DIPLOMARBEIT

Correlation of liver fibrosis stages with the serum biomarker ST2 in patients with non-alcoholic fatty liver disease.

An explorative retrospective study.

Zur Erlangung des akademischen Grades

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Danksagung

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1.) ZUSAMMENFASSUNG

Hintergrund

Die nicht-alkoholische Fettlebererkrankung umfasst ein Krankheitsspektrum von einfacher Fettleber über Steatohepatitis und Fibrose bis hin zur Leberzirrhose und hepatozellulärem Karzinom. Sie stellt ein großes und wachsendes Gesundheitsproblem dar, das bis zu 20-30% der allgemeinen Bevölkerung betrifft.

Fragestellung & Zielsetzung

Das Fehlen von verlässlichen nicht-invasiven Biomarkern für die Diagnose des Schweregrades der Erkrankung ist eines der Hauptprobleme im klinischen Management von Patienten mit nicht-alkoholischer Fettlebererkrankung.

Das Ziel dieser Studie war es, einen Überblick über die Rolle des Serummarker ST2 im Rahmen der nicht-alkoholischen Fettlebererkrankung zu bekommen.

Unsere Hypothese war, dass ST2 mit der Schwere der Leberfibrose korreliert, und dass Patienten mit schwerer Leberfibrose (Grad 3-4) höhere ST2 Werte haben, als Patienten mit keiner oder leichter Fibrose (Grad 0-2).

Methoden & Statistik

Dies ist eine retrospektive, explorative Studie. Es wurden PatientInnen eingeschlossen, die im Zeitraum von September 2011 bis März 2016 eine Leberbiopsie zur Erhebung des Fibrosegrades und eine Blutabnahme zur Messung des ST2 Levels mittels ELISA erhalten haben.

Ergebnisse

Die Serumlevel von ST2 korrelierten positiv mit dem Leberfibrosegrad der NAFLD-Patienten (r_s= 0,360, p-Wert = 0,009).

Patienten mit schwerer Leberfibrose (Grad 3-4) hatten signifikant höhere ST2 Werte als Patienten mit keiner oder leichter Fibrose (Grad 0-2), (p-Wert = 0,019).

Schlussfolgerung

Die höheren ST2 Werte bei Patienten mit schwerer Leberfibrose können auf dessen Involvierung im Rahmen der hepatischen Fibrogenese hinweisen und möglicherweise als diagnostischer Marker genutzt werden.

2.) ABSTRACT

Background

The prevalence of NAFLD in the general population in Europe is estimated at 20-30%. About 5 to 20% of patients with fatty liver develop a non-alcoholic-steato-hepatitis (NASH), which in 10 to 20 % of cases progresses to higher stages of fibrosis or even cirrhosis in less than 5%. There is lack of reliable non-invasive biomarkers for diagnosis and pathophysiological mechanisms of disease severity and prediction of prognosis is one of the major drawbacks in the clinical management of patients with NAFLD.

Aim

The current proposal was expected to result in the identification of novel non-invasive diagnostic and prognostic parameters for the management of NAFLD.

We hypothesized that the levels of the serum biomarker ST2 correlate with liver fibrosis stage and that patients with severe liver fibrosis have higher levels of ST2 than those with mild liver fibrosis.

Methods

51 patients participated in our retrospective cohort study. The retrospective data consisted of a liver biopsy for diagnosis of the NAFLD and determination of liver fibrosis stage and blood sampling for measurement of the biomarker ST2 by ELISA.

Results

Serum levels of ST2 correlated positively with the liver fibrosis stages of patients with non-alcoholic fatty liver disease (r_s= 0,360, p-value = 0,009).

Patients with severe liver fibrosis (stage 3-4) had significantly higher serum levels of ST2 than patients with mild liver fibrosis (stage 0-2), (p-value = 0,019).

The mean value of ST2 was 630,17pg/ml in fibrosis group 1 compared to 750pg/ml in fibrosis group 2.

A cut-off value of serum ST2 of >572,5 pg/ml had a sensitivity of 81,8% and a specificity of 41,4% for diagnosis of severe liver fibrosis (stage 3 or 4).

Conclusion

The higher levels of ST2 in patients with severe liver fibrosis underline the hypothesis, that ST2 is involved in liver fibrogenesis during nonalcoholic fatty liver disease and possibly can be used as a diagnostic biomarker in NAFLD.

3.) NONALCOHOLIC FATTY LIVER DISEASE

3.1. DEFINITION

Non-alcoholic fatty liver disease (NAFLD) is defined as hepatic steatosis, characterized by simple accumulation of triglycerides in at least 5% of hepatocytes, with no other cause for liver damage due to alcohol consumption, which must be less than <20g/d for females and <30g/d for men, steatogenic drugs or other hepatic diseases, such as viral hepatitis, hemochromatosis or Wilson disease.(1)(2)(3)

NAFLD comprises a disease spectrum ranging from simple fatty liver over non-alcoholic steatohepatitis (NASH) and fibrosis to liver cirrhosis and hepatocellular carcinoma(HCC).(2)(1) Non-alcoholic steatohepatitis is defined by additional inflammation with hepatocyte injury.(2) In patients with NASH hepatocytes exhibit inflammatory signs, such as baloonic degeneration, necroapoptosis and fibrosis.(4)

3.2. EPIDEMIOLOGY

NAFLD is the most frequently reason of chronic liver disease(4) and in the United States the most commonly cause of asymptomatic elevations of liver enzymes.(1) The incidence of NAFLD is esteemed to be about 28 up to 58/1000 person-years.(2) The prevalence of NAFLD in the general population in Europe is estimated at 20-30%.(3) In children the prevalence is about 5-10%.(5) This high prevalence is directly related to the worldwide epidemic of obesity and diabetes.(1) Both excessive body mass index (BMI) and visceral obesity are recognized risk factors for NAFLD.(1) In patients with morbid obesity the prevalence is increased up to 75%.(6) Additionally, a high prevalence of NAFLD (> 65 %) is observed in individuals with type 2 diabetes.(3) Diabetes seems to lead to an even higher risk of severe liver diseases, such as fibrosis, cirrhosis or hepatocellular carcinoma but also cardiovascular and vascular diseases.(7)

About 5 to 20% of patients with fatty liver develop NASH, which in 10 to 20 % of cases progresses to higher stages of fibrosis.(4) Progression to cirrhosis is seen in less than 5% of these cases.(4) An estimation based on these scopes of progression results in a prevalence of cirrhosis caused by NAFLD cirrhosis of 0.05 to 0.3 % in the general population.(4) Two separate groups appear to exist, where one group of patients (probably the majority) may not progress beyond steatosis and have a good liver prognosis while being at increased cardiovascular and metabolic risk. Conversely, once NASH and fibrosis has occurred, 7-25% of patients may progress to liver cirrhosis and beyond, with considerable liver related death up

to 11% within 8-10 years (figure 1).(4)(8)(9) Recently, stages of liver fibrosis were identified as only factor associated with long-term overall mortality, liver transplantation, and liver-related events.(10) The metabolic co-morbidity is a major driver of progression to NASH and fibrosis.(1) The higher prevalence of NASH cirrhosis in patients with obesity and type 2 diabetes or patients with morbid obesity undergoing bariatric surgery highlights this concept.(11–13) Alarmingly, HCC does increasingly occur in pre-cirrhotic stages of NAFLD and NASH.(1)

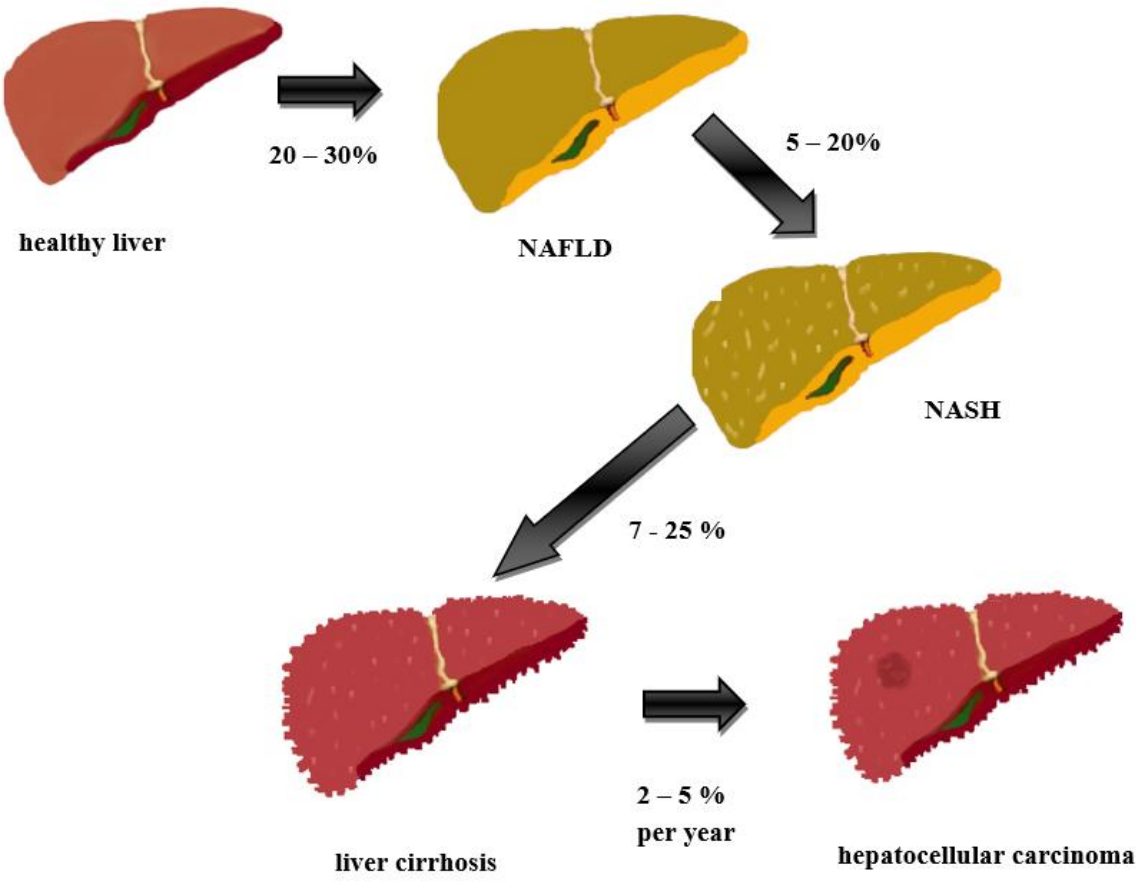


Figure 1: Prevalence of NAFLD in the general population is about 20-30%, progression of NAFLD to NASH, liver cirrhosis and hepatocellular carcinoma occurs in 5-20%, 7-25% and 2-5% respectively. (modified according to (5))

3.3. PATHOGENESIS AND COURSE OF DISEASE

NAFLD is characterized by triglyceride accumulation in hepatocytes, whereas underlying mechanisms are not completely understood.(5) Insulin resistance seems to play a major role in disease genesis, as NAFLD patients have a decreased insulin sensitivity.(5) Insulin resistance leads to an enhanced flux of fatty acids to the liver, due to increased lipolysis in white adipose tissue.(5) These free fatty acids are responsible for about two-thirds of the hepatic fat accumulation.(14) Moreover hyperinsulinemia causes an increased de novo hepatic lipogenesis due to heightened activity of transcription factors like peroxisome proliferator-activated receptor (PPAR-γ), sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP).(5) Also serum fatty acids, hepatic de novo lipogenesis(5)and diet-related ingested fatty acids(15) as well as a low very low density lipoprotein-triglyceride (VLDL) secretion of the liver(14) leads to hepatic accumulation of triglycerides.(5) The hepatic accumulation of triglycerides itself does not seem to be harmful for the liver tissue, but may be a sign of an overloading of the liver with fatty acids, which are suspected to cause lipotoxicity (figure 2).(5) An inhibition of diacylglycerol acyltransferase 1 and 2, the enzymes catalyzing the last step of triglyceride synthesis, led to an improvement of liver steatosis in mice, but intensified liver injury(14), suspecting the less catalyzed free fatty acids to be the harmful agent.(5) Particularly, accumulation of free fatty acids in mitochondria induces liver damage due to tumor necrosis factor alpha (TNF-α) and creation of reactive oxygen species (ROS)(14), leading to inflammation and a hepatic stellate cell mediated induction of fibrogenesis.(5) Free fatty acids related lipotoxicity can harm normal cell signaling, manifesting in cellular dysfunction or induction of cell death.(5) Saturated fatty acids are able to induce an upregulation of death receptors like tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas consequently leading to an activation of proapoptotic pathways and can lead to a destabilization of lysosomes followed by an activation of nuclear factor 'kappa-light-chain-enhancer' (NF-κ-B) and TNF-α as well as activation of c-jun N-terminal-kinase-dependent proapoptotic protein Bax.(5)

There are studies suggesting, that reduced levels of adiponectin and enhanced levels of TNF-α and hepatic leptin are involved in hepatic triglyceride accumulation, as being able to affect insulin sensitivity.(5) Conversely, a treatment with an anti-TNF-α antibody reduced steatosis in a mouse model.(14)

Liver fibrosis is one important complication of NAFLD and appears to be the only histological feature associated with mortality.(10) This is mediated through an activation of hepatic stellate cells, causing a hepatic deposition of extracellular matrix, such as collagen or proteoglycans.(5)

Hepatic stellate cells can be activated by the profibrotic protein transforming growth factor β (TGF-β), which can be upregulated by fatty acids and co-activated by hyperinsulinemia (figure 2).(5) The abovementioned adipokine leptin can also lead to an upregulation of TGF-β as well as to a direct activation of hepatic stellate cells, whereas adiponectin serves as an opponent to leptin.(5) Proliferation of hepatic stellate cells can also be promoted through T helper 2 cells (Th2 cells) mediated cytokines like interleukin- (IL-) 6, IL-4 and Il-13.(16) Moreover, the IL-33 is involved in hepatic fibrogenesis via the suppression of tumorigenicity 2 (ST2) receptor(16), which is explained in detail below in the text.

