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Microbiome-related biomarkers for early diagnosis of liver fibrosis

Bachelor Research Project

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Abstract

New evidence showed the critical role of human gut microbiota in liver diseases, recognised as translocation of microbiota from the gut to the liver. These findings are consistent with the evidence that bacterial translocation is involved in the pathogenesis of liver fibrosis. Therefore, this study will explore the possibility of using bacterial translocation to the blood or the microbiome's composition as a diagnostic tool to detect liver fibrosis at an early stage. Here, we review the current evidence on the relationship between gut microbiota and liver diseases and the alterations in microbiome composition. Researchers assessed a detailed analysis of 16S rRNA in the blood or faeces to characterise microbial profiles and determine the best new microbiome-related biomarker for the early diagnosis of liver fibrosis at stage F1 or F2. A better understanding of the gut microbiome to liver diseases could make an early diagnosis of liver fibrosis possible. In which further disease progression toward clinical consequences can stop and initiates appropriate therapeutic regimens. During this literature study, I found that it might be possible to diagnose liver fibrosis using microbiome-related biomarker in blood and faeces in stage F2 more likely than in F1. Still, further research is necessary, but the findings are promising.

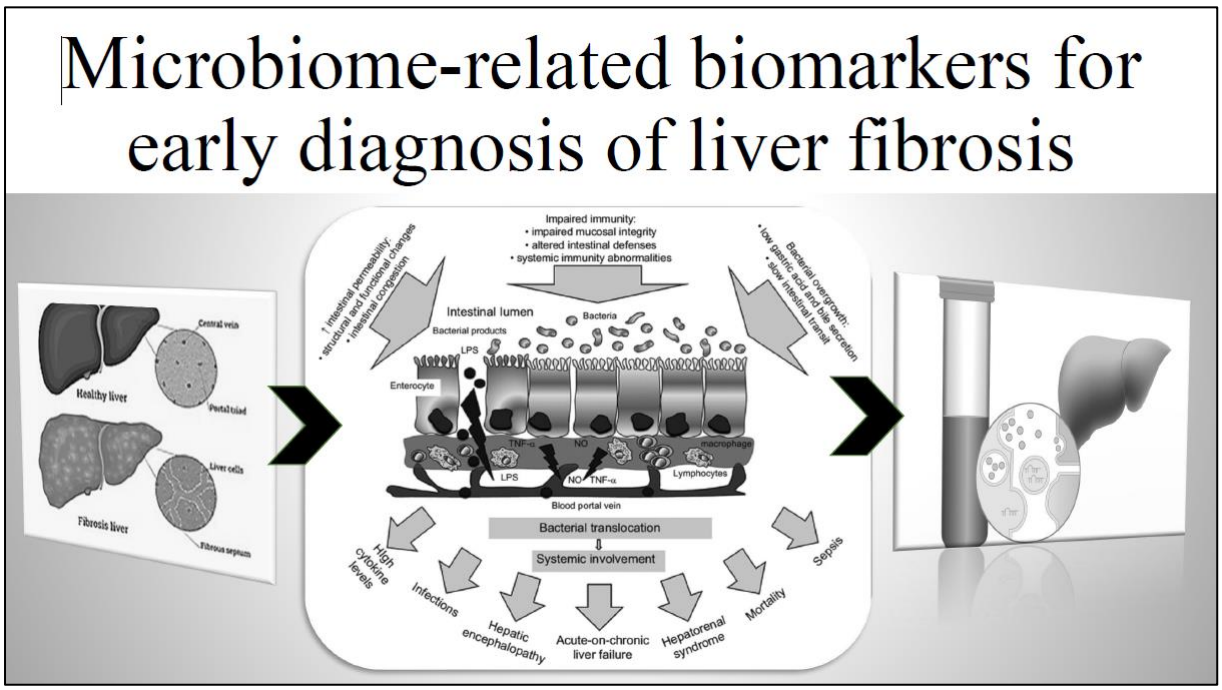

Table of content

1. Introduction

Liver fibrosis is a chronic liver disease characterised by an excessive accumulation of collagen and extracellular matrix proteins, which will form scar tissue. It can damage the structure and regenerating function of the hepatocytes. Nowadays, no effective antifibrotic therapy is available for liver fibrosis. When patients suffer from liver fibrosis, the quality of life reduces, and the only treatment option these days is liver transplantation. More investigation is needed to explore the possibility of early diagnosing of liver fibrosis. At stage one or two, detection of liver fibrosis is because there is higher proof that liver fibrosis is reversible [1.4].

Increasing evidence shows that bacteria and bacterial products from the gut stimulate the fibrotic process. Therefore, the communication between the gut and the liver plays a vital role in the early detection of liver fibrosis. The cross-talk between the liver and the gut increases called the gut-liver axis. Due to this direct communication, microbiota underlies a modulatory effect of gut microbiota on the liver. With this evidence, this study will explore the possibility of using bacterial translocation or microbiome composition as a diagnostic tool to diagnose liver fibrosis at an early stage. This study will focus on both blood / faecal bacteria and bacterial products to represent which method could be possible used in the future. Consequently, the research question of this study will be: *Is it possible to use bacterial translocation from the intestine to the blood or the microbiome's composition as a diagnostic tool to diagnose liver fibrosis at stage F1 or F2 as an early marker?*

For answering this question and testing the hypothesis set, a literature study is performed. The task accomplishes because a significant total of the patients with liver fibrosis remains in the early stage (F1 and F2), the reversible state of the disease. Approximately 5% of the patients with non-alcoholic steatohepatitis (NASH) will progress into fibrosis or cirrhosis. However, it is challenging to expect who will get fibrosis or cirrhosis, which means many patients enter the clinic too late. Certain patients will undergo transition, in which bacterial translocation perform an essential role in the pathology. To identify and quantify different microbial genomes, qPCR can be used to determine bacterial DNA for a particular region of the 16S rRNA gene via nucleotide sequencing in patients with liver fibrosis. Therefore, with a detailed analysis of 16S rRNA in the blood or faeces, investigators can assess the characterisation of microbial profiles. Hopefully, after finding new diagnostic tools, liver fibrosis can be diagnosed at early stages, in which disease progression and hospitalisations will be prevented.

2. Literature review

2.1. Liver fibrosis

In general, liver fibrosis is an excessive accumulation of collagen and extracellular matrix proteins (ECM). Fibrosis represents the consequences of sustained wound healing, which will be influenced by different factors like infections, metabolic disorders, drug/alcohol abuse, or autoimmune disorders. These factors can cause chronic liver diseases. When there is an accumulation of ECM, the liver structure is disturbed, and a fibrous scar is formed [1]. As a result of the formation of fibrous scar tissue, the organ's ability to regenerate and the function of the liver will be dramatically reduced. When damage to the liver continues, liver cirrhosis can also occur. Liver cirrhosis is the end stage because of liver fibrosis. Both are the result of sustained wound healing, but when damaging the hepatocytes continues, the internal structure of the liver destroys, and scarring continues over a more extended period. Thus, it is called liver cirrhosis [2]. Detection of liver fibrosis is complex because it almost shows no symptoms until the liver damage becomes severe enough, which is liver cirrhosis. The clinical manifestations of cirrhosis vary widely. Symptoms the patient can experience in the stage of cirrhosis are extreme fatigue, confusion, swelling in the abdomen, and bleedings [2]. Major complications will happen when a patient suffers from liver cirrhosis—for instance, renal failure, variceal bleedings and ascites [1]. Progression of liver cirrhosis can lead to hepatocellular carcinoma (HCC). HCC is the most predominant form of primary liver cancers [9].

The formation of scar tissue is a typical liver reaction when the liver regenerates after acute injury. However, when the liver's damage continues, the liver reacts with a progressive and uncontrolled accumulation of the ECM proteins. In the process of accumulation of ECM proteins, different growth factors, cells and signals are involved. In a healthy liver, 10% of the total liver volume consists of ECM. In patients with chronic liver diseases, the ECM protein synthesis increases in which the importance of ECM can be eight times higher than a healthy liver [5]. Scar tissue consists of different components: ECM proteins, collagen type I and III, sulphated proteoglycans and glycoproteins [3]. Hepatic stellate cells (HSC) can produce ECM proteins. HSC are present between the hepatocytes and endothelial cells. Hepatocytes and activated HSC excrete fibroblast growth factor receptor (FGF-R) excrete different fibroblast growth factors (FGF). The function of FGF in the body is wound healing and repair. Therefore, FGF shows regeneration in the liver. When there is overexpression of FGF in the liver, ECM can accumulate, which can cause liver fibrosis [6].

Four different stages accompany liver fibrosis. The Metavir scoring system measures development of fibrosis. In figure 1 shows the four different liver fibrosis stages. F1 means portal fibrosis with no septa (thick connective tissue bands). The microscopic picture in figure 1 shows there is no connective tissue present. However, there is some expansion of the portal tract by fibrosis. F2 means portal fibrosis with

infrequent septa. Figure 1 shows in this stage, there are some connective tissue bands visible. F1 and F2 are the most critical stages for early diagnosing of liver fibrosis. In these stages, the disease is still reversible. Liver fibrosis stage F3 means numerous septa but no cirrhosis yet. Liver fibrosis stage F4 means cirrhosis. When a patient is in stage F4, the only treatment option will be liver transplantation, which should be prevented. Figure 1 shows bridges of fibrous tissue connecting two portal tracts in the liver [8]. The Metavir scoring system assesses the severity of fibrosis based on liver biopsy from patients who suffer from liver diseases. It is essential to grade the stage of liver disease because, with the Metavir grade, choices do make on who will treat, and it is critical to supervise the development of chronic liver disease [43]. In addition, the Metavir score indicates the degree of liver inflammation and the prognosis of the disease.

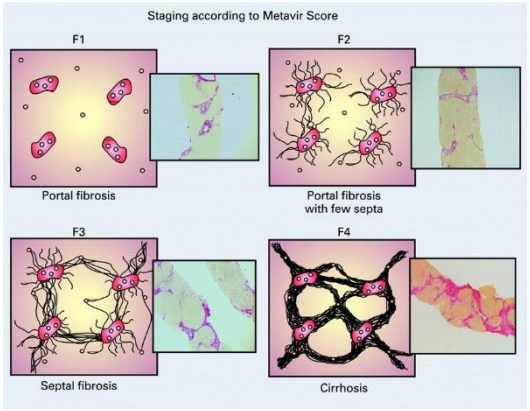

Figure 1: The Metavir scoring system to progress the stages of liver fibrosis. The drawn images represent a schematic representation of the liver fibrosis stages. The microscopic view represents a view from a liver biopsy.

