Diploma Thesis

Morphometric analysis of hepatic steatosis and fibrosis in patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

submitted by

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Graz, 5th August 2015

AFFIDAVIT (EIDESSTATTLICHE ERKLÄRUNG)

I hereby declare that the following diploma thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, 5. August 2015 Lukas Peter Binder, eh.

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ABSTRACT

Morphometric analysis of hepatic steatosis and fibrosis in patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

BACKGROUND & AIMS: NAFLD (non-alcoholic fatty liver disease) has the potential to progress to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis which paves the way for the development of hepatocellular carcinoma. Over the last decades the prevalence of NAFLD has reached an epidemic dimension and therefore is to be considered a major problem of public health. Several formal scoring systems have been developed for the semiquantitative evaluation of the histological activity (grade) and degree of fibrosis (stage), however, as has been shown by a number of studies these scoring systems are prone to inter- as well as intra-observer bias, which might be improved by the use of computer assisted digital image analysis.

METHODS: Liver samples from biopsies of 41 patients with non-cirrhotic NASH (recruited from the University of Virginia liver clinic) were analysed using Definiens™ Tissue Studio V3.51to assess their levels of hepatic fibrosis and hepatocellular steatosis. Results were compared with semiquantitative histological scoring systems as well as magnetic resonance imaging (MRI).

RESULTS: In hepatocellular steatosis detection, digital image analysis showed better correlation ($r = 0{,}794$; $p < 0{,}001$) with the gold standard method (Dixon modificated MRI measurement), than semiquantitative histological scoring ($\rho = 0{,}704$; $p \leq 0{,}001$). The detection of hepatic fibrosis (which was much harder to perform), only showed significant correlation to the semiquantitative histological scoring results of one of the two observers ($\rho = 0{,}517$; $p \leq 0{,}01$).

CONCLUSIONS: Digital image analysis is a useful tool to assess hepatocellular steatosis as well as hepatic fibrosis in liver specimen. It is able to detect minimal changes in histologic features very precisely. Still, this method is currently still unable to provide important information like the diseases‘ activity, which clearly suggests that digital image analysis can only assist, but never replace the well-trained eye of a histopathologist.

ZUSAMMENFASSUNG

Morphometrische Analyse von Lebersteatose und –fibrose bei Patientinnen und Patienten mit nicht-alkoholischer Fettleber-Erkrankung und nicht-alkoholischer Steatohepatitis

ZIELE & HINTERGRUND: Nicht-alkoholische Fettlebererkrankung (NAFLD) kann zu nicht-alkoholischer Steatohepatitis (NASH), Leberfibrose und Zirrhose führen, welche ihrerseits Risikofaktoren für Leberzellkarzinome darstellen. Vor allem in den letzten Jahrzehnten entwickelte sich NAFLD zu einer weitverbreiteten Volkskrankheit. Die bisher entwickelten histopathologischen Scoringsysteme unterliegen zum Teil großen intra- als auch inter-observer Variationen, welche durch das Hinzuziehen computergestützter Analyseverfahren verringert werden könnten.

METHODEN: Leberbiopsien von 41 Patienten mit nicht-zirrhotischer NASH (rekrutiert durch die Leberklinik der University of Virginia) wurden mithilfe von Definiens™ Tissue Studio V3.51 digital analysiert, um deren Fettgehalt, als auch den Grad ihrer Fibrosierung zu messen. Die Ergebnisse der digitalen Bildanalyse wurden sowohl mit histologischen Scoringsystemen, als auch mit Magnetresonanz-Messmethoden verglichen.

ERGEBNISSE: Für die Lebersteatose konnte die digitale Bildanalyse eine bessere Korrelation ($r = 0{,}794$; $p < 0{,}001$) zum Goldstandard (Magnetresonanz-Modifikation nach Dixon) erreichen, als das semiquantitative, histologische Scoring ($\rho = 0{,}704$; $p \leq 0{,}001$). Für die Fibrose hingegen konnte nur mit einem der beiden Histologen, welche die semiquantitative Befundung durchgeführt hatten, eine Übereinstimmung im Sinne einer signifikanten Korrelation ($\rho = 0{,}517$; $p \leq 0{,}01$) gefunden werden.

SCHLUSSFOLGERUNGEN: Die digitale Bildanalyse vermag präzise Aussagen über die Menge an steatotisch veränderten Leberzellen und Fibrosegewebe innerhalb eines Biopsiezylinders zu treffen und unterliegt augenscheinlich eher geringen intra-observer Variationen. Allerdings ist es bisher nicht möglich, mit dieser Methode weitere wichtige Informationen wie die Aktivität der Erkrankung zu messen, weshalb sie die Arbeit eines geschulten Histopathologen niemals zu ersetzen, allenfalls jedoch zu bereichern imstande sein kann.

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1. INTRODUCTION

1.1. Non-alcoholic fatty liver disease

1.1.1. Definition

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most common cause of chronic liver disease in industrialized countries over the last decades. Its prevalence has reached an epidemic dimension of reportedly 20-30% in the adult population and therefore this disease is considered to be a major problem of public health.(1) Additionally, NAFLD is emerging as an important liver disease in children, because of the rising prevalence of childhood obesity worldwide.(2)

Fatty liver disease (FLD) is strongly linked to increased cardiovascular risk.(3) FLD represents one of the most common causes of elevated liver enzymes and is closely linked to insulin resistance and the development of diabetes and the metabolic syndrome.(4)

NAFLD comprises a spectrum of liver diseases, defined by histology, ranging from non-alcoholic fatty liver to non-alcoholic steatohepatitis (NASH) and NASH-associated cirrhosis which paves the way to hepatocellular carcinoma. NASH is considered the progressive lesion of NAFLD, associated with inflammation and the development of fibrosis (see below) (1,5)

1.1.2. Histopathological findings

Although a number of non-invasive tests for diagnosing features of NAFLD, (steatosis, NASH and cirrhosis) have recently emerged, histological assessment of liver tissue has to be considered as the reference method providing at the same time the diagnosis of NAFLD type, assessment of the disease's severity and additional risk factors like excessive iron storage as well as evidence or exclusion of additional liver diseases.(6)

Steatosis, a hallmark of NAFLD, is histologically characterized by the accumulation of triglycerides within hepatocytes which exceeds 5% of the parenchymal area(7). Steatosis, termed after the ancient Greek word for fat, "stear", is typically present in the perivenular, acinar zone 3 location of the hepatic lobules but may - in severe cases - comprise most of the lobular parenchyma or the entire acinus.(8) The hepatocellular steatosis in NAFLD is usually macrovesicular (figure 1), with either large, intracellular triglyceride droplets, displacing the

nucleus to the cell periphery, or of a mixed type including larger and smaller droplets (figure 2). (9) Rarely, macrovesicular steatosis can be accompanied by microvesicular steatosis.(8)

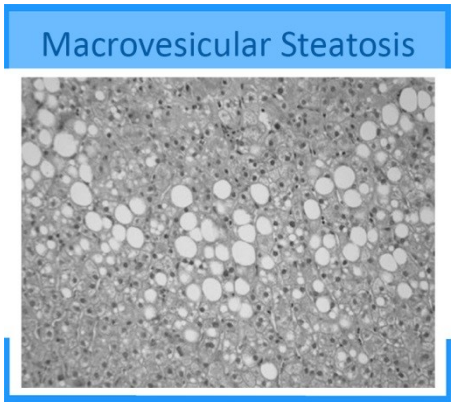


Figure 1 NAFLD: macrovesicular steatosis (haematoxylin and eosin, ×100).

The semiquantitative histological assessment of steatosis is usually executed based on routine stains.

Non-alcoholic steatohepatitis (NASH) is the progressive form of NAFLD. The diagnosis of NASH in adults is defined by hepatocellular steatosis, signs of hepatocellular injury in the form of hepatocellular ballooning (see figure 2) and lobular inflammation on histology.(10)

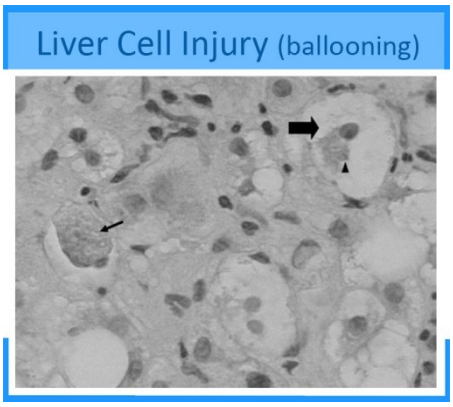


Figure 2 NASH: liver cell injury (ballooning; thick arrow) and apoptotic body (thin arrow). The ballooned hepatocyte contains a Mallory–Denk body (MDB; arrowhead) (haematoxylin and eosin, ×400).

Injured (ballooned) hepatocytes, usually located among steatotic ones, show an enlarged shape with rarefied cytoplasm, that may contain Mallory-Denk bodies (MDB).(8)The usually mild lobular inflammatory infiltrate consists of mononuclear cells and eventually admixed neutrophilic granulocytes. Typically, the key lesions of NASH (steatosis, lobular inflammation and hepatocellular ballooning) are accentuated in the central areas of the hepatic lobules.

Hepatic fibrosis occurs as a consequence of steatohepatitis and marks disease progression.

Histologically, fibrosis represents excess extracellular matrix, rich in fibril-forming collagens. Fibrosis may be seen as a result of a wound healing process and scarring of the liver resulting from the activation of a variety of cell types, most importantly stellate cells and Kupffer cells.(11,12)

Liver parenchyma tends to react with the activation of mechanisms of fibrogenesis to acute injury as well, but only severe, chronic disruptive factors are able to achieve the accumulation of significant fibrosis.(13,14)

Early in the progression of NASH deposition of extracellular matrix in the vicinity of hepatocytes in the centrolobular areas in response to sonic hedgehog signalling leads to the so-called "chicken wire" pattern or pericellular type of fibrosis, which is quite typical for fatty liver diseases.(14,15) During the diseases progression, portal and periportal fibrosis develops, followed in some cases by bridging (either central-portal or portal-portal) fibrosis and finally cirrhosis,(16) as displayed in figure 3.

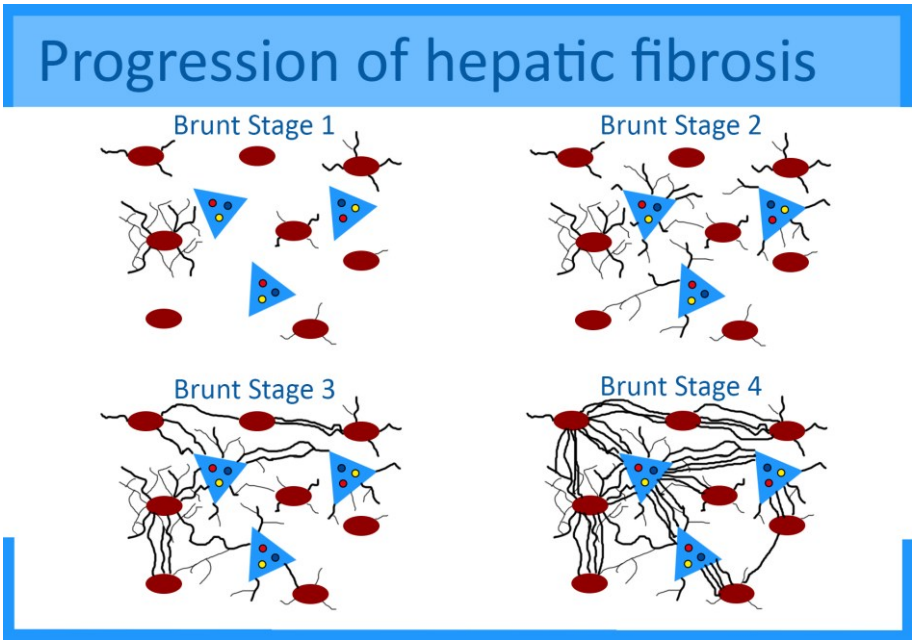


Figure 3 **Typical pattern of fibrosis progression due to affection in NAFLD (according to Brunt's fibrosis stages: 1 –** zone 3 perisinusoidal fibrosis**; 2 –** as stage 1, with portal fibrosis**; 3 –** as stage 2, with bridging fibrosis**; 4 –** cirrhosis**) (16)**