Furthermore hepatic stellate cells can be directly activated by Fas and TRAIL pathways during apoptosis of hepatocytes, but those also are activating Kupffer cells, which in turn ultimately activate hepatic stellate cells.(17)

The renin-angiotensin-system is suggested to be involved in hepatic fibrogenesis via angiotensin II mediated activation of hepatic stellate cells.(5)

Additionally, dietary factors, such as fructose intake, may be able to increase de novo lipogenesis in liver and to reduce lipid oxidation.(5) Massive fructose intake can activate the ChREBP pathway mentioned above.(5) Augmented fructose intake is linked to enhanced inflammation and fibrosis of the liver.(14)

Fatty acids may exist in a non-steatotic liver as well (14) and may be involved in disease progression to NASH by producing oxidative stress, which causes ultrastructural lesions in mitochondria, leading to decreased adenosine triphosphate (ATP) recovery, that were found in patients with steatohepatitis but not in patients with simple steatosis or healthy volunteers (figure 2).(1) As well genetic variants of the patatin-like phospholipase domain-containing 3 protein (PNPLA3), also called adiponutrin, enhance the risk for developing NAFLD.(5) PNPLA3 is a homologue of the adipose triglyceride lipase, responsible for mobilization of hepatic triglycerides by hydrolyze of fatty acids.(5) PNPLA3 comprises a single nucleotide polymorphism that changes an isoleucine to methionine.(18) Carriers of the PNPLA3 variant are predisposed to have more fat accumulation in liver and to have an increased risk for developing NASH.(19) Obesity seems to amplify the risk, as studies showed a prevalence of NAFLD of 84% in humans homozygous for PNPLA3 and adiposity.(18) Moreover it was identified as a risk factor for hepatocellular carcinoma in patients with NASH and there was an association found regarding advanced fibrosis.(20)

The nuclear receptor farnesoid X receptor (FXR) may play a role in disease development and progression.(21) FXR is expressed by enterohepatic tissues but also outside of gastrointestinal tissues.(21) It is activated by bile acids leading to a reduction of gluconeogenesis, an inhibition

of hepatic de-novo lipogenesis and a reduction of bile acid synthesis.(21) FXR may also be involved in disease development by interfering with the gut microbiome, as it leads to a secretion of antimicrobial peptides.(21) Moreover, FXR activation seems to suppress nuclear factor κ-B, which is induced by lipopolysaccharides of gut bacteria leading to an activation of inflammatory cells by TNF and Interleukine-1β.(22) Studies found that FXR-deficient mice were predisposed for developing NAFLD and NASH.(21)

The gut microbiome is able to modulate the immune system and is involved in glucose metabolism, vitamin synthesis, bile salts and liberation of short-chain fatty acids, for example butyrate, which helps to keep up the intestinal barrier(23) by tight junctions, or acetate and propionate, which play a role in hepatic gluconeogenesis and lipogenesis.(22) NAFLD patients were found to have an enhanced gut permeability, which was linked to the scope of liver steatosis.(14) Due to the enhanced permeability lipopolysaccharides can pass the intestinal barrier more easily and activate toll-like receptors 4 of hepatic stellate cells and Kupffer cells leading to liver fibrosis (figure 2).(22)

Lipopolysaccharides, expressed by many bacteria of our gut microbiome can be modulated by dietary factors and are increased at high-fat diet.(14) There are studies indicating NAFLD patients have an altered microbiome, as a reduced amount of Bacteroidetes and an increase of Clostridium coccoides was associated with steatohepatitis.(22)

Another microbiome-related target is the trimethylamine-N-oxide (TMAO).(24) TMAO is metabolized out of trimethylamine by hepatic flavin-containing monooxygenases.(24) Trimethylamine in turn is metabolized out of choline by the gut microbiome.(24) TMAO is involved in the metabolism of glucose and lipids and can lead to a reduction of the bile acid pool.(24) High TMAO plasma levels were associated with the presence and severity of NAFLD.(24)

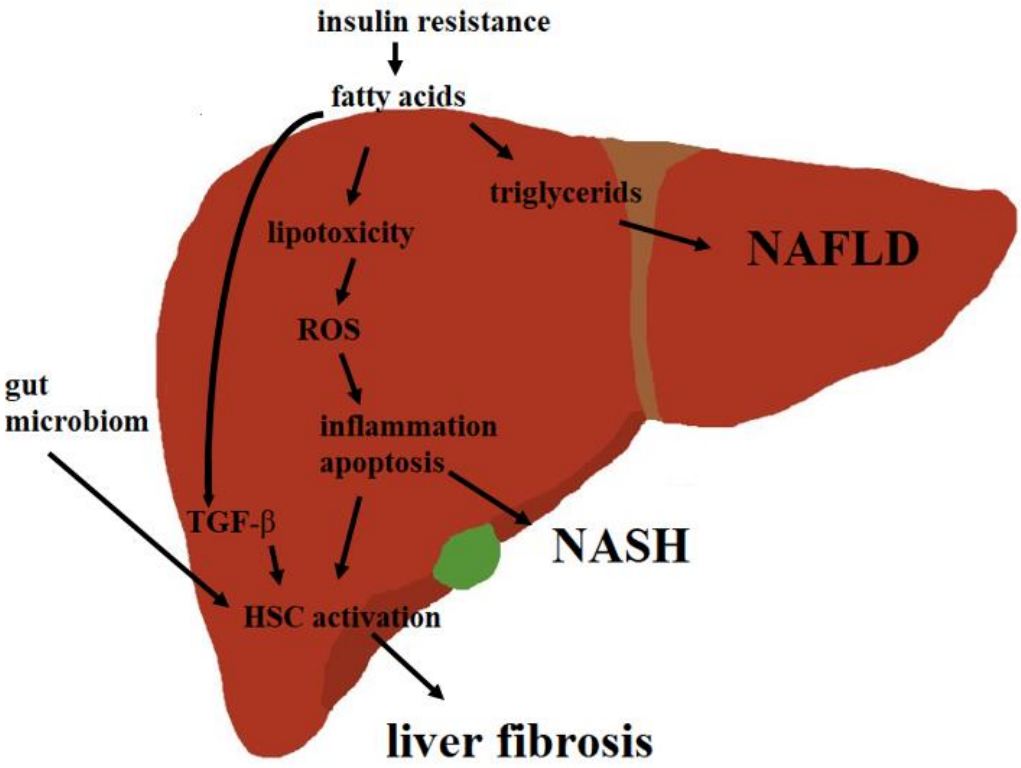


Figure 2: Insulin resistance leads to an overload of the liver with fatty acids, which, on the one hand, cause the accumulation of triglycerides in hepatocytes and consequently lead to the development of NAFLD. On the other hand, fatty acids can cause lipotoxicity, thus leading to ROS, which are responsible for inflammation and apoptosis of hepatocytes leading to NASH. Furthermore, fatty acids as well as apoptosis and gut microbiome can lead to TGF-β and hepatic stellate cell (HSC) activation, ultimately leading to liver fibrosis.

3.4. DIAGNOSIS

Usually patients with non-alcoholic fatty liver disease do not present with any symptoms. Some may feel an abdominal fullness in the region of the liver or fatigue.(1) In physical examination hepatomegaly can be found in most patients.(1)

An elevation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which cannot be explained due to other diseases, often leads to diagnosis of NAFLD. (1) The de-Ritis-quotient (AST/ALT) is normally less than 1, but enhances in advanced liver fibrosis.(1) Also serum alkaline phosphatase, γ-glutamyl transferase (γ-GT) and serum ferritin levels can be increased.(1)

Steatosis and fibrosis in patients with NALFD can be detected by ultrasound examination, where it is characterized by a higher echogenicity in comparison to those of the kidneys.(1) In a computed tomography (CT) scan the parenchyma of a fatty liver may appear less dens than

a normal liver.(1) Magnetic resonance imaging (MRI), to be more exact a magnetic resonance elastography (MRE) and a magnetic resonance imaging-proton density fat fraction (MRI-PDFF) can be used to determine liver stiffness(25) and to quantify the fatty deposit of the liver(1). MRE showed a high accuracy in quantifying liver stiffness and liver fibrosis, with a sensitivity of 85% and a specificity of about 93% for detection of advanced fibrosis (stage > 3).(25) MRI-PDFF-determined steatosis values correlate strongly with those examined by liver biopsy.(26) Moreover these MRI methods were found to be more accurate than transient liver elastography measurements.(27) However, a widespread performance is limited by the high costs, long performance time, availability and patients applicability.(25)

Liver biopsy:

Nevertheless, the golden standard to diagnose NAFLD is still liver biopsy.(28) Moreover it remains the only diagnostic possibility for the differentiation between NAFLD and NASH.(19) Histological NASH appears with hepatocyte ballooning and necrosis, inflammatory-cell infiltration and steatosis, with possible fibrosis and Mallory's hyaline while portal tracts are not affected from fibrosis.(19)

Liver biopsy allows to classify the severity of disease(1), for example by using different scoring systems, as follows:

Table 1: NAFLD and NASH Scoring System (29)

steatosis:	
grade 1	<33% of hepatocytes affected
grade 2	33% to 66%
grade 3	>66%
staging for fibrosis	
stage 1:	zone 3 perivenular, perisinusoidal, or pericellular fibrosis; focal or extensive
stage 2:	as above, with focal or extensive periportal fibrosis
stage 3:	bridging fibrosis, focal or extensive
stage 4:	cirrhosis

steatohepatitis	
grade 1, mild	➢ steatosis: predominantly macrovesicular, involves up to 66% of lobules
	➢ ballooning: occasionally observed; zone 3 hepatocytes
	➢ lobular inflammation: scattered and mild acute inflammation (polymorphonuclear cells) and occasional chronic inflammation (mononuclear cells)
	➢ portal inflammation: none or mild
grade 2, moderate	➢ steatosis: any degree; usually mixed macrovesicular and microvesicular
	➢ ballooning: obvious and present in zone 3
	➢ lobular inflammation: polymorphonuclear cells may be noted in association with ballooned hepatocytes; pericellular fibrosis; mild chronic inflammation may be seen
	➢ portal inflammation: mild to moderate
grade 3, severe	➢ steatosis: typically involves >66% of lobules (panacinar); commonly mixed steatosis
	➢ ballooning: predominantly zone 3; marked
	➢ lobular inflammation: scattered acute and chronic inflammation; polymorph nuclear cells may be concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis
	➢ portal inflammation: mild to moderate

A modified variant for disease classification including the NAFLD activity score is shown in table 2(30):

Table 2: NAFLD activity score

score for steatosis	
0	<5% of hepatocytes
1	>5% - 33%
2	>33% - 66%
3	>66%

score for lobular inflammation	
0	no foci
1	<2 foci per 200x field
2	2 - 4 foci per 200x field
3	>4 foci per 200x field
score for ballooning	
0	none
1	few balloon cells
2	many cells / prominent ballooning
staging for fibrosis	
stage 0:	none
stage 1:	periportal or perisinusoidal
- 1A	mild, zone 3, perisinusoidal
- 1B	moderate, zone 3, perisinusoidal
- 1C	portal, periportal
stage 2:	perisinusoidal and portal/periportal
stage 3:	bridging fibrosis
stage 4:	cirrhosis

According to this categories, the NAFLD activity score (NAS) can be calculated using the score for steatosis, lobular inflammation and ballooning resulting in a value between 0 and 8, whereby 0-2 is clarified as 'no steatohepatitis existent' and a score ≥5 is an indicator for the presence of steatohepatitis.(30) Compared to the algorithm given in table 1, fibrosis stage 1 is divided into 3 further subclassifications, whereas the description of ballooning is reduced to "none", "few" and "many".(30)

Another tool for the histological assessment of NAFLD is the steatosis, activity, fibrosis score (SAF score), as shown in table 3.(31)

The steatosis score describes the quantity of steatosis affected hepatocytes. The activity score describes the quantity of hepatocyte ballooning and lobular inflammation.(31) Inflammatory cells within the lobule are defined as lobular inflammation, whereby no present inflammatory cells are classified as "none lobular inflammation = 0", ≤2 inflammatory foci per 20x magnification is classified as "grade 1" and ≥ 2 foci are classified as "grade 2".(31) Ballooning is also graded from 0 to 2, whereas "grade 0" is defined by normal hepatocytes, "grade 1" comprises clusters of hepatocytes with a rounded shape and a reticulated and pale

cytoplasm, “grade 2” additionally implies enlarged hepatocytes.(31) The fibrosis activity is classified according to the scoring system mentioned in table 2.(31)

For the activity grade the values mentioned above are added, resulting in the following activity score (table 3).(31)

Table 3: SAF scoring system	
steatosis score (S)	
0	<5% of hepatocytes affected (no steatosis)
1	5-33% (mild steatosis)
2	34-66% (moderate steatosis)
3	>67% (marked steatosis)
activity score (A)	
0	no activity
1	mild activity
2	moderate activity
3	severe activity
fibrosis score (F)	
0	no fibrosis
1	1a / 1b – perisinusoidal zone 3 fibrosis
	1c – portal fibrosis
2	perisinusoidal and periportal fibrosis without bridging
3	bridging fibrosis
4	cirrhosis

The SAF score results in an algorithm tree, whereby NAFLD is diagnosed by grade 1 steatosis. If at least grade 1 is classified in each of the features steatosis, lobular inflammation and ballooning, then the tissue sample is categorized as NASH.(31)

Considering that liver biopsy is an invasive method, its disadvantage involves a risk of complications like bleeding(32) or visceral injury(25), making it problematical to perform it repetitive for an observation of disease progression.(33) Moreover, its accuracy can be affected by several variations concerning sampling variability and evaluation.(32) Due to the small sample size of only about 1/50,000 of the liver and the heterogeneously distribution of liver fibrosis(34), a sample bias can occur.(35) Therefore it is necessary to collect an adequately size

including enough portal tracts.(36) A study showed, if biopsy was taken of both liver lobes, discordant results regarding fibrosis stage appeared in up to 50%.(35)
Regarding the evaluation studies showed a difference in biopsy classification between expert liver pathologists and general pathologists.(37) Though, using scoring systems, like the SAF score, can reduce the interobserver variability significantly.(38)

Transient elastography:

A non-invasive technology allowing the measurement of liver fibrosis and steatosis is the transient elastography. Typical cut-off values for fibrosis staging, including measurements using XL-probe in obese patient, are shown in table 4(32):

Table 4: fibrosis grading score for fibroscan examinations(32)

M-Probe	**XL-Probe**
Stage ≥ 2: 7,0 kPa	Stage ≥ 2: 6,2 kPa
Stage ≥ 3: 8,7 kPa	Stage ≥ 3: 7,2 kPa
Stage = 4 10,3 kPa	Stage = 4 7,9 kPa

Liver steatosis is measured through the proprietary controlled attenuation parameter (CAP) acquisition protocol(39), quantifying the attenuation of emitted ultrasound waves as they move through the liver tissue.(40)
A meta-analysis showed a sensitivity and specificity of 79 % and 75% for the diagnosis of fibrosis stage ≥ 2, 85% and 85% for fibrosis stage ≥ 3, 92% and 92% for fibrosis stage 4 respectively.(41)
Advantageous transient elastography measurement involves an approximately 4cm² cylinder of the liver parenchyma, which is an about 100 times bigger part of the liver investigated than examined with liver biopsy samples.(33)
Results can be affected by liquid, which is why measurement must not be above large vessels(28) and is impossible in patients with ascites.(42) There are also limitations regarding obese patients, because adipose tissue can attenuate the shear and ultrasound waves strongly.(42) Moreover, a too small intercostals space can impede stiffness measurement.(42)
As pressure exerted on the device by the investigator also influences results, a software shows warnings on the computer when pressure is not appropriate.(28)

NAFLD fibrosis score:

Another non-invasive tool to discriminate fibrosis stages is the NAFLD fibrosis score. This score is composed of the parameters age, BMI, diabetes, AST, ALT, platelets and albumin and has a predictive positive value of 82-90 % and a negative predictive value of 88-93 %.(43) Results include two cut-off values, <-1,455 allows to exclude the presence of severe liver fibrosis (stage 3 or higher) and >0,676 suggest the presence of advanced fibrosis.(25) NAFLD fibroses score is of considerable prognostic value for predicting long term outcome in addition to predict the presence of fibrosis, emphasizing the relevance of metabolic comorbidity.(43)

3.5. THERAPY

Considering obesity and diabetes are common co-morbidities in patients with NAFLD, weight reduction is recommended as an important step of therapy.(44) Studies showed, particularly in patients with NASH, that a weight loss of 7-10%, as a result of lifestyle intervention, including diet and physical training, reduces histologic findings of NASH, such as steatosis, inflammation and ballooning injury, and levels of ALT, whereas fibrosis was not influenced by this amount of weight loss.(45) Whereby an augmented weight loss, thus more than>10% showed an improvement of fibrosis too.(46)

3.5.1. BARIATRIC SURGERY

Bariatric surgery and the subsequent weight loss led to a reduction of ALT and AST in multi-morbid obese patients.(47) NAFLD patients receiving a Roux-en-Y gastric bypass showed a significant decrease of NAFLD-Fibrosis Score.(12)

A Meta-Analysis of fifteen studies found a complete resolution of NASH after bariatric surgery (mainly Roux-en-Y gastric bypass) in 69,5% and an improvement of NASH in even 81,3%. Moreover, those data showed an improvement of liver fibrosis in 65,5% and steatosis in 91,6% quantified by liver biopsy.(48)

