AUS DEM LEHRSTUHL
FÜR INNERE MEDIZIN I
PROF. DR. MED. MÜLLER-SCHILLING
DER MEDIZINISCHEN FAKULTÄT
DER UNIVERSITÄT REGENSBURG

HEPATIC STEATOSIS CAUSES INDUCTION OF THE CHEMOKINE RANTES IN THE ABSENCE OF SIGNIFICANT HEPATIC INFLAMMATION

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

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I. INTRODUCTION

1. Non-alcoholic fatty liver disease (= NAFLD)

1.1 Definition, etiology and histology

Definition

Non-acloholic fatty liver disease (NAFLD) is a broad term used to describe a whole spectrum of liver diseases that ranges from simple steatosis to non-alcoholic steatohepatitis, the extreme form of NAFLD which is regarded as major cause of non-alcoholic, non-cholestatic cirrhosis of the liver and probably hepatocellular cancer (Clark 2003, Bugianesi 2002). The daily consumption of alcohol must not exceed 10g for female or 20g for male patients with NAFLD (Younossi 2008).

Although often being used synonymly steatosis, fatty liver and steatohepatitis are three different, histologically well defined terms: if less then 50% of all hepatocytes contain fat storage, the histological finding is steatosis. In accordance, a stake of more than 50% of all hepatocytes containing fatty storage or a fat portion exceeding 5% of the liver tissue is defined as fatty liver. Steatohepatitis is a combination of hepatocyte damage (e.g. obeseness, ballooning, apoptosis) and an inflammatory compoment (Neutrophilic infiltration, Monocytes) or fibrosis (Dancygier 2006).

Etiology

The exact pathogenesis of non-alcoholic steatohepatitis is not exactly clarified. The current explanation model for the pathogenesis of NASH is a two-hit-hypothesis that was proposed in 1998 by Day and James (Day 1998). The first hit in the progress of NAFLD is hepatocellular steatosis based on insulin resistance, the second hit means necroinflammatory mechanisms that lead to liver injury (Charlton 2002).

To understand insulin resistance, the basic physiology of insulin metabolism is explained in the following: Insulin is the most potent hormone within the human organism to lower serum glucose levels. It is secreted by β-cells, i.e. special cells in Islets of Langerhans located in the pancreas if blood glucose levels exceed around 5mmol/ l. Physiologically, insulin binds to a special protein expressed on the cell surface, the insulin receptor. By binding to this transmembrane receptor, a signalling pathway is started that mediates the fusion of vesicles containing special receptors for glucose transportation, i.e. GLUT-4-transporters. These GLUT-4-transporters are not expressed permanently only in presence of insulin receptor activity. This is an effective mechanism to lower blood glucose levels to approximately 5mmol/ l (Shepherd 1999).

Within the human organism, there are different types of glucose transporters. This is important to understand why insulin is still highly effective in hepatic cells but not in peripheral muscle- and adipose tissue in insulin resistance. While muscle cells and fat cells express mainly above mentioned insulin dependent GLUT-4 transporters, hepatic cells mainly express insulin independent GLUT-2-transporters. That means, GLUT-2-transporters are expressed independently from insulin levels.

Figure 1 illustrates the development of insulin resistance. A combination of **exogenous factors** like overnutrition and lower muscular activity, **hormonal changes** – especially insulin resistance – and possibly **genetic disposition** may lead to a non-alcoholic hepatocellular steatosis . As this combination is common in patients with metabolic syndrome, the epidemiologic correlation of NAFLD and the metabolic syndrome can be easily understood.

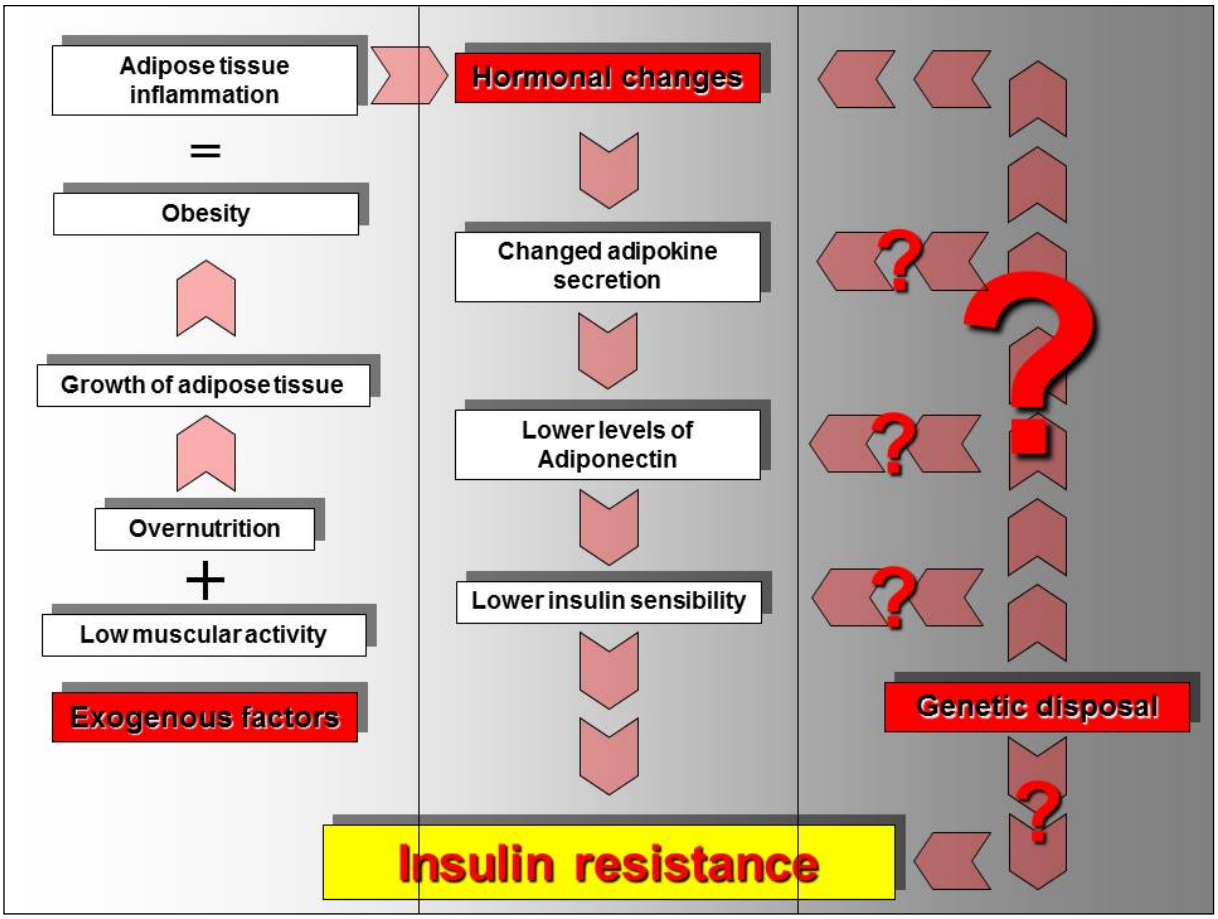


Fig. 1: Etiology of insulin resistance

Overnutrition and fewer muscular activity lead to an increase of adipose tissue and with it to obesity. Proteohormons built in adiopocytes, i.e. spezial cells in fatty tissue, are called adipokines. The most important member of this cytokine family is adiponectin. Adiponectin enhances cellular sensibility to insulin. Obesity is considered as low-grade-inflammation of fatty tissue with higher infiltration of activated macrophages. Macrophages may inhibit the differentiation of adipocytes and by this lead to a modified adipokine secretion and by this to lower levels of adiponectin (Heilbronn 2008). Lower levels of adiponectin lead to lower sensibility to insulin and with it to insulin resistance (Renaldi 2009).

Other cytokines secreted by adipose tissue are TNF-α (tumor necrosis factor), Interleukin-6, leptin and resistin. Higher levels of TNF-α and IL-6 can be found in fat

tissue of obese patients. These proinflammatory cytokines also disrupt normal insulin action in fat- and muscle cells. TNF-α worsens insulin resistance via activation of IKK-β, c-Jun N-terminal kinase (Farrell 2005). Adiponektin is a potent TNF-α-neutralizing and anti-inflammatory adipokine. Furthermore, adiponketin induces anti-inflammatory cytokines, like Interleukin-10 (Tilg 2006). This, again, shows the importance of adiponektine for prevention of insulin resistance.

In the beginning of insulin resistance β-cells compensatorily secrete more insulin to decrease high blood glucose levels. This itself leads to insulin resistance by downregulation of the insulin receptor and with it to downregulation of GLUT-4 (type four glucose transporter) (Flores-Riveros 1993). Therefore, peripheral glucose uptake is reduced and serum glucose levels increases again. As the GLUT-2-transporter expressed on hepatic cells is not insulin dependent and consequently not downregulated, hepatic cells take up glucose permanently. Within a hepatocyte, glucose binds to the ChREBP-transcription factor (carbohydrate response element binding protein) and induces glucose degradation to Acetyl-CoA, the starting product of fatty-acid synthesis (Iizuka 2008).

Insulin itself activates the SREBP-1c-transcription factor (sterol regulatory element binding protein) and induces the synthesis of enzymes of the fatty-acid-synthesis (Guillou 2008).

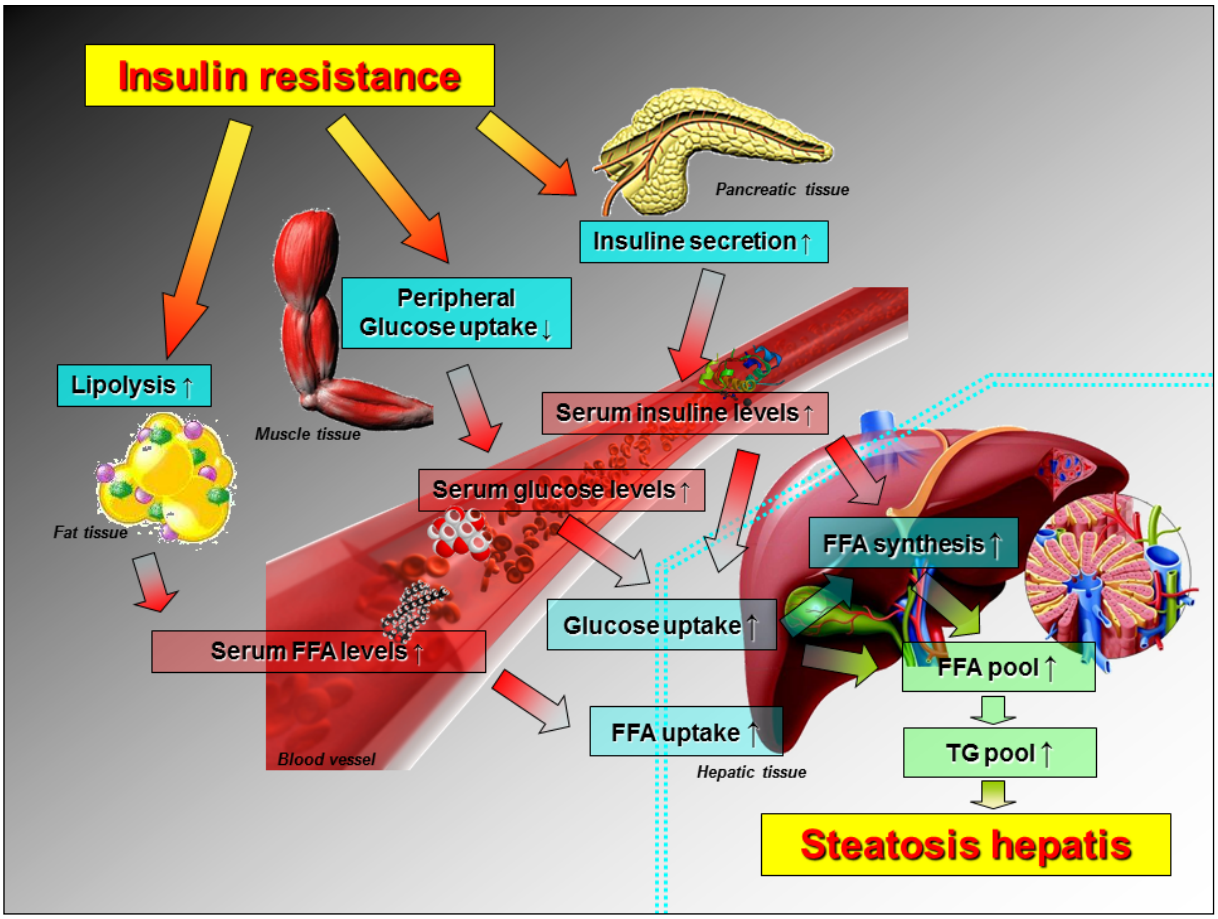


Fig. 2: Insulin resistane. FFA = free fatty acids, TG = triglycerides

Peripheral insulin resistance leads to upregulated lipolysis in fatty tissue. Physiologically, insulin mediates downregulation of lipolysis. Due to the downregulation of insulin receptors and reduced insulin sensibility lipolysis can not be downregulated. Higher lipolysis entails higher levels of free fatty acids (FFA) in serum. Increased serum-FFA-levels lead to higher FFA-uptake in hepatocytes. Intracellular FFA bind to the PPARα-transcription factor which induces the synthesis of enzymes for oxidative degradation of FFA to their building blocks Acetyl-CoA, i.e. β-oxidation. Thereby, the increased intracellular concentration of FFA can be compensated. In obese patients, the PPARα-transcription factor is fewer expressed, probably because of lower adiponectin-levels. Therefore, the intracellular FFA-concentration increases. Intracellular FFA, either de-novo-synthesized or directly uptaken, are processed to triglycerides.

To achieve a new stady steate and to regulate the intracellular triglyceride concentration, surplus triglycerides are either bound to and secreted as very low density lipoproteins (VLDL) or degradated by increased β-oxidation.

Another pathway for decreasing high FFA levels is exporting them by lipoproteins. Lipoproteins are special proteins for transportation of lipophil substances like triglycerides, fatty acids and cholesterin in hydrophil blood. For stabilisation of VLDL-chylomicrons Apolipoprotein B100 is needed. High insulin levels lead to increased intracellular degradation of Apolipoprotein B100 (Charlton 2002). In obese patients with permanent high insulin levels, VLDLs can not be stabilized and by this intracellular triglycerides can not be transported away and accumulate in hepatocytes and lead to hepatic steatosis. Figure 2 summaries the link between insulin resistance and steatosis hepatis.

The above mentioned increased β-oxidation is an alternative pathway do limitate further lipid accumulation in hepatic cells. Increased mitochondrial β-oxidation could be proven in NASH patients (Miele 2003).

As already said, hepatic steatosis represents the first step of the commonly accepted two-hit-hypothesis of non-alcoholic steatohepatitis. The second step in this explanation model is the development of an inflammatory steatohepatitis. The two main pathways in the pathogenesis of steatohepatitis is the effect of oxidative-stress induced lipid peroxidation and cytokine mediated injury (Stewart 2001).

Reactive oxygen species (ROS) are built as a by-product of the respiratory chain. In this energy producing metabolic pathway electrons are transported to different

enzyme complexes to finally reduce molecular oxygen to H_2O. ROS are built if a small fraction of these transported electrons erroneously react with oxygen to reactive oxygen species. Normally, these highly active metabolites are neutralized by enzymatic or non-enzymatic anti-oxidants. Increased β-oxidation leads to high input of electrons to the respiratory chain, by this to high ROS-formation, and consequently, to a high consumption of anti-oxidants. A state in which ROS-formation exceeds the cellular repair- and detoxification leads to injury of cellular and extracellular macromolecules. This state is called oxidative stress (Schmidt 2007).

A possible explanation for the increased building of ROS is the decreased activity of respiratory chain complexes (Pérez-Carreras 2003). Both mechanisms, the increased input of electrons by up-regulated β-oxidation and the impaired outflow of electrons by lower enzyme activity lead to accumulation of electrons within the respiratory chain and thus, to increased ROS formation (Pessayre 2004).

A second source of mitochondrial ROS could be hepatic CYP2E1, which is increased in patients with NASH. CYP2E1 can produce ROS and trigger lipid peroxidation (Chalasani 2003). CYP2E1 is responsible for microsomal ω1- and ω2-oxidation of free fatty acids, which leads to formation of cytotoxic decarboxylated acids and of free radicals (Woodcroft 2002). If the formation of ROS and free radicals exceeds their enzymatic and non-enzymatic metabolism, mitochondrial DNA, mitochondrial enzymes and proteins of the respiratory chain are damaged.

