NON-INVASIVE ELASTOGRAPHY-BASED STUDY IN PATIENTS WITH
CHRONIC LIVER DISEASES IDENTIFIES VARIANT ADIPONUTRIN AS
COMMON FIBROSIS RISK FACTOR

DISSETATION
ZUR ERLANGUNG DES GRADES EINES DOKTORS DER MEDIZIN
DER MEDIZINISCHEN FAKULTÄT
DER UNIVERSITÄT DES SAARLANDES
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vorgelegt von
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1. **SUMMARY**

1.1 Deutsche Zusammenfassung

1.2 Abstract

Liver fibrosis is the common consequence of chronic liver injury and inevitably leads to liver cirrhosis, which is the tenth most common cause of death in Germany. To date no effective preventive and therapeutic options, except for liver transplantation, are available for patients suffering from cirrhosis. Recent genome-wide association studies have identified the p.I148M adiponutrin (PNPLA3) variant as risk factor for severe forms of non-alcoholic and alcoholic liver diseases. The PNPLA3 allele p.148M confers an increased risk for fatty liver disease and elevated serum aminotransferase activities reflecting liver injury. In the current elastography-based observational cross-sectional study we aimed to investigate adiponutrin as a genetic determinant of liver fibrosis, the hallmark of all chronic liver diseases. Therefore we staged 899 patients with different chronic liver diseases non-invasively by transient elastography (Fibroscan) and genotyped them for variant adiponutrin (rs738409) by PCR-based assays. A subgroup of 229 patients consented to percutaneous liver biopsy, validating the accuracy of elastography in staging fibrosis ($\rho = 0.743, P < 0.01$). Genotyping results proved that carriers of distinct p.I148M adiponutrin genotypes display significant ($P = 0.017$) differences in liver stiffness determined by elastography. In particular, individuals carrying the allele p.148M are at higher risk of developing liver cirrhosis defined by stiffness values $\geq 13.0$ kPa (OR = 1.56, $P = 0.005$). Of note, the PNPLA3 risk variant advances fibrosis in the total cohort as well as in the subgroups of patients with viral hepatitis and non-viral liver diseases and contributes 16% of the total cirrhosis risk. In conclusion, our study shows that the adiponutrin risk variant is a common genetic determinant of progressive liver fibrosis. This is the first study showing that clinically relevant genetic investigations in patients with chronic liver disease can be performed in cohorts phenotyped with transient elastography, and based on these results non-invasive follow-up strategies for these individuals can be envisioned.
1.3 Abbreviations

ALD, alcoholic liver disease; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AUC, area under the curve; BMI, body mass index; CI, confidence interval; FFA, free fatty acid; gamma-GT, γ-glutamyl transferase; GWAS, genome-wide association study; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HWE, Hardy-Weinberg equilibrium; I, isoleucine; INR, international normalised ratio; kPa, kilopascal; M, methionine; MELD, model of end-stage liver disease; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; PAF, population attributable fraction; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; PNPLA3, patatin-like phospholipase domain containing 3 (adiponutrin); PPAR, peroxisome proliferator activated receptor; PSC, primary sclerosing cholangitis; SNP, single nucleotide polymorphism; SREBP1c, sterol response element binding protein 1c; TE, transient elastography; TG, triglycerides; TNF, tumor necrosis factor.
2. Introduction

