KLINIK UND POLIKLINIK FÜR INNERE MEDIZIN I
PROF. DR. MARTINA MÜLLER-SCHILLING
DER MEDIZINISCHEN FAKULTÄT
DER UNIVERSITÄT REGENSBURG

ANALYSIS OF SYSTEMIC MONOCYTE CHEMOATTRACTANT PROTEIN-1 LEVELS IN PATIENTS WITH AND WITHOUT SONOGRAPHIC EVIDENT HEPATIC STEATOSIS

Inaugural – Dissertation
zur Erlangung des Doktorgrades
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Tag der mündlichen Prüfung:	19.02.2013

Vergleich der Serum MCP-1-Spiegel bei Personen mit und ohne sonographisch evidenter Steatosis Hepatis

Einleitung - Nicht-alkoholische Fettlebererkrankung und MCP-1

Die Nicht-alkoholischen Fettlebererkrankungen (NAFLD) werden je nach Fortschreiten der Erkrankung in die drei Schweregrade der reinen Fettleber (Steatosis hepatis), der Nicht-alkoholischen Steatohepatitis (NASH) und der Fettleberzirrhose unterteilt.

Die Entstehung der NASH beruht dabei auf zwei Schritten. Im ersten Schritt (first hit) kommt es zur Fetteinlagerung in der zuvor gesunden Leber, getragen vor allem durch ungesunde Ernährung und gestörte Zuckerverwertung im Rahmen des metabolischen Syndroms [1, 2]. Anschließend (second hit) entsteht durch einen entzündlichen Prozess eine Fettleberhepatitis, im Zuge derer Leberzellen zerstört werden. Dabei spielen mehrere Signalwege, darunter auch Chemokin-vermittelte eine zentrale Rolle. Insbesondere das Chemokin Monozyten-chemoattractant Protein-1 (MCP-1) kann bei diesem Schritt vermehrt nachgewiesen werden [3, 4].

Die vorliegende Arbeit zeigt, dass MCP-1 nicht erst im Entzündungsprozess, sondern bereits im Stadium der Leberverfettung vermehrt sein könnte. Relevant wird dieses Ergebnis, wenn man berücksichtigt, dass im Rahmen von Routine-Ultraschalluntersuchungen des Abdomen häufig eine Fettleber detektiert wird.

Patienten und Methode

In einer Gemeinschaft von 3 Studenten wurden von Januar 2008 bis Januar 2009 506 Patienten, die aufgrund unterschiedlicher Indikationen dem Ultraschallzentrum des Uniklinikums Regensburg zugewiesen wurden, untersucht. Nach einer ausführlichen Anamnese und körperlichen Untersuchung wurden standardisierte Ultraschalluntersuchungen durchgeführt. Dabei wurde insbesondere auf Zeichen der Leberverfettung geachtet: die Echogenität, die Struktur der Leber, ihre Oberfläche und eventuell vorhandene Leberraumforderungen wurden beurteilt. Außerdem wurden der Pfortaderfluss, sowie die Lebergröße ausgemessen.

Nach einer nüchtern durchgeführten Blutentnahme bei den Studienteilnehmern wurden zudem unterschiedliche Serumparameter bestimmt. Mittels ELISA-Tests wurden die MCP-Spiegel bestimmt.

Ergebnisse

In dieser Studie konnte kein Zusammenhang zwischen den gemessenen MCP-1-Werten und dem Alter, Geschlecht und Zigarettenkonsum festgestellt werden. Allerdings fand sich unter den Rauchern eine Korrelation zwischen erhöhten MCP-1-Werten und der Menge sowie der Dauer des Nikotinkonsums ($p=0,012$).

Bei Patienten mit metabolischem Syndrom wurden signifikant erhöhte MCP-1-Spiegel bei erhöhtem BMI ($p=0,018$), Taillenumfang ($p=0,028$), erhöhter Blutglukose ($p<0,001$) und bei erniedrigtem HDL-Cholesterin ($p=0,048$) gemessen.

Erhöhte MCP-1-Werte zeigten sich ebenfalls bei erhöhten ALT-Werten ($p=0,047$).

Bei sonographisch nachgewiesener NAFLD konnten höhere MCP-1-Werte im Vergleich zur Kontrollgruppe ohne NAFLD festgestellt werden (*p=0,042*). Keine Korrelation bestand zwischen MCP-1-Serum-Spiegeln und Pfortaderfluss, beziehungsweise der Lebergröße.

Diskussion

Zusammenfassend konnte eine Korrelation des MCP-1-Spiegels mit verschiedenen Merkmalen des metabolischen Syndroms bestätigt werden [5, 6]. Wie bereits durch Haukeland und Kollegen [3] beschrieben, wurden in dieser Studie erhöhte MCP-1-Werte bei Patienten mit sonographisch nachgewiesener Nicht-alkoholischer Fettlebererkrankung gemessen. Auch die Serum-Transaminasen zeigten eine positive, signifikante Korrelation mit MCP-1 [5].

Obwohl histologische Untersuchungen zur Abgrenzung einer reinen Nicht-alkoholischen Fettlebererkrankung zur Hepatitis fehlen, kann aufgrund von epidemiologischen Studien [7] jedoch davon ausgegangen werden, dass erhöhte MCP-1-Werte bereits in einem frühen Stadium der Leberverfettung auftreten.

In diesem Stadium der Fetteinlagerung kann man die erhöhten MCP-1-Werte zum einen aus dem Lebergewebe selbst erwarten, zum anderen auch durch eine vermehrte MCP-1-Produktion im Bauchfett erklären. Diese Chemokin-Sekretion aus dem Fettgewebe kann die Leber direkt über die Pfortader erreichen und somit zusätzlich den weiteren Prozess zur Steatohepatitis entscheidend fördern.

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Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CCL2	Chemokine (C-C motif) ligand 2 = MCP-1
CCR2	Chemokine (C-C motif) receptor 2
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acid
GLUT 4	Glucose transporter type 4
GOT	Glutamate Oxalacetate Transaminase
GPT	Glutamate Pyruvate Transaminase
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HIV	Human Immunodeficiency Virus
HSC	Hepatic stellate cells
IDF	International Diabetes Federation
LDL	Low density lipoprotein
MCL	Medioclavicular line
MCP-1	Monocyte-chemoattractant-protein-1
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
TNF-α	Tumor-necrosis factor α
VLDL	Very low density lipoprotein
WHO	World Health Organisation
γ-GT	γ-Glutamyl Transferase

1 Introduction

1.1 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver (NAFL) is recognized as the most common cause of chronic liver disease, especially in the “Western world” [1, 2, 8, 9].

Ludwig et al described the non-alcoholic fatty liver disease (NAFLD) already in 1980 [10], and prosperity leads to the increasing importance of this disease. Today NAFLD is even considered as part of the metabolic syndrome or as its “hepatic manifestation”, respectively [1, 2].

NAFLD is defined by a significant lipid accumulation in hepatic tissue in the absence of chronic alcohol consumption [11, 12] (less than 20g/d for men, and 10g/d ethanol for women [8]). It includes simple steatosis, its most benign form, non-alcoholic steatohepatitis (NASH) and liver cirrhosis [2].