Lipid peroxidation means a chain reaction mechanism that is initiated by the reaction of ROS and fatty acids which are commonly found in lipid membranes. Fatty acid radicals, very instable products of this reaction, react spontaneously with oxygen to form again other organic oxygen radicals. Most important representatives of lipid peroxidation products are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). If

this organic oxygen radical chain reaction can not be stopped by antioxidants like vitamin E, they cause structural and functional modifications of lipid double layers and biomembranes by interaction with their unsattured lipids. This leads to an vicious circle because by affecting mitochondrial biomembranes and with it mitochondrial respiratory chain, even more ROS are built. This again leads to oxidative stress and mitochondrial and cellular damage. Sanyal et al. could show that NASH patients show high levels of lipid peroxidation (Sanyal 2001). On the other side, glutathione levels, which serve as a marker of an organism's antioxidant capacity, are decreased (Vendemiale 2001).

Firstly, ROS can directly damage mtDNA, respiratory chain polypeptides and mitochondrial cardiolipin, the latter effect relases reactive lipid peroxidation products, which also damage mitochondria. These effects further block the flow of electrons within the respiratory chain and with it increase again ROS formation.

Secondly, ROS activate NF-κB, which induces the hepatic synthesis of TNF-α, which also damages mitochondria and increases again ROS formation by increasing the outer mitochondrial membrane permeability for cytochrome c and thus to again impaired electron flow within the respiratory chain (Chalasani 2003). Crespo et al. could show that patients with NASH have high hepatic TNF-α mRNA levels (Crespo 2001).

As a result, the production of ATP (adenosinetriphosphate), the main intracellular energy storage, is decreased. The impaired energy homeostasis contributes to cell damage (Sanyal 2001).

Thirdly, high production of ROS lead to a consumption of anti-oxidants which further aggravates ROS-induced damages. Indeed low vitamin E levels are found in obese children with steatohepatitis (Strauss 1999).

Both, lipid peroxidation products and TNF-α may damage mitochondria and lead to mitochondrial dysfunction by different mechanisms. The hepatic mitochondria of NASH patients exhibit ultrastructural lesions with the presence of para-crystalline inclusions in **megamitochondria** (Caldwell 1999).

Mitochondria have their own mitochondrial DNA (mtDNA). This DNA encodes for 13 polypeptides of mitochondrial respiratory chain. Haque et al. could show that mtDNA is severly depleted in NASH patients (Haque 2002). Although the exact mechanism is still not exactly clarified, a possible explanation for this depletion could be due to lipid peroxidation products and ROS, which both can damage mtDNA (Hruszkewycz 1988).

TNF-α also impairs the respiratory chain by partially blocking the electron flow. TNF-α increases the permeability of mitochondrial membranes and by this releases cytochrome c from the intermembrane space of the mitochondria into the cytosol. This partially blocks the cytochrome c mediated electron flow from complex III to complex for of the mitochondrial respiratory chain (Pessayre 2004). Indeed, patients

with NASH have an impaired in-vivo ability to re-synthesize ATP after a fructose challenge (fructose transiently depletes hepatic ATP) (Cortez-Pinto 1999).

The cellular damage and oxidative stress cause an **inflammatory reaction** mediated by cytokines like TNF-α, Interleukin-8 (IL-8) and Interleukin-6 (IL-6). The expression of these cytokines is mediated by ROS themselves and by activated aldehydes like 4-HNE (Pessayre 2001). IL-8 as chemoattractant mediates immigration of leukocytes. As one third of the proinflammatory and leukocyte attracting Interleukin-6 is built in adipose tissue the correlation of obesity an steatohepatitis can be comprehended. This might be a reason why obesity also favors the transition from steatosis to steatohepatitis.

Another source of cytokine production is the activation of resident hepatic macrophages called Kupffer cells. Kupffer cells are activated by endotoxines that reach the liver from the intestine via the portal vein. Endotoxins are structural molecules of the outer bacterial membrane. Prototypical examples of endotoxin are lipopolysaccharide (LPS) and lipooligosaccharide (LOS). High intestinal endotoxin production is the result of bacterial overgrowth, a state which is associated with NASH (Wigg 2001). Endotoxins activate the Toll-like-receptor 4 (TLR-4) and CD14, both endotoxine receptors on the cell surface of Kupffer cells. This activates the transcription of pro-inflammatory cytokines, like TNF-α or IL-6 (Michael 2004).
Cytokines induce the immigration of leukocytes out of the blood vessels and are capable of producing all of the classical histological features of NASH (see below).

As already discussed, there are different sources of TNF-α. It can be released by fat-engorged adipocytes, by ROS-stimulated hepatocytes or by endotoxin-stimulated

Kupffer-cells. TNF-α interacts with its receptor TNF-receptor 1 (TNFR1). The hepatic expression of both, TNF-α mRNA and TNFR1 is increased in patients with NASH (Crespo 2001). This interaction of TNF-α and TNFR1 activates a apoptotic pathway by activating a casade of proteases (caspases), which make the outer mitochondrial membrane leaky for proteins (cytochrome). Further mitochondrial damage also opens the inner mitochondrial membrane for proteins which exaggerates apoptosis. Caspase levels are also increased in patients with NASH or alcoholic liver disease (Ramalho 2006).

A second pathway to initiate a apoptose pathway is the interaction of Fas, a membrane receptor, with its ligand, the Fas ligand. Normally, hepatocytes express Fas, but not Fas ligand, which prevents the from killing their neighbour hepatocytes. Similar to TNFR1, Fas expression is also increased in NASH patients (Feldstein 2003). Above described conditions in hepatocytes leading to increased ROS formation can cause Fas ligand expression by hepatocytes, so that Fas ligand on one hepatocyte can now interact with Fas on another hepatocyte, to cause its apoptosis (Pessayre 2000).

A major drop of hepatocellular ATP levels prevents apoptosis, which requires energy, and blocks plasma membrane pumps. This causes cell and organelle swelling (**ballooning**) and later plasma membrane rupture and necrotic cell death (**necroinflammation**) (Leist 1997). Hepatocellular ballooning (see below) refers to enlarged hepatocytes with rarefied, reticular cytoplasm indicating cell injury. It is considered as the result of alterations in intermediary filament cytoskeleton (Tiniakos 2010).

Kupffer cells, which ingest apoptotic bodies, release TGF-β, which activates hepatic stellate cells (Ito-cells) into collagen-producing myofibroblastic cells. Cytokines and 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) also induce liver **fibrosis** via activation of stellate cells and result in increased production of transforming growth factor-beta (TGF-β). Leptin, another member of the adipokine family, which is – in contrast to adiponectin – up-regulated in NASH patients contributes to insulin resistance and might even stimulate fibrogenesis in animal models of NAFLD (Ikejima 2001).

Lipid peroxidation products activate fibrogenesis in two ways: Firstly, lipid peroxidation products enhance the production of TGF-β by macrophages (Leonarduzzi 1997). Second, lipid peroxidation products directly induce collagen production by activated stellate cells (Parola 1993).

TGF-β also induces tissue transglutaminase. This enzyme is associated with the cytoskeleton, including intermediary filaments. The induction of tissue transglutaminase could partizipate in the generation of **Mallory bodies**, which are formed of aggregated cytoskeletal proteins, in particular polymerized cytokeratins (Pessayre 2001).

Histology

Non-alcoholic fatty liver disease

The minimal histological change of NAFLD is hepatocellular steatosis which means accumulation of triglycerides within spezial liver tissue cells called hepatocytes. This hepatocellular steatosis is present in all cases and has generally benign course.

According to the American Association fort the study of liver diseases (AASLD) NAFLD is defined as the accumulation of fat in the liver tissue exceeding 5 to 10% by weight (Neuschwander-Tetri 2003).

This steatosis is commonly macrovesicular, that means a singular large fat droplet or smaller well-defined intracytoplasmatic doplets displacing the nucleus. Rarely, groups of hepatocytes with microvesicular steatosis may also be found. In this case, these hepatocytes are filled with many tiny lipid droplets that do not displace the nucleus (Tiniakos 2010). Figures 3 and 4 are supposed to show this difference between macro- and microvesicular steatosis.

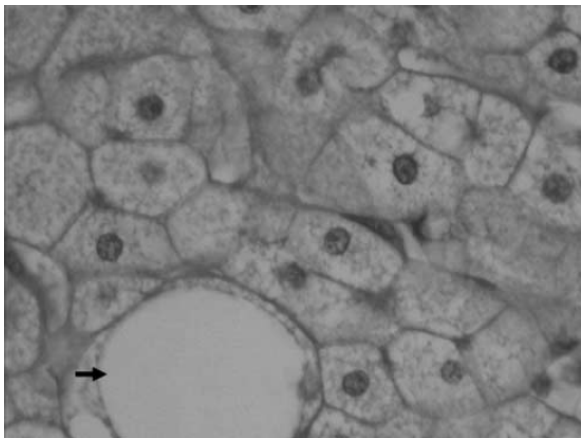


Fig.3: Nonalcoholic fatty liver disease: microvesicular steatosis in most hepatocytes, while one shows macrovesicular steatosis (arrow) (hematoxylin and eosin, _400).
Source: Tiniakos 2010

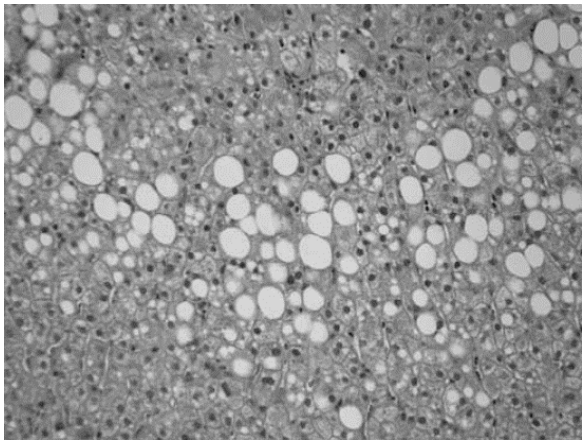


Fig. 4: Nonalcoholic fatty liver disease: macrovesicular steatosis (hematoxylin and eosin, _100).
Source: Tiniakos 2010

Non-alcoholic steatohepatitis

The histological finding of NASH is often a trias of steatosis which was described above, hepatocellular balloning and lobular inflammation. This trias represents the minimal criteria for adult NASH. In 10-15% of NASH patients progressive fibrosis can also be found, but just like in other chronic liver diseases, fibrosis is not required for the diagnosis of steatohepatitis. However, there are no histological differences between alcohol induced and non-alcoholic fatty liver diseases.

Hepatocellular ballooning means enlarged hepatocytes with rarefied, reticular cytoplasm indicating cell injury. It is considered as the result of alterations in intermediate filament cytoskeleton. Other forms of hepatocellular injury like apoptotic bodies and lytic necrosis may also be present (Tinakos 2010). Loss of keratin 8/ 18 immunostaining might be a possible marker for intermediate filament alterations (see Figure 5).

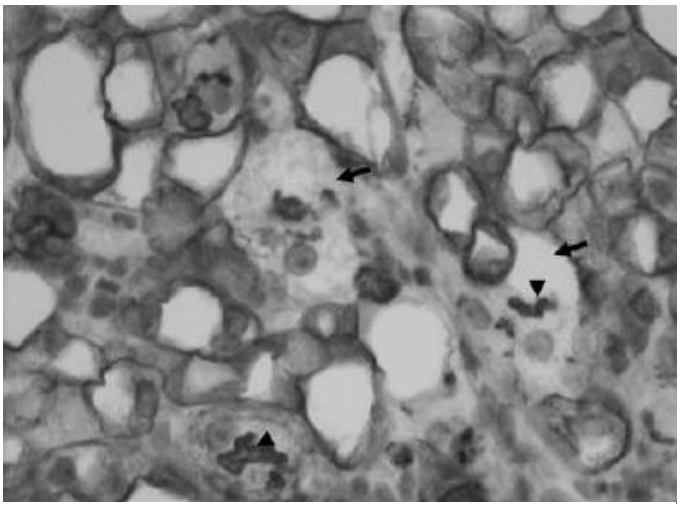


Fig. 5: Nonalcoholic steatohepatitis: immunohistochemistry for Keratin 18, highlights absence of staining in ballooned hepatocytes (arrows) and positive staining in Mallory–Denk bodies (arrowheads), _400.
Source: Tiniakos 2010

Inflammation is a possible feature of NASH histology. If present it is usually mild and consists of mixed inflammatory cell infiltrate. “Satellitosis”, a lesion often seen in NASH histologies, means polymorphs around ballooned hepatocytes. Inflammation can be observed in lobular and portal areas. In untreated patients, portal inflammation can be considered as a marker of advanced disease, as it correlates with a diagnosis of definite steatohepatitis and advanced fibrosis (Tiniakos 2010).

Fibrosis in patients with non-cirrhotic NASH is typically perisinusoidal/ pericellular (chickenwire). Fibrosis is associated with active lesions of NASH but may also be seen without. With progression of NASH, portal and periportal fibrosis may be observed, in some cases by bridging fibrosis (Tiniakos 2010).

Mallory-Denk bodies - another histological feature that can be found in NASH - are irregularly shaped, eosinophilic, intracytoplasmatic inclusions composed of keratins 8 and 18, ubiquitin, heat shock proteins and p62. Mallory-Denk bodies are usually found in ballooned hepatocytes (see Figure 6) (Zatloukal 2004).

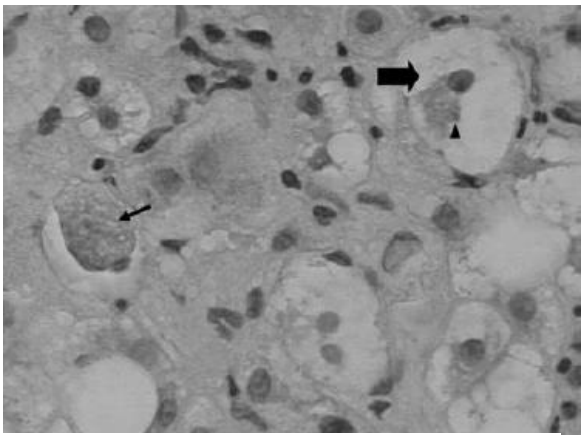


Fig. 6: Nonalcoholic steatohepatitis: liver cell injury in the form of ballooning (thick arrow) and apoptotic body (thin arrow) is evident. The ballooned hepatocyte contains a Mallory–Denk body (arrowhead) (hematoxylin and eosin, _400).
Source: Tiniakos 2010

Megamitochondria (see etiology) appear in the histological image as round eosinophilic structures within hepatocytes and (as discussed above) are a result of lipid peroxidation. On electron microscopy, these abnormal mitochondria show paracrystalline and loss of cristae (Sanyal 2001, Caldwell 2004).

Glycogenated nuclei are vacuolated nuclei and are typically located in periportal hepatocytes (Sorrentino 2004). Some investigators consider them as characteristic for NASH because they are found very rarely in biopsies of alcoholic steatohepatitis.

Grading and Staging

Matteoni et al. initially proposed a classification system that correlated features of NAFLD with disease outcome: they descriped four types of NAFLD: simple steatosis (type 1), steatosis and lobular inflammation (type 2), steatosis and hepatocellular ballooning (type 3), and steatosis with ballooning and either Mallory-Denk bodies or fibrosis (type 4). In this classification type 3 and 4 histologically and clinically resembled NASH as diagnosed now and were associated with subsequent

development of cirrhosis. This system did not include assessments of disease severity and was intended mainly for clinical use (Matteoni 1999).

Brunt et al. proposed another **grading** classification system based on the major histopathological lesions of NASH, that is steatosis, hepatocellular ballooning and inflammation (see Table 1). They also proposed a **staging** method for the characteristic pattern of fibrosis in NASH (see Table 2) (Brunt 1999).

Table 1. Brunt grading system for NASH

Grade	Steatosis	Ballooning	Inflammation
Mild (grade I)	1-2	Minimal	L= 1-2 P= 0-1
Moderate (grade II)	2-3	Present	L= 1-2 P= 1-2
Severe (grade III)	2-3	Marked	L= 3 P= 1-2

Steatosis:	**Lobular inflammation (L):**	**Portal inflammation (P):**
1: 0-33%, 2: 33-66% 3: 66-100%	0= none, 1= < 2 foci/ 20x field, 2= 2-4 foci/ 20x field, 3= > 4 foci/ 20x field.	0= none, 1= mild, 2= moderate, 3= marked.