1.1.3. Diagnosis of NAFLD.(17)

The clinical diagnosis of NAFLD requires the evidence of hepatic steatosis (either histological or by imaging) and the absence of any causes of secondary fat accumulation in the liver, in particular excessive alcohol consumption (16). Other causes of hepatic steatosis are compiled in table 1:

Common Causes of Secondary Hepatic Steatosis

- Macrovascular steatosis

- Excessive alcohol consumption
- Hepatitis C (genotype 3)
- Wilson's disease
- Lipodystrophy
- Starvation
- Parenteral nutrition
- Abetalipoproteinemia
- Medications (e.g. amiodarone, methotrexate, tamoxifen, corticosteroids)

- Microvascular steatosis

- Reye's syndrome
- Medications (e.g. valproate, anti-retroviral drugs)
- Acute fatty liver of pregnancy
- HELLP Syndrome
- Inborn errors of metabolism (e.g. LCAT deficiency, cholesterol ester storage diseases, Wolman disease)

Table 1 Causes for secondary hepatic steatosis. (10)

NAFLD Types

NAFLD is clinically divided into two different subtypes: Non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NAFL shows hepatic steatosis without presence of notable signs of inflammation (in the form of ballooning of hepatocytes) or fibrosis, whereas in NASH, as mentioned above, hepatic steatosis occurs together with hepatic inflammation and signs of hepatocyte injury (ballooning), with or without fibrosis (see table 2). Histologically, NASH may be indistinguishable from alcoholic steatohepatitis (ASH).(18,19)

NAFLD and related definitions	
NAFLD	Covers the whole spectrum of fatty liver diseases without significant alcohol consumption.
NAFL	Hepatic steatosis, but no evidence of hepatocellular injury - minimal chance of progression to cirrhosis.
NASH	Presence of hepatic steatosis and inflammation with hepatocellular injury. Can progress to cirrhosis.
cryptogenic fibrosis	Presence of fibrosis without signs of any etiology. These patients may show many metabolic risk factors such as obesity and metabolic syndrome.

Table 2 **Definitions of NAFLD and related entities.**

1.1.4. Pathogenesis and risk factors

The exact pathway of pathogenesis in NAFLD is still not well defined. One of the most important factors contributing to pathogenesis is insulin resistance contributing to the development of hepatic steatosis and NASH. Other possible pathogenic factors include oxidative stressors like excess storage of iron in hepatocytes (20), decreased leptin levels (21) or intestinal bacteria overgrowth(22).

T**riglyceride accumulation** within hepatocytes resulting in steatosis may arise from different mechanisms:

- Excessive influx of free fatty acids (FFA) from adipose tissue, either caused by rapid weight loss, overnutrition and insulin resistance.
- Decreased hepatic export of FFA due to impaired VLDL secretion or synthesis, caused by protein malnutrition(23), drug toxicity (as seen with tetracycline and amiodarone(24) or several assimilation deficiencies.
- Impaired beta-oxidation of FFA (to ATP) in the course of for example excessive alcohol consumption, vitamin B5- (pantothenic acid) or coencyme A deficiency.

Insulin resistance is associated with obesity and type 2 diabetes(25). All of these conditions are frequently observed amongst patients with NAFLD.(18) Still a number of patients with NAFLD or NASH shows insulin resistance even without obesity and with physiological glucose tolerance levels.(26) The fact that NASH can occur without insulin resistance suggests that there may also be other yet poorly defined mechanisms responsible and that NASH is a heterogeneous syndrome of diverse aetiology(27,28).

In a clinical study from 2009, *C. Argo et al.* studied 187 patients with NAFL or NASH using paired biopsies with a mean interval of 4.2 years. They found, that patients with hepatic inflammation in the first biopsy were at increased risk to develop advanced fibrosis (49%) compared to those without inflammation at baseline (17%). *Argo et al.* identified inflammation and age as independent risk factors for the development of advanced fibrosis. Along with these independent risk factors, a wide range of other contributory factors associated with the development and progression of NAFLD has been identified over the last decades: Diabetes mellitus(29), elevated serum aminotransferases(29,30), obesity (body mass index >28 kg/m²)(30) and high visceral adiposity index (which includes BMI, waist circumference, HDL- and triglyceride-levels)(31) were among the most important factors found.

On the contrary, coffee consumption was associated with lower risk of disease progression.(32)

1.1.5. Epidemiology

The **incidence** of NAFLD varies depending on the population studied (33) but may still be underreported in some populations due to the difficulty to diagnose the condition with certainty using non-invasive methods.(4)

In 2005 two Japanese studies reported on the incidence and risk factors of NAFLD. Hamaguchi et al. studied routine health care reports of Japanese government employees and found an overall incidence of non-alcoholic hypertransaminasemia of 31 cases per 1000 person-years,(34). Suzuki et al. observed 3147 persons without NAFLD at baseline and found the development of signs of NAFLD in 308 (~10%) of these cases over an observation period of 14 months.(35)

In contrast, more recent European studies show far lower rates, like 29 cases per 100.000 person-years,(33) The findings about the global incidence of NAFLD are summarized in Figure 4.

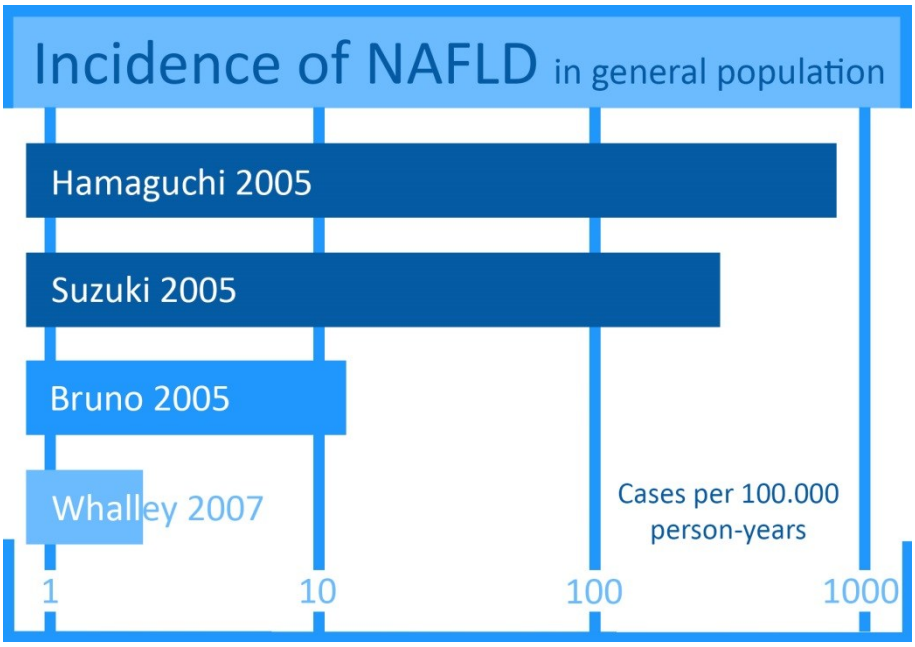


Figure 4 Comparison of 4 clinical studies on the incidence of NAFLD. (36)

The **prevalence** of NAFLD and NASH has been examined in many studies all over the world. In the US, where one third of the population is considered obese, biopsy series estimate the prevalence of NASH between 3% and 5%, while the prevalence of NAFLD is likely to be around 30% according to the number of obese people in the country.

Studies from other countries all over the world show prevalence rates from 6% to 35%, with a median of 20%.(4) Still it needs to be considered, that these studies used different diagnostic tools to diagnose NAFLD: Few studies used the current gold standard – liver biopsy, which, however as an invasive procedure associated with a small but not negligible risk of mortality and morbidity(37) cannot be used for population-based studies. However, this tool was used for cohorts of liver donor patients, who were considered to be in good health: A US study revealed, that a number of 20% of the examined individuals were ineligible for a donation, because their hepatic steatosis level was over 30%.(38)

Other studies used tools such as autopsy, or non-invasive methods like imaging via MRI, ultrasonography or scores based on liver enzymes. Although these non-invasive tools allow us to draw some interesting conclusions, because they give a population-wide overview, their results usually provide less definitive and less accurate information than the histology-based evaluations.

1.1.6. Mortality

Although cardiovascular diseases are the predominant cause of death in patients with NAFLD, patients with NASH are at increased risk for liver-related death compared with patients without NASH.(39)

NAFLD patients seem also to have an increased overall mortality compared to the general population, however the results are contradictory. Some studies found an increase in mortality. However, a large study to date including 11371 US adults found no association between NAFLD and increased mortality.(40)

1.1.7. Distinction between NAFLD and NASH

The best way to distinguish NAFLD from NASH is – equal to the one to diagnose one of the diseases – histological examination. For reasons of standardized reporting and for use in clinical studies, the **NAFLD activity score (NAS)** was introduced by the *Pathology Committee of the NASH Clinical Research Network* (CRN) in 2005. The NAS is a scoring system, that can be used for grading (i.e, assessing severity of inflammation and hepatocellular damage) of NAFLD. Three key morphological features of NAFL/NASH are assessed semiquantitatively by the application of numerical scores: steatosis (score 0-3), lobular inflammation (0-3) and hepatocellular ballooning (0-2). The NAS represents the unweighed sum of the scores and can have a range from 0 to 8. A diagnosis of NASH is made if the NAS exceeds 5, while scores from 3-4 indicate so called " borderline NAS" and scores below 3 are considered consistent with the diagnosis of "no NASH". The CRN also issued a scoring system for the assessment of fibrosis ranging from 0 (no fibrosis) to 4 (cirrhosis) .(36)

In contrast to the NAS the recently introduced Steatosis (S) Activity (A) Fibrosis (F) scoring system focuses on the definitive categorization of NAFLD into NAFL and NASH (41). (Example in figure 5)

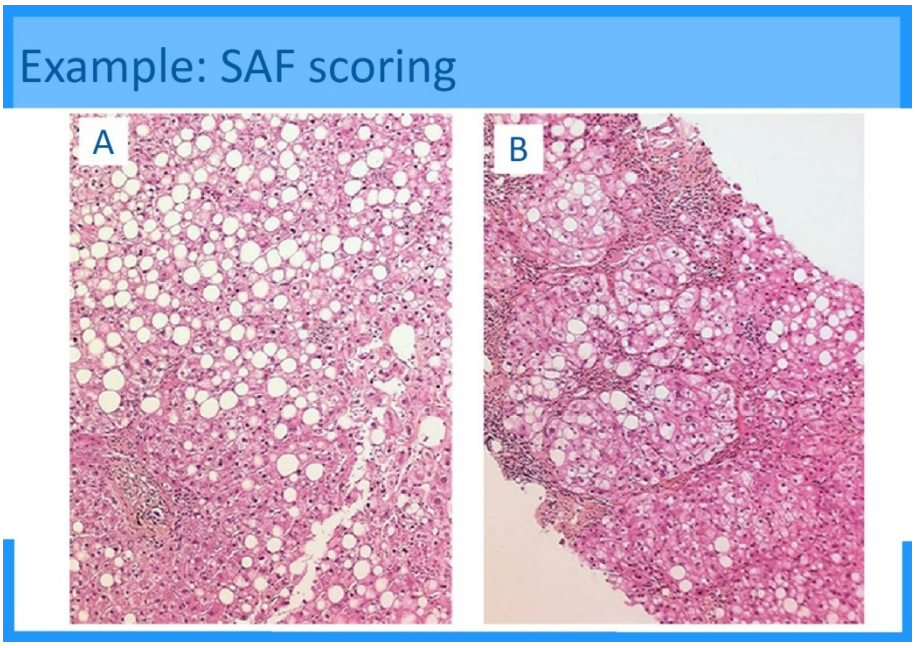


Figure 5 Liver biopsies of obese patients. (A) SAF score $S_3A_2F_1$; marked steatosis, moderate activity, and mild fibrosis. (B) SAF score $S_1A_3F_4$; cirrhosis with severe activity and mild steatosis. (40)

1.2. From fibrosis to cirrhosis

1.2.1. Hepatic fibrosis

Hepatic fibrosis is a process in liver parenchyma that marks the reaction to several sources of irritation that have the potential to affect liver tissue. It can be seen as a reversible wound-healing reaction due to liver-cell impairments of all kinds, a balance between parenchymal repair and scar formation.(13) The process of development of hepatic fibrosis is similar to scarring in other tissues like skin or kidneys, as it involves very similar cell types and mediators. (42) The predominant location of fibrosis-appearance is always the region of injury. For example, patients, suffering from alcohol-induced liver diseases, show pericentral injury already in earlier stages of the disease.

