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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

We encourage authors to submit their manuscripts to *WJH*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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MINIREVIEWS

Limitations and opportunities of non-invasive liver stiffness measurement in children

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Abstract

Changes in liver structure are an important issue in chronic hepatopathies. Until the end of the 20th century, these changes could only be determined by histological analyses of a liver specimen obtained *via* biopsy. The well-known limitations of this technique (*i.e.*, pain, bleeding and the need for sedation) have precluded its routine use in follow-up of patients with liver diseases. However, the introduction of non-invasive technologies, such as ultrasound and magnetic resonance imaging, for measurement of liver stiffness as an indirect marker of fibroses has changed this situation. Today, several non-invasive tools are available to physicians to estimate the degree of liver fibrosis by analysing liver stiffness. This review describes the currently available tools for liver stiffness determination that are applicable to follow-up of liver fibrosis/cirrhosis with established clinical use in children, and discusses their features in comparison to the "historical" tools.

Key words: Children; Transient elastography; Liver fibrosis; Liver biopsy

Core tip: Non-invasive liver stiffness measurement is a new and helpful tool for assessing liver fibroses in children, but it cannot yet replace liver biopsy.

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INTRODUCTION

Until the end of the 20th century structural changes of the liver could only be determined by histological analyses of a liver specimen obtained by percutaneous liver biopsy. The well-known limitations of this technique (*i.e.*, pain, bleeding and the need for sedation), however, precluded its routine use in follow-up of patients with liver diseases, and it has only been used routinely in studies[1]. The introduction of non-invasive imaging technologies, such as ultrasound and magnetic resonance imaging, has changed this situation, allowing for measurement of liver stiffness as an indirect marker of fibroses. Today, several non-invasive tools are available to physicians to estimate the degree of liver fibrosis by analysing liver stiffness.

This review will describe the currently available tools for liver stiffness determination that are applicable to follow-up of liver fibrosis/cirrhosis with established clinical use in paediatric patients (children between 0 and 18-year-old), and discusses their features in comparison to the "historical" tools.

Liver fibrosis is a dynamic reaction of the healthy liver towards chronic cell injury[2]. It is frequently observed in patients with chronic liver disease, regardless of aetiology[3] and patient age. Structural changes of liver architecture usually appear slowly, within years or decades, and accompanied by a continual development from low-grade fibrosis to liver cirrhosis. Liver cirrhosis, itself, represents the end-stage of fibrotic liver diseases.

Development of fibrosis leads to an increase in liver stiffness, detectable by non-invasive methods. Progression from liver fibrosis to cirrhosis may be preventable, if the fibrosis is detected early in the course. Examples of preventable fibrosing liver diseases are hepatitis B or hepatitis C infections[4,5], liver transplantation[6] or Wilson' s disease. For other fibrosis aetiologies, a close follow-up is recommended to detect changes in liver structure in a timely manner and to determine the disease course. This holds true for post-liver transplant patients and patients with autoimmune liver diseases. Today, histology is the gold standard for the diagnosis of liver fibrosis.

Liver biopsy

Liver biopsy remains the method of choice for clarification of the aetiology of hepatopathies. It has the advantage of obtaining direct information, not only on the degree of fibrosis but also on the presence of inflammation, necrosis, steatosis, and iron or copper deposits. However, the histopathologic examination of a liver specimen also has limitations. Studies have clearly indicated that liver biopsy is prone to sampling errors and may underestimate the amount of liver fibrosis. As such, cirrhosis could be missed on a percutaneous liver biopsy, reportedly affecting an estimated 30% of cases[7,8]. Liver biopsy has further technical limitations. There is a small risk of clinically relevant bleeding (0.3%) and mortality due to the intervention, shown to affect 0.04%-0.07% in a large case series[9]. In a paediatric series, major complications occurred in 1.5% and minor complications in 25% of 275 liver biopsies[10]. Another drawback of this method is the size of the specimen obtained[8]. A single liver biopsy reportedly has a 20%-30% chance of missing the relevant area of interest, thereby underestimating liver diseases[11]. Paediatric patients have an additional risk due to the need of sedation for the biopsy procedure. Therefore, in clinical practice liver histology is almost exclusively used for diagnoses and only in certain settings, such as liver transplantation, and for therapy control[1,12].

On the other hand, liver biopsy has some clear advantages. A recent study of a cohort of patients with either histologically-proven non-alcoholic steatohepatitis (NASH) or non-alcoholic fatty liver disease (NAFLD) showed that outcome (*i.e.*, death, liver transplantation or severe liver disease) was directly dependent upon the degree of fibroses[13]. Another recent study by Mann *et al*[14] demonstrated an association of portal inflammation, metabolic syndrome and fibrosis in 430 obese children. These findings support the current tenet that portal inflammation and exact degree of fibrosis are best determined by liver biopsy.

Histological assessment of liver biopsy

The liver biopsy specimen is recommended to have length of at least 10 mm and width of at least 1 mm (obtained with > 18 gauge needle)[15]. Several histological scoring systems have been established for grading (necroinflammatory activity) and staging (fibrosis) of structural liver damage in patients[16]. The Desmet score[17] is used to evaluate adult hepatitis C patients, and the METAVIR[18,19] and Ishak score[20] are used in cases of chronic viral hepatitis (B and C). The SSS-score of Chevallier[21] was developed to quantify fibroses irrespective of the underlying disease. Some of these scores have been evaluated in children (Table 1), and a detailed break-down of each (in children and adults) is provided below: (1) the METAVIR score[18] assesses fibrosis qualitatively on a 0-4 scale, with F0 indicating absence of fibrosis, F1 indicating portal fibrosis without septa, F2 indicating portal fibrosis with a few septa, F3 indicating architectural distortion with numerous septa without cirrhosis, and F4 indicating cirrhosis. This score has been used to evaluate adult patients with hepatitis B and C[19] and paediatric patients after liver transplantation[22], biliary atresia[23], intestinal failure[24] and total parenteral nutrition[25]; (2) the grading score of Ishak *et al*[20] assesses fibrosis qualitatively on a 0-6 scale. The Ishak score has been used in paediatric populations with various liver diseases, and including children after liver transplantation[26] or cardiovascular surgery[27]; (3) the grading score of Desmet *et al*[17] assesses fibrosis qualitatively on a 0-4 scale, with F0 indicating absence of fibrosis, F1 indicating portal fibrosis, F2 indicating fibrosis with septa without distortion of the liver architecture, F3 indicating septal fibrosis with severe

Table 1 Comparison of the 4 main histological scoring systems used in the evaluation of fibrosis in paediatric liver diseases today

Scoring system	Staging	Evaluated in adults with	Evaluated in children with
METAVIR	F0-F4	Hepatitis B and C	Biliary atresia, intestinal failure, total parenteral nutrition and post-liver transplantation
Ishak	F0-F6	Hepatitis B and C	Post-liver transplantation and after cardiovascular surgery
Desmet	F0-F4	Hepatitis C	No
SSS-score	0- > 15	Hepatitis B and C	Hepatitis B

distortion of the liver architecture, and F4 indicating cirrhosis. It has been used to evaluate adult patients with chronic hepatitis C[28]; and (4) the semi-quantitative severity score of Chevalier *et al*[21] has been used in children[29] and adults with hepatitis B[30] and C[31].

Aminotransferases

Numerous attempts have been made to determine liver fibroses by non-invasive means. One of the oldest is measurement of serum aminotransferases, which remains the most widely used, and convenient, tool to measure liver cell integrity. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are inexpensive laboratory values. They can be easily obtained from a patient and are stable in serum specimen. ALT, especially, is highly liver specific.

Unfortunately, aminotransferases poorly reflect the stage of liver fibrosis or cirrhosis. If they are elevated, a more detailed examination of the liver is obligate. But, ALT and AST may even be normal or only slightly elevated in fibrotic or cirrhotic liver diseases. The positive predictive value of aminotransferases for NAFLD or NASH is low. In a series of 222 patients with histologically-proven NAFLD, 37% of the patients with advanced fibrosis or NASH presented with normal ALT levels. This phenomenon was also recently demonstrated in children, in a study of paediatric cases of NAFLD conducted by Molleston *et al*[32].

Aminotransferases may serve as a first screening tool for detection of fibrosis, but even normal levels of aminotransferases do not exclude severe liver disease with changes in liver structure. Some of the techniques that have been developed to identify NAFLD in adult patients have been tested in children, including the AST to platelet ratio index (APRI) score, the NAFLD fibrosis score[33] and the Fibrosis-4 index score. Yet, recent data have indicated that only the APRI score and the paediatric NAFLD fibrosis score reliably reflect fibrotic changes of the liver. Alkhouri *et al*[34] have developed and published a new paediatric NAFLD fibrosis score based on a model using ALT, alkaline phosphatase, platelet counts and gamma-glutamyl transferase, and demonstrated its predictive ability of fibroses as good.

Collectively, these tests are reliable in detecting severe fibrosis or cirrhosis (grade 2 or greater for the Desmet score). Thus, while they can reliably show if the patient suffers from a change in liver structure they cannot reliably predict the exact degree of fibrosis.

SONOELASTROGRAPHY

Transient elastography

Transient elastography (TE) is a technique based on the measurement of the velocity of a shear wave that is induced to the liver by a mechanical impulse. To apply that impulse to the liver, the probe has to be pressed onto the skin with a certain force, and the thoracic wall prevents the liver from being compressed by the probe. Therefore, TE can only be measured reliably in the right lobe of the liver and not in other organs or in other parts of the liver.

The velocity of the shear wave is directly proportional to the stiffness of the liver. Stiffness mainly depends on the amount of fibrotic material in the liver. Therefore, liver elasticity is measured in kilopascal (kPa) and liver stiffness increases with liver fibrosis. The probe is placed in the 7th or 8th intercostal space in the right ventral axillary line. The patient lies in supine position, with the right arm in maximal abduction. This technique has been described in detail elsewhere[35]. A mechanical impulse of 50 Hz induces an elastic shear wave that passes through the liver tissue. The speed of this wave is measured *via* ultrasound. For more detailed information on the basic physical principle, the Young Modules, see Frulio *et al*[36].

TE reliably detects liver fibroses, as demonstrated in numerous studies and meta-analyses comparing the technology to liver biopsy[35-42]. The median liver stiffness in adults varies between 4.4 and 5.5[43,44]. In addition, there is evidence that stiffness is greater in males, increases with body mass index in adult patients, and tends to increase with age but not to a statistically significant extent[44]. In children, the median liver stiffness significantly rises with age, starting with 4.4 in preschool children and rising to 5.1 in pubertal children. Liver stiffness in children has also been shown to differ according to sex, with girls showing significantly less (4.7) than boys (5.6)[45]. In split liver transplants of left liver, which is the main transplantation technique used in infants, toddlers and preschool children, liver stiffness measurement cannot be used because it is technically performable only in the right liver lobe (as detailed above). A clinical example of TE use in a paediatric patient is presented in Figure 1.

Introduction of the small TE-probe that is also suitable for use with infants and very young children has made TE possible for every age group. But liver stiffness measurement can only be performed in a patient that is laying calmly in supine position. This is usually not an

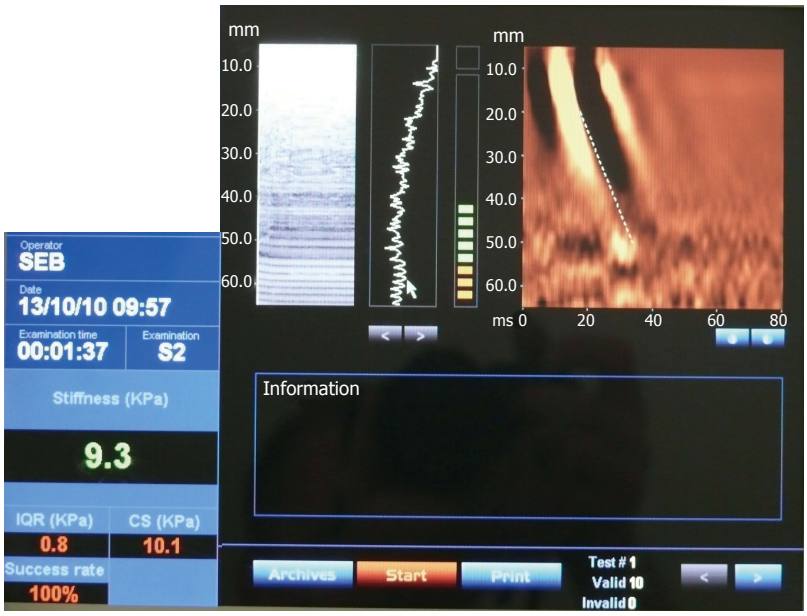

Figure 1 Transient elastography findings for a 10-year-old female suffering from Wilson's disease. The patient's brother had previously developed acute liver failure, which triggered routine monitoring of the patient thereafter. The patient was clinically completely healthy. The transient elastography shows 9.3 kPa, which is above the 6.5 kPa upper limit of normal. Histology findings for the patient showed the liver to be cirrhotic.

attainable state in toddlers without sedation. Therefore, the problem of invalid liver stiffness measurement due to moving and crying of the patients makes this method questionable in infants.

Another general drawback of this method is the price. The technique is reliant on hardware that ultrasound machines do not come equipped with normally. Therefore, an extra-device is required to accompany the ultrasound machine and this produces extra-costs of more than 50000 Euros. Finally, the capacity for integrated measurement in B-mode ultrasound images is not yet available.

Findings from a recent Cochrane analysis of adult patients with alcoholic liver disease led to the recommendation of TE as a useful tool to exclude fibroses and, in cases of liver stiffness measurement above 12.5 kPa, to suggest cirrhosis. These data, however, still have to be confirmed in further studies[46], especially for their applicability to the paediatric age group. It is well accepted that TE enables the investigator to clearly exclude severe changes in liver architecture, but it remains a matter of debate whether TE can also enable clear staging of fibrosis. As such, TE is routinely used to assess liver fibrosis in adult patients with chronic hepatitis C, and this use is confirmed in the EASL Clinical Practice Guidelines 2011[47]. With the increasing application of TE in children with viral hepatitis, however, TE has the capability to gain more relevance for detection of liver fibrosis.

Acoustic radiation force impulse

Acoustic radiation force impulse (ARFI) is a point shear wave elastography that measures tissue elasticity independent of an external mechanical impulse to the tissue. Therefore, this method is not only useful for liver stiffness measurement but also for determination of changes in stiffness of the spleen[48], testis[49], thyroid[50], breast[51], placenta[52], pancreas in chronic pancreatitis[53] and transplanted kidney[54]. The technique is based on an acoustic impulse and measurement of the speed of the shear wave induced by it; results are displayed in m/s. The stiffer the organ, the faster the shear wave.

The ARFI method has two advantages. First, it can be performed by an additional technical tool for a high-end ultrasound system, providing integrated B-mode images. Second, the tissue is not compressed by the probe, as in TE. Compression itself causes changes in stiffness, and this feature of ARFI enables measurement of stiffness in numerous tissues. Many studies have shown the reliability and reproducibility of this technique in adult patients[55] and in children[56]. The correlation of ARFI and fibroses is in a good range[57], comparable to that of TE[58], and control-values have been established for children[56,59] and adults[36] (Table 2). Moreover, ARFI was demonstrated as effective in paediatric patient groups with biliary atresia or severe fibrosis[60,61] and in follow-up after liver transplantation[62]. A clinical example of ARFI use in a paediatric patient is presented in Figure 2.

Children with biliary atresia could gain particular benefit from non-invasive examinations for assessment of timing of liver transplantation after kasai-portoenterostomy[63,64]. According to METAVIR or SSS-score, ARFI shows overlap of shear wave velocity values in different fibrosis stages, as shown in the study by Hanquinet *et al*[65]. ARFI might offer diagnostic advantages over B-mode imaging in terms of combining stiffness measurement with sonomorphological parameters as the qualitative sonomorphological aspect becomes

Table 2 Control and normal values of non-invasive liver stiffness measurement

	Normal values (ULN is defined as mean + 1.64 SD)		
	Children	Adults	Impulse generation
TE	ULN: 6.47 kPa[40]	8.3/7.83 (m/f)[39]	Mechanical
RTE	Median: 106 a.u.[67]	127 a.u.[78]	Aortal pulsing
MRE	Mean: 2.71 kPa[79] -2.93 kPa[71]	3.45 kPa[80]	Acoustic
ARFI	ULN: 1.39 m/s (mean + 1.64 SD)[59,81]	1.35 m/s[36]	Ultrasound

Normal values are defined as mean + 1.64 times SD, while control values are expressed as mean. ARFI: Acoustic radiation force impulse; MRE: Magnetic resonance elastography; RTE: Real-time tissue elastography; TE: Transient elastography; ULN: Upper limit of normal.

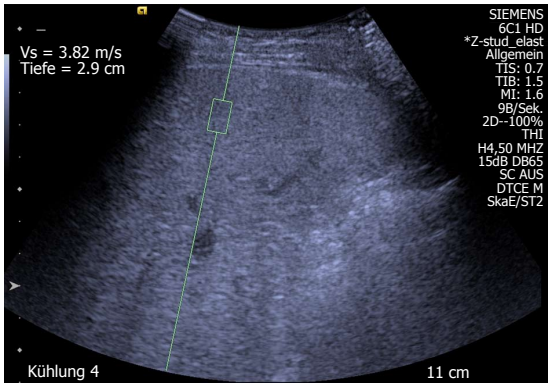

Figure 2 Acoustic radiation force impulse measurement of the liver in a 16-year-old female patient with cystic fibrosis. Hyperechoic liver parenchyma with irregular liver surface in fibrotic liver parenchyma was revealed. The shear wave velocity was 2.3-3.82 m/s in multiple measurements, significantly above normal values. The same patient had undergone a Fibroscan and the results showed a stiffness of 21.3 ± 2.5 kPa. Six months previously, another Fibroscan had shown a value of 20.4 ± 2.8 kPa.

quantitative[61]. This makes comparison in patients easier.

Similar to TE, increased application of ARFI in children could lead to an implementation of this type of measurement in the routine clinical work flow, especially for patients with specific paediatric diseases, such as cystic fibrosis or biliary atresia.

Real-time tissue elastography

Real-time tissue elastography (RTE) examinations can be performed with an ultrasound device and a standard linear transducer[66]. The RTE software captures images of tissue motions caused by heartbeats or respiration. These images are then transferred into colour-coded plane and the system calculates a histogram of strain elasticity values of the matrix in arbitrary units (a.u.), ranging from 0 to 255[67]. The method can be performed without extra-hardware, but data on the value of this method in children are scarce. Morikawa *et al*[67] analysed RTE in 101 adult patients with hepatitis c and found a good correlation of the RTE values with the histologic grading of fibroses. In contrast, data obtained from children in another study[68] showed only a moderate correlation, and it was concluded that RTE could not be recommended for a clear differentiation of fibrosis stages while the difference between stage Ⅳ fibrosis and normal liver tissue or stage I fibrosis was significant.

Other studies of adult patients[69] have concentrated

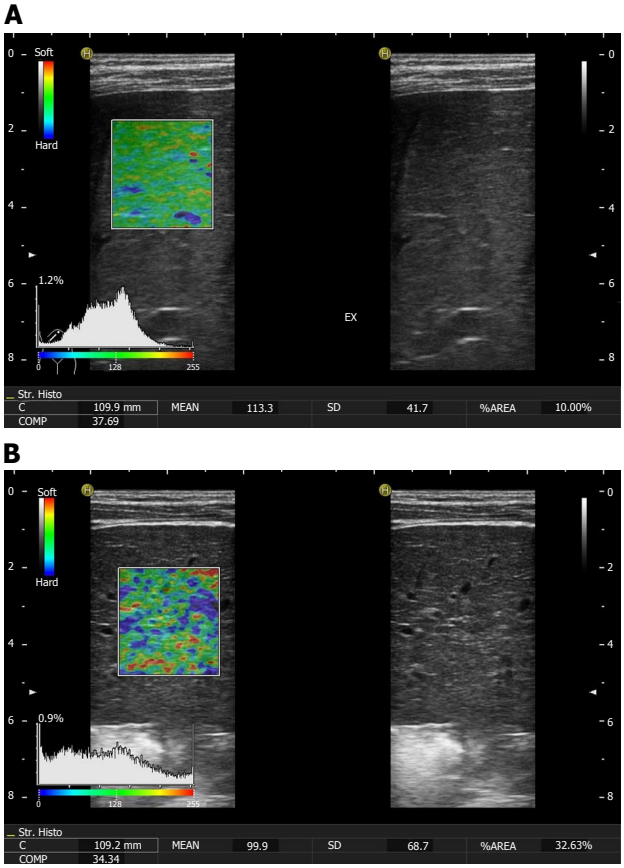

Figure 3 Real-time tissue elastography in a normal and cirrhotic liver. A: RTE with a normal strain histogram (mean: 113.3 a.u.; %AREA: 10%) in a 8-year-old female patient with cystic fibrosis and nearly normal liver structure; B: RTE with pathological strain histogram in a 6-year-old female patient with tyrosinemia type 1 and liver cirrhosis with small nodules. The mean value was 99.9, and the peak of histogram shifted to the left to lower values of the mean. The percentage of stiffer areas (colour-coded in blue; %AREA) increased up to 23.6%. This histogram is more flattened in comparison to the normal strain histogram. RTE: Real-time tissue elastography.

on the elastic or fibrosis index values, which have not been adequately studied in the paediatric age group. In a meta-analysis of RTE conducted by Kobayashi *et al*[70], the authors concluded that RTE has low accuracy for detecting any stage of fibrosis. Today, we would not recommend the use of single statistical parameters as the mean elasticity value of strain histogram or %AREA in children alone to predict the histological fibrosis stage. Differentiation of high fibrosis stages to normal tissue is possible, but application in young infants can be

difficult. Clinical examples of RTE use in two paediatric patients are presented in Figure 3.