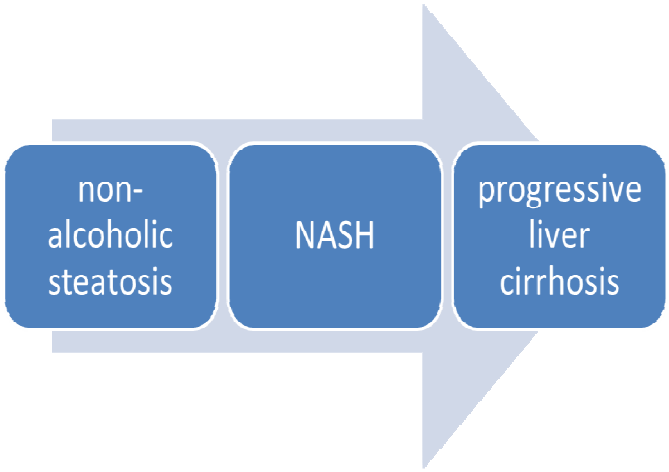

Figure 1: NAFLD includes non-alcoholic steatosis, NASH and progressive liver cirrhosis

Epidemiology and risk factors

Approximately 20%-30% of our population have NAFLD [9, 11, 13, 14]. The prevalence of NASH is 2% to 3% [13, 15]. Only in the Caucasian race, the prevalence in men is higher than in women [14]. Overall, this disease increases with preceding age [13].

Aetiology

Causes of fatty liver are manifold, and categorize the NAFLD in “primary” or “secondary” [8]: Primary NAFLD is associated with obesity, insulin resistance, and the metabolic syndrome [13].

Association with the metabolic syndrome

Although the metabolic syndrome is an increasing disease in the “Western world” and associated with a high risk of cardiovascular morbimortality [9, 16], there is no uniform definition of this syndrome. Some medical organisations like the World Health Organisation (WHO) or the International Diabetes Federation (IDF, Fig. 2) have tried to summarise different criteria: The most important factor, which needs to be included, is the presence of central obesity with a waist circumference of more than 94cm for men and more than 80cm for women [17]. Lifestyle and prosperity lead to raised blood pressure (over 130mmHg systolic or over 85mmHg diastolic) and raised fasting plasma glucose over 100mg/dl, respectively 110mg/dl, or type-2-diabetes mellitus [17]. In consequence to the renal damage, urine samples often show micro albuminuria. Furthermore, raised triglycerides over 150mg/dl and reduced high-density lipoprotein (HDL-) cholesterol (less than 40mg/dl for males and less than 50mg/dl for females) can be analysed in blood samples of adipose individuals [17].

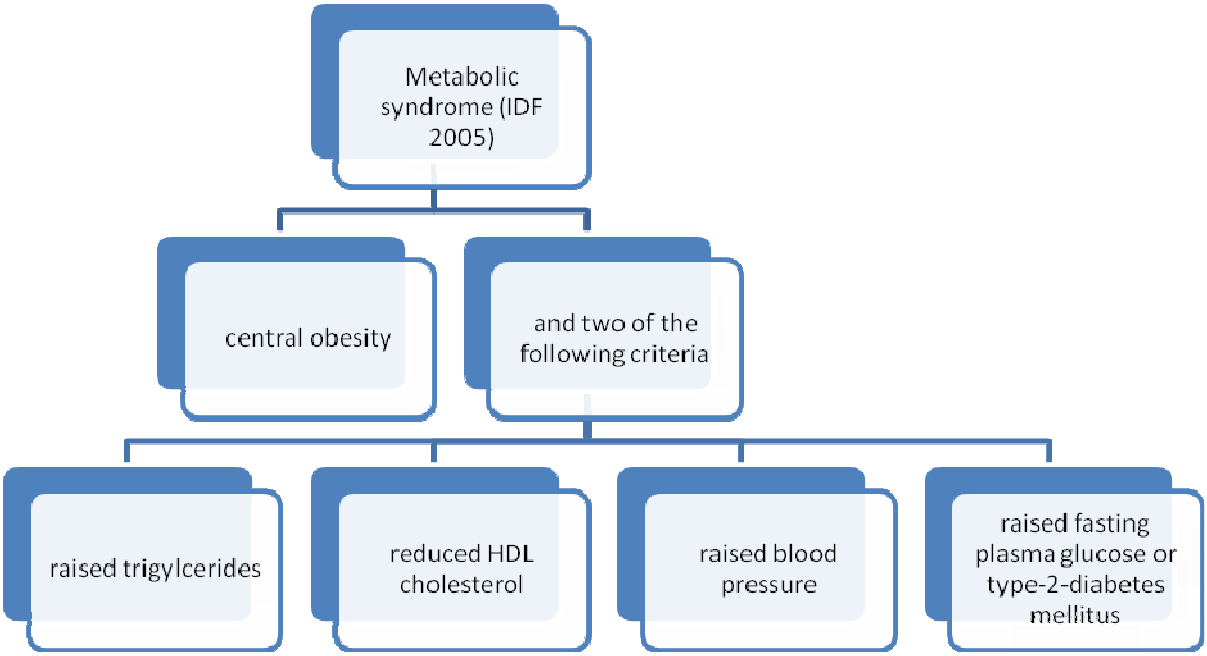

Figure 2: Definition of the Metabolic Syndrome according to the International Diabetes Federation of 2005: Beside central obesity, two of the following four criteria are needed: raised triglycerides, reduced HDL cholesterol, raised blood pressure or raised fasting plasma glucoe or type-2-diabestes mellitus.

The NAFLD is considered to be the “hepatic manifestation of metabolic syndrome” [1, 2].

On the one hand, patients with NAFLD are commonly obese [18], have hypertriglyceridemia, decreased levels of high density lipoprotein (HDL) cholesterol and hypertension [9, 11, 12, 13, 19]. Moreover, patients with a fatty liver might encounter a higher risk of developing Type 2 diabetes [20, 21].

On the other hand, a high number of patients with metabolic syndrome have steatotic livers [22]. These two perspectives affirm both the metabolic cause of primary NAFLD.

Secondary NAFLD includes viral infections, autoimmune or hereditary diseases, hepatotoxins and drugs such as glucocorticoids or antiestrogens. Furthermore, endocrine metabolic conditions, nutritional causes and jejuno-ileal bypass surgery may cause NAFLD [13].

Clinic

Most patients are asymptomatic or have mild, non-characteristic right upper quadrant complaints [18, 20], some complain of fatigue [18]. Apart from hepatomegaly, the clinical examination is often unremarkable [18]. In this case, it is helpful to measure the waist circumference or to diagnose the BMI in order to prove central obesity.

Blood test results

In the initial stage of liver disease there is a short rise in γ-Glutamyl Transferase (γ-GT), and subsequently in Glutamate Pyruvate Transaminase (GPT) and Glutamate Oxalacetate Transaminase (GOT) [18, 23]. Afterwards only a fraction of NASH patients have elevated transaminases [24].

Elevated GPT and degree of abnormal liver lipid levels are predictive of severe NAFLD graded by ultrasound [1, 11, 23]. The GOT:GPT ratio is usually less than 1 [18, 23], a ratio greater than 1 suggests advanced fibrosis [11, 13].

In addition, hyperglycaemia caused in context with diabetes is present in approximately one-third of patients [18]. Furthermore, hyperlipidaemia is often present [18].

Sonography

A referral to a sonography is often precipitated by abnormal liver enzyme levels, detected by routine evaluations [11]. An increased parenchymal echogenicity can be

considered as a reliable criterion for diagnosing fatty liver [1, 25, 26] although a sensitivity of approximately 83% has to be admitted [27]. Although, sonography does not distinguish between fatty liver and steatohepatitis [12], it represents a valid method to detect hepatic steatosis [1, 25, 26].

Histology

Liver biopsy remains the “gold standard” for the diagnosis of NAFLD [18]. It allows the simple steatosis to be kept apart from NASH and to stage the disease [12]. The initial lesion of NAFLD is steatosis, but can be accompanied by early mild diffuse lobular inflammation [11, 23, 28]. Afterwards, an increasing inflammation combined with features of liver cell injury, characterise the steatohepatitis [29, 30]. Subsequent liver fibrosis is the result of progressive collagen deposition [30].