Besides FLD, other chronic liver diseases have the potential to lead to hepatic fibrosis as well: Viral hepatitis, biliary diseases and metabolic disorders should be mentioned in this context.(13)

Fibrosis of liver parenchyma usually shows a slow progression and in most cases it takes a long time from the first appearance of histologically significant fibrosis to the development of severe cirrhosis. It generally evolves over many years up to decades.(5,43) Exceptions are typically hepatitis C infections after liver transplantations,(44) co-infections of human immunodeficiency virus (HIV) and hepatitis C(45) and serious drug injury. In these cases, fibrosis may be rapidly progressive over weeks or months.

1.2.2. Recent research on hepatic fibrosis

The main effectors of fibrosis are hepatic stellate cells (HSC; also known as perisinusoidal cells or „*Ito*“ cells) and portal fibroblasts. Hepatic stellate cells are resident perisinusoidal cells in the subendothelial space between hepatocytes and sinusoidal endothelial cells.(5) Especially the activation of resident hepatic stellate cells into proliferative, contractile and fibrogenic so-called „activated stellate cells“ is considered one of the most important players mediating the fibrogenic process.(46) Hepatic resident cells (mainly Kupffer cells) and infiltrating inflammatory cells produce mediators during chronic hepatic diseases, that activate the stellate cells.

Inflammatory processes and several types of hepatocellular injury are linked to the production of reactive oxygen species (ROS). (47) Derived from hepatocytes, macrophages, stellate cells and inflammatory cells throughout lipid peroxidation, ROS mediate the classical pathway of cytochrome P450 2E1 induction in hepatocytes (this pathway is relevant for the pathogenesis of many acute and chronic liver diseases and also in alcoholic steatohepatitis (ASH) and NASH(48,49)). In particular, ethanol, polyunsaturated fatty acids and iron showed an enhancing effect on the release of ROS.(50)

Recent research also shows links to other pathomechanisms that are likely to be connected to the development of hepatic fibrosis. The reduction of nicotinamide adenine dinucleotide phosphate oxidase or the induction of nitric oxide synthase 2 throughout mitochondrial injury in hepatocytes(51,52) are subject of recent studies and should therefore be mentioned in this context.

In addition, hepatocellular apoptosis emerged as a very important inflammatory stimulus,(53) that can activate stellate cells which have been shown to take up apoptotic hepatocytes thereby reducing nicotinamide adenine dinucleotide phosphate oxidase.(11)

Based on these findings, new antifibrotic strategies are being developed that counteract hepatocyte-apoptosis.(54) Another potentially helpful strategy could be the induction of selective apoptosis in stellate cells rather than hepatocytes (mediated for example by proteasome inhibitors).(12)

In contrast to apoptosis, (oncotic) necrosis is no more considered another important contributory factor to hepatic fibrosis.(55) While ten years ago, hepatic fibrosis was considered a progressive activity in liver parenchyma, that could at best be detained at its current state, but would never be able to be taken into regression of some kind, it is now seen in a more comprehensive way. Hepatic fibrosis may increase, remain constant or even regress over time, depending on the severity and persistence (or non-persistence) of liver damage, that results in fibrogenesis on the one hand and the efficiency of hepatic repair mechanisms and medical treatment, which both enhance intrahepatic fibrinolysis on the other.(13)

Clinical studies already showed partial or even complete dissolution of hepatic fibrosis in numerous cases of chronic liver diseases, such as seen in the following examples:

- chronic hepatitis B – following lamivudine therapy(56)
- chronic hepatitis C – following eradication of HCV(57)
- autoimmune hepatitis after immune-suppression(58)
- non-alcoholic steatohepatitis – following massive weight loss(59)
- hemochromatosis after excess iron removal(60)
- alcoholic steatohepatitis – following alcohol abstinence (61)

1.2.3. Cirrhosis

1.2.3.1. Development of cirrhosis research

Cirrhosis is defined as the presence of parenchymal nodules surrounded by fibrous septa. Depending on the etiology of liver disease the nodules and septa may vary in size. (1)

The diversity of cirrhosis is related to the different possible etiologies and the duration of the hepatic injury. These different morphological expressions of hepatic cirrhosis also show clinical manifestations of variable severity, ranging from impaired liver function, portal hypertension or hepatic encephalopathy, to liver failure. (43)

Cirrhosis used to be seen as the endstage of fibrosis-development, as an irreversible state of the destruction of the lobular architecture of the liver. However, recent results indicate that cirrhosis may be a more dynamic and still treatable process of diverse etiology with the potential to regress.(13,43)

1.2.3.2. Complications of cirrhosis

Albeit the potential of curability in cirrhosis has changed, there are still many possible complications that definitely have to be considered during cirrhosis progression (table 3).

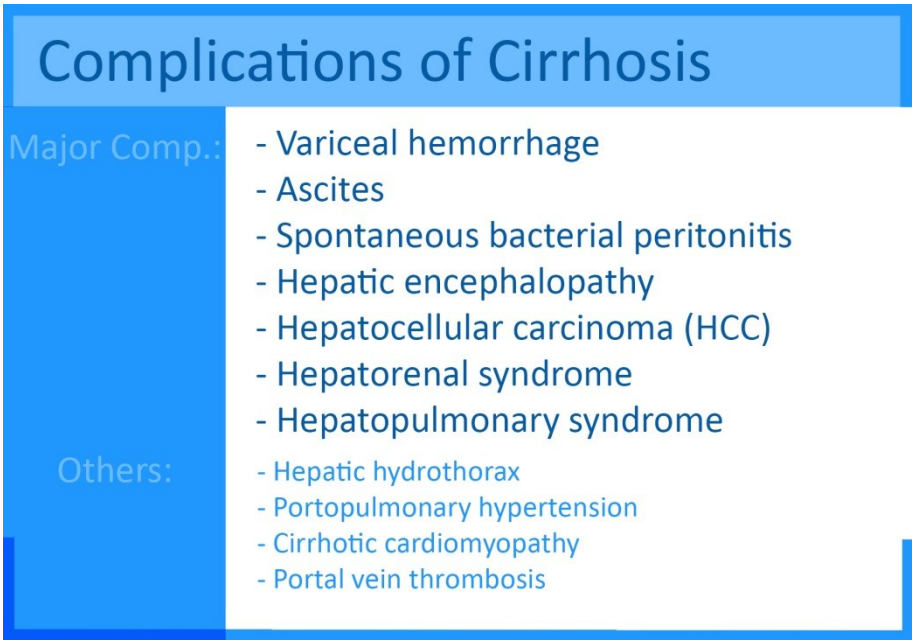


Table 3 Common complications that occur in patients with hepatic cirrhosis.(8)

As soon as one of these complications develops, patients are considered to suffer from decompensated cirrhosis. Risk factors for the disease to steer a such course include obesity, infections, bleeding, alcohol consumption, dehydration and constipation.(62–64) Amongst these, obesity has even been found out to be an independent risk factor, associated with a very aggressive course of cirrhosis development.(65)

1.3. Treatment of NAFLD

After decades of intensive investigation, weight loss combined with increased physical activity is the only therapy for patients with NAFLD, that has shown reasonable positive effects as well as sufficient safety for the patient throughout treatment.(66,67)

In addition, optimization of blood sugar control for patients with diabetes and control of hyperlipidaemia are part of the treatment in order to minimize cardiovascular risk factors. In that context, statin therapy was proven to be a safe way of cardiovascular event prevention for patients with NAFLD.(68)

Insulin-sensitizing agents are likely to lessen the contribution of insulin resistance to the development of NAFLD. While thiazolidinediones like pioglitazone have shown histologic improvements in patients with NASH, metformin has not.(69) Still, thiazolidinediones are not routinely used in NAFLD management because they are associated with significant side effects like weight gain, painful swollen legs and even heart failure.(70,71)

The treatment of both patients with hepatic fibrosis and also those, who already have developed cirrhosis focuses on slowing down or even reversing the progression of the liver disease itself.

In patients with cirrhosis, the prevention of superimposed insults to the liver and generally of the complications of cirrhosis (see table 3) is the main goal of treatment.

2. OBJECTIVES

Recently, computer assisted digital image analysis has become commercially available and is accessible at the Institute of Pathology of the Medical University of Graz. The idea of using computer assisted technologies is, that intra- as well as inter-observer variations can be minimized: While even the most experienced histopathologist may not be able to reproduce the exact same estimations of steatosis or fibrotic tissue area within a series of liver samples, a computer arguably can do this – in theory and under perfect conditions.

In addition, a standardized, automated procedure of steatosis and fibrosis quantification could eliminate inter-observer variations and therefore make results from different centres more comparable, providing that the same technology is used.

The objectives of our study are to investigate the applicability of the Definiens™ image analysis software (Definiens AG, Munich) for the quantification of hepatocellular steatosis and hepatic fibrosis (morphometrical evaluation) in histological sections of patients with NAFLD and to compare the results with conventional, semiquantitative histological scoring and radiological fat estimate (Dixon modification of magnetic resonance imaging) and to evaluate the utility of this software in a clinical study of NAFLD.

3. MATERIALS & METHODS

3.1. Patients

The patients' information for this examination derives from a study on NASH patients that intended to assess the hepatic and systemic metabolic effects of supplementing N-3 fatty acids versus placebo.

Eligible patients were recruited from the University of Virginia liver clinic. All of the subjects underwent conventional laboratory evaluation to exclude viral hepatitis, autoimmune or cholestatic liver disease, hemochromatosis, and Wilson disease. Initial diagnostic biopsy was accepted as the study entry biopsy provided it was performed within 6 months of enrolment without interval change in clinical or therapeutic interventions. For study entry, the biopsy was required to show steatohepatitis, defined as steatosis with inflammation, hepatocellular ballooning and/or fibrosis(7,72). Subjects were required to have ethanol consumption less than 30 g/day for males or 20 g/day for females. Subjects diagnosed with cirrhosis or secondary forms of steatohepatitis (i.e. methotrexate, tamoxifen) or those treated with thiazolidinediones were excluded.(73)

During this study, patients were each going through a liver biopsy at baseline and at the end of the study.

3.2. Histological assessment

Blinded histological assessment was performed separately by two experienced observers: one hepatologist (SC) and one pathologist (CL), both of whom have previously published on histological aspects of NASH.(74,75) Biopsy samples (cores with mean length of 1.9 ± 0.6 cm) were deemed adequate for diagnosis in all cases. The samples were independently and blindly scored to calculate the NAS (NASH Activity Score, 0-8) using H&E stained slides according to the NASH CRN score for grade of steatosis (0-3), inflammation (0-3) and hepatocellular ballooning (0-2)(36), staged for fibrosis using Masson trichrome according to the Brunt fibrosis stages (0-4, as given in table 4)(16) by one observer (SC) and using the CRN-Score for fibrosis (0-4, as given in table 5)(36) by the other (CL).

Brunt scorig system for hepatic fibrosis staging

Stage	zone 3 peri-sinusoidal fibrosis	periportal fibrosis	bridging fibrosis	cirrhosis
1	focal or extensive	0	0	0
2	focal or extensive	focal or extensive	0	0
3	+ or -	+ or -	+	0
4	+ or -	+ or -	extensive	+

Table 4 The scoring system used by one of the two histopathological observers (SC) for the staging of hepatic fibrosis.