3.5.2. DRUG TREATMENT

Drug treatment is indicated in patients with progressive NASH or NASH with high necroinflammation.(19) Therapy is used off-label, as there are no admissions of regulatory agencies due to absent phase III trials concerning patients with NASH.(19) Most medications

aim for co-morbidities of NAFLD, such as the metabolic syndrome, including dyslipidemia or hypertension.(49)

Vitamin E

Vitamin E is a controversial seen antioxidant. There are studies showing it could decrease ALT levels, steatosis and inflammation in patients with NASH.(49)

For example, the PIVENS trial investigated the changes in NAFLD Activity Score due to an intake of 800mg Vitamin E per day. Results showed a significant decrease of the NAFLD Activity score, serum levels of ALT and liver steatosis, whereas it failed in reduction of liver fibrosis stage or curing NASH.(49) On the other side, there is a lively discussion about an increased overall mortality(19) and a higher relative risk for prostate cancer, caused by Vitamin E intake.(49) The SELECT study resulted in an enhanced relative risk for prostate cancer of 17%, though this may be due to a single nucleotide polymorphism concerning the metabolism of Vitamin E, as there was found an lower absolute risk for prostate cancer.(49) Moreover, studies reported a higher relative risk for hemorrhagic stroke (22%) but a lower risk for ischaemic stroke (10%).(49)

Pioglitazone

Pioglitazone is an agonist of peroxisome proliferator-activator receptor γ, normally used in the treatment of diabetes mellitus.(49)

The PIVENS study did not result in an improvement of histologic findings of NASH, but patients receiving pioglitazone showed lower serum levels of aspartate transaminase.(50) In contrast, a meta-analysis, also including the PIVENS trial, showed an improvement of liver steatosis, ballooning, necroinflammation as well as lobular inflammation.(51) Side effects of pioglitazone include a gain of weight(51), an enhanced risk for congestive heart failure, bladder cancer and osteoporosis, why usage has to be considered carefully in treatment of NASH.(49) However, a recent meta-analyses found no association of bladder cancer and pioglitazone.(52)

PUFA

A diet with high amounts of polyunsaturated fatty acids (PUFA) showed an enhanced insulin sensitivity in rats and a decrease of triglycerides and NASH in mice models.(53) Clinical trials showed distinct results regarding the resolution of NASH and reduction of fat accumulation in the liver.(53) A double-blinded placebo controlled trial found no significant improvement of NASH, whereby liver fat content was ameliorated by n-3-PUFA.(54)

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) showed an enhancement of biochemical markers, but not of liver histology.(19) Nor-UDCA, a side chain shortened variant of UDCA, exhibited a decrease of liver injury signs, such as fibrosis, steatosis, inflammation or apoptosis in mouse models.(55) It is contemporary tested in a phase 2 trial in NAFLD.(55)

3.5.3. DRUGS TESTEDIN CLINICAL TRIALS FOR THE TREATMENT OF NAFLD AND NASH

Obeticholic acid

A multicenter phase IIb clinical trial (FLINT study), concerning the effect of obeticholic acid, a farnesoid X receptor (FXR) agonist, in patients suffering from NASH, showed an improvement of NAFLD activity score in liver biopsy without worsening of liver fibrosis compared to placebo, whereas resolution of NASH was not significantly higher compared to placebo.(56)

Obeticholic acid also led to a decreased level of high-density lipoprotein cholesterol, while low-density lipoprotein cholesterol was heightened(56), leading to an uncertainness concerning long-term concomitant metabolic effects.(57) Another main adverse event, affecting up to 33% of probands, was pruritus.(56)

Further FXR agonists are currently tested in randomized clinical trials with NAFLD patients.(21) The synthetic, nonsteroidal agonist GW4064 exhibited an amelioration of insulin resistance and sugar metabolism in mice.(21) It reduced levels of free fatty acids, triglycerides and cholesterol in mouse studies and moderated the fat accumulation in hepatocytes.(21) The agonist WAY-362450 showed a benefit in experimental NASH by decreasing fibrosis and inflammation.(21) However, there are data suggesting nonsteroidal FXR agonist can be allied with weight gain, impairment of liver steatosis and glucose metabolism.(21)

Elafibranor

Elafibranor is an agonist of PPAR α and δ. Former trials demonstrated a PPAR- δ-activation mediated increase of hepatic and peripheral insulin resistance.(58) Also Elafibranor was able to improve lipid profiles and liver enzymes compared to placebo. Furthermore, in NASH patients it led to a dose-depended reduction of hepatic fibrosis and steatosis(58).

Except of a reversible increase of serum creatinine levels the drug was well-tolerated in previous studies. Elafibranor is currently undergoing a phase III trial.(58)

Cenicriviroc

Cenicriviroc is an antagonist of C-C chemokine receptors type 2 and 5, whose ligands are involved in inflammatory cell recruitment and led to an activation of hepatic stellate cells in the liver.(49)

A randomized, double-blinded multinational phase 2b study concerning patients with NASH, showed a reduction of fibrosis stage ≥1 compared to placebo after a treatment period of one year.(59) Furthermore it led to a decreased level of cluster of differentiation (CD)14, which is participating in inflammatory cell activation and a decrease of fibrosis-4-score in patients with HIV.(60)

SGLT2-inhibtors

In mice suffering from NAFLD sodium glucose co-transporter 2 inhibitors (SGLT2-Inhibitors) showed an enhancement of steatosis, inflammation and fibrosis. In humans SGLT2-Inhibitors helped at weight loss, reduced levels of ALT and fatty liver index score.(60)

Liraglutide

Liraglutide, a glucagon-like peptide-1 receptor agonist, usually used for patients with type 2 diabetes, showed an improvement of hepatic steatosis and insulin resistance due to an activation of adenosine monophosphate (AMP)-activated protein kinase in animal models.(61) In a small randomized trial of patients with NASH Liraglutide ameliorated histological signs of steatohepatitis.(61)

Semiglutide is another upcoming glucagon-like peptide-1 receptor agonist, that gained a positive opinion in the EU for type 2 diabetes and is currently tested for treatment of NAFLD too.(62)

3.5.4. TREATMENT OF NAFLD-ASSOCIATED LIVER CIRRHOSIS

In the United states liver cirrhosis due to NAFLD is one of the most 3 reasons for liver transplantation.(63) The 3- and 5-year survival after liver-transplantation is not decreased in NAFLD patients compared to those without NAFLD receiving a liver transplantation. Moreover, graft failure is lower, whereas the risk of death due to sepsis or cardiovascular problems is increased.(19) Also there is an association between overall mortality after transplantation and BMI and diabetes in NAFLD patients, as reports show a 1-year mortality rate of 50% in patients with BMI >35kg/m^2.(19)

3.6. ASSOCIATED DISEASES

NAFLD is considered to be a hepatic manifestation of the metabolic syndrome(64), including an increased waist circumference, low high-density lipoprotein (HDL-) cholesterol levels and high triglyceride levels, an impaired fasting glucose or type 2 diabetes mellitus as well as increased blood pressure.(19) NAFLD and components of the metabolic syndrome seem to influence each other bi-directional.(65) Suffering from components of the metabolic syndrome enhances the probability of developing NAFLD, as well as NAFLD seems to be a precursor of the metabolic syndrome.(65) About 90% of NAFLD patients additionally suffer from more than one constituent of the metabolic syndrome too.(66)

As mentioned above, up to 70% of patients with type 2 diabetes are developing NAFLD, but there are studies showing that conversely NAFLD patients without type 2 diabetes have a twofold higher risk of developing type 2 diabetes, independent of other risk factors like obesity.(65) Metformin remains first line therapy for patients with type 2 diabetes and NAFLD, though it could not show any improvement of liver histology or serum transaminases.(60) Furthermore NAFLD patients have a higher risk for cardiovascular diseases including early carotid intima-media thickness, coronary plaques and endothelial dysfunction.(66) Up to 50% of NAFLD patients suffer from arterial hypertension.(65) Moreover they have an increased prevalence of ischaemic heart disease, which is the leading cause of death in patients with NAFLD.(67)(66) This risk might especially be increased with advanced fibrosis.(68) Co-morbidities of NAFLD, like arterial hypertension should be treated according to guidelines, whereby angiotensin receptor blockers may be advantageous by operating anti-fibrotic concerning liver fibrosis.(60)

Most recently, the grade of hepatic steatosis was identified as a risk factor for developing endothelial dysfunction in patients with NAFLD(69) supporting the role of sinusoidal endothelial dysfunction in inflammation and fibrosis in NAFLD.(70) The patho-mechanisms are manifold, not only comprising common risk factors of these two diseases like obesity, type 2 diabetes mellitus, hyperlipidemia, hypertension or physical inactivity, but also including other mechanism like oxidative stress, which is common in the genesis of NASH as mentioned above, leading to oxidation of LDL, then incorporated by macrophages, also induced to adhesion to the arterial wall by ROS.(71) Regarding this, dyslipidemia should be treated with statins, which can reduce mortality in patients with NASH.(60)

Also higher levels of inflammation markers, such as c-reactive protein (CRP) and prothrombotic mediators like plasminogen-activator-inhibitor 1 are reported in patients with NAFLD.(72).

Moreover, NAFLD is also suggested to be associated with chronic kidney disease, up to 55% of patients with NAFLD were found to suffer from chronic kidney disease as well.(73)

4.) ST2

4.1. DEFINITION & PATHWAY

Suppression of tumorigenicity 2 (ST2) belongs to the Toll-like/Interleukin- 1 (IL-1) receptor family and arbitrates the biological effects of Interleukin-33 (IL-33), which is a member of the IL-1 cytokine family. Due to alternative splicing 3 isoforms exist: the trans-membrane receptor ST2L, a soluble ST2 and a variant form.(74)

The trans-membrane receptor ST2L:

The ST2 receptor is found in different cells, such as hematopoietic cells, macrophages, lymphocytes, neutrophil granulocytes, but also in osteoclasts and osteoblasts, endothelial cells, cardiomyocytes and adipocytes.(74)

Binding of the ligand IL-33 leads to a recruitment of the myeloid differentiation primary response protein 88 (myD88), the Il1-receptor associated kinase 1 and 4 due to the IL-1 receptor accessory protein(75), consequently leading to an inflammatory response by activation of NF-κB of activated B-cells, mitogen activated protein (MAP)-kinases, activator protein 1 (AP-1) and other mediators causing inflammation (figure 4).(76) Furthermore it causes chromatin compaction and transcription inhibition, by acting as an intracellular nuclear factor.(74)(76) IL-33 is suggested to be released during necrosis serving as an endogenous danger or alarming signal (figure 4).(76)

It also plays a role in the activation of T-helper 2 (Th2) cells immune responses, as IL-33 binding at the ST2 receptor of Th2 cells leads to a production of IL-5 and IL-13 (figure 3).(76)Also, it is responsible for the differentiation of naive T-cells into Th2 cells (figure 3).(75) Moreover, ST2 is suggested to be involved in bacterial immune defense, as studies showed an IL-33 induced and ST2 receptor mediated increase of the lipopolysaccharide-induced inflammatory cytokine production of macrophages in a mouse model (figure 3).(77) Also other hematopoietic cells, like B-cells, neutrophil and eosinophil granulocytes seem to be affected by the ST2 / IL-33 pathway.(76) More precisely, the IL-33 mediated release of IL-5 and IL-13 mentioned above leads to an increased production of anti-ox-low density lipoprotein (LDL) antibodies by B-cells. Those are able to prevent the binding of LDL to macrophages, consequently resulting in a reduced plaque inflammation in atherosclerotic arterial walls in a mouse model (figure 3).(76) In neutrophils IL-33 prevents the Toll-like-receptor mediated inhibition of chemotaxis, besides studies proved an IL-33 induced migration of neutrophils to

joints affected by rheumatoid arthritis.(76) Basophil granulocytes respond to IL-33 by cytokine production, adhesion and degranulation (figure 3).(76)

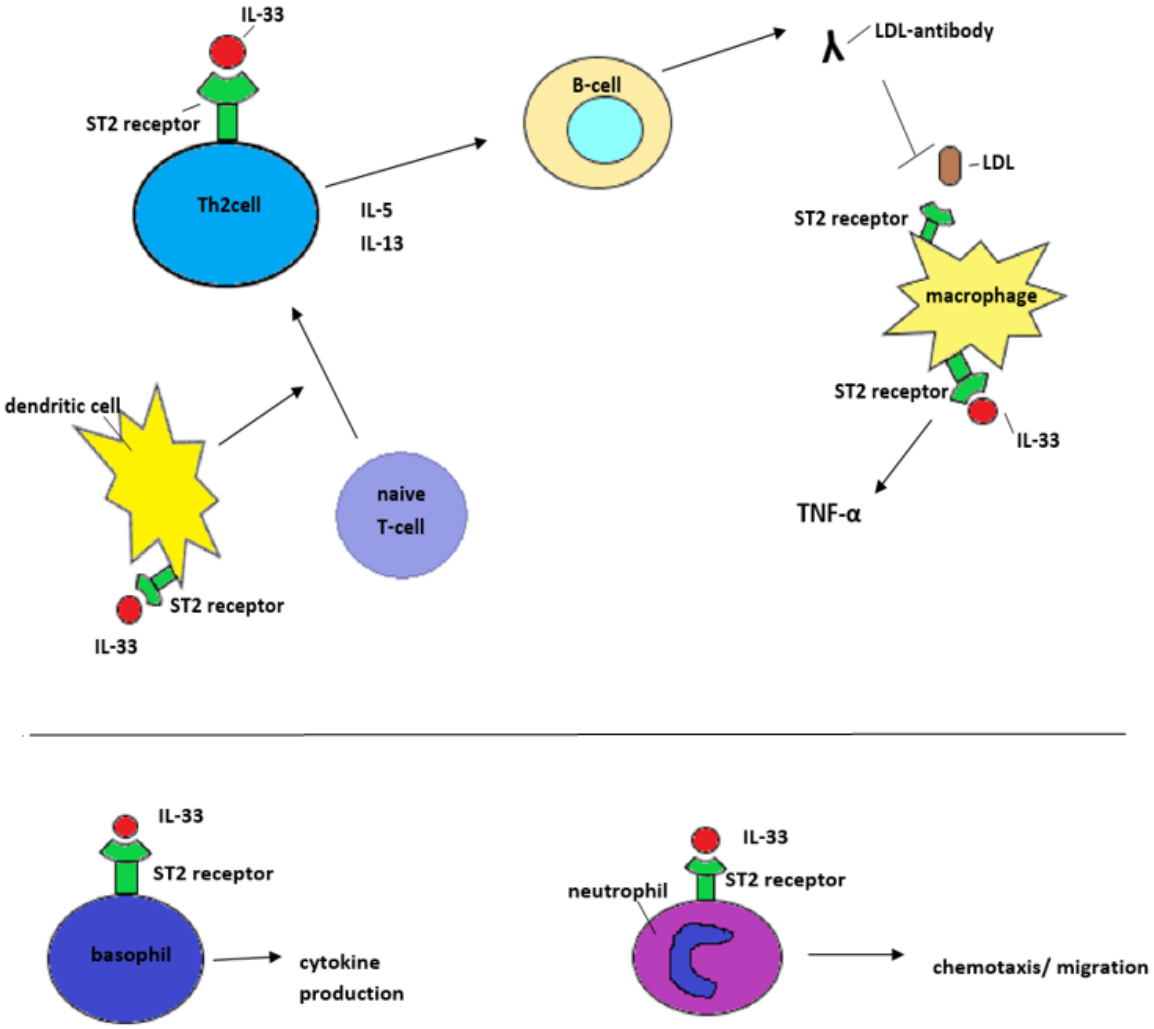


Figure 3: dendritic cells lead to an IL-33 mediated switch of naïve T-cells to Th-2-helper cells; IL-33 induces to an IL-5 and IL-13 production by Th2-helper cells; IL-5 and IL-3 lead to a B-cell mediated production of LDL-antibodies, which prevent LDL from binding to macrophages resulting in less atherosclerotic plaque inflammation; IL-33 stimulates TNF-α production of macrophages;
basophil granulocytes produce cytokines when activated by IL-33; neutrophil migration is induced by IL-33