Nowadays, no effective antifibrotic therapy is available for liver fibrosis [4]. When patients suffer from liver cirrhosis, the quality of life dramatically reduces, and the only treatment option is liver transplantation. Thus, more investigation is needed to explore the possibility of early detection of liver fibrosis. With the use of non-invasive markers, liver fibrosis could be diagnosed. There is an urgent need for several reasons. For instance, millions of patients worldwide are infected with the hepatitis C virus, of which 25% of them are likely to develop liver fibrosis [3]. Another reason for detecting liver fibrosis at stage one or two is because there is more evidence that liver fibrosis is reversible [7].

2.2. Influence of the liver on the gut

The communication between the gut and the liver plays a vital role in the progression of liver fibrosis. The cross-talk between the two organs is called the gut-liver axis [10]. The Gut-liver axis refers to the bidirectional relationship between the gut, microbiota, and the liver, due to interaction between different signals. Due to this direct communication, microbiota underlies a modulatory effect of gut microbiota on the liver [16]. The liver and the gut communicate via bidirectional links through the portal vein, biliary tract and systemic circulation. However, the way of communication between the two organs differs. The liver communicates with the gut via the biliary tract; it can secrete primary bile acids, immunoglobin A and antimicrobial molecules. Bile acids are the most critical factors in the communication of the liver to the gut. There are two different types of bile acids, primary and secondary bile acids. Primary bile acids synthesise in the liver, and secondary bile acids result from bacterial actions in the gut. Therefore, the secondary bile acids are essential in the other directional communication via the gut to the liver [21].

Via this pathway, bile acids play another critical role in the control of microbiota. Bile acids cover the signalling function via nuclear receptors [11]. The two processes of bile acids are related to each other. Bile acids act on the farnesoid X receptor (FXR); FXR is a nuclear receptor expressed in the liver and the intestine. The function of the FXR receptor is the regulation of the homeostasis of bile acids [18]. Keeping homeostasis of bile acids goes via an indirect mechanism. When bile acids bind to FXR in the enterocytes, there is an increase in the enterokinase fibroblast growth factor (EFGF). An increase in transduction results in a down-regulating feedback loop for the forming of bile acids synthesis.

Also, when bile acids bind to FXR, there is an increase in the production of antimicrobial peptides by the liver. These antimicrobial peptides are released when there is an overgrowth of microbes [19]. However, there are qualitative and quantitative changes in microbes (dysbiosis). Dysbiosis causes a shift in the balance between primary and secondary bile acids. Patients with liver fibrosis showed an increase in intestinal dysbiosis. Due to this increase in intestinal microbes, there is an induction of secondary bile acids. These secondary bile acids disrupt the down-regulating feedback loop of bile acids. They are resulting in an increase in bile acids and a decrease in antimicrobial peptides. This imbalance in bile acids and microbes causes a cascade of inflammatory reactions and progression of liver fibrosis [12,20].

The communication of the liver with the gut goes via three ways: primary bile acids, antimicrobial peptides and immunoglobulin A (IgA). The function of IgA in the intestinal lumen is to maintain microbial homeostasis [22]. The transport of IgA does regulate via the intestinal poly-immunoglobulin receptor (PIgR) [23], and it protects against pathogens and microbes in the intestine by blockading epithelial receptors [12]. Immunoglobulin A forms together with mucus and microbial peptides the first-line defence in the intestinal epithelium, further discussed in the next chapter. Recent results [24] suggest that IgA may be involved in the pathogenesis of liver fibrosis. The level of IgA's is elevated compared to healthy people. IgA, which the liver secretes to communicate with the gut, is promoted because of deference against mucosal or surface infection [24]. Therefore, serum IgA can serve as an independent marker to detect liver fibrosis at an early stage.

2.3. Influence of the gut on the liver

The gut communicates with the liver via the portal vein. For bidirectional cross-talk, the gut can secrete secondary bile acids and dietary metabolites [12]. The portal vein is the venous outflow from the intestine. In the gut, the intestinal barrier has a vital function. The intestinal wall has a high permeability, resulting in microbes and molecules move from the gut lumen across the tight junctions to the blood [12]. The intestinal barrier consists of four different lines of defence. When there is a breakdown of one of these barrier lines of protection, it compromises gut-barrier integrity. This gut-barrier integrity results in the liver exposed to several toxic factors derived from the intestine [25]. An association is shown between gut permeability and chronic liver diseases. An increase in gut permeability will be promoted

by factors that negatively influence the liver, like alcohol or drug abuse and viral infections. In addition, increased intestinal permeability contributes to the development of bacterial translocation [26].

Qualitative and quantitative changes of microbes, which is called dysbiosis, can cause an increase in intestinal permeability [16]. When there are disturbances in the intestinal barrier, a portal influx of bacteria is shown in the liver. The bidirectional links through the gut and the liver affects the portal influx of bacteria [11]. The bidirectional links result in an association between gut microbiota, inflammation, and gram-negative bacteria, for example, A. Muciniphila[14]. A high permeability for the gram-negative bacteria A. Muciniphila ensures thinning of the mucus layer, which will lead to increased inflammation of the liver. Thinning the gut's mucus layer makes sure microbes can translocate from the gut to the liver [14,15]. The microbes are transported to the portal vein, in which they activate several inflammatory cytokines in the liver. To discuss this more on the cellular level: When the microbes bind to the toll-like receptors (TLR), Kupffer cells (KC) are activated. They activate the innate immune response via different cytokines. Also, the microbes can induce localised inflammation via pattern recognition receptors on hepatic stellate cells [12]. Activation of the TLR is the leading cause of immune response in the liver. When the TLR activate the KC, there is a downstream pro-inflammatory cascade. This cascade reaction will cause activation of a major transcription factor that regulates genes, called nuclear factor-kB (NF-kB) [12]. Therefore, microbes can cause exacerbation of liver diseases because of cytokines and oxidative stress [16].

In patients with liver fibrosis, there is an increase shown in bacterial translocation and LPS levels. Recent studies showed TLR4 plays an essential role in activating the innate immune response due to binding to LPS [26]. LPS is the major component of the outer membrane of gram-negative bacteria [27]. For binding of LPS on the TLR4, co-receptors will introduce downstream signalling of the TLR. The pathway is shortly described above and can lead to the expression of cytokines and oxidative stress. Under pathologic conditions, in which the gut-barrier is disrupted, and homeostasis is impaired, activation of immune cells (especially Kupffer cells) ensue. This results in the fact that LPS-stimulated activation of the Kupffer cells is considered the key mechanism for the pathogenesis of chronic liver diseases [26].

As described above, increased intestinal permeability contributes to the development of bacterial translocation. Thus, qPCR can measure bacterial DNA concentration for a particular region of the 16S rRNA gene via nucleotide sequencing in patients with liver fibrosis. The 16S-rRNA gene is responsible for the translation of mRNA into proteins. For eukaryotes, the active centre for protein synthesis is 18Sr-RNA. Thus, 16Sr-RNA sequence analysis becomes an essential tool in recognition relationships between bacteria and the progression of liver diseases [28].

2.4. Mechanisms of bacterial translocation

As mentioned above, liver diseases like liver fibrosis are related to an increase in intestinal permeability. An increase in intestinal permeability means a rise in the movement of material from inside the gastrointestinal tract via the epithelial cells covering the gut wall into the rest of the body. Usually, there is a balance between the absorption of nutrients and the translocation of intraluminal bacteria during homeostasis. However, some conditions can affect the barrier permeability and thus indirect homeostasis of the intestinal epithelium. It is possible to see an increase in efflux in bacteria and pathogen-associated molecular pattern (PAMPs) from the gut lumen. Impaired intestinal permeability can have different causes: disrupted tight junctions, dysbiosis or small intestinal bacterial overgrowth [29]. In normal conditions, the translocation of PAMPs is into the gut lumen is low. The result of an increase in intestinal permeability is bacterial translocation. The intestine-derived bacterial products consist of LPS, and bactDNA will transport via the portal vein into the liver [30]. Indirect due to an increase in bacterial translocation, activation of the mucosal immune system and secretion of inflammatory mediators will occur. These factors contribute to liver disease progression [31].

Many pre-cirrhotic liver diseases are related to a rise in intestinal permeability and disrupt tight junctions in the intestinal barrier. Although, is dysfunction of the intestinal wall, so an increase in intestinal permeability, caused by dysbiosis of microbial products? Initial, it is essential to realise that there are different stages in the fibrotic process. In the first place, fatty liver disease can occur, meaning an accumulation of fat in the liver, called steatosis or non-alcoholic fatty liver disease (NAFLD). Secondly, when there is a combination of fat accumulation and inflammation of the liver, the inflammatory cells become activated because hepatocytes active the Kupffer cells (KC). This stage is called steatitis or non-alcoholic steatohepatitis (NASH). In addition, KC activation can cause a transition from F2 to F3. In the third phase, there is scar tissue formation within an inflamed liver called fibrosis. Lastly, scarred tissue replaces healthy tissue in the liver, which is called cirrhosis [32].

As mentioned before, liver diseases are associated with dysfunction of the intestinal barrier. The first stage of liver disease is called NAFLD. For example, obesity is associated with an increased risk of NAFLD. The prevalence of developing NAFLD in the general population is around 15%-30%, and the majority of developing NAFLD in obese patients is close to 50-90% [33]. Obese patients are at higher risk to develop NAFLD, and this is because obese patients deal with an inflammation reaction in the colorectal mucosa. This inflammation response in the colorectal mucosa generates changes in the correlated inflammatory genes [34]. The current situation will test on obese mice, which showed that an increase in TNF-alfa and NF-kB in the small intestine took place [35].

Additionally, different kind of cytokines belongs to the TNF-family. When the NF-kB pathway becomes activated, there is an initiation of transcription genes engaged in the inflammation process in the small intestine. Indirect, this will lead to inflammation reactions of the liver [36,37].