CRN scorig system for hepatic fibrosis staging

Stage	Definition
0	none
1A	Mild, zone 3, perisinusoidal fibrosis
1B	Moderate, zone 3, perisinusoidal f.
1C	Portal / periportal fibrosis
2	Perisinusoidal and portal/periportal f.
3	Bridging fibrosis
4	Cirrhosis

Table 5 Fibrosis scoring system by the NASH Clinical Research Network

Recognizing that inter- and intra-observer variability in scoring histological activity in NASH is well-documented and an inherent limitation,(16,76–78) the authors elected to average the scores in the analysis for the histological parameters with the intention of capturing a range of disease activity and to reflect common variation in histological interpretation especially relevant to hepatocellular ballooning.(79)

3.3. Digital image analysis

3.3.1. Digital image analysis in general

Digital image analysis, also known as “morphometry”, a tool, that previously has been used in liver research mainly according to HBV- and HCV-studies(80–82), can be seen as an improvement, that allows even more precise examination of liver specimens gained from biopsies or autopsies and therefore offer highly accurate results according to hepatic steatosis- and fibrosis-levels.

The technique of digital image analysis has been known for decades, before it began to be used on medical contents. Basically, digital image analysis means just the use of computer algorithms to perform image processing on digital images. In this way, it is possible to find, calculate and describe certain elements within a picture both automatically and precisely.

As technical standards rose and new techniques on the sector of digital images were invented, it became possible to use analysis tools for digital images in more and more fields of research, until the method finally made its way into medicine. According to the high standards of modern computing, it is now possible to see and assess visible changes with the size of a micrometre within an organ and to use this tool to improve both the diagnosis standards and the possibility to predict a patients prognosis with a certain disease.

In hepatology this means, that the major histological signs of many liver diseases can be measured and – in spite of a semiquantitative histological scoring – minimal changes can be made visible in order to show the effect of a therapy as well as the progression of a disease earlier and more easily.

3.3.2. Performance of digital image analysis in this study

To be able to perform the digital image analysis, a further tissue section was cut and stained with sirius red. The reproductability of results deriving from consecutively cut sections has already been proven in literature.(81)

The sirius red stained slides were now scanned using Aperio™ Scan Scope tissue slide scanner. The pictures gained from the tissue slides were converted into high resolution digital „*.svs“-files (with an average resolution of 40.000 x 60.000 pixels per slide) for further analysis. The scanned images were then analysed using Definiens™ Tissue Studio V3.51 (see figure 6), a basic surface for digital image analysis, which is part of Definiens Developer XD® product line, provided by Definiens™ AG from Munich, Germany.

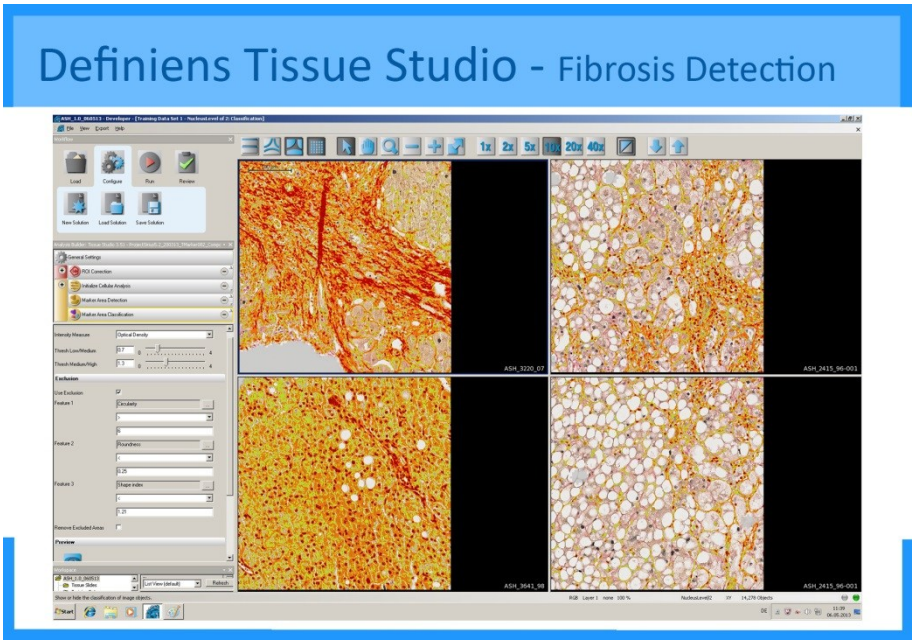


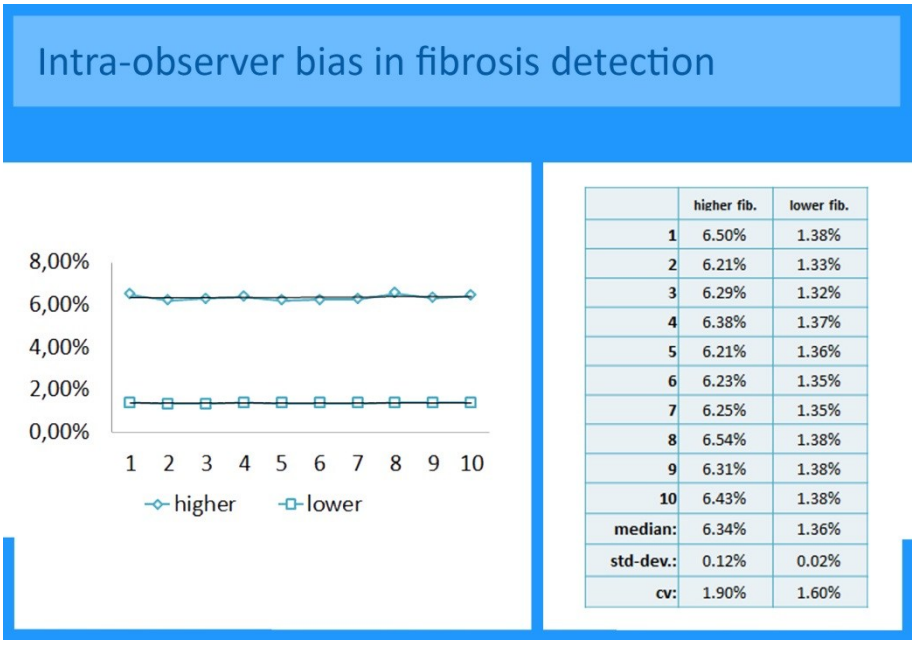
Figure 6 Screenshot of Definiens Tissue Studio during fibrosis detection.

3.3.3. Assessment of fibrosis via digital image analysis

In this particular case, Tissue Studio was used, to assess the collagen proportionate area (CPA) within the liver tissue. This method for the quantification of hepatic fibrosis was used in cases of hepatitis B(80) and hepatitis C(83) before.

First, the liver capsule and large portal tracts were excluded manually, because these areas do not represent disease-related collagen. To get the correct size of the whole tissue area (in µm²), it was automatically separated from the background by the software, in rare cases manually corrected and then recorded as "region of interest" (ROI). Now the optimum threshold for sirius red positive pixels was manually adjusted and the correctness of the recognised areas was verified by a pathologist (CL). The results for the sirius red stained area (in µm²), was recorded. To avoid the recognition of wrongly positive areas, a filter, excluding elements of certain roundness (nuclei) was added. Finally, the calculated area of the ROI divided by the area of sirius red stain equalled the CPA.

To eliminate an arguable intra-observer bias during digital fibrosis assessment, two different biopsy-cores (one with moderate and one with more significant fibrosis) were examined ten times each (figure 7) and showed very similar results with a standard deviation of 0.12% in higher, respectively 0.02% in lower fibrosis.



	higher fib.	lower fib.
1	6.50%	1.38%
2	6.21%	1.33%
3	6.29%	1.32%
4	6.38%	1.37%
5	6.21%	1.36%
6	6.23%	1.35%
7	6.25%	1.35%
8	6.54%	1.38%
9	6.31%	1.38%
10	6.43%	1.38%
median:	6.34%	1.36%
std-dev.:	0.12%	0.02%
cv:	1.90%	1.60%

Figure 7 Data and graphic for the evaluation of intra-observer bias in morphometrical fibrosis detection.

3.3.4. Assessment of steatosis via digital image analysis (DIA)

Unlike in fibrosis detection, the assessment of hepatocellular steatosis can be performed based on routine stains such as haematoxylin/eosin staining.

The first step of steatosis evaluation, equal to the beginning of fibrosis detection, is to find and define the relevant tissue areas in order to separate them from other structures like contamination or irrelevant tissue pieces. The area classified as relevant in this first step is now quantified (in µm²) to provide the basis for a calculation of steatosis area within the sample.

The next step is to find all steatosis-related areas in the picture. Steatotic areas are characterized with a very low optical density, because fat tissue appears white in H/E stained samples. This is done by a simple cellular analysis tool where the software is being trained to find and mark all areas with low optical density and a certain, manually adjusted minimal size.

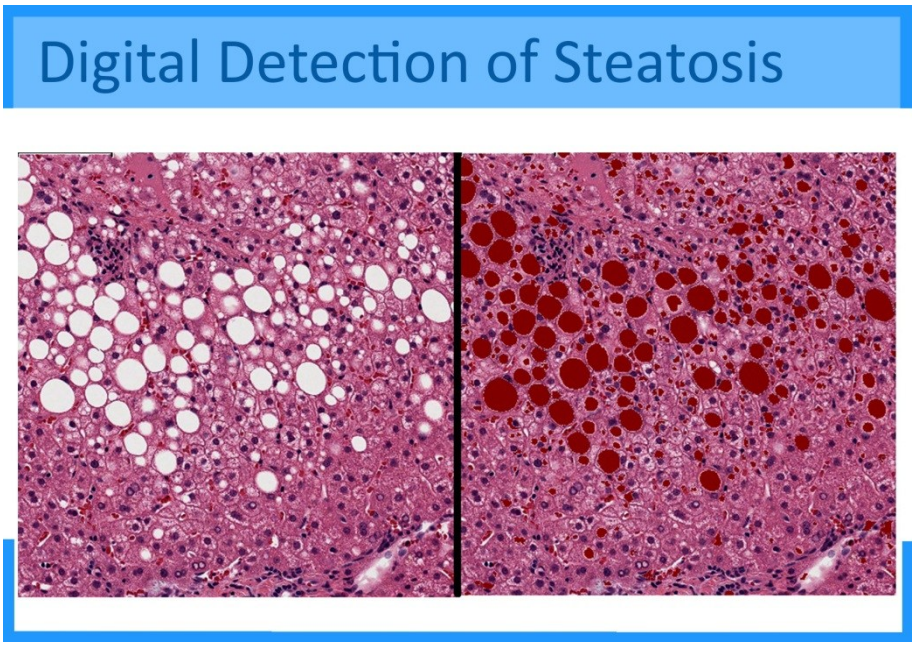


Figure 8 Morphometrical detection of hepatocellular steatosis. Left: Histological image of a steatotic liver specimen. Right: Steatotic hepatocytes are marked red via DIA.

To get unbiased results, all non-fatty areas the software had found during the previous step, have to be excluded in an additional step before running the calculation. This is necessary, because other structures, especially small vessels, could misleadingly be recognized as steatotic hepatocytes by the software.

So in order to exclude vessels that could appear like fat cells, these two structures were divided by their homogeneity (while fat cells appear just white inside, you might easily find structures such as erythrocytes inside a vessel), typical size and wall-thickness. In a second exclusion all areas that have a different shape than the fat cells were removed by geometrical criteria such as roundness, length/width-proportion and a special "shape index" the software provides.

Finally, the calculation can be run and the software gives an area of steatotic tissue all over the sample (in μm^2), the percentage of steatosis in the sample and the number of detected steatotic hepatocytes as a result.