2.1 Hepatic fibrosis – a complex process determined by genetic factors

Liver fibrosis (or liver scarring), a hallmark of chronic liver diseases, is characterized by excessive deposition of collagen and of other extracellular matrix proteins in the liver leading to a progressive deterioration of hepatic function (Bataller R 2005, Friedman SL 2008). According to the latest studies chronic hepatitis C infections, alcoholic liver disease (ALD) and non-alcoholic steatohepatitis represent the main causes of liver fibrosis in developed countries. Based on the underlying liver disease, the distribution of lesions and the fibrotic mechanisms, distinct patterns of liver fibrosis have been described (Hernandez-Gea V 2011). However, regardless of the causative factor triggering hepatic fibrogenesis, chronic liver injury leads inevitably to progressive fibrosis (Friedman SL 2010). Once in the cirrhotic phase, the natural history of liver destruction includes a progression from compensated to decompensated disease. The latter one is characterised by portal hypertension, liver failure and ultimately the death of the patient, if not transplanted. It is worth noting that in the past decades cirrhosis mortality rates have increased dramatically in Europe and currently reach 33 cases per 100,000 German males yearly (Leon DA 2006). In developing countries cirrhosis mortality rates are also increasing and according to the latest data they exceed 23 cases per 100,000 Polish males yearly (Zatoński WA 2010).

Liver is one of the most complex organs with a high level of functional variability. Among several liver functions clearance of xenobiotics and endobiotics, synthesis of proteins and energy storage represent only some of its multiple tasks. Thus, only a
minor number of liver conditions are monogenic. The most common liver diseases are complex, as they are determined by a combination of genetic and environmental risk factors. The complexity of the genes expressed in the hepatic cells, their interactions and function is a major challenge in determining the pathogenesis of complex hepatopathies. In the recent years, genotyping of over 500,000 single nucleotide polymorphisms (SNPs) that cover more than 75% of the human genome, in large cohorts of individuals with specific liver conditions, have led to the identification of several loci. Indeed, such an approach used in genome wide association studies (GWAS), has enabled the identification of the causative genes involved in several liver diseases. Among hepatic conditions with unraveled genetic background, fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), viral hepatitis, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) result in hepatic fibrosis as a result of a chronic liver injury.

Conceptually liver fibrogenesis is a complex (i.e. multifactorial) process governed by multiple genetic factors and their interactions with numerous environmental determinants. The non-genetic triggers leading to liver fibrogenesis (e.g. viral hepatitis, excessive alcohol consumption, fatty liver disease, autoimmune liver diseases) have been well described; nevertheless as the progression of liver fibrosis varies markedly between individuals with similar risk profiles, genetic factors are likely to represent critical determinants of this process (Hillebrandt 2005). Hence, the identification of profibrogenic genetic variants is crucial for detecting at-risk individuals and for designing tailored preventive strategies (Krawczyk M 2010b). As shown in Figure 1, based on the prevalence and penetrance of genetic variants, several approaches can be applied. For example, an Australian candidate-gene study (Richardsom MM 2005) identified an
association between polymorphisms in six loci (APOE, CCR5, CTLA4, HFE, MTTP, SOD2) and progression of liver fibrosis in patients with chronic hepatitis C virus (HCV) infection. On the other hand, Huang et al. (Huang H 2007) used a machine-learning approach to analyse results from a large genome wide-association study (GWAS) in 1020 HCV patients and identified a polygenic risk score of hepatic fibrosis including seven polymorphisms (AP3S2, AQP2, AZIN1, DEGS1, STXBPS, TLR4, TRPM5). The OR (odds ratio) among carriers of at least five risk alleles from the first study (Richardson MM 2005) reached 24, and area under the curve (AUC) statistics of the cirrhosis risk score from the second study (Huang H 2007) was 0.73. Of note, both studies identified different variants as putative determinants of liver fibrosis in comparable cohorts (Huang H 2007, Richardson MM 2005). Hence, the results remain inconclusive, attempts to unravel the host genetic background of hepatic fibrosis in humans have met with limited success, and no common susceptibility variants have been identified so far (Weber S 2008, Guo J 2009, Marcolongo M 2009, Wasmuth HE 2009).
Figure 1. Relationship between frequencies of susceptibility variants and phenotypes. In case of Mendelian diseases, which are mostly caused by rare mutations, the causative variant confers a high risk for developing a disease. The low-frequency alleles that associate with intermediate risk as well as rare variants with low disease risk can be identified by resequencing techniques. Genetic risk variants identified by GWAS represent the common alleles that are associated with a low disease risk. Adapted from Krawczyk M 2010b.
2.2 Measurement of liver fibrosis: the invasive approach