(Brunt 1999)

Table 2. Brunt staging system for NASH

Stage	Perisinusoidal fibrosis	Periportal fibrosis	Bridging fibrosis	Cirrhosis
1	**Focal or extensive**	**0**	**0**	**0**
2	**Focal or extensive**	**Focal or extensive**	**0**	**0**
3	**+/-**	**+/-**	**+**	**0**
4	**+/-**	**+/-**	**extensive**	**+**

(Brunt 1999)

1.2 Epidemiology and risk factors

Epidemiology

Non-alcoholic fatty liver disease has become the most common liver disease in Western countries. The **prevalence** in of NAFLD is rapidly increasing worldwide in parallel with the rising prevalence of obesity and type 2 - diabetes (Björnsson 2007). The prevalence of NAFLD is estimated to be 20-30% in adult population in industrialized countries. It is important to note that these numbers are always dependant on the method used to diagnose fatty liver, the method to assess alcohol consumption and the cut-off used to exclude alcoholic liver disease. The prevalence of NASH in common population is estimated to be 2-3% (Bellentani 2004).

The prevalence of NAFLD varies according to age, gender and weight status:

Gender

In contrast to early studies emphasizing that NAFLD is more common in women, recent studies show that NAFLD may be even more prevalent among males. A possible reasond for this might be the postulation that female hormones protect against NAFLD. This postulation is supported by evidence showing that NAFLD is twice as common in post-menopausal women as in premenopasual women. Women receiving hormone replacement therapy are significantly less like to have NAFLD compared with women without hormone therapy (Clark 2002, Carulli 2006, McKenzie 2006).

Age

NAFLD can be found in all age groups. However, as a rule, the prevalence appears to increase with age. An old study out of 1977 showed a prevalence of 1% in people

under 20 years, 18% in the group of 20 to 40 years and 39% in people among 60 and older (Hilden 1977).

Type 2 Diabetes

Several studies have described a higher prevalence of NAFLD among type 2 diabetes patients compared with non-diabetics with prevalence estimates ranging from 40% to 69.5% (Targher 2007, Angelico 2005, Kelley 2003). Patients with type-2-diabetes do not only have higher prevalence of NAFLD, but also appear to have more severe forms of the disease, including NASH and fibrosis (Angulo 1999, Dixon 2001).

Weight

In their review of twelve studies on prevalence of NAFLD, Machado et al found a prevalence of NAFLD up to 90% in morbidly obese patients with a body mass index (BMI) of more than 40kg/ m² (Machado 2006). 37% of these patients undergoing bariatric surgery had NASH.

There is very few data available on the **incidence** of NAFLD in the general population. In a 8.5-year-follow-up of the Dionysos Study a incidence of 18.5 per 1000 person-years was found (Bedogni 2007).

Insulin resistance

As described above, insulin resistance is considered to be the pathophysiological cause of NAFLD. Several studies have found strong associations between direct measures of insulin resistance and NAFLD. In addition, different studies showed a good correlation between NAFLD and metabolic syndrome which is considered to be the clinical construct of insulin resistance. A long-term prospective study demonstrated that the presence of metabolic syndrome is associated with increased odds for NAFLD (OR= 4.0 for men and 11.2 for women) (Hamaguchi 2005). Another cross-sectional study found that the presence of metabolic syndrome was associated with higher odds of NASH (OR = 3.2) and fibrosis (OR = 3.5) (Marchesini 2003).

Obesity

Worldwide, obesity remains the most important and well-described risk factor for NAFLD. Obesity is defined as a body mass index (BMI) greater tha 30 kg/ m². In the Dionysos study, NAFLD was present in 94% of obese population, 67% in overweight population and 24.5% in normal weight population (Bellentani 2004). Regardless of BMI, patients with truncal obesity are at greater risk of fatty liver disease.

Genetic factors

A genetic susceptibility to NAFLD, NASH and its complications has been postulated. Environmental factors like obesity cause simple steatosis in the majority of people. However, in a minority of the expsed, more advanced forms of NAFLD and hepatocellular cancer can be found. This leads to the hypothesis that susceptibility is inherently determined. Epidemiologic studies showing familial aggregation of NFALD and insulin resistance also support a genetic basis for NAFLD. Furthermore, studies have found inter-ethnic-variations in the prevalence of NAFLD (Lazo 2008).

Other risk factors to develop NAFLD are disorders of lipid metabolism, total parenteral nutrition, iatrogenic medication with diltiazem, amiodarone, tamoxifen, steroids and highly-active anti-retroviral therapy.

1.3 Metabolic syndrome: definition and components

The metabolic syndrome is defined by the presence of 3 or more of the following criteria: increased waist circumference, hypertriglyceridemia, hypertension, high fasting glucose or a low high-density lipoprotein (HDL) level. Most NAFLD patients carry at least one feature of metabolic syndrome and/ or are obese. Conversely, it has been shown that a high number of patients with metabolic syndrome have steatotic livers. NAFLD is recognized to be the hepatic manifestation of the metabolic syndrome.

1.4 Clinical manifestation and diagnosis

Diagnosis of NAFLD is based on clinico-pathological criteria. A diagnosis of NAFLD can be achieved by excluding other causes of abnormal liver function tests and after performing appropriate imaging. Liver biopsy is still considered as the gold standard for the diagnosis of NAFLD. But still, in the clinical setting, there is no consensus, about whether or not liver biopsy is required to confirm a diagnosis of NAFLD.

History and physical examination

Most NAFLD patients are asymptomatic. They are mostly identified while undergoing imaging examination for evaluation of abnormal liver function test results. Patients might complain about light right upper quadrant abdominal pain, nausea and other non-specific symptoms concerning the gastrointestinal tract (Lewis 2010).

In the physical examination hepatomegaly is common. In patients with cirrhotic livers, of course, all signs of portal hypertension may occur (i.e. ascites, edema, spider angiomas, varices, gynecomastia). As metabolic syndrome is the most common association, increased waist circumference is common.

Serology

There is no single biochemical marker that can confirm a diagnosis of NAFLD or distinguish between steatosis, NASH and cirrhosis. Liver function test (LFT) abnormalities are common in NAFLD patients, with (if present) slightly elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT), usually not exceeding four times the upper limit of normal with ALT higher then AST. A ratio of AST/ ALT greater then 2 suggests an alcoholic cause (Pratt 2000). In addition, some patients can present with isolated elevations of alkaline phosphatise (Pantsari 2006).

Alkaline phosphatase levels can also be elevated in NASH patients. Hyperlipidemia, elevations of serum ferritin, iron and decreases of transferring saturation may also occur in patients with NASH.

Poynard et al. proposed the **SteatoTest**, a battery of biochemical markers including ALT, α2-macroglobulin, apolipoprotein A-I, haptoglobin, total bilirubin, γ-glutamyl

transpeptidase (GGT), cholesterol, triglycerides, glucose, age, gender, and BMI, that predicted >30% steatosis with sensitivity of 90%, specifity of 90%, negative predictive value of 93% and positive predictive value of 63% (Poynard 2005).

The **FibroTest**, another test to diagnose advanced fibrosis in NASH by biochemical markers, including α2-macroglobulin, apolipoprotein A-I, haptoglobin, total bilirubin, γ-glutamyl transpeptidase (GGT) and ALT. It is useful to find predictors of fibrosis because patients with elevated markers of fibrosis might be at risk for developing cirrhosis and its complications. The positive prediction value of this test ist 73%, the negative predictive value for severe fibrosis is 90%, but the test does not differentiate among stages of fibrosis. Similarly, the NAFLD fibrosis score, an index consisting of age, hyperglycaemia, body mass index, platelet count, albumin ans AST/ ALT ratio, showed a positive prediction value of 82% and a negative predictive value of 93% (Ratziu 2006).

Leptin, a hormone secreted by adipose tissue (as discussed above), has shown profibrotic effects in animal models (Ikejima 2001). Even though human studies reported that elevated leptin levels are found in patients with steatosis and NASH, absolute leptin levels have not been shown to correlate with degree of steatosis or fibrosis (Uygun 2000).

TNF-α, another inflammatory mediator involved in the “multi-hit-hypothesis” (see above), is elevated in patients with NASH and absolute TNF- α correlate with severity of inflammation and fibrosis (Crespo 2001). Other circulating markers of inflammation, in particular interleukin-6 (**IL-6**), CC-chemokine ligand 2 (**CCL-2**) and hyaluronic acid (**HA**) are elevated in NASH patients (Haukeland 2006, Suzuki 2005).

Imaging

Ultrasound is the main imaging procedure to detect NAFLD related liver changes. The ultrasound correlate of steatosis is increased echotexture, called a “bright liver”. The hepato-renal contrast predicts steatosis and fibrosis more accurately. Non-steatotic hepatic parenchyma exhibits an echotexture similar to that of renal parenchyma. In steatotic livers, the liver parenchyma appears “brighter” because fat appears white in ultrasound imaging. Saadeh et al. found the sensitivity of ultrasound scan to be 100% (Saadeh 2002). A possible criticism of ultrasound is that it is not able to evaluate inflammation and fibrosis.

Liver elasticity is an non-invasive measure of liver fibrosis that is performed by special ultrasound machines, called Fibro-Scan. When ultrasound is used, the ultrasound probe emits vibrations that creates a shear wave within the liver. This shear wave corresponds to liver stiffness (Lewis 2010, Castera 2005). Takeda found a good correlation of liver elasticity measured by Fibro-Scan and Brunt fibrosis score. Liver stiffness was significantly higher in patients with stage 3 or stage 4 fibrosis (Takeda 2007). Fukuzawa et al. measured liver stiffness by Fibro-Scan of biopsy proved NAFLD of 135 patients and showed that liver elasticity accurately predicted fibrosis and that Fibro-Scan was able to distinguish each of the Brunt classification stages (Fukuzawa 2007).

CT-scan

CT scans are also used to evaluate liver architecture. Piekarski et al. measured CT numbers of normal subjects. They found an average CT number of the normal liver of 24.9 Hounsfield units (HU) and an average CT number of the spleen of 21.1 HU. In this study fatty livers were associated with lower CT numbers (Piekarski 1980).

Lee et al. studied liver-to-spleen attenuation and visual grading on non-enhanced CT scans of 703 liver donor candidates and compared these values with findings of liver biopsies. Both measures proved accurate the diagnosis of steatosis exceeding 30% of the liver (Lee 2007). It appears that non-contrast CT scans are more useful in steatosis diagnostic (Jacobs 1998).

Magnetic resonance tomography (MRI)

Magnetic resonance tomography is able to detect steatosis reliably. Fatty changes are assessed by differential chemical shifts between fat and water (Lewis 2010). Fishbein et al. found in their study good correlation between MRI, ultrasound and histology in NAFLD patients. In the same study, they showed that MRI detected lower levels of steatosis more accurately than CT scans and ultrasound (Fishbein 2005). But MRI assessment remains a more expansive and less accessible technology.

2. Chemokines and its receptors

2.1 Chemical properties and classification

Chemokines are a big family of chemotactical cytokines with structural similarities in their secondary structure. Chemokines can be synthesized by almost every human cell. Their name is derived from their main function: the ability to induce directed chemotaxis in nearby responsive cells. Till now, more then 44 chemokines and 21 chemokine-receptors have been described. Chemokines are small molecules with a weight of 8-10 kDa and 20-70% homologies in their amino acid sequence.

Their tertiary structure is stabilized by disulfide bonds between condensated cysteine residues (Fernandez 2002).

Depending on four of these invariant cysteine residues chemokines are classified into four subclasses: the C-, CC-, CXC- and CX3C-subfamily. In any case the first cysteine residue binds covalently to the third and the second cysteine residue to the forth.

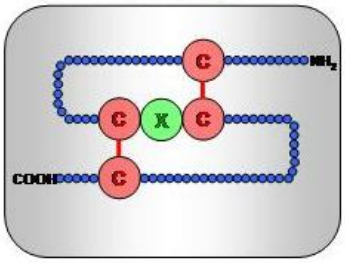


Fig. 7: CXC-chemokines

Chemokines that have one amino acid between the first two N-terminal cysteines are subclassified as CXC- or α-chemokines (see Figure 7). This subfamily is further divided into two groups depending on another special amino acid sequence (i.e. motif) consisting of three amino acids: glutamic acid ("E" in single letter code) – leucine ("L" in single letter code) – arginine ("R" in single letter code) (Fernandez 2002).

Chemokines showing this motif immediately before the first N-terminal cysteine are called ELR-positive chemokines or ELR^{+}-chemokines (see Figure 8), those without this motif are called ELR-negative chemokines or ELR^{-}-chemokines. Both groups also show differences in chemoattractant functions and bind to different types of receptors (2).

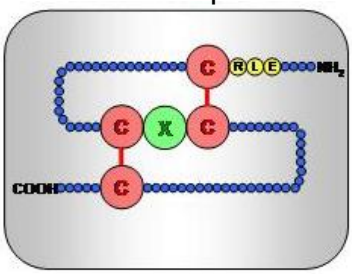


Fig. 8: ELR^{+}-CXC-Chemokines

If the first two N-terminal cysteines are adjacent that means they follow each other directly in the primary sequence, the chemokines are called CC- or β-chemokines (see Figure 9).

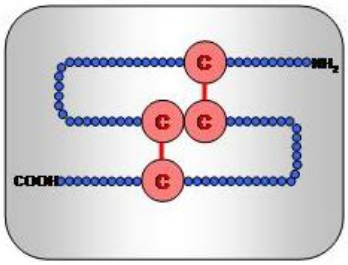


Fig. 9: CC-chemokines

This subfamily is also divided into two subgroups: CC-chemokines containing four cysteines are called C4-CC chemokines and chemokines with six cysteines C6-CC chemokines. The latter subgroup is represented by only a small number of chemokines, for example CCL21.

Similar to the CXC- or α-subfamily, chemokines belonging to the CX_3C-or γ-subfamily have three intervening amino acids between the first two cysteines (see Figure 10). This subfamily - so far – is represented by only one member called fractalkine or CX_3CL1.

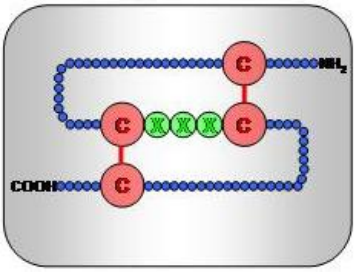


Fig.10: CX_3C-chemokines

In contrast to the members of the first three subfamilies, the chemokines of a fourth subfamily – the C- or δ-subfamily shows only two cysteine residues in its primary sequence (see Figure 11) (Fernandez 2002). This group is represented by two members: XCL1 or lymphotactin-α and XCL2 or lymphotactin-β.

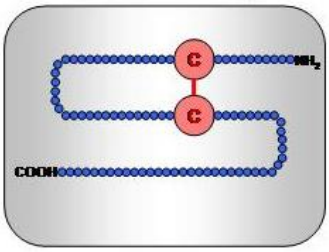


Fig. 11: C-chemokines

2.2 Function and clinical relevance of Chemokines

As already said, chemotaxis of immune competent cells is the main function of chemokines. As chemoattractant they guide the migration of cells. In accordance to their main function, two main groups of Chemokines are distinguished: pro-inflammatory or inducible and homeostatic or constitutive chemokines. Most Chemokines are pro-inflammatory and are released in response to bacterial infection, viruses and agents that cause physical damage. Homeostatic Chemokines are produced continuously and are involved in surveillance of normal tissue, such as directing lymphocytes to lymph nodes.

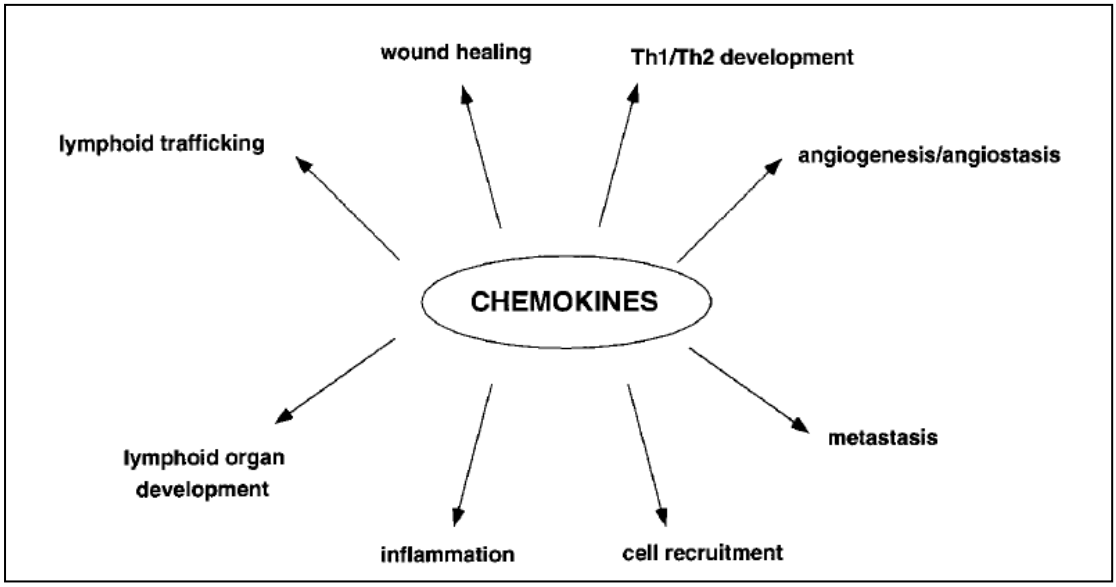


Fig. 12: Biological functions of chemokines and chemokine receptors
Source: Schall T. Biology of the RANTES/ SIS cytokine family. Cytokine 1991 May; 3(3): 165-83.