Initiation of inflammation reaction of the liver will cause an increase in TNF-alfa and NF-kB in the small intestine. But, to give a more precise answer, whether the intestinal barrier is directly dependent on the microbiome and dysbiosis of bacteria, studies on mice deficiency for inflammasome NLRP3 and NLRP6 were done [35,37]. Inflammasomes show an essential function in recognising and responding to pathogens like LPS or microbial DNA. Inflammasomes can activate cytokines, which are involved in host defence against pathogens [38]. For example, cytokine interleukin-1 beta (IL-1B) activation can initiate NF-kB, inducing transcription genes' initiation in the inflammation process [39]. Inflammasome components are highly expressed in the intestinal epithelium and play an essential role in the host defence against pathogens leaking from the intestinal barrier [40]. As a result of deficiency of the inflammasome NLRP3 and NLRP6, there is less recognition of pathogens (PAMPs or DAMPs) in the intestinal barrier. Less recognition of pathogens results in mice with deficiency for NLRP3/6. As a result, there is more intestinal dysbiosis, which causes inflammation of the colon. An increase in bacterial products (like LPS and bactDNA) in the portal vein is shown [41]. Therefore, dysbiosis induces bacterial translocation, resulting in the progression of NAFLD into NASH. Because disruption of the intestinal barrier is associated with liver diseases and dysbiosis, bacterial translocation induced disruption depends on the microbiome and dysbiosis of bacteria [37].

The progression of NAFLD into NASH can induce further bacterial translocation. About 30% of the general population deal with NAFLD. Only up to 5% of that population progress in NASH [44]. Approximately 5% of the patients with NASH will evolve into fibrosis or cirrhosis [44]. However, it is challenging to predict who will get fibrosis or cirrhosis. Certain patients will go to F3 and F4, in which bacterial translocation performs an essential role in the pathology as described above. To be completed, measuring bacterial DNA by qPCR and detailed analysis of 16S rRNA, characterisation of microbial profiles can be assessed. Hopefully, after analysing, 5% of the population with NASH that will evolve into fibrosis/cirrhosis can be identified hopefully and therefore prevented from developing liver fibrosis at a late stage. A literature study was performed to answer the following research question: *Is it possible to use bacterial translocation from the intestine to the blood or the microbiome's composition as a diagnostic tool to diagnose liver fibrosis at stage F1 or F2 as an early marker?*

3. Research design

3.1. Research question and approach

In this part of the research, a literature review does help answer the research question: *Is it possible to use bacterial translocation from the intestine to the blood or the microbiome's composition as a diagnostic tool to diagnose liver fibrosis at stage F1 or F2 as an early marker?*

To answer this question, the following hypothesis will be tested: The gut-liver axis plays an essential role in the pathogenesis of liver fibrosis. Dysbiosis can cause an increase in paracellular and transcellular permeability, which can lead to gut barrier dysfunction and an increase in translocation of bacteria and bacterial-related products. *Therefore, it can be expected that the presence or absence of bacterial DNA can be determined by qPCR for a particular region of the 16S rRNA gene via nucleotide sequencing in patients with liver fibrosis. Therefore, using detailed analysis of 16S rRNA in the blood or faeces, the characterisation of microbial profiles can be assessed. However, I expect that detecting liver fibrosis at the early stage (F1 or F2) is complex because of slight differences in bacteria community composition between the early stages*.

4. Detection of bacteria and bacterial species in blood

4.1. 16S-rRNA gene sequencing analysis

Bacterial translocation is an essential process in developing liver fibrosis. Due to an enhanced intestinal permeability, bacterial overgrowth can happen [46]. To identify and quantify different microbial genomes, 16S-rRNA gene sequencing analyses are performed, primarily in microbial classification [47]. The overall goal of 16S-rRNA sequencing is to discriminate bacterial taxa isolated from the human gut microbiome to estimate the expansion and reduction of bacterial taxa in liver fibrosis. Identifying the unknown bacterial taxa short regions from the 16S rRNA genes of the bacteria is read off [47]. Bacteria and archaea comprise 16S-rRNA sequences. The 16S-rRNA gene is responsible for the translation of mRNA into proteins. For eukaryotes, the active centre for protein synthesis is 18S rRNA. Prokaryotes consist of 16SrRNA, 23SrRNA and 5SrRNA, according to their sedimentation rate and sequence length [28]. Thus, 16S-rRNA sequence analysis becomes an essential instrument in discovering relationships between bacteria taxa because it is more rapidly and easily sequenced. It also contains enough genetic information about bacterial taxa [28]. The 16S-rRNA gene has nine variable regions and ten conserved regions in total. The highly conserved regions are highly similar in all prokaryotes, making universal amplification possible [48].

In contrast, the highly variable regions are conserved within species and identify specific bacterial species possible [48]. The workflow of 16S rRNA sequencing comprises four different phases: sample preparation, library construction, sequencing and data analysis [49]. After DNA isolation, the DNA is selectively PCR magnified utilising primers targeting the 16S rRNA gene. Common following invention sequencing platforms encompasses 100-600 base pairs per single read with varying degrees of precision. In total, the 16S rRNA gene consists of around 1500 base pairs [28]. Therefore, primers will choose that can cover only a fraction of the 16S rRNA gene. There are numerous primer pairs accessible. A suitable primer does apply to a specific region. As referred to above, the 16S-rRNA gene comprises nine variable regions. The V1-V3 part does positively identify as the most helpful region for classifying different bacterial taxa in the full-length 16S rRNA sequences [50]. After sequencing, the high put sequencing is filtered and clustered into operational taxonomic units (OTU). Functional taxonomic units categorise bacteria species founded on sequence comparison of the 16S-rRNA marker gene [52]. When there is 97% uniqueness of the regions, species can detect using databases [51]. The three standard databases used for 16S-rRNA sequences are Greengenes, SILVA and RDP [53]. Operational taxonomic unit clusters will define by a 97% identity threshold of the 16S gene sequences, resulting in distinguishing bacteria at the genus level [52].

4.2. Altered microbiome in blood

Several studies (Li, Z. et al. 2020; Liu, Y. et al. 2020; Lelouvier, B. et al. 2016; Campion, C. et al. 2020) have studied changes in blood or faecal microbiota profiles as a strategy to modelling early onset of liver fibrosis. All four different studies sequence the 16S-rRNA or 16S rDNA gene to reveal the change of microbiota composition at different liver fibrosis stages. Research carried out by Campion et al. (2020) sequenced the 16Sr-DNA instead of 16S-rRNA. The 16S-rDNA gene codes for the small ribosomal region of the bacterial rRNA, which reads the messenger RNA. The advantage of using 16S-rDNA is that the DNA sequence in the particular areas of the 16S-rDNA gene is more conserved in bacteria related to 16S-rRNA, meaning mutations do not appear rapidly in these regions, and universal primers can be utilised [54].

4.2.1. Study 1: Campion, C. et al. (2020); *Human liver microbiota modelling strategy*. [45]

The most recent and most hopeful study to be discussed is the one conducted by C. Campion et al. (2020) [45]. NAFLD is mainly the result of obese patients or patients with diabetes. Campion (2020) is an observational cohort study involving patients with obesity. The cohort study involved 82 Caucasian patients, divided into different liver fibrosis stages based on liver biopsy. The patients suffered from morbid obesity with a mean BMI of 42.6. Thirty-four patients of the total population were diagnosed with liver fibrosis stage F0, 37 patients with stage F1 and 11 patients with stage F2. Campion et al. (2020) performed both biological and statistical analysis. First, by metagenomic sequencing, 16Sr-DNA of the V3 and V4 regions were amplified using two-step PCR analysis. After sequencing, the high put sequencing was filtered and clustered into operational taxonomic units (OTU).

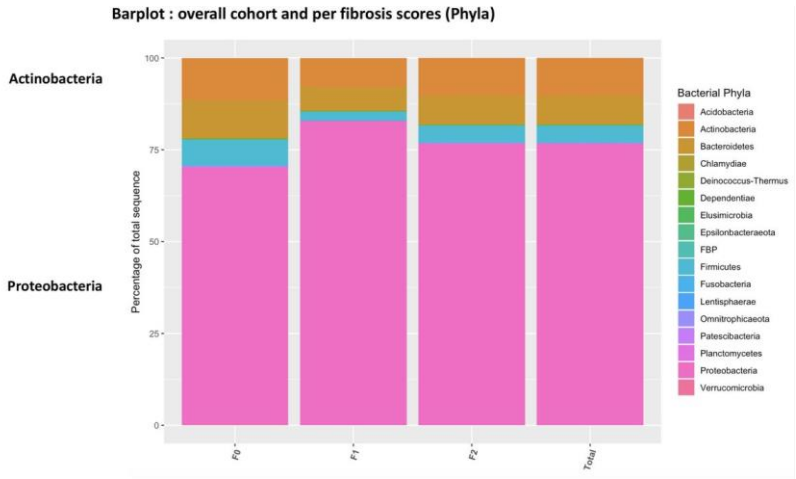

Figure 2: Different bacterial phyla and their percentage of liver sequence per liver fibrosis stage [45].

Additionally, with statistical analysis, the bacterial profiles compared three different groups of liver fibrosis (F0, F1 and F2). For discussion of the results of this study, the most important statistical analysis was the combination of linear discriminant analysis (LDA) with effect size measurements (LEfSe). LEfSe verified the characteristics of bacteria that could explain most likely the differences of bacteria taxa when the statistical analysis was linked with bacterial analysis (16Sr-DNA) [55]. LEfSe was performed on the OTU table, whereas cladograms were produced. Also, with the use of the OTU table, the less plenty OTUs were filtered out of the analysis. After that, principal component analysis (PCA) was performed on the various OTUs. In this way, data

was reduced, and PCA investigation was conducted to if the three stages of liver fibrosis are related to the differential liver bacterial DNA [45].

Research carried out by Campion et al. (2020) showed a considerable heterogeneity of bacteria between the participated NAFLD patients at the phyla level. However, showed in figure 2, the liver microbiota of the total cohort consisted of more than 70% of the phyla *Proteobacteria*. Besides the fact that phyla Proteobacteria was overall abundant in the liver microbiota, the entire sequence of the liver microbiota for the phyla *Actinobacteria* was around 22%. Around the last 8% of the whole series could be linked to the phyla *Firmicutes*. Besides the fact the phyla *Proteobacteria* was overall abundant in the liver microbiota, figure 2 showed the percentage of the total sequence of *Proteobacteria* increased when there was a transition from stage F0 into stage F1. Additionally, a reduction was demonstrated in the entire sequence of *Actinobacteria* and *Firmicutes* by the transition from F0 into F1. However, movement from stage F1 into F2 showed a slight decrease in the total series of the phyla *Proteobacteria* and a slight increase in the entire sequence of the phyla *Actinobacteria*.

Bacterial taxonomic families were studied to investigate changes in blood microbiota profiles as a strategy for modelling the early onset of liver fibrosis. Figure 3 showed the different bacterial families at different liver fibrosis stages.