In non-hematopoietic cells, like endothelial cells, IL-33 leads to morphologic differentiation and migration resulting in impacts on angiogenesis.(76) Moreover, endothelial cells are able to

regulate the local effects of IL-33 as they can secrete the soluble form of ST2.(76)

Soluble ST2:

Studies found that soluble ST2 is a decoy receptor of IL-33, which is able to neutralize the effects of IL-33, as it is no more able to bind at the ST2 receptor (figure 4).(76) The highest occurrence of soluble ST2 (sST2) was found in the lung, but also potential sources are found in the cardiovascular system, more precisely the cardiac myocytes and endothelial cells.(76)

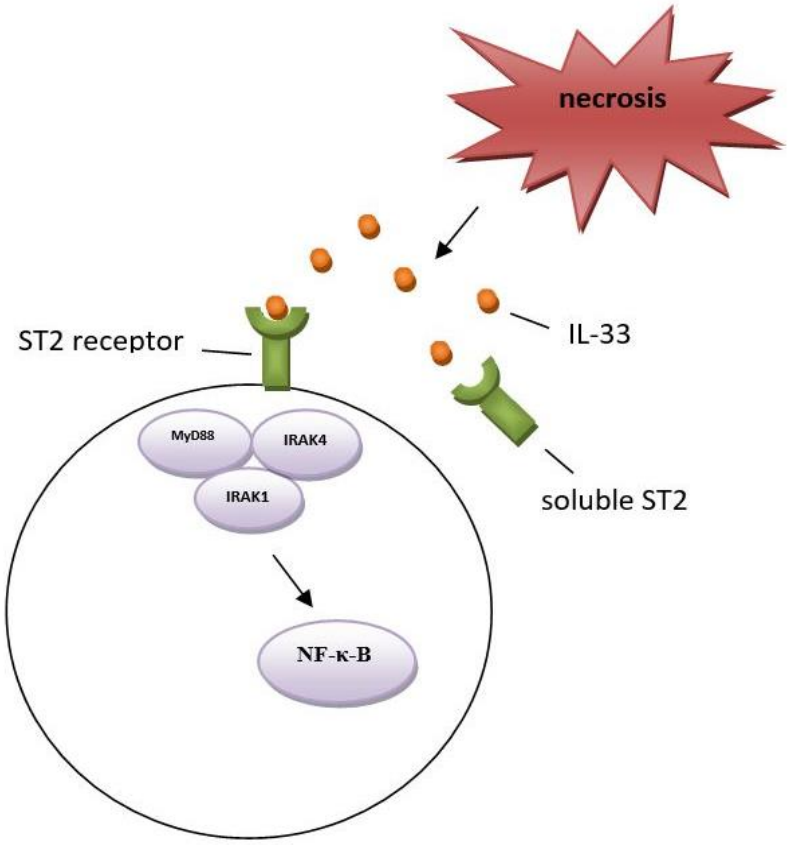


Figure 4: Interleukine-33 (IL-33) is released during necrosis, leading to an inflammatory response by activation of nuclear factor 'kappa-light-chain-enhancer' -B (NF-κB). Soluble ST2 functions as a decoy receptor and neutralizes the effects of IL-33.

In patients with chronic hepatitis C serum concentrations of IL-33 and also of soluble ST2 were significantly higher compared to those of healthy controls.(78) Although IL-33 levels did not correlate with soluble ST2 levels, IL-33 correlated positively with AST and ALT. Moreover, a treatment with interferon decreased IL-33 levels significantly.(78)

4.2. ST2 AND LIVER FIBROSIS

ST2 is involved in hepatic fibrogenesis via its ligand IL-33, which is set free due to hepatocellular stress leading to an IL-13 production by innate lymphoid cells ultimately activating hepatic stellate cells, which then are causing an excessive deposition of extracellular matrix, particularly collagen, in the liver (figure 5).(79)(80)

In immunohistochemical analyses of liver tissue from NASH patients IL-33 was found in hepatic stellate cells, endothelial cells and hepatic sinusoids.(81)

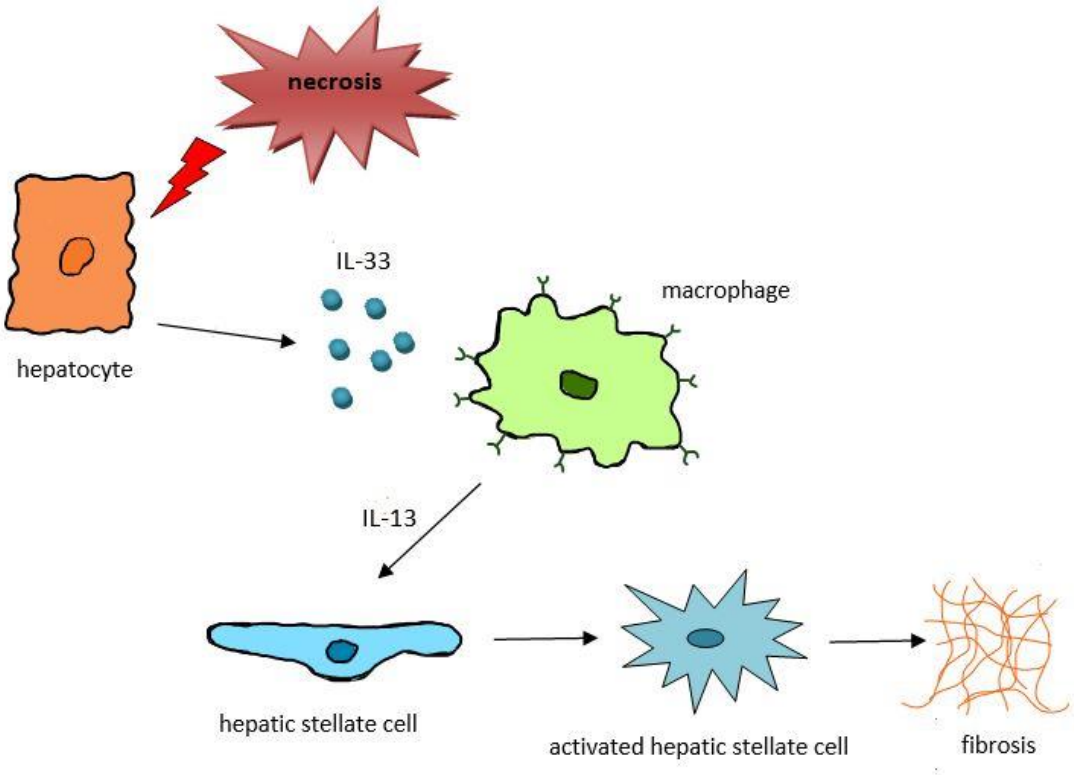


Figure 5: Interleukine-33 (IL-33) is released due to necrosis, leading to an inflammatory response by macrophages, leading to a release of IL-13, which is able to activate hepatic stellate cells. Those are involved in liver fibrogenesis by accumulation of extracellular matrix.

Also the Th2 cells seem to have an impact on developing liver fibrosis, as T helper 1 (Th1) - type mouse strains developed liver fibrosis after a high-fat diet and had higher levels of IL-33, IL-13 compared to Th2 cells mouse strains.(64)

Moreover, ST-receptor deficient mice showed less liver fibrosis, steatosis and inflammation and lower levels of IL-33, IL-13 and a reduced deposition of collagen compared to wild type mice.(64)

Mice with diet induced fatty liver disease showed significant higher levels of IL-33 and ST2 compared to controls.(81) Moreover, treatment of these mice with a recombinant IL-33 led to improved values of blood sugar, ALT and triglycerides and a reduction of weight.(81) Additionally, treatment with IL-33 reduced steatosis but aggravated liver fibrosis.(81) Also, a switch from a Th1 to a Th2 cell immunological profile of the liver was observed in this study.(81)

There are references, that Galectin-3 interacts with the IL-33/ST-2 pathway.(64) Galectin-3 is a circulating β-galactosidase-binding lectin, which is secreted by immune and inflammatory cells, such as activated macrophages and is also already used as a biomarker in cardiology, a sGal-3 it is associated with premature myocardial infarction.(82) Hepatocytes of high-fat diet fed Galectin-3 knockout mice expressed less IL-33 and hepatic IL-13 than those of wild type mice. Macrophages of those mice could not respond to IL-33, which led to fewer CD11b+ myeloid cells and less ST2 receptor upregulation and IL-13 production (figure 6).(64)

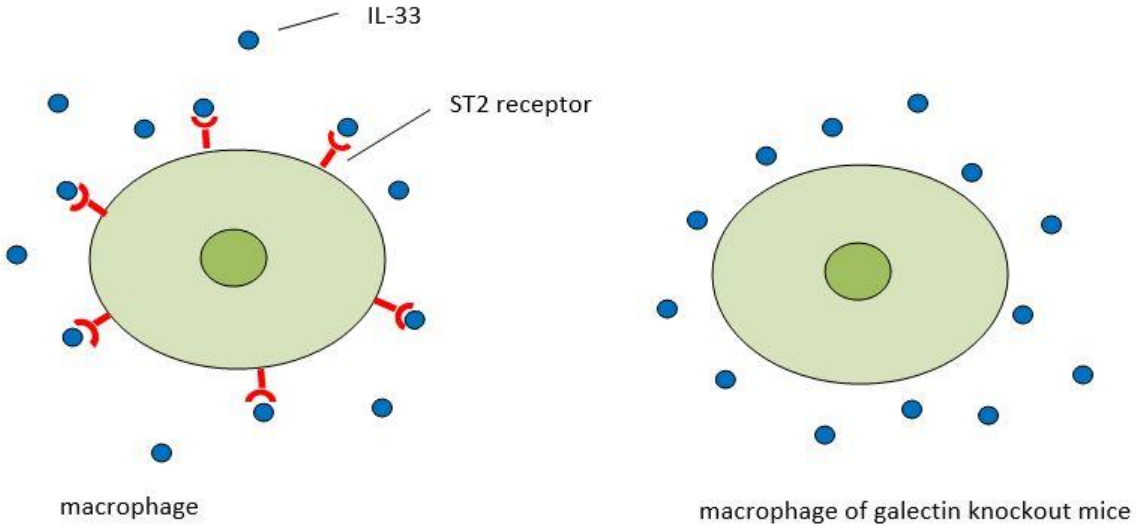


Figure 6: Macrophages of Galectin-3 knockout mice cannot respond to IL-33, subsequently upregulation of the ST2 receptor an IL-13 production is decreased.

Interestingly in chronic alcohol fed mice the ST2 pathway was protective regarding ALT levels as ST2 receptor deficient mice showed higher levels of ALT. Conversely, inducing cell death consequently leading to a release of IL-33 led to lower levels of inflammation markers in ST2

receptor deficient mice.(83) This data suggest, that there are two different mechanism of acting regarding ST2 in the liver.(83) Binding of IL-33 to the ST2 receptor leads to a formation of an IL-33/ST2 receptor complex including the IL-1 receptor accessory protein (IL-1RAcP). These protein is essential for evolving the IL-33 effects.(84) An activation of the ST2 receptor by other toll like receptor does not lead to a recruitment of IL-1RAcP and consequently NF-κ-B is inhibited, causing the protective effects described above.(83)

4.3. ST2 AND CARDIOVASCULAR DISEASES

In atherosclerosis the ST2 receptor is involved by its effect on Th2 cells. T cells have different effects on the vessel wall.(76) While Th1 cells enhance inflammation in the plaques of atherosclerotic vessels, Th2 cells reduce the inflammatory response.(76) IL-33 administration led to a shift from Th1 cells to Th2 cells resulting in significantly smaller plaques compared to controls in mouse models.(85) Backwards, treatment with the soluble form of ST2 resulted in larger atherosclerotic plaques, underlining the neutralizing effect of sST2.(76) Above, IL-33 reduces the formation and accumulation of foam cells in atherosclerotic plaques by reducing the LDL uptake and enhancing the cholesterol efflux.(86)

In the heart, more precisely in cardiomyocytes and cardiac fibroblasts IL-33 is produced due to biomechanical strain. (87)In vitro studies showed that IL-33 antagonizes angiotensin-II-induced hypertrophy(87) and reduces cardiomyocyte apoptosis and fibrosis(76), whereas treatment with sST2 led to more hypertrophy.(87) Levels of soluble ST2 can be increased by endothelin-1.(76) Echocardiography results revealed that ST2 receptor deficient mice showed greater cardiac hypertrophy and a reduced systolic function.(87) Analysis of rats showed an IL-33 / ST2 mediated decrease of apoptosis of cardiomyocytes and an improvement of cardiac contractility after myocardial infarction through reducing of caspase-3 and enhancement of antiapoptotic proteins.(88)

4.4. ST2 AND OTHER ORGANS

In obesity IL-33 had protective effects, it led to a decrease of adiposity and enhanced the glucose and insulin tolerance, while reducing the fasting glucose.(89) Furthermore, expression of IL-33 and ST2 in human adipose tissue is increased in severe obesity predominantly in endothelial cells leading to increased adhesion, inflammation and angiogenesis as well as vascular permeability.(90) Plasma levels of soluble ST2, but not those of IL-33, were elevated in those patients.(90) Studies indicate that the IL-33/ST2 pathway plays a protective role in obesity,

adipose tissue inflammation and atherosclerosis, but it seems to be profibrotic regarding the genesis of NASH.(64)

Also in fibrotic processes of other organs ST2 seems to be involved, as mice treated with bleomycin to induce pulmonary fibrosis showed significant higher levels of ST2 compared to controls.(91) Regarding the lung, patients suffering from asthma had increased levels of ST2 and IL-33 compared to healthy controls too.(92)

Moreover, patients with chronic kidney disease showed significant higher levels of the soluble form of ST2 than controls, whereas the levels of IL-33 did not differ.(93)

Interestingly, high concentrations of soluble ST2 in patients undergoing hematopoietic cell transplantation were a prognostic marker for the occurrence of graft versus host disease.(94) Blocking the soluble ST2 with a monoclonal antibody and thereby its trapping of IL-33 led to a decreased severity of graft versus host disease and its mortality.(94) Furthermore, $CD4^+$-T cells and intestinal endothelial and stromal cells were identified as major sources of soluble ST2.(94)

4.5. DIFFERENT EFFECTS OF IL-33

To give an overview, some of the most important IL-33 mediated effects are specified repeatedly, as follows:

In general IL-33 is set free during necrosis as a danger or alarming signal, via the ST2 receptor it causes inflammation.(76)

In the cardiovascular system IL-33 arbitrates a Th2 cell mediated production of IL-5 and IL-13, consequently leading to an increased production of anti-ox-LDL antibodies by B-cells, which are able to prevent the binding of LDL to macrophages(76) resulting in significantly smaller plaques of atherosclerotic vessels.(85) IL-33 antagonizes angiotensin-II-induced hypertrophy(87) and reduces cardiomyocyte apoptosis and fibrosis.(76)

In hepatic fibrogenesis IL-33 leads to an IL-13 production by innate lymphoid cells ultimately activating hepatic stellate cells, which then are causing an excessive deposition of extracellular matrix, particularly collagen, in the liver.(79)(80)

4.6. CURRENT USAGE OF ST2 AS A BIOMARKER

Soluble ST2 is elevated in patients with stable coronary artery disease and impaired left ventricular diastolic function(95) also linked to cardiac remodeling(96) and chronic inflammation and is used as a prognostic biomarker in chronic heart failure.(97) As a biomarker

of myocardial fibrosis ST2 is a predictor for hospitalization and death in patients with heart failure.(98) As a biomarker of myocardial fibrosis, clinical usage of ST2 is recommended during acute situations and ambulatory settings in the guidelines of the American College of Cardiology Foundation and the American Heart Association(98), though the level of evidence for its benefit was degraded in the latest guidelines.(99) It is now suggested to be advantageous in patients with stage C or D heart failure or hospitalized patients with acute decompensation of heart failure with a weak (class IIb) recommendation, compared to the biomarker N-terminal prohormone of brain natriuretic peptide (NT-pro-BNP), which is strongly recommended.(99)

5.) AIM OF THIS STUDY

Issue:

The lack of reliable non-invasive biomarkers for diagnosis and pathophysiological mechanisms of disease severity and prediction of prognosis is one of the major drawbacks in the clinical management of patients with NAFLD. There are no specific diagnostic and prognostic markers, which allow an adequate assessment of the NAFLD-associated complications, such as liver fibrosis.