Leukocyte trafficking

Cells attracted by chemokines follow a signal of increasing chemokine concentration towards the source of the chemokine. By this they direct lymphocytes to lymph nodes so they can screen for invasion of pathogens by interacting with antigen-presenting cells like dendritic cells.

Chemokines also play a central role in the process of extravasation of leukocytes. In the scheme of rolling, adhesion and transmigration of immune competent cells chemokines are involved in every step including interactions of adhesion molecules and the chemoattractant function of these proteins:

As part of the inflammatory response, activated thrombocytes or endothelial cells release chemokines which serve as signal for rolling leukocytes. These leucocytes “crawl” along the endothelial membrane following the chemokine gradient. This process is called haptotaxis. In this process RANTES seems so play a crucial role. At the same time leukocytes up-regulate their integrine expression on their outer cell membrane and endothelial cells express P-selectines, the corresponding ligand for integrines. This forms the basis for extravasation of leukocytes. Chemokine induced matrix-metalloproteases cause lysis of the basal membrane which eases the transmigration.

Angiogenesis and angiostasis

Angiogenesis is a biological process through which blood vessels are generated. Angiogenesis is not only physiological in wound healing, it is also associated with several chronic inflammatory diseases, such as psoriasis, rheumatoid arthritis as well as tumor growth and metastasis (Arenberg 1997). It is well established that ELR+-CXC-chemokines are potent angiogenic factors, able to stimulate endothelial cell chemotaxis, whereas most non-ELR-CXC-chemokines are strong angiostatic factors which inihibit the endothelial cell chemotaxis (Strieter 1995).

Metastasis

Besides their role in angiogenesis chemokines also seem to be involved in the process of tumor cell migration, invasion and metastasis. It is known that certain tumors exhibit patterns of metastasis to certain organs, in other words tumor cells do not migrate randomly. One possible explanation for this is that this specific migration of tumor cells may be determined by the Chemokine receptors they express and the chemokines expressed by target organs. As a proof for this hypothesis, Youngs et al have reported that different breast carcinoma cell lines respond differentially to distinct chemokines (Youngs 1997).

Anti-tumor activity

Although the presence of chemokines in some chronic inflammatory diseases may not be beneficial, in other diseases, like cancer, it is desirable that the immune response is promoted. In theory, any chemokine that is capable of inducing the migration of T-cells, natural killer cells (NK-cells), dendritic cells or macrophages could promote regression or even eradication of a tumor mass by boosting the immune response against the tumor. In a mouse model, lymphotactin (XCL1) in combination with Interleukin-2 (IL-2) has shown anti-tumor activity. The most likely explanation for this is, that lymphotactin induces T-cell and natural-killer cell infiltration to the tumor site while IL-2 expands the T-cell clones and enhances a specific immune response (Hedrick 1997).

Organogenesis

Although traditionally associated with the development and response of the immune system chemokines also play an important role in organogenesis: knock-out mice leaking genes for CXCL12 (SDF-α) or its receptor CXCR4 have suffer from impaired

fetal development of the cerebellum, the cardiac septum, gastric vasculature and B-cell lymphopoesis. These mice die either in utero or at birth (Nagasawa 1996, Zou 1998).

2.3 Structure and ligands of chemokine receptors

Approximately 20 signalling chemokine receptors and 3 non-signalling Chemokine receptors have been reported. All Chemokine known receptors are seven-transmembrane receptors (7TM) with seven helical membrane-spanning regions that are connected by extra-membranous loops. The N-terminus of the amino acid chain and three extracellular loops are exposed outside of the cell, the C-terminus of the amino acid chain and again three extracellular loops face to the intracellular loop (Allen 2007). Figure 13 is a good scheme to clarify the structure of a 7TM-

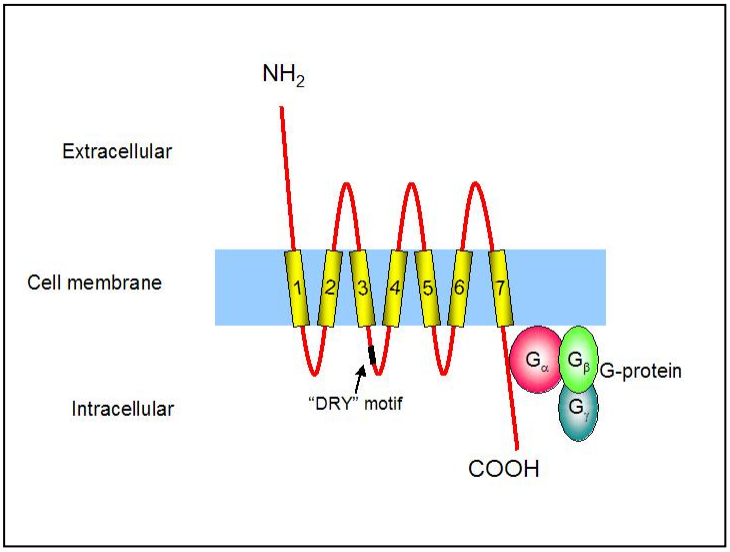


Fig. 13: Structure of a 7TM-chemokine receptor.
Source: Wikipedia, the free encyclopedia

Table 7 shows a good overview of known Chemokine receptors and their chemokine ligands and illustrates again the fact that different ligands bind the same receptor and some ligands bind different receptors.

Table 7: Chemokine receptors and their ligands	
Receptor	**Ligands**
CCR-1	CCL3, CCL5, CCL7, CCL13, CCL14, CCL15, CCL16, CCL23
CCR-2	CCL2, CCL7, CCL8, CCL13, CCL16
CCR-3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL24, CCL26, CCL28
CCR-4	CCL17, CCL22
CCR-5	CCL3, CCL4, CCL5, CCL8, CCL11, CCL14, CCL16
CCR-6	CCL20
CCR-7	CCL19, CCL21
CCR-8	CCL1
CCR-9	CCL25
CCR-10	CCL27, CCL28
CXCR-1	CXCL6, CXCL7, CXCL8
CXCR-2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
CXCR-3A	CXCL9, CXCL10, CXCL11
CXCR-3B	CXCL4, CXCL9, CXCL10, CXCL11
CXCR-4	CXCL12
CXCR-5	CXCL13
CXCR-6	CXCL16
CXCR-7	CXCL12
XCR-1	XCL1, XCL2
CX_3CR-1	CX3CL1
CCX-CKR	CCL19, CCL21, CCL25
D6	CCL2, CCL31, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22
DARC/ Duffy	CCL2, CCL7, CCL8, CCL11, CCL13, CCL14, CCL16, CCL17, CXCL1, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL11, CXCL13

.

2.4 Signalling pathways of chemokine receptors

Activation

Chemokine receptors are G-protein coupled receptors (GPCR). This name is derived from their signal transduction mechanism. A heterotrimeric G-Protein is bound to the intracellular loops of the 7TM-receptor (see Figure 14a). The heterotrimeric G-protein consists of three subunits: Gα-, Gβ- and Gγ-subunit. The Gα-subunit directly interacts with the intracellular loops and with the Gβ-subunit which in turn forms a tight complex with the Gγ-subunit.

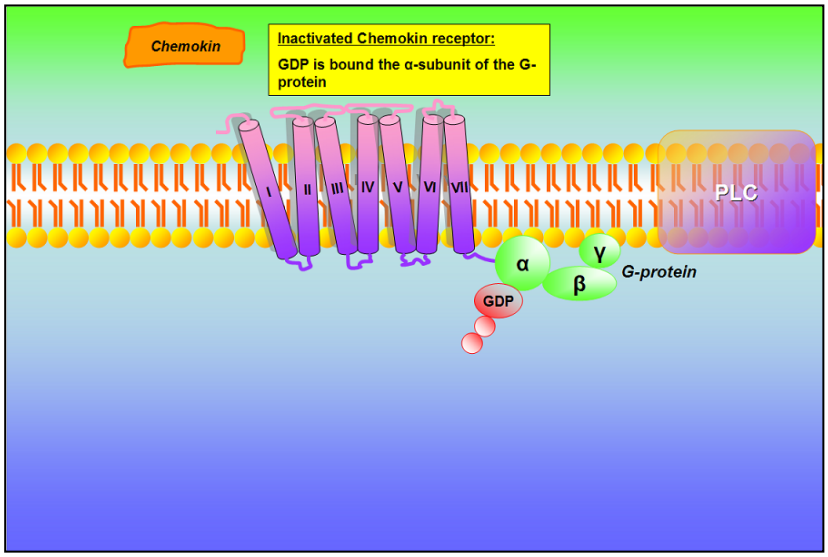


Fig. 14a: Signalling pathway of chemokine receptors

The Gα-subunit contains a GTPase domain which is involved in binding and hydrolysis of GTP (=guanosin triphosphate). In the inactive state, the Gα-subunit binds GDP (=guanosin diphosphate) (see Figure 14a).

When a ligand binds to the GPCR receptor, the receptor itself changes its conformation and by this activates the intracellular G-Protein causing dissociation of GDP from the Gα-subunit and its replacement by GTP.

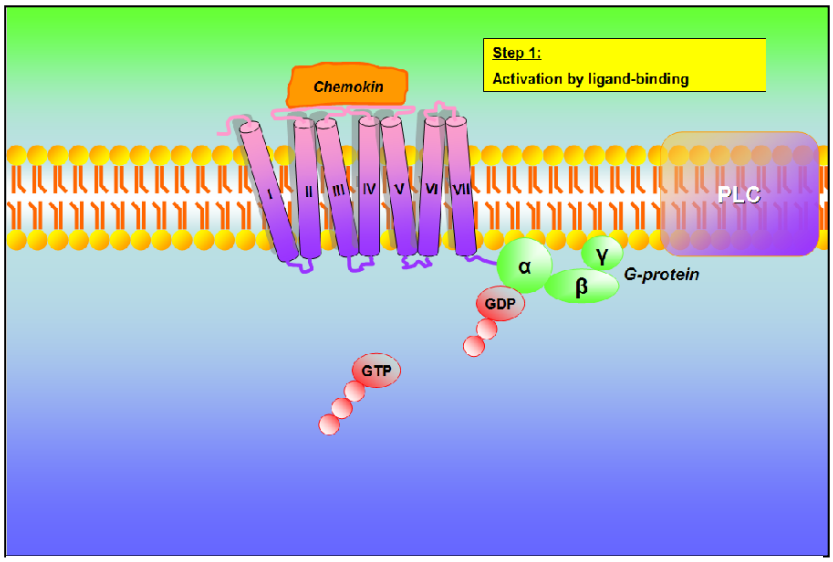


Fig. 14b: Signalling pathway of chemokine receptors

The Gα-subunit then dissociates from the receptor and from the Gβγ-heterodimer (see Figure 14c-e).

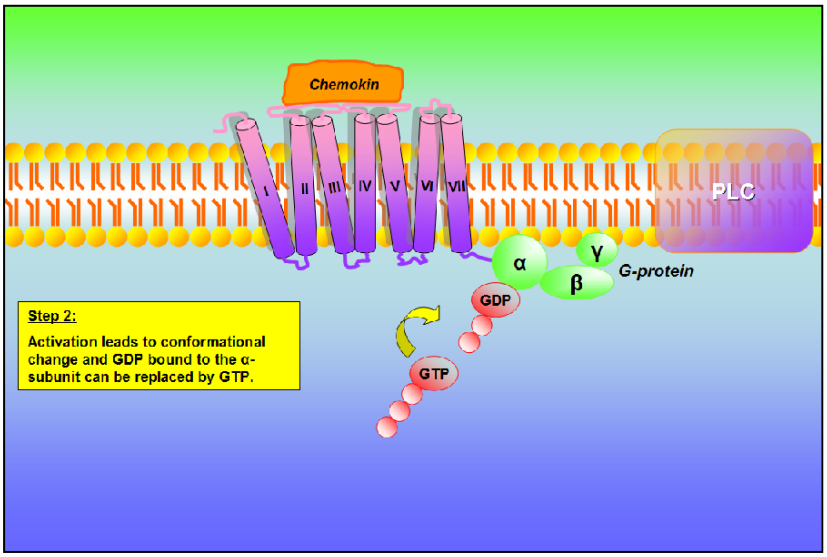


Fig. 14c: Signalling pathway of chemokine receptors

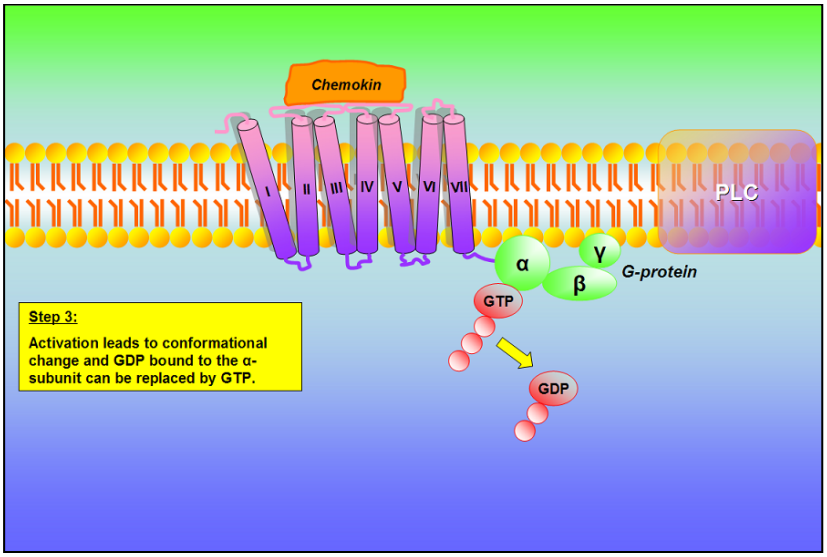


Fig. 14d: Signalling pathway of chemokine receptors

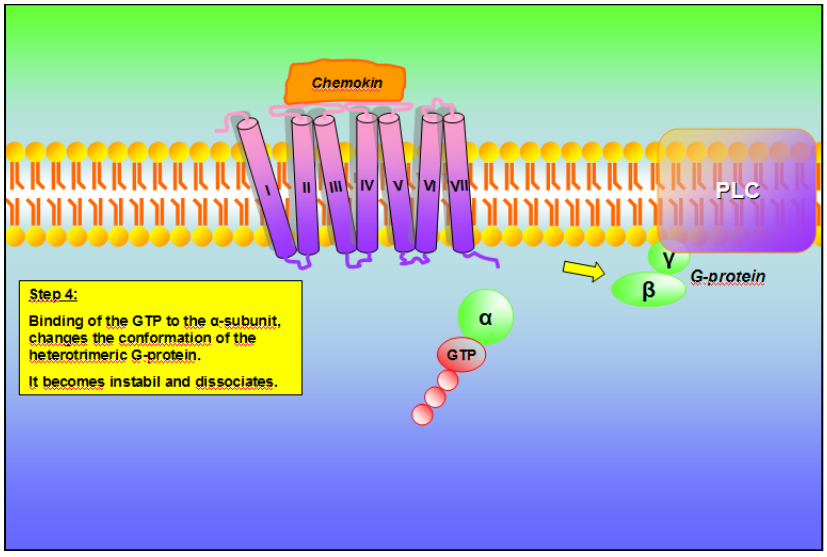


Fig. 14e: Signalling pathway of chemokine receptors

Both complexes, the Gα-GTP-complex as well as the Gβγ-heterodimer-complex, then activate downstream effectors with different physiological responses: The Gβ-subunit activates an enzyme called Phospholipase C (PLC) which is part of the cell membrane. PLC cleaves Phosphatidylinositol(4,5)-bisphosphate (PIP2) to form two second messenger molecules called inositol-triphosphate (IP3) and diacylglycerol (DAG). As second messenger DAG activates protein kinase C, IP3 triggers the release of calcium from intracellular stores, for example the sarcoplasmatic reticulum. These effects promote different cascades effecting different cellular responses.