Further studies on the use of the elastic index in paediatric patients should be conducted. High fibrosis stages can be differentiated from low fibrosis stages, but no clinical recommendations exist as of yet.

MR-elastography

MR-elastography (MRE) is an elastography technique using an acoustic impulse to produce a shear wave. The impulse is produced by an audio subwoofer and subsequently transmitted to the liver *via* a connecting-tube that is placed on the skin of the patient. Then, the shear wave induced by this acoustic impulse is measured and stiffness is calculated in kPa[71]. Studies of MRE in adult patients with hepatitis C have shown good relation of MRE-measured liver stiffness, as compared to Child-Pugh score[72]. In another study of adult patients with cystic fibrosis[73] the liver stiffness measurement was shown to correlate well with serum levels of amino-transferases and also with ultrasound findings, but there were insufficient data to make any conclusions regarding histopathologic changes.

A new and promising application of MRE involves the differentiation of NASH from NAFLD. Both diseases can occur in obese patients, but there is yet no non-invasive method capable of distinguishing between the two. Patients with NASH develop cirrhosis in 10% of cases, while patients with NAFLD do not. Neither aminotransferases[32] nor ultrasound can differentiate these two diseases. Recent studies have suggested that MRE might be able to reliably determine the presence of NASH in an obese patient[74]. Future studies may prove that MRE, therefore, is useful, even in clinical analysis of obese patients, for defining relevant end-points.

DISCUSSION

ARFI does not replace liver biopsy for staging of liver fibroses or cirrhosis, neither do TE, RTE or MRE[75,76]. The limitations of these non-invasive techniques are low specificity and high cost, the latter being especially relevant for TE.

Liver structure changes can be excluded by each of these non-invasive techniques, with an acceptable sensitivity but an unacceptable low specificity. TE, ARFI and MRE have the potential to exclude severe liver structure changes. For RTE, however, the data are conflicting and do not support a recommendation; certainly, further studies are necessary. For diagnosing liver disease, none of these non-invasive techniques is useful. But, in many patients, the ethology is quite clear due to readily assessable clinical or laboratory aspects, such as the presence of obesity, chronic viral hepatitis or alpha-1 antitrypsin deficiency. In cases of the patient being post-liver transplantation or with an already-obtained liver biopsy, the analysis of liver structure changes is of greater importance.

A possible diagnostic approach to patients with liver disease in 2016 is to first perform clinical examinations to obtain anthropometric data, ultrasound images and standard laboratory measures. If then there is evidence for liver disease, ARFI or TE should be performed. If those findings then suggest liver structure changes, a biopsy should be obtained in any case. If the findings suggest normal liver structure, the biopsy may be delayed and further laboratory studies may be performed first. If there is no change in aminotransferase levels after 6 mo, a liver biopsy should be performed. Non-invasive liver stiffness measurement can be used for follow-up after liver biopsy if the stage of fibrosis has been determined based on histopathological criteria[77].

In patients with obesity, MRE possibly offers a new approach by which to define patients at risk for NASH or even to diagnose NASH in obese patients. Therefore, in the setting of an obese patient, MRE presents a real advantage over the classical methods of hepatology and future studies will show if this promising technique is suited to becoming part of the routine diagnostic workup in obese patients early in their clinical course and also in follow-up.

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ORIGINAL ARTICLE

Basic Study

Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver

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Author contributions: Schneider ACR and da Silveira TR conceived the project and designed the experiments; Gregório C, Guizzo R and Longo L carried out genetic assays and zebrafish liver histology; Malysz T and Faccioni-Heuser MC performed ultrastructural analysis; Schneider ACR and Uribe-Cruz C analysed the data; the manuscript was written by the first author Schneider ACR and reviewed by an English grammar professor; all authors contributed clarifications and guidance on the manuscript; all authors were involved in editing the manuscript; all authors read and approved the final manuscript.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at schneiderac@gmail.com. Participants gave informed consent for data sharing. No additional data are available.

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Abstract

AIM

To evaluate the effects of chronic exposure to ethanol in the liver and the expression of inflammatory genes

in zebrafish.

METHODS
Zebrafish (n = 104), wild type, adult, male and female, were divided into two groups: Control and ethanol (0.05 v/v). The ethanol was directly added into water; tanks water were changed every two days and the ethanol replaced. The animals were fed twice a day with fish food until satiety. After two and four weeks of trial, livers were dissected, histological analysis (hematoxilin-eosin and Oil Red staining) and gene expression assessment of adiponectin, adiponectin receptor 2 (*adipor2*), sirtuin-1 (*sirt-1*), tumor necrosis factor-alpha (*tnf-a*), interleukin-1b (*il-1b*) and interleukin-10 (*il-10*) were performed. Ultrastructural evaluations were conducted at fourth week.

RESULTS
Exposing zebrafish to 0.5% ethanol developed intense liver steatosis after four weeks, as demonstrated by oil red staining. In ethanol-treated animals, the main ultrastructural changes were related to cytoplasmic lipid particles and droplets, increased number of rough endoplasmic reticulum cisterns and glycogen particles. Between two and four weeks, hepatic mRNA expression of *il-1b*, *sirt-1* and *adipor2* were upregulated, indicating that ethanol triggered signaling molecules which are key elements in both hepatic inflammatory and protective responses. *Adiponectin* was not detected in the liver of animals exposed and not exposed to ethanol, and *il-10* did not show significant difference.

CONCLUSION
Data suggest that inflammatory signaling and ultrastructural alterations play a significant role during hepatic steatosis in zebrafish chronically exposed to ethanol.

Key words: Ethanol; Hepatic steatosis; Inflammation; Zebrafish; Alcoholic fatty liver

Core tip: Excessive alcohol consumption remains one of the most important causes of liver disease worldwide. Alcoholic steatosis results from the deposition of fat in liver cells and is the earliest stage of alcohol-related liver disease. Usually inflammation is associated with steatohepatitis, however our results demonstrate that chronic ethanol exposure increased the expression of the inflammatory gene interleukin-1b. Paradoxically the expression of adiponectin receptor-2 and sirtuin-1 also increased for attenuating the liver injury. Ultrastructural abnormalities were observed showing early alterations in liver cells. Knowledge of alcohol injury mechanisms will contribute to the development of novel therapies in the treatment of alcoholic liver disease.

Schneider ACR, Gregório C, Uribe-Cruz C, Guizzo R, Malysz T, Faccioni-Heuser MC, Longo L, da Silveira TR. Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver. *World J Hepatol* 2017; 9(8): 418-426 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i8/418.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i8.418

INTRODUCTION

Alcoholic liver disease (ALD) encompasses a wide spectrum of injury, ranging from simple steatosis, leading to steatohepatitis, fibrosis and finally to cirrhosis[1,2]. Hepatic steatosis is the first and most common consequence of alcohol abuse, develops in about 90%-95% of individuals who drink heavily, is usually asymptomatic and self limited; but may also occur in individuals who drink moderately[2]. Several studies have suggested that progression to more severe liver disease occurs in about 5%-20% of alcohol consumers[1]. As a consequence, it is important to better understand the pathogenesis of hepatic steatosis and the relationship between steatosis and liver injury.

Excessive accumulation of triglycerides in hepatocytes is the hallmark of hepatic steatosis. The source of triglyceride in the liver of ethanol consumers may be originated from disturbances of fatty acid oxidation mechanisms, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, alterations in lipogenic and lipolytic pathways and immune responses to ethanol[3-7]. A number of molecular mediator pathways regulating the synthesis, export, and oxidation of lipids have been discovered to be altered by ethanol: Sterol regulatory element-binding proteins, peroxisome proliferator-activated receptors alpha and gamma, adiponectin, sirtuins and others[8].

Zebrafish is increasingly used as an *in vivo* model system for translational research, since zebrafish have a high degree of genetic conservation and their morphological and molecular basis of tissue and organ development is either identical or similar to other vertebrates including humans[9,10]. In previous studies regarding to hepatic diseases related to ethanol, zebrafish proved to be a valuable strategy for identifying lipogenic mechanisms, genes and pathways that influence hepatic steatosis[11-14]. Studies focused in inflammatory pathways in steatosis are scarce and the issue is not completely elucidated. Chronic ethanol consumption results in the activation of innate immunity and an inflammatory state, which contributes to the pathogenesis of ethanol-induced liver injury. The expression of tumor necrosis factor - alpha (*tnf-a*), interleukin-1b (*il-1b*), interleukin-10 (*il-10*), adiponectin, adiponectin receptor 2 (*adipor2*) and sirtuin-1 (*sirt-1*) were evaluated and histological and ultrastructural evaluations were performed in liver of adult zebrafish after chronic ethanol exposure.

MATERIALS AND METHODS

Animal care and use statement

Wild-type, adult zebrafish (*Danio rerio*), male and female, were purchased from a commercial distributor (Fish Flower, Porto Alegre, RS). The animals were of

Table 1 Primers and probes identification assays

Gene	Assay ID
adiponectin	F: 5′-AGG CTT AGA CTG TGA ACG GTG GGA C-3′ R: 5′-AGC AGG TGT GTC CAG ATG TTT CCA G-3′
adipor2	dr0342657
sirt-1	ENSDART00000098209
tnf-a	dr03126848
il-1b	dr03114368
il-10	dr03103209
ef-1a	dr03432748

tnf-a: Tumor necrosis factor-alpha; *il*: Interleukin; *adipor2*: Adiponectin receptor 2; *sirt-1*: Sirtuin-1; *ef-1α*: Elongation factor-α gene.

heterogeneous wild type stock from the standard short-fin phenotype and were housed for 2 wk before the experiments in order to acclimate to the laboratory facility. The animal protocol was designed to minimize pain or discomfort to the animals. Fish were maintained in aerated water at 28 ℃ ± 2 ℃, 6.8-7 pH, on a 12/12 light/dark photoperiod cycle (lights on at 7:00 am). Biochemical parameters and quality of the water were monitored regularly: pH, presence of nitrates and nitrites, oxygen and ammonia levels. The animals were fed twice a day with fish food until satiety. Experiments were performed using a total of 104 animals. All fish used in this study were healthy and free of any signs of disease.

After acclimation period, the fish were randomly allocated into experimental tanks, density of 1 fish per liter of water. The following groups were performed (n = 52/group): Control (C) and ethanol (E). E group received 0.5% (v/v) of ethanol (Merck KGaA, Germany) directly added into water; tank water was changed every two days and the ethanol replaced[15]. This ethanol dose was chosen due to the liver damage observed by Schneider and coworkers in zebrafish exposed to 0.5% of ethanol[15]. The tank water of C group was also changed in same days of E group. At 2 and 4 wk, fish were euthanized by hypothermal shock[16] and livers were completely removed for molecular and histological analysis.

The protocols were approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre, Brazil (No. 10.0327), and conducted in accordance with international guidelines for the care and use of laboratory animals.

Histological analysis

Livers of zebrafish dissected at 2nd and 4th weeks were stained with hematoxylin and eosin (n = 5/group) or Oil Red (n = 5/group). Livers were fixed in 10% formalin, embedded in paraffin wax, sectioned (5 μm), and slices were stained with hematoxylin and eosin. Livers embedded in Tissue-Tek OCT Compound (Sakura Finetek, United States) were cryosectioned (8 μm thick) and stained with Oil Red (Sigma-Aldrich, United States) to assess fatty droplet accumulation.

For ultrastructural evaluation, livers of 2 animals (male) of each group (C and E) were fixed in 2.5% glutaraldehyde diluted by 0.12 mol/L phosphate buffer (pH 7.2-7.4) for 3 h at 4 ℃. The material was washed three times in the same buffer at 30-min intervals and then post-fixed for 30 min in 1% buffered osmium tetroxide followed by a phosphate buffer (0.1 mol/L) wash three times at 15-min intervals. Livers were dehydrated using ascending grades of alcohols and embedded in an epon-araldite mixture. Ultrathin sections (90 nm) were stained with 2% uranyl acetate and 1% lead citrate[17]. The ultrathin sections were examined under JEM 1200 FX Ⅱ transmission electron microscope.

Gene expression assessment

The liver samples (pool = 3 livers; n = 5/group/period) were immediately immersed in liquid nitrogen and stored in ultrafreezer (-80 ℃). Total RNA was extracted using the TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol and the concentrations were quantified by Nanodrop (Thermo Fisher Scientific, United States) at 260 nm. RNA purity was verified by a 260/280 nm ratio of 1.8 or greater. First-strand cDNA was synthesized from 3 μg of total RNA using the Superscript ™ Ⅱ RT system (Invitrogen, United States). Gene expression analysis of *adiponectin*, *adipor2*, *sirt-1*, *tnf-a*, *il-1b* and *il-10* were performed from 5 μL of cDNA and run in duplicate using TaqMan Gene Expression Assays (Life Technologies, United States) (Table 1).

PCR amplifications were run on a Step One™ Real time PCR System (Applied Biosystems, United States) and performed starting with a 2 min denaturation step at 50 ℃, 10 min at 95 ℃ followed by 40 cycles with 15 s at 95 ℃ and 1 min at 60 ℃.

Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ (threshold cycle) method and normalization was done using the *elongation factor-α gene* (*ef-1α*).

Statistical analysis

Log-transformed data was tested with Kruskal-Wallis test and Dunn as *post hoc* test for multiple comparisons. Results with $P < 0.05$ were considered statistically significant. All analysis were performed using the Statistical Package for the Social Sciences (SPSS 18.0) software.

RESULTS

Ethanol effects on zebrafish liver histology

Sections of livers from control animals stained with hematoxylin-eosin showed well-preserved liver cells without signs of fat deposits (Figure 1A). After 2 wk of ethanol exposure, the liver appearance of animals from E group were similar to the C group (Figure 1B), however at 4 wk, the hepatocytes of animals from E group showed an expressive enlargement and presented nuclei displaced to the periphery of the cytoplasm due to fatty infiltration (Figure 1C). Livers of the control animals stained with

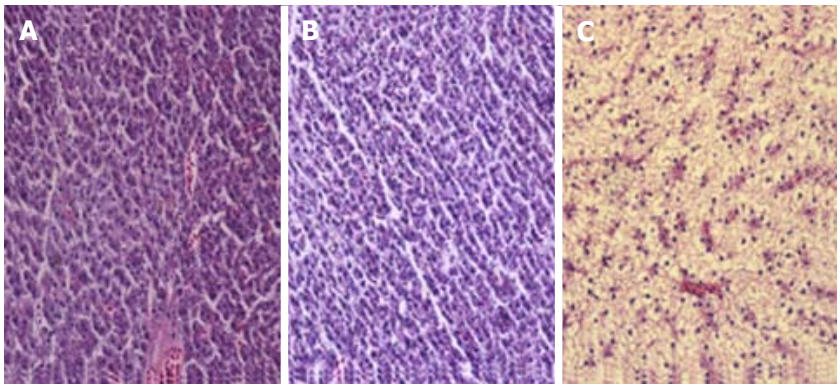

Figure 1 Hematoxylin-eosin staining of liver sections from zebrafish. A: C group (2 wk), the hepatocytes are aligned in cords, absence of fat droplets; B: E group (2 wk), without apparent changes compared with the C group; C: E group (4 wk), enlarged hepatocytes due to fatty infiltration. Magnification: 400 ×.

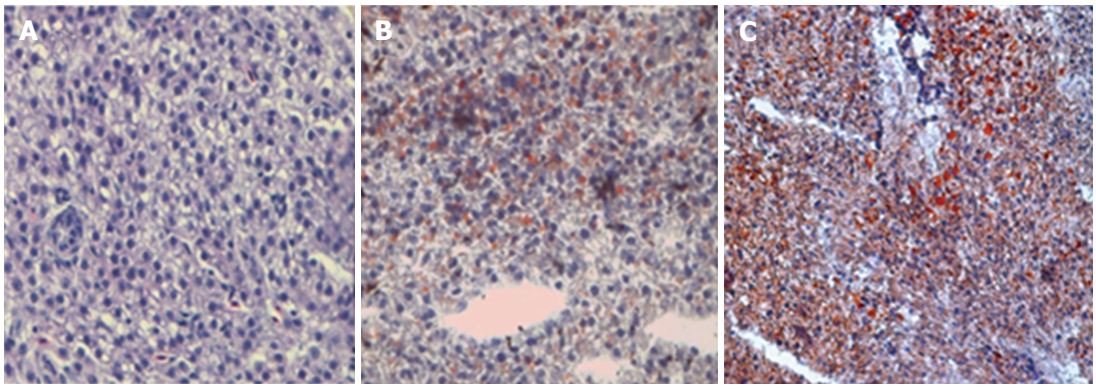

Figure 2 Oil red staining sections of zebrafish liver. A: C group (4 wk), absence of lipid droplets; B: E group (2 wk), mild presence of lipid droplets; C: E group (4 wk), intense lipid accumulation induced by ethanol in hepatocytes. Magnification: 400 ×.

Oil Red did not present any lipid droplets (Figure 2A). However, ethanol-treated animals presented a light steatosis at 2nd week (Figure 2B) which increased severely in the 4th week (Figure 2C).

The supplemental file contains the results of ultrastructural evaluations. Control group showed hepatocytes with hexagonal shape, evident nucleoli of moderate size and located in the centre of the spherically shaped nuclei (Figure 3A), intracellular duct with microvilli (Figure 3C), rough endoplasmic reticulum (RER) contained few cisternae and were closely associated with mitochondria (Figure 3E). Compared to control group, hepatocytes of ethanol-treated fish showed a large amount of glycogen associated with numerous lipid droplets (Figure 3B); the intracellular canaliculi often showed signs of degeneration with aspects of myelin figures therein (Figure 3D); and augmented number of RER cisterns (Figure 3F).

Gene expression assessment

At 2nd week the genes evaluated did not present statistical difference in mRNA expression between E and C groups, except for *tnf-a,* which was decreased. An increase of expression of *tnf-a, il-1b, adipor2* and *sirt-1* was observed between two and four weeks in E group, demonstrating that time to ethanol exposure had influence on expression of these genes. The *il-10* expression did not reach significant statistical difference between groups at any period (data not shown). *Adiponectin* mRNA was not detected in liver of animals from C and E groups.

The effect of ethanol on the expression of cytokines mRNA

The hepatic *tnf-a* expression in E group was lower than in C group at 2 wk (P = 0.018). The *il1-b* expression was significantly increased between C and E groups at 4th week (P = 0.024) (Figure 4).

Ethanol effects on mRNA expression of adipor2 and sirt-1

The expression of *adipor2* increased in E group between 2 and 4 wk (P < 0.0001) and was higher in E compared to C group (P = 0.006) at 4th week (Figure 5). *Sirt-1* showed an increased expression in E group along time until the 4th week (P = 0.001) (Figure 5).