Therapy

Initial therapy for all NAFLD patients should be a complete change of lifestyle [8, 18, 20, 31]. Ten per cent weight loss can improve steatosis [12], but more consistent weight loss may also improve necroinflammation and overall activity in NASH [8]. In addition, there are only experimental but no clinically established therapies [8, 23, 32]. There are no established therapeutic agents, which can reverse fibrosis [32].

Prognosis

NASH develops into fibrosis or cirrhosis within five to ten years in about 10% to 40% of all cases [23]. Cirrhosis related to NASH has also been shown to be associated with an increased risk of Hepatocellular carcinoma [33].

1.2 Pathogenesis of NASH

NAFLD encompasses a spectrum of hepatic pathology, ranging from simple steatosis (NAFL), its most benign form, to non-alcoholic steatohepatitis (NASH) and liver cirrhosis [2]. The two-hit hypothesis separates the pathway to steatohepatitis into two different parts [23]:

The first hit symbolizes the accumulation of triglycerides in the liver [23, 34], called steatosis. According to the fat content, one can subdivide the fatty degeneration in two groups: “Liver steatosis” is defined as a condition in which disseminated liver cells have a fat content of 3-10% of the liver wet weight [23]. In cases where the fat storage is over 10% of the liver wet weight in over 50% of the hepatocytes it is called “fatty liver” [23]. Steatosis alone is considered to be relatively innocuous and is usually reversible [34].

The second hit involves an inflammatory insult in combination with deregulated cytokine production and oxidative stress to the liver [34], leading to steatohepatitis. It is characterized by hepatocellular injury and death.

The development of inflammation determines whether a patient progresses to irreversible liver damage and fibrosis or not [34].

1.2.1 First hit - exogenous and endogenous factors

Exogenous and endogenous pathogenic factors which disturb the lipid metabolism may be responsible for the development of liver steatosis [2, 23].

The most important exogenous factor is the increase in lipid uptake from the intestine [2, 8], associated with hyperalimentation especially in high-fat and high-cholesterol diets which lead to lipid accumulation in the liver [35].

This malnutrition leads to obesity [23] and insulin resistance [2], which both change the physiological lipid utilisation, symbolizing the endogenous factor to liver steatosis.

Insulin resistance leads to central elevated serum glucose levels with a concomitant lack of glucose in peripheral cells. An elevated supply of glucose (or similar energy sources) or better glucose utilization can improve this situation:

On the one hand, in peripheral adipose tissue, insulin resistance and glucose deficiency enhance peripheral lipid mobilization with more lipolysis. This increases the delivery of adipose-derived FFAs to the liver [2, 36, 37]. Additionally, an inhibition of lipid processing in hepatocytes and lower liver export rates lead to fat accumulation in the liver [2, 23, 38].

On the other hand, more insulin is produced in order to improve the peripheral glucose uptake. However, elevated serum levels of insulin also augment the feeling of hunger, closing the circle to the increased lipid uptake, peripheral lipid mobilization and the metabolic syndrome with obesity.

1.2.2 Second hit – necroinflammatory response

Several factors are involved in the progression of Steatosis to Steatohepatitis. This process, called “necroinflammatory response” [3] includes systemic inflammation, cytotoxic effects of free fatty acids, oxidative stress and an augmented adipocytokine production.

Systemic inflammation and TNF-α

Patients with NAFLD are characterized by a chronic low-grade systemic inflammation [39]. This inflammation symbolizes the protective attempt of the liver to remove the injurious stimuli of fat infiltration. It contains a balance between healing factors and destruction. Macrophages secrete tumor necrosis factor-α (TNF-α), a cytokine that plays an important pathogenetic role in the development of NASH [3, 4, 8, 39]. It induces oxidative stress, apoptosis and cytokine production, such as the secretion of MCP-1 (explained in detail below). Moreover TNF-α acts as insulin antagonist, leading to peripheral lipolysis and in consequence elevates lipid offer to the liver.

In conclusion, NAFL progresses to NASH if the regulation of the inflammation process overbalances. Adaptive mechanisms that protect hepatocytes become overwhelmed, and rates of hepatocyte death begin to outstrip mechanisms that normally regenerate dead hepatocytes [2].

Free fatty acids and oxidative stress

The phenomenon of peripheral insulin resistance leads to an increased delivery of free fatty acids (FFAs) from fat deposits. These FFAs can be directly toxic to hepatic cells [8, 11], and also the delivery causes abnormal quantities of reactive oxygen and free radical production that can no longer be detoxified [40, 41].

Due to subsequent cytokine induction, hepatocellular injury especially in mitochondria further progresses [23, 42].

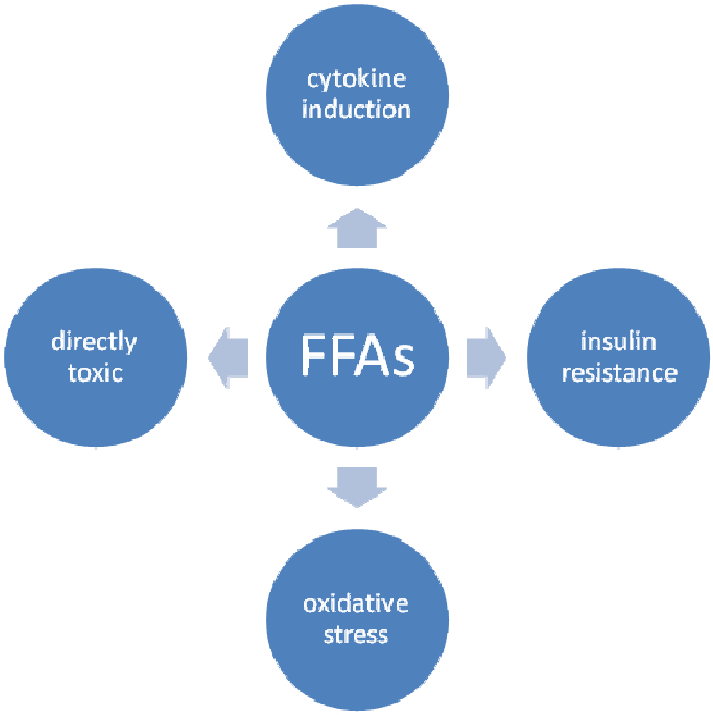

Figure 3: Effects of free fatty acids: FFAs are directly toxic, induce cytokine production, cause insulin resistance and produce oxidative stress.

1.3 Chemokines and MCP-1

The chemokine monocyte chemoattractant protein (MCP-1) plays an important role in the conversion from simple steatosis to NASH [(3)]. This cytokine is specifically implicated in the inflammatory process but also sustains liver steatosis and fibroinflammatory reactions.

Definition of chemokines

Chemokines or chemotactic cytokines (Greek *-kinos*, movement) are a family of more than 50 small proteins with a molecular weight in the range of 8 to 12 kD [(43)] and mediate chemo-attraction to regulate cell trafficking [(44)]. Chemokines are released by infected or damaged cells forming a concentration gradient. The attracted cells move through the gradient towards the higher local concentration of chemokines [(44)].

Classification of chemokines

Chemokines are subdivided into four families, based on the number and spacing of the conserved cysteine residues in the N-terminus of the protein [(44)], named “CXC”, “CC”; “CX3C” and “C” (Fig. 4).

One major chemokine subfamily is called “CXC” because the two amino-acids which are located next to the N-termini of these proteins are separated by a single amino-acid.