Assessment of liver fibrosis in patients with chronic liver diseases is crucial for diagnosis and prognosis. To date this is accomplished mostly by liver biopsy, which represents the “gold standard” for staging hepatic fibrosis (Rockey DC 2009). In this procedure liver specimens are obtained either percutaneously or via a vascular (transjugular) route. In rare cases a laparoscopic liver biopsy is performed. After the procedure the achieved hepatic specimens are examined microscopically by a pathologist to assess liver injury. Depending on the underlying disease distinct histological patterns of liver fibrosis can be recognised. Therefore, pathologists use various scoring systems, for example the Desmet and Scheuer score (Desmet VJ 1994) in patients with chronic hepatitis, whereas in NAFLD the Brunt scoring system (Brunt EM 1999) has been applied. Although liver biopsy is regarded as the mainstay for staging hepatic fibrosis, it has limitations such as procedure-related bleeding (Terjung B 2003), sampling errors, observer variability, and lack of subclassification of cirrhosis (Bedossa P 2003, Castéra L 2010b). As a result, previous genetic or intervention studies of hepatic fibrosis have been hampered by small study sizes, since participants had to undergo invasive liver biopsies for histological assessment of liver status (Bataller R 2003, Weber S 2010). Lately the International Fibrosis Group has recommended that non-invasive surrogate markers reproducibly detecting changes in fibrosis would be helpful to reduce the complexity and increase the size of antifibrotic trials (McHutchison J 2006). Therefore, non-invasive methods for the assessment of liver fibrosis including direct and indirect (surrogate) markers are being evaluated (Grühnage F 2009, Castéra L 2010b).
2.3 Quantification of liver fibrosis: the non-invasive approach

Among alternative approaches, transient elastography (TE) has emerged as a non-invasive and accepted tool of quantifying liver fibrosis, since it is a rapid, pain- and complication-free method with high intra- and interobserver agreement (Pinzani M 2008, Castéra L 2010c). In short, TE is performed with an ultrasound transducer probe (Figure 2) that is mounted on a vibrator. The tip of the transducer is placed on the skin between the rib bones at the level of the right lobe of the liver. The vibration of the probe induces a shear wave that propagates through the liver. These propagating waves are followed by pulse-echo ultrasound acquisitions, and their velocity is directly related to tissue stiffness. In other words, the harder and the more fibrotic is the liver, the faster the shear wave propagates. The measurement depth ranges between 25 and 65 mm below the skin surface. The results are expressed in [kPa], and ten measurements are performed in each patient. The median value is taken as representative. This approach has several advantages in comparison to liver biopsy. The procedure does not last more than 10 – 15 minutes, it can be performed by an experienced nurse or a clinician and is virtually complications-free. Moreover it is suitable for an ambulant follow-up of patients with chronic liver diseases. To date, prospective studies in patients with HBV (Marcellin P 2009) and chronic HCV (Castéra L 2005) or non-viral hepatitis (Obara N 2008) as well as meta-analyses have documented that liver stiffness correlates significantly with histological fibrosis stages, in particular the most common technique known as transient elastography (Fibroscan®) (Friedrich-Rust M 2009), and exactly defined cut-off values for staging of liver fibrosis are being developed (Friedrich-Rust M 2008). In general, the cut-off of 13.0 kPa has been proposed that is the most suitable one to distinguish between individuals with liver fibrosis and cirrhosis (Friedrich-Rust M 2008).
2.4 Variant Adiponutrin (PNPLA3) – a genetic risk factor for fatty liver