DISCUSSION

Hepatic metabolic derangements are key components in the development of steatosis, considered the first hit for development of ALD. Until recently, the role of inflammation was linked to the presence of steatohepatitis, and scarce evidences have shown the precocity

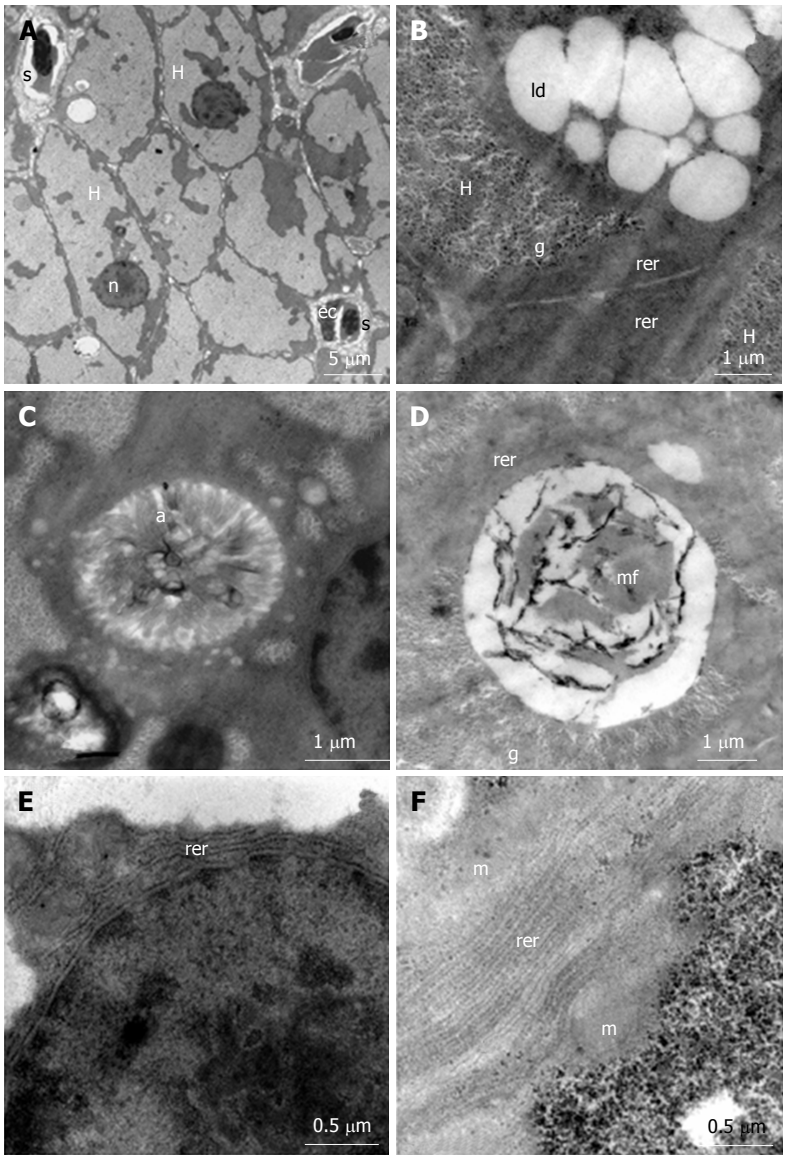

Figure 3 Electron micrographs of liver sections of control (A, C and E) and ethanol exposed groups (B, D and F). A: Polygonal hepatocytes (H), spherical nucleus (n) sinusoid (s), endothelial cell (ec); B: Presence of large amount of glycogen (g) and lipid droplets (ld) in the hepatocytes cytoplasm; C: Intracellular canaliculus with large number of microvilli (a) within; E: It is noted the parallel arrangement of rough endoplasmic reticulum (rer) around the core; D: Myelin figure (mf) inside an intracellular canaliculus; F: Rough endoplasmic reticulum (rer) composed by 8-12 parallel cisterns; H: Hepatocytes; ec: Endothelial cell.

of inflammatory signaling during steatosis[18].

Important histological and ultrastructural abnormalities in liver of chronic ethanol-exposed zebrafish were seen at 4th week in this study. A light steatosis was detected by oil red staining at 2nd week, which increased severely at 4th week. Electron transmission microscopy revealed concurrent marked accumulation of glycogen and lipid droplets in cytoplasm, committed intracellular canaliculi, and increased RER cisterns. Our findings were similar to those described in alcoholic humans: Increased glycogen and fat deposits in the cytoplasm, abnormalities in endoplasmic reticulum[19]. Howarth *et al*[13] observed abnormalities of endoplasmic reticulum and biliary canaliculi in acutely ethanol treated zebrafish larvae. Mitochondrial and ER abnormalities were seen in a model of non-alcoholic fatty liver disease (NAFLD) induced by fructose in zebrafish[20].

In accordance to histological findings, there were changes in hepatic mRNA expression of *il-1b, tnf-a, sirt-1* and *adipor2*. At fourth week, in the presence of more advanced steatosis, *il-1b* showed an expressive increase. Growing evidence indicates that increased pro-inflammatory cytokines are involved in the progression of alcohol-induced liver injury[21,22]. The activation of innate immunity also stimulates the release of hepato-protective and anti-inflammatory cytokines, which play a compensatory role against liver damage and inflammation[22]. In our study the *il-10* expression was not different between C and E groups. *The il-10* is produced by macrophages, lymphocytes, and Kupffer cells, and the liver is considered to be the main source of *il-10* production in response to lipopolysaccharides (LPS) stimulation[23].

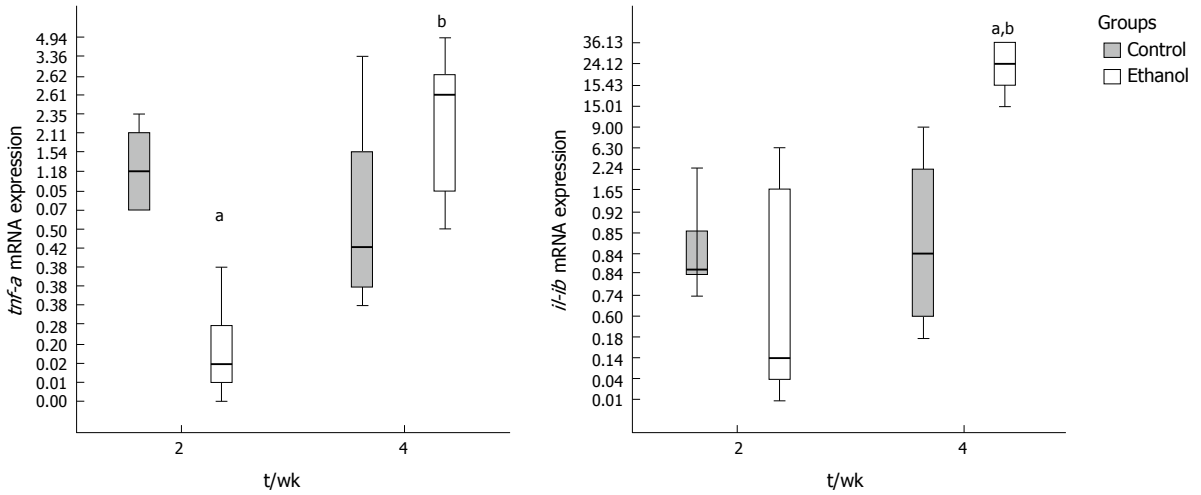

Figure 4 Effect of ethanol on mRNA liver expression of tumor necrosis factor-alpha and interleukin-1b. *tnf-a* was decreased significantly in E group compared to C at 2nd week ($P = 0.018$) and increased along time up 4th week ($P < 0.001$), reaching C group levels. *Il1-b* expression increased between 2 and 4 wk ($P = 0.001$) and at 4th week there was a significant difference between C and E groups ($P = 0.024$). Statistical data were determined by the Kruskal-Wallis test and Dunn as post hoc test. Values significantly different where indicated: [a]Significant statistical difference between 2 and 4 wk; [b]Significant statistical difference between C and E groups. $P < 0.05$ was considered. *tnf-a*: Tumor necrosis factor-alpha; *il*: Interleukin.

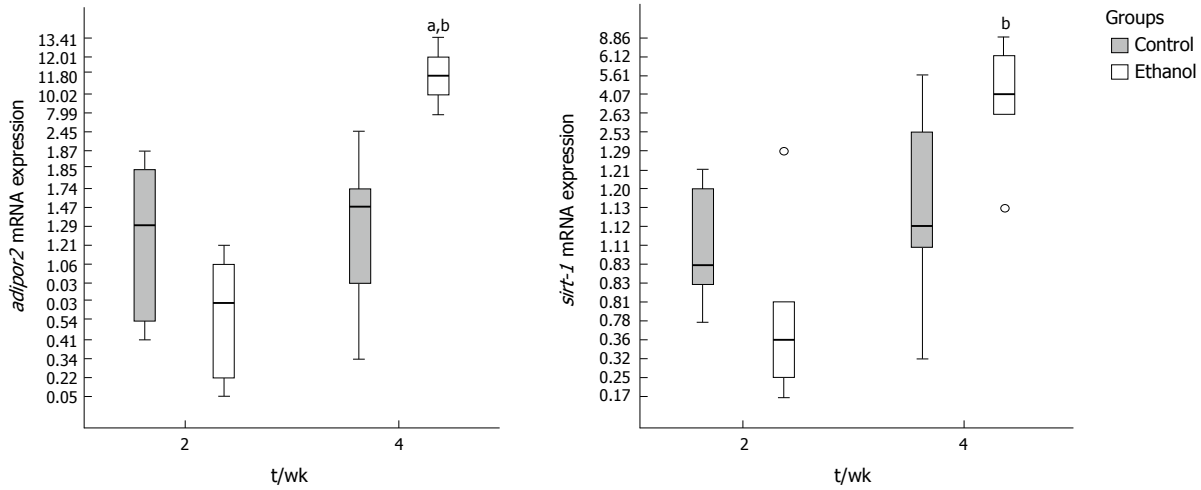

Figure 5 Effect of ethanol on mRNA liver expression of adiponectin receptor 2 and sirtuin-1 of zebrafish. *adipor2* and *sirt-1* expression increased in E group between 2 and 4 wk; $P < 0.0001$ and $P = 0.001$, respectively. At 4th week *adipor2* of E group was increased compared to C, $P = 0.006$. [a]Significant statistical difference between C and E groups; [b]Significant statistical difference between 2 and 4 wk. $P < 0.05$ was considered significant. *adipor2*: Adiponectin receptor 2; *sirt-1*: Sirtuin-1.

Sepulcre *et al*[24] demonstrated that zebrafish responds to LPS with much lower sensitivity than mammals, what can explain the absence of difference in hepatic expression of *il-10*, between C and E groups.

Elevated circulating levels of *TNF-*α and *IL-1b* have been observed in human patients and animal models of ethanol-induced liver injury[25,26]. The expression levels of these cytokines correlate well with the progression of the disease. In our study the *tnf-a* liver expression was initially decreased in E group compared to C at 2nd week and increased significantly along 2nd and 4th week, reaching the C expression levels at 4th week. Liu *et al*[27] demonstrated that only zebrafish with previous intestinal inflammation presented elevated *tnf-a* expression in liver compared to healthy animals after LPS exposure. Zebrafish are indeed able to respond to LPS, however with much lower sensitivity than mammals and *via* a *tlr4/myd88*-independent signaling pathway[24,28]. Among the few studies that evaluated *tnf-a* expression in the liver of zebrafish, Sapp observed an elevation of *tnf-a* in fructose-treated larvae and Hammes in thiocetamide-treated fish[20,29]. Although there was no direct evidence in our study, these cited findings conducted us to the following conclusions: *tnf-a* is not promptly induced by LPS in zebrafish exposed to ethanol as occurring in mammals and its activation mechanism seems to be associated to more aggressive hepatotoxicants.

Differently, the hepatic expression of *il-1b* increased significantly over the period considered and at 4th week it was significantly higher in E group compared to C. *Interleukin-1*, the "gatekeeper" of inflammation, is the apical cytokine in a signaling cascade that drives the

early responses to injury or infection[30]. *Il-1b* production requires caspase-1 activation by inflammasomes-multiprotein complexes that are assembled in response to danger signals. Vojtech *et al*[31] have described the cleavage of zebrafish *il-1b* by the caspase-1 homologues caspase-A and caspase-B, implying that the basic facets of the inflammasome platform of immune activation are conserved in zebrafish. The induction of *il-1b* demonstrated an early response to inflammatory stimuli in the present study. The up regulation of *il-1b* did not occur synergistically with *tnf-a* expression, as seen in mammals with ALD[26]. This result may suggest that *il-1b* is up regulated during chronic alcohol induced steatosis in zebrafish in a LPS independent pathway.

Adiponectin is a hormone that is secreted exclusively by adipocytes and has anti-inflammatory and hepatoprotective activities[32]. Circulating adiponectin is decreased in mammals with alcoholic disease[32,33]. In our study, *adiponectin* mRNA did not amplify in the livers of animals of both groups. Amali and collaborators observed elevated expression of *adiponectin* in liver of zebrafish treated with thioacetamide, but not in control animals[34].

In this study, the *adipor2*, a receptor of adiponectin, was over expressed in liver of animals exposed to ethanol, during the period that hepatic steatosis became more severe. To date, very few data are available regarding the effect of chronic ethanol exposure on hepatic *adipor2*. Hammes *et al*[29] observed decreased mRNA expression of hepatic *adipor2* and *sirt-1* and increased *tnf-a* in a model of NAFLD induced by thioacetamide in zebrafish. Possibly, thioacetamide, a more aggressive liver toxicant, contributed to down regulate *adipor2*. In humans, it was observed by Kaser that in presence of nonalcoholic steatohepatitis, *adiponectin receptor 2* expression was decreased compared to simple steatosis[35]. Neumeier *et al*[36] showed that animals (rodents) with liver steatosis presented elevated liver expression of *adipor2.* The increased expression of *adipor2* may be related to hepatic protection during steatosis.

SIRT-1 is a NAD^+-dependent class Ⅲ protein deacetylase that regulates lipid metabolism by deacetylation of modified lysine residues on histones, and targets a number of transcription factors involved in the regulation of gluconeogenesis, mitochondrial biogenesis, resistance to oxidative stress, adipogenesis and lipolysis, glycolysis, inflammation, apoptosis, cell differentiation, and angiogenesis[37]. To date, little is known about the function of *SIRT-1* in innate immunity and host defense. Studies in mammals have indicated that *SIRT-1* suppresses innate inflammatory responses[38]. Other authors have shown an expressive increase of *sirt-1* in liver of zebrafish chronically exposed to ethanol (0.5% vv)[39,40]. In our study occurred a significant increase of *sirt-1* between second and fourth weeks in fish treated with ethanol. We can speculate that the *sirt-1* hepatoprotective role might be involved in this process.

Ethanol effectively induced hepatic lipid accumulation and ultrastructural abnormalities in liver of zebrafish. Augmented expression of *il-1b* suggests that inflammatory signaling plays a significant role in hepatic steatosis and *adipor2* and *sirt-1* increased expression appears to represent compensatory efforts to alleviate consequences of ethanol liver injury, probably, indicating a hepatoprotective reaction. Hepatic steatosis is considered the first hit of chronic progressive ALD. The investigation of earliest events linked to ALD requires multiple strategies to reverse the damage effects of ethanol to the liver and to contribute to development of new therapies.

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COMMENTS

Background

Alcohol abuse is an acute health problem throughout the world and alcohol consumption is related to the occurrence of chronic liver disease. Hepatic steatosis is the first step of liver damage, and in spite of being considered a benign event, may progress to alcoholic steatohepatitis and more severe liver disease. The zebrafish has been proposed for the study of the effects of ethanol on several organs and has been helpful to unravel the pathways of liver damage by alcohol.

Research frontiers

The zebrafish (*Danio rerio*) is increasingly recognized as an important model system for studying liver development and human liver disease. Despite differences in the anatomical architecture of the zebrafish liver from mammals, alcoholic liver damages are similar to those of human beings, including alcoholic steatosis. This animal model will likely be a useful tool to further elucidate the pathogenesis and related disorders of alcoholic liver disease, as well as to discover new treatments.

Innovations and breakthroughs

Proinflammatory cytokines were frequently linked to steatohepatitis, however, this study describes early ultrastructural alterations in hepatocytes and cytokines increase in the onset of ethanol-induced liver damage.

Applications

The major advantage of zebrafish as a model system for hepatic processes is the ability to perform screening (genetic or chemical) in a vertebrate organism. The investigation of earliest events linked to alcoholic liver disease can contribute to the development of new strategies to prevent the advance of such disease.

Terminology

Hepatic steatosis: Or fatty liver. It is caused by an excessive fat deposition in the liver; Steatohepatitis: It is a type of fatty liver disease characterized by the presence of inflammation; Fibrosis: Scars produced in a reparative or reactive process in the liver; Ultrastructure: The detailed structure of a biological specimen, such as a cell, that can be observed by electron microscopy; Histology: The study of the microscopic anatomy of tissues. The cell of the tissue can be observed under a light microscope.

Peer-review

The paper is well-written.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Factors associated with long-term survival after liver transplantation: A retrospective cohort study

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Abstract

AIM

To identify predictive factors associated with long-term patient and graft survival (> 15 years) in liver transplant recipients.

METHODS

Medical charts of all *de novo* adult liver transplant recipients (n = 140) who were transplanted in Hamburg between 1997 and 1999 were retrospectively reviewed. In total, 155 transplantations were identified in this time period (15 re-transplantations). Twenty-six orthotopic liver transplant (OLT) recipients were early lost to follow-up due to moving to other places within 1 year after transplantation. All remaining 114 patients were included in the analysis. The following recipient factors were analysed: Age, sex, underlying liver disease, pre-OLT body mass index (BMI), and levels of alanine aminotransferase (ALT), bilirubin, creatinine and gamma-glutamyltransferase (gamma-GT), as well as warm and cold ischemia times. Furthermore, the following donor factors were assessed: Age, BMI, cold ischemia time and warm ischemia time. All surviving patients were followed until December 2014. We divided patients into groups according to their underlying diagnosis: (1) hepatocellular

carcinoma (*n* = 5, 4%); (2) alcohol toxic liver disease (*n* = 25, 22.0%); (3) primary sclerosing cholangitis (*n* = 6, 5%); (4) autoimmune liver diseases (*n* = 7, 6%); (5) hepatitis C virus cirrhosis (*n* = 15, 13%); (6) hepatitis B virus cirrhosis (*n* = 21, 19%); and (7) other (*n* = 35, 31%). The group "other" included rare diagnoses, such as acute liver failure, unknown liver failure, stenosis and thrombosis of the arteria hepatica, polycystic liver disease, Morbus Osler and Caroli disease.

RESULTS

The majority of patients were male (*n* = 70, 61%). Age and BMI at the time point of transplantation ranged from 16 years to 69 years (median: 53 years) and from 15 kg/m^2 to 33 kg/m^2 (median: 24), respectively. Sixty-six OLT recipients (58%) experienced a follow-up of 15 years after transplantation. Recipient's age (*P* = 0.009) and BMI (*P* = 0.029) were identified as risk factors for death by χ^2-test. Kaplan-Meier analysis confirmed BMI or age above the median as predictors of decreased long-term survival (*P* = 0.008 and *P* = 0.020). Hepatitis B as underlying disease showed a trend for improved long-term survival (*P* = 0.049, χ^2-test, *P* = 0.055; Kaplan-Meier analysis, Log rank). Pre-transplant bilirubin, creatinine, ALT and gamma-GT levels were not associated with survival in these patients of the pre-era of the model of end stage liver disease.

CONCLUSION

The recipients' age and BMI were predictors of long-term survival after OLT, as well as hepatitis B as underlying disease. In contrast, donors' age and BMI were not associated with decreased survival. These findings indicate that recipient factors especially have a high impact on long-term outcome after liver transplantation.

Key words: Liver transplantation; Age; Body mass index; Long-term survival; Hepatitis B

Core tip: Due to organ shortage and epidemiological developments, the number of older potential orthotopic liver transplant (OLT) recipients increased greatly over the last decades. In order to identify predictors for long-term survival after liver transplantation, we analysed all adult, first OLTs performed at the University Medical Center Hamburg-Eppendorf between 1997 and 1999 and compared these findings with the Eurotransplant database. Our study shows that recipient's age and body mass index as well as hepatitis B as underlying disease are predictors of long-term survival after OLT.

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INTRODUCTION

Survival after liver transplantation has strongly improved in the last decades, but factors associated with long-term survival have not been well defined yet. Research on the factors associated with best long-term outcome is, therefore, essential for an optimal use of the donated organs. This is even more relevant since age of donors and recipients is increasing. This development is mostly due to the organ shortage as well as epidemiological developments.

The majority of deaths after older potential orthotopic liver transplant (OLT) occur within the first months after transplantation. This is predominantly caused by pulmonary infections, sepsis or multiple organ failure[1]. An analysis of a large cohort from the Eurotransplant database included more than 90000 patients that were liver transplanted between 1968 and 2009[1]. Within this cohort the early mortality was 6%, 9% and 12% for 1-, 3- and 6-mo mortality in patients who were liver transplanted after the year 2000[1].

Although several transplant centres worldwide now have more than 20 years of clinical experience in the field of liver transplantation, only few studies have analysed the long-term outcomes in OLT recipients[2,3].

Several donor and recipient factors, including age and body mass index (BMI), are well-known to influence short-term survival[4]. Their relevance for long-term outcome has not been studied in detail yet. However, the negative influence of obesity on survival in non-transplant recipients is a well-known fact, since the Framingham study of the 1990s[5]. The World Health Organization has defined obesity as a condition of excessive accumulation of body fat, causing severe damage to health (http://www.who.org). In fact, the prevalence of obesity is increasing worldwide and is a major threat to liver transplant recipients as well as the health of the general population. Common co-morbidities associated with obesity are hypertension, coronary heart disease, heart failure, stroke, hyperuricemia, dyslipidemia, insulin resistance and glucose intolerance. In addition, within the Framingham study, it was shown that fluctuations in body weight in non-transplant patients were associated with an increased mortality, independent of obesity and the trend of body weight over time[5].