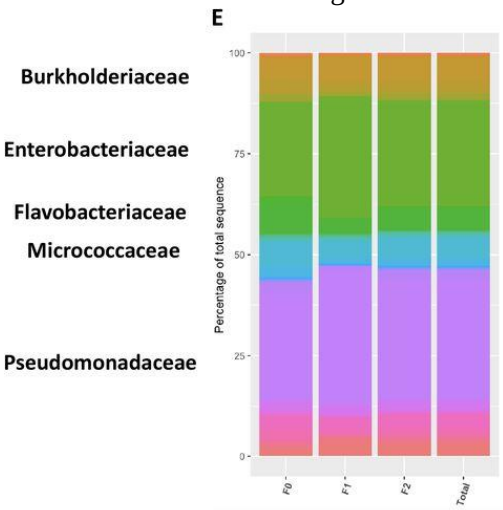

Figure 3: Different bacterial families and their percentage of liver sequence per liver fibrosis stage [45].

Figure 3 showed the most abundant bacteria at the family taxonomic level: the *Pseudomonadaceae* and the *Enterobacteriaceae*. Together they covered the entire sequence with a percentage of around 60%. Both the *Pseudomonadaceae* and the *Enterobacteriaceae* belong to the phyla *Proteobacteria*, and both were subdivided into class 3 (the *Gammaproteobacterial)*. *Pseudomonadaceae* belongs to family one and order 8 *(Pseudomonales)*. *Enterobacteriaceae* belongs to family one and order 7 *(Enterobactriales)* [56].

Figure 3 showed by comparing stage F0 and F1, the *Caulobacteraceae*, *Flavobacteriaceae* and *Propionibacteriaceae* family taxa were significantly different. *Caulobacteraceae* showed a slight decrease in the percentage of the total sequence when there is a transition from F0 to F1. In contrast, *Flavobacteriaceae* showed a significant decline, and *Propionibacteriaceae* showed a slight increase in the percentage of the total sequence when there is the transition from F0 to F1 [45].

The different bacterial families and their percentage of liver sequence per liver fibrosis stage showed in figure 3 indicated a slight difference between F1 and F2 compared to F0. When the three different groups were analysed together, the three groups could not be classified. Using principal coordinate analysis

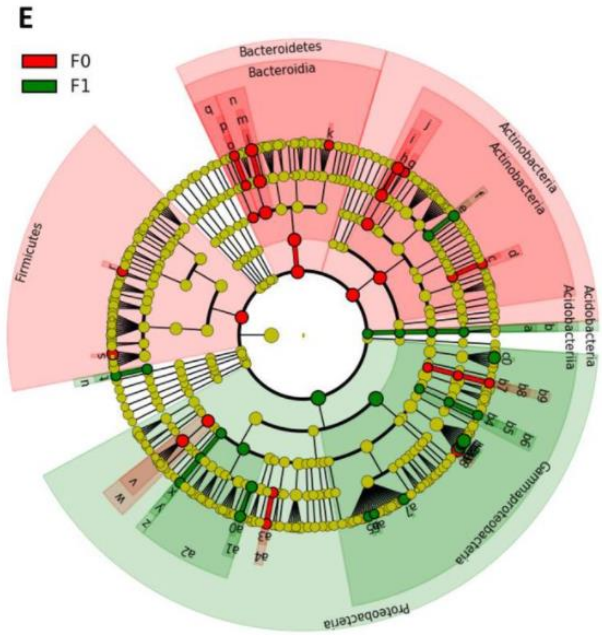

Figure 4: The cladogram with the bacterial phyla on the outer ring and bacterial genera on the inner ring. The bacterial taxa are shaded based on their abundance in either stage F0 or F1 [45].

(PCoA) proved that the F0 group differed from the F1 and F2 groups, but the F1 and F2 groups did not significantly differ from each other. That might indicate that the two liver fibrosis stages had similar liver microbiota. To identify the signature of the bacteria per liver fibrosis score, in this study, F1 and F2 had been combined. Figure 2 and 3 showed the identification of bacterial phyla and bacterial family. However, to identify microbial signatures regarding different liver fibrosis phases, it should give more factual information. Therefore, Campion et al. (2020) performed linear discriminant analysis (LDA) combined with effect size measurements (LEfSe). LDA is an effect size method that used to compare bacterial taxa between F0 and F1/2 groups. The LEfSe scores could be interpreted as the degree of consistent difference in relative abundance between features in the two groups [55]. The LEfSe scores were expressed into a cladogram and a boxplot.

Figure 4 showed the cladogram. The cladogram is a taxonomic representation of statistically and biologically differences between liver-stage F0 and F1. The cladogram appeared in different rings, in which the outer ring is bacterial phyla, and the inner ring is bacterial genera. Each circles diameter is proportional to the taxon's abundance. The higher the diameter, the higher the quantity per liver fibrosis stage. As indicated in figure 4, the phylum *Firmicutes*, *Bacteroidetes* and *Actinobacteria* showed the highest abundance in stage F0 compared to F1. The phyla *Proteobacteria* showed the highest quantity in stage F1 compared to F0. As indicated in figure 4, the largest abundant phyla in liver fibrosis stage F0 was the phyla *Actinobacteria.* The three genera within this phylum, with the largest diameter, were *Cutibacterium (h), Marmoricola (m)* and *Rhodococcus (c),* which can be read off from figure 3E of the article conducted by Campion et al. (2020) [45]. It showed a high abundance within the phyla *Actinobacteria* in liver fibrosis stage F0. The second most abundant phyla in liver fibrosis stage F0 was the phyla *Firmicutes.* The two genera within this phylum, with the largest diameter, were *Faecalibacterium (s)* and *Lactococcus (r).* They show a high abundance within the phyla *Firmicutes* in liver fibrosis stage F0. The last most abundant phyla in liver fibrosis stage F0 was the phyla *Bacteroidetes.* The two genera within this phylum, with the largest diameter, were *Flavobacterium (l)* and *Prevotella (k).* They showed a high abundance within the phyla *Bacteroidetes* in liver fibrosis stage F0.

As indicated in figure 4, the phyla *Proteobacteria* showed the largest abundant classes in liver fibrosis stage F1. The five genera within this species, with the largest diameter, were *Fusobacterium (t), Reyranella (x), Delftia (a5), Tepidimonas (a7)* and *Bradyrhizobium (a0),* could read it off from figure 3E in the article [45]. Thus, these five genera showed a high abundance within the phyla *Proteobacteria* in liver fibrosis stage F1.

As shown in figure 3E in the article [45], the boxplot described the significantly different taxa based on effect size. A negative LDA score indicates enriched bacterial taxa in F0, and a positive LDA score shows enriched taxa in F1. The boxplot explains the most significant differences between bacterial communities [52].

Campion et al. (2020) could make conclusions. Altogether, the combined use of 16S-rDNA sequencing and statistical analysis could deliver an essential role in concealing information regarding the early detection of liver fibrosis in patients suffering from NAFLD. The most abundant phyla in patients with liver fibrosis at an early stage (F1/F2) was the *Proteobacteria,* which also could be seen in figure 2. Patients with low grades of liver fibrosis could be sorted by biomarkers from the *Proteobacteriaceae* family within the liver. The most abundant phyla in patients with liver fibrosis at stage F0 were the *Firmicutes, Bacteroidetes* and *Actinobacteria,* as shown in figure 4.

4.2.2. Study 2: Lelouvier, B. et al. (2016); *Changes in blood microbiota profiles associated with LF in obese patients.* [57]

The second study to be discussed is the one conducted by Lelouvier, B. et al. (2016). This study is a cross-sectional cohort study accomplished on patients with severe obesity to recognise liver fibrosis in patients with NAFLD. The study aimed to describe the relationship between gut microbiota and NASH. At the start of the cohort study, 50 Spanish obese patients were involved and afterwards, Lelouvier et al. (2020) engaged 71 Italian patients to confirm the cohort study. Some inclusion and exclusion criteria were dedicated to this study. The most important criteria were that the patients should have a BMI above 40 kg/m2 and not consume alcohol. As discussed earlier in the study conducted by C. Campion et al. (2020) [45] also performed this study analysis in biological and statistical ways. First, sequencing of the 16S rDNA was performed. Again, the V3-V4 hypervariable regions of the 16S rDNA were amplified. Subsequently, the hypervariable regions were quantified and analysed with the use of qPCR. Similarly, afterwards, the bacterial taxa were classified according to their operational taxonomic units (OTUs). After quantifying and analysing the qPCR data, statistical analysis was performed. Using Mann-Whitney tests and Fisher's exact tests, data from qPCR were compared with patients with or without liver fibrosis [57].

Research carried out by Lelouvier et al. (2016) indicated that NAFLD patients with liver fibrosis deal with an increased concentration of 16S rDNA. Figure 5 shows the 16S rDNA concentrations in obese patients with or without liver fibrosis. Compared to Campion et al. (2020) [45], liver fibrosis stage F1, F2 and F3 have been merged under the section' fibrosis'. As shown in figure 5, patients with liver fibrosis have, on average, almost three times higher blood 16S rDNA concentration compared to patients without liver fibrosis. This rise in concentration could assess that blood microbiota can be considered as a biomarker to detect liver fibrosis by patients at risk, in this case by obese patients. In this way, researchers can accomplish early recognition of liver fibrosis in patients with NAFLD. However, this study also engaged patients afterwards to confirm the observations—this will be discussed later in this section.

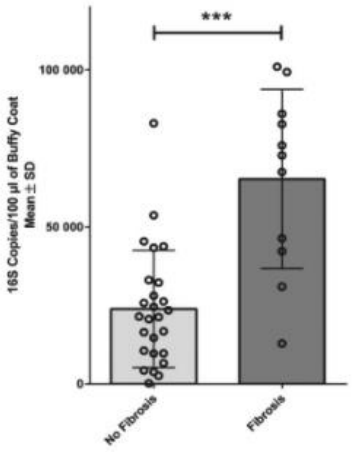

Figure 5: The concentrations of 16S rDNA assessed by qPCR. The light gray indicates the patients without fibrosis and the dark gray with fibrosis. The bar plot indicaties the mean 16S rDNA concentrations [57].

Furthermore, Lelouvier et al. (2016) also indicated a much lower diversity of bacterial 16S rDNA in the blood of obese patients with liver fibrosis than patients without liver fibrosis. The blood phyla of all patients consist of *Proteobacteria (87.9%), Actinobacteria (7.3%), Firmicutes (3.7%) and Bacteroidetes*

(1.1%). Again, in these patients, *Proteobacteria* and *Actinobacteria* were the most dominating bacterial taxa.