This is in contrast to the other major subfamily which is called “CC”; these two cysteines are adjacent.

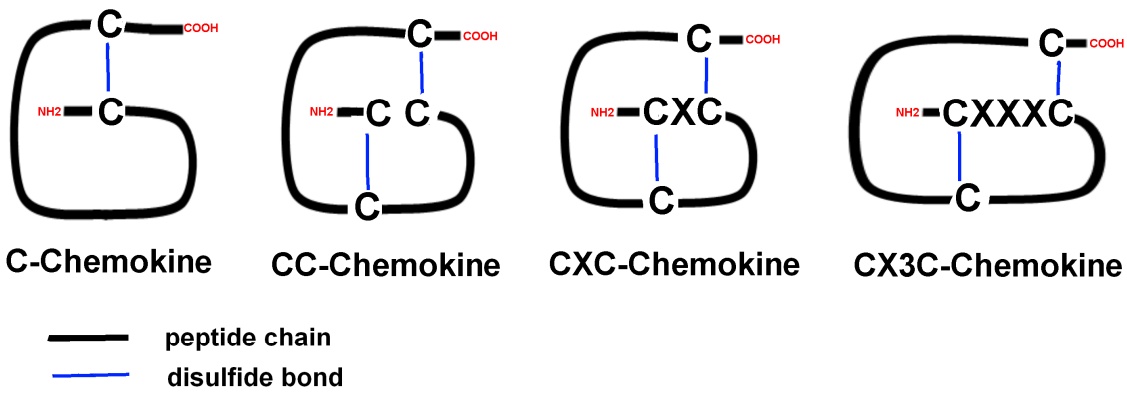

Figure 4: Chemokines and their structure

Monocyte chemoattractant protein-1

Monocyte chemoattractant proteins (MCPs) are responsible for the development of inflammatory responses by recruiting immune cells to sites of inflammation [45].

Monocyte chemoattractant protein-1 (MCP-1) was first identified as chemo-attractant for monocytes, which are the precursors of macrophages, the “scavenger cells” of cellular debris [44, 46]. MCP-1 is a CC-chemokine, composed of 76 amino acids and 13kDa in size [47]. After different stimuli such as TNF-α, macrophages and endothelial cells produce MCP-1 [46].

MCP-1 interacts as “molecular signal transmitter” in the process of liver steatosis as well as liver inflammation.

MCP-1 and fat accumulation

The monocyte chemoattractant protein-1 may induce steatosis by acting directly on hepatocytes [37]. It increases the hepatic expression of different liver genes that regulate enzymes to induce fat accumulation [37, 46].

MCP-1 and inflammation

MCP-1 leads to an increased hepatic expression of proinflammatory cytokines [4] and is a potent activator of leukocytes, macrophages and other cell types at the site of inflammation [48].

Specialized macrophages in the liver, called Kupffer cells, are activated by MCP-1 [4]. These stimulated macrophages themselves may secrete a variety of chemokines and other cytokines which further promote the local inflammatory reaction [46].

MCP-1 and adipose tissue

Adipose tissue, especially in obesity, is recognized as a metabolic active endocrine organ which also has the capability of secreting cytokines like MCP-1 [2]. MCP-1 leads to macrophage infiltration into the foci of inflammation, especially in the more inflammatory visceral adipose tissue, as compared with subcutaneous adipose tissue [2, 49, 50].

MCP-1 and insulin resistance

Additionally, MCP-1 can induce and boost insulin resistance [5, 45, 51] as a link between inflammation and metabolic disease [35]. In this context, MCP-1 inhibits insulin-stimulated glucose uptake as well as the adipocyte expression of metabolically important genes like glucose transporter type 4 (GLUT 4) [51].

A reduction in adipose tissue inflammation via inhibition of the MCP-1 receptor CCR2 function ameliorates both insulin resistance and hepatic steatosis [45, 52, 53].

Fibroinflammatory response

Several lines of evidence indicate that MCP-1 plays a role in the recruitment and maintenance of the inflammatory infiltrate, but also in repair mechanism during liver injury [45, 46, 54]. After destruction of liver tissue, hepatic stellate cells (HSCs), also known as Ito-cells, undergo a transdifferentiation to myofibroblast-like cells [2, 55, 56]. They are responsible for the secretion of extracellular matrix components [57] and lead to fibrosis or cirrhosis [58, 59]. Additionally, hepatic myofibroblasts express and secrete different members of the CC chemokine subfamily, including MCP-1 [55, 60]. MCP-1 itself promotes the attraction of other HSCs [54], potentiating the fibrogenic properties of the first HSC. This leads to an increasing distortion of the hepatic architecture [8, 58].

1.4 Aim of the study

The study's aim is to analyse serum MCP-1 levels in patients with sonographically diagnosed fatty liver and to correlate serum MCP-1 levels with serological and clinic-pathological parameters.

2 Patients and Methods

2.1 Patients

Initially, 506 randomly selected patients from different Departments of the University Hospital Regensburg were included in this study. The cohort consisted of outdoor patients and hospitalized patients, who were referred to the interdisciplinary ultrasound department for sonographic examination of the abdomen from January 2008 to January 2009. The participants were divided into two groups according to the results of a standardized ultrasound examination: (i) a control group with normal US liver appearance and (ii) a group of patients with US-diagnosed fatty liver.

All patients took part in a face-to-face interview, based on a standard questionnaire including a detailed medical history (see annexe 2).

Patients with any of the following criteria were excluded from the study: 1. hepatobiliary diseases, 2. malignancies, 3. ascites, 4. medications known to cause hepatic steatosis (as estrogens, corticosteroids, amiodarone, valproate; at present or within the last 2 years), 5. inflammatory bowel disease, 6. infection with the human immunodeficiency virus (HIV), 7. chronic drug or alcohol abuse (more than 20 g/day), 9. known (familial) hyperlipidaemia, and 10. acute medical conditions with confounding effect on laboratory measurements.

Consequently, the remaining study population consisted of 104 patients.

2.2 Methods

The collection of patients' data, examination of patients and collection of patients' material was performed in collaboration with two other MD students (Catrin Beer and Christoph Niessen). The data pool was used for theses addressing different aspects of NAFLD.

2.2.1 Questionnaire

Each patient was asked to answer some questions about their medical history. The questionnaire included questions to epidemiological facts like date of birth, gender, nationality and treatment (ambulant or hospitalized). Moreover, the patients were asked about their medical history, why they were coming to the clinic, what past medical history they had, what medication they were taking and what diseases were known in their families. Furthermore, questions were related to known diabetes, liver disease, problems with the lipid metabolism or cancer.

Further, the history of daily alcohol consumption and nicotine abuse was analysed. The daily consumption of alcohol was mainly answered in litres of beer or glasses of wine per day and then converted into mg of alcohol. Furthermore, patients were asked for their drug consumptions.

2.2.2 Anthropometric measurements and clinical examination

After filling out the consent form (see annexe 4) and the questionnaire, each patient was checked by a general physical examination (see annexe 1). The person's height was measured by a fixed scale on the wall. The patients were weighed on a standardized scale without shoes and wearing only thin clothes.

Their blood pressure and heart rate were taken in a seated position on the right and left upper arm, located at about the same height as the heart. The blood pressure cuff had been adjusted to the circumference of the upper arm. Before the blood pressure was measured, the patient had to rest for a minimum of five minutes.

The waist and hip circumferences were measured on the standing patient with a flexible tape. The waist circumference was measured at the smallest level of the torso while the subject was at minimal respiration. The hip circumference was taken at the largest circumference between the edge of the iliac bone and the upper part of the thigh bone, at the level of the anterior superior iliac spine.