In contrast to the general population, the role of bodyweight in liver transplant recipients is less clear. Werneck *et al*[6] demonstrated in a study including 136 liver transplant recipients that there was no significant difference between obese and normal weight patients regarding length of stay in the Intensive Care Unit or in 2-year survival. On the other hand, Sawyer *et al*[4] demonstrated a decreased short-term survival in obese

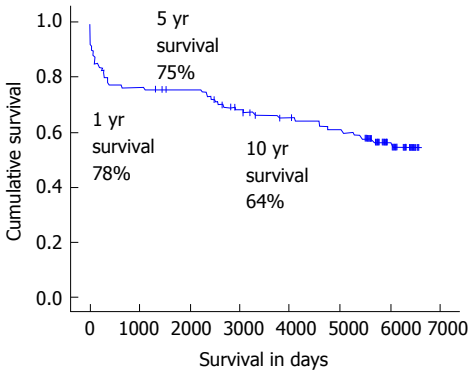

Figure 1 Overall survival of liver transplant recipients, monitored for 15 years.

patients in comparison to normal weight liver transplant recipients.

In addition to BMI, ages of donor and recipient have been discussed controversially within the last years[7]. Recipients' age is also known to have an influence on the outcome of liver transplantation. Schoening *et al*[2] studied the 20-year survival rate of 313 liver transplant recipients. Those authors divided their cohort into three sub-groups: Patients below the age of 30, between 30 years and 55 years, and patients above 55 years. Patients below the age of 30 lived significantly longer after transplantation, as compared to the other two groups. However, no analysis was performed in which the patients were divided according to the median age in that study. Furthermore, the long-time survival of transplant recipients was compared with a "virtual control group", based on the life expectancy in the general population. While patients younger than 55 years showed a decreased survival, as compared to the general population, there was no difference in life expectancy between patients older than 55 years and the general population.

The aim of the present study was to identify factors associated with long-term patient and graft survival (> 15 years) in liver transplant recipients and compare these to the Eurotransplant database. This study focused specifically on recipient's age and BMI, as the influence of these factors is still not well defined.

MATERIALS AND METHODS

This study was performed at the University Medical Center Hamburg-Eppendorf, a tertiary centre in North Germany. Since the first liver transplantation in Hamburg was performed in 1984, more than 2000 liver transplantations have been performed at this centre.

Medical charts of all *de novo* adult liver transplant recipients (n = 140), who were transplanted in Hamburg between 1997 and 1999, were retrospectively reviewed (Figure 1). In total, 155 transplantations were identified in this time period (15 re-transplantations). Twenty-six OLT recipients were early lost to follow-up due to moving to other places within 1 year after transplantation (Figure 1). All remaining 114 patients were included in the analysis. The following recipient factors were analysed: Age, sex, underlying liver disease, pre-OLT BMI, and levels of alanine aminotransferase (ALT), bilirubin, creatinine and gamma-glutamyltransferase (gamma-GT), as well as warm and cold ischemia times. Furthermore, the following donor factors were assessed: Age, BMI, cold ischemia time and warm ischemia time. All surviving patients were followed-up until December 2014. We divided patients into groups according to their underlying condition (Table 1): (1) hepatocellular carcinoma (HCC) (n = 5, 4%); (2) alcohol toxic liver disease (n = 25, 22.0%); (3) primary sclerosing cholangitis (n = 6, 5%); (4) autoimmune liver diseases (n = 7, 6%); (5) hepatitis C virus (HCV) cirrhosis (n = 15, 13%); (6) hepatitis B virus (HBV) cirrhosis (n = 21, 19%); and (7) other (n = 35, 31%). The group "other" included rare diagnoses, such as acute liver failure, unknown liver failure, stenosis and thrombosis of the arteria hepatica, polycystic liver disease, Morbus Osler and Caroli disease.

In addition to patient survival, the graft survival was also analysed. By definition, graft loss resulted in re-transplantation or death. The factors that were significantly associated with graft survival in our cohort were then compared with a large cohort of 2971 patients from Eurotransplant, which had been transplanted within the same period (1997-1999).

Statistical analysis

Categorical variables were compared using χ^2 test. Metric data were compared using the non-parametric Mann-Whitney test. Survival analysis was performed utilizing Kaplan-Meier analysis. All investigated factors were tested utilizing univariate and multivariate models.

As metric values did not fulfil the criteria for a normal distribution (Kolmogorov Smirnov test $P < 0.01$), median values instead of mean values were depicted. All statistical analyses were performed utilizing SPSS (version 13.0) and P-values < 0.05 were considered to be statistically significant.

For this retrospective, observational study neither informed consent nor approval of the ethics committee was needed according to the Professional Code of the German Medical Association (article B.Ⅲ. § 15.1) and to the recommendations of our local ethical committee (Ethikkommission der Ärztekammer Hamburg).

Control cohort

To discuss the survival of transplant patients with an age below and above the median of age (53 years) we constructed an imaginary control cohort. Therefore, we analysed the survival of historical data (https://www.destatis.de) of an age-matched cohort of the healthy German population.

In addition, to improve reliability of data, we compared our results with data from a cross-sectional Eurotransplant cohort including 2971 patients who underwent liver transplantation between 1997 and 1999. Eurotransplant kindly supported us with de-personalized

Table 1 Patient characteristics directly before transplantation

	Patients who survived (n = 68)	Patients who died (n = 46)	P-value (χ^2 test)
Male	39 (57%)	31 (67%)	NS
Age, yr (median, SD)	16-65 (50.5, 13)	17-69 (56.0, 12)	0.009
BMI, range kg/m^2 (median, SD)	18-33 (23.1, 3)	15-29 (25.9, 4)	0.029
Pre-LTx creatinine, mg/dL (median, SD)	0.4-3.5 (1.0, 0.5)	0.3-2.9 (1.1, 0.6)	NS
GFR, mL/min (median, SD)	15.3-230.2 (73.3, 38.1)	22.6- 240.4 (62.5, 48.0)	NS
ALT, U/L (median, SD)	4-2610 (35.5, 449.5)	6-1566 (19.5, 339.0)	NS
Gamma-GT, U/L (median, SD)	7-374 (47.0, 8)	13-184 (43.0, 45)	NS
Bilirubin, mg/dL (median, SD)	0.4-28.1 (2.4, 5.7)	0.4-28.3 (2.4, 5.8)	NS
Warm ischemia time, min (median, SD)	25-100 (50.0, 18)	22-75 (54.0, 15)	NS
Cold ischemia time, min (median, SD)	242-940 (542.5, 157)	174-825 (521.0, 146)	NS
Donor age, yr (median, SD)	12-70 (36.5, 16)	13-75 (41.0, 1)	NS
Donor BMI, kg/m^2 (median, SD)	17-30 (23.5, 3)	18-31 (24.2, 2)	NS
Underlying diagnosis n (%)			
HCC	2 (3)	3 (6)	NS
Alcohol toxic liver cirrhosis	12 (18)	13 (27)	NS
PSC	4 (6)	2 (4)	NS
Autoimmune	5 (8)	2 (4)	NS
HCV cirrhosis	9 (14)	6 (13)	NS
HBV infection	16 (24)	5 (10)	0.049
Other	18 (27)	17 (35)	NS

ALT: Alanine aminotransferase; BMI: Body mass index; OLT: Orthotopic liver transplantation; HCV: Hepatitis C virus; HBV: Hepatitis B virus; NS: No statistically significant difference; GFR: Glomerular filtration rate; HCC: Hepatocellular carcinoma; PSC: Primary sclerosing cholangitis.

data that were already arranged and categorized according to our median values of age and BMI and to status of HBV positivity. To compare this cohort with our own cohort, survival of these patients was analysed up to the same time point (until December 2014).

RESULTS

Patient characteristics

Overall, 114 OLT recipients were included in the study (Table 1). The majority of the patients were male (n = 70, 61%). The age and BMI at the time of transplantation ranged from 16 years to 69 years (median: 53 years) and from 15.1 kg/m^2 to 33.3 kg/m^2 (median: 24 kg/m^2), respectively. See Table 1 for an overview of the overall investigated factors. The median follow-up was 5139 d. Sixty-six (58%) OLT recipients experienced a follow-up of 15 years after OLT (Figure 1). The 1-, 5- and 10-year patient survival rates were 78%, 74% and 64% (Figure 1).

Follow-up and graft survival

Graft survival 15 years post-OLT was 53%. Fifty-three patients experienced a graft loss either by death (34%) or re-transplantation (13%). Characteristics of patients with graft survival and those with graft loss are depicted in Table 1.

Association between patient survival and recipient's age

During the observational period, the mortality rate was significantly higher in patients with an age above the median (53 years) at transplantation as compared to patients younger than the median (P = 0.009). The Kaplan-Meier analysis confirmed that older patients had a decreased patient survival rate (P = 0.008; Figure 2). Furthermore, the median age at the time of transplantation was higher in patients who deceased within 15 years of follow-up in comparison with patients who were still alive at the end of the study period (P = 0.006, Mann-Whitney test; Figure 3). These findings were confirmed in the cross-sectional Eurotransplant cohort (n = 2973) transplanted in the same period, with a follow-up of 15-17 years. In this cohort, 625/1145 (55%) patients with an age above 53 years died within the 15-year to 17-year follow-up period, while only 653/1809 (36%) patients with an age below 53 years died (P < 0.001; Table 2).

In a sub-analysis, we defined age above 60 years as "old" and analysed the groups of transplant younger (n = 89) and older (n = 25) than this threshold, separately. In patients older than 60 years, the patient survival rate was significantly lower as compared to younger patients (χ^2 test P = 0.007, Kaplan-Meier analysis P = 0.002). Donor age (12-75 years, median: 40) was not significantly correlated with patient survival. A multivariate analysis confirmed age as an independent factor associated with graft survival (P < 0.01).

Association between graft survival and age

Patients with an older age at the time of transplantation had a significantly worse graft survival, compared to patients younger than the median (Figure 4). This was confirmed by χ^2 test (P = 0.017) and Mann-Whitney test (P = 0.017). Looking at the subgroup of patients older than 60 years, there was a significantly lower graft survival according to Kaplan-Meier survival analysis (P = 0.05) but not according to the χ^2-test. Donor's age was not related to graft survival in this study (P = ns). There was no significant association between patients

Table 2 Comparison of survival according to age, body mass index and hepatitis B virus status in a Eurotransplant control cohort (*n* = 2973)[1]

	Patients who survived *n* (%)	Patients who died *n* (%)	*P*-value (χ^2 test)
Age below 53 yr (*n* = 1809)	1156 (64)	653 (36)	
Age above 53 yr (*n* = 1145)	520 (45)	625 (55)	< 0.001
BMI below 24 kg/m^2 (*n* = 1454)	880 (61)	574 (39)	
BMI above 24 kg/m^2 (*n* = 1493)	796 (53)	697 (47)	< 0.001
Hepatitis B as underlying disease (*n* = 255)	170 (67)	85 (33)	
Non-hepatitis B patients (*n* = 1705)	946 (55)	759 (45)	< 0.001

[1]Data for age, BMI and hepatitis B virus status were not available for the total cohort. BMI: Body mass index.

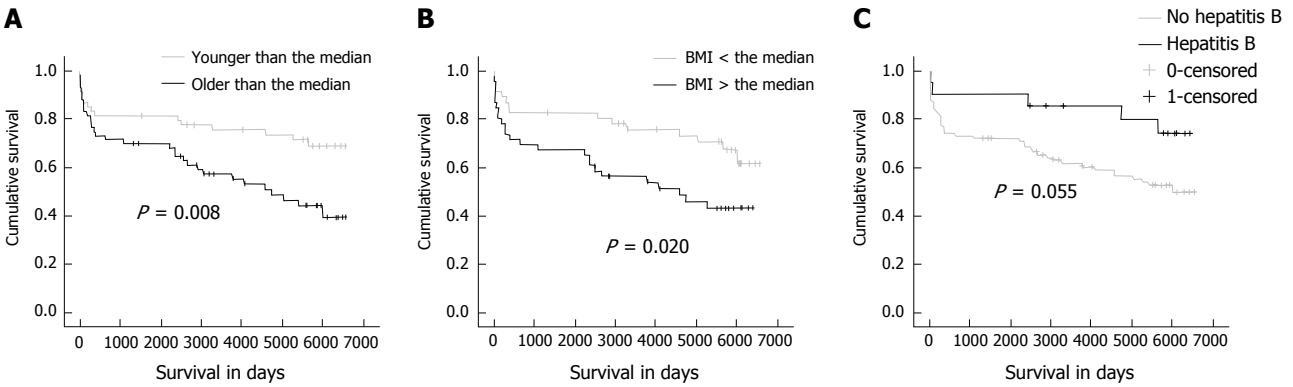

Figure 2 Kaplan-Meyer survival analysis reveals increased survival for patients younger than the median (53 years) (A), with body mass index lower than the median (24 kg/m^2) (B) and hepatitis B as underlying disease (C).

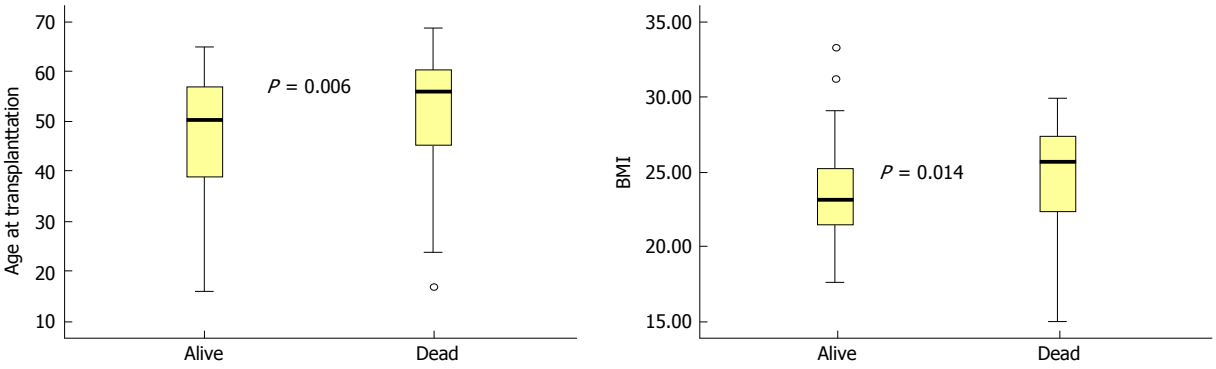

Figure 3 Age and body mass index at the time point of transplantation were higher in deceased patients in comparison to patients who survived.

who survived more than 1 year and had age above the median (χ^2 test P = 0.498).

Association of patient survival and BMI

Patients with a BMI above the median (24 kg/m^2) displayed a higher mortality than patients with a BMI below the median (P = 0.029). This reduced survival rate was confirmed by the Kaplan-Meier analysis (P = 0.020; Figure 2). Additionally, BMI at the time of transplantation was higher in patients who died within 15 years of follow-up in comparison to patients who survived (P = 0.014; Figure 3). This was confirmed in the Eurotransplant control cohort (n = 2971). Patients with a BMI below 24 kg/m^2 showed an improved survival rate in comparison with patients with a BMI above this threshold ($P \leqslant 0.001$). In detail, 61% with a BMI below 24 kg/m^2 survived, while 53% with a BMI above 24 kg/m^2 survived (Table 2).

A sub-analysis of patients with severe obesity and a BMI above 30 kg/m^2 was not possible as only two patients fulfilled this criterion.

There was no significant association between patients who survived more than 1 year and had BMI above the median (χ^2 test P = 0.449). Notably, there was no significant association between age and BMI of the recipient (R = 0.114, P = 0.278), so that BMI seemed to be independent of age. Unfortunately, a multivariate analysis did not confirm BMI as an independent factor associated with decreased survival; perhaps, significance was missing due to the limited number of factors.

In contrast, the BMI of the donor was not associated

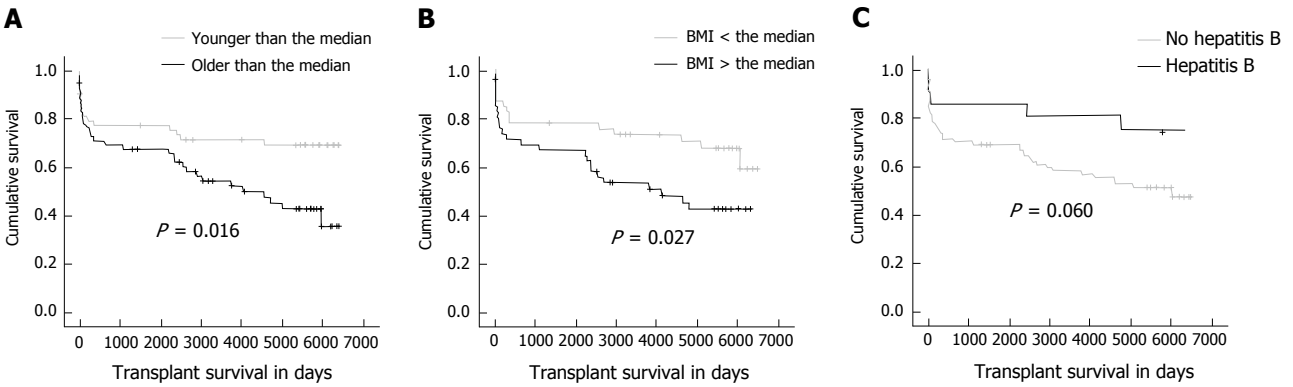

Figure 4 Kaplan-Meyer survival analysis reveals increased transplant survival for patients younger than the median (53 years) (A), with body mass index lower than the median (24 kg/m^2) (B) and hepatitis B as underlying disease (C). BMI: Body mass index.

with survival of the recipient (P = ns).

Association of graft survival and BMI

Patients having a BMI above the median (24 kg/m^2) had a significantly worse graft survival, compared to patients with a BMI lower than the median (χ^2 test: 0.009, Mann-Whitney test: 0.047). On the other hand, in this study, donor's BMI did not have an influence on graft survival.

Association of patient and graft survival with the underlying liver diseases

The only underlying aetiology of cirrhosis which was statistically significantly associated with outcome was hepatitis B. Patients with hepatitis B as an underlying disease tended to have an improved patient survival in comparison to patients with other underlying diseases (P = 0.049 in the categorical analysis and P = 0.055 in the Kaplan-Meier analysis; Figure 2C). Three out of 21 liver transplant recipients with hepatitis B suffered from acute, fulminant hepatitis B, leading to acute liver failure and transplantation, while the majority (n = 18) had been transplanted due to chronic hepatitis B with cirrhosis. Regarding the BMI, there was no difference between HBV-positive and HBV-negative patients (t-test, 2-sided, unequal variance, P = 0.38), so that other reasons must be responsible for the survival benefit.

All HBV-positive liver transplant recipients received intravenous immunoglobulins, hepatitis B immune globulin (HBIG), to avoid reinfection of the graft.

In addition to patient survival, graft survival of patients with hepatitis B as underlying disease was also improved, compared to patients with other diagnoses (χ^2 test: 0.018, Mann-Whitney test: 0.018). The Eurotransplant control cohort confirmed that patients with hepatitis B had an improved survival in comparison to the remaining patients (Table 2).

Remaining factors

Neither recipient's laboratory parameters prior to transplantation (ALT, gamma-GT, bilirubin, creatinine) nor warm ischemia time or cold ischemia time influenced patient survival significantly.

DISCUSSION

In the current situation of tremendous organ shortage, it is important to identify patients who benefit most from a liver transplantation and also to detect risk factors associated with poor outcome. The main findings of this study were that recipients' age and BMI are relevant for prediction of long-term patient survival as well as graft survival. Interestingly, neither other recipient factors such as bilirubin, creatinine, ALT nor donor factors, such as age and BMI, were associated with decreased survival. Another interesting finding was that OLT recipients with hepatitis B as underlying disease had improved survival rates.

The association of recipients' age and BMI and patient and graft survival was proven by univariate analysis for both factors. However, in multivariate analysis only age remained a significant predictor. On the other hand, it was unexpected that there was no significant association between survival of recipient's and donor's age and BMI. This finding is in contrast to numerous previous studies which demonstrated a significantly decreased survival in recipients of older donations within a large ET-DRI study[8]. Recently, a large analysis of more than 41000 liver transplant recipients receiving a donation after circulatory death showed that recipients of livers from donors with an age below 50 years had a higher survival rate, compared to recipients of livers from donors with an age above 60. However, several studies indicated that older grafts can be used safely with a careful selection of patient and donor in the majority of cases[9-13]. Based on the published literature, strict recommendations for the acceptance or refusal of potential liver donors cannot be made. The authors concluded that careful donor organ and recipient selection can lead to excellent results[14].