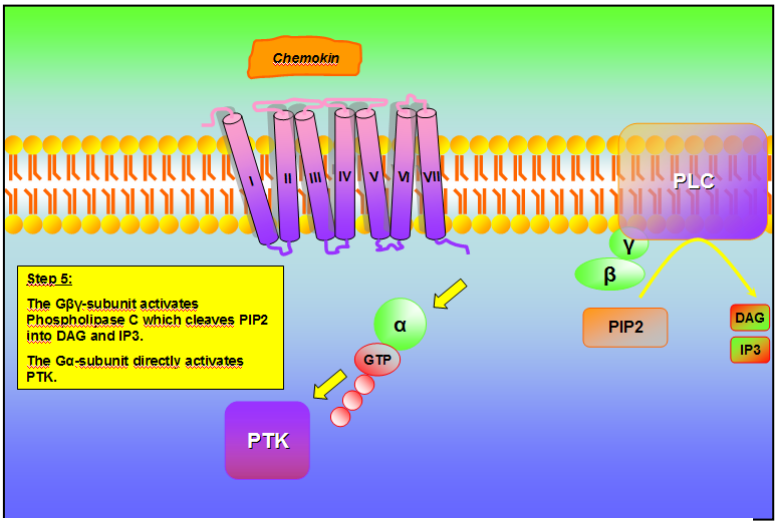


Fig. 14f: Signalling pathway of chemokine receptors

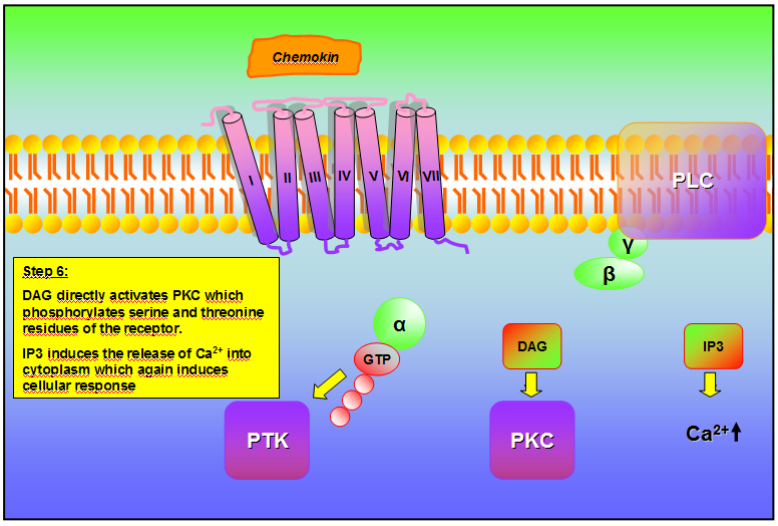


Fig. 14g: Signalling pathway of chemokine receptors

The Gα-.subunit directly activates an enzyme called protein tyrosine kinase (PTK) which phosphorylates serine and threonine residues in the tail of the chemokine receptor causing its desentisation or inactivation (see Figure 14g) (Murdoch 1995).

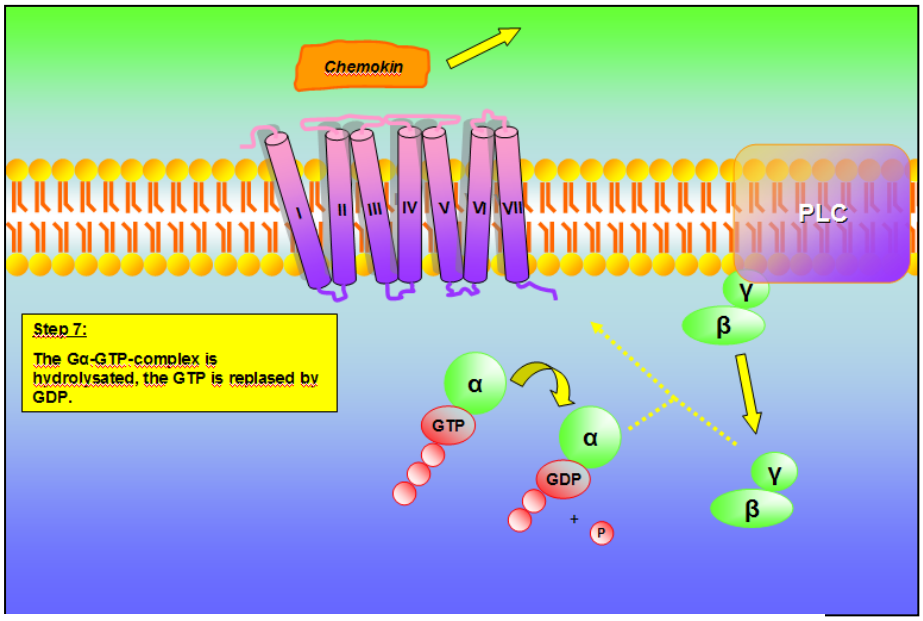


Fig. 14h: Signalling pathway of chemokine receptors

The Gα-GTP-complex is hydrolysated into a Gα-GDP-complex with fewer intrinsic energy. This complex then binds the Gβγ-complex and as new-formed heterotrimeric complex to the GPCR receptor. By this the GPC-receptor is regenerated.

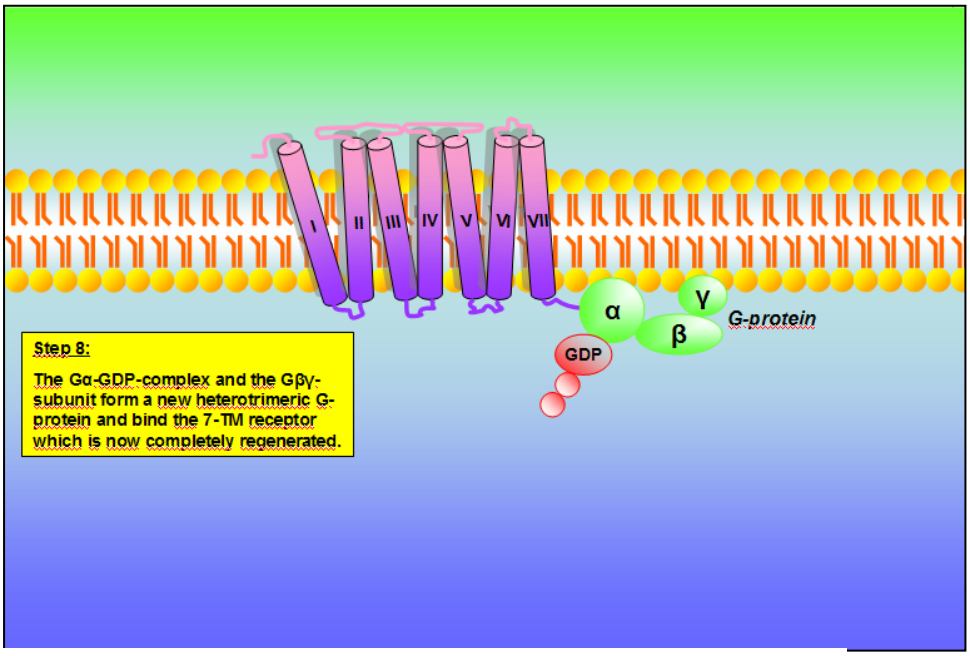


Fig. 14i: Signalling pathway of chemokine receptors

Inactivation

The GPCR is inactivated by agonist-dependent phosphorylation of the C-Terminus of the amino acid chain. Receptor phosphorylation subsequently promotes binding of arrestins which blocks further interaction with G-proteins and mediates receptor internalization through clathrin-coated pits. Receptor phosphorylation is consequence of continued stimuli by corresponding ligands.

3. RANTES/ CCL-5-Chemokin

Chemokine (C-C motif) ligand 5 (CCL5) is also known as **R**egulated upon **A**ctivation, **N**ormal **T**-cell **E**xpressed and **S**ecreted (RANTES). With its two pairs of adjacent cysteine residues it belongs to the CC-chemokine subfamily. It was originally identified by Schall et. al. via subtractive hybridisation, a screening technology based on PCR reaction to identify differentially expressed genes (Schall 1988, Diatchenko 1996). RANTES is also called SIS-delta, SCYA5, EoCP-1 (eosinophil chemotactic polypeptide) or TCP228 (T-cell-specific-protein).

3.1 Coding Gene, structure and synthesis

The coding gene for RANTES has been shown to be located on Chromosome 17, position 17q11.2-q12 (Donlon 2006).

In its primary structure, RANTES shows a cleaved signal peptide consisting of about 20 to 24 amino acids and a mature secreted protein of 68 to 76 amino acids. The RANTES protein is a highly basic polypeptide with a molecular weight of 8kDa. Figure 15 shows the complete primary structure of RANTES, Figure 16 its tertiary structure (Schall 1991). A large amount of basic residues in the amino acid chain renders it a highly basic polypeptide. This might be a possible explanation for the ability of RANTES to bind negatively charged endothelial cells of blood vessels (Krensky 1995).

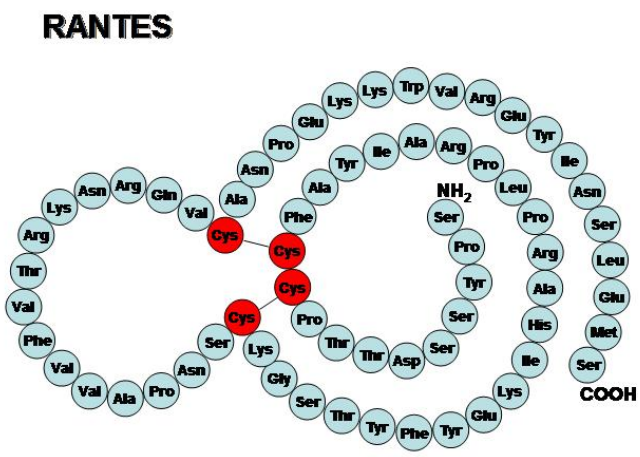


Fig. 15: Primary structure of RANTES

As already said, RANTES belongs to the CC-subfamily of chemokines with the first two N-terminal cysteine residues adjacent to each other.

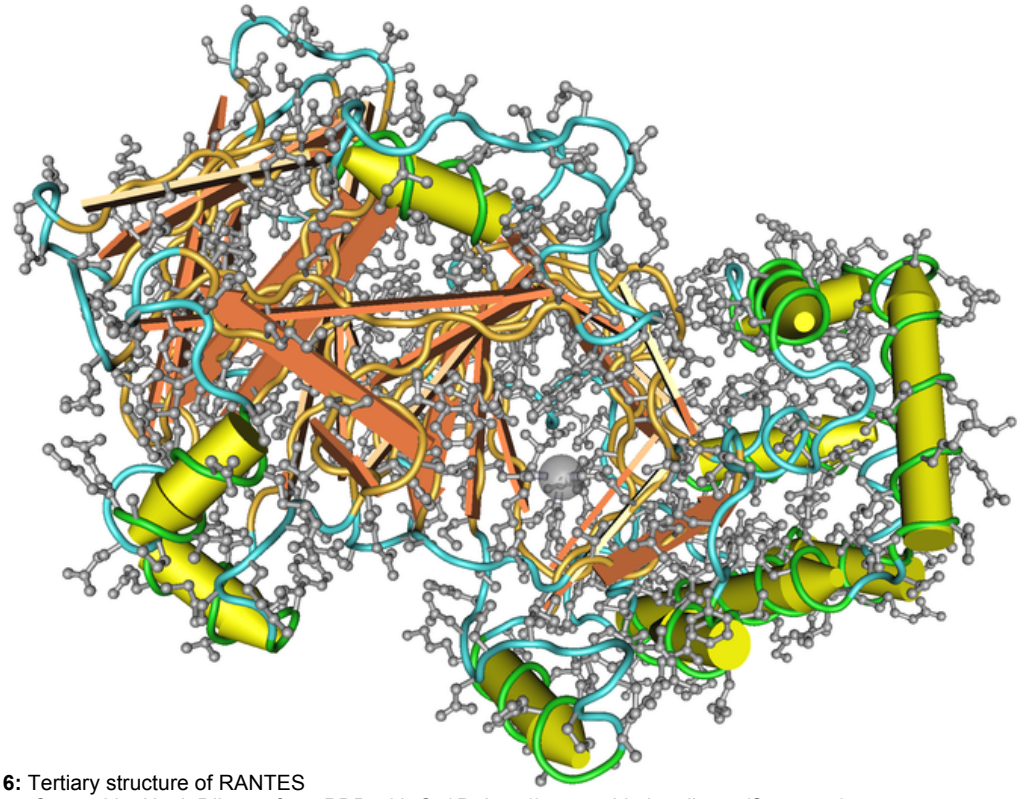


Fig. 16: Tertiary structure of RANTES
Source: Created by Nevit Dilmen, from PDB with Cn3D, http://www.ncbi.nlm.nih.gov/Structure/

The RANTES gene structure exhibits a three exon/ two intron strucuture. The first exon contains the 5' untranslated sequence and coding nucleotides for the leager peptide, the second exon encodes the N-terminal half of the mature protein. The third exon consists of carboxyl-region-coding nucleotides and the 3' untranslated region (Schall 1991).

The RANTES monomer structure is similar to the structure of Interleukin-8 and MIP-1β. But in their quartery structure RANTES and MIP-1β differ from Interleukin-8. RANTES dimers are elongated and cylindrical with dimension of approximately 66 by 37 angstroms (Krensky 1995).

Before releasing RANTES undergoes different post-translational modifications like O-glykolisation of the serine-residues or oxygenation of the methionine residue.

3.2 RANTES expression and its regulation

RANTES is a proinfammatory cytokine and is normally not expressed by most tissues. Although RANTES was originally indentified in activated T lymphocytes, different cell types are able to express this cytokine upon stimulation. These cell types include renal tubular epithelialium and mesangium, synovial fibroblasts, endothelial cells, platelets, monocytes, T lymphocates and dendritic cells, natural killer cells.

It was found that RANTES was significantly upregulated upon stimulation with TNF-α, CD40L, LPS and PMA (Zhou 1995, Sallusto 1999).

The infiltration of mononuclear cells, on of the hallmarks of organ transplant rejection, may be mediated by expression of RANTES, in part at least. Also inflamed tissue, like infected tonsils has been shown to express RANTES. Also in extramedullar hematopoesis, a clinical situation where blood forming cells are found in the spleen, high RANTES expression in megakaryocytes can be seen. Megakaryocytes are capable of platelets production. RANTES expression can also be found in lymph nodes showing delayed type hypersensitivity lesions (DTH) found in sarcoidosis and tuberculosis.

RANTES is not only expressed by inflammatory tissue, but also by transformed tissue. RANTES mRNA is expressed by solid tumor cell lines such as

rhabdomyosarcoma, RD and osteosarcoma cells for examples. The observation that RANTES is expressed in relatively undifferentiated tumor tissues, like in Wilms` tumor and generally not expressed in relatively differentiated cell types, which make up the renal cell carcinomas and also that RANTES is expressed by tumors but not by normal tissue leads to the suggestion that RANTES expression may be developmentally regulated.

While most of the RANTES/ SIS cytokine family are massively inducible in hematopoietic cells, RANTES is not rapidly induced in hematopoietic cells (Schall 1991). RANTES expression in T lymphocytes is regulated by Kruppel like factor 13 (KLF13) (Song 2010).

3.3 In-vivo effects and associated diseases

Inflammatory activities

The migration of neutrophils and mononuclear phagocytes is a hallmark of inflammation. Once at the inflamed site, these cells are activated to destroy microorganisms and cellular debris, remove injured and degraded endogenous tissues and mount an immune response. The complex cascade of inflammatory events is mediated through various cells near the inflamed site, which are in turn attracted to that site by various chemotactic substances including bacterial membrane components, complement protein fragments and lymphokines.

To appreciate the significance of RANTES and other chemokines one must understand the broader context of leukocyte/endothelial interaction. Leukocyte migration from the circulation into tissue involves a series of interactions between molecules on the surface of a leukocyte and the endothelial surface. Within the

scheme of "**rolling, adhesion and transmigration**" of leukocytes chemokines play a central role as they interfere on nearly all steps of the extravasation process of immune competent cells: In sites of inflammation activated thrombocytes and endothelial cells release and present cytokines which act as signal for rolling leukocytes (Springer 1995).

In the **first step,** a leukocyte loosely binds to the endothelium via members of the selectin family. This loose tethering slows the rate of leukocyte passage through the vessel and allows cells interaction with chemoattractant molecules (such as RANTES) which are displayed on the endothelium. In the **second step**, the chemokines induce upregulation of integrins on the surface of leukocytes. This leads to the **third step** whereby the integrins on the surface of leukocytes bind their ligands on endothelium, i.e. selectins, which induces a firm adhesion and resulting in arrest of the rolling leukocyte.