2.2.3 Collection and storage of serum samples

After a close disinfection of the skin and a minimized congestion, blood samples were taken from each patient: one EDTA tube of 5ml and one serum tube of 7.5ml after an overnight fast.

Plasma was obtained by centrifugation at 700g RCF (Relative Centrifugal Force) for nine minutes and was stored in aliquots at minus 20 degree Celsius for upcoming analysis.

The following laboratory analyses were performed by the certified Institute for Clinical Chemistry and Laboratory Medicine of the University Hospital Regensburg: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl-transferase (γ-GT), bilirubin, ALP, total serum protein, albumin, cholinesterase, triglycerides, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), very-low-density lipoprotein (VLDL) and fasting glucose.

2.2.4 Ultrasound examination

In addition to a complete abdominal ultrasound examination all patients underwent a standardized ultrasound examination (see annexe 3). The following ultrasound equipment was used: Siemens Sonoline Elegra, Siemens ACUSON Sequoia 512 (Siemens, Erlangen, Germany), GE Healthcare Logic 9 (GE Medical Systems, Wisconsin, USA) or Hitachi EUB-8500 (Hitachi Medical Corporation, Tokyo, Japan) with a 3.5-7.5 MHz transducer.

First, the liver size was measured as a subcostal, vertical diameter in the medioclavicular line by using the 3-5 MHz probe (Fig. 5). Hepatomegaly was defined as a liver size above 155 mm.

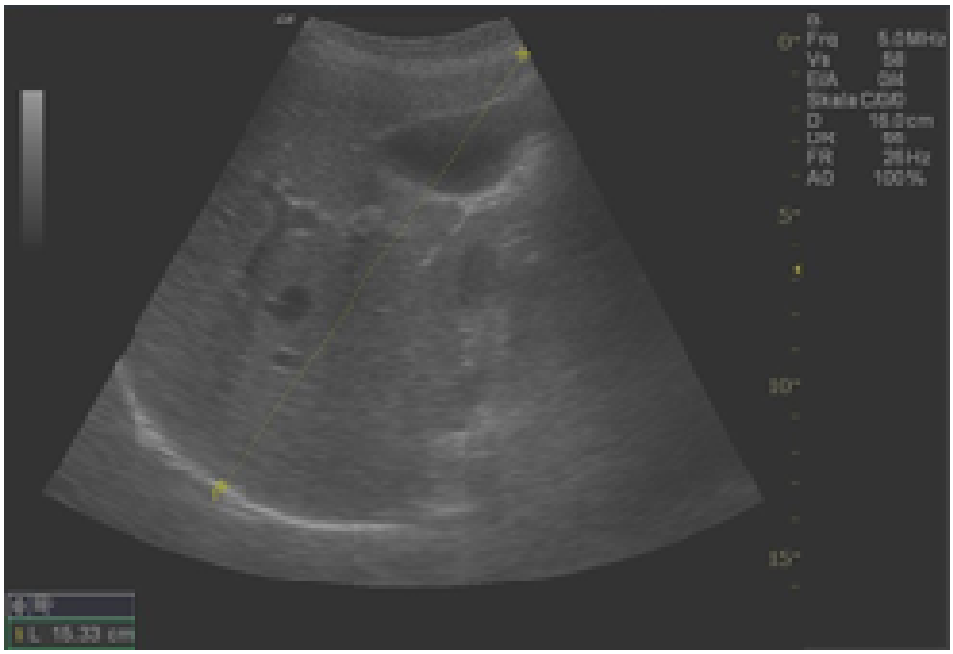

Figure 5: Ultrasound-picture of the liver in the right mediclavicular line. Hepatomegaly was defined as size above 155mm.

Further, we looked for ascites in the Morison pouch (Recessus hepatorenalis), the part between the liver and the right kidney, one of the lowest points in the upper abdomen. The Koller-Pouch (Recessus splenorenalis), between the spleen and the left kidney, and the Douglas pouch were also checked for free fluid.

The echogenicity of the liver was diagnosed via comparison of the liver parenchyma with the parenchyma of the kidney and classified in normal, slight or intense augmentation of the echogenicity (Fig. 6). The increase of intensity and frequency of echoes yields the image of a “bright (white) liver” [23], due to the high number of water-to-fat interfaces in livers with steatosis and can be considered as a sensitive non-invasive method for steatosis quantification [61].

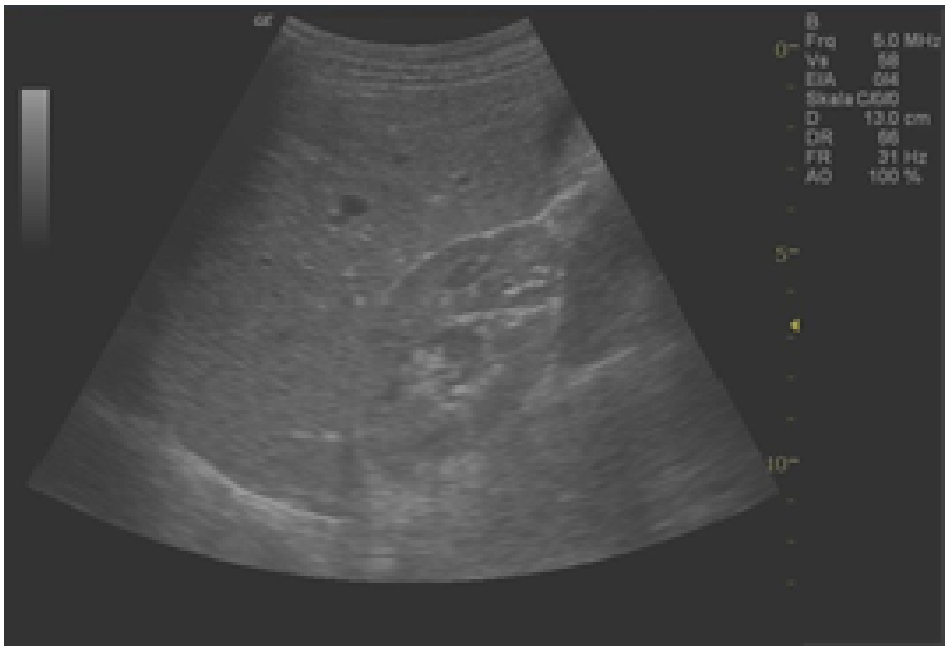

Figure 6: Ultrasound-picture of the liver and right kidney: Echogenity and liver steatosis can be analysed.

Further, the structure of the liver parenchyma was staged after a complete examination of the whole liver. Scans were obtained in transverse and longitudinal planes from the midline in the hypochondrium [62] to the right costal margin and right posterior axilla line. We separated homogenous from inhomogeneous parenchyma.

Focal sparing or focal fat infiltrations of a diameter of five mm upwards can be identified with the knowledge of their common areas, often near the gallbladder bed. Moreover, we looked for other focal lesions, like haemangiomas or metastases.

Additionally, subcutaneous fat thickness in the periumbilical and right subcostal regions (Fig. 7) was assessed using high frequency transducers (Sonoline Elegra: 7.5L40/5-9 MHz, ACUSON Sequoia 512: 15L8w-S 14 MHz, GE Healthcare Logic 9: 7L/3-7 MHz or 10L/4-10 MHz, Hitachi EUB-8500: EUP-L53/5-10 MHz).