In contrast to donor's age, our study highlighted the value of recipient's age as a predictor of survival. We identified a threshold of 53 years for recipient's age and a BMI of 24 kg/m^2 as relevant risk factors. These findings were confirmed in the analysis of the Eurotransplant

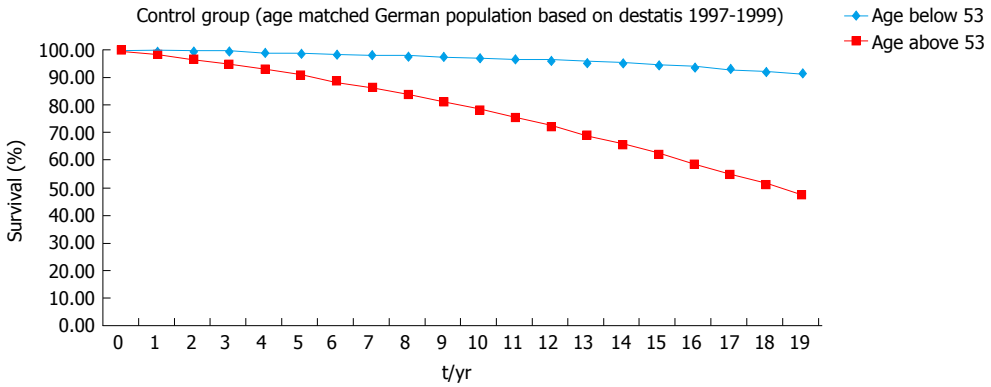

Figure 5 Percentage of survival in an age-matched control of the German general population (age range: 18-67 years). This age-matched control cohort was constructed baseding on historical data about the German healthy population (https://www.destatis.de).

cohort of 2971 patients. Perhaps a larger cohort might also confirm a relevant aspect of donor age on survival. However, our study did not find such an association.

Within a previous German study with a follow-up period of 20 years and 313 liver transplant recipients, the survival of elderly transplant recipients (> 55 years) was reduced within the first year after transplantation, but long-term survival was similar to the general population[2]. Our observation that there is a relevant difference regarding survival between OLT recipients above and below the median age of 53 years (Figure 2A) is well in line with this study. However, we could not find a significant association between 1-year patient survival and age or BMI above the median. Therefore, these factors might be associated with long-term but not with short-term survival. Further studies are needed to elucidate this aspect.

Earlier studies showed inconsistent results concerning BMI and survival. A study by Fujikawa *et al*[15] investigated the impact of obesity on clinical and financial outcome after liver transplantation and showed no influence on either patient survival or hospital costs. Also, it is conceivable that obese recipients were selected more carefully with respect to other risk factors. In contrast, the study by Rustgi *et al*[16] observed a worse survival rate in patients having a BMI > 35. Our study confirms the finding that a higher BMI of the recipient is associated with a decreased survival. Only three of the patients in our study displayed malnutrition with a BMI < 18; thus, no interpretation of a possible effect of malnutrition and survival was possible for our cohort.

In order to strengthen our data, we compared the survival rate of our patients (younger or older than the median of 53 years) with two control groups (as described in the methods). There were no significant differences between all three groups (Figure 5 and Table 2). However, these are hypothetical control cohorts and more detailed statistical analyses were not possible.

Three independent statistical tests (Kaplan-Meier survival analysis/Log rank, χ^2 test, Mann-Whitney test) confirmed the association between recipient's age or BMI and decreased patient and graft survival rates. However, there was no correlation between age and BMI indicating that these factors are independently associated with lower survival. Unfortunately, a multivariate analysis makes no sense due to the low number of significant factors in the univariate analysis. It is not surprising that older or overweight patients depict a shorter survival. This has been a well-known fact for many years.

Interestingly, hepatitis B was associated with an improved long-term patient survival in our cohort. This should be interpreted carefully as there are only 21 HBV patients in our study population. However, this observation might be due to the regularly applied immunoglobulin preparations, HBIG, that these patients still get at our institution[17-19]. However, currently this is only one hypothetical explanation of the observed survival benefit of hepatitis B patients.

In addition, our study cohort analysis of the Eurotransplant control cohort also shows an increased survival for transplant recipients with underlying hepatitis B in comparison to the remaining patients ($P < 0.001$). This observation is in line with an analysis of the survival of liver transplant recipients with hepatitis B, based on the European liver transplant registry[20]. Within this study investigating the outcome of liver transplant recipients with hepatitis B as underlying disease within a period of approximately 20 years (1988-2010), it could be shown that the survival of HBV-positive transplant recipients strongly improved within these 2 decades[20]. This has been assumed to be caused by the prevention of hepatitis B re-infection by immunoglobulins[20]. However, this hypothesis still needs to be confirmed by further studies.

The results of this study might be helpful to identify patients with better chances of long-term survival. Our overall 15-year patient survival rate (Figure 1) of 58% is well in line with previous reports depicting a 20-year survival rate of approximately 50% after liver transplantation[2,3]. However, in the current era of model of end-stage liver disease (MELD)-allocation, which favours the sickest patients, such survival rates might not be met in future studies. Upcoming studies are needed to investigate not only short but also long-term survival of patients who received a liver transplantation in the

MELD-era. Perhaps the MELD score is a valuable tool for identifying the sickest patients, but it might not be the best predictor of long-term outcome. Furthermore, according to previous studies, it has been shown that prognosis of the patient is far more related to clinical parameters than laboratory data[17]. The study of Aloia *et al*[18] also showed a decreased value of the MELD score in contrast to parameters such as ventilator status, diabetes mellitus, HCV, creatinine levels and recipient's and donor's age.

Our study has some limitations. It is based on patients who underwent liver transplantation in the pre-MELD era and at a time when less patients received organs with extended donor criteria. Furthermore, the number of patients with HCC was only 4% (5/114, 4%). In our study, at present these numbers are much higher.

Unfortunately, multivariate analysis of our data was prone to errors due to the small number of patients in comparison to the multiple variables. Thus, it can be said that the analysed cohort was too small for the investigation of the variables. This was a retrospective analysis and, therefore, there is some lack of information considering the long time period of observation (15-17 years). However, there are not many studies dealing with such long-term data as presented in this collective. In the future, more research, especially on the potential influence of immunoglobulins on the HBV patient's outcome, is necessary.

In conclusion, age and BMI of OLT recipients were predictors of long-term survival, while pre-transplant bilirubin, creatinine, ALT and gamma-GT were not associated with patient survival or graft survival (pre-MELD era). Age and BMI of the donor had no relevant influence on patient or graft survival in this cohort. OLT recipients with hepatitis B as underlying disease displayed an improved survival. The relevance of this observation still needs to be determined.

ACKNOWLEDGMENTS

We thank Eurotransplant for providing data for the control cohort of 2971 patients.

COMMENTS

Background

Predictive factors associated with long-term patient and graft survival (> 15 years) in liver transplant recipients are not well defined. This study evaluates the possible association between various factors and survival.

Research frontiers

The role of age and body mass index (BMI) for the outcome of liver transplant recipients still needed to be shown.

Innovations and breakthroughs

This is the first study demonstrating a relevant association between age above 53 years or a BMI above 24 kg/m^2 with decreased graft survival. These thresholds were confirmed in an independent large Eurotransplant cohort to be associated with decreased graft survival. Furthermore, there was a weaker association between underlying hepatitis B and improved graft survival. The pathological mechanism and relevance of this finding still needs to be shown.

Applications

Future studies will focus in detail on patients with an age above 53 years or a BMI above 24 kg/m^2 to verify the authors' findings. If their data can be confirmed, this will help transplant physicians worldwide to predict the risk of liver transplant recipients.

Terminology

Liver transplant recipients and their survival as well as graft survival, defined as period until death or re-transplantation were studied.

Peer-review

Pischlke *et al* analyzed the clinical data of the patients who underwent liver transplantation during 1997 to 1999. This article is interesting.

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ORIGINAL ARTICLE

Observational Study

Concordance of non-invasive mechanical and serum tests for liver fibrosis evaluation in chronic hepatitis C

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Institutional review board statement: The study was reviewed and approved by the CAPPesq, the Ethics Committee for researches of the institution (CAAE number: 1276/09).

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Abstract

AIM

To determine the sensitivity and specificity of liver stiffness measurement (LSM) and serum markers (SM) for liver fibrosis evaluation in chronic hepatitis C.

METHODS

Between 2012 and 2014, 81 consecutive hepatitis C virus (HCV) patients had METAVIR score from liver biopsy compared with concurrent results from LSM [transient elastography (TE) [FibroScan®/ARFI technology (Virtual Touch®)] and SM [FIB-4/aspartate aminotransferase-to-platelet ratio index (APRI)]. The diagnostic performance of these tests was assessed using receiver operating characteristic curves. The optimal cut-off levels of each test were chosen to define fibrosis stages F $\geqslant$ 2, F $\geqslant$ 3 and F = 4. The Kappa index set the concordance analysis.

RESULTS

Fifty point six percent were female and the median age was 51 years (30-78). Fifty-six patients (70%) were

treatment-naïve. The optimal cut-off values for predicting F ⩾ 2 stage fibrosis assessed by TE were 6.6 kPa, for acoustic radiation force impulse (ARFI) 1.22 m/s, for APRI 0.75 and for FIB-4 1.47. For F ⩾ 3 TE was 8.9 kPa, ARFI was 1.48 m/s, APRI was 0.75, and FIB-4 was 2. For F = 4, TE was 12.2 kPa, ARFI was 1.77 m/s, APRI was 1.46, and FIB-4 was 3.91. The APRI could not distinguish between F2 and F3, *P* = 0.92. The negative predictive value for F = 4 for TE and ARFI was 100%. Kappa index values for F ⩾ 3 METAVIR score for TE, ARFI and FIB-4 were 0.687, 0.606 and 0.654, respectively. This demonstrates strong concordance between all three screening methods, and moderate to strong concordance between them and APRI (Kappa index = 0.507).

CONCLUSION

Given the costs and accessibility of LSM methods, and the similarity with the outcomes of SM, we suggest that FIB-4 as well as TE and ARFI may be useful indicators of the degree of liver fibrosis. This is of particular importance to developing countries.

Key words: Elastography; Serum markers; Hepatitis C virus; Liver stiffness; Liver biopsy

Core tip: Liver fibrosis evaluation in hepatitis C virus (HCV) patients has critical impact on prognosis and treatment strategies. Despite liver biopsy (LB) remains the gold standard for its evaluation, non invasive methods has improved in recent years. We evaluated 81 HCV patients with elastography methods [Fibroscan and acoustic radiation force impulse (ARFI)] and serum markers (APRI and FIB-4) compared to LB, and found that Fibroscan, ARFI, and FIB-4 independently identify advanced fibrosis. We suggest that FIB-4 alongside Fibroscan and ARFI may be good tools for the prediction of severity of liver fibrosis. This may be of particular importance to developing countries.

Paranaguá-Vezozzo DC, Andrade A, Mazo DFC, Nunes V, Guedes AL, Ragazzo TG, Moutinho R, Nacif LS, Ono SK, Alves VAF, Carrilho FJ. Concordance of non-invasive mechanical and serum tests for liver fibrosis evaluation in chronic hepatitis C. *World J Hepatol* 2017; 9(8): 436-442 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i8/436.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i8.436

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most frequent etiologies of cirrhosis, and is therefore responsible for most of its complications, including hepatocellular carcinoma, which is the sixth most common cancer worldwide[1]. Despite the recent advances in HCV therapy, the prevalence of advanced liver disease will continue to increase as well as the corresponding healthcare burden[2]. In Brazil, although the hepatitis C viremic prevalence is about 1%, only 15% of the estimated infected patients are diagnosed, usually with advanced fibrosis. This is partly explained by the scarcity of specialist centers compared with the societal needs. Of those who are diagnosed, only 60% receive specialized treatment[3,4].

The stage of liver fibrosis in HCV patients is associated with prognosis, and has a resulting impact on treatment strategy and follow-up. Liver biopsy (LB) is still the gold standard procedure for fibrosis assessment, but non-invasive new approaches have been strongly recommended for evaluation of fibrosis, mainly in HCV. They require less operator expertise, have no complications and have good diagnostic accuracy[5-7]. The most extensively used non-invasive mechanical methods based on ultrasound are transient elastography (TE or FibroScan®) and acoustic radiation force impulse (ARFI) technology, Virtual Touch®. There are several laboratorial markers in development, and validated scores such as aspartate aminotransferase-to-platelet ratio index (APRI) and FIB-4 [based on age, platelet count, aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], that are easily calculated with routine laboratory tests.

Use of liver biopsy has decreased following the introduction of non-invasive tests, especially among chronic HCV patients[8]. Although, according to the recently published EASL Guideline for the evaluation of HCV patients, a perfect marker (AUROC > 0.90) for liver disease could not be achieved, the use of non-invasive tests reduce, but do not abolish the need for liver biopsy[8].

Many biological tests, including APRI, PGA index, Forns' index, Fibrotest, FIB-4 and Hepascore have been compared with TE and/or ARFI and LB for initial evaluation of liver fibrosis in HCV patients[9]. They have compared favorably, although some are difficult to calculate and others use specialized expensive commercially produced markers. However, APRI and FIB-4 are more accessible and easier to apply than others. As TE and ARFI show a representative result of different parts of the liver, accuracy studies have been developed to evaluate performance compared to LB, which evaluates only a small sample of the liver. As the best cut-off points of each fibrosis stage varies according to different cirrhosis etiologies and populations, these cut-off points need to be validated.

The aim of this study is to identify optimal cutoff values for TE, ARFI, APRI and FIB-4 compared with LB in a Brazilian HCV cohort, according to levels of significant fibrosis (F ⩾ 2), advanced fibrosis (F ⩾ 3) and cirrhosis (F = 4).

MATERIALS AND METHODS

Ethical considerations

The Ethics Committee of the Hospital das Clínicas (CAPPesq number 1276/09) reviewed and approved this study,

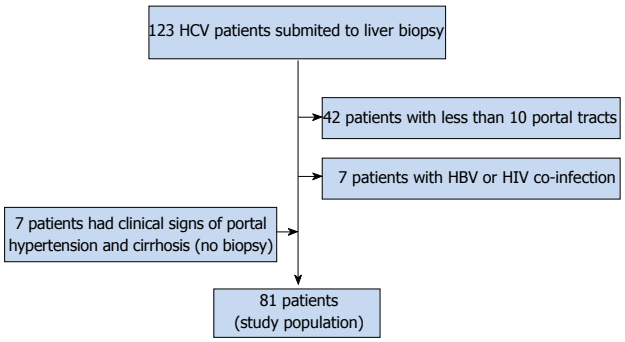

Figure 1 Flowchart of study population enrollment. HCV: Hepatitis C virus; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus.

that was conducted following the ethical guidelines of the 1975 Declaration of Helsinki. The requirement for informed written consent was waived.

Study design

We performed an observational study of diagnostic accuracy for TE, ARFI, APRI and FIB-4 compared with LB. Between 2012 and 2014, 123 consecutive HCV patients followed by Hepatology Outpatient Center of Hospital das Clínicas, University of São Paulo School of Medicine, Brazil, that would be submitted to liver biopsy had liver stiffness measurement (LSM) [FibroScan®, EchoSens, Paris, France)/ARFI, Siemens AG, Erlangen, Germany) and serum markers (SM) [FIB-4/APRI] exams done, in order to compare the data with METAVIR score.

Inclusion criteria were: (1) HCV polymerase chain reaction (PCR) RNA positivity for at least 6 mo, and clinical or histopathological diagnosis of chronic HCV; and (2) representative liver biopsy (minimum of 10 portal spaces, non subcapsular fragment) carried out until 30 d prior to LSM and SM. Exclusion criteria were: (1) patient under 18 years of age; (2) hepatitis B virus (HBV) or human immunodeficiency virus (HIV) co-infection; (3) other chronic liver disease (cholestasis, non-alcoholic steatohepatitis, autoimmune hepatitis, hemochromatosis, Wilson's disease); (4) decompensated cirrhosis; (5) biopsies performed for more than 30 d of the evaluation; and (6) non-representative liver biopsy.

Results from TE and ARFI® were blinded for the results from LB. FibroScan® and ARFI were performed by an experienced ultrasonographist with more than 80000 liver ultrasounds, more than 2000 FibroScan® and more than 2000 ARFIs.

Forty-nine patients were excluded, as shown in Figure 1. Seven patients without LB, but with clinical signs of portal hypertension and cirrhosis (Metavir F = 4) were included. In the end, 81 were selected for the study. Three patients had hepatocellular carcinoma, with less than 2 cm. They were included in the study.

Clinical and biological data

Anthropometric, clinical and laboratorial data were collected: Gender, age, weight, height, body mass index (BMI), smoking status, alcohol consumption, hypertension, diabetes, dyslipidemia, and serum enzymes such as AST, ALT, bilirubin, albumin, glucose levels and platelet count, all taken from medical charts.

Transient elastography

LSM were performed using the FibroScan® 402 device powered by VCTE (EchoSens, Paris, France), equipped with the standard M probe. The examination procedure have been previously described[9-11]. A valid LSM examination included 10 valid measurements, a success rate of 70%, and an interquartile range of measurements (IQR) below 30% of the median value. Controlled attenuation parameter (CAP) was also evaluated.

ARFI

ARFI technology measures the shear wave speed in a precise anatomical region, with a predefined size, provided by the system. Measurement value and depth are also reported and elasticity results are represented in m/s[12]. ARFI elastography was performed using a Siemens Acuson S2000® ultrasound system, a Virtual Touch® quantification elastography technology (Siemens AG, Erlangen, Germany). The patients were examined in dorsal decubitus, with the right arm in maximum abduction. Scans were performed in a right inferior intercostal space over the right liver lobe (*e.g.,* segment 8), 2 cm under the capsule, with minimal scanning pressure applied by the operator, while patients were asked to stop breathing temporarily. Ten measurements per patient were performed and a median and IQR values were calculated by the machine. Only when an IQR 30% was reached was the median value accepted.

APRI and FIB-4

APRI and FIB-4 were calculated through the following scores: APRI score = {[AST/upper limit of normal (ULN)] 100}/platelet count 10^9/L. FIB-4 score = {[age (yr) x AST (U/L)] / [platelet count (10^9/L) x ALT (U/L)]}.

LB

LB was performed in all but 7 patients, who had clinical or ultrasonographic signs of portal hypertension and cirrhosis. They were judged to have Metavir F4 histology. The LB was guided with 14 G - TruCut needle (Medical Technology, Gainsville, FL, United States). LB fragments including at least 10 portal tracts were considered adequate for pathological interpretation, and were included in our study. Liver specimens were fixed in formalin and embedded in paraffin. Two micron sections were stained with hematoxylin-eosin, Masson's trichrome and Sirius red for histological assessment. The liver biopsies were assessed according to the METAVIR score, by a senior pathologist and classified as: F0 - no fibrosis; F1 - portal fibrosis without septa; F2 - portal fibrosis and few septa extending into lobules; F3 - numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4 - cirrhosis.

Statistical analysis

Statistical analyses were performed by using R statistics

Table 1 Demographic, laboratory and liver fibrosis characteristics

Characteristics	Patients (n = 81)
Gender (male/female)	40 (49.4%)/41 (50.6%)
Age (yr) - median (Q1;Q3)	51 (30-78)
BMI (kg/m^2) - median (Q1;Q3)	26.5 (24.3-29.6)
ALT (IU/L) - median (Q1;Q3)	50 (32.5-85)
AST (IU /L) - median (Q1;Q3)	42 (28.5-61.5)
Platelet count (× 10^3/mm^3) - median (Q1; Q3)	202 (148-247)
Histological fibrosis stage, n (%)	
F0	5 (6.2)
F1	33 (40.7)
F2	20 (24.7)
F3	12 (14.8)
F4	11 (13.6)
TE (kPa) - median (Q1; Q3)	6.9 (5-12.2)
TE success rate (mean ± SD)	0.92 ± 0.21
CAP (dB/m) - mMedian (Q1; Q3)	237 (204-263)
ARFI (m/s) - median (Q1; Q3)	1.25 (0.65-2.89)
APRI - median (Q1; Q3)	0.66 (0.41-1.29)
FIB-4 - median (Q1; Q3)	1.41 (0.90-2.45)

BMI: Body mass index; ALT: Alanine amino transferase; AST: Aspartate amino transferase; TE: Transient elastography; CAP: Controlled attenuation parameter; ARFI: Acoustic radiation force impulse; APRI: Score index (AST/LSN AST/platelet); FIB-4: Score index (age × AST/platelet × ALT).

version 3.2.5. (R Core Team, Vienna, Austria). The STARD Statement guidelines were followed. Quantitative characteristics were expressed as mean (SD), median (first and third quartile) and range. Variables were compared using non-parametric Wilcoxon test. P value < 0.05 was considered significant.

The ANOVA or Kruskal-Wallis tests were used for comparison of two or more groups, whether or not the data were normally distributed, respectively. We set that mild disagreement was when only one class was different, and severe disagreement when two or more class were wrongly misclassified.

The diagnostic performance of FibroScan®, ARFI, APRI and FIB-4 tests was assessed using receiver operator curves (ROC). The optimum cut-off levels, defined as area under ROC (AUROC), of each test was chosen to define fibrosis stages F ⩾ 2, F ⩾ 3 and F = 4. The Kappa index set the concordance analysis. The best sensitivity values (> 80%) have been chosen in order to identify all HCV patients with METAVIR F ⩾ 3 (prioritized for treatment according to Brazilian Ministry of Health recommendations)[13]. Positive and negative predictive values (PPV and NPV) were calculated using the prevalence of liver fibrosis stages (F > 2) in the Hepatology Outpatient Center from Hospital das Clinicas of the University of Sao Paulo School of Medicine, Brazil. A statistical review of the study was performed by a biomedical statistician (João Ítalo França).