The bacterial diversity assessed by 16s rDNA sequencing is measured using the Shannon index. Shannon index is a statistical measure that can characterise species diversity within a community [58]. Lelouvier et al. (2016) assessed the Shannon index on six different levels of bacterial taxa: phylum, class, order, family, genus and OTU. All the six different levels of bacterial taxa showed a lower Shannon index by patients with liver fibrosis than patients without liver fibrosis. These results indicate the blood 16S rDNA diversity is lower in patients with liver fibrosis.

Once 16S rDNA is sequenced, the taxonomic assignment was completed. This study suggested new information about the increased levels of 16S rDNA in obese patients with liver fibrosis. By completing the taxonomic work, differences and similarities between bacterial genera can be compared, for instance, with the study conducted by Campion et al. (2020). Figure 2C from the article conducted by Lelouvier (2016) [57] showed the bar plot of different blood bacterial taxa, using the Mann-Whitney test after analysing 16S-rDNA sequencing. The bar plot indicates the percentage of reads of the entire sequence at varying levels of bacterial taxa. First, the phylum *Actinobacteria* doubled in size in patients without liver fibrosis compared to patients with liver fibrosis. Second, the phylum *Proteobacteria* showed a higher percentage of reads of the entire sequence in patients with liver fibrosis. The proportion of these two phyla in obese patients with liver fibrosis yields the same results as Campion et al. (2020).

As mentioned above, this study showed that obese patients with liver fibrosis deal with an increased concentration of 16S rDNA, as shown in figure 5. To consider the results, Lelouvier et al. (2016) afterwards engaged 71 Italian patients to confirm the cohort study, which the results can be seen in figure 6. Figure 6A demonstrated the correlation between an increased concentration of 16S rDNA in the blood of patients with liver fibrosis. An increase in blood in 16S rDNA concentration was measured in the Italian population. Table 2B, given in Lelouvier (2016) [57], described that the difference between SD and mean in both groups is 1.4, in which the SD is lower than the mean.

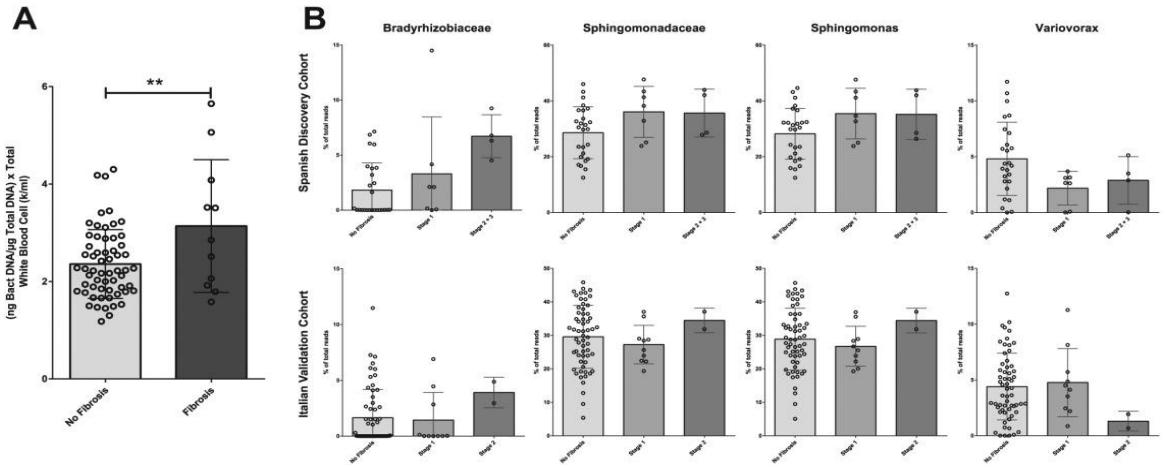

Figure 6: Comparative percentages of relevant blood bacterial taxa in Spanish and Italian patients with liver fibrosis (dark grey) or without liver fibrosis (light grey). On the y-axis, the percentage of reads of the entire sequence is shown [57].

However, the comparative percentages of relevant blood bacterial taxa in Italian obese patients with liver fibrosis differ in some taxa regarding the blood signature in Spanish patients. For instance, in the Spanish cohort study, increased reads from the totals sequence of the genera Sphingomonas were shown. In the Italian validation cohort, this percentage of the genera *Sphingomonas* was decreased. Furthermore, the genera *Variovorax* reduced reads from the entire sequence in the Spanish discovery cohort. On the other hand, the genera *Variovorax* increased the percentage of reads of the whole sequence in the Italian validation cohort. However, the relative abundances of the bacterial taxa in stage F2 to F0 are in line in both cohorts. From figure 6, it can be seen that stage F1 did not match between the cohorts and stage F2 did. The microbiome composition in stage F1 differs too much, which aligns with Lelouvier (2016) results.

Altogether, an increase in the blood concentration of 16S rDNA was measured in patients with liver fibrosis. This increase could be designated as a biomarker to diagnose the presence or absence of liver fibrosis in obese patients. In this way, early recognition of liver fibrosis in patients with NAFLD can be accomplished. The rise in the concentration of 16S rDNA designated bacterial translocation from the gut to the liver took place. Also, Lelouvier et al. (2016) assessed the bacterial taxa profiles. The most abundant phyla in obese patients with liver fibrosis at an early stage is the *Proteobacteria*. In contrast, the most abundant species in obese patients without liver fibrosis is the phyla *Actinobacteria*. These observations were in line with the results from the study conducted by Campion et al. (2020). Besides the increase in the blood concentration of 16S rDNA, differences in bacterial taxa in stage F1 can be seen between the two cohorts. Stage F1 did not match between the two cohorts, while F2 did. The differences in microbiome composition are too much, as earlier described in Lelouvier (2016).

5. Detection of bacteria and bacterial species in faeces

5.1. Altered microbiome in faeces

5.1.1. Study 3: Li, Z. et al. (2020); *Gut microbiota and LF: one potential biomarker for predicting LF* [59]

The third study to be discussed is the one conducted by Li, Z. et al. (2020). This study is more focused on the altered microbiome in faeces. This study deal with the induced liver fibrosis rat model. In total, 131 specific pathogen rats were implicated in this study. The liver fibrosis group rats (n=66) were injected with a mixed solution of CCl4 and olive oil. The liver fibrosis group includes 15 rats in stage F1, 22 rats in F2, 11 rats in F3 and 18 rates in F4. The control rats (n=65) were treated with saline. CCl4 is subcutaneously injected and can encourage liver damage through the formation of free radicals. The reactive free radicals can stick to macromolecules which ensures the construction of nucleic acid. Due to the excessive formation of nucleic acid, there is less methylation of rRNA. That has led to a decrease in protein synthesis which finally cause steatosis, inflammation, and apoptosis. Reduced repair of the liver ensures the progression of fibrosis [60]. After collecting and storing the samples of both the liver fibrosis group and the control group, DNA was extracted, and the V4 regions of the 16S rRNA gene were amplified. Subsequently, the hypervariable regions were quantified and analysed with the use of PCR. Again, afterwards, the bacterial taxa were classified according to their operational taxonomic units (OTUs), based on the OTU tables. After quantifying and analysing the PCR data, statistical analysis was performed [59].

Research carried out by Li et al. (2020) assessed statistical differences between the control group and the liver fibrosis group. Based on the OTU tables, Chao1, Simpson and Shannon were calculated. These measurements can describe the alpha diversity in ecology. Alpha diversity means the variation of microbes in a single sample [61]. Chao1 index estimates the richness of species based on abundance data. The Shannon function estimates the diversity of species within a community and increases as the diversity increases. Simpson index is a similarity index of bacterial community diversity. The higher the value of the Simpson index, the lower the diversity. [62,63].

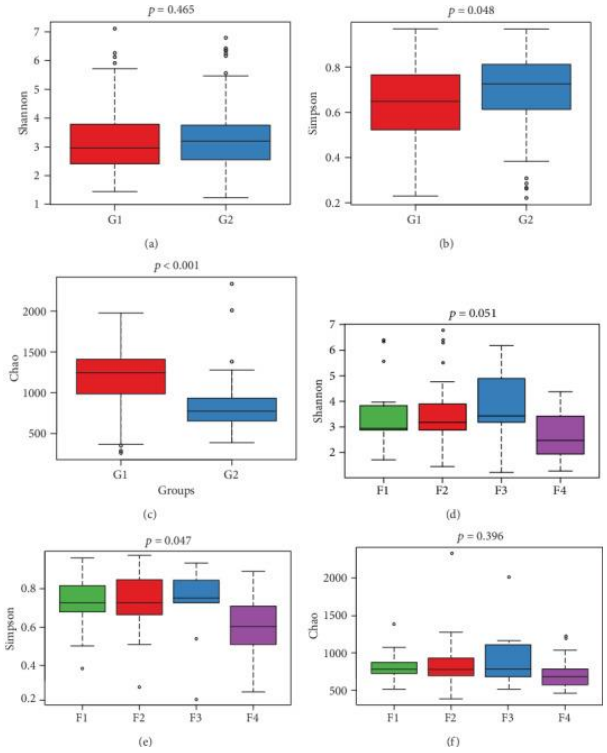

Figure 7: Shannon function, Simpson index and Chao1 index to describe the alpha diversity in the liver fibrosis group, control group and the different liver fibrosis stages. The red bar (G1) indicates the control group and the blue bar (G2) indicates the liver fibrosis group [59].

Figure 7 showed the statistical analysis of the liver fibrosis group rats and the control group. Figure (a) describes the Shannon function. As shown in figure 7, no differences in Shannon were found between the control and liver fibrosis group rats. Figure (b) describes the Simpson index; the Simpson index was higher in the liver fibrosis group compared to the control group. Meaning the community diversity is lower in rats with liver fibrosis compared to the control group. Figure (c) describes the Chao1 index, which is higher in the control group than liver fibrosis rats. The increase in index estimates a higher richness of microbes in the control group compared to the liver fibrosis group. Figure (d, f) describes no significant differences in alpha diversity between the control and liver fibrosis groups measuring the Shannon function and Chao1 index. However, figure (e) illustrates the Simpson index between the four different liver fibrosis stages. It indicated a much lower Simpson index value in liver fibrosis stage F4. The Simpson index average value was in the F1, F2 and F3 liver fibrosis stages almost equal. Meaning, the community diversity in rats with liver fibrosis in stage F4 was higher than the community diversity in rates with lower liver fibrosis stages. The study also confirmed these findings in humans using results from other studies [59].