Aim:

The current proposal was expected to result in the identification of a novel non-invasive diagnostic and prognostic parameter for the management of NAFLD. Currently used non-invasive tools are for example scoring systems like the Fibrosis 4 (FIB-4) index (regarding age, AST, ALT and platelet count)(100), the above mentioned NAFLD-fibrosis score(43) and the enhanced liver fibrosis (ELF-) test.(101) The ELF test is based on quantitative values of hyaluronic acid, amino-terminal propeptide of type III procollagen and tissue inhibitor of metalloproteinase 1 in human serum.(101) These scores might be appropriate for the differentiation between advanced and non-advanced liver fibrosis as well as the prediction of long-term outcome and mortality.(19) Moreover, they exhibit high negative predictive values for excluding severe fibrosis, wherefor they can be used for first-line risk stratification in patients unlikely to have advanced liver fibrosis.(19) However, they are not recommended as diagnostic tools for patients with higher stages of liver fibrosis and for follow-up, where liver biopsy remains necessary.(19)

Hence, our aim was to determine, whether the biomarker ST2 is elevated in patients with different stages of histological proven liver fibroses due to non-alcoholic fatty liver disease. Our hypothesis was that the level of the serum biomarker ST-2 correlates with liver fibrosis stage and its progression.

We hypothesized that the level of the serum biomarker ST2 is significantly higher in patients with severe liver fibrosis (fibrosis stage 3 or 4) than in those with mild liver fibrosis (fibrosis stage 0,1 and 2).

Furthermore, it was tested, if results of fibrosis staging with transient elastography accord to fibrosis stages determined by liver biopsy. Another aim was the comparison of the serum biomarker with a non-invasive assessing tool, the NAFLD-fibrosis score.

6.) METHODS

6.1. STUDY DESIGN

This study was a retrospective explorative single-center cohort study to evaluate the correlation of liver fibrosis with the serum level of ST2 in patients with non-alcoholic fatty liver disease (NAFLD). 53 patients participated in our cohort study. All patients are under medical treatment at the outpatient clinic at the Division of Gastroenterology and Hepatology in the Department of Internal Medicine III at the general hospital of Vienna.

This study was performed at the department of Internal Medicine III mentioned above and the study protocol was confirmed by the ethical review committee of the Medical University of Vienna. (ethics vote number: 2186/2017)

Retrospective data included a liver biopsy to prove the diagnosis of NAFLD and to measure liver fibrosis stages and hepatic steatosis and a blood sampling to measure the biomarker level and other parameters regarding liver functions. Some of the patients also had fibroscan examinations. Demographic and physical data, including sex, age, height, weight and body mass index were examined through anamneses and physical examinations. All data were examined between September 2012 and March 2016.

6.2. STUDY POPULATION

Patients between 18 and 85 years (at the time of liver biopsy and blood sampling) with histological proven NAFLD in the abovementioned period have been included, provided they did not consume alcohol (the cut-off was <20g/d for females and <30g/d for men). Other exclusion criteria were liver diseases, such as active Hepatitis B or C, Morbus Wilson, Haemochromatosis, or infectious diseases (such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), Tuberculosis), a HbA1c higher than 9%, pregnancy or breast-feeding and a participation in another clinical trial.

For the comparison of transient elastography and ST2 serum levels, patients with a valid fibroscan result within 6 months to liver biopsy have been included. For the comparison of the fibrosis score and ST2 serum levels patients with available data needed for the calculation of the score were included.

Patients who were considered suitable by the investigator have been asked whether they are interested in participating in this study.

6.3. LIVER BIOPSY AND HISTOLOGY

After determination of blood coagulation, percutaneous liver biopsy was performed ultrasound assisted in fasting conditions and on patients lying in supine position. Prior to the biopsy, all patients got a local anesthesia at puncture site.

Histological analysis was performed by a board of certified liver pathologist. Therefor the samples got formalin-fixed and paraffin-embedded, followed by a hematoxylin-eosin and chrome aniline blue staining to evaluate fibrosis, steatosis and inflammation. A Prussian blue staining was made for iron assessment.

Steatosis, ballooning, lobular inflammation and fibrosis were classified according to the NASH Clinical Research Network Scoring System definition as follows (30): Steatosis involving <5% of cells is referred as S0 (no steatosis), steatosis of 5-33% as S1 (mild steatosis), >33%-66% as S2 (moderate steatosis) and >66% as S3 (severe steatosis) of hepatocytes.(30)

Ballooning was staged as none (0), few balloon cells (1) and many cells/prominent ballooning (2). Lobular inflammation was graded as no foci = 0 (no lobular inflammation), <2 foci per 200x field = 1 (mild), 2-4 foci per 200x field = 2 (moderate) and >4 foci per 200x field = 3 (marked).(102)

Fibrosis was divided into no fibrosis (F0), perisinusoidal or periportal fibrosis (F1), perisinusoidal and portal/periportal fibrosis (F2), bridging fibrosis (F3) and cirrhosis (F4).(102)

According to the SAF score mentioned above (see point 3.5. Diagnosis of NAFLD), for the diagnosis of NASH, it was necessary that steatosis was ≥5% and ballooning and lobular inflammation of at least grade 1 were found.(31)

For the statistical analyses fibrosis stages were divided into 0-2, classified as no/mild fibrosis, and stages 3-4, classified as advanced fibrosis.

6.4. BLOOD SAMPLING

Venous blood sampling was performed at the time of liver biopsy (±1day). It was used to determine the differential blood count, serum chemistry, lipid profile, blood coagulation, inflammatory markers, virology and blood sugar levels. Out of one tube serum for the measurement of the biomarker ST2 was prepared and meanwhile stored at -80°C.

6.5. ST2-ENZYME-LINKED-IMMUNOSORBENT-ASSAY (ELISA)

To quantify the level of ST2 in our patient's serum, we used the "Human IL1RL1/ST-2 ELISA Kit" of Invitrogen by Thermo Fisher Scientific. The ELISA was performed according to the product information sheet.

Before use, all components reached room temperature.
First the 1X Wash Buffer was prepared by diluting 15ml of Wash Solution Concentrate (20X) with 285ml of distilled water. Afterwards it was stored in the refrigerator.
Then the 1X Assay Buffer was prepared by diluting 14ml of Assay Buffer (5X) with 56ml of distilled water, afterwards it was also stored in the refrigerator.
Before use our calculated amount of 1X Assay Buffer needed (3ml) was separated and10µl of a protease inhibitor (100X) cocktail and 1µl of phenylmethane sulfonyl fluoride (PMSF) (100X) were added.
The serum samples were diluted 1:5 in 1X Assay Buffer with protease inhibitor and 100mM PMSF.
For the dilute standards 500µl of 1X Assay Buffer was added to one vial of IL1RL1/ST-2 Standard to reconstitute 2000pg/ml. After mixing and a standing time of 10 minutes to ensure complete reconstitution, 200µl of 1X Assay Buffer were added to each of 7 tubes. Serial dilutions of the standard were made as follows: 1000, 500, 250, 125, 62,5, 31,25 and 0 pg/ml interleukin-1-receptor-like-1 (IL1RL1)/ST-2.

Performance:

At first 50µl of standards and samples were added to the appropriate wells. Then the plate was covered with plate sealer and incubated for 60 minutes at room temperature on a plate shaker at 300 rpm. Afterwards the solution was removed, and the wells were washed 4 times with 300µl of 1X Wash Buffer.
After that, 50µlIL1RL1/ST-2 Detection Antibody were added into each well. Again, the plate was covered with plate sealer and incubated for 60 minutes at room temperature on a plate shaker at 300 rpm. Afterwards the solution was removed, and the wells were washed 4 times with 300µl of 1X Wash Buffer.

50µl Streptavidin-Peroxidase Conjugate were added into each well and the plate was covered with plate sealer and incubated for 30 minutes at room temperature on a plate shaker at 300rpm. Afterwards the solution was removed, and the wells were washed 4 times with 300µl of 1X Wash Buffer.

Finally, 100µl of TMB Substrate were added to each well and the substrate began to turn blue. After incubation for 30 minutes at room temperature 50µl Stop Solution were added to each well. It was gently mixed by tapping the side of the plate. The solution in the wells changed from blue to yellow.

Now the absorbance was read at 450nm with a spectrophotometer. After calculating a standard curve, the correct concentrations of the serum-levels were calculated.

According to the manual of the 'Human IL1RL1/ST-2 ELISA Kit' of invitrogen by Thermo Fisher Scientific, the analytic sensitivity of this assay was 30.5ph/ml human IL1RL1/ST2. Specificity for human IL1RL1/ST2 was 100%.

6.6.FIBROSCAN

Patients had to lie in supine position with the right arm abducted behind the head and measurement was performed through the intercostals space on the right side.(42) Patients were told not to eat at least 2 hours prior to examination.(28)

This method utilizes transverse shear wave velocity to evaluate liver stiffness.(28) It consists of a probe including a low-frequency vibrator device(42) coupled to a 3,5Mhz ultrasound system(40), a control unit and a computer.(42) Through vibration, a small piston generates a low-frequent shear wave (< 50m/s), which is quickly attenuated by the liver parenchyma, as the wave propagation is perpendicular to the motion of the affected liver tissue.(28) This attenuation is depended on the liver elasticity. Liver stiffness can be calculated with the help of Hook's law, which claims, that velocity of shear waves, moving through an elastic object, is proportional to the objects stiffness.(28) To determine the shear waves velocity, fast ultrasound waves are generated by the probe afterwards, allowing to detect the position of the shear waves in relation to time.(28)

The M-Probe (adult probe) with an ultrasound frequency of 3,5MHz and a measuring depth between 25 to 65mm under the skin, is used for most of adults, provided they have a thoracic perimeter larger than 75cm.(40)

The XL-Probe is more sensitive with an ultrasound frequency of 2,6MHz, allowing a deeper penetration up to 75mm under the skin and is used for patients with a skin-capsula distance of more than 2,5cm.(40)

Results of liver elasticity are presented as the mean of ten valid measurements and are to be expressed by stiffness (kPa) and success rate (%).(33) Valid results are characterized by the interquartile range, showing the variability of the validated measures, which should be below

30% of the median and the success rate, describing the number of successful measurements in relation to the overall number of measurement, which should be at any rate 60%.(33)
Most studies concern patients with viral hepatitis.(28) Summarized cut-off values of eight studies concerning fibrosis staging in NAFLD patients resulted in following ranges(28):

- fibrosis stage ≥2 – 6,2 to 11 kPa
- fibrosis stage ≥ 3 – 8 to 12 kPa
- fibrosis stage 4- 9,5 to 20 kPa

Hepatic steatosis can be assessed by the proprietary controlled attenuation parameter (CAP) acquisition protocol, using the designated 3.5 MHz probe of the Fibroscan device. CAP measurements can only be obtained after liver stiffness measurement acquisition has been assessed as valid.(39)
Steatosis hepatis is classified according to the following grades(39):

Table 5: Classification of steatosis measured by CAP fibroscan

steatosis	hepatocytes with fatty accumulation	optimal cut-off value	sensitivity	specificity
grade 0:	< 10%:	< 238 $dB.m^{-1}$		
grade 1:	11-33%:	≥ 238 $dB.m^{-1}$	91%	81%
grade 2:	34-66%:	≥ 259 $dB.m^{-1}$	89%	86%
grade 3:	67-100%:	≥292 $dB.m^{-1}$	100%	78%

6.7. STATISTICS

This study was explorative in nature. The aim was to get an overview of the biomarker ST2 and his role in liver fibrosis in NAFLD.
Thus, no formal sample size calculation was performed. All available data of patients, which conformed to the inclusion and exclusion criteria, were used.
After exerting data into an Excel file, statistical analyses and generation of graphic representations were conducted using the Statistical Package for the Social Sciences (SPSS 24.0 for Windows).
For the following two major issues a Bonferroni correction was calculated. Consequently, a p-value of <0,025 was regarded as statistically significant.

Primary outcome:

The main purpose was to show if there is any correlation between liver fibrosis stage and serum ST-2 levels.

Therefor bivariate Spearman rank correlations were conducted to detect relation between liver fibrosis stage (F0-4) and ST2 serum levels. A p-value of 0,025 was regarded as statistically significant.

Secondary outcome:

The second major purpose was to examine, whether the mean value of ST2 serum levels differ between patients with mild fibrosis including stage 0-2 and those with severe fibrosis including stage 3-4.

Patients were divided into two groups according to their fibrosis stage:

- Group 1: fibrosis stage 0,1 and 2
- Group 2: fibrosis stage 3 and 4.

After determination whether our data are distributed normal using the Kolmogorov-Smirnov-test, a two-tailed t-test was performed. A p-value of 0,025 was regarded as statistically significant.

A receiver operating characteristic curve (ROC-curve) was performed to determine a cut-off value for detection of severe liver fibrosis (stage 3-4) and the area under the curve value was calculated.

To compare our cut-off values to former study results(103), a second ROC curve, using another classification, was performed. Therefor severe liver fibrosis was already assumed at fibrosis stage 2, whereas no/mild fibrosis was defined at fibrosis stage 0-1.

Explorative testing:

For other explorative examinations no adaption of the p-value was performed. A p-value of 0,05 was regarded as statistically significant.

Patients fibrosis score was calculated using the "NAFLD fibrosis score online calculator" by Angulo P. *(http://www.nafldscore.com/index.php)*.Therefor parameters regarding age, bodymass index, diabetes mellitus, AST, ALT, thrombocytes and albumin were used. A bivariate Pearson correlation was chosen to determine a correlation between serum ST2 levels and NAFLD fibrosis score. Therefor the metric value of the score value was used.

For the creation of graphics the authors classification for mild and severe fibrosis(43) was used as follows:

- < -1.455: predictor of absence of significant fibrosis (F0-F2 fibrosis)
- ≤ -1.455 to ≤ 0.675: indeterminate score
- > 0.675: predictor of presence of significant fibrosis (F3-F4 fibrosis)

Moreover, a ROC curve for the detection of the diagnostic accuracy regarding advanced fibrosis (stage ≥3) was performed and compared with those of ST2 and Fibroscan. Also, a bivariate Pearson correlation was performed for the examination of any correlation between serum ST2 and liver fibrosis stage determined by transient elastography (stiffness kPa).
A ROC curve concerning the diagnosis of severe liver fibrosis (stage ≥3) was conducted here, too.

Several bivariate Pearson rank correlations were made to examine correlations between ST2 levels and:

- liver steatosis
- levels of AST
- levels of ALT
- levels of bilirubin
- levels of cholinesterase
- levels of γ-GT
- levels of normotest
- levels of thrombocytes
- levels of HbA1C
- Body mass index
- levels of albumin

To calculate if there were any statistically significant differences of these parameters between the two fibrosis groups, t-tests were performed, and differences were represented with boxplots.

7.) RESULTS:

53 patients have been selected to participate in this study. Two patients were excluded due to hemolytic serum probes, which led to disproportionally higher results in de ST2-ELISA.
Baseline characteristics of the 51 patients, who could be included, are summarized in table 6.