RESULTS

A total of 81 patients with HCV were included, 41 (50.6%) were female. Anthropometric and laboratorial characteristics are shown in Table 1. The median age was 51 years (30-78). Eleven (13.6%) patients had diabetes, 22 (27.2%) hypertension, 20 (24.7%) were smokers and 11 (13.6%) consumed alcohol (> 20 g /d). The median BMI was 26.5 (24.3-29.6), and 70% of the patients were HCV treatment-naïve. Most of the patients had Metavir F1 on LB fibrosis stage (33 patients, 40.7%), followed by F2 (20 patients, 24.7%). The mean success rate of TE was 92%.

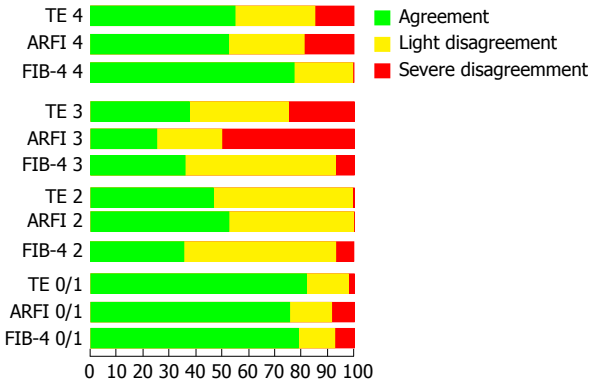

Figure 2 Rate of agreement of transient elastography, acoustic radiation force impulse, aspartate aminotransferase-to-platelet ratio index and FIB-4 according to Metavir fibrosis stage (%). TE: Transient elastography; ARFI: Acoustic radiation force impulse; FIB-4: Score index (age × AST/platelet × ALT).

The best cut-off values of each test (LSM and SM) are found in Table 2. For predicting F ⩾ 2 stage fibrosis with TE was 6.6 kPa, for ARFI 1.22 m/s, for APRI 0.75 and for FIB-4 1.47. For F ⩾ 3, TE was 8.9 kPa, ARFI was 1.48 m/s, APRI was 0.75, and FIB-4 was 2. For F = 4, TE was 12.2 kPa, ARFI was 1.77 m/s, APRI was 1.46, and FIB-4 was 3.91. The APRI could not distinguish between F2 and F3 (P = 0.92). The NPV for F = 4 for TE and ARFI was 100%. Kappa Index values for F ⩾ 3 METAVIR score for TE, ARFI and FIB-4 were 0.687, 0.606 and 0.654, respectively. This demonstrates strong concordance between the TE, ARFI and FIB-4 methods, but moderate concordance between them and APRI (Kappa index = 0.507). Figure 2 shows the rate of agreement of TE, ARFI, APRI and FIB-4 according to Metavir stage on LB. Since alcohol consumption and severity of liver inflammation could affect TE measurements, patients were also analyzed individually. Of the 11 patients with alcohol consumption, 2 patients had discordant results between TE and liver biopsy. One had Metavir F1A1 and TE of 10 kPa and the other had Metavir F1A2 and TE of 16.3 kPa. With regard to patients with more intense inflammatory activity on hepatic biopsy (Metavir A3-4), we had 6 patients with Metavir A3, and none with Metavir A4. Of these Metavir A3 patients, 5 were Metavir F3 and 1 had cirrhosis. TE discordance with liver biopsy could be found in all Metavir F3A3 patients, with overestimation of TE results (mean TE results: 25.3 kPa). One Metavir F3A3 patient had ALT of 148 U/L and a TE of 28.4 kPa. All other patients with Metavir F3A3 had ALT results between 32 and 86 U /L.

Table 2 Summary of cut-off values, area under de curve, sensitivity, specificity, positive predictive value, negative predictive value and accuracy

Method	AUC	AUC CI (95%)	Se	Sp	PPV	NPV	Accuracy
TE (kPa)							
> F2 (6.6)	0.8716	0.7953-0.948	82.90%	77.50%	89.30%	66.70%	80.20%
> F3 (8.9)	0.9187	0.8319-1.000	87%	86.20%	87.30%	85.90%	86.40%
= F4 (12.2)	0.9675	0.9321-1.000	100%	87.10%	79.30%	100%	88.90%
ARFI (m/s)							
> F2 (1.22)	0.7701	0.6653-0.8749	78%	70%	85.50%	58.40%	74.10%
> F3 (1.48)	0.8669	0.7756-0.9583	82.60%	82.80%	83.90%	81.40%	82.70%
= F4 (1.77)	0.9188	0.8592-0.9784	100%	85.70%	77.50%	100%	87.70%
APRI							
> F2 (0.75)	0.8107	0.7136-0.9077	75.60%	87.50%	93.20%	61.30%	81.50%
> F3 (0.75)	0.8272	0.7140-0.9405	87%	72.40%	77.40%	83.60%	76.50%
= F4 (1.46)	0.9143	0.8387-0.9899	81.80%	90%	80.10%	91%	88.90%
FIB-4							
> F2 (1.47)	0.8652	0.7844-0.9461	78%	82.50%	91%	62.40%	80.20%
> F3 (2.0)	0.8703	0.7634-0.9773	82.60%	86.20%	86.70%	82%	85.20%
= F4 (3.91)	0.9636	0.9211-1.000	90.90%	95.70%	91.30%	95.50%	95.10%

AUC: Area under de curve; CI: Confidence interval; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

DISCUSSION

Our results show that three methods, ARFI, TE and FIB-4, independently identify advanced fibrosis. Non-invasive methods have been studied and compared to other methods of liver fibrosis evaluation in order to diminish complications of liver biopsy and costs involved[8]. We evaluated the AUROC and the inter-agreement of LSM (TE and ARFI) as well as SM (APRI and FIB-4) compared with liver biopsy in a population of HCV-infected patients. The best cut-offs were established based on METAVIR fibrosis stages F $\geqslant$ 2, F $\geqslant$ 3 and F = 4, considering not only the recent international consensus (EASL-ALEH 2015) but the recommendation for HCV treatment in Brazil, which prioritize F $\geqslant$ 3 patients according to the 2015 Brazilian Protocol for HCV treatment[4,8,13]. The results of this study do not conflict with previous findings (Table 2). The TE sensitivity (Se) and specificity (Sp) in a recent FibroScan® meta-analysis[14] ranged from about 0.70 and 0.81 for F $\geqslant$ 2, 0.80 and 0.85 for F $\geqslant$ 3, and from 0.86 and 0.88 for F = 4. These are similar to our results. Although TE had better results for F $\geqslant$ 2, the overall accuracy for ARFI and TE were comparable, as previously demonstrated by Crespo *et al*[15]. For the F4 group we found an almost perfect correlation between ARFI, TE and FIB-4, suggesting that only one method is sufficient to identify cirrhosis. It is important to note that on 2 out of 11 patients who reported alcohol consumption (> 20 g/d), TE values were overestimated. The influence of alcohol intake on liver stiffness measurement should be taken into account when interpreting TE results, as shown by Bardou-Jacquet *et al*[16]. Hepatic inflammation can also be a confounding factor when evaluating liver fibrosis by TE[17]. We could demonstrate that all Metavir F3A3 patients had overestimation of liver fibrosis by TE, with median values of 25.3 kPa.

APRI is a good reproducible marker of cirrhosis, with a high applicability (> 95%), it is easy to perform and is a non-patented score[18]. In our study APRI could not differentiate between F = 2 and F = 3. This is possibly because it uses fewer variables than FIB-4. APRI uses AST and platelet count, while FIB-4 also incorporates ALT and age of the patient.

The F2 group is less clearly defined than other stages of fibrosis, as shown in the literature by Rizzo *et al*[19], and all methods identified it less accurately. In our study however, there was less disagreement than Afdhal *et al*[20] which shows that for F2 group, application of both methods, TE and ARFI are necessary to identify these patients.

Although LB is the reference standard, its reproducibility is poor, owing to heterogeneity in liver fibrosis, operator bias and sample size. This can account for an margin of error of up to 20% in disease staging[20].

A limitation of this study is that it identifies and selects cut-off points, but is not prospectively validated, warranting further studies to confirm these results. However, from this study, we can consider that a combined use of FibroScan® and FIB-4 or FibroScan® and ARFI in the follow-up of HCV patients can be a surrogate for fibrosis assessment through LB, which can be held in reserve for cases with significant diagnostic doubt. This is especially important in the intermediate stages of fibrosis (F2 and F3), where each individual non-invasive method is not sufficiently accurate to make a diagnosis, and so should be performed in combination. Further studies are necessary to identify whether they should be performed simultaneously or in parallel, and identify the best cutoffs for each combination of methods.

In conclusion, given the higher cost and reduced accessibility of LSM methods, and the similarity with the outcomes of SM in evaluation of liver fibrosis, we suggest that FIB-4 used alongside TE and ARFI may be good tools for the prediction of severity of liver fibrosis. This may be of particular importance to developing countries.

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COMMENTS

Background

The evaluation of liver fibrosis is not a simple task and demands the use of different methods. Liver stiffness measurement (LSM) and serum markers (SM) provide a non-invasive source of diagnosis with good correlation with the gold standard method, liver biopsy.

Research frontiers

The F2 group is less clearly defined than other stages of fibrosis. In the authors' study there was however less disagreement, which shows that for F2 group, application of both methods, transient elastography (TE) and acoustic radiation force impulse (ARFI) is necessary to identify these patients. Further studies are necessary to identify whether they should be performed simultaneously or in parallel, and identify the best cutoffs for each combination of methods.

Innovations and breakthroughs

This results show that three methods, ARFI, TE and FIB-4, independently identify advanced fibrosis.

Applications

Given the higher cost and reduced accessibility of LSM methods, and the similarity with the outcomes of SM in evaluation of liver fibrosis, the authors suggest that FIB-4 used alongside TE and ARFI may be good tools for the prediction of severity of liver fibrosis. This may be of particular importance to developing countries.

Terminology

Non-invasive tests for liver fibrosis evaluation: (1) Mechanical markers: liver stiffness measurement according to transient elastography (FibroScan®) or ARFI; and (2) Serum markers: Aspartate aminotransferase-to-platelet ratio index (APRI) and FIB-4 (based on age, platelet count, aspartate aminotransferase and alanine aminotransferase). Liver biopsies were assessed according to the METAVIR score classified as: F0 - no fibrosis; F1 - portal fibrosis without septa; F2 - portal fibrosis and few septa extending into lobules; F3 - numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4 - cirrhosis.

Peer-review

This study is addressed to evaluate the diagnostic performance and the concordance of different noninvasive methods for the evaluation of liver fibrosis (APRI, FIB-4, transient elastography, ARFI) in 81 patients with chronic hepatitis C, most of them with biopsy-proven diagnosis. The authors concluded that FIB-4, ARFI and transient elastography are useful tests for the noninvasive assessment of liver fibrosis, with a good concordance.

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META-ANALYSIS

Meta-analysis reveals up-regulation of cholesterol processes in non-alcoholic and down-regulation in alcoholic fatty liver disease

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Abstract

AIM

To compare transcriptomes of non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) in a meta-analysis of liver biopsies.

METHODS

Employing transcriptome data from patient liver biopsies retrieved from several public repositories we performed a meta-analysis comparing ALD and NAFLD.

RESULTS

We observed predominating commonalities at the transcriptome level between ALD and NAFLD, most prominently numerous down-regulated metabolic pathways and cytochrome-related pathways and a few up-regulated pathways which include ECM-receptor interaction, phagosome and lysosome. However some pathways were regulated in opposite directions in ALD and NAFLD, for example, glycolysis was down-regulated in ALD and up-regulated in NAFLD. Interestingly, we found rate-limiting genes such as *HMGCR*, *SQLE* and *CYP7A1* which are associated with cholesterol processes adversely regulated between ALD (down-regulated) and NAFLD (up-regulated). We propose that similar phenotypes in both diseases may be due to a lower level of the enzyme CYP7A1 compared to the cholesterol synthesis enzymes HMGCR and SQLE. Additionally, we provide a compendium of comparative KEGG pathways regulation in ALD and NAFLD.

CONCLUSION

Our finding of adversely regulated cholesterol processes in ALD and NAFLD draws the focus to regulation of cholesterol secretion into bile. Thus, it will be interesting to further investigate CYP7A1-mediated cholesterol secretion into bile - also as possible drug targets. The list of potential novel biomarkers may assist differential diagnosis of ALD and NAFLD.

Key words: Non-alcoholic fatty liver disease; Alcoholic liver disease cholesterol; Bile; Alcohol dehydrogenase; CYP7A1

Core tip: With a meta-analysis of newly published liver biopsy-derived transcriptome datasets we identified multiple key genes and pathways in common and mutually exclusive in alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). We provide a compendium of comparative regulation for all KEGG pathways in both diseases and propose a list of biomarkers distinguishing both diseases. One surprising finding was that cholesterol metabolism was up-regulated in NAFLD and down-regulated in ALD although leading to the same steatosis phenotype which might be explained by an insufficient conversion rate to bile acids under both conditions.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) have nearly identical symptoms and in the first report non-alcoholic steatohepatitis (NASH) was described as histologically mimicking alcoholic hepatitis[1]. While the cause of ALD is excessive alcohol, the cause of NAFLD is excessive fat resulting from an imbalance between diet and physical activity often associated with insulin resistance and obesity.

We are working on the hypothesis that alcohol is metabolized to fat and beyond this pathway both diseases share a common phenotype. Therefore we place special emphasis on alcohol metabolism which naturally plays a crucial role in ALD. Associations of variants in alcohol and aldehyde dehydrogenases with alcoholism have already been proposed[2]. Most variants protective against alcoholism result in a higher acetaldehyde level either by accelerating alcohol dehydrogenase (most common variants in *ADH1B*) metabolizing alcohol to acetaldehyde or by reducing aldehyde dehydrogenase (most common variants in *ALDH2*) metabolizing acetaldehyde to acetic acid. Acetaldehyde is a carcinogen and causes severe reactions such as flushing, accelerated heart rate and nausea. These severe reactions will impose on most carriers of these variants to abstain from alcohol and thus reduce their risk of becoming alcohol addicts. Furthermore, it has been reported that aldehyde dehydrogenases are down-regulated in alcoholics[3] or animals continually exposed to alcohol had lower ethanol elimination rates[4]. However, this is a matter of debate as no significant down-regulation of aldehyde dehydrogenases was reported by Vidal *et al*[5] but instead a down-regulation in cirrhotic livers independent of alcoholism. Acetic acid - the product of ethanol metabolism, can be further metabolized by acyl-CoA synthetases (ACSS1 and ACSS2) to acetyl-CoA, the substrate for fatty acid synthesis[6]. The expression and activity of Acyl-CoA synthetases in turn are controlled by the sterol regulatory element-binding protein which has been reported to be activated by ethanol[7].

The progression of NAFLD from mild steatosis up to severe NASH or from ALD to alcoholic hepatitis varies widely between individual patients. Oxidative stress and dysregulation of cytokines as a basis for inflammation appear to foster progression to NASH[8] as well as alcoholic hepatitis (AH)[9]. A two-hit progression from simple steatosis to steatohepatitis and fibrosis has been proposed[10], and suggests that after fat accumulation in the liver, lipids are peroxidized by oxidative stress induced by factors such as CYP2E1. The microsomal enzyme CYP2E1 metabolizes ethanol to acetaldehyde under conditions of alcohol dehydrogenase overload and generates oxidative stress as a by-product, however fatty acids also can be a substrate of CYP2E1[9].

Recently the role of the gut has attracted attention. Under alcoholic or high-fat conditions lipopolysaccharides can pass the border of the intestine to the portal vein and circulate to the liver where they trigger inflammation in ALD[11] and in NAFLD[12].

Some studies have already compared ALD and NAFLD[13], *e.g.*, Wilfred de Alwis and Day[14] compared the genetics of both diseases addressing the question why only a small percentage of heavy drinkers and obese people progress from steatosis to severe liver disease. Here, we provide an analytical comparison of transcriptomic and metabolic processes involved in the progression of ALD and NAFLD. Employing transcriptome data derived from patient liver biopsies retrieved from several public repositories we performed a meta-analysis and report a signature of biomarkers distinguishing AH from NASH samples. Furthermore, we found predominating commonalities between both diseases at the level of biological pathways thus implying a large mechanistic similarity between both diseases.

MATERIALS AND METHODS

Transcriptome data analysis

Datasets of microarray gene expression data from liver biopsies were downloaded from the public repositories at NCBI GEO and EBI Array-Express. The compendium consisted of the ALD datasets GSE28619[15] and E-MTAB-2664[16] and the NAFLD datasets GSE61260[17], GSE59045[18], GSE48452[19] and GSE46300[12]. Illumina data was processed *via* R/Bioconductor[20] and packages lumi[21], limma[22] and qvalue[23]. Background-corrected log2-transformed data was normalized *via* quantile

normalization from the lumi package. Affymetrix data was processed *via* R/Bioconductor and packages affy[24], limma, qvalue employing the *rma* normalization method.

Measurements from the multiple platforms were brought together in terms of mean ratios between ALD cases and controls and between NAFLD cases and controls. As controls, healthy liver biopsies or liver biopsies with a low grade of fat accumulation were used. For details we refer to the methods sections of the publications associated with the employed datasets[12,15-19]. Heterogeneity of the datasets was assessed *via* the meta-analysis R package metafor[25] generating forest and funnel plots (supplementary Figure 1A and B). The ratios were transformed to a log2 scale and normalized *via* quantile normalization. The results were again assessed with forest and funnel plots (supplementary Figure 1C and D).

Pathway analysis

In order to disentangle commonalities and differences between ALD and NAFLD, KEGG pathways[26] were analysed with respect to common pathways, up- and down-regulation and discordant up- and down-regulation. The ratios between ALD and control and NAFLD and control were employed to count the numbers of up- and down-regulated genes for each pathway. A pathway was considered up-regulated when it contained more up- than down-regulated genes. Genes with a ratio > t were termed up-regulated and genes with a ratio < 1/t were termed down-regulated. The threshold t was determined at the 95-quantile of the mean ratios between ALD and NAFLD *vs* control and was set accordingly to t = 4/5. Up- and down-regulation of a pathway was determined *via* the ratio of numbers of up-and down-regulated genes and *via* a binomial test assuming an equal probability of $P = 0.5$ for a gene to be up- or down-regulated.

$$n_{up,pw,case} = |\{g|(\frac{x_{g,case}}{x_{g,control}} > t) \wedge (g \in g_{pw})\}|, \text{case} \in \{\text{ALD, NAFLD}\} \quad (1)$$

$$n_{down,pw,case} = |\{g|(\frac{x_{g,case}}{x_{g,control}} < 1/t) \wedge (g \in g_{pw})\}|, \text{case} \in \{\text{ALD, NAFLD}\} \quad (2)$$

$$n_{pw,case} = n_{up,pw,case} + n_{down,pw,case} \quad (3)$$

$$r_{pw,case} = \frac{n_{up,pw,case}}{n_{down,pw,case}} \quad (4)$$

Here, $n_{up,pw,case}$ and $n_{down,pw,case}$ are the numbers of up- and down-regulated genes in a pathway *pw*, g_{pw} are the gene symbols associated with a pathway, $x_{g,case}$ is the gene expression value in a case which can be *ALD* or *NAFLD*, $x_{g,control}$ is the gene expression value in the control case, $r_{pw,case}$ is the ratio indicating up-regulation ($r_{pw,case} > 1$) or down-regulation ($r_{pw,case} < 1$) of pathway *pw*. Significance of up- or down-regulation of a pathway is assessed *via* the Binomial test with the Null hypothesis $H_0: p \leqslant p_0$ and the test statistic $B(p_0, n_{pw,case})$. Because of assumed equal distribution of up- and down-regulation the probability for the binomial distribution is set to $p_0 = 0.5$.

Pathway charts of KEGG pathways indicating up- and down-regulation of genes in ALD and NAFLD were generated *via* the R/Bioconductor package pathview[27].