In the **fourth step**, leukocytes then cross the endothelial lining of the blood vessel to enter the tissue: Matrix-metalloproteases which are up-regulated by chemokines ease the transmigration of leukocytes by lysis of the basal membrane. After extravasation the leukocytes then follow a soluble chemokine gradient to sites of the highest chemokine concentration. This process is called haptotaxis (Rot 1993). In this process RANTES seems to play a decisive role (Weber 2001).

RANTES plays an active role in recruiting leukocytes into inflammatory sites. RANTES induces leukocyte migration by binding specific receptors CCR1, CCR3, CCR4 and CCR5 (see above). With the help of particular cytokines like Interleukin-2 (IL-2) and Interferon-γ (IFN- γ) released by T cells, RANTES also induces the

proliferation and activation of certain natural-killer (NK) cells to form CHAK (CC-Chemokine-activated killer) cells (Maghazachi 1996) .

Although RANTES was initially considered to be T cell-specific it has since been found to have a high chemotactic activity towards multiple immune cells like eosinophils, basophils, mast cells, monocytes, cytotoxic T cells (CTLs), naïve CD4+ T cells. These cells express some of the CCL5 receptors like CCR1, CCR3, CCR4, CCR5 (Lapteva 2010). It has also been shown to attract monocytes as well as very specific T cell subsets, but not neutrophils (Schall 1990).

It causes the release of histamine from basophils and activates eosinophils. This cytokine is one the major HIV-suppressive factors produced by CD8+ cells (Rot 1992).

It can also be involved in direct antimicrobial (anti-trypanosomal) activity by inducing NO in macrophages (Villalta 1998).

However, RANTES can have detrimental effects via the recruitment of immune cells that enhance inflammatory processes such as arthritis, atopic dermatitis, nephritis, colitis, and other disorders (e.g., arteriosclerosis, pulmonary hypertension, asthma, nasal polyps, endometriosis, nephropathy, and perhaps Alzheimer's disease). High levels of RANTES can also be found in allogenic transplant rejection (Appay 2001).

RANTES levels with alcohol-induced hepatitis

Berres et al. could show that RANTES plays a pro-fibrotic role in human and murine liver fibrosis. In human HCV infected livers, RANTES mRNA expression was significantly associated with higher stages of liver fibrosis and NASH. Genetic deletion of RANTES in fibrosis prone mice led to a significantly reduced degree of fibrosis as assessed by liver histology and hydroxyproline content in both murine models of fibrosis (Berres 2008).

II. PATIENTS AND METHODS

1. Patients

1.1 Patient recruitment

The collection of patients' data started in February 2009. Originally, data of 506 randomly selected fasting patients from different Departments of the University Hospital Regensburg was collected and included in this study. Non-fasting patients did not take part in this study examination. The cohort consisted of both outdoor patients and hospitalized patients who were referred to the interdisciplinary ultrasound department for sonographic examination of the abdomen.

All patients took part in a face-to-face interview. The interview was carried out by one of three students from the Department of Inner Medicine of the University Hospital Regensburg. For these interviews a standard questionnaire (see figure 18) was built and provided including a short history of patients' medical history (see below).

1.2 Exclusion criteria

Patients with any of the following criteria were excluded from the study:

1. History of malignancies of any kind
2. Chronic hepatobiliary diseases
3. Ascites
4. Medications known to affect hepatic steatosis
   (as estrogens, corticosteroids, amiodarone, valproate either 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),

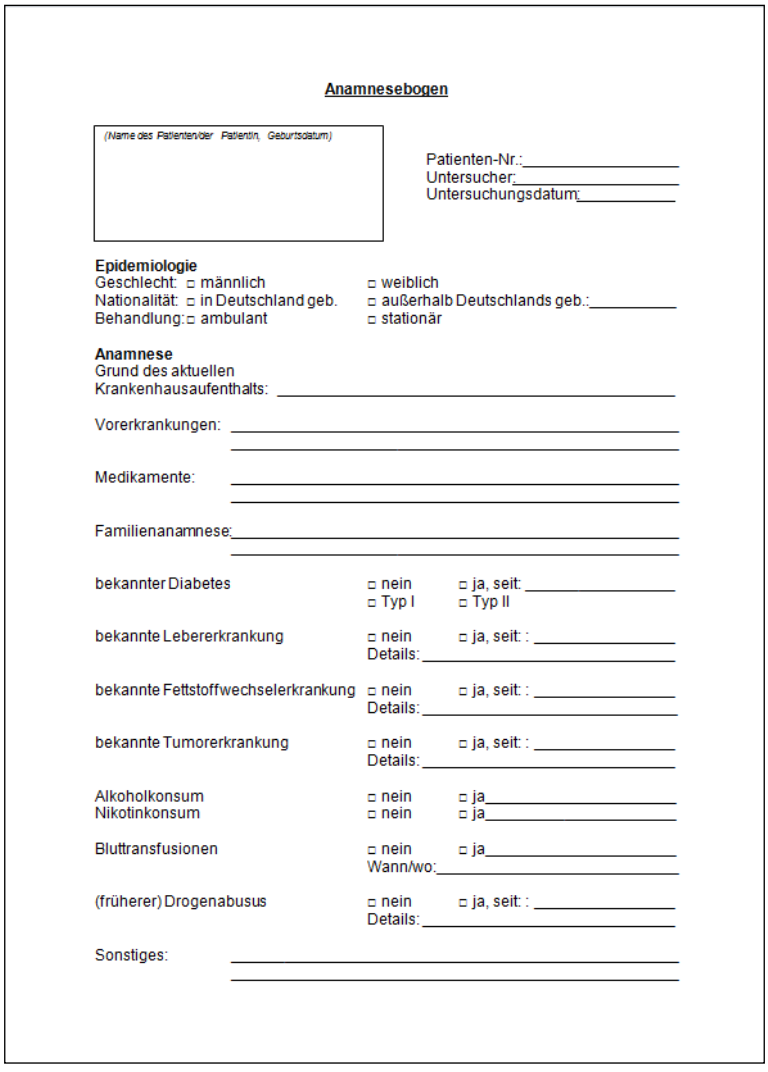
8. Known (familial) hyperlipidemia, and
9. Acute medical conditions with confounding effect on laboratory measurements and RANTES serum levels.

After exclusion of all patients who did not meet our requirements the remaining study population consisted of 155 patients.

2. Methods

2.1 Questionnaire

The questionnaire also included patient information and epidemiological data as sex, date of birth, nationality and treatment (ambulant or hospitalized). The patients' medical history included questions about the reasons for the actual hospitalisation or ambulant referral to the ultrasound department, the past medical history, actual medication, and diseases known in his family and particular questions related to a known diabetes, liver diseases, problems with the lipid metabolism or a known cancer disease and other questions like history of blood transfusions.



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: _____

Fig. 17: Questionnare of this study about epidemiologic data and medical history of the patient (diseases, medication, family history, diabetes, liver diseases, malignant or metabolic diseases, usage of alcohol, nicotin and drugs, blodd transfusions)

The patients were also asked about their daily alcohol consumption and their nicotine consumption. The daily consumption of alcohol was mainly answered in “bottles of beer per day” or “glasses of wine per day” and then converted in “mg of ethanol per day”. Furthermore, the patient was asked about any history of drug consumptions. Figure 17 shows the original questionnaire that was used in this study.

2.2 Anthropometric measurements and clinical examination

After signing the consent form and the finishing interview using the above printed standard questionnaire (see Fig. 18), each patient underwent a general physical examination.

Height and weight

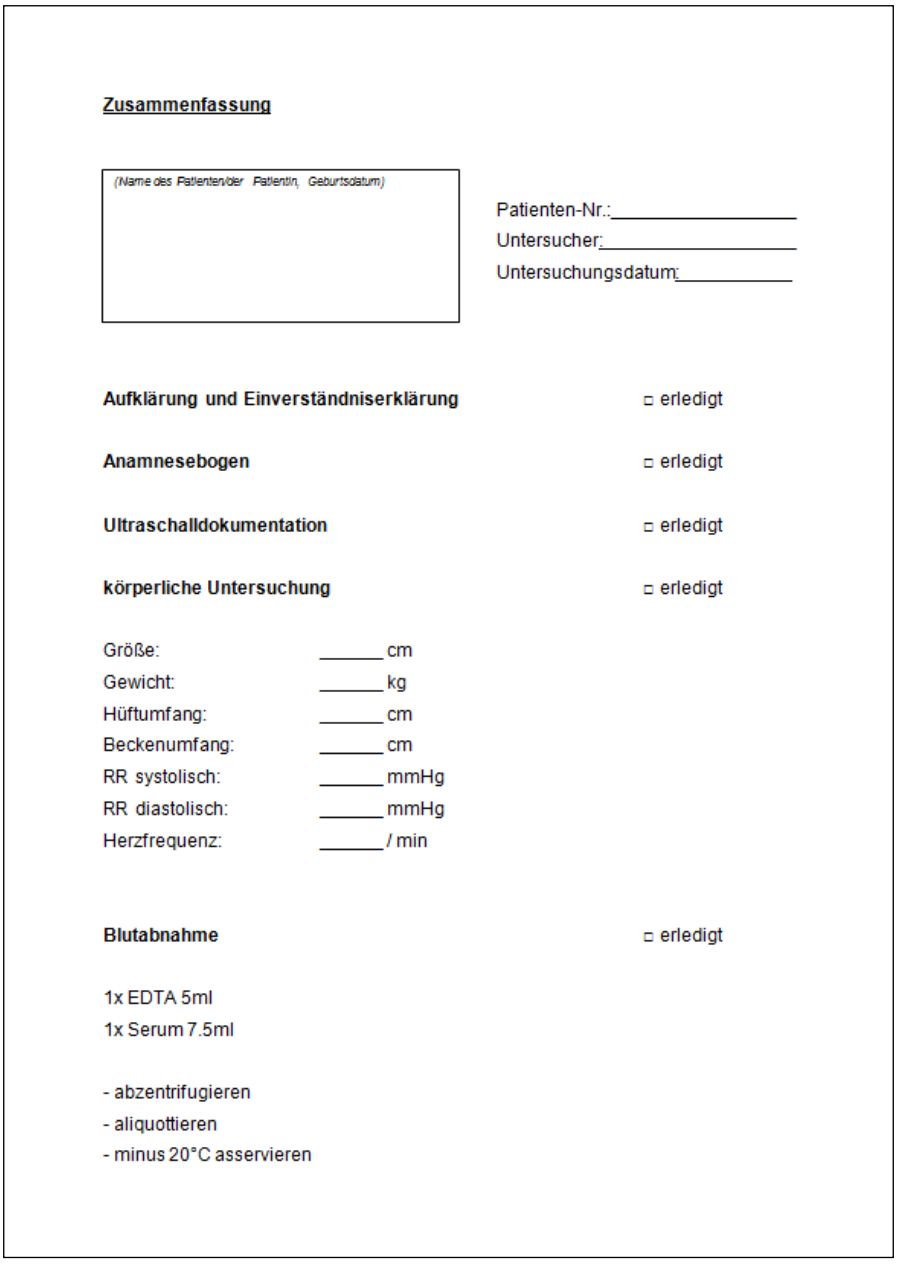
The height of the standing patient was measured using a fixed scale on the wall in centimeters. The weight of each patient was measured using the same scale. The patients were asked to undress except for thin clothes with their shoes off.

Blood pressure and heart rate

Blood pressure and heart rate of a seating patient were measured on the right and left upper arm, the same level as the patients’ 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 at least 5 minutes, and during the measurement the patient was asked not to talk. The heart rate was detected by palpation of the patient’s right radial artery and counting the puls waves for 15 seconds. The result was multiplied by 4 to get the heart rate per minute.

Waist and hip circumference

The waist and hip circumference was measured on the standing patient using a flexible measuring band. The patients waist circumference was measured at the smallest level of the torso during minimal inspiration. 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. Figure 18 shows the form that was used for documentation of anthropometric measurement and clinical 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°C asservieren

Fig. 18: Documentation of physical examination (weight, heigt, hip and waist circumference, blood pressure and heart rate)

Collection and storage of serum samples

For the collection of patients serum blood was taken either from the right or the left upper arm of the patient. After a close skin disinfection with alcohol swabs and venous congestion by using a tourniquet around the upper arm blood samples were taken from each patient mostly out of the cubital vein: one red EDTA tube of 5ml and one white serum tube of 7.5 ml.

The plasma was obtained by centrifugation of the serum tube at 3,000 rpm for 10 minutes. The plasma then was stored in aliquots at minus -20° Celsius for subsequent analysis.

2.3 Biochemical analysis of serum parameters

The following laboratory analyses were performed by the same laboratory, 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);
- Triglycerides,
- Very-low-density lipoprotein cholesterol (VLDL-C),
- Low-density lipoprotein cholesterol (LDL-C),
- High-density lipoprotein cholesterol (HDL-C) and
- Total cholesterol;
- Albumin,
- Total serum protein,
- Bilirubin,

- Choline esterase,
- Akaline phosphatase (ALP) and
- Glucose.

2.4 Ultrasound examination of the liver and fat tissue

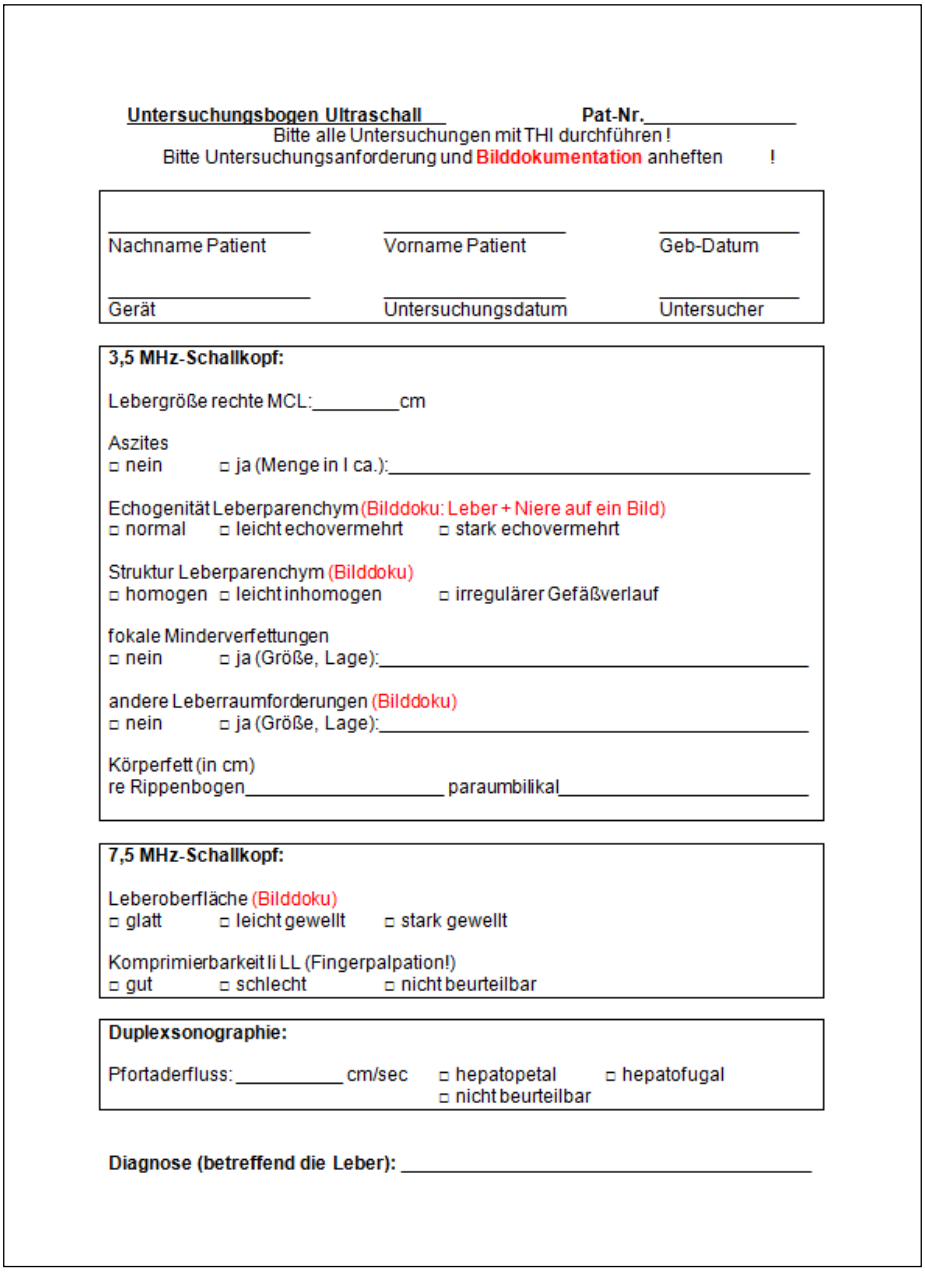
In addition to a complete abdominal ultrasound examination all patients underwent a standardized ultrasound examination performed by the same three investigators (Hanna Huber, Catrin Beer and Christoph Nießen).