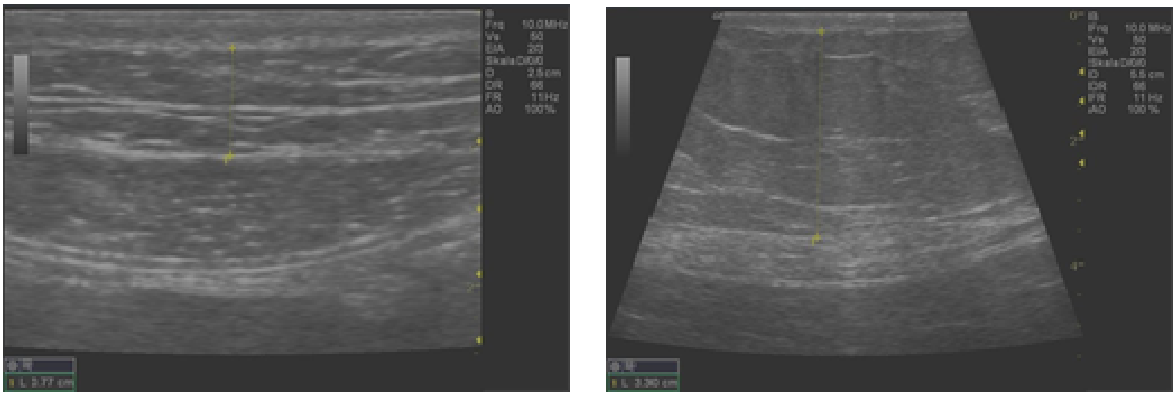

Figure 7: Ultrasound-picture of the subcutaneous adipose tissue: periumbilical and in the right subcostal region

The liver surface was inspected with a 7.5 or 10 MHz transducer and graduated in smooth, slightly waved or strongly waved (Fig. 8).

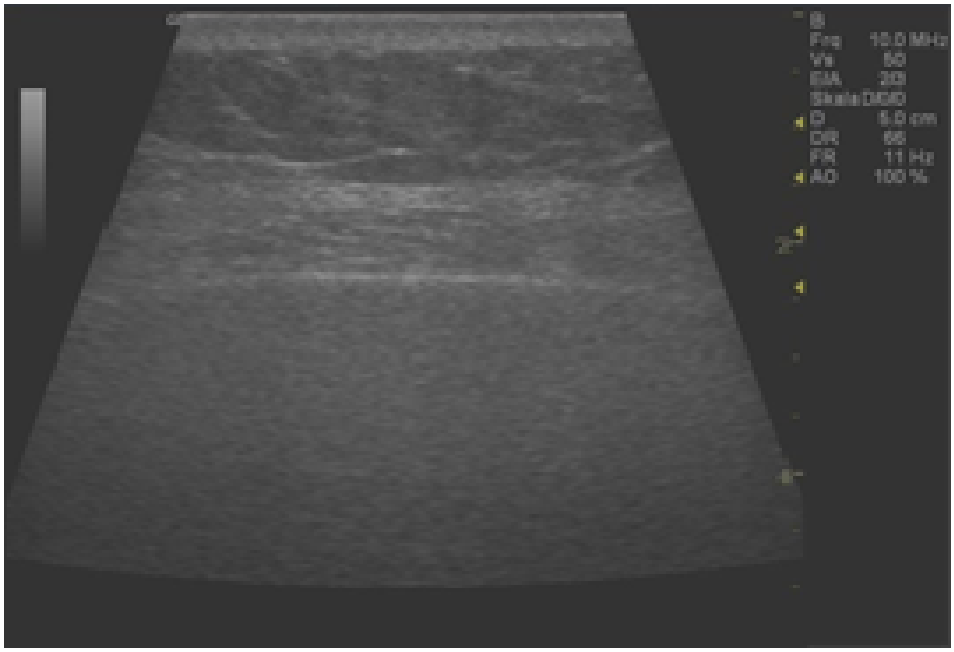

Figure 8: Ultrasound-picture of the liver surface

Further, we examined the compressibility of the liver via palpation of the left and right lobe through the abdominal wall.

With the portal duplex and colour Doppler sonography the maximum velocity and the direction of the portal vein flow were measured, especially with a right lateral intercostals approach (Fig. 9). Then it was achieved, that the portal vein passes almost directly towards the transducer, giving an optimal beam-vessel angle. In general, the mean velocity of the portal vein blood flow decreases as the severity of fatty infiltration increases [(63)].

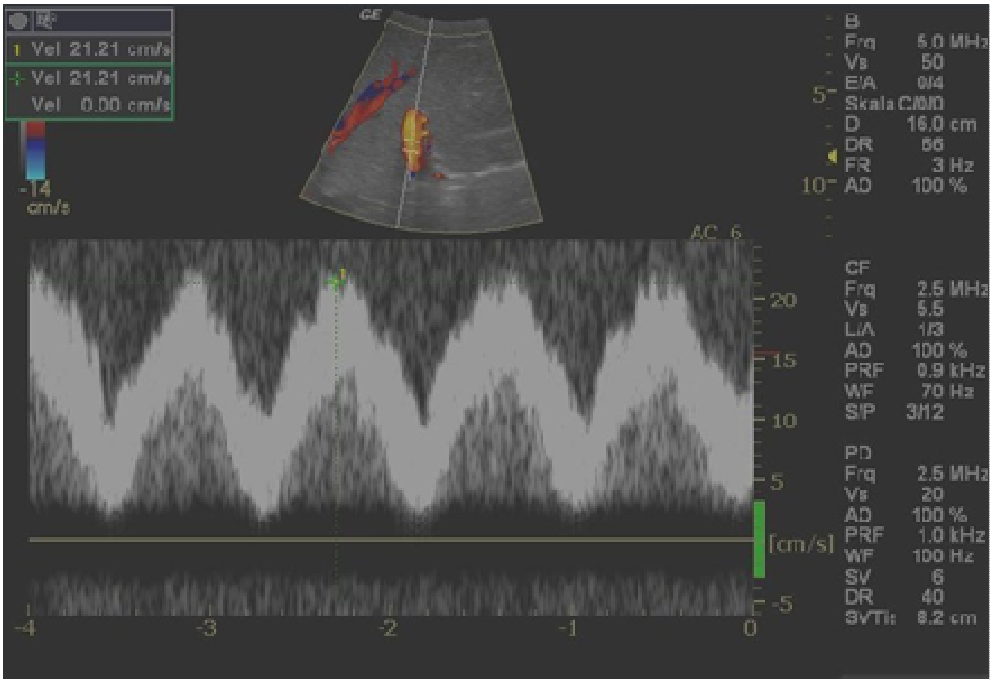

Figure 9: Ultrasound-picture with colour Doppler: Measurement of the portal vein flow

2.2.5 Serum analysis of MCP-1 levels

MCP-1 in human sera was assessed by sandwich enzyme-linked immunosorbent assay (ELISA) using the human CCL2 DuoSet ELISA development kit (R&D Systems Inc., catalog number DY279), following the manufacturer's instructions.

Plates were coated with a known quantity of capture antibodies, which bind to MCP-1 in the samples. The undiluted samples were added and any antigen (MCP-1) present bound to a capture antibody. In a next step detecting antibodies were added and bound specifically to the antigens. The antibody was conjugated to an enzyme, according to the manufacturer's instructions. Between the last steps the plate had to be washed to remove unbound antigen and antibodies. In the final step a substrate

was added and was converted by the enzymes to a detectable form. The colour was measured to determine the quantity of MCP-1.

2.2.6 Statistical analysis

Results of continuous variables are expressed as means ± standard deviation. Comparisons between groups were made using one way analysis of variance (with Bonferroni correction for multiple comparisons) for variables with normal distribution, and the Mann-Whitney U test for other variables (serum MCP-1 levels in patients). Correlation between MCP-1 and serum parameters was assessed by Spearman's test. P-values <0.05 were considered as statistically significant.