Table 6: baseline characteristics of included patients

		sex			**age**
		female	male	total	mean
fibrosis stage	**0**	3	4	7	45,43
	1	6	7	13	43,54
	2	5	4	9	46,56
	3	3	9	12	55,50
	4	4	6	10	58,40
	total	21	30	51	50,06

For age descriptive statistics, including mean, standard deviation, median, minimum and maximum, were calculated. The mean age of the participants was 50,06 years, the median was 51 years, the minimum was 19 and the maximum was 83 years.
58,82% of the patients were male, 41,18% were female.

7.1. PRIMARY OUTCOME:

At first it was calculated if there is any correlation between ST2 serum levels and histological defined fibrosis stage.
Bivariate spearman rank correlation studies showed a significant positive correlation between liver fibrosis stage and ST2 serum levels (correlation coefficient r_s= 0,360, p-value = 0,009 (2-tailed)), see figure 7.

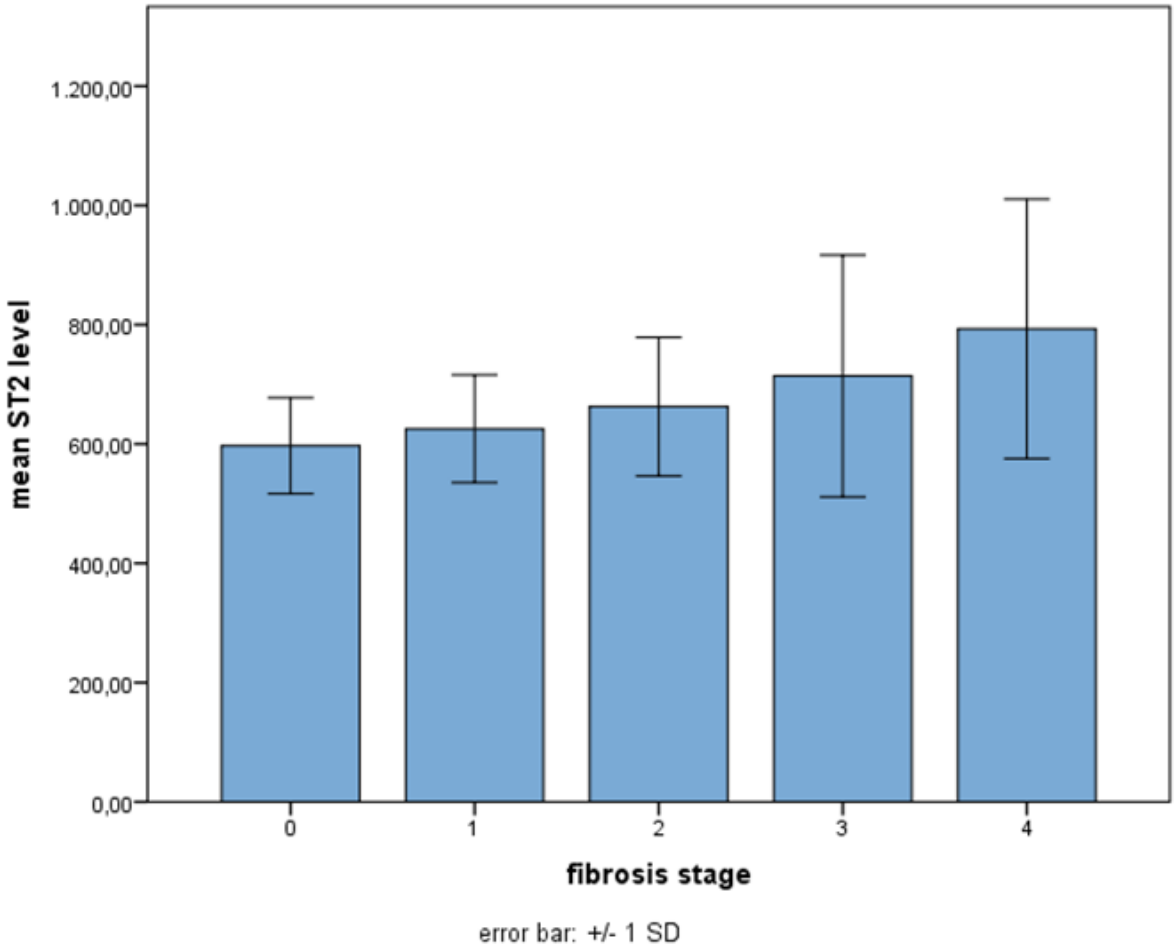


Figure 7: mean values of serum ST2 referred to fibrosis stage 0-4

Descriptive statistics regarding ST2 levels in different stages of liver fibrosis are shown in table 7.

The mean value of ST-level was:

- 597,14pg/ml in patients with fibrosis stage 0
- 625,38pg/ml in those with fibrosis stage 1
- 662,78pg/ml in fibrosis stage 2
- 714,17pg/ml in fibrosis stage 3
- 793pg/ml in fibrosis stage 4

Table 7: descriptive statistics of ST2 levels depending on liver fibrosis stage

		ST2 -levels				
		mean	**SD***	**SEM***	**minimum**	**maximum**
fibrosis stage	**0**	597,14	80,41	30,39	510,00	730,00
	1	625,38	90,33	25,05	460,00	765,00
	2	662,78	116,22	38,74	545,00	880,00
	3	714,17	202,73	58,52	505,00	1170,00
	4	793,00	217,40	68,75	535,00	1200,00
	total	681,86	164,51	23,04	460,00	1200,00

*SD = standard deviation; SEM = standard error of the mean

7.2. SECONDARY OUTCOME:

To detect whether patients with severe liver fibrosis have higher serum levels of ST2 than patients with no or mild liver fibrosis, patients were divided into 2 groups: fibrosis group 1 and fibrosis group 2.

Fibrosis group 1 included patients with liver fibrosis stage 0, 1 and 2, while fibrosis group 2 included those with fibrosis stage 3 and 4.

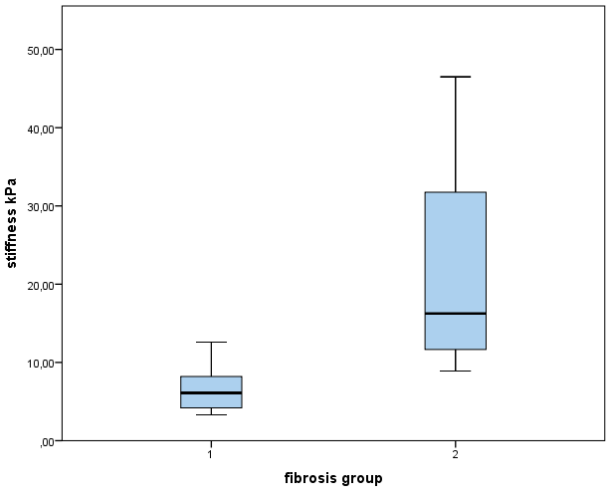
Baseline characteristics of these two groups are shown in table 8.
29 patients were referred to fibrosis group 1, of those 51,72% were male and 48,28% were female. The mean age in this group was 45 years.

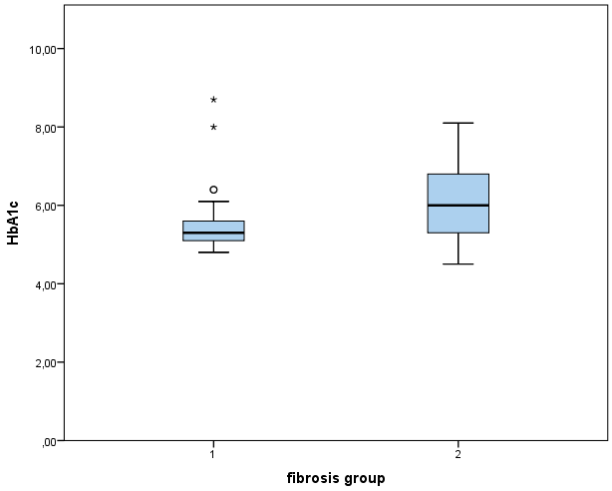
22 patients were referred to fibrosis group 2, of those 68,18% were male, 31,82% were female. The mean age in this group was 57.

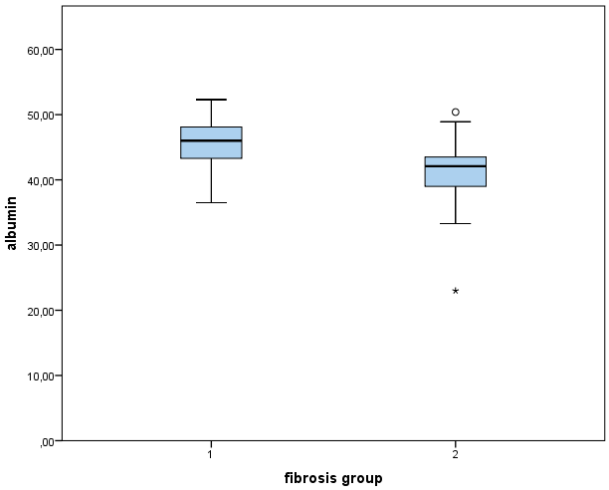
The value of steatosis, body mass index HbA1c, ALT, AST, albumin, gamma-GT, bilirubin, cholinesterase, normotest and thrombocytes are given in table 8.

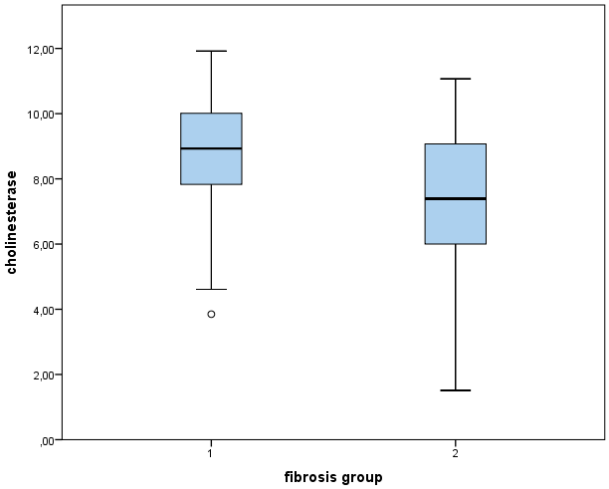
Patients of fibrosis group 2 had significant higher levels of liver stiffness measured by fibroscan ($p = 0,021$), HbA1c ($p = 0,021$) and significant lower levels of albumin ($p = 0,001$), cholinesterase ($p = 0,034$) and normotest ($p = 0,001$) compared to patients of fibrosis group 1 (figure 8-12).

There were no other differences regarding other demographic parameters shown in table 8. Although patients with severe liver fibrosis tended to have a higher body mass index, more liver steatosis and higher levels of AST, γ-GT and a lower number of thrombocytes, those differences between the two groups were not statistically significant.









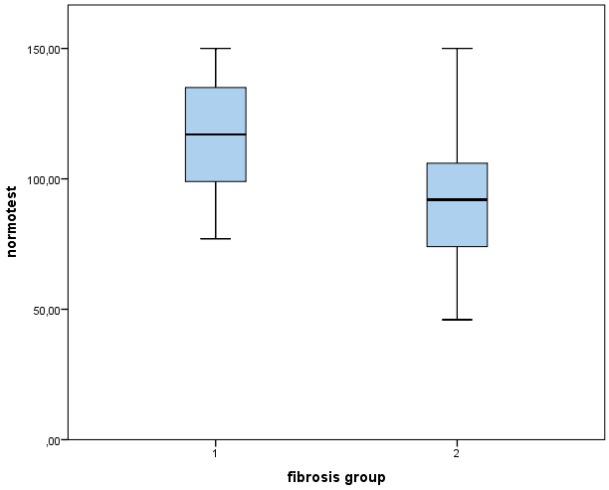


Figure 8-12: boxplots showing different mean values of stiffness kPa measured by

- fibroscan
- HbA1c
- albumin
- cholinesterase
- normotest

concerning fibrosis group 1 vs. group 2.

Table 8: baseline characteristics of patients in fibrosis group 1 and 2

		fibrosis_group	
		1 (n=29)	2 (n=22)
		mean (±SD)	mean (±SD)
sex	**male/female[a]**	15 (51,72%) / 14 (48,28%)	15 (68,18%) / 7 (31,82%)
age		45 (±11)	57 (±14)
steatosis[1]		48,39 (±26,53)	55,24 (±17,43)
body mass index[2]		30,66 (±7,69)	33,45 (±8,22)
Hba1c		5,56 (±0,88)	6,21 (±1,07)*
ALT[3]		77,21 (±50,30)	69,68 (±45,67)
AST[4]		51,89 (±45,19)	73,95 (±53,95)
albumin[3]		45,56 (±3,88)	41,06 (±5,47)**
γGT[4]		155,26 (±195,66)	250,41 (±286,34)
bilirubin[5]		0,94 (±0,80)	1,21 (±1,43)
cholinesterase[3]		8,75 (±1,86)	7,46 (±2,38)*
normotest[3]		116,59 (±22,73)	91,67 (±24,51)**
tnhrombocytes[3]		219,79 (±64,55)	195,45 (±76,04)

data are presented as mean and standard deviation
[a] data are given by absolute numbers and percent
[1]n=49; [2] n=29; [3] n=38; [4] n=36; [5] n=34
ALT = Alanine transaminase; AST=Aspartate transaminase
*p = <0,05
**p = <0,01

The mean value of ST2 was 630,17pg/ml in fibrosis group 1 compared to 750pg/ml in fibrosis group 2. Descriptive statistics are shown in table 9.

Table 9: descriptive statistics regarding ST2 levels in fibrosis group 1 and 2

		ST2			
		mean	**standard deviation**	**minimum**	**maximum**
fibrosis group	**1**	630,17	96,78	460,00	880,00
	2	750,00	208,32	505,00	1200,00

A t-test for independent samples to compare the mean of serum ST levels in our two fibrosis groups, showed a significant difference between ST2 serum levels in patients with fibrosis stage 0-2 and those with fibrosis stage 3-4 (p-value = 0,019). Equal variance could not be assumed, as Lavene test was significant with a p-value of 0.001. Patients with severe liver fibrosis showed significant higher levels of ST2 than patients with no or mild fibrosis (figure 13).