This way of assessing hepatocellular steatosis in liver specimen has already been used for several clinical studies(84–86). Most of the studies evaluated steatosis amounts of patients with HCV or HCC, evaluations on patients with FLD(87) have rarely been published so far.

3.4. Assessment of physiological amount of collagen area in liver specimen

To be able to recognize changes in pathological amounts of collagen proportionate area in liver specimen, it is necessary to be aware of the physiological amounts of collagen that cannot be affected by therapy. As it is part of the organs structure, collagen is found around large vessels and in liver septa in every liver specimen.

For that reason, 15 samples (deriving from healthy liver areas from resections of benign liver tumors) of livers histologically as well as clinically proven to be healthy were sent through digital image analysis in order to assess their (physiological) amount of collagen. This data was used to distinguish between physiological and pathological amounts of fibrosis in further calculations and the statistical analysis.

3.5. Assessment of hepatic fat content via MRI

Magnetic Resonance Imaging (MRI) for assessing hepatic fat content was performed using a 1.5 Tesla scanner (Siemens Medical Systems, Inselin, NJ) at entry and completion of the study.(88–92)

Paired conventional in-phase/out-of-phase (IP/OP) gradient echo imaging was performed consisting of 15-30, 8 mm thick slices covering the whole liver [*Repetition time (TR) 112 ms, echo time (TE) 4.85 ms (IP)/2.43 ms (OP), flip angle 70 degrees, number of excitations (NEX) 1; for Dixon-based (DB) fat-only and water-only images: TR 7.48, TE 4.76/2.38, flip angle 10 degrees*].

For IP/OP, the liver signal intensity (SI) was measured by manually drawing regions-of-interest (ROIs) for the entire liver on each image with exclusion of vessels after which the "fat fraction" was calculated using the formula *(SIIP – SIoP)/2*SIIP*. The same region-of-interest was used for each matching set of in- and out-of-phase images at the same slice position. For the Dixon-based (DB) method, fat content was calculated from similar ROIs drawn at the pre-marked biopsy site on the fat-only and water-only images, and expressed as a value between 0 (no fat) and 1 (all fat). Comparison between IP/OP and DB was done with Pearson correlation. Each subject was used as his/her own control to compare the difference in SI from between baseline and end of treatment studies. Five subjects (two placebo and three N-3 treated) lacked paired values due to claustrophobia (3), metal in the orbit (1), and equipment malfunction (1). At each MR imaging session, a cross-sectional image at the level of L4-L5 vertebral bodies was also obtained to measure visceral fat (cm^2) at the beginning and end of the study.

3.6. Statistical analysis

Categorical (non-continuous) data were summarized as frequencies and percentages. Continuous data were summarized by the mean, the standard deviation, and the range of the measurement distribution. Data was tested for a normal curve of distribution. Frequency tables were used to display results of the histopathological scoring.

The correlation between several variables was evaluated by the bivariate Spearman's rank correlation coefficient *(*interpretation given in table 6). P-values are given as two-sided probabilities. The level of tolerated significance was below 0.05. Correlations and significances were calculated using IBM SPSS software, Version 21. The data is displayed graphically using scatterplots and boxplots.

Distributions of the tested parameters (results from several semiquantitative histological scorings as well as from quantitative methods such as digital image analysis or MRI) were examined using nonparametric tests (Mann Whitney test) to find significant differences between the treated and the placebo group.

All analyses were performed for the whole collective of samples or patients, except for those, where complete data wasn't available.

Interpretation of Correlation Coefficient	
.00 - .19	Slight, almost negligible correlation
.20 - .39	Low, quite small correlation
.40 - .69	Moderate correlation
.70 -.89	**High correlation**
.90 - 1.00	Very high correlation

Table 6 **Help for interpretation of the given correlation coefficients.**

4. RESULTS

4.1. Histological assessment of fibrosis

As mentioned, the histological scoring of fibrosis was executed by two different observers (LC, CS) and two slightly different scoring systems (CRN-Score and Brunt-Score). The different scoring results (examples displayed in table 7) showed notable inter-observer variations.

Histological Scoring of Fibrosis

Pt. No.	Observer	Baseline	2nd Biopsy
1	LC	4	3
	CS	3	3
2	LC	2	2
	CS	3	3
3	LC	1C	1C
	CS	1	2
4	LC	X	0
	CS	2	2
5	LC	4	1B
	CS	3	2
6	LC	3	1B
	CS	3	2
7	LC	4	4
	CS	3	3
8	LC	2	1A
	CS	2	2
9	LC	2	1A
	CS	2	2
10	LC	1B	3
	CS	2	3
11	LC	1B	3
	CS	2	3
12	LC	2	2
	CS	1	2

Table 7 **The scoring results from the two observers (CS & LC) show notable variations in some cases.**

Such variations in histological staging of hepatic fibrosis are seen quite often and well-described in literature(93,94), mostly described in HCV and HIV cases.

4.2. Fibrosis evaluation via digital image analysis

Digital image analysis provides the size of fibrotic areas within a histological slide (CPA), expressed as a percentage of the whole liver tissue area. To evaluate the quality of these measurements, the results were compared to those of the semiquantitative histological scorings of two different observers.

While the results of observer 1 (CS) did not show a significant correlation with the results gained from DIA for hepatic fibrosis content, those of observer 2 (LC) did (figure 9).

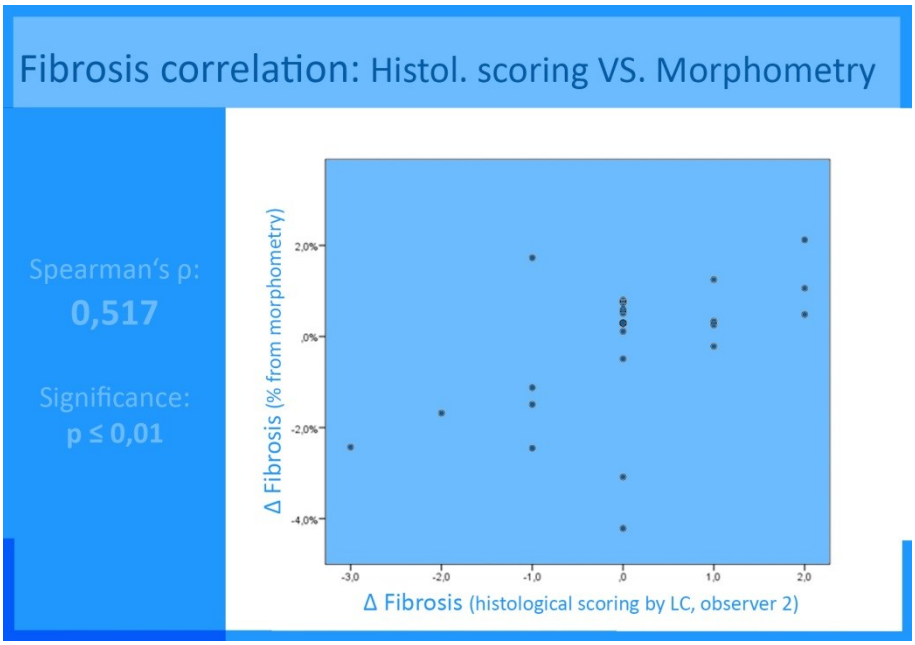


Figure 9 Histological scoring by observer 2 (LC) shows good correlation to DIA in fibrosis assessment.

The Spearman correlation of the change in fibrosis between the two biopsies showed a non-significant correlation ($p > 0.1$) with the results from digital image analysis. (Spearman's $\rho = 0{,}274$) The correlation of observer 2's scoring results with the same DIA-measurements yielded to a significant ($p \leq 0{,}01$) correlation ($\rho = 0{,}517$).

The fact that the data for all histological scores (on the specimen of both biopsies and both observers) shows a significant ($p < 0{,}001$) and good correlation ($\rho = 0{,}698$) to the DIA results (figure 10) indicates, that there is a general correlation between digital fibrosis assessment and semiquantitative histological scorings. By showing better correlation to one of two or more observers, DIA could be able to give a hint on whose scores on the same range of liver specimen are more accurate.

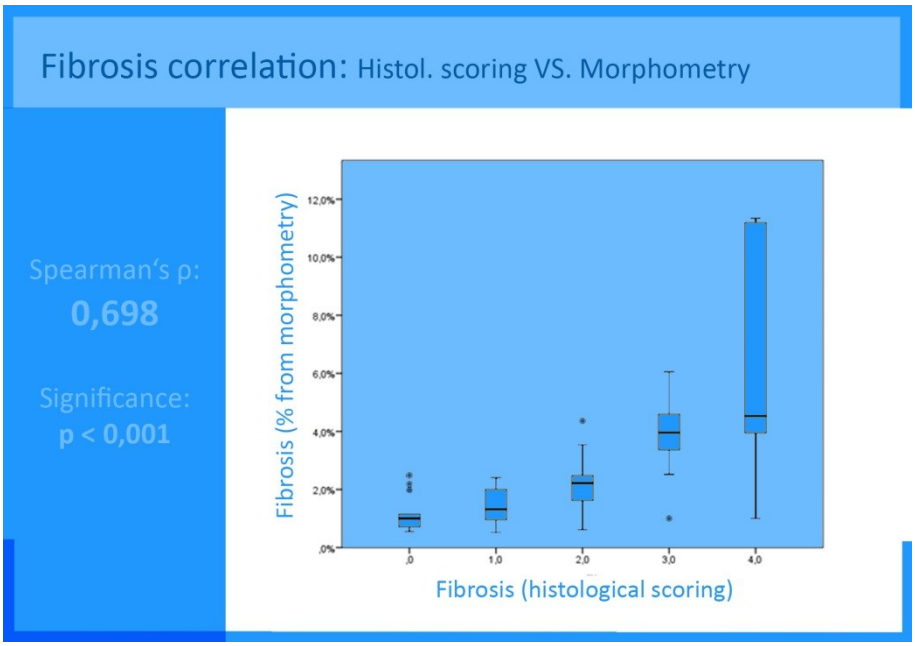


Figure 10 Correlation between fibrosis scores and the results from DIA given as a boxplot.

The original study this essay is based on was unable to show any regression of hepatic fibrosis during treatment. Although we were hoping to be able to show at least a minimal decrease of fibrosis via DIA, the statistical evaluation showed no significant results. Though, DIA showed a non-significant trend towards higher decrease in fibrosis of the group treated with fish oil (-0.54% against only -0.10% in the placebo group) – a difference, the histopathological scoring wasn't able to detect.

One possible reason for the unsatisfactory result of this part of the statistical analysis might be the physiological amount of fibrosis in every liver sample. Even a healthy liver contains certain amounts of connective tissue (collagen) - the physiological appearance of fibrosis. This 'brace' of the liver won't regress during anti-fibrotic therapy and is therefore a potential bias in the detection of fibrosis changes – especially in patients with low amounts of fibrosis, such as in this study.

As mentioned in the “methods” section, the physiological amount of fibrosis was assessed within 15 samples deriving from healthy livers. The mean amount of fibrosis in these samples was 3.43% with a standard deviation of 0.45% (figure 11).

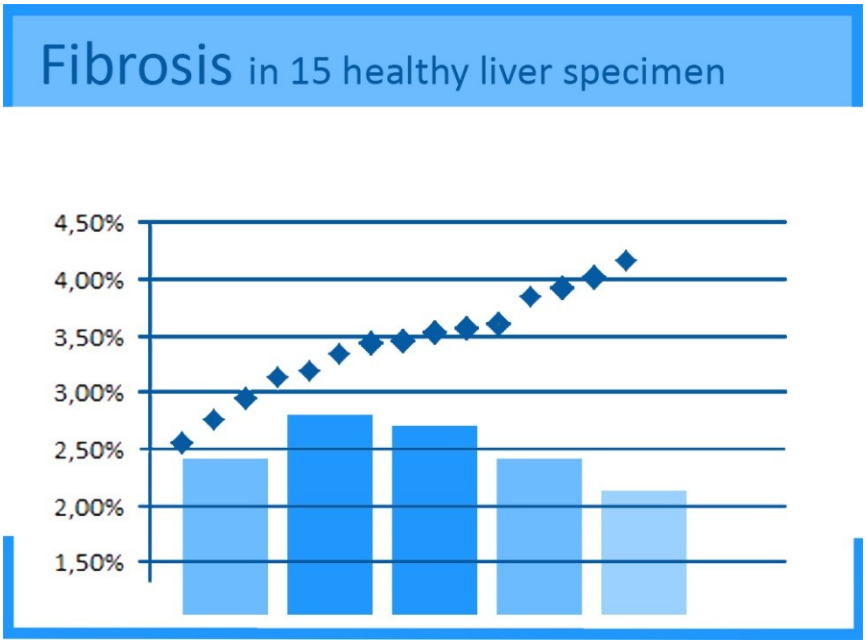


Figure 11 Fibrosis amounts of healthy liver specimen assessed by morphometrical analysis.