In 2008, a genome-wide association scan (GWAS) using a dense map of single nucleotide polymorphisms (SNPs) (see Figure 1) revealed for the first time an intriguing association between the adiponutrin (PNPLA3) gene locus and plasma levels of liver enzymes in the general population (Yuan X 2008). Afterwards, another GWAS in 2111 individuals identified the p.I148M (rs738409) variant of PNPLA3 as genetic risk factor for non-alcoholic fatty liver disease (NAFLD) (Romeo S 2008), a finding subsequently...
confirmed in additional cohorts (Kantartzis K 2009, Kotronen A 2009a, Romeo S 2010, Sookoian S 2009, Wagenknecht LE 2011), and extended to alcoholic liver disease (ALD) (Tian C 2010, Stickel F 2011). In particular, carriers of the [G] allele showed higher hepatic fat contents (as determined by proton magnetic resonance spectroscopy) as well as serum activities of alanine aminotransferase (ALT) (Romeo S 2008, Kollerits B 2010) and in case of ALD, were at risk of developing liver cirrhosis (Tian C 2010). Moreover, adiponutrin variant has been related to glucose metabolism. For example analysis Johansson et al. (Johansson LE 2008) demonstrated that carriers of the p.I148 polymorphism are in general more insulin-resistant at lower BMI whilst carriers of the methionine allele are prone to decreased insulin secretion after oral glucose challenge.

Adiponutrin, a membrane-bound enzyme encoded by the PNPLA3 gene, is a 481-amino acid protein that contains a highly conserved patatin-like domain at the N terminus. It is expressed predominantly in the liver and in the adipose tissue in response to lipogenic factors (Kershaw EE 2006, Huang Y 2010). In vitro studies have demonstrated that adiponutrin possesses lipid hydrolase and transacylase activities (Lake AC 2005, Wilson PA 2006, He S 2010), whereas in vivo it has been shown to hydrolyze emulsified triglycerides in lipid droplets (He S 2010). In contrast to constitutive PNPLA3 expression in adipose tissue, transcription in the liver is driven by carbohydrate overnutrition via the sterol response element binding protein 1c (SREBP1c). As postulated by Huang et al. (Huang Y 2010), feeding with carbohydrates increases insulin level which subsequently activates SREBP1c through the heterodimer nuclear receptors LXR/RXR and leads to transcriptional activation of PNPLA3. Conversely, degradation of adiponutrin is inhibited by accumulation of C16:0, C18:0 and C18:1 fatty acids (Huang Y 2010). Functional analysis of the rs738409 adiponutrin NAFLD/ALD risk variant showed
that the resulting amino acid substitution of isoleucine by methionine change at position 148 disrupts triglyceride emulsifying activity of PNPLA3 \textit{in vivo}. As a result, carriers of the PNPLA3 variant p.148M are at an increased risk of developing hepatic inflammation and fatty liver \cite{Krawczyk2010a}.

2.5 Fatty liver – the most prevalent liver condition

The currently experienced increase in liver fibrosis and cirrhosis rates in developed countries is in part related to the epidemics of fatty liver disease. Indeed fatty liver, also known as non-alcoholic fatty liver disease (NAFLD), is the most common liver disorder in the Western world \cite{Krawczyk2010a}. It is characterised by an excessive hepatic fat deposition and encompasses benign and more severe patterns of lipid accumulation. Non-alcoholic steatohepatitis (NASH) represents the severe, necroinflammatory form of NAFLD, which can lead to liver fibrosis and cirrhosis. Currently the incidence of NAFLD is raising dramatically with an estimated prevalence of 20 – 40 \% in Western countries \cite{Loguercio2004}. Obesity and the so-called ‘metabolic syndrome’ are the most prominent non-genetic risk factors for NAFLD \cite{Portincasa2006}. Indeed, although only 2 – 3\% of lean individuals are affected by NASH \cite{Neuschwander-Tetri1996}, some reports state that up to 50\% of morbidly obese patients may suffer from steatohepatitis \cite{Dam-Larsen2004}. Of note, up to 10\% of NASH patients will develop liver cirrhosis within 5 years \cite{Cortez-Pinto2003}. Interestingly, most of the patients with fatty liver present with no specific symptoms. In some cases enlarge liver can be found at the examination of the patient, asymptomatic elevation of transaminases is also characteristic for patients with NAFLD. Liver ultrasonography detects the so called ‘bright liver’ in these individuals. Nevertheless ultrasonography does not allow
differentiation between benign forms of hepatic fat accumulation and NASH. When NASH is suspected, liver biopsy is performed to confirm or exclude the diagnosis. According to Brunt et al (Brunt EM 1999) liver status in NASH patients than staged as follows: S1 = perivenular, perisinusoidal or pericellular fibrosis, S2 = periportal fibrosis; S3 = bridging fibrosis; S4 = cirrhosis.