Furthermore, Li et al. (2020) also suggested various community composition at OUT-level when performing the principal coordinate analysis (PCoA) between the liver fibrosis group, the control group and the different liver fibrosis stages. After performing PCoA, Li et al. (2020) performed LEfSe analysis. The LEfSe scores can be interpreted as the degree of consistent difference in relative abundance between features in the two groups [55]. The LEfSe scores can be expressed into a cladogram and a boxplot, as shown in figure 8/9.

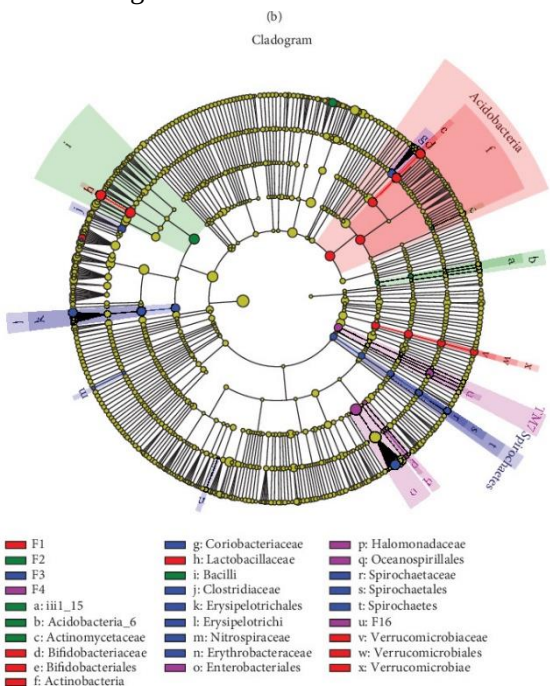

Figure 8: The cladogram with the bacterial phyla on the outer ring and bacterial genera on the inner ring. The bacterial taxa are shaded based on their abundance in either stage F1, F2, F3 or F4 [59].

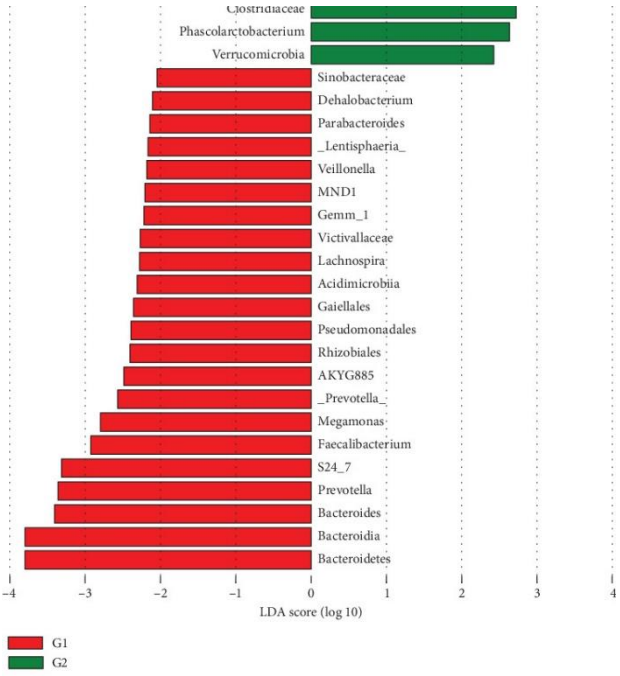

Figure 9: The boxplot on the right with on the x-axis the LDA score and on the y-axis the different bacterial taxa. Red bar (G1) indicates the control group and the green bar (G2) indicates the liver fibrosis group [59].

Figure 8 showed the cladogram. The cladogram is a taxonomic representation of statistically and biologically differences between the four different liver stages. The cladogram shows other rings, in which the outer ring is bacterial phyla, and the inner ring is bacterial genera. Each circles diameter is proportional to the taxon's abundance. As shown in figure 8 and 9, the phylum *Actinobacteria, Saccharibacteria (TM7)* and *Firmicutes* showed an increase in quantity in the liver fibrosis group. The phyla *Actinobacteria* showed, according to figure 8, a higher abundance in liver fibrosis stage F1. The phyla *TM7* showed a higher quantity in liver fibrosis stage F4. However, as shown in figure 8 the diameter of the phyla *Verrucomicrobia* is lower. Figure 9 showed that phyla *Bacteroidetes* had the most negative LDA score, which indicates a high abundance of this phylum in the control group. The abundance of both species *Verrucomicrobia* and *Bacteroidetes* are lower in liver fibrosis stage F1 compared to F0. Meaning there is a decreased abundance of the species *Verrucomicrobia* and *Bacteroidetes* in the liver fibrosis group. The species differs among the four different liver fibrosis groups.

To give a more comprehensive description of the results. At the genus level, six genera showed reduced abundance, and eight genera showed increased abundance. From the phyla *Actinobacteria*, the two genera which have an increased abundance in the liver fibrosis group are *Bifidobacterium* and *Adlercreutzia*. The six genera with a high quantity of the phyla *Firmicutes* in the liver fibrosis group are *Uricibacter, Clostridium, Dorea, Ruminococcus, Phascolarctobacterium* and *Allobaculum.* From the phyla *Bacteroidetes,* the genera *Bacteroides* and *Provotella* have a decreased abundance in the liver fibrosis group. From the phyla *Firmicutes*, the genera *Faecalibacterium* and *Megamonas* have a reduced quantity in the liver fibrosis group. From the phyla *Verrucomicrobia,* the genera *Akkermansia* have a decreased abundance in the liver fibrosis group.

Li, Z. et al. (2020) studied the changes in the microbiome of liver fibrosis rats and control rats. Altogether, statistical analysis showed a significant difference in community diversity and community richness between the two groups. Liver fibrosis rats showed lower community diversity and lower community richness compared to control rats. Also, the community diversity in rats with liver fibrosis in stage F4 was higher than the community diversity in rates with lower liver fibrosis stages. Using LEfSe analysis, investigators identified the differences in relative abundance between features. The three phylum *Actinobacteria*, *Saccharibacteria (TM7)* and *Firmicutes* showed an increase in quantity in the liver fibrosis group.

On the other hand, there is a decreased abundance of *Verrucomicrobia* and *Bacteroidetes* in the liver fibrosis group. Thus, the species differ among the four different liver fibrosis groups. By comparing the bacterial taxonomic profiles with the study conducted by Campion et al. (2020) and Lelouvier et al. (2016), the research conducted by Li. et al. (2020) revealed the association between liver fibrosis and gut microbiota. In Campion et al. (2020) and Lelouvier et al. (2016), the phyla *Actinobacteria* showed

the highest abundance in liver fibrosis stage F0. While within the study conducted by Li et al. (2020), the phyla *Actinobacteria* showed higher abundance in the group of rats with liver fibrosis. In Campion et al. (2020), the phyla *Firmicutes* showed the highest abundance in liver fibrosis stage F0. Within the study conducted by Li et al. (2020), the phyla *Firmicutes* showed an increase in abundance in the group of rats with liver fibrosis. In Campion et al. (2020) and Li et al. (2020), the phyla *Bacteroides* showed the highest abundance in liver fibrosis group F0.

5.1.2. Study 4: Liu, Y. et al. (2020); *Early prediction of liver disease using conventional risk factors and gut microbiome-augmented gradient boosting* [64]

The last study to be discussed is the one conducted by Liu, Y. et al. (2020). This longitudinal cohort study includes 7115 participants who have, on average, a follow-up time of 15 years, an electronic health records follow-up. The study investigates the association and predictive function of the gut microbiome on various liver diseases using metagenomic sequencing. During this study, several risk factors are taken into account. This study makes predictions based on multiple models. First, stool samples were sequenced using shotgun metagenomics, and with the use of the genome taxonomy database (GTDB), taxonomic classification took place. The most abundant taxa have been filtered and analysed from the database. Second, results were based on machine learning models to predict liver diseases. With logistic regression and ridge regression, the Gradient boosting classifier optimises and develops the prediction learning models for any liver diseases.

Research carried out by Liu et al. (2020) indicated that higher prediction presentation for liver diseases and alcoholic liver diseases were remarked at lower taxonomic levels by using Gradient boosting classifier, as shown in figure 10. The Gradient boosting classifier was identified after performing ridge regression and logistic regression.

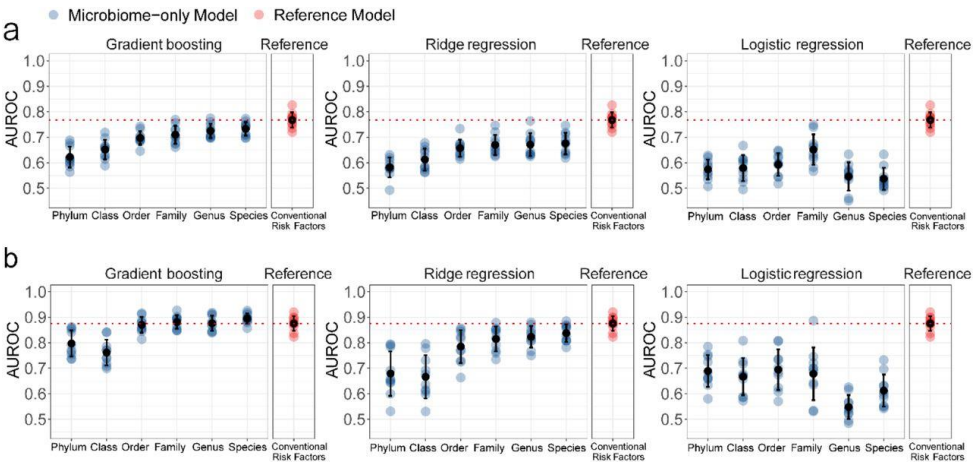

Figure 10: Multivariable logistic regression and ridge regression at different taxonomic levels to indicate the prediction performance using gut microbial features. Row (a) predicts any liver disease and row (b) predicts alcoholic liver disease [64].