However, this value cannot be used as an upper limit of normal fibrosis amounts in liver sections, because the ballooned and steatotic hepatocytes in NAFLD are characterised by an elevated cell volume, so the amount of physiological collagen is proportionately reduced in steatotic liver specimen. It is hard to estimate the effect of this gain of volume to the physiological amount of collagen, but as many of the liver specimen examined in this study showed morphometrical fibrosis levels far lower than the ones we found in healthy livers, this effect should not be underestimated.

To be sure, not to exclude any pathologically elevated levels of fibrosis, but to get rid of the cases without elevation at the same time, a cut-off level of 2.5% of fibrosis at baseline biopsy was introduced for a second series of statistical evaluation.

In this second evaluation, every patient except one showed a clear decrease of fibrosis (figure 12). Still, it was impossible to detect statistically significant differences between the treated and the placebo group.

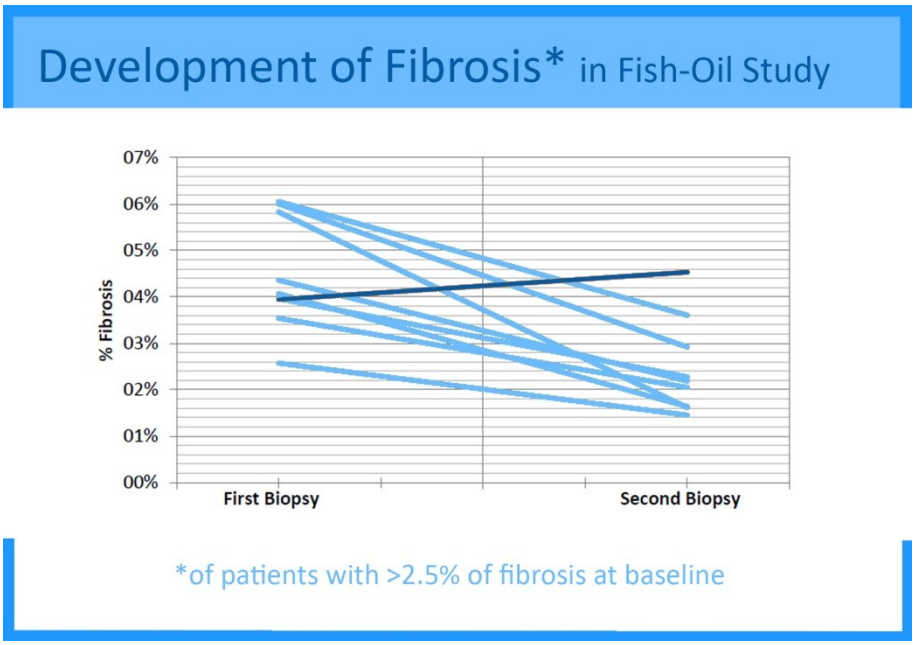


Figure 12 Cases with >2,5% fibrosis in baseline morphometrical assessment showed a decrease of fibrosis in 9 out of 10 cases.

4.3. Steatosis evaluation via digital image analysis

The evaluation of steatosis in liver specimen is much easier than the assessment of fibrosis. There is no special staining required for steatosis measurement – a simple H/E stained slide works perfectly for such evaluations.

Concerning steatosis, the main goal of our testings was to introduce digital image analysis as a powerful tool for its accurate assessment.

For that reason, the histopathological scores of all NAFLD patients that were included in the study were compared to the results for steatosis gained from Dixon-modificated MRI – the gold standard for the assessment of hepatocellular steatosis – by the calculation of Spearman's rank correlation coefficient 'ρ' (figure 13).

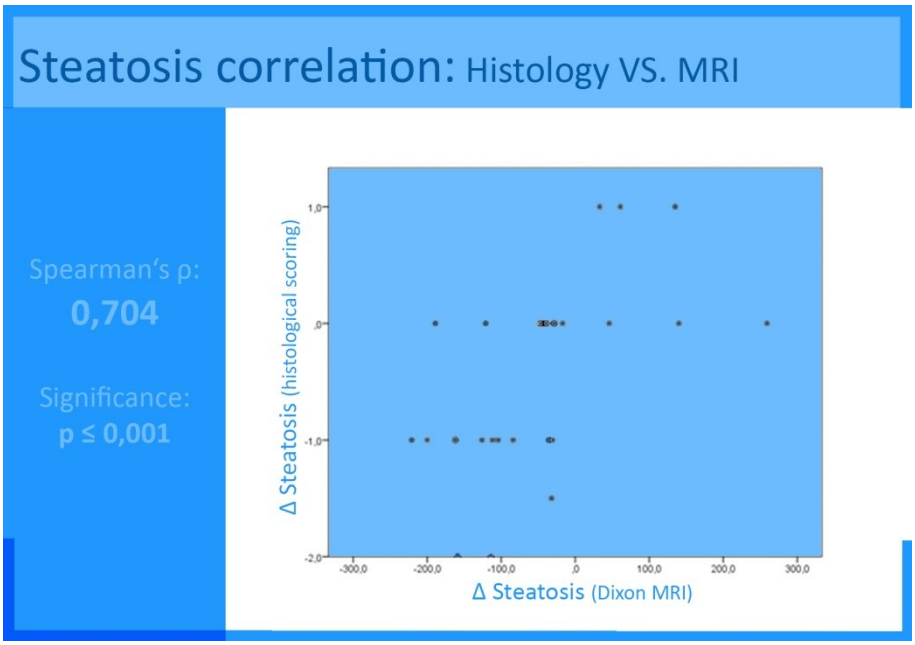


Figure 2 Histological steatosis scoring in correlation with Dixon modificated MRI measurements showed significant results.

As expected, this calculation showed a good correlation ($\rho = 0{,}704$) between histological scoring and Dixon MRI for steatosis detection, that was statistically significant ($p \leq 0{,}001$).

The next step was to investigate the correlation between the results gained from DIA and the ones examined previously. The statistical tests, that were used to compare the delta values (from baseline biopsy to second biopsy), showed a very good correlation of DIA with the histological scorings ($\rho = 0{,}838$; $p < 0{,}001$, scatterplot given in figure 14.).

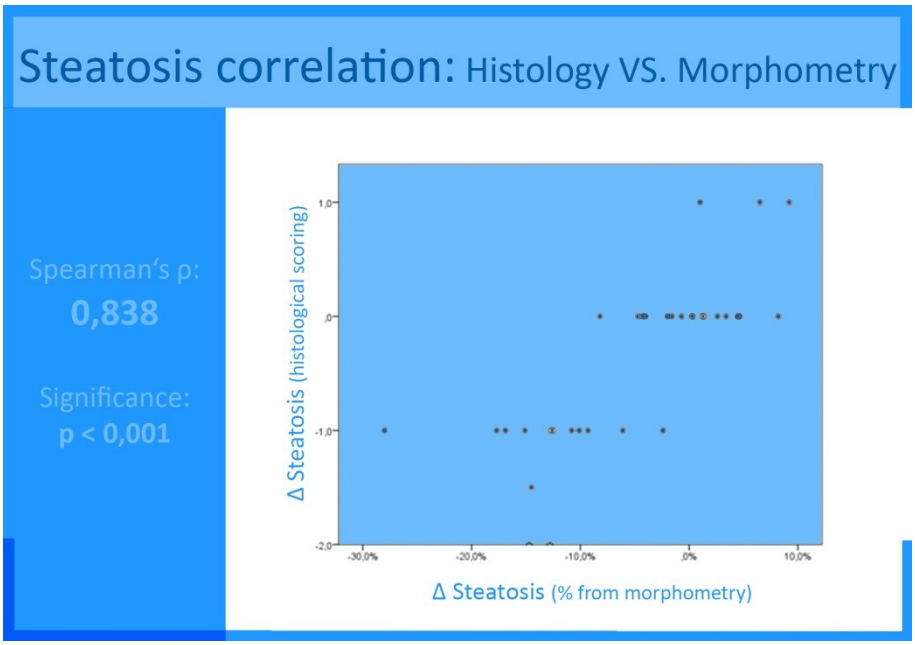


Figure 14 Correlation of histological scorings with the results from morphometrical analysis showed a very high correlation between the two methods of steatosis assessment.

A second evaluation was even able to show, that the results of DIA correlate better with the gold standard of Dixon MRI fat measurement (Pearson's r = 0,794 , p < 0,001), than the well-established method of semiquantitative histological scoring (fig. 15).

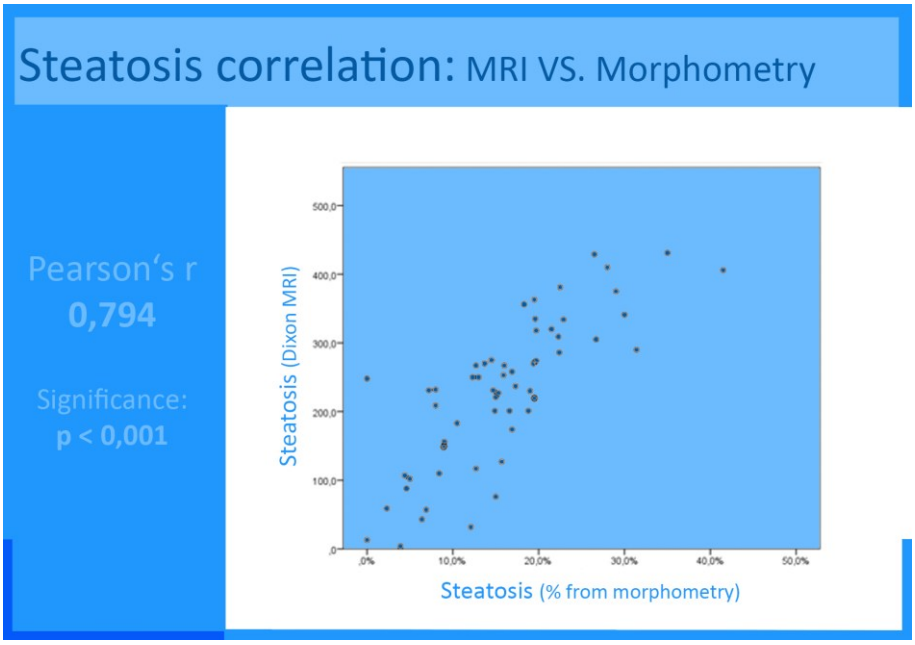


Figure 15 Digital image analysis shows better correlation to the gold standard for steatosis assessment, the Dixon modification of MR imaging, than semiquantitative histological scoring.

Interestingly, examinations on hepatic fat content were also able to show, what was published several times before: The histologists' eye tends to overestimate the amount of hepatocellular steatosis and the overestimation generally increases with the severity of steatosis.(95)

In contrast to fibrosis evaluation, the measurement of delta steatosis showed a significant difference between the placebo group and the group of patients treated with N-3 fatty acids. The statistical evaluation (Wilcoxon signed-rank test) determined a significant ($p \leq 0{,}01$) difference between the two tested groups (figure 16).