The natural course of fatty liver diseases can be highly variable, it has been therefore for long suspected that common gene polymorphisms increase the risk of fibrosis in a subgroup of subjects with fatty liver. Indeed studies investigating family clustering show that at least 20% of individuals with NASH have first degree relatives who are also affected with this condition (Wilner IR 2001). Moreover, a high degree of concordance in families with respect to non-alcoholic steatohepatitis and cryptogenic cirrhosis has been documented (Struben WM 2000). Figure 3 presents the complexity of NAFLD pathogenesis. Excessive accumulation of fat in the liver is determined by overweight, obesity, increased visceral adiposity, and insulin resistance. In this constellation of risk factors, intrahepatic lipid accumulation results from an excessive influx of free fatty acids (FFAs) from the adipose tissue, decreased hepatic export of FFAs, or impaired FFAs ß-oxidation (Krawczyk M 2010a). Several metabolic abnormalities at the hepatic level have been observed in patients with NAFLD. These include oxidative stress (Stewart SF 2004, Palmieri VO 2006), impaired mitochondrial oxidation (Sanyal AJ 2001, Pessayre D 2001), generation of free oxygen radicals, and increased activity of the cytochrome P-450 system (Robertson G 2001). All of these contribute to the progressive hepatic accumulation of lipids and to liver fibrosis in NASH (Portincasa P 2006).
Figure 3. Pathogenesis of fatty liver disease (see text for explanation).

Abbreviations: FFA, free fatty acids; HCC, hepatocellular carcinoma; PPARs, peroxisome proliferator activated receptors; TG, triglycerides; TNF, tumor necrosis factor. Symbols: increased (↑); decreased (↓); stimulatory effect (+); inhibitory effect (-).

Adapted from Krawczyk M 2010a.
2.6 Study outline and specific aims

Hepatic fat accumulation, either due to NAFLD or ALD, represents one of the most frequent conditions leading to liver fibrosis and cirrhosis worldwide (Everhart JE 2009). Previous adiponutrin genetic association studies focused on hepatic fat accumulation and were exclusively based on cohorts with NAFLD and ALD (Romeo S 2008, Tian C 2010). Systematic analyses in patients with fatty liver reported that the PNPLA3 variant p.I148M is also associated with histological severity (Sookoian 2009) and more particularly with fibrosis in an additive pattern as well (Valenti L 2010, Rotman Y 2010, Speliotes EK 2010). In the aforementioned studies (Kantartzis K 2009, Kotronen A 2009a, Sookoian S 2009, Romeo S 2010, Tian C 2010, Wagenknecht LE 2011) patients with liver diseases other than NAFLD and ALD have not been investigated with respect to the adiponutrin polymorphism, hence its role as a common genetic determinant of hepatic fibrosis has remained speculative (Weber S 2010).