RESULTS

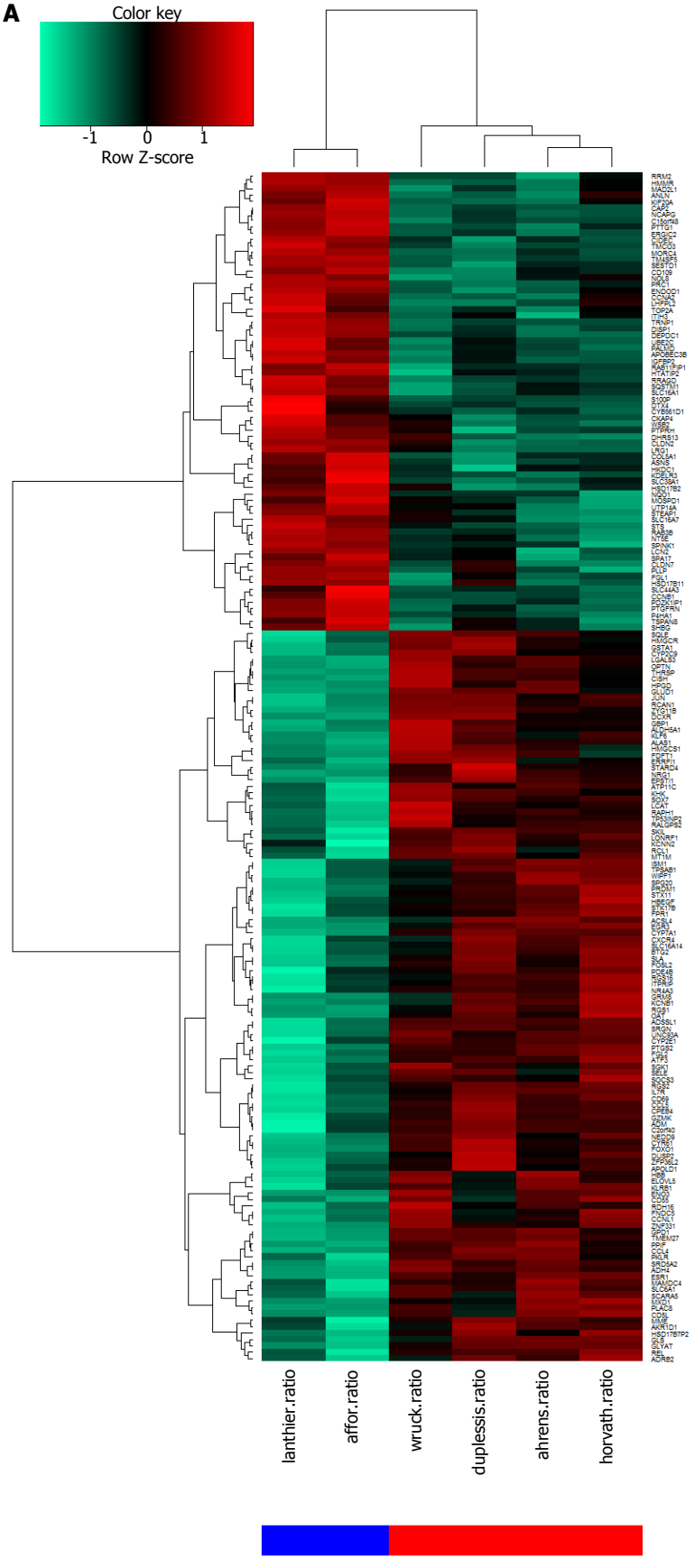
A gene signature distinquishes ALD from NAFLD

The differences between ALD and NAFLD at the transcriptome level could be condensed to a signature of 187 genes which are differentially expressed between both conditions with a *P*-value < 0.01 from the limma test and a ratio > 3/2 or a ratio < 2/3. The heatmap in Figure 1A shows a cluster analysis of this signature of gene expression data from ALD liver biopsies (blue bar) and NAFLD liver biopsies (red bar). The table in Figure 1B shows the 20 most up-regulated and 20 most-down-regulated genes from the signature indicating their log2-ratios and their *P*- and *Q*-values for the comparison ALD *vs* NAFLD. The most up-regulated gene between ALD and NAFLD was SPINK1. SPINK1 is secreted in the pancreatic juice to reversibly inhibit activated trypsin thus preventing pancreatic auto-digestion[28] and variants in this gene have been associated with pancreatitis[29]. Obesity and more prominent alcohol abuse are other causative factors for pancreatitis[28] which by its effects on insulin may contribute to liver disease. Lanthier *et al*[16] revealed the association of SPINK1 with inflammation and proliferation *via* correlation with the inflammatory macrophage marker CD68 and the cell cycle markers Cdk1 and CyclinB1. At the lower part of the table in Figure 1B two RGS (regulator of G-protein signalling) encoding genes, RGS1 and RGS2 are down-regulated in ALD but up-regulated in NAFLD. Nunn *et al*[30] reported reduced fat deposits, decreased serum lipids, and low Leptin levels in RGS2 deficient mice.

Genes regulated in common between ALD and NAFLD

Analysis of the common genes between ALD and NAFLD was subdivided into analysis of down- and up-regulated genes. Figure 2A shows that 104 genes are down-regulated in ALD and NAFLD (ratio < 0.8) while 638 genes are exclusively down-regulated in ALD and 285 in NAFLD. Figure 2B shows that 97 genes are up-regulated in ALD and NAFLD (ratio > 1.25) while 519 genes are exclusively up-regulated in ALD and 362 in NAFLD. There are more distinctly expressed than overlapping genes - in contrary to the KEGG pathways where most pathways overlap (Figure 2E and F). Gene regulation was further restricted with a threshold for the limma test for differential expression of $P < 0.05$. Figure 2C shows a venn diagram of the four resulting sets of up/down-regulated genes in ALD and NAFLD. Here most genes are exclusively regulated but interestingly from the genes regulated in both diseases more genes are oppositely than commonly regulated: 61 genes are up-regulated in NAFLD but down-regulated in ALD and 12

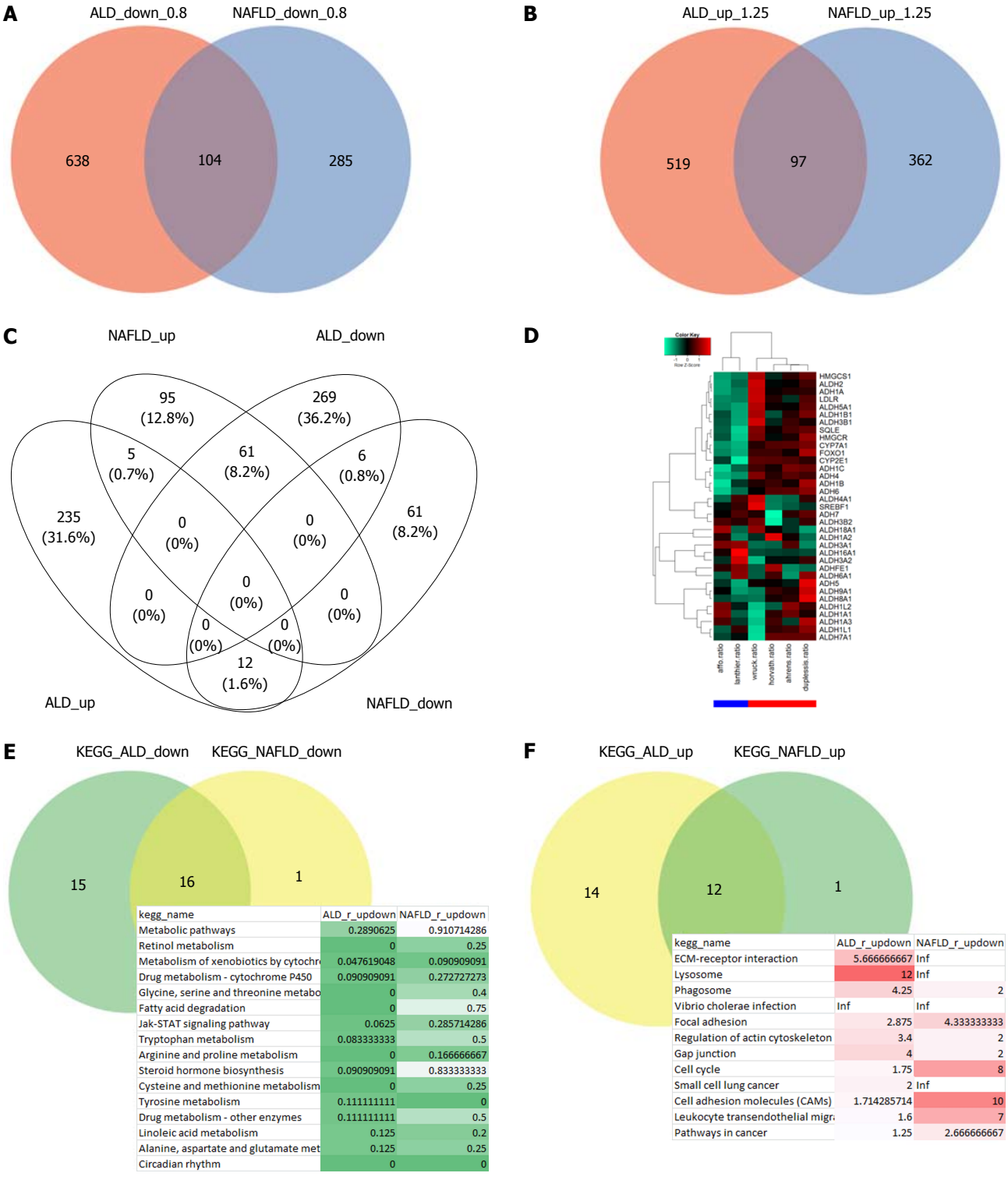
A



B

Gene	log2ratio_ ALD_NAFLD	limma_p	limma_q
SPINK1	3.1120	0.00086043	0.18404683
IGFBP2	2.0876	0.00021163	0.11364535
S100P	2.0475	0.00076244	0.1814015
LCN2	1.8578	0.00157791	0.20177258
P4HA1	1.6796	6.30E-5	0.09050201
PDZK1IP1	1.5916	8.43E-5	0.09050201
HKDC1	1.4752	0.0099653	0.27847194
NQO1	1.4712	0.00077208	0.1814015
SHBG	1.4032	0.003424	0.22085368
TRNP1	1.3509	1.28E-5	0.09050201
RAB3B	1.3441	0.0001067	0.09050201
RRM2	1.3100	0.0005424	0.15563552
CLDN2	1.2816	0.0023388	0.21111468
STEAP1	1.2667	0.00207474	0.20878205
C15orf48	1.2369	5.77E-5	0.09050201
SLC16A7	1.1967	0.00684988	0.25828645
TMCO3	1.1675	9.41E-5	0.09050201
SQSTM1	1.1231	0.00140252	0.20177258
FGL1	1.0544	0.00104332	0.18745735
CD109	1.0035	0.0081723	0.1814015
SGK1	-1.4274	0.00386065	0.23007365
HBEGF	-1.4634	0.00616426	0.25461827
THRSP	-1.5061	0.00141011	0.20177258
IL7R	-1.5111	0.00126129	0.19647757
ACSL4	-1.5548	0.00302525	0.2146298
ENO3	-1.6119	0.00193989	0.20878205
CD69	-1.6346	0.00050895	0.15352702
JUN	-1.6946	0.00050895	0.11364535
KCNN2	-1.7040	0.0019417	0.27677322
CXCR4	-1.7057	0.00958215	0.27677322
SQLE	-1.8663	0.00937092	0.18404683
PTGS2	-1.8778	6.27E-5	0.09050201
ATF3	-1.9327	0.00026863	0.12722548
SRD5A2	-2.0482	3.49E-5	0.09050201
NR4A3	-2.0615	0.00436311	0.23545747
CYR61	-2.0653	0.00104554	0.18745735
CYP7A1	-2.0965	1.68E-5	0.09050201
RGS2	-2.1605	0.00069501	0.17342654
HMGCS1	-2.1724	0.0057275	0.25288411
RGS1	-2.2757	0.00128147	0.19647757

Figure 1 A gene signature distinquishes alcoholic liver disease from non-alcoholic fatty liver disease. A: The heatmap shows a cluster analysis of logarithmic ratios of gene expression data from ALD liver biopsies *vs* control (blue bar) and NAFLD liver biopsies *vs* control (red bar); B: The table shows the 20 most up-regulated and 20 most-down-regulated genes from the signature indicating their log2-ratios and their *P*- and *Q*-values for the comparison ALD *vs* NAFLD. The full list of these genes can be found in Supplementary Table 2. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.



kegg_name	ALD_r_updown	NAFLD_r_updown
Metabolic pathways	0.2890625	0.910714286
Retinol metabolism	0	0.25
Metabolism of xenobiotics by cytochr	0.047619048	0.090909091
Drug metabolism - cytochrome P450	0.090909091	0.272727273
Glycine, serine and threonine metabo	0	0.4
Fatty acid degradation	0	0.75
Jak-STAT signaling pathway	0.0625	0.285714286
Tryptophan metabolism	0.083333333	0.5
Arginine and proline metabolism	0	0.166666667
Steroid hormone biosynthesis	0.090909091	0.833333333
Cysteine and methionine metabolism	0	0.25
Tyrosine metabolism	0.111111111	0
Drug metabolism - other enzymes	0.111111111	0.5
Linoleic acid metabolism	0.125	0.2
Alanine, aspartate and glutamate met	0.125	0.25
Circadian rhythm	0	0

kegg_name	ALD_r_updown	NAFLD_r_updown
ECM-receptor interaction	5.666666667	Inf
Lysosome	12	Inf
Phagosome	4.25	2
Vibrio cholerae infection	Inf	Inf
Focal adhesion	2.875	4.333333333
Regulation of actin cytoskeleton	3.4	2
Gap junction	4	2
Cell cycle	1.75	8
Small cell lung cancer	2	Inf
Cell adhesion molecules (CAMs)	1.714285714	10
Leukocyte transendothelial migr	1.6	7
Pathways in cancer	1.25	2.666666667

Figure 2 Most biological pathways are regulated in the same direction in alcoholic liver disease and non-alcoholic fatty liver disease but a subset of metabolism-associated genes are oppositely regulated. A: Compares ALD and NAFLD in terms of down-regulated genes (ratio < 0.8); B: In terms of up-regulated genes (ratio > 1.25). There are more distinct than overlapping genes - in contrary to the KEGG pathways where most pathways overlap (E and F); C: Interestingly, when regulation is further restricted with a *P*-value < 0.05 more genes are oppositely than commonly regulated - but most are exclusively regulated. Many of the oppositely regulated genes are associated with cholesterol processes, *e.g.*, HMGCR, SQLE and CYP7A1, and are co-expressed with alcohol (ADH) and aldehyd dehydrogeneases (ALDH) as seen in the heatmap (ALD: Blue bar, NAFLD: Red bar) (D). A pathway is considered down-regulated (E) when it contains more down- than up-regulated genes as tested by the binomial test and the ratio, analogously up-regulated pathways are determined (F). The table of common down-regulated pathways includes metabolic, retinol, cytochrome and fatty acid degradation pathways, the up-regulated include ECM-receptor, lysosome and phagosome. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

are up-regulated in ALD and down-regulated in NAFLD while only 5 were commonly up and 6 commonly down-regulated. Supplementary Table 1 shows the corresponding gene sets. The genes up-regulated in

NAFLD but down in ALD refer to major players in cholesterol processes such as *HMGCS1, HMGCR, SQLE, CYP7A1* and *LDLR*. This would confirm the involvement of cholesterol biological processes in the etiology of NAFLD as we previously reported[31] and which distinguish it from the etiology of ALD. The opposite regulation of cholesterol processes as down in ALD and up in NAFLD can also be observed in the corresponding KEGG pathways Steroid biosynthesis, Primary bile acid biosynthesis and Terpenoid backbone biosynthesis (Supplementary file 1, p22, 34 and 84). These findings are in line with reports of a 29% decrease in HMGCR and a 56% decrease in cholesterol 7α-hydroxylase alias CYP7A1 by Lakshmanan *et al*[32], they suggested that increased ethanol leads to a reduced rate of cholesterol degradation to bile acids and accumulation of cholesterol in the liver. We also found (Supplementary Table 2) a stronger down-regulation of *CYP7A1* (log2-ratio = -0.95) than of the upstream cholesterol genes *HMGCR* (log2-ratio = -0.429) and *SQLE* (log2-ratio = -0.33) in ALD while in NAFLD, *CYP7A1* (log2-ratio = 1.15) was weaker up-regulated than *HMGCR* (log2-ratio = 1.57) and *SQLE* (log2-ratio = 1.53). Thus although oppositely regulated in ALD and NAFLD in both diseases more cholesterol is produced than can be secreted by the bile *via* CYP7A1.

Amongst the genes up-regulated in ALD but down in NAFLD are *TNFSF14* in line with the major role of TNF-alpha in ALD[11] and *SPINK1* which was described above in "a gene signature distinguishes ALD from NAFLD".

To further investigate the mechanisms by which ethanol induces these changes in cholesterol processes we analysed expression clusters of genes involved in ethanol and cholesterol related processes. The analysis revealed a cluster of genes down-regulated in ALD and up-regulated in NAFLD including among others the genes encoding for ALDH2, ADH1A, LDLR, SQLE, HMGCR, CYP7A1, CYP2E1 and FOXO1 (Figure 2D). FOXO Transcription factors such as FOXO1, whose expression has been reported to be altered by ethanol[33] and may play a role in the regulation of several genes from this cluster. Interestingly, the heatmap (Figure 2D) shows a much higher degree of co-regulation of *FOXO1* with the rate-limiting cholesterol synthesis enzymes *HMGCR* and *SQLE* than of *SREBF1* which is known as the main regulator of cholesterol[34].

The five genes up-regulated in common between ALD and NAFLD include two collagen encoding genes - *COL1A1* and *COL3A1*, thus demonstrating overlapping disease pathology in the development of fibrotic tissue. The six down-regulated genes in ALD and NAFLD include *HPRT1* which has been reported to be down-regulated in severe liver disease[35].

Pathway analysis

Most biological pathways are regulated in the same direction in ALD and NAFLD. A pathway is considered down-regulated (Figure 2E) when it contains more down- than up-regulated genes as tested by the binomial test and the ratio is less than 1. Up-regulated pathways are determined accordingly (Figure 2F). The table of common down-regulated pathways includes metabolic, retinol, cytochrome and fatty acid degradation pathways, the up-regulated pathways include ECM-receptor, lysosome and phagosome.

Common pathways down-regulated in ALD and NAFLD

Sixteen common pathways are down-regulated in ALD and NAFLD. A pathway with high relevance to both diseases is Fatty acid degradation which is down-regulated in ALD and NAFLD but more so in ALD. The KEGG graph in Figure 3A shows down-regulation (green) in nearly all genes for ALD (left part of the gene boxes) while for NAFLD (right part of the gene boxes) there are up-regulated genes such as *ACSL1* and *ACAT1* but more are down-regulated. Interestingly, in the alcohol metabolism at the bottom of the chart, genes are down-regulated in ALD. At the bottom of Figure 3A, alcohol metabolism is shown in a schematic view. In a more detailed view we examined the behaviour of the alcohol dehydrogenase (ADH) encoding genes in the heatmap in Figure 3B and in the aldehyde dehydrogenase genes in Figure 3C. This resulted in a clear image for the ADHs which were down-regulated in ALD. The heatmap for the ALDHs (Figure 3C) looked more complex showing consistently ALD-down-regulated ALDHs only in a cluster at the top including *ALDH2* while most genes were heterogeneously regulated between ALD and NAFLD.

Common pathways up-regulated in ALD and NAFLD

Few pathways (12) were up-regulated in ALD and NAFLD. One of these is ECM-receptor interaction (Supplementary file 1, p. 142). Up-regulation of this pathway might indicate the onset of fibrosis which is accompanied by excessive accumulation of extracellular matrix proteins including collagen[36]. Here, the involvement of the collagen *COL1A1* is shown.

Pathways oppositely regulated in ALD and NAFLD

Of the oppositely regulated pathways, sixteen were down-regulated in ALD and up-regulated in NAFLD while only one was up-regulated in ALD and down in NAFLD (Supplementary Table 3). The Glycolysis pathway was down-regulated in ALD and up-regulated in NAFLD. The KEGG graph (Supplementary file 1, p. 11) shows more down- (green, *e.g.*, *PGM1, ENO1*) than up-regulated (red, *e.g.*, *PFKL*) genes for ALD (left part of gene boxes) while for NAFLD (right part of gene boxes) up-regulated genes predominate. Reduction of glycolysis by ethanol has been brought into context with consumption of oxygen for the alcohol metabolism and has been reported by several authors[37,38]. Berry *et al*[38] reported that ethanol oxidation inhibits glycolysis in rat hepatocytes *via* competition of the reducing equivalents generated during ethanol oxidation with those arising in glycolysis for transfer to the mitochondria.