With the use of figure 10, researchers could make predictions about liver diseases. The prediction performances were measured in the area under the receiver operating characteristic (AUROC). AUROC scores suggest the probability of experiencing an event. The higher the average AUROC score, the higher the ability to discriminate between taxes and prediction diseases [65]. As shown in figure 10, the highest average AUROC score for predicting any liver disease with the used Gradient boosting model is at the species level with the highest score of 0.733. The highest average AUROC score for predicting alcoholic liver disease is also at the species level, with the highest score of 0.895. Thus, discrimination

between species to predict alcoholic liver disease is higher than any liver diseases. Logistic regression is the most frequently used statistical tool for the use of prediction models. In this case, ridge regression was used because it is more suitable for correlated microbiome features. With ridge regression, a penalty added up to the loss function. As shown in figure 10, row (a), the highest average AUROC score (0,675) was generated at species level using ridge regression for any liver disease. The average AUROC scores for alcoholic liver disease was higher, as shown in row (b). The highest average AUROC score (0,838) was generated at species level using ridge regression. With the use of logistic regression, lower average AUROC scores were assessed. The highest average AUROC score (0,651) was generated at the family level using logistic regression for any liver disease. For alcoholic liver disease, the highest average AUROC score (0,694) was developed at the order level using logistic regression. Form analysis performed in figure 10, Gradient boosting classifier showed the highest average AUROC score at the species level and was used in subsequent analysis.

To investigate the association and predictive function of the gut microbiome on any liver diseases and alcoholic liver diseases, bacterial taxa that contributed to the optimal Gradient boosting classifier were investigated. As shown in figure 5 in the study conducted by Liu et al. (2020) [64], the most abundant gut microbiome signatures associated with liver diseases were the phylum *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes.*

This study reported the different families that most contributed to other kinds of liver diseases based on previous studies. In patients with alcoholic liver disease (ALD), the families *Chitinophagaceae, Steptococcaceae* and *Enterobacteriaceae* have been frequently enriched. For patients who suffer from acute-on-chronic liver failure, NAFLD and cirrhosis, the families *Steptococcaceae, Erysipelotrichaceae* and *Enterobacteriaceae* have been frequently enriched. Also, a negative association with liver diseases were shown. From the phyla *Verrucomicrobia,* the genera *Akkermansia* showed prediction on the liver function.

As mentioned before, patients with liver fibrosis showed qualitative and quantitative changes in microbes (dysbiosis). These changes cause a shift in the balance between primary and secondary bile acids and can cause an increase in intestinal permeability. These patients showed an increase in intestinal dysbiosis. Due to this increase in intestinal microbes, there is an induction of secondary bile acids and a reduction of primary bile acids, leading to liver insufficiency. Induction of secondary bile acids are associated with genera *Clostridium* and *Eubacterium, and* they can produce secondary bile acids. Also, association with an increase in intestinal permeability is shown with bacterial taxa. The genera *Ruminococcus, Dorea, Faecalibacterium* and *Blautia* are related to an increase in intestinal permeability.

Conclusions could be made from the study conducted by Liu, Y. et al. (2020). This study showed an investigation of the gut microbiome to predict various liver diseases. With the use of Gradient boosting

classifier, predictions could be made. The most abundant gut microbiome signatures associated with different liver were the phylum *Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes.* In Campion et al. (2020), Lelouvier et al. (2016) and Li et al. (2020), these species were also associated with the progression of different kind of liver diseases. The family *Enterobacteriaceae* have been enriched in patients with ALD, NAFLD and cirrhosis. It belongs to the species *Proteobacteria* and showed in Campion et al. (2020) also an induction in abundance by a transition from F0 to F1. A negative association with liver diseases is indicated by the species *Verrucomicrobia.* This result is in line with Li et al. (2020). It showed there is a decreased abundance of the phyla *Verrucomicrobia* and *Bacteroidetes* in the liver fibrosis group. The class differs among the four different liver fibrosis groups [64].

5. Discussion

Gut microbiota plays a role in the pathogenesis of metabolic liver diseases. Microbiota are related and involved in the communication between the liver and the gut, called the gut-liver axis. Additionally, there is growing evidence that disruption of the gut-liver axis is involved in the progression of chronic liver diseases. These findings are consistent with the evidence that bacterial translocation is involved in the pathogenesis of liver fibrosis.

A significant number of patients with liver fibrosis remain in the early stage of the disease, the reversible state of the disease. Around 5% of the patients with non-alcoholic steatohepatitis (NASH) will progress into fibrosis or cirrhosis. It remains a challenge for the patients to get fibrosis or cirrhosis, which means many patients enter the clinic too late. When patients undergo the progression of liver fibrosis, microbiome alteration plays an essential role in pathogenesis. In the present study, I investigated the possibility of using bacterial translocation from the intestine to the blood or the microbiome's composition as a diagnostic tool to diagnose liver fibrosis at an early stage. Researchers can assess a detailed analysis of 16S rRNA in the blood or faeces, characterisation of microbial profiles. Early diagnosis of liver fibrosis stops further disease progression toward clinical consequences and initiates appropriate therapeutic regimens. Besides helping hospitalisations, bacterial infections and liver-related complications in cirrhosis are functional regions of investigation.

Recently, several studies have studied changes in bacterial translocation from the intestine to the blood or the composition of the gut microbiome. They determined the faecal and blood microbiota profiles and specified different gut and blood microbiota profiles. After analysing the studies, I should give advice on which profile microbiota investigators should look at. Based on previous studies, there are some disadvantages of using faecal microbiota. First, the bacterial diversity is higher in faecal samples than in blood samples. These high quantities of bacteria might make it challenging to see slight fluctuations in these bacteria [69]. Also, several factors affect gut microbiota and modulate species abundances, like age and delivery pattern, diet, exposure to pathogens, and lifestyle [70]. The last disadvantage of the faecal microbiome is that faecal samples do not reflect the microbiota in the intestine. However, faecal samples are less burden for the patients, and no doctors visit is needed. Measuring the microbiome in blood samples means a lower bacterial diversity, and fluctuations are better visible. However, it is more burden for the patients. After weighing up the pros and cons of measuring the microbiome in blood or faecal, I aimed to investigate the potential role in only blood samples.

The study aimed to investigate a diagnostic tool to diagnose liver fibrosis at stage F1 or F2. It is essential to distinguish mild (F1) correctly, and moderate fibrosis (F2) stages from the normal liver (F0) because liver fibrosis can be treatable and is reversible in the early stages. Champion (2020) observed that no obvious classification was observed when analysing the three groups (F0/F1/F2). With the use of

principal coordinate analysis (PCoA), Lelouvier (2020) had proved that the F0 group differed from the F1 and F2 groups, but the F1 and F2 groups do not significantly differ from each other. That might suggest the two liver fibrosis stages had comparable liver microbiota [45]. Also, the results of Lelouvier (2016) deal with this phenomenon. This study showed that the relative abundances of the two cohorts differ from each other in stage F1 by comparing it to F0. While, However, the relative abundances of the bacterial taxa in stage F2 to F0 are in line in both cohorts. This means stage F1 did not match between the cohorts, and stage F2 did. This also suggests that the microbiome composition in stage F1 differs too much, which is in line with Lelouvier (2020) results. However, the geographical area between the two cohorts varies among each other, which could also be a reason for the differences in bacterial abundances between F1 and F0. I aimed that detection of liver fibrosis detection is more likely in liver fibrosis stages F2 than F1.

First of all, Lelouvier et al. (2016) also indicated that patients with liver fibrosis have, on average, an almost three times higher blood 16S rDNA concentration compared to patients without liver fibrosis. This observation was expected because by increasing bacterial translocation from the gut to the blood, the concentration of bacterial DNA in the blood increases. Based on this analysis, I propose that a rise in blood 16S rDNA concentration can be considered a general biomarker in blood samples to assess whether a patient suffers from chronic liver disease. This literature review described the most unspecified biomarker, but it can accomplish early recognition of chronic liver diseases. With qPCR, blood bacterial DNA could be used successfully to identify the existence or lack of patients with liver fibrosis. Moreover, it could function as a general biomarker. Only in the case of blood 16S rDNA concentration can something be said about this potential marker's reliability. The SD and the mean for blood 16S DNA (copies/ul) are calculated for the Italian confirming cohort population. The SD for both the control and the liver fibrosis group deviates from the mean by a difference of 1.4 copies/ul. Therefore, the SD is smaller than the mean of blood 16S DNA. A smaller SD indicates that more data is clustered about the mean. This means that there is a smaller spread, which is advantageous for the patient. Therefore, the rise in blood 16S rDNA concentration is the most potential biomarker but still a general and non-disease specific biomarker.

Furthermore, all four studies (Li, Z. et al. 2020; Liu, Y. et al. 2020; Lelouvier, B. et al. 2016; Campion, C. et al. 2020) described the most abundant gut microbiome signatures. The most associated phyla in different liver fibrosis stages were *Proteobacteria, Actinobacteria and Bacteroidetes.* In both studies, Campion et al. (2020) and Lelouvier et al. (2016), the phyla *Proteobacteria* showed a higher percentage of the total sequence reads in NAFLD patients with liver fibrosis at an early stage (F1/2). An increase abundance of *Proteobacteria* means, in general, that biomarkers can sort NAFLD patients with early stages of liver fibrosis (F1/F2). Therefore, based on this analysis, I propose that an increased abundance of the bacterial phylum *Proteobacteria* can be a microbiome-related biomarker in blood samples for a potential early diagnostic criterion of liver fibrosis in patients suffering from NAFLD. The increase in

abundance was expected because this family belong to gram-negative bacteria. As demonstrated in the literature, alterations in gut-microbiota raise intestinal permeability and LPS. The release of LPS from the gram-negative bacteria in the gut to the blood can cause LPS-associated toxicity. In patients with liver fibrosis, there is an increase shown in bacterial translocation and LPS levels. Recent studies showed Toll-like receptor 4 (TLR4) plays an essential role in activating the innate immune response. For binding of microbes on the TLR4, co-receptors will induce downstream signalling of the TLR. The TLRs activate the KC, causing a downstream pro-inflammatory cascade. [26]. This cascade can lead to the expression of cytokines and oxidative stress. Expression of these factors results in the fact that LPS-stimulated activation of the Kupffer cells is considered the key mechanism for the pathogenesis of chronic liver diseases. *Proteobacteria* make a significant contribution to it [26]. The reliability of the *Proteobacteria*, a potential microbiome-related marker, is still a matter of dispute. The reliability of the biomarkers should in future be described by standard deviations (SD). A patient does not benefit from a large spread, which indicates the likelihood of getting or not getting liver fibrosis. About the dispersion, like SD, nothing is described in the four articles.