In the current study it was our aim to investigate the role of the adiponutrin variant p.I148M as a common determinant of liver cirrhosis and liver fibrosis in general. Therefore we recruited a large cohort of patients with different chronic liver diseases, who we all staged non-invasively by transient elastography. Our genetic study is the first to employ elastography for phenotyping fibrosis, which allowed the recruitment of a large cohort of patients. In this elastography-based study, we demonstrate that the adiponutrin variant not only predisposes to severe forms of NAFLD and ALD but also markedly enhances hepatic fibrosis across different chronic liver diseases. Our results prove that carriers of the risk variant are at an increased risk of developing liver cirrhosis in general. Hence, we suggest a new surveillance strategy focusing on a genetically defined subgroup of patients at risk for advanced liver injury.
3 PATIENTS AND METHODS

3.1 Patients
In this observational cross-sectional study, we recruited 1,000 consecutive European individuals (age 17 - 83 years, 547 males) with chronic liver diseases who were referred to our department for transient elastography. All patients underwent careful clinical examination. Blood samples were drawn from fasted subjects, and liver functions tests including serum ALT activities were determined by standard clinical-chemical assays in the central laboratory.

Table 1 summarizes the baseline clinical characteristics of our cohort. We recorded data on estimated daily alcohol consumption, and individuals with non-alcoholic liver disease who also reported a regular high alcohol intake of > 40 g/d in the last year were excluded from the study, as described previously (Hillebrandt S 2005, Geier A 2004).

Informed consent was obtained from all patients, and the study was approved by the Research Ethics Committee of the University of Bonn.
### Table 1
Clinical characteristics of the study cohort

<table>
<thead>
<tr>
<th>Variables</th>
<th>Subject characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (male / female)</td>
<td>899 (547 / 352)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 (17 - 83)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 (14.9 - 45.2)</td>
</tr>
<tr>
<td>Primary liver disease (N)</td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td>541 (60.2%)</td>
</tr>
<tr>
<td>ALD</td>
<td>112 (12.5%)</td>
</tr>
<tr>
<td>HBV</td>
<td>67 (7.5%)</td>
</tr>
<tr>
<td>PBC / PSC / AIH</td>
<td>67 (7.5%)</td>
</tr>
<tr>
<td>NAFLD / NASH</td>
<td>64 (7.1%)</td>
</tr>
<tr>
<td>Haemochromatosis</td>
<td>25 (2.8%)</td>
</tr>
<tr>
<td>Other liver diseases*</td>
<td>23 (2.6%)</td>
</tr>
<tr>
<td>Alcohol consumption (g/24 h)§</td>
<td>0 (0 - 40), mean ± SD: 6.2 ± 11.0</td>
</tr>
<tr>
<td>Overt cirrhosis</td>
<td>163 (18.1%)</td>
</tr>
<tr>
<td>Transient elastography</td>
<td></td>
</tr>
<tr>
<td>Liver stiffness (kPa)</td>
<td>6.8 (2.2 - 75.0)</td>
</tr>
<tr>
<td>≥ 13.0 kPa (N)</td>
<td>201 (22.3%)</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.6 (0.1 - 6.0)</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.9 (0.5 - 5.1)</td>
</tr>
<tr>
<td>INR</td>
<td>1.0 (0.9 - 2.1)</td>
</tr>
<tr>
<td>MELD</td>
<td>7 (6 - 31)</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>43 (3 - 67)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>38 (12 - 889)</td>
</tr>
<tr>
<td>γ-GT (U/L)</td>
<td>56 (8 - 2597)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>94 (60 - 195)</td>
</tr>
<tr>
<td>Platelets (×10⁹/l)</td>
<td>205 (23 - 887)</td>
</tr>
</tbody>
</table>

Values are given as medians (ranges), unless stated otherwise. * Other liver diseases include cryptogenic liver disease (N = 13), biliary atresia (N = 3), Wilson Disease (N = 2), amyloidosis (N = 1), α₁-antitrypsin deficiency (N = 1), Budd-Chiari syndrome (N = 1), congenital liver disease (N = 1), and sarcoidosis (N = 1). § during the 12 months prior to transient elastography.

