in cholestasis and hepatitis B/C, it is expected that these diseases also show an increase in the concentration of 16S rDNA in the blood.[32][25] Furthermore, the number of *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Bacteroidetes* differed among previous studies. It is expected to find a more clear pattern of these bacteria in the different diseases, leading (hopefully) to a specific change that happens in each liver disease. Therefore, it is expected that an increase in 16S rDNA concentration together with a change in *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and/or *Actinobacteria* in the blood can be used to diagnose stage F2 of liver fibrosis in all liver diseases that lead to cirrhosis.

Study population

In the study, the patients with early liver fibrosis will be divided into 4 groups: early NAFLD, early ALD, early cholestasis and early hepatitis B/C. These patients will have stage F2 of liver fibrosis, which is confirmed with a liver biopsy. Also, a control group will be made with individuals that are healthy.

The study population by Champion et al.[31], Lelouvier et al.[13], Puri et al.[32] and Li et al.[28] ranged from 11 individuals to 65 individuals per group, and therefore this study will use around 50 individuals in each group. All groups will be equally large ($N \approx 50$) and the characteristics of the persons between the groups need to be similar. This means that an equal number of women and men will be present in each group, and the mean age in each group will be approximately the same. In that case, the real effects of liver fibrosis on the blood microbiome will be measured. So, in total 250 individuals will be included in this study and these individuals will be divided into the groups based on their cause of liver fibrosis. These individuals must meet the inclusion criteria and not have the exclusion criteria.[37]

The participants will be collected from the research programme Lifelines, in the northern region of the Netherlands. In this research programme, around 167.000 inhabitants (10% of the population) are followed over 30 years.[38] A study by Van Den Berg et al.[39] studied the prevalence of NAFLD and fibrosis in the northern region of the Netherlands, using Lifelines. They examined 38.243 patients and measured if they have a form of liver fibrosis. They concluded that 22% of the population in the north of the Netherlands were suspected to have a form of NAFLD.[39] Therefore, around 36.740 participants in the Lifelines programme (22% of 167.000) will have a form of NAFLD, meaning that there will be enough patients to fulfil the group of 50 individuals having stage F2 liver fibrosis caused by obesity or metabolic syndrome.

Furthermore, it is estimated that 0.34% of the people in the Netherlands have a hepatitis B infection and 0.16% have a hepatitis C infection.[40] Therefore, it can be expected that around 567 people (0.34% of 167.000) will have HBV and around 267 people (0.16% of 167.000) will have HCV in the Lifelines cohort. This means that it might be challenging to gather 50 participants for this study, as not all patients will have stage F2 of liver fibrosis. Therefore, a smaller amount of patients can be expected.

In the Netherlands, around 300.000 people drink alcohol excessively and are labelled as alcoholics.[41] As not all excessive alcohol users are labelled as alcoholics (yet), it can be expected that this number is even higher. Therefore, it can be assumed that a large number of people will have a form of alcoholic liver disease in the Lifelines cohort. This means that 50 participants having stage F2 of liver fibrosis due to alcoholism is feasible.

Finally, the prevalence of cholestatic liver diseases is of importance. For example, PBC has a prevalence of 1 in 1000 women over 40 years.[42] Therefore, it might be difficult to predict the amount of patients with PBC in the Lifelines cohort, and it might be difficult to collect 50 patients. A smaller amount can be expected and should be accepted.

Inclusion criteria

The participants of this study must be registered in Lifelines and live in the northern region of the Netherlands. The patients included in this study need to have liver fibrosis in stage F2 and the cause of the liver fibrosis must be known. The healthy individuals that will be included in the study must not have liver fibrosis. These criteria will be confirmed with liver biopsies, which might lead to a small sampling error. As only a small part of the liver will be examined, the liver biopsy can be seen as a small part of the whole picture. Therefore, higher stages of liver fibrosis can be present in other parts of the liver, which was not foreseen in the liver biopsy. Also, it is possible that the interpretation of the biopsy is different among different observers, or it is possible that the patient does not want to have a liver biopsy at all.

In a study by Sajja et al.[43], 2048 patients with cirrhosis were examined. These patients ranged from 19 to 94 years old, with a mean age of 52 years old ($\pm$ 11 years).[43] As liver fibrosis happens before liver cirrhosis, the mean age of patients with liver fibrosis will be lower. Also, some diseases might occur more frequently at a higher age, meaning that a maximum age for a study can be beneficial.[44] Therefore, the age of the participants must be between 18 and 65 years old. Furthermore, all participants need to fill in an informed consent.

Exclusion criteria

The patients that were used in this study must suffer from liver fibrosis (stage F2) and not have any other disease that influences the permeability of the gastrointestinal tract. Examples of these diseases are inflammatory bowel disease and irritable bowel syndrome.[45] If a higher stage of liver fibrosis is established, these patients should be excluded from the study. Also, the individuals from the control group should not have other diseases that increase the permeability of the intestinal wall, as this might influence the blood microbiome. Furthermore, women that are pregnant are excluded from this study.