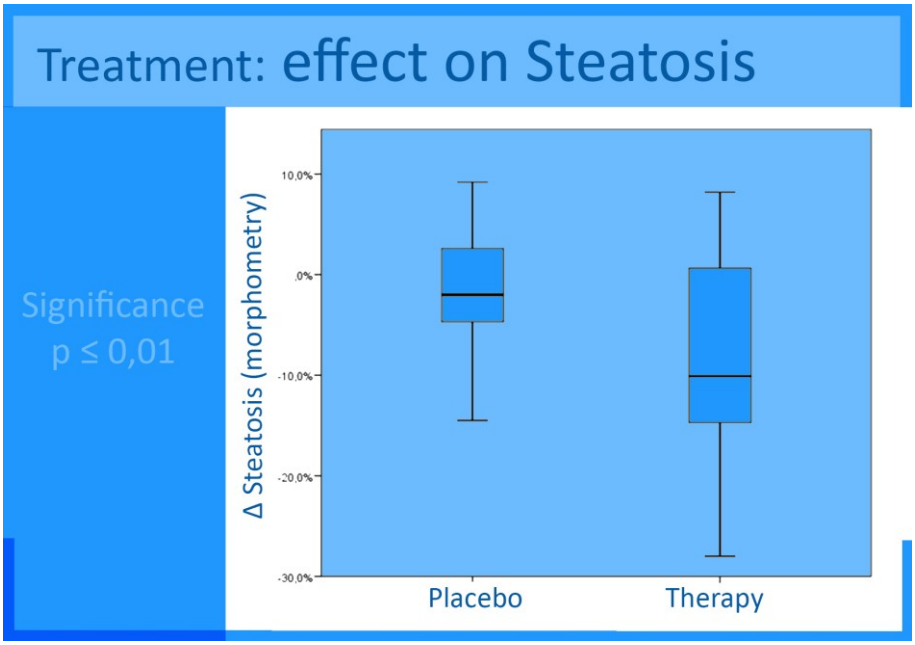


Figure 16 The difference in hepatocellular steatosis amounts from morphometrical analysis (delta values [changes] from 1st to 2nd biopsy) between treated and placebo group was statistically significant.

4.4. The idea of a digital NAFLD image scoring (NIS)

The correlation analysis between the semiquantitative histological scorings for fibrosis and the results of digital image analysis has to deal with the problem of comparing percentages with a histological score, that implies many features on the one hand and can only be expressed as one out of five numbers (0-4) on the other.

Because these two parameters are hard to compare (while histological scoring of fibrosis focuses on features such as architecture and activity, the digital image analysis just calculates the collagen proportionate area, without consideration of such parameters), we tried to introduce a different (morphometrical) scoring method, including both the change of hepatocellular steatosis and hepatic fibrosis, in order to find a digital scoring-scheme

comparable to the NASH activity score (NAS) – of course without measuring any activity in that score, because evaluation of activity marks a clear limitation of digital image analysis.

This newly introduced NAFLD imaging score (NIS) aimed to describe the level of change in both fibrosis and steatosis between two biopsies. Therefore, the percent change of the two observed parameters was scored from +2 (high increase) to -2 (high decrease), as displayed in table 8.

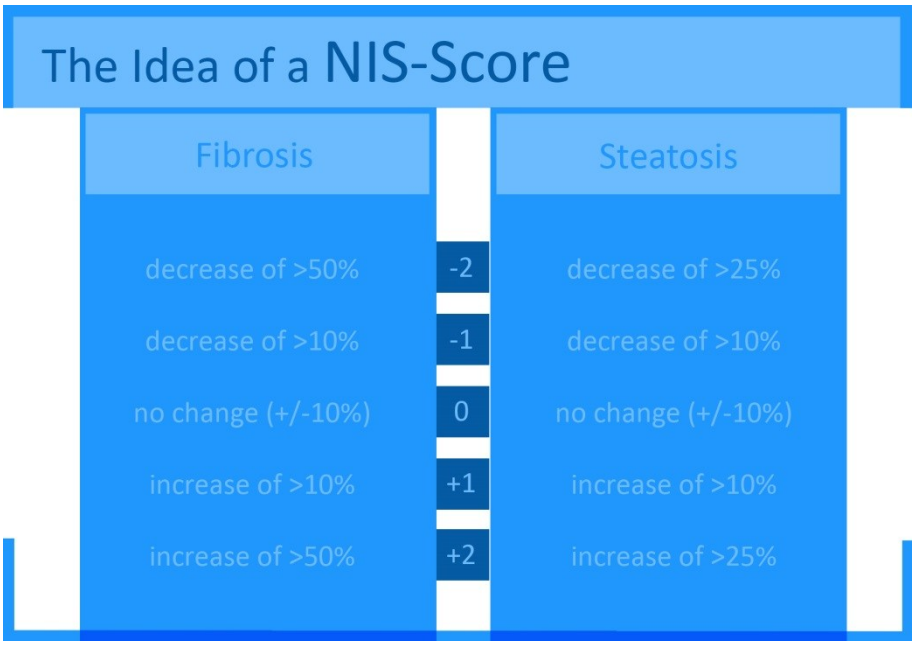


Table 8 Scoring scheme for the introduced NAFLD imaging score (NIS).

Unfortunately, the idea of a morphometrical scoring system for NAFLD and NASH did not seem to work out well yet – there was no significant correlation between this score and the actual NAS results.

Still, as the advances in digital image analysis continue to improve, this idea should be considered again in the future.

5. DISCUSSION

5.1. Potential problems and biases in digital image analysis

Digital image analysis may be a promising novel technology to assess morphological features in histological sections. However, some shortcomings have to be considered.

Of course, intra-, as well as inter-observer variations do exist for this method as well, because the assessment cannot be performed in a fully automated way due to potential pitfalls like uneven stains of the tissue samples. Different users may pick slightly varying cut-off values leading to different results in fibrosis detection. In addition, the same operator may not be able to adjust the software to achieve an identic cut-off value in a series of samples, that aren't stained exactly the same. Especially histologic samples of diverging age or coming from different centres may increase the chance of significant intra-observer variation.

Nevertheless, we were able to show that the results of DIA are reproducible and of very low fluctuation, which indicates very little intra-observer variations. Data about inter-observer variations in DIA are not available yet, but we already seek cooperation with other research groups performing DIA of liver samples, in order to acquire suitable data for such calculations.

5.2. Conclusion

Digital image analysis is a powerful tool to acquire huge amounts of accurate data in a very short time in simple analyses like steatosis measurement. Even for the fibrosis calculation (the one harder to perform), DIA showed a very low intra-observer variation, which makes this tool very reliable and its results reproducible.

In steatosis detection, DIA was able to confirm the overestimation of hepatic fat content by semiquantitative histological scoring, which has already been described in literature several times. By displaying this constant overestimation (while the frequently used CRN score distinguishes between 33-66% of steatosis and >66% of steatosis for severe cases of hepatocellular steatosis, even the most steatotic liver samples in this study hardly reached the 40% mark), DIA could be able to contribute to a new way of interpreting hepatocellular steatosis and maybe improve histopathological judgements.

Because of the need for adjustment on every single specimen, DIA may be more time-consuming than the examination by a well-trained histopathologist for more specific

measurements like the one for hepatic fibrosis. Also, morphometry is currently unable to provide several pieces of information necessary for treatment and probably also for prognosis issues, like the diseases' activity.

Still, DIA may be able to detect minimal changes in histologic features, which could never be revealed by a histopathologists' eye that precisely. As DIA showed very good correlations to histopathological scoring of fibrosis ($\rho = 0{,}698$) and steatosis ($\rho = 0{,}838$) and even to MR imaging ($r = 0{,}794$), this technique deserves to be seen as one possible way to assess the degree of damage dealt to the liver during chronic liver diseases.

As a side effect our examinations also showed, that this technology may provide results quite comparable to histological scorings, in terms of fibrosis and steatosis detection. A fact that may qualify DIA to settle discordance between histologists or pathologists by re-evaluating their results.

Another potential goal of the research done for this essay was the hope to be able to show an effect of a therapy that could not be shown in other methods of NAFLD examination. As mentioned above, this goal has not been reached. Still, we were able to show a non-significant trend towards higher change of fibrosis content of the treated group in contrast to the placebo group. This fact gives hope, that on the one hand an effect of this therapy could eventually be shown in a study with longer observation time and on the other hand that advances in digital image analysis could be able to reveal even such small changes more precisely, able to gain statistically significant results.

6. APPENDIX

6.1. Figures and tables

Figure 1: Histological image of macrovesicular steatosis by Tiniakos D. et al.(1)

Figure 2: Histological image of ballooned hepatocytes in NASH by Tiniakos D. et al.(1)

Figure 3: Progression of fibrosis in chronic liver disease. Adapted from (16)

Figure 4: Incidence of NAFLD. Adapted from (4)

Figure 5: Examples for SAF-Scoring Bedossa P. et al.(41)

Figure 6: Screenshot from the Tissue Studio by Definiens AG, Munich.

Figure 7: Evaluation of intra-observer bias in DIA.

Figure 8: Morphometrical steatosis detection via Definiens Tissue Studio.

Figure 9: Scatterplot and data from correlation: fibrosis scores of observer 2 (LC) VS DIA fibrosis assessment. (Data from observer 1 is not displayed graphically because of its non-significant result in calculation of correlation.)

Figure 10: Boxplot: Correlation of morphometrical and histological results for fibrosis.

Figure 11: Results from healthy liver fibrosis evaluation via digital image analysis.

Figure 12: Fibrosis development of cases with definite non-physiological amount of fibrosis.

Figure 13: Data from correlation analysis between histol. scorings and MRI for steatosis.

Figure 14: Data from correlation analysis between histol. scorings and morphometrical evaluation for steatosis.

Figure 15: Data from correlation analysis between morphometrical evaluation and MRI for steatosis.

Figure 16: Therapy-effect of N-3 fatty acids on hepatocellular steatosis measured by digital image analysis.

Table 1: Causes of secondary hepatocellular steatosis. Adapted from (10)

Table 2: Definition of NAFLD and related entities. Adapted from (10)

Table 3: Complications of hepatic cirrhosis. Generated with information from (8,43,65,96)

Table 4: Brunt scoring system for hepatic fibrosis. Adapted from (8)

Table 5: CRN scoring system for hepatic fibrosis. Adapted from (36)

Table 6: Typical interpretation scheme for a Spearman's rank correlation.

Table 7: Scoring results from the two histopathological observers.

Table 8: Scoring system of the NAFLD imaging score (NIS).