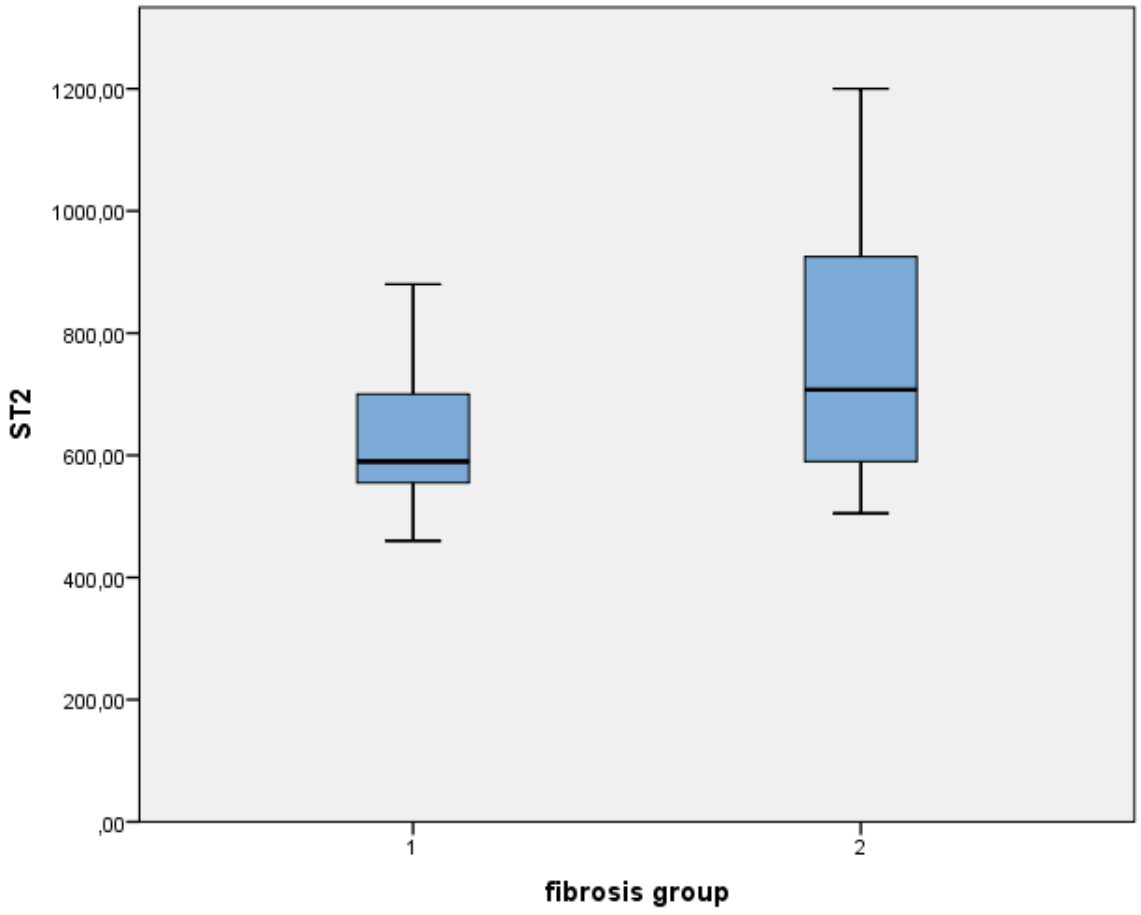
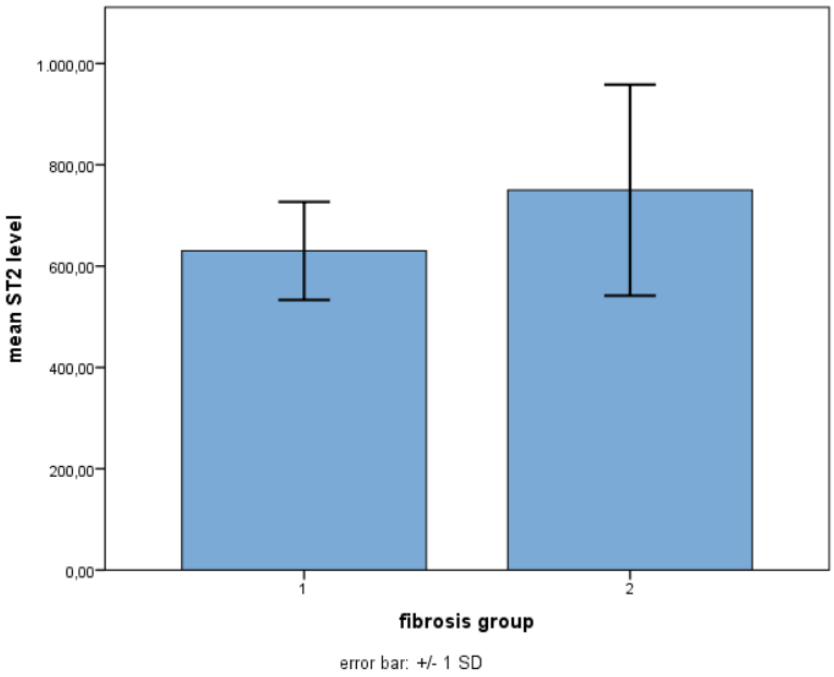
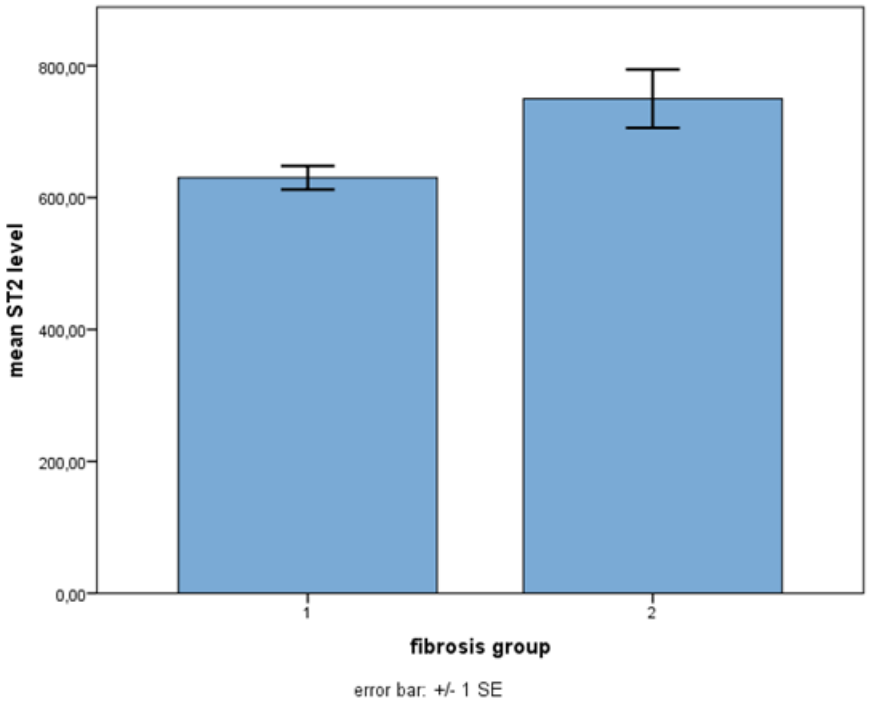


Figure 13: ST-2 levels of fibrosis group 1 vs. fibrosis group 2

The standard deviation (SD) and the standard error of the mean (SE) of the ST2 levels in fibrosis group 1 and 2 are shown in graphic 14 and 15 as follows:





The ROC-curve for distinction between no/mild and severe fibrosis showed an area under the curve (AUC) value of 0,676 with a significance of 0,033. (95% Confidence interval 0,520 – 0,831), see table 10.

Table 10: results of the ROC curve regarding ST2 for the diagnosis of advanced fibrosis

	standard	asymptotic	asymptotic 95% confidence interval	
area	**error**	**significance**	**lower Bound**	**upper Bound**
0,676	0,079	0,033	0,520	0,831

A cut-off value of serum ST2 of >572,5 pg/ml had a sensitivity of 81,8% and a specificity of 41,4% for diagnosis of severe liver fibrosis (stage 3 or 4), see figure 16.

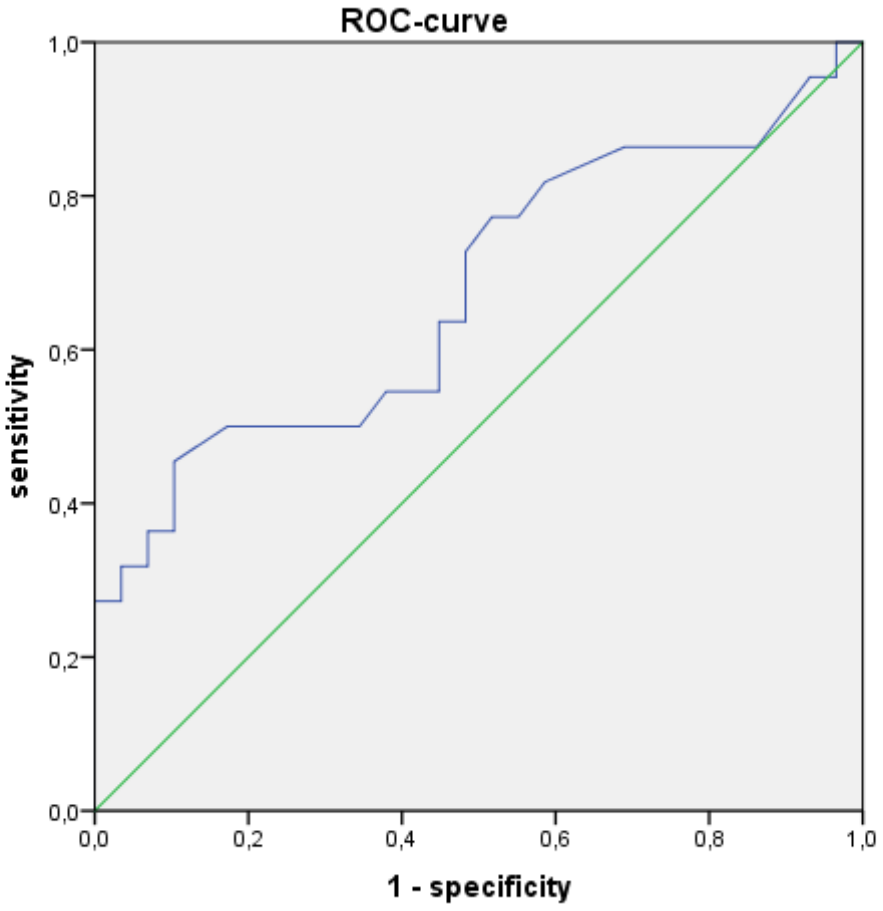


Figure 16: Receiver operating curve of ST2 levels for the diagnosis of advanced liver fibrosis. AUC-value 0,676 (p=0,033)

If fibrosis stage 2-4 were clarified as severe liver fibrosis the ROC curve showed an AUC-value of 0,683 with a significance of 0,029. (95% Confidence interval 0,5238 – 0,828). A cut-off value of serum ST2 of >572,5 pg/ml had a sensitivity of 77,4% and a specificity of 45% for diagnosis of severe liver fibrosis (stage 2-4), see figure 17.

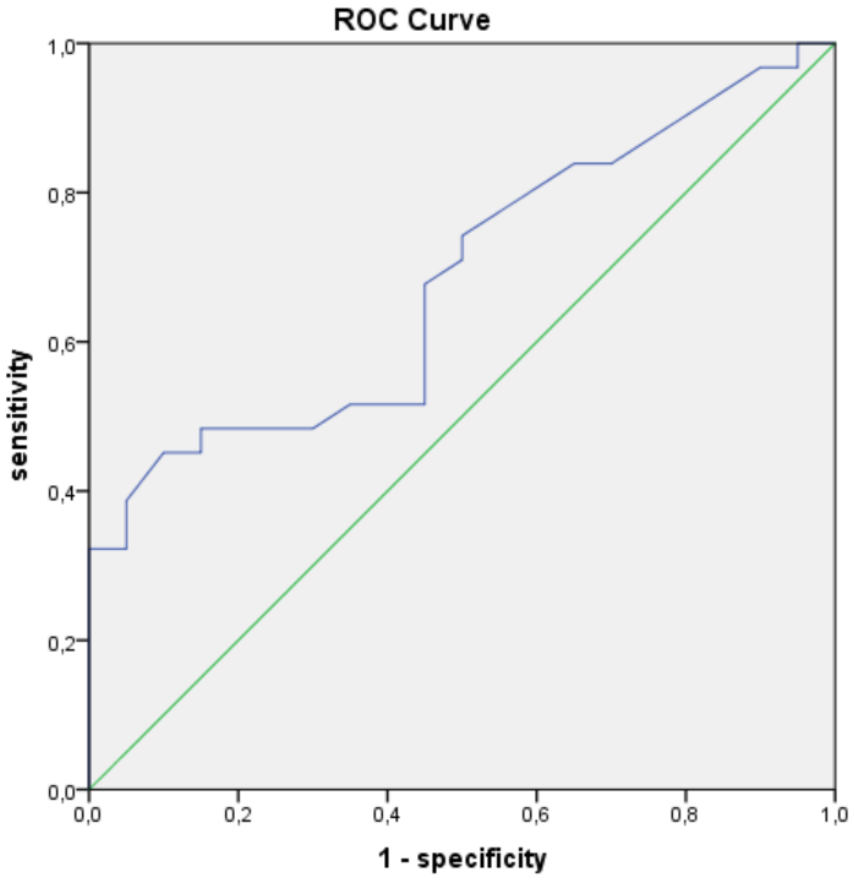


Figure 17: Receiver operating curve of ST2 levels for the diagnosis of advanced liver fibrosis (fibrosis stage 2-4). AUC-value 0,683(p=0,029)

7.3. EXPLORATIVE STATISTICS:

For explorative statistics the p-value was not adjusted. A p-value <0,05 was regarded as statistically significant.

Fibrosis Score & ST2:

Of 28 of our patients, data needed for the calculation of the fibrosis score were available. ST2 levels showed a significant positive correlation with the fibrosis score (r=0,581, p-value=0,005), see figure 18.

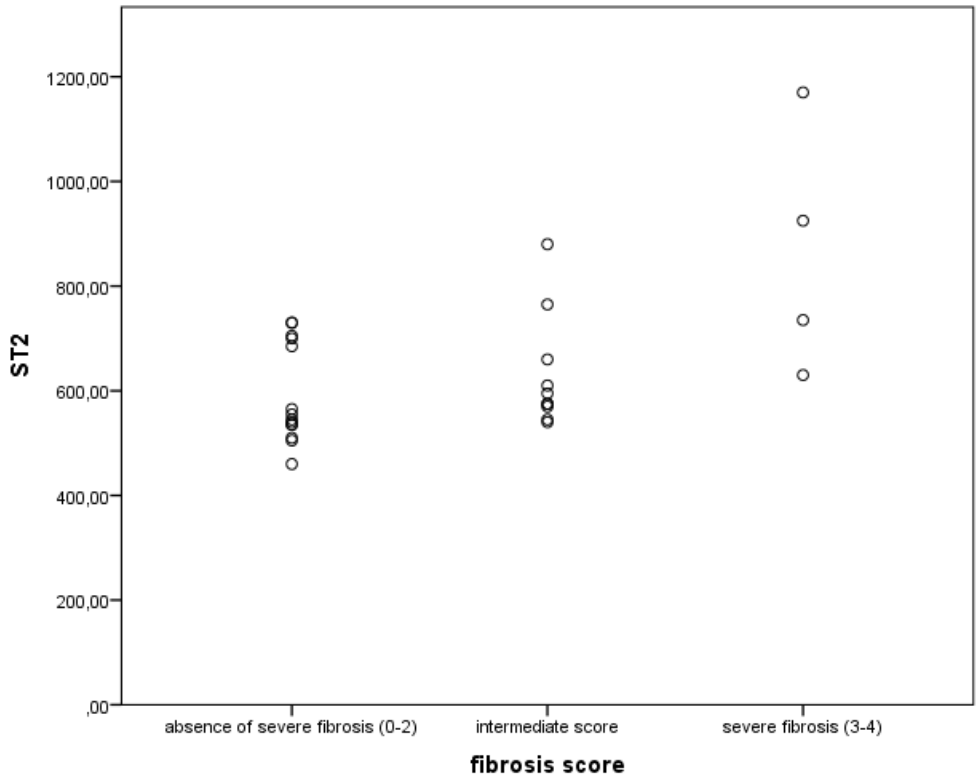


Figure 18: scatterplot showing the correlation of ST2 levels with NAFLD fibrosis score

The fibrosis score itself showed 100% specificity and only 40% sensitivity regarding the detection of advanced fibrosis (stage 3-4), compared to the results of the liver biopsy. Concerning lower fibrosis stages (0-2) the specificity was 80% and sensitivity 66,66%.

In comparison with the serum marker ST2, the AUC for the detection of advanced fibrosis (≥3) was superior, with an AUC level of 0,872, p-value = 0,001 (table11, figure 19).