For this assessment the following high-end ultrasound machines were used:

- Siemens Sonoline Elegra (Siemens, Erlangen, Germany)
- 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).

<u>Ultrasound examination of liver</u>

For the ultrasound assessment of the liver the patients were asked to lie on their back with their arms folded behind their heads. All results of the ultrasound assessment were written down on a standard form for the study's ultrasound examination (see Figure 19). In addition, ultrasound pictures of each measurement were printed for documentation.



Untersuchungsbogen Ultraschall **Pat-Nr.**_____

Bitte alle Untersuchungen mit THI durchführen!

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): _____

Fig. 19: Ultrasound examination form used in this study. Liver size measured in medioclavicular line, ascites, echogenity of the liver, liver structure, liver masses, thickness of the fat layer over the right costal arch and periumbilical, liver surface, incompressibility and maximum flow in the portal vein.

The liver was examined in transverse and longitudinal section planes using a 3.5 MHz transducer with the patient lying on his back. For the longitudinal scan the transducer was put on the epigastric angle, left to the midline in the hypochondrium in longitudinal direction. From there, the transducer was moved slowly to the right costal margin and right posterior axilla line. With these longitudinal planes it is possible to analyse **size** (longitudinal size measured in the medioclavicular line), **shape**, **internal structure** and **compressibility** of the left and right lobes. To

optimise the view of the liver, compliant patients can be asked to suspend in deep inspiration during short periods. At the same time it is possible to get an impression of the gall bladder, hepatic porta, portal vein, inferior vena cava and common hepatic duct.

Transverse scans can be useful for analysis of the left lobe of the liver, the hepatic porta and the falciform ligament. Oblique subcostal scans show the internal structure of the right lobe with the portal vein, portal vein branches and hepatic veins with the segmental anatomy. Additionally gall bladder and intrahepatic bile ducts are pictured. The right lobe with the subphrenic region can be demonstrated with intercostals sections. For these scans the transducer can be put on patients intercostal spaces, especially if the subcostal, epigastric view is limited due to bowel gas.

To evaluate the liver echogenity, the echo of the liver parenchyma was compared with the echogenity of the kidneys. Normally, the liver echogenity is very similar to the echogenitiy of the kidney parenchyma. In patients with steatotic livers, the liver parenchyma is hyperechoic and seems so be whiter then kidney parenchyma. Figure 20 shows a steatotic liver compared to a normal kidney.

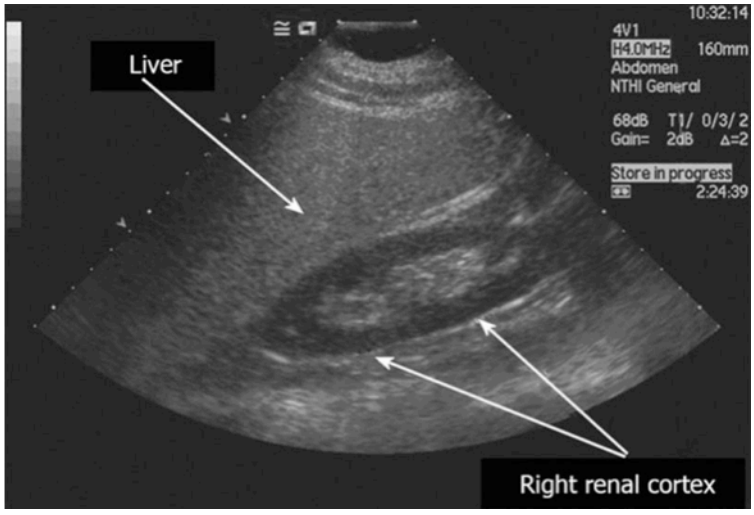


Fig. 20: Ultrasound image with steatotic liver and normal kidney parenchyma.
Source: World J Gastroenterol. 2008 June 14; 14(22): 3476-3483.

To determine the maximum flow in the portal vein a duplex sonography was also performed. Duplex sonography uses the effect of moving fluid within a vessel that causes a shift of the echoreflex frequency. This shift can be detected by the 3.5 MHz transducer and the ultrasound machine can convert this signal into a color-coded scale. By this scale and its typical continuous duplex signal the portal vein can be identified and direction and flow strength of the portal vein can be examined. Figure 21 shows an ultrasound image showing the typical flow signal of the portal vein.

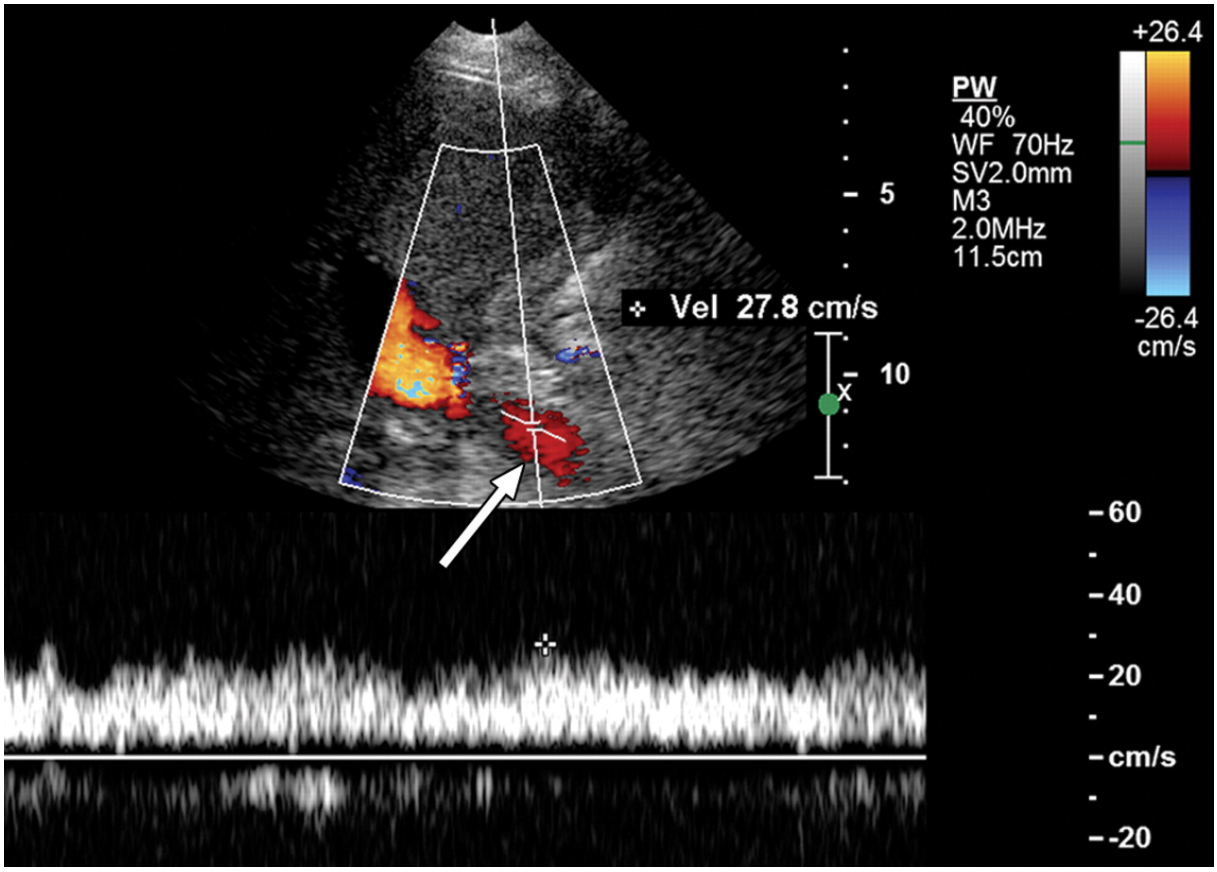


Fig. 21: Duplex ultrasound with color-coded scale and time-flow diagram of the portal vein.
Source: Mullan C P et al. AJR 2010;195:1438-1443

After identification of the portal vein the mean and the maximum flow of portal vein can be detected by using the power duplex ultrasound. For that a detector is placed into the ROI (region of interest) and the portal flow is converted into a time-flow-

diagram (see Fig. 21). To avoid wrong results the angle of the flow direction has to be corrected (see the white line on red background which represents the flow direction). After correction of the flow angel the flow velocity can be read from the flow-time-scale (see the vertical scale on the right side of the ultrasound images, Fig. 21). The measurement is performed in expiratory position with the transducer in normal pressure on the patient. Normal flow velocity in the portal vein is
15 – 30 cm/ s.

Using the 7.5 MHz the thickness of the fat layer over the right costal arch and periumbilcal was measured and documented in the examination form of this study, together with all the other results of the examination using the 3,5 MHz transducer.

2.5 Serum analysis of RANTES

RANTES in human sera was assessed by sandwich enzyme-linked immunosorbent assay (ELISA) using the human CCL5 DuoSet ELISA development kit (R&D Systems Inc., MN, USA, catalog number DY278). In this ELISA test samples were used undiluted.

An ELISA test, also known as enzyme immunoassay (EIA), is a biochemical technique to detect the presence of an antibody or antigen in a sample based on an enzymatic color change reaction. With the help of an ELISA test, proteins, viruses as well as antibodies, hormons, toxins and other can be detected in a sample.

In a double-antibody-sandwich-ELISA two antibodies are used which both specifically bind the antigen to be detected. According to the manufacturers' instructions the implementation of this test is as follows: In the first step, the first monoclonal antibody – the coating or capture antibody – (in this case specific for RANTES) is tied to a microtiter plate (in this case a 96 well polystyrene microplate coated with a mouse monoclonal antibody against RANTES). In the second step the sample with the antigen is added to that plate and given some time to adhere to the plastic. In this time the coating antibody bound to the plate binds the antigen in the sample. In the third step, the plate is washed to remove all unbound proteins. Thus, only the antigens bound to the coating antibody remain on the plate. In the fourth step, the second polyclonal antibody - the detection antibody – is added to the plate (in this case specific for RANTES). The detecting antibody binds another epitope of the antigen than the capture antibody. The detecting antibody has an attached enzyme (in this case horseradish peroxidase). After incubating a short time an antibody-antigen-antibody-complex exists when the detecting antibody binds the antigen-

capture antibody complex. That is where the name “sandwich-ELISA” comes from. After a second washing in order to remove unbound antibodies, the suitable enzyme substrate is added to the plate. Often, this substrate changes its colour upon reaction with the enzyme. The color change shows that the second antibody has bound to primary antibody. The higher the concentration of the antigen in the probe the stronger the color change. Then a spectrometer is used to give quantitative values for the color strength. For clarification, figure 22 shows a scheme of a sandwich ELISA (Description of Human CCL5/ Rantes DuoSet (Catalogue Number DY279, R&D Systems Inc.).

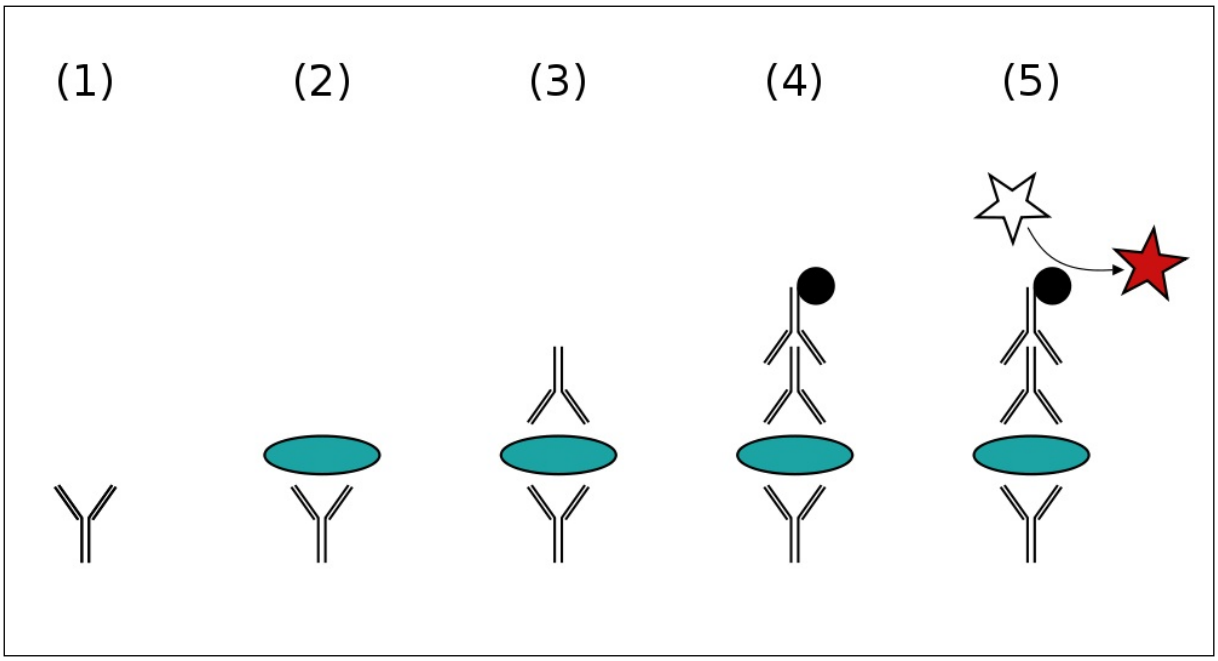


Fig. 22: (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.
Quelle: Jeffrey M. Vinocur 2006, (Multi-license with GFDL and Creative Commons CC-BY 2.5)

2.6 Statistical analysis

For continuous variables results are expressed as means ± standard deviation. One-way analysis of variance (F test) was used to compare means. Correlations between categorical variables were assessed constructing contingency tables and applying the χ^2 test. To identify independent predictors of NAFLD, the significance value of the

Wald statistic in a multivariate logistic regression model was used. A p-value < 0.05 was considered significant. Statistical analysis was performed on a PC using SPSS 15.0 software.

Statistical analysis were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA) and GraphPad Prism Software (GraphPad Software, Inc., San Diego, USA). Results are expressed as means ± standard error (range). Comparisons between groups were made using one way analysis of variance (with Bonferroni correction for multiple comparisons). P-values <0.05 were considered as statistically significant.

The data of this study were correlated with analysis of murine models of NAFLD and an in vitro model of hepatic lipid accumulation. Together these data have been successfully published (Kivovski 2010).

In the following, the data of the human study, which are the basis of this dissertation, are presented (in results) and discussed (in discussion) together. Methods applied for in vitro studies and assessment of the murine model are described in detail in the above mentioned publication (Kivovski 2010).

III. RESULTS

Increased RANTES serum levels in patients with non-alcoholic fatty liver

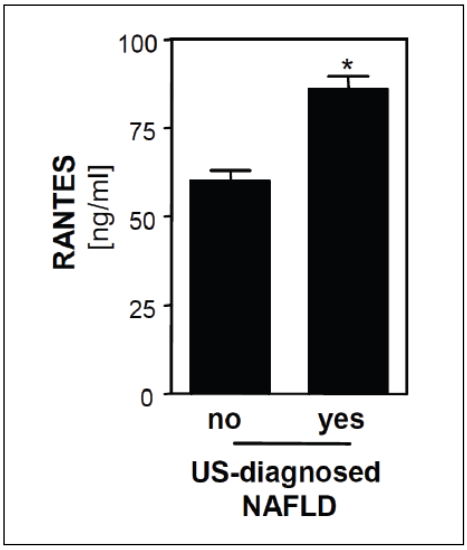


Fig. 23: Serum RANTES levels in patients with NAFLD. Serum RANTES levels in patients with ultrasound (US) diagnosed fatty liver compared to a control (ctr.) group of patients with normal sonographic liver appearance. (*p<0.05 compared to control).

Recently, we have described a cohort of patients with referral for sonographic examination of the abdomen and ultrasound-diagnosed NAFLD (Kivovski 2010). Analysis of serum levels of RANTES revealed slightly but significantly elevated RANTES levels in patients with ultrasound-diagnosed NAFLD (n=45; mean: 85.9 ng/ml, SEM 3.7 ng/ml,) in comparison to the control group (n=61; 60.1 ng/ml, SEM 2.9 ng/ml; p<0.001), (Fig. 23).