Statistical analysis was performed on a PC using SPSS 15.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism Software (GraphPad Software, Inc., San Diego, USA).

The data of this study was correlated with analysis of murine models of NAFLD. Together these data have been successfully published (see reference 64). Methods applied for the assessments of the murine model are described in detail in the above mentioned publication (see reference 64).

In the following, the data of the human study, which are the basis of this dissertation, are presented and discussed together with the data obtained in the murine models.

3 Results

Baseline characteristics of the 104 subjects included in the study are summarized in Table 1.

Table 1: Correlation of MCP-1 serum levels with age, gender, and alcohol and nicotine consumption

Variables	Mean ± SD (or %)	Correlation coefficient (ρ)	p
age (years)	54.4 ± 17.7	0.08	0.423*
male gender (%)	52.3%	---	0.821†
smoking (%)	46.8%	---	0.271†
pack years (n)	15.5 ± 23.6	0.29	**0.012***

Correlation between MCP-1 levels and numerical variables was assessed by Spearman´s test (*).
Correlation between MCP-1 levels and categorical variables was assessed by ANOVA (†).
Bold-face figures indicate significant differences ($p<0.05$)

Analysis of serum levels of MCP-1 in this group revealed no significant correlation with age or gender. Further, MCP-1 levels were similar between smokers and non-smokers, however and interestingly, the number of pack years correlated with MCP-1 serum levels in the group of smokers.

Next, we assessed the association between MCP-1 serum levels and liver related serum parameters and found a significant correlation between ALT and MCP-1 serum levels (Table 2).

Table 2: Correlation of MCP-1 serum levels with liver related serum parameters

Variable	Mean (±SD)	Correlation coefficient (ρ)	p*
AST [U/l]	28.6 ± 12.6	0.08	0.384
ALT [U/l]	19.7 ± 20.7	0.20	**0.047**
γ-GT [U/l]	64.7 ± 92.5	0.14	0.172
bilirubin [mg/dl]	0.61 ± 0.44	-0.21	0.838
ALP [U/l]	73.8 ± 32.7	-0.02	0.840
total serum protein [g/l]	74.6 ± 7.7	0.05	0.675
albumin [g/l]	46.7 ± 6.7	-0.01	0.923
cholineesterase [kU/l]	8.69 ± 2.39	0.03	0.771

NAFLD = non-alcoholic fatty liver disease; AST = aspartate aminotransferase; ALT = alanine aminotransferase; γ-GT - gamma-glutamyl-transferase; ALP = alkaline phosphatase
* by Spearman´s test
Bold-face figures indicate significant differences ($p<0.05$)

Moreover and interestingly, MCP-1 serum levels were significantly higher in patients with ultrasound-diagnosed NAFLD (median: 67.3 ng/ml; 25^{th}-75^{th} percentile: 30.2-100.3 ng/ml) in comparison to the control group (median: 41.3 ng/ml; 25^{th}-75^{th} percentile: 16.1-100.3 ng/ml; p=0.042), (Fig. 10). No correlation was found between MCP-1 serum levels and portal vein flow or liver size as assessed by ultrasound examination (data not shown).

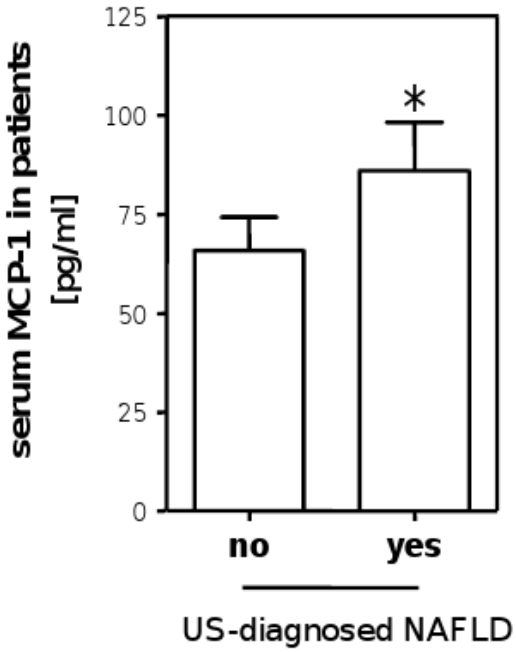

Figure 10: Serum MCP-1 levels in patients with ultrasound (US) diagnosed fatty liver compared to a control (ctr.) group of patients with normal sonographic liver appearance. (*p<0.05 compared to control).

After hepatic parameters, we analyzed MCP-1 serum levels in relation to biochemical and anthropometric features of the metabolic syndrome. Spearman´s test revealed that MCP-1 serum levels correlated with serum levels of glucose and HDL-cholesterol (inverse), and also with body-mass index (BMI) and waist circumference (Table 3).

Table 3: Correlation of MCP-1 serum levels with features of the metabolic syndrome.

Variable	Mean ± SD (or %)	Correlation coefficient (ρ)	p
body mass index (kg/m²)	25.9 ± 4.4	0.23	**0.018***
waist circumference (mm)	92.2 ± 15.4	0.22	**0.028***
hip circumference (mm)	97.6 ± 13.4	0.06	0.544*
waist to hip ratio	0.94 ± 0.09	0.26	**0.007**
triglycerides (mg/dl)	124.1 ± 58.3	0.17	0.095*
total cholesterol (mg/dl)	199.1 ± 46.2	-0.09	0.392*
HDL cholesterol (mg/dl)	55.6 ± 19.8	-0.22	**0.048***
LDL cholesterol (<150 mg/dl)	105.4 ± 36.8	-0.02	0.840*
VLDL cholesterol	38.4 ± 19.4	0.18	0.073*
reported diabetes mellitus type II (%)	20.2 %	---	**0.046**†
fasting glucose (mg/dl)	106.7 ± 32.9	0.37	**<0.001***
reported hypertension (%)	37.5 %	---	0.632†
systolic blood pressure (mmHg)	130.8 ± 16.4	0.11	0.263*
diastolic blood pressure (mmHg)	78.9 ± 10.7	-0.11	0.259*

HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very-low-density lipoprotein
Correlation between MCP-1 levels and numerical variables was assessed by Spearman´s test (*).
Correlation between MCP-1 levels and categorical variables was assessed by ANOVA (†).
Bold-face figures indicate significant differences (p<0.05)

4 Discussion

The contribution of MCP-1 to inflammatory and fibrogenic processes in chronic liver disease has long been recognized [59, 65]. The pathophysiological role played by MCP-1 has been demonstrated by the finding that MCP-1 inactivation abrogates hepatic inflammation and fibrosis in an experimental murine model of chronic liver injury [66].

Recently, it has been shown that hepatic MCP-1 expression is up-regulated in patients with NAFLD [67]. Further, Haukeland and collegues reported elevated MCP-1 serum levels in NAFLD patients and found that circulating MCP-1 levels correlated with the severity of liver damage [3]. In line with this report, we found in the present study higher MCP-1 serum levels in patients with ultrasound-diagnosed NAFLD, and circulating MCP-1 levels correlated significantly with serum transaminases. Still, despite the lack of histological examination in this hospital cohort of randomly selected patients, it can be estimated from epidemiological studies [7], that only a minority of cases had significant hepatic inflammation (i.e. criteria for NASH), which may have caused elevated MCP-1 levels.

Animal and human studies show a correlation of serum MCP-1 with serum transaminases [5], HDL-cholesterol, body-mass index (BMI) and waist-hip ratio [6] as indicative of systemic insulin resistance and metabolic syndrome. In our study, serum MCP-1-levels correlated with serum ALT and glucose levels as well as BMI and waist circumference.