Pathway-based functional gene annotation

In "genes regulated in common between ALD and NAFLD" we described that after filtering genes with a

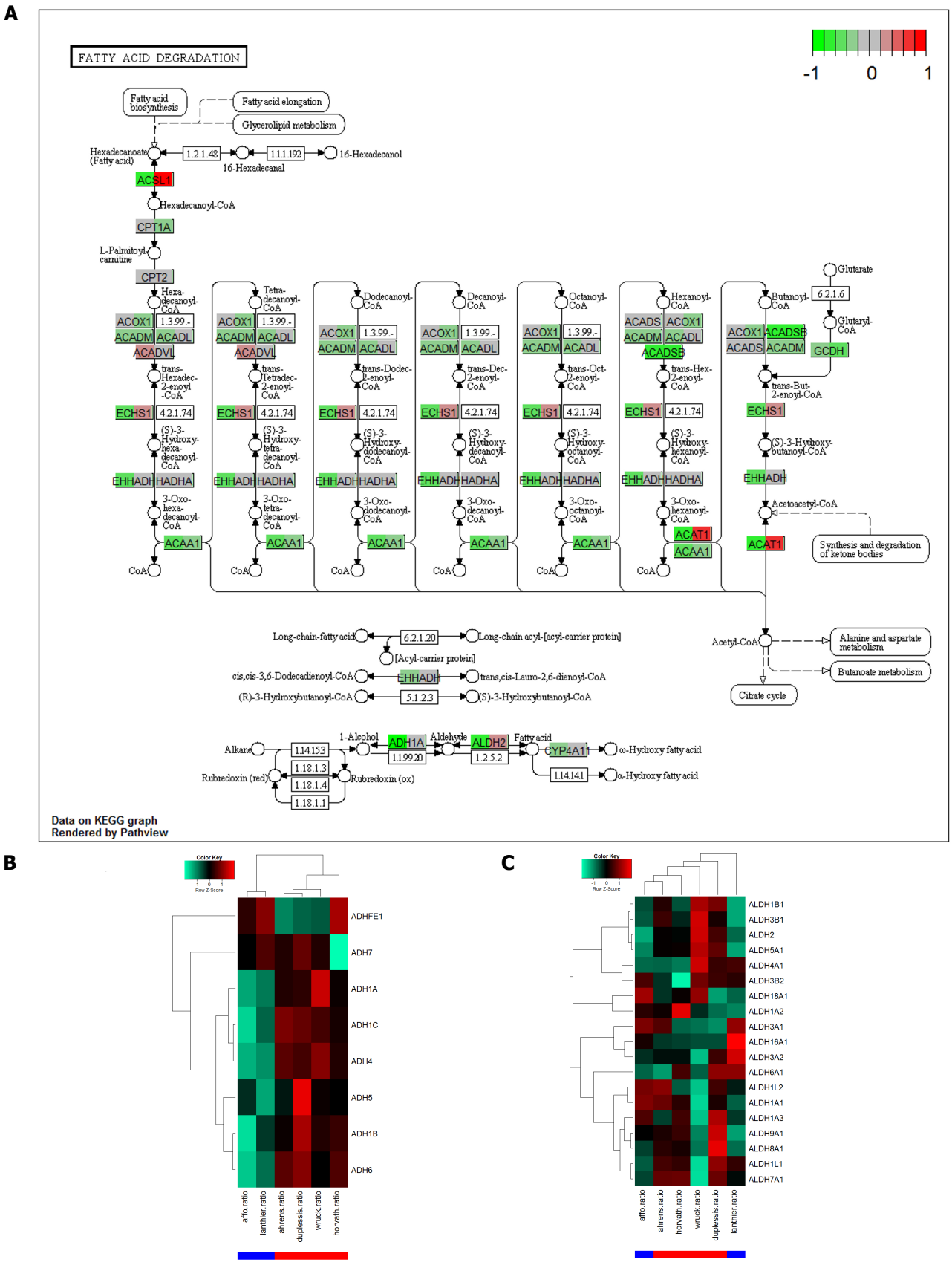


Figure 3 Fatty acid degradation is down-regulated in alcoholic liver disease and non-alcoholic fatty liver disease but more pronounced in alcoholic liver disease. A: The KEGG graph shows down-regulation (green) in nearly all genes for ALD (left part of the gene boxes) while for NAFLD (right part of the gene boxes) there are up-regulated genes such as ACSL1 and ACAT1 but more are down-regulated. Interestingly, in alcohol metabolism at the bottom of the chart, genes are down-regulated in ALD. Alcohol metabolism at the bottom of (A) is shown in detail in the alcohol dehydrogenase (ADH) genes in the heatmap in (B) and in the aldehyde dehydrogenase genes in (C). ADHs are down-regulated in ALD while only dedicated ALDHs, *e.g.*, ALDH2 are down-regulated in ALD. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

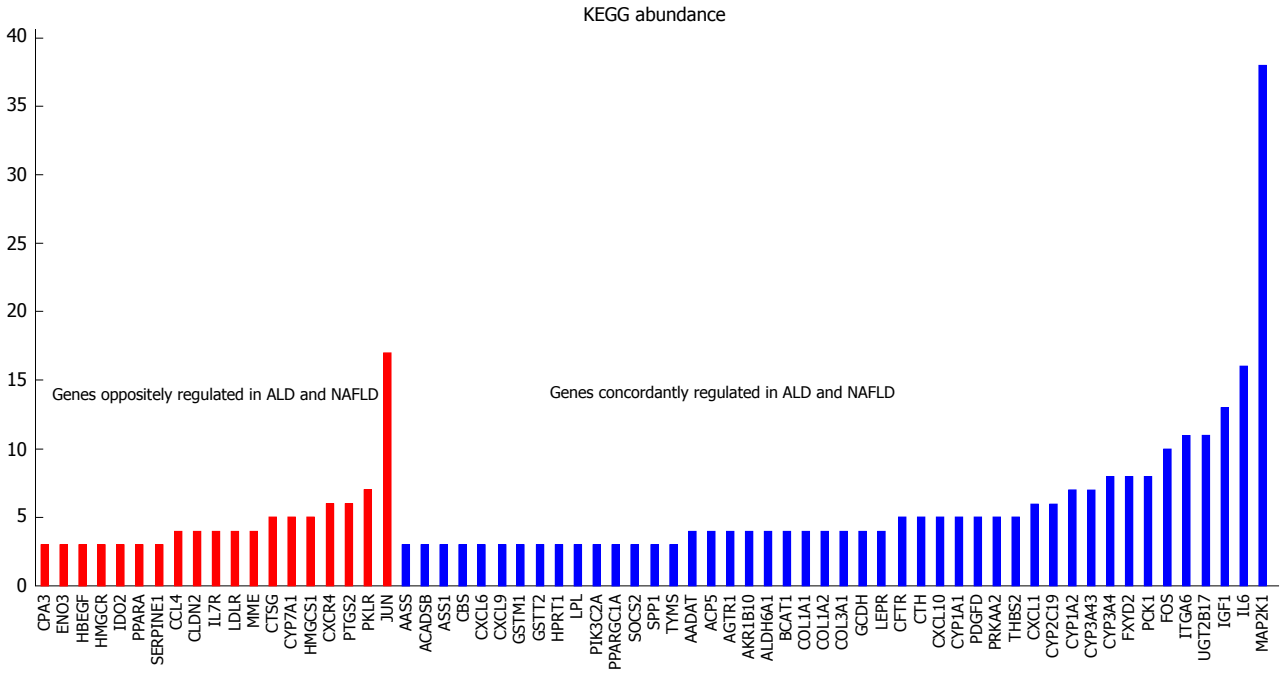


Figure 4 More genes are concordantly than oppositely regulated in alcoholic liver disease and non-alcoholic fatty liver disease. The chart shows the abundance of concordantly and oppositely regulated genes in KEGG pathways (for abundances > 3). The most abundant MAP2K1 (MEK1) refers to the MAPK/RAS-signalling module acting in many KEGG-pathways. JUN which is appearing in 17 KEGG pathways and is down-regulated in ALD and up-regulated in NAFLD shows that there are mechanistic differences in disease pathologies. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

P-value < 0.05 for differential expression more genes were oppositely than concordantly regulated in ALD and NAFLD. This filtering revealed the interesting genes described above but was very restrictive due to the low number of replicates in the condensed ratios - the *P*-values were relatively high. However, the condensed ratios were themselves based on numerous replicates so we consider them as reliable. In a second approach, we filtered genes only by fold change 1.25 and checked on the pathway-level if there were significantly more up- or down-regulated genes based on the binomial test. With this method more genes were concordantly than oppositely regulated in ALD and NAFLD. Figure 4 shows the abundance of concordantly and oppositely regulated genes in KEGG pathways (for abundances > 3). The most abundant MAP2K1 (MEK1) refers to the MAPK/RAS-signalling module acting in many KEGG-pathways. JUN which appears in 17 KEGG pathways and is down-regulated in ALD and up-regulated in NAFLD shows that there are mechanistic differences in molecular basis of these diseases. JUN which is directly connected to c-Jun N-terminal kinase (JNK) was down-regulated in ALD and up-regulated in NAFLD. The up-regulation of JUN in NAFLD is in line with reports from Samuel *et al*[39] showing that activated PKC-ε and JNK can induce insulin resistance *via* impaired IRS1 and IRS2 tyrosine phosphorylation in rats fed with high fat diet.

Pluripotent stem cell-based models of ALD and NAFLD

We recently described a disease-in-a-dish model of steatosis[40]. Pluripotent stem cells, both human embryonic stem cells and induced pluripotent stem cells were differentiated into hepatocyte-like cells and afterwards challenged with ethanol (E) and oleic acid. In order to test how close these models are to the modeled disease we applied our gene signature distinguishing ALD from NAFLD to gene expression data described in Graffmann *et al*[40]. Figure 5 demonstrates that our gene signature can clearly separate two clusters of the ALD and the NAFLD model in a heatmap generated from this gene expression dataset. Furthermore, relevant regulating or rate-limiting genes described above such as *CYP7A1, CYP2E1, HMGCS1, FOXO1* are down-regulated in the ALD-model and up-regulated in the NAFLD-model similar to the liver biopsy-derived dataset.

DISCUSSION

In this comparative analysis of gene expression in ALD and NAFLD liver biopsies we unveiled many commonalities in pathways regulated in the same direction in both diseases. However, there were also pathways regulated in the opposite direction and maybe even more important, essential rate-limiting or regulating genes were adversely regulated. This adverse effect was unexpected as in our working hypothesis, we stated that alcohol is metabolized to fat and beyond this pathway both diseases share a common phenotype. It could hardly be brought together with the common phenotype that of the genes significantly dysregulated between ALD and NAFLD there were more genes regulated in the opposite than in the same direction. One major complex within the adversely regulated genes were cholesterol-related processes including the rate-limiting genes *HMGCR, SQLE, CYP7A1* and *LDLR*. These

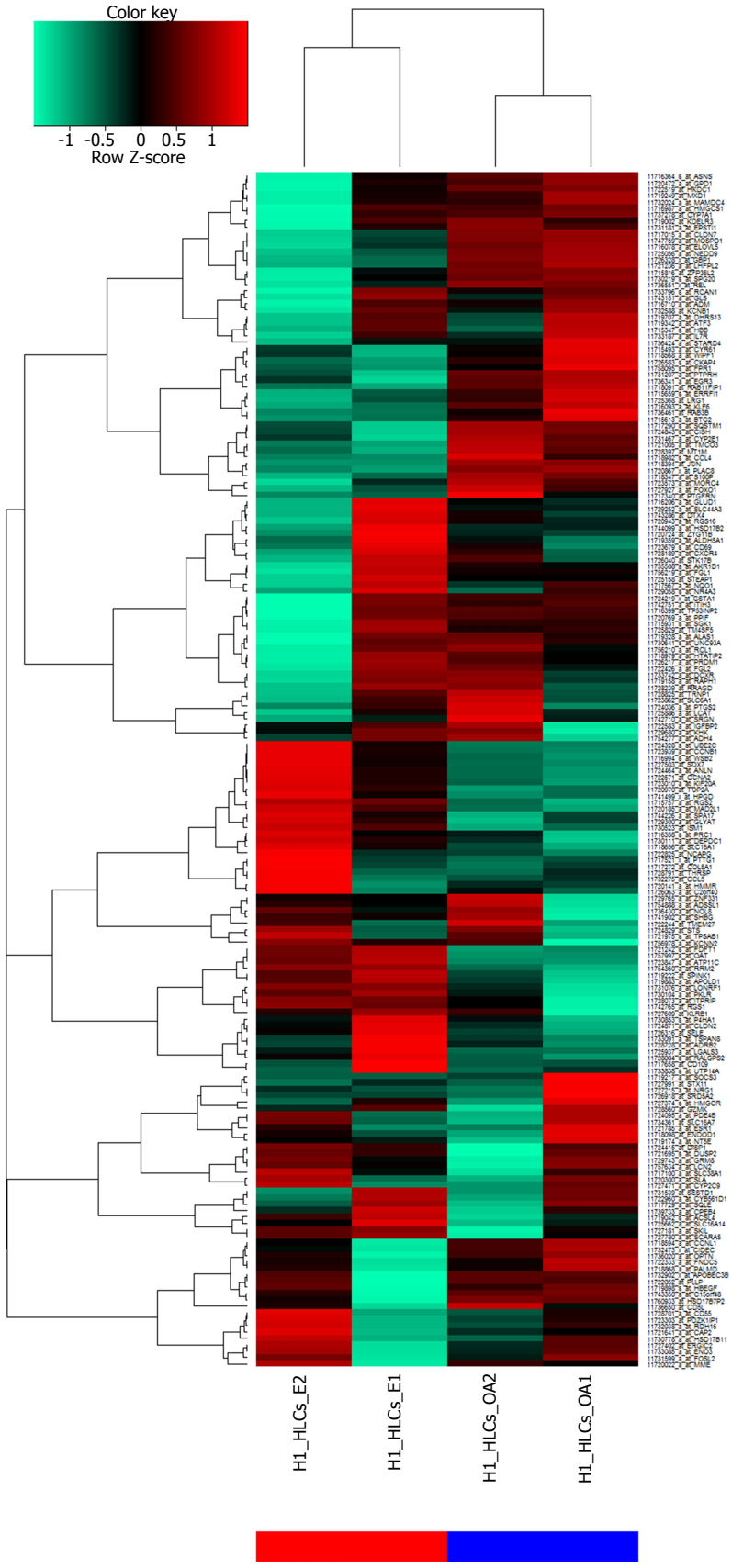


Figure 5 The pluripotent stem cell models of alcoholic liver disease and non-alcoholic fatty liver disease reflect the characteristics of the biopsy-derived gene signature. The gene signature condensed from the meta-analysis of multiple ALD and NAFLD gene expression datasets was applied to the steatosis-model by (Graffmann *et al*[40]) where pluripotent-stem-cell-derived hepatocyte-like cells (HLCs) were challenged with ethanol (E) and oleic acid (OA). The cluster analysis shows a clear separation into the ethanol model (red bar) and the oleic acid model (blue bar). ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

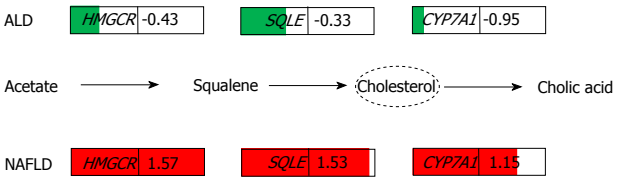


Figure 6 Rate-limiting genes of cholesterol metabolism are down-regulated in alcoholic liver disease and up-regulated in non-alcoholic fatty liver disease. This schematic figure shows the log2-ratios of *HMGCR*, *SQLE* and *CYP7A1* indicating down-regulation in ALD (green) and up-regulation in NAFLD (red). There was stronger down-regulation of *CYP7A1* (log2-ratio = -0.95) than of the upstream cholesterol genes *HMGCR* (log2-ratio = -0.429) and *SQLE* (log2-ratio = -0.33) in ALD while in NAFLD, *CYP7A1* (log2-ratio = 1.15) was weaker up-regulated than *HMGCR* (log2-ratio = 1.57) and *SQLE* (log2-ratio = 1.53). The size of the arrows points to a disequilibrium between cholesterol production and secretion into the bile *via* CYP7A1 in both diseases despite the opposite regulation in ALD and NAFLD. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

were down-regulated in ALD and up-regulated in NAFLD (each compared *vs* healthy control). However, we found in both cases that the gene encoding CYP7A1 - the enzyme responsible for cholesterol removal by catalysing the conversion of cholesterol to bile acids was regulated at a lower level than the genes encoding for the cholesterol synthesis determining enzymes *HMGCR* and *SQLE*. This would explain cholesterol accumulation in the liver because more cholesterol is produced than secreted into bile - regardless if the cholesterol processes are down-regulated in total (in ALD) or up-regulated (in NAFLD). Moreover, the strong down-regulation of *CYP7A1* in ALD might be a clue for the higher risk of cholestasis in ALD than in NAFLD[41]. Briefly, these findings emphasize the importance of cholesterol efflux from the liver *via* CYP7A1 and may suggest that the cause of the disease might be that the rate of cholesterol efflux is too low. Negative feedback loops down-regulating CYP7A1 by bile acids have already been described[42]: Bile acids can down-regulate CYP7A1 *via* (1) FXR and SHP; or (2) by interaction with liver macrophages (Kupffer cells) whose role in fibrosis has been established as they produce cytokines such as transforming growth factor beta leading to the transformation of stellate cells into myofibroblasts[43]. Furthermore, Kupffer cells secrete cytokines, *e.g.*, tumor necrosis factor (TNFα) and interleukin (IL-1β) which in turn induce protein kinase, c-Jun N-terminal kinase and thus inhibit hepatocyte nuclear factor and consequently CYP7A1[44,45]. This gives rise to the question if the lower CYP7A1 levels are a cause of steatosis or are a consequence of the profibrotic stage. Here, systems biology modelling of cholesterol fluxes in the liver including bile acids and regulatory mechanisms of CYP7A1 could be useful in determining under which condition efflux rates are too low.

Beside the differences in cholesterol processes we could also confirm effects which had been much disputed before such as the ethanol-mediated down-regulation of glycolysis and of alcohol and aldehyde dehydrogenases.

The common up-regulated pathways might provide synergies for research into ALD and NAFLD. We found similar mechanisms underlying the progression of both diseases and could identify the common up-regulated ECM-receptor interactions and also associated collagen encoding genes *COL1A1* and *COL3A1* which indicate development of fibrotic tissue.

Finally, we provide a comprehensive compendium displaying comparative regulation of all KEGG pathways in ALD *vs* NAFLD which may serve as an encyclopaedic tool to lookup regulation of dedicated pathways associated with ALD and NAFLD.

In the current study we performed a meta-analysis of gene expression data of liver-derived biopsies from ALD and NAFLD patients, and report a gene signature which clearly separates the transcriptomes of ALD and NAFLD derived liver biopsies. Furthermore, we uncovered predominating commonalities between both diseases at the level of biological pathways, *e.g.*, common down-regulation of the Fatty acid degradation pathway and common up-regulation of the ECM-receptor interaction pathway which may explain common progression of both diseases by cytokines being exchanged between hepatocytes, Kupffer cells and stellate cells at the fibrosis stage. This is confirmed by the common expression of *COL1A1* and *COL3A1* which are associated with fibrotic tissue.

Interestingly, we found rate-limiting genes of cholesterol processes such as *HMGCR*, *SQLE* and *CYP7A1* adversely regulated (Figure 6) between ALD (down-regulated) and NAFLD (up-regulated). The fact that both diseases have the same phenotype may be due to a lower level of the enzyme CYP7A1 compared to the cholesterol synthesis enzymes HMGCR and SQLE. Thus, it will be interesting to further investigate CYP7A1-mediated cholesterol secretion into bile - possibly by systems biology modeling of cholesterol fluxes in the liver. For future therapy, drugs able to adjust CYP7A1 to levels amenable with cholesterol synthesized in or transported to the liver will be useful.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) are highly prevalent liver diseases and in an increasing number of developed countries NAFLD is becoming the most common cause of liver disease. Although NAFLD and ALD have distinct etiologies the manifestation and the potential progression of both diseases to hepatitis, cirrhosis and cancer is similar.

Research frontiers

A two-hit hypothesis is the established explanation for disease progression to alcoholic hepatitis (AH) and non-alcoholic steatohepatitis (NASH). After steatotic fat accumulation due to metabolic disorders such as insulin resistance (NAFLD) or due to alcohol (ALD) oxidative stress and dysregulation of cytokines initiate inflammation and hence the progression to NASH as well as AH.

Innovations and breakthroughs

The authors found that rate-limiting enzymes of cholesterol metabolism such as HMGCR, SQLE and CYP7A1 are down-regulated in ALD and up-regulated in

NAFLD compared to a healthy control. However, in ALD and NAFLD CYP7A1 - associated with conversion of cholesterol into bile acids - is regulated at a lower level than HMGCR and SQLE. That might explain the accumulation of cholesterol by the reduced efflux into bile acids.

Applications

CYP7A1 is a potential drug target and the proposed gene signature distinguishing ALD from NAFLD consists of biomarkers which may be exploited for diagnostic tests. The compendium of KEGG pathway regulation in ALD and NAFLD and the finding of the adverse regulation of cholesterol metabolism in ALD and NAFLD are promising start points for future research.

Terminology

NAFLD is the disease related to fat accumulation (steatosis) in the liver in the absence of alcohol abuse (usually the threshold is set at 30 g/d of alcohol for men and 20 g/d for women). It ranges from the relatively benign steatosis to NASH, cirrhosis and hepatocellular carcinoma.

Peer-review

This manuscript was informative. The authors found commonalities between both ALD and NAFLD at the level of biological pathways implying some mechanistic similarity between both diseases.

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