Correlation between fat metabolism markers and RANTES serum levels

Table 8 shows the correlation coefficients of different fat metabolism markers and RANTES serum levels using Spearman's Rho test.

Table 8: Correlation between fat metabolism markers and RANTES

Triglyceride	Correlation Coefficient	-0.021
	Sig. (2-tailed)	0.811
	N	137
Full cholesterol	Correlation Coefficient	0.038
	Sig. (2-tailed)	0,661
	N	138
HDL-Cholesterol	Correlation Coefficient	-0.024
	Sig. (2-tailed)	0.804
	N	111
LDL-Cholesterol	Correlation Coefficient	0.121
	Sig. (2-tailed)	0,157
	N	138
VLDL-Cholesterol	Correlation Coefficient	-0.238
	Sig. (2-tailed)	0.005
	N	137

We could not find any significant relations between RANTES serum levels and triglyzeride concentration, full cholesterol, HDL-Cholesterol and LDL-Cholesterol. Interestingly, there was a weak, negative correlation between VLDL-Cholesterol and RANTES serum levels. This might be a statistical phenomenon because of our low number of cases or increased RANTES levels go along with decreased VLDL-Cholesterol serum concentration by so far unknown mechanisms.

Correlation between liver function tests and RANTES serum levels

Table 9 shows the correlation coefficients of different liver function tests and RANTES serum levels using Spearman's Rho test.

Table 9: Correlation between liver function tests and RANTES

GOT (AST)	Correlation Coefficient	-0.044
	Sig. (2-tailed)	0.607
	N	138
GPT (ALT)	Correlation Coefficient	0.073
	Sig. (2-tailed)	0.397
	N	138
Gamma-GT	Correlation Coefficient	-0.126
	Sig. (2-tailed)	0.162
	N	124
Alcalic Phosphatase	Correlation Coefficient	0.094
	Sig. (2-tailed)	0.275
	N	136
Bilirubin	Correlation Coefficient	0.134
	Sig. (2-tailed)	0.118
	N	136
Lipase	Correlation Coefficient	-0.097
	Sig. (2-tailed)	0.315
	N	110
Total protein	Correlation Coefficient	0.175
	Sig. (2-tailed)	0.068
	N	109
CHE	Correlation Coefficient	0.136
	Sig. (2-tailed)	0.156
	N	111

We could not find significant correlations between RANTES serum levels on the one hand and GOT (AST), GPT (ALT), Gamma-GT, alcalic phosphatase, Bilirubin, Lipase, total protein and cholinesterase on the other.

Correlation between iron metabolism markers and RANTES serum levels

Table 10 shows the correlation coefficients of some iron metabolism markers and RANTES serum levels using Spearman's Rho test.

Table 10: Correlation of iron metabolism markers and RANTES

Fe^{3+}	Correlation Coefficient	0,036
	Sig. (2-tailed)	0,839
	N	34
Ferritin	Correlation Coefficient	0,146
	Sig. (2-tailed)	0,279
	N	57
Transferrin	Correlation Coefficient	0,133
	Sig. (2-tailed)	0,305
	N	61
Transferrin saturation	Correlation Coefficient	-0,145
	Sig. (2-tailed)	0,266
	N	61

We did not find any significant correlations between RANTES serum levels and typical iron metabolism markers like Fe^{3+}, ferritin, transferring and transferrin saturation.

Correlation between physical examination findings and RANTES serum levels

Table 11 shows the correlation coefficients of physical examination findings and RANTES serum levels using Spearman's Rho test.

Table 11: Correlation between physical examination findings and RANTES serum levels

Age	Correlation Coefficient	-0.158
	Sig. (2-tailed)	0.063
	N	139
Hip circumference in cm	Correlation Coefficient	0.016
	Sig. (2-tailed)	0.854
	N	138
Waist circumference in cm	Correlation Coefficient	0.096
	Sig. (2-tailed)	0.264
	N	138
waist to hip ratio	Correlation Coefficient	-0.07
	Sig. (2-tailed)	0.414
	N	138
Systolic blood pressure in mmHg	Correlation Coefficient	-0.205
	Sig. (2-tailed)	0.015
	N	140
Diastolic blood pressure in mmHg	Correlation Coefficient	-0.1
	Sig. (2-tailed)	0.24
	N	140
Heart rate	Correlation Coefficient	0.034
	Sig. (2-tailed)	0.688
	N	140

There was no significant correlation between diastolic blood pressure and RANTES serum levels. However, we found a weak negative correlation between RANTES serum levels and systolic blood pressure. Also here, the most likely reason for this is a statistical phenomenon due to a too low sample count.

Correlation between ultrasound examination findings and RANTES serum levels

Table 12 shows the correlations coefficients of ultrasound examination findings and RANTES serum levels using Spearman's Rho test.

Table 12: Correlation between ultrasound examination findings and RANTES serum levels

Liver size in right MCL	Correlation Coefficient	0.069
	Sig. (2-tailed)	0.418
	N	140
Fat layer on right costal arch	Correlation Coefficient	0.109
	Sig. (2-tailed)	0.197
	N	141
Fat layer paraumbilical	Correlation Coefficient	0.143
	Sig. (2-tailed)	0.092
	N	141
Portal vein flow in cm/ s	Correlation Coefficient	-0.08
	Sig. (2-tailed)	0.347
	N	139

We could not find any significant correlation between RANTES serum levels and ultrasound examination findings, i.e. liver size measured in medioclavicular line, thickness of the fat layer on the right costal arch, fat layer measured in the paraumbilical region and portal vein flow.

IV. DISCUSSION

The chemokine RANTES has been shown to play a central role in the pathogenesis and progression of chronic liver disease, however, present information regarding its expression in nonalcoholic fatty liver disease (NAFLD) is sparse (Wasmuth 2010).

RANTES has been shown to be expressed by hepatocytes and is up-regulated in response to inflammatory conditions (Zeremski 2007, Afford 1998, Rowell 1997, Ohashi 2009). Further, our group has shown that lipid accumulation does also dose-dependently induced RANTES expression in primary human hepatocytes *in vitro*. Thus, pure hepatocellular steatosis leads to increased hepatic RANTES expression. In line with this, we observed that feeding a high-fat diet induced hepatic RANTES expression in the absence of significant hepatic inflammation or fibrosis in mice. This finding further confirms steatotic hepatocytes but not infiltrating inflammatory cells or activated hepatic stellate cells as main cellular sources of elevated hepatic RANTES expression in high-fat diet fed mice (Kivovski 2010).

Nevertheless, it has to be considered that obesity frequently leads to up-regulation of cytokines as a part of a systemic state of low inflammation not only in the liver but also in (visceral) adipose tissue, which is another important source of circulating cytokines including RANTES (Skurk 1009). However, in our study, RANTES expression in visceral fat of mice, which were fed with a high-fat diet, was similar to the control group. This suggests that at least under these experimental conditions the fatty liver but not adipose tissue is the source of elevated (circulating) RANTES levels (Kivovski 2010). Taken together this data and the known pathophysiological role of RANTES in other chronic liver diseases indicate that steatosis-triggered RANTES

production by hepatocytes is an early event during the natural course of NAFLD and may be involved, along with other factors/cytokines, in the progression of fatty liver to significant inflammation, e.g. NASH.

Interestingly, patients in the present study with US-diagnosed NAFLD had significantly elevated RANTES serum levels compared to the control group, although this increase was only moderate compared to the induction of RANTES mRNA expression in *in vitro* lipid loaded hepatocytes and murine fatty livers. However, despite US examination can be adequately used to assess hepatic steatosis (Joseph 1991, Saverymuttu 1986), it has to be considered that the reliable threshold is approximately 30% fat in the hepatic tissue (Saadeh 2002). Thus, it is likely that a significant number of patients in the control group also had hepatic steatosis below the US-detection level. NAFLD is generally defined by lipid deposition greater than 5-10% of the liver weight (Neuschwandner-Tetri 2003, Caldwell 2004). Further, the above mentioned *in vitro* data (Kivovski 2010) indicate that even minimal hepatocellular lipid accumulation leads to increased RANTES levels. Conversely, despite the lack of histological examination in this hospital cohort of randomly selected patients, it can be estimated from epidemiological studies (Bellentani 2010), that only a minority of cases had significant hepatic inflammation (e.g. criteria for NASH), which may have caused elevated RANTES levels.

In summary, this study indicates that hepatic steatosis causes an up-regulation of hepatic RANTES expression. Noteworthy, increased expression of RANTES in response to hepatocellular lipid accumulation appears to occur in the absence of relevant hepatic inflammation. This finding further indicates that hepatic steatosis *per se* has pathophysiological relevance and should not be considered as benign.

V. Appendix

1. Bibliography

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2. Summary

Einführung

Die **nicht-alkoholische Fettlebererkrankung** beschreibt ein weites Spektrum von Erkrankungen, das von einfacher Leberverfettung (Steatosis) bis zu nicht-alkoholischer Steatohepatitis, die mit als Hauptursache der nicht-cholestatischen Leberzirrhose und damit als Vorstufe eines Leberzellkarzinomes gilt.

Die genaue Ursache der nicht-alkoholischen Fettleberhepatitis ist nicht genau bekannt. Am wahrscheinlichsten gilt die sog. Zwei-Schritte-Hypothese, die berets 1998 von Day und James vorgestellt wurde: der erste Schritt ist dabei die Insulinresistenz, der zweite Schritt sind necroinflammatorische Mechanismen, die dann zum Leberparenchymschaden führen.

Chemokine sind eine große Familie chemotaktischer Zytokine, deren Mitglieder strukturelle Ähnlichkeiten in ihrer Sekundärstruktur aufweisen. Man unterscheidet zwei Gruppen von Chemokinen: pro-inflammatorische und homöostatische Zytokine.

RANTES (**R**egulated upon **A**ctivation, **N**ormal **T**-Cell **E**xpressed and **S**ecreted) oder CCL-5 gehört zu der Gruppe der CC-Chemokine, da es zwei aufeinanderfolgende Cystein-Resten in seiner Sekundärstruktur aufweist. RANTES ist ein pro-inflammatorisches Chemokin, welches von den meisten Geweben physiologischerweise nicht produziert wird.

Berres et al. konnten in ihrer Studie zeigen, dass RANTES pro-fibrotische Eigenschaften bei Entstehung der Leberfibrose hat. Die Expression von RANTES mRNA zeigte einen signifikanten Zusammenhang mit höheren Stadien der Leberfibrose und der NASH (Nicht-alkoholische Steatohepatitis).

Ziel dieser Studie war es, den Zusammenhang zwischen dem Grad der Steatosis hepatis und RANTES-Serumspiegeln zu evaluieren.

Patienten und Methoden

Beginn der Datensammlung war im Februar 2009. Stationäre und ambulante von verschiedenen Abteilungen des Uniklinikums Regensburg, die rountinemäßig im Ultraschallzentrum des Uniklinikums untersucht werden sollten, wurden in die Studie eingeschlossen. Nach Ausschluss aller Patienten mit Ausschlusskriterien (Patienten mit bösartigen Erkrankungen, anderen Lebererkrankungen, mit Aszites oder chronisch-entzündlichen Darmerkrankungen, mit Alkhol- oder Drogenabusus oder Patienten, die Medikamente einnehmen, die eine Steatosis hepatis induzieren können) blieb eine Gruppe von 155 Patienten.

Mit allen Patienten wurde ein Anamnesegespräch durchgeführt und die Ergebnisse anschließend in einem Anamnesebogen dokumentiert (Geschlecht, Vorerkrankungen, Medikamente, Familienanamnese, Diabetes, Lebererkrankungen, Fettstoffwechselerkrankungen, Tumorerkrankungen, Alkohol-, Nikotin- und Drogenkonsum). Anschließend wurde eine körperliche Untersuchung durchgeführt (Bestimmung von Größe, Gewicht, Hüft- und Beckenumfang, Blutdruckmessung und Bestimmung der Herzfrequenz.

Außerdem wurde eine Blutabnahme durchgeführt. Die weißen Serumröhrchen wurden unmittelbar nach Blutabnahme zentrifugiert und das Zentrifugat zunächst in 10 Eppendorff-Cups aliquotiert und anschließend tiefgefroren. Durch das Institut für klinische Chemie des Uniklinikums Regensburg wurden folgende Blutwerte bestimmt: AST, ALT, γ-GT, Blutstoffwechselparameter, Albumin, Gesamtprotein, Bilirubin, Cholinesterase, alkalische Phosphatase und Nüchternglukose).

Anschließend erfolgte eine Ultraschalluntersuchung und Dokumentation in einem Standard-Untersuchungsprotokoll. Es wurden dabei folgende Merkmale untersucht: Lebergröße in der MCL, Ausschluss von Ascites, Bestimmung der Leberechogenität, der –binnenstruktur, der Leberoberfläche sowie ihrer Kompressibilität, Suche noch fokalen Minderverfettungen und anderen Leberraumforderungen, Bestimmung des Körperfettes über dem rechten Rippenbogen und periumbilikal und Bestimmung des Pfortaderflusses.

Die Serumanalyse von RANTES erfolgte in den Forschungslaboren von Herrn Prof. Hellerbrand mittels einem Sandwich-ELISA (CCL5 DuoSet ELISA development kit; R&D Systems).

Ergebnisse

Die RANTES-Serumspiegeln waren in der Patientengruppe mit Ultraschall-diagnostizierter Fettleber gering, aber signifikant ($p< 0,001$) erhöht (85,9 ng/ ml) gegenüber der Kontrollgruppe mit normalem Leberparenchym in der Ultraschalluntersuchung (60,1 ng/ ml).

Für die Fettstoffwechselwerte (Triglyzeride, Gesamtcholesterin, HDL, LDL) und RANTES sowie für die Leberwerte (ALT, AST, γGT, alkalische Phosphatase, Bilirubin, Lipase, Gesamtprotein und Cholinesterase) und RANTES sowie für Eisenstoffwechselparameter (Fe^{3+}, Ferritin, Transferrin und Transferrinsättigung) und RANTES konnten keine signifikanten Zusammenhänge nachgewiesen werden.

Es konnte auch kein siginifikanter Zusammenhang zwischen den Ergebnissen der körperlichen Untersuchung (Alter, Hüft- und Beckenumfang, Blutdruck und Herzfrequenz) und RANTES sowie den übrigen Befunden der Ultraschalluntersuchung (Lebergröße, Fettschicht über dem rechen Rippenbogen und periumbilikal, Pfortaderfluss) und RANTES nachgewiesen werden.

Diskussion

RANTES scheint eine zentrale Rolle in der Entstehung und der Fortschreiten der chronischen Lebererkrankungen zu spielen. Es konnte bereits gezeigt werden, dass RANTES von Hepatozyten vermehrt bei Vorliegen einer Entzündung exprimiert wird.

Wir konnten in unserer Studie zeigen, dass die Ansammlung von Fett in-vitro ebenfalls die vermehrte Expression von RANTES bewirkt. Im Mausmodel konnte gezeigt werden, dass RANTES in Abwesenheit von hepatischer Entzündung oder Leberfibrose vermehrt exprimiert wird. Dies bestätigt, dass RANTES hauptsächlich von verfetteten Hepatozyten und nicht von pro-entzündlichen Zellen oder aktivierten Sternzellen produziert wird.

Skurk konnte zeigen, dass auch das Fettgewebe ein wichtiger Produktionsort für zirkulierende Zytokine, unter anderem auch RANTES, ist. Dennoch war die RANTES-Expression im Mausmodel bei Mäusen mit NASH gegenüber der Kontrollgruppe nicht erhöht. Dies zeigt möglicherweise, dass die Fettleber und nicht das Fettgewebe die Hauptquelle für erhöhte RANTES-Serumspiegel ist.

Zusammenfassend lässt sich sagen, dass unsere Studie darauf hin deutet, dass die Steatosis hepatis zu einer Erhöhung der RANTES-Expression führt. Diese Erhöhung der RANTES-Expression tritt auch in Abwesenheit von relevanter hepatischer Entzündung auf. Dies deutet daraufhin, dass die Fettleber an sich schon eine pathophysiologische Relevanz hat und nicht als benigen erachtet werden sollte.

4. Declaration on oath

Eidesstattliche Erklärung

Familienname: Nießen
Vorname: Christoph
Geb.: 11. Mai 1985

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich/unentgeltlich geholfen:

1. _____

2. _____

3. _____

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eine Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit gesagt und nichts verschwiegen habe.

Vor Aufnahme der obigen Versicherung an Eides Statt wurde ich über die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung belehrt.

_____ _____
(Ort, Datum) (Unterschrift)
Unterschrift des die Versicherung an Eides Statt aufnehmenden Beamten.

_____ _____
(Ort, Datum) (Unterschrift)