Table 11: results of the ROC curve regarding NAFLD fibrosis score the diagnosis of advanced liver fibrosis

area	standard error	asymptotic significance	asymptotic 95% confidence interval	
			lower bound	upper bound
0,872	0,068	0,001	0,740	1,000

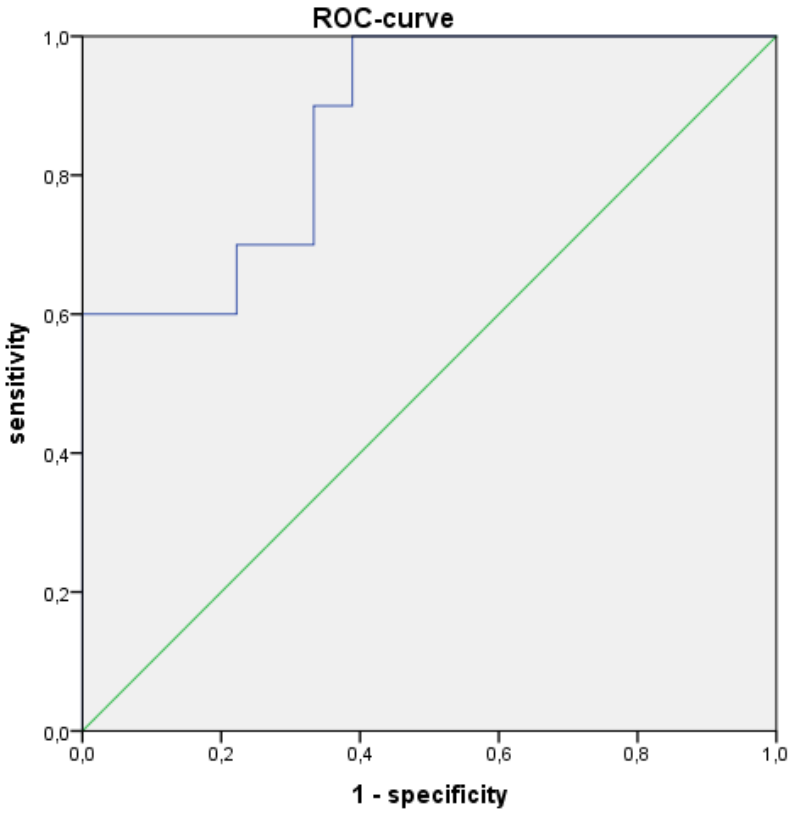


Figure 19:ROC curve regarding the NAFLD fibrosis score for diagnosis of advanced liver fibrosis. AUC-value 0,872 (p=0,001).

Fibroscan & ST2:

It was an aim to calculate the correlation between ST2 levels and liver stiffness measured by fibroscan.

Only 19 of our 51 patients had a valid fibroscan result within ±6 month of the blood sampling. Pearson correlation did not correlate significantly. (r=0,286, p=0,236)

Regarding the accuracy of the detection of advanced fibrosis, a ROC curve was performed too (figure 20), showing the highest diagnostic precision in comparison with ST2 and NAFLD fibrosis score. The AUC value was 0,966 (p-value=0,001), see table 12.

Table 12: results of the ROC curve regarding fibroscan for the diagnosis of advanced liver fibrosis

area	standard error	asymptotic significance	asymptotic 95% confidence interval	
			lower bound	upper bound
0,966	0,036	0,001	0,895	1,000

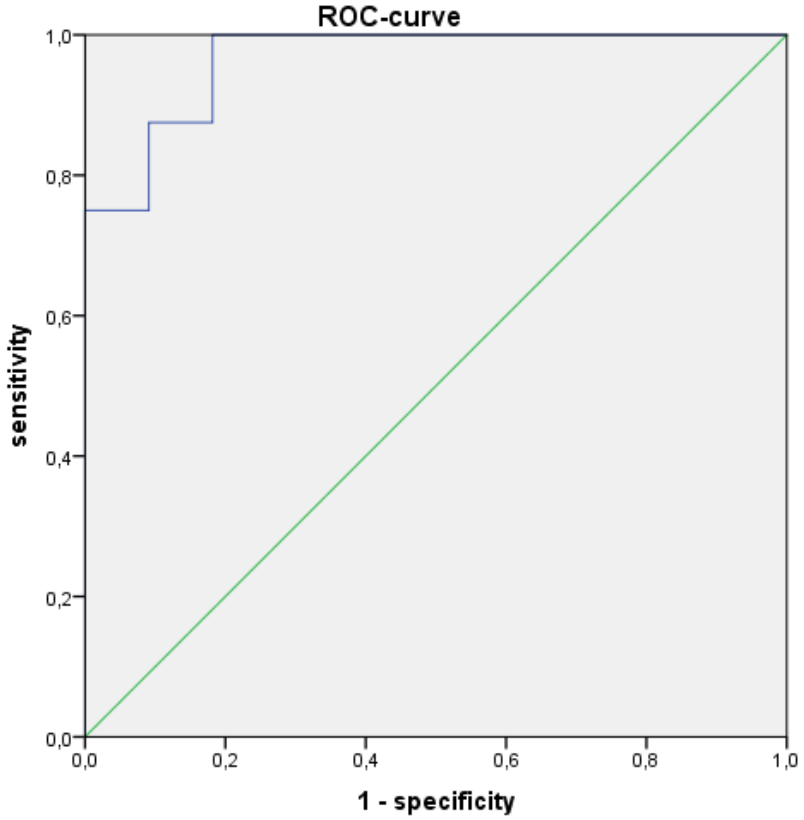


Figure 20: ROC curve regarding fibroscan results for diagnosis of advanced liver fibrosis, AUC value 0,966 (p=0,001).

Several correlation studies regarding ST2 and liver synthesis parameters were calculated. ST2 levels correlated positively with levels of AST (r=0,337, p=0,018), gamma-GT (r=0,698, p= 0,00000000248) and bilirubin (r=0,454, p=0,001). A negative correlation was found between ST2 levels and albumin (r= -0,530, p=0,000064), thrombocytes (r = -0,361, p=0,009), cholinesterase (r = -0,509, p = 0,000136) and normotest (r = -0,439, p = 0,002561), Those correlation studies were statistically significant.

Correlation between ST2 levels and steatosis (r=0,243. P=0,092), as well as HbA1c (r=0,047, p=0,743), body mass index (r=0,116, p=0,431) and ALT (r=0,011, P=0,94) were not statistically significant.

8.) DISCUSSION

Due to the high prevalence of non-alcoholic fatty liver disease, a non-invasive biomarker for diagnosis of disease and progression of associated liver fibrosis would be an enrichment, as the current standard, the liver biopsy does not only comprise a risk for complications such as bleeding(32), also the small probe can lead to sampling errors(35), as the histologic lesions are distributed unequally in liver parenchyma.(34) Additionally, narrow follow up measurements are therefore not possible.

Several studies have investigated the potential of the biomarker ST2 as a diagnostic tool. There are presumptions, that serum ST2 is expressed due to inflammatory mediators like Il-1β, IL-6 or TNF-alpha, wherefore soluble ST2 is assumed to be an anti-inflammatory opponent as it leads to an inhibition of Toll-like receptor signaling and NF-kappa-B downregulation.(104) This theory of a down-regulating mechanism of inflammation of soluble ST2 is supported by studies showing increased soluble ST2 levels in patients with sepsis or dengue virus infection, as well as liver failure.(104)

Serum ST2 levels were increased in patients with acute liver failure and acute-on-chronic-liver failure compared to patients with chronic hepatic failure, again those with chronic hepatic failure present higher ST2 levels than healthy controls.(104) Furthermore, serum ST2 levels correlated significantly with serum levels of IL-33, the ligand of the ST2-receptor.(104)

Another study found significant higher concentrations of soluble ST2 levels in patients with hepatocellular carcinoma and liver cirrhosis compared to healthy controls.(105) Moreover the levels of soluble ST2 correlated with overall survival of patients with hepatocellular carcinoma. Higher levels of soluble ST2 were a negative prognostic marker of overall survival of HCC. (105)

It is discussed whether soluble ST2 leads to an impairment of antitumoral immunity, as it attenuates the effect of IL-33, and therefor is set free from necrotic tumor cells, which stimulates antitumoral immunity due to recruitment of $CD4^+$ and $CD8^+$ cytotoxic lymphocytes and natural killer cells by interferon-γ.(105)

This theory is supported by mouse models studies, finding increased tumor growth of colon cancer after antagonizing the soluble ST2.(106)

Regarding this a comparison of ST2 levels in NAFLD patients with steatosis and liver fibrosis and those already progressed to hepatocellular carcinoma, would be a point of interest.

Impact on liver fibrosis:

As ST2 is involved in hepatic fibrogenesis(79), we wanted to demonstrate an association of ST2 and liver fibrosis. It is observed, that patients suffering from NASH had significant higher levels of the ST2 ligand IL-33 in the serum and also messenger ribonucleic acid (mRNA) of IL-33 and ST2 was enhanced in liver tissue of these patients compared to healthy controls.(81)

As the clinical classification of mild and severe liver fibrosis is uneven, we correlated the ST2 levels with each fibrosis stage (0-4).

ST2 showed a mild positive correlation (r_s= 0,360, p = 0,009) with liver fibrosis stage in patients suffering from non-alcoholic fatty liver disease.

For comparison of mild and advanced fibrosis we chose stage 3 as cut-off value. Patients with severe liver fibrosis (stage 3 and 4) had significant higher levels of ST2 than those with mild fibrosis (stage 0-2), with a mean value of 750 pg/ml compared to 630,17 pg/ml (p=0,019).

Likewise, a study investigated the usefulness of ST2 as a marker of fibrosis in patients with chronic hepatitis B infection.(103) Chronic hepatitis B patients with either no cirrhosis or hepatitis B related cirrhosis were compared to healthy controls, respectively. The results showed a statistical difference of serum ST2 levels between the control group and the patients with chronic hepatitis B.(103)

ST2 showed a moderate correlation with the METAVIR fibrosis score in the patient group. The ROC curve for differentiation of no/mild fibrosis (F0, F1) from severe fibrosis (F≥2) with an under the curve value of 0,68 of serum ST2, was used to set a cutoff value for severe fibrosis. (103)

A cutoff value of 674pg/ml of serum ST2 levels showed a sensitivity of 91,7% and specificity of 40% for the diagnosis of fibrosis greater or equal stage 2 (p = 0,009).(103)

A multiple regression analysis detected the METAVIR fibrosis score as an independent predictor of serum ST2 enhancement. (103)

In our study the AUC value of our ROC was similar (0,676) to this, but our cut-off value of >572,5 pg/ml for the diagnosis of severe fibrosis showed a decreased sensitivity (81,8%) and equal specificity (41,1%), whereas in our study, the cut off for severe liver fibrosis was determined at stage 3 in difference to the study mentioned above.

Compared with NAFLD fibrosis score and fibroscan examination ST2 examination was inferior regarding the AUC values of 0,872, 0,966 and 0,676, respectively. Though, for the analysis of

fibroscan only data of 18 patients and for NAFLD fibrosis score only 28 data were available compared to 51 for the analysis of ST2.

On the contrary examinations did not find a significant difference between ST2 and FIB-4 score, which is a similar noninvasive scoring system including age, AST, ALT and platelets(100), regarding diagnosis of significant liver fibrosis in patients with chronic hepatitis B infection.(103)

Regarding liver synthesis parameters, in our study ST2 levels correlated positively with AST, γ-GT, bilirubin, and negatively with cholinesterase, albumin and thrombocytes. Similar to our results, bilirubin and prothrombin time correlated significantly in the study mentioned above too. (103)

As there is evidence of ST2 changes in patients suffering from cardiovascular diseases such as heart failure(97), those could be confounders regarding the ST2 levels in our study, as cardiovascular co-morbidities were not set as exclusion criteria. Although in preexisting patient charts of the department of Internal Medicine III none of our patients had documented cardiovascular diseases, two of them were suspected but not proved to suffer from cardiovascular diseases, i.e. myocardial infarction and cardiomyopathy. An exclusion of those patients leads to a mild change of the positive correlation (r_s= 0,383, p = 0,006) between ST2 levels and liver fibrosis stage compared to r_s= 0,360, p = 0,009. The t-test for comparison of mild and advanced fibrosis was similar, showing that patients with severe liver fibrosis had significant higher levels of ST2 than those with mild fibrosis, with a mean value of 751,75 pg/ml compared to 630,17 pg/ml (p=0,023), again compared to a mean value of 750 pg/ml compared to 630,17 pg/ml (p=0,019.
Also, the small number of included patients and the retrospective character might be a limitation of our study.

In conclusion ST2 is elevated in several hepatologic diseases including liver fibrosis, such as hepatitis, but also in other fibrotic diseases like pulmonary fibrosis or cardiac remodeling due to infarction or cardiac insufficiency.(103) This data may indicate that ST2 is a general marker of fibrosis, but data also show an impact of the IL-33/ST2 pathway on inflammatory diseases as well.(107) Hence, it might be difficult to establish ST2 as a specific marker for NAFLD but long term data on patients with NAFLD and the metabolic syndrome might add up to a better metabolic risk stratification regarding both liver and cardiac endpoints.
In the management of heart failure ST2 is already used as a biomarker of myocardial fibrosis,

recommended for patients with stage C or D heart failure or decompensated hospitalized patients(99). However, using it as a biomarker in non-alcoholic fatty liver disease is not yet established.

Actual practice guidelines for the management of NAFLD of the European association for the study of the liver, the European association for the study of diabetes and the European association for the study of obesity, released in 2016, judge biomarkers acceptable for an usage for the identification of patients having a small risk of advanced fibrosis or cirrhosis with an evidence level A2, while for advanced fibrosis liver biopsy is still recommended as necessary for a proper diagnosis, as the biomarkers are too inaccurate.(19)

Summarized, our study showed a mild correlation between ST2 levels and liver fibrosis stage in patients with NAFLD as well as significant higher levels of ST2 in patients with advanced liver fibrosis in comparison to those with no or mild fibrosis, underlining the hypothesis, that ST2 is involved in liver fibrogenesis during nonalcoholic fatty liver disease. However, for the diagnosis of liver fibrosis ST2 level determination was not superior to other noninvasive tools like NAFLD fibrosis score or fibroscan examination. Larger studies might be needed to evaluate the potential utility of ST2 as a biomarker in the disease management of nonalcoholic fatty liver disease and the metabolic syndrome.

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10.) TABLE OF FIGURES

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12.) LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
AMP	adenosine monophosphate
AP-1	activator protein 1
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
BMI	body mass index
CAP	controlled attenuation parameter
CD	cluster of differentiation
ChREBP	carbohydrate response element binding protein
CMV	cytomegalovirus
CRP	c-reactive peptide
CT	computer tomography
ELF	enhanced liver fibrosis
ELISA	Enzyme-linked-Immunosorbent-Assay
FIB-4	fibrosis-4
FXR	farnesoid X receptor
γ-GT	γ-glutamyl transferase
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HIV	human immunodeficiency virus
IL	interleukin
IL-1RAcP	interleukine-1 receptor accessory protein
IL1RL1	interluekine-1-receptor-like-1
LDL	low density lipoprotein
MAP	mitogen activated protein
MRE	magnetic resonance elastography
MRI	magnetic resonance imaging

MRI-PDFF	magnetic resonance imaging-proton density fat fraction
mRNA	messenger ribonucleic acid
myD88	myeloid differentiation primary response protein 88
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	non-alcoholic steatohepatitis
NT-pro-BNP	N-terminal prohormone of brain natriuretic peptide
NF-κ-B	nuclear factor 'kappa-light-chain-enhancer' B
PNPLA3	patatin-like phospholipase domain-containing 3 protein
PMSF	phenylmethane sulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
ROC	receiver operating
SAF-score	steatosis, activation, fibrosis – score
SD	standard deviation
SE	standard error of the mean
SGLT	sodium glucose co-transporter 2 inhibitors
SREBP-1c	sterol regulatory element-binding protein 1c
sST2	soluble suppression of tumorigenicity 2
ST2	suppression of tumorigenicity 2
TGF-β	transforming growth factor β
Th2 cells	T helper 2 cells
Th1 cells	T helper 1 cells
TMAO	trimethylamine-N-oxide
TNF-α	tumor necrosis factor α
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
UDCA	Ursodeoxycholic acid
VLDL	very low-density lipoprotein-triglyceride