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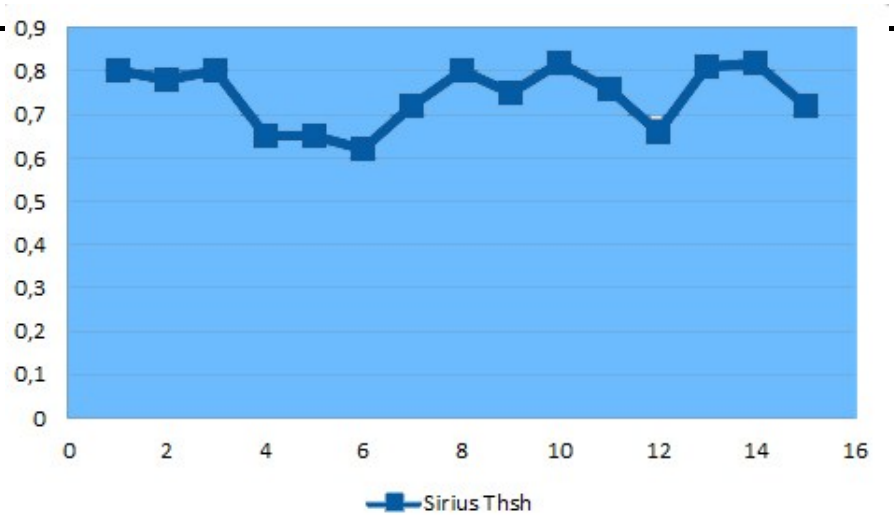
6.3. Attachments

Results from assessment of healthy liver fibrosis:

No	Ncl. Thsh	Sirius Thsh	Fibro (%)
1	0,05	0,8	3,35%
2	0,05	0,78	3,61%
3	0,05	0,8	3,85%
4	0,05	0,65	3,58%
5	0,05	0,65	4,01%
6	0,05	0,62	3,93%
7	0,05	0,72	4,16%
8	0,05	0,8	2,75%
9	0,05	0,75	2,55%
10	0,05	0,82	3,14%
11	0,05	0,76	3,19%
12	0,05	0,66	3,53%
13	0,05	0,81	2,95%
14	0,05	0,82	3,44%
15	0,05	0,72	3,46%
		Mean:	3,43%
		Std.Dev.:	0,45%



			Histo: Fibrosis		
Pt. No.	Observer	Rx?	Baseline	2nd Biopsy	Δ HistoF
1	CS	0	1	1	0
4	CS	1	3	3	0
5	CS	0	1	1	0
6	CS	1	1	2	1
7	CS	0	3	2	-1
8	CS	0	3	3	0
10	CS	1	2	2	0
11	CS	1	3	3	0
12	CS	0	1	0	-1
13	CS	1	1	3	2
14	CS	0	1	1	0
15	CS	1	4	3	-1
17	CS	0	1	1	0
18	CS	0	3	2	-1
19	CS	1	3	4	1
21	CS	0	2	3	1
22	CS	1	1	1	0
23	CS	0	1	1	0
24	CS	0	3	3	0
25	CS	1	2	1	-1
26	CS	1	1	1	0
27	CS	0	1	2	1
28	CS	1	2	2	0
29	CS	0	2	2	0
30	CS	1	3	2	-1
31	CS	0	1	1	0
32	CS	1	1	2	1
33	CS	1	2	3	1
35	CS	1	2	2	0
36	CS	0	1	2	1
37	CS	0	2	3	1
38	CS	0	1	2	1
39	CS	1	3		
41	CS	1	2	2	0
1	LC	0	1	1	0
4	LC	1	2	2	0
5	LC	0	0	1	1
6	LC	1	1	1	0
7	LC	0	4	1	-3
8	LC	0	4	4	0
10	LC	1		0	
11	LC	1	3	4	1
12	LC	0	0		
13	LC	1	1	3	2
14	LC	0	0	0	0
15	LC	1		3	
17	LC	0	0	1	1
18	LC	0		1	

19	LC	1	4	4	0
21	LC	0	1	3	2
22	LC	1	1	1	0
23	LC	0	0	0	0
24	LC	0	4	3	-1
25	LC	1	1	1	0
26	LC	1	1	1	0
27	LC	0	2	2	0
28	LC	1	2	1	-1
29	LC	0	2	1	-1
30	LC	1	3	1	-2
31	LC	0	0	1	1
32	LC	1	0	0	0
33	LC	1	1	3	2
35	LC	1	2	1	-1
36	LC	0	0	1	1
37	LC	0	1	3	2
38	LC	0		1	
39	LC	1	4		
41	LC	1	0	1	1

	Morpho: Fibrosis			Histo: Steatosis		
Pt. No.	Baseline	2nd Biopsy	Δ MorphoF	Baseline	2nd Biopsy	Δ HistoS
1	0,5%	1,3%	0,8%	2,5	1,0	-1,5
4	6,0%	2,9%	-3,1%	1,0	2,0	1,0
5	1,2%	0,9%	-0,2%	1,0	1,0	0,0
6	5,8%	1,6%	-4,2%	3,0	2,0	-1,0
7	4,1%	1,6%	-2,4%	1,0	1,0	0,0
8	3,9%	4,5%	0,6%	2,0	1,0	-1,0
10	4,4%	2,2%	-2,2%	3,0	2,0	-1,0
11		3,3%		1,0	1,0	0,0
12	0,6%	1,1%	0,6%	1,0	1,0	0,0
13		6,0%		2,0	2,0	0,0
14	0,6%	0,8%	0,3%	1,0	1,0	0,0
15		4,6%		2,0	3,0	1,0
17	0,7%	1,0%	0,3%	1,0	1,0	0,0
18		1,2%		2,0	2,0	0,0
19	1,0%	11,3%	10,3%	2,0	1,0	-1,0
21	2,0%	2,5%	0,5%	1,0	1,0	0,0
22	1,5%	1,0%	-0,5%	2,0	1,0	-1,0
23	0,7%	0,8%	0,1%	2,0	1,0	-1,0
24	6,1%	3,6%	-2,5%	1,0	2,0	1,0
25	1,3%	2,0%	0,7%	2,0	1,0	-1,0
26	1,0%	1,3%	0,3%	2,0	1,0	-1,0
27	2,1%	2,4%	0,3%	1,0	1,0	0,0
28	3,5%	2,1%	-1,5%	1,0	1,0	0,0
29	2,6%	1,5%	-1,1%	3,0	1,0	-2,0
30	4,0%	2,3%	-1,7%	2,0	1,0	-1,0
31		1,8%		1,0	1,0	0,0
32	2,0%	2,5%	0,5%	2,0	1,0	-1,0
33	2,2%	4,3%	2,1%	3,0	1,0	-2,0
35	0,6%	2,3%	1,7%	2,0	1,0	-1,0
36	1,0%	1,3%	0,3%	1,0	1,0	0,0
37	2,3%	3,4%	1,1%	2,0	2,0	0,0
38		1,7%		1,0	1,0	0,0
39	11,2%			1,0		
41	1,2%	2,4%	1,2%	3,0	3,0	0,0
1	0,5%	1,3%	0,8%	2,5	1,0	-1,5
4	6,0%	2,9%	-3,1%	1,0	2,0	1,0
5	1,2%	0,9%	-0,2%	1,0	1,0	0,0
6	5,8%	1,6%	-4,2%	3,0	2,0	-1,0
7	4,1%	1,6%	-2,4%	1,0	1,0	0,0
8	3,9%	4,5%	0,6%	2,0	1,0	-1,0
10	4,4%	2,2%	-2,2%	3,0	2,0	-1,0
11		3,3%		1,0	1,0	0,0
12	0,6%	1,1%	0,6%	1,0	1,0	0,0
13		6,0%		2,0	2,0	0,0
14	0,6%	0,8%	0,3%	1,0	1,0	0,0
15		4,6%		2,0	3,0	1,0
17	0,7%	1,0%	0,3%	1,0	1,0	0,0
18		1,2%		2,0	2,0	0,0

19	1,0%	11,3%	10,3%	2,0	1,0	-1,0
21	2,0%	2,5%	0,5%	1,0	1,0	0,0
22	1,5%	1,0%	-0,5%	2,0	1,0	-1,0
23	0,7%	0,8%	0,1%	2,0	1,0	-1,0
24	6,1%	3,6%	-2,5%	1,0	2,0	1,0
25	1,3%	2,0%	0,7%	2,0	1,0	-1,0
26	1,0%	1,3%	0,3%	2,0	1,0	-1,0
27	2,1%	2,4%	0,3%	1,0	1,0	0,0
28	3,5%	2,1%	-1,5%	1,0	1,0	0,0
29	2,6%	1,5%	-1,1%	3,0	1,0	-2,0
30	4,0%	2,3%	-1,7%	2,0	1,0	-1,0
31		1,8%		1,0	1,0	0,0
32	2,0%	2,5%	0,5%	2,0	1,0	-1,0
33	2,2%	4,3%	2,1%	3,0	1,0	-2,0
35	0,6%	2,3%	1,7%	2,0	1,0	-1,0
36	1,0%	1,3%	0,3%	1,0	1,0	0,0
37	2,3%	3,4%	1,1%	2,0	2,0	0,0
38		1,7%		1,0	1,0	0,0
39	11,2%			1,0		
41	1,2%	2,4%	1,2%	3,0	3,0	0,0

	Morpho: Steatosis			Dixon: Steatosis		
Pt. No.	Baseline	2nd Biopsy	Δ MorphoS	Baseline MRI	2nd MRI	Δ DixonS
1	31,4%	16,9%	-14,5%	290	258	-32
4	15,9%	22,4%	6,5%	253	286	33
5	15,7%	16,0%	0,3%	127	267	140
6	43,0%	26,1%	-16,9%			
7	12,3%	8,0%	-4,3%	250	209	-41
8	18,8%	12,7%	-6,1%	201	117	-84
10	41,5%	29,0%	-12,5%	406	375	-31
11	12,7%	13,0%	0,3%	267	250	-17
12	8,0%	6,4%	-1,6%	232	43	-189
13	26,4%	25,7%	-0,7%			
14	19,4%	14,7%	-4,7%	270	231	-39
15	21,5%	22,5%	1,0%	320	381	61
17	16,9%	19,5%	2,6%	174	220	46
18	18,2%	19,6%	1,4%			
19	19,5%	4,4%	-15,1%	219	107	-112
21	15,0%	19,6%	4,6%	76	335	259
22	18,3%	9,0%	-9,3%	356	156	-200
23	17,3%	14,9%	-2,4%	237	201	-36
24	10,5%	19,7%	9,2%	183	318	135
25	26,7%	16,6%	-10,1%	305	201	-104
26	19,7%	8,9%	-10,8%	274	148	-126
27	14,5%	19,0%	4,5%	275	230	-45
28	7,2%	8,4%	1,2%	231	110	-121
29	26,5%	13,7%	-12,8%	429	270	-159
30	22,3%	4,6%	-17,7%	309	88	-221
31	12,1%	3,9%	-8,2%	32	4	-28
32	28,0%	0,0%	-28,0%	410	248	-162
33	30,0%	15,3%	-14,7%	341	227	-114
35	15,0%	2,3%	-12,7%	221	59	-162
36	9,0%	5,0%	-4,0%	150	102	-48
37	19,5%	22,9%	3,4%	363	334	-29
38	12,7%	10,7%	-2,0%			
39	6,9%	0,0%	-6,9%	57	13	-44
41	35,0%	43,2%	8,2%	431		
1	31,4%	16,9%	-14,5%	290	258	-32
4	15,9%	22,4%	6,5%	253	286	33
5	15,7%	16,0%	0,3%	127	267	140
6	43,0%	26,1%	-16,9%			
7	12,3%	8,0%	-4,3%	250	209	-41
8	18,8%	12,7%	-6,1%	201	117	-84
10	41,5%	29,0%	-12,5%	406	375	-31
11	12,7%	13,0%	0,3%	267	250	-17
12	8,0%	6,4%	-1,6%	232	43	-189
13	26,4%	25,7%	-0,7%			
14	19,4%	14,7%	-4,7%	270	231	-39
15	21,5%	22,5%	1,0%	320	381	61
17	16,9%	19,5%	2,6%	174	220	46
18	18,2%	19,6%	1,4%			

19	19,5%	4,4%	-15,1%	219	107	-112
21	15,0%	19,6%	4,6%	76	335	259
22	18,3%	9,0%	-9,3%	356	156	-200
23	17,3%	14,9%	-2,4%	237	201	-36
24	10,5%	19,7%	9,2%	183	318	135
25	26,7%	16,6%	-10,1%	305	201	-104
26	19,7%	8,9%	-10,8%	274	148	-126
27	14,5%	19,0%	4,5%	275	230	-45
28	7,2%	8,4%	1,2%	231	110	-121
29	26,5%	13,7%	-12,8%	429	270	-159
30	22,3%	4,6%	-17,7%	309	88	-221
31	12,1%	3,9%	-8,2%	32	4	-28
32	28,0%			410	248	-162
33	30,0%	15,3%	-14,7%	341	227	-114
35	15,0%	2,3%	-12,7%	221	59	-162
36	9,0%	5,0%	-4,0%	150	102	-48
37	19,5%	22,9%	3,4%	363	334	-29
38	12,7%	10,7%	-2,0%			
39	6,9%			57	13	-44
41	35,0%	43,2%	8,2%	431		

(black areas = no results available)

Wenn du ein richtiger Wissenschaftler sein willst, denke wenigstens eine halbe Stunde am Tag das Gegenteil von dem, was deine Kollegen denken.

– **Albert Einstein**