Methods

Blood samples

To analyse the 16S rDNA concentration in the blood and measure the quantity of *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, and *Proteobacteria*, blood samples must be taken from each individual. The study from Champion et al.[31] and Puri et al.[32] did not mention how many millilitre blood was sampled, however the study from Lelouvier et al.[13] did mention this. They used 200 µl of blood from the individuals to extract DNA and quantify/qualify 16S rDNA sequences.[13] As 200 µl is an extremely small amount, errors can be made very easily. Therefore, this study will sample 1 ml of blood from each individual.

Analysis

The concentration of 16S rDNA in the blood of individuals will be measured using the PCR technique. As mentioned before, the study from Johnson et al.[27] evaluated the use of 16S rRNA gene sequencing in the identification of bacteria. They found that full 16S rRNA sequencing leads to better results, compared to short-read sequencing.[27] Short-read sequencing was used in the studies that were mentioned previously, however full 16S rRNA gene sequencing could lead to more accurate results. Therefore, this study will use the complete 16S rDNA genome to identify the *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* in the blood of patients with early liver fibrosis. The full 16S rDNA gene will be amplified and quantified by the PCR technique.[13]

The quantity of 16S rDNA in the blood of patients with early liver fibrosis will be compared to healthy controls. A distinction will be made between the four groups of liver fibrotic patients, to see if there are differences between these patients. Furthermore, the *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* in the blood will be qualified and quantified. These numbers will also be compared to the healthy controls and to the other groups with liver fibrosis. If the concentration of 16S rDNA in all groups containing patients with liver fibrosis will be higher than the control group and/or if similar bacterial patterns can be seen of *Bacteroidetes*, *Proteobacteria, Fusobacteria* and/or *Actinobacteria* between these patients, the study can be performed on a larger scale.

Future

If the follow-up study has success and a general biomarker was found, further research is still necessary. It should be investigated whether this biomarker is specific for liver diseases, and does not indicate that there are other diseases present. When these studies are performed and it can be concluded that the concentration of 16S rDNA together with the number of *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and/or *Actinobacteria* in the blood can be used to diagnose early liver fibrosis, a general bacterial biomarker is found. This general bacterial biomarker needs to be applied in practice and will be used as a 'screening tool' for patients that are at risk of developing liver diseases. As liver fibrosis has several causes, each person that is at high risk of developing a liver disease can get an invitation to get their blood examined. Therefore, all known alcoholics, all known persons with obesity or a metabolic syndrome, all persons that are suspected to get cholestasis (for example due to pregnancy) and all persons with a hepatitis B or C infection will receive this invitation. These persons can go to their general practitioner, where 1 ml of their blood will be taken. This blood can be examined, and the concentration of 16S rDNA together with the number of *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and/or *Actinobacteria* will be measured. If the concentration of 16S rDNA is above a certain threshold (established by previous studies) and if the number of *Bacteroidetes*, *Proteobacteria* and/or *Actinobacteria* is above/below a certain threshold (also established priorly), this person likely has liver fibrosis. This can be confirmed with liver biopsies, where the exact stage

of liver fibrosis will be determined. Hopefully, patients with early stages of liver fibrosis will be detected sooner, leading to a higher survival rate and more treatment options.

However, when the studies are performed and it cannot be concluded that the 16S rDNA concentration together with the number of *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and/or *Actinobacteria* in the blood can be used as a general diagnostic tool, other research is necessary. It might be advised to investigate the liver diseases separately, and see if a diagnostic tool for each liver disease can be found individually. For example, the phyla *Proteobacteria* (together with 16S rDNA concentration) can be investigated for the diagnosis of early NAFLD, as an increase is mentioned in the study by Champion et al.[31] and Lelouvier et al.[13] Next, the phyla *Fusobacteria* (together with 16S rDNA concentration) can be investigated for the diagnosis of early ALD, as an increase in this phyla is mentioned in the study by Puri et al.[32] In this way, it might be possible to use different biomarkers for different causes of liver fibrosis. These biomarkers should be investigated further to see if they are not indicating other diseases.

Conclusion

It can be concluded that it is possible to diagnose liver fibrosis in the early stages of the disease, using a bacterial biomarker in the blood and in the stool of patients. It is more likely that a biomarker for liver fibrosis can be found in stage F2 of the disease, as stage F1 has too many variations between individuals. When comparing the advantages and disadvantages of a bacterial biomarker in the blood and in the stool of patients, it can be concluded that a biomarker in the blood is more beneficial.

Furthermore, it can be concluded that an increase in the concentration of 16S rDNA in the blood might be a promising general bacterial biomarker. Further research is necessary on the specific bacterial changes in the blood of patients with liver diseases. The number of *Bacteroidetes* decreases in most studies, but no clear pattern was seen in the number of *Proteobacteria*, *Fusobacteria* and *Actinobacteria*. Therefore, the phylum *Bacteroidetes* shows the most promising results as a general biomarker, while the phyla *Proteobacteria*, *Fusobacteria* and *Actinobacteria* might be more useful as disease-specific biomarkers. Further research is necessary to confirm these findings, especially in patients with cholestatic liver diseases and hepatitis B/C infections, as not much information on the possibility of a bacterial biomarker in the blood to diagnose these diseases is known. So, to confirm the expectations *and* see if early diagnosis of liver fibrosis using a bacterial biomarker in the blood is possible, more research is necessary. Nevertheless, the findings from previous studies are promising and a bacterial biomarker for liver fibrosis may not be far away.

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