Effects on the progression of simple steatosis to NASH may take place in addition to - or possibly even prior to - hepatic MCP-1 expression in response to hepatocellular lipid accumulation.

In summary with data obtained in murine model of hepatosteatosis without inflammation (reference 64), our data indicate both the liver and adipose tissue as cellular sources of elevated circulating MCP-1 levels already in the early phase of hepatic steatosis. Since MCP-1 derived from visceral adipose tissue reaches the liver via portal circulation at high concentrations, it may significantly contribute to the progression of simple steatosis to NASH.

ACKNOWLEDGEMENT

I would like to thank Prof. Dr. Claus Hellerbrand for his guidance, understanding and patience.

I would like to express my gratitude to the collaboration with Catrin Niessen, Christoph Niessen, the members of the ultrasound department, the Institute for Clinical Chemistry and Laboratory Medicine and the research group of Experimental Hepatology of the University Hospital Regensburg.

Special thanks go to my twin sister Susanne and to my parents, who supported me during my studies and who made this dissertation possible.

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Annexe 1: Cover and examination

Zusammenfassung

(Name des Patienten/der Patientin, Geburtsdatum)	Patienten-Nr.:_____ Untersucher:_____ Untersuchungsdatum:_____

Aufklärung und Einverständniserklärung □ erledigt

Anamnesebogen □ erledigt

Ultraschalldokumentation □ erledigt

körperliche Untersuchung □ erledigt

Größe:	_____ cm
Gewicht:	_____ kg
Hüftumfang:	_____ cm
Beckenumfang:	_____ cm
RR systolisch:	_____ mmHg
RR diastolisch:	_____ mmHg
Herzfrequenz:	_____ / min

Blutabnahme □ erledigt

1x EDTA 5ml, 1x Serum 7.5ml

- abzentrifugieren
- aliquottieren
- minus 20℃ asservieren

Annexe 2: Medical history - **Anamnesebogen**

(Name des Patienten/der Patientin, Geburtsdatum)

Patienten-Nr.:_____
Untersucher:_____
Untersuchungsdatum:_____

Epidemiologie

Geschlecht: □ männlich □ weiblich
Nationalität: □ in Deutschland geb. □ außerhalb Deutschlands geb.:_____
Behandlung: □ ambulant □ stationär

Anamnese

Grund des aktuellen
Krankenhausaufenthalts: _____

Vorerkrankungen: _____
_____

Medikamente: _____
_____

Familienanamnese:_____
_____

bekannter Diabetes □ nein □ ja, seit: _____
□ Typ I □ Typ II

bekannte Lebererkrankung □ nein □ ja, seit: : _____
Details: _____

bekannte Fettstoffwechselerkrankung □ nein □ ja, seit: : _____
Details: _____

bekannte Tumorerkrankung □ nein □ ja, seit: : _____
Details: _____

Alkoholkonsum □ nein □ ja_____
Nikotinkonsum □ nein □ ja_____

Bluttransfusionen □ nein □ ja_____
Wann/wo:_____

(früherer) Drogenabusus □ nein □ ja, seit: : _____
Details: _____

Sonstiges: _____
_____

Annexe 3: Ultrasound examination - **Untersuchungsbogen Ultraschall**

Bitte alle Untersuchungen mit THI durchführen! **Pat-Nr.**_____
Bitte Untersuchungsanforderung und Bilddokumentation anheften!

_____	_____	_____
Nachname Patient	Vorname Patient	Geb-Datum
_____	_____	_____
Gerät	Untersuchungsdatum	Untersucher

3,5 MHz-Schallkopf:

Lebergröße rechte MCL:_____cm

Aszites
□ nein □ ja (Menge in l ca.):_____

Echogenität Leberparenchym (Bilddoku: Leber + Niere auf ein Bild)
□ normal □ leicht echovermehrt □ stark echovermehrt

Struktur Leberparenchym (Bilddoku)
□ homogen □ leicht inhomogen □ irregulärer Gefäßverlauf

fokale Minderverfettungen
□ nein □ ja (Größe, Lage):_____

andere Leberraumforderungen (Bilddoku)
□ nein □ ja (Größe, Lage):_____

Körperfett (in cm)
re Rippenbogen_____ paraumbilikal_____

7,5 MHz-Schallkopf:

Leberoberfläche (Bilddoku)
□ glatt □ leicht gewellt □ stark gewellt

Komprimierbarkeit li LL (Fingerpalpation!)
□ gut □ schlecht □ nicht beurteilbar

Duplexsonographie:

Pfortaderfluss: _____ cm/sec □ hepatopetal □ hepatofugal
□ nicht beurteilbar

Diagnose (betreffend die Leber): _____

Annexe 4: Information and agreement

Leber auf den Prüfstand

Patientenaufklärung Pat-Nr.:_____

Sehr geehrte Patientin, sehr geehrter Patient,

Im Rahmen unserer wissenschaftlichen Forschung beschäftigen wir uns u.a. mit den zugrundeliegenden Mechanismen der Gewebeschädigung bei unterschiedlichen Lebererkrankungen.

Wir bitten Sie daher darum, 15 ml Blut (zwei Röhrchen) für Studienzwecke abnehmen zu dürfen.
Wie bei jeder Blutentnahme aus einer Vene kann es zu geringen Schmerzen, einem Bluterguß, einer Entzündung oder Gerinnselbildung kommen. Durch sorgfältige Ausführung läßt sich die Häufigkeit dieser Komplikationen auf ein Minimum reduzieren.
Außerdem würden wir Sie um einige anamnestische Angabe bitten und einige Daten der Ultraschalluntersuchung auswerten. Die Untersuchung verlängert sich dadurch um einige Minuten.

Vertraulichkeit der Unterlagen:
Um die ärztliche Schweigepflicht und den Datenschutz zu wahren, werden die für die wissenschaftlichen Untersuchungen benötigten Krankheitsdaten anonymisiert, d.h. die nötigen Einzelheiten über Ihre Krankheit werden bei den Untersuchungen ohne Ihren Namen verwendet.

Wir möchten Sie darauf hinweisen, dass Ihre Einwilligung auf freiwilliger Basis erfolgt. Sie haben das Recht, Ihre Einwilligung jederzeit zurückzuziehen, ohne dass dies Ihre übliche ärztliche Versorgung oder Ihr Verhältnis zu dem Sie betreuenden Arzt/Personal beeinflussen würde.

Mit Ihrer Einverständniserklärung unterstützen Sie die wissenschaftliche Arbeit auf dem Gebiet des Leberleidens.

Wir danken für Ihre Mithilfe.

(Name des Patienten/der Patientin, Geburtsdatum)

Ich erkläre hiermit, dass ich die Patientenaufklärung gelesen habe und mir verbliebene Fragen erläutert wurden.

Regensburg, den

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(Unterschrift des Patienten) (Unterschrift des/der aufklärenden Arztes/Ärztin)

Leber auf den Prüfstand

Einverständniserklärung Pat-Nr.:_____

(Name des Patienten/der Patientin, Geburtsdatum)

Ich bin damit einverstanden, dass Blut, das im Rahmen der Routinediagnostik von mir gewonnen wird, und Ergebnisse der Ultraschalluntersuchung zu wissenschaftlichen Analysen verwendet wird. Über die Risiken der routinediagnostischen Maßnahmen wurde ich aufgeklärt. Mir wurde zugesichert, dass meine Daten - unter Berücksichtigung der gesetzlichen Datenschutzbestimmungen - anonym behandelt werden und ich meine Einwilligung jederzeit zurückziehen kann.

Regensburg, den

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(Unterschrift des Patienten) (Unterschrift des/der aufklärenden Arztes/Ärztin)