Moreover, LPS-producing bacteria are also associated with obesity, which is a significant risk for developing NAFLD [66]. Likewise, in both studies, Campion et al. (2020) and Lelouvier et al. (2016), the percentage of *Actinobacteria* of the 16S rDNA bacterial sequences is lower in liver fibrosis patients than in the control group. It decreases the proportion and hence the abundance of *Actinobacteria* in patients with liver fibrosis. A decrease in the percentage of the total sequence was expected in both studies because the phyla *Actinobacteria* belongs to gram-positive bacteria. Patients who have a significant risk for developing NAFLD have a high-fat diet, which increases many gram-negative bacteria in the gut at the expense of gram-positive bacteria [66].

The study conducted by Li et al. (2020) showed a higher abundance of *Actinobacteria* in the liver fibrosis group after investigating the rats' gut microbiome composition. In this study, rats were used for human gut microbiome research, and the rats were treated with carbon tetrachloride. Li et al. (2020) used germ-free rats because they resemble most human gut microbiota composition. Nevertheless, there are features of the human pathology that did not recapitulate in rats. Rats have lacked some human-specific gut bacteria, and the proportion of bacterial species is different compared with humans. Meaning, the relative abundances of most of the major genera in rats and humans are relatively diverse, and the rats' models cannot wholly recapitulate human systems [67]. However, it is not only through the use of a different model that results may differ. Also, differences in species between different cohorts can affect results between studies. Likely reflect variations in environmental factors, dietary factors, imbalance in BMI between other groups, age among patients and treatment options, affecting results between different cohort studies. Despite the differences in models used, I propose that a decreased abundance of the bacterial phylum *Actinobacteria* can be a microbiome-related biomarker in blood samples for liver fibrosis's potential early diagnostic criterion in patients suffering from NAFLD. Whether the

decline in abundance of *Actinobacteria* could only link detection of liver fibrosis from patients only suffering from NAFLD, further investigation is needed, in which similar models should be used.

The last striking phenomenon is a relative decrease in abundance of the species *Bacteroidetes* in the liver fibrosis group, suggested by Campion et al. (2020) and Li et al. (2020). *Bacteroidetes* belong to the largest group of LPS producers. LPS-producing bacteria were associated with a cascade of pro-inflammatory responses and promote the progression of liver diseases. As described above, the release of LPS can activate KC, which positively influences the liver's fibrotic activity. Therefore, it was expected to observe an increase in the relative abundance of *Bacteroidetes*. However, a decrease in the percentage of the total sequence in *Bacteroidetes* was observed in both studies. Previously published studies demonstrated a lower quantity of *Bacteroidetes* in patients with a higher BMI [68]. This result can explain the relative decrease in abundance of *Bacteroidetes* in patients suffering from NAFLD, as Campion et al. (2020) described. Thus, a dynamic linkage could be given between gut microbial ecology and the results from patients suffering from NAFLD concerning *Bacteroides*. Based on this analysis, I propose that a decreased abundance of the bacterial phylum *Bacteroidetes* can be a microbiome-related biomarker in blood samples for a potential diagnostic criterion of liver fibrosis in patients suffering from NAFLD. Additionally, Li et al. (2020) suggested a decrease in baseline abundance of the genera *Bacteroides*, indicating a significant correlation with the liver fibrosis group of rats. Whether the decline in abundance of *Bacteroides* could link any chronic liver disease and early detection needs further investigation.

Ultimately, there are some limitations shown in this literature study. First of all, all the four described literature reviews used 16S rRNA amplicon sequencing. It makes it easier to compare the highly conserved sequenced regions of the 16S rRNA gene between studies. However, limitations of this method are that the annotation was based on the presumed association of only the 16S rRNA gene with bacterial taxa, which were already classified as one OTU. It results in the fact that specific genes are not directly sequenced but predicted based on the OTUs. Meaning, less accuracy at species level occur [72]. Furthermore, 16S rRNA sequencing can be highly biased. 16S rRNA sequencing uses databases for the classification of bacterial taxa. When these are incomplete, it can lead to bias. Furthermore, it has a high sensitivity to disturbances. Also, varying PCR amplification can disturb the sequence analysis [73]. An alternative method is whole shotgun sequencing. In this way, more accuracy at species level occurs, and both use different databases for the classification of taxa. However, it is expensive, and the results are hard to interpret [72].

Overall, it can be conducted that the ideal situation in which further investigation is needed is to develop a disease-specific biomarker to diagnose liver fibrosis at an early stage.

6. Future directions

A literature study was performed to investigate the possibility to use bacterial translocation from the intestine to the blood or the microbiome's composition as a diagnostic tool to diagnose liver fibrosis at an early stage. A new microbiome-related biomarker can be developed for liver fibrosis detection at stage F2, more likely than F1. The research here is relatively recent, and not much literature has been done yet. Accordingly, after a literature review, a plan for follow-up research for a specific study population is the next step in broadening the knowledge towards early detection of liver fibrosis. Improving evidence and understanding how the microbiota affects liver fibrosis observed in patients suffering from NAFLD, ALD, hepatitis B/C and cholestasis liver disease are the drivers for diagnostic and prognostic tools. In the future, this will be assessed with improved computational techniques and experimental designs.

This literature study contains both biological and statistical results. Nowadays, animal models perform a crucial role to investigate the causalities between microbiota and liver diseases. However, as earlier discussed, rats, in this case, have lacked some human-specific gut microbiota, and the proportion of bacterial species is different compared with humans. This can result in diverse relative abundances between rats and humans of the major genera. In the future, large-scale clinical trials are required to successfully interpret and utilise the outcomes from bench to bedside. Developing microbiome-related biomarkers of liver fibrosis is an essential objective in experimental hepatology and will assist the construction of clinical trials. There are several things which need further investigation. As described in the discussion section, more research is needed towards the specific increase in 16S rRNA concentration in the blood of patients with liver fibrosis. Can a particular percentage of increase in 16S rRNA concentration linked to a particular liver fibrosis stage? Differ the 16S rRNA concentration between patients with different liver diseases? Also, more investigation is needed towards the relative increase in abundance of the *Proteobacteria* and the relative decrease in abundance of *Actinobacteria* and *Bacteriodetes*. Is a change in abundance specific related to specific liver disease? Or does the transformation of the bacteria mentioned above seen in all patients with liver fibrosis? Can these bacterial phyla serve as a potential microbiome-related biomarker?

This study is focused moreover on patients suffering from NAFLD. For the follow-up study, patients suffering from ALD, hepatitis B/C and cholestasis liver disease should be included. First of all, all the patients provided informed consent for participation. After that, a liver biopsy is taken to determine which stage of liver fibrosis a patient is in. In total, 50 participants are involved in each clinical trial, in which there is equal distribution of men and women. Incapacity, 250 participants are involved (5 different liver diseases). Also, after taking a biopsy, almost equal distribution between the different liver fibrosis stages is needed. In total, three additional studies need to perform to give more reliable results. The inclusion criteria for the cohort study in patients with NAFLD were that patients are: (1) aged

between 30 and 60 years, (2) patients with histologically confirmed fatty liver disease, (3) suffer from morbid obesity with a mean BMI above 40 kg/m2. The exclusion criteria are: (1) history of significant alcohol consumption, (2) viral/autoimmune hepatitis, (3) metabolic diseases, (4) hepatotoxic medication, (5) weight change of 3kg in the previous six months and (6) pregnancy. The inclusion criteria for the cohort study in patients with NASH were that patients are: (1) aged between 30 and 60 years, (2) liver biopsy consistent with NASH of cirrhosis (F4) according to CRN classification. The exclusion criteria are: (1) other causes of liver diseases, (2) history of liver implantation, (3) history of hepatocellular carcinoma (HCC) and (4) pregnancy. The inclusion criteria for the cohort study in patients with ALD are: (1) aged between 30 and 60 years, (2) ongoing consumption of more than 40 gr alcohol/day (female) of 60 gr alcohol/day (male) for six months. The exclusion criteria are: (1) patients with severe liver disease, (2) viral/autoimmune hepatitis, (3) organ failure, (4) uncontrolled GI-bleedings, (5) HCC, (6) pregnancy.

After performing consent, taking a liver biopsy, selecting the study population and phenotyping the metadata, biochemical analysis is performed. From the patient, a 1 ml blood sample is taken and will be examined. Investigation of the relative abundances of *Proteobacteria, Actinobacteria* and *Bacteroidetes* should give more information about a possible new microbiome-related biomarker to diagnose liver fibrosis at stage F1/F2. Importantly, it is easier to determine an increase of relative abundances than a decrease of relative quantities.

Towards detection of liver fibrosis at stage F1 or F2 also further investigation is needed. As earlier discussed, I propose that diagnosing liver fibrosis at early stages is possible, but not precisely for stage F1 or F2 separately. The microbiome-related biomarkers developed for liver fibrosis detection can represent liver fibrosis stages F2 more likely than F1. However, with more specific results, relative abundances of bacterial taxa per liver fibrosis stage can be given and assessed whether F1 only showed potential to diagnose liver fibrosis early.

At the end of the cohort study, hopefully, for each specific liver disease, a disease-specific biomarker is developed. It becomes a challenge, but microbiome-related biomarkers will reduce the patient's burden, stop further disease progression toward clinical consequences, and initiate appropriate therapeutic regimens.

7. General conclusion

Due to disruption of the gut barrier and impairing homeostasis, bacterial translocation occurs. Alterations in the microbiota profiles have been reported to contribute to the further progression of chronic liver diseases. Overall, it can be concluded that it is possible to diagnose liver fibrosis in the early stages of the disease. Both in the blood and the faeces, a microbiome biomarker can be used. However, based on the disadvantages of stool samples, it might be more beneficial to have a microbiome biomarker in blood. To diagnose at an early stage of the disease, it is not likely that it can happen in stage F1 instead of in F2.

The most potential and general biomarker is the increase in 16S rRNA concentration in the blood of patients suffering from liver fibrosis. In this literature study, it was the most unspecific biomarker, but it can accomplish early recognition of chronic liver diseases. In addition, more investigation is needed towards the microbiome-related biomarkers. Although, some observations of bacterial abundance have great potential in the early detection of liver fibrosis. The increased abundance of *Proteobacteria* and the decreased abundance of *Actinobacteria* can be a microbiome-related biomarker in blood samples for liver fibrosis's potential early diagnostic criterion in patients suffering from NAFLD. Whether the change in microbiome composition of *Proteobacteria* and *Actinobacteria* could link more chronic liver diseases and their early detection, further investigation is needed. Lastly, a decrease in abundance of *Bacteroidetes* was also measured in two NAFLD studies. Therefore it can also be a microbiome-related biomarker in blood samples for early diagnosis of liver fibrosis in patients suffering from NAFLD. However, also on this bacterial biomarker, further research is needed on different liver diseases.

8. References

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