Abbreviations: AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; BMI, body mass index; γ-GT, γ-glutamyl transferase; HBV, chronic hepatitis B virus infection; HCV, chronic hepatitis C virus infection; INR, international normalised ratio; MELD, model of end-stage liver disease; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.
3.2 Non-invasive phenotypic characterization of liver fibrosis

Liver fibrosis was assessed by transient elastography (Fibroscan®, Echosens SA, Paris, France). In patients with tense ascites, elastography was preceded by large-volume paracentesis. Following the criteria by Castéra et al. (Castéra L 2010a), the results were called unreliable if the success rate (i.e. the ratio of valid shots to the total number of shots) was < 60% or the interquartile range was > 30% of the median value. The procedure was considered to have failed when no value was obtained after 10 shots (Castéra L 2010a). According to previous studies (reviewed in Pinzani M 2008) and an authoritative meta-analysis (Friedrich-Rust M 2008), individuals with liver stiffness ≥ 13.0 kPa were regarded to suffer from liver cirrhosis (fibrosis stage F4). Overt cirrhosis was diagnosed if there were unequivocal clinical, ultrasound, and/or endoscopic findings.

3.3 Invasive assessment of liver fibrosis

A subgroup of individuals (presented in Table 3) was scheduled for liver biopsy. The indications of liver biopsy followed recent consensus recommendations (Schirmacher P 2004, Rockey DC 2009), and the most common reasons were staging of viral hepatitis and abnormal liver tests of unknown aetiology or suspicion of cirrhosis. In most cases the intercostal technique approach the percutanous Menghini technique with 1.8 mm needles (Hepafix® G15) under ultrasound guidance was employed. The biopsy was performed under local anaesthesia in the 8th or 9th intercostal space in the mid-axillary line. In patients with coagulopathies or ascites a transjugular liver biopsy was performed. After the procedures patients were observed for 24 hours to exclude complications. For histological scoring of liver fibrosis, paraffin embedded 2- to 4-μm liver sections were
stained with haematoxylin an eosin as well as Sirius red for collagen (Hillebrandt S 2005). The pathologist (Prof. Dr. H. P. Fischer, University Hospital Bonn), who was blinded to the elastography results, staged hepatic fibrosis according to Desmet and Scheuer as follows: F0 = absence of fibrosis; F1 = perisinusoidal and/or portal fibrosis; F2 = perisinusoidal and portal/periportal fibrosis; F3 = septal or bridging fibrosis; and F4 = cirrhosis (Desmet VJ 1994).

3.4 DNA isolation from EDTA anticoagulated blood
Genomic DNA was isolated from EDTA anticoagulated blood according to the membrane-based QIAamp DNA extraction protocol (Qiagen, Hilden, Germany). In short, defrosted blood samples of 200 µl were mixed with 20 µl QIAGEN Protease and 200 µl Buffer AL by pulse-vortexing for 15 seconds. After incubation at 56 °C for 10 minutes and brief centrifugation, 200 µl of 96-100 % ethanol was added to the sample pulse-vortexed. The mixture was centrifuged at 8000 rpm for 1 minute and 500 µl of Buffer AW1 was added. After centrifuging at 8000 rpm for 1 minute 500 µl of Buffer AW2 was added and the mixture was centrifuged at 14000 rpm for 3 minutes. The yielded concentrations of DNA were measured using spectrophotometer.

3.5 Genotyping of the adiponutrin (PNPLA3) variant
The adiponutrin coding single nucleotide polymorphism (SNP) p.I148M (rs738409) (Romeo S 2008, Tian C 2010, Sticke F 2011 ) was genotyped using a solution-phase hybridization reaction with 5'-nuclease and fluorescence detection in the 7300 Real-Time PCR system (Applera, Norwalk, CT). Primer and probe sequences are listed in Table 2.
The PCR reactions (25 µL) contained:

- 20 ng of DNA
- 900 nM of each primer
- 1 × *TaqMan* Universal Master Mix
- 200 nM of VIC-labelled MGB/NFQ probes
- 200 nM of FAM-labelled MGB/NFQ probes.

*Abbreviation: MGB/NFQ – minor groove binder/non-fluorescent quencher.***

**Table 2**

<table>
<thead>
<tr>
<th>Primer / probe</th>
<th>Sequence</th>
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<tr>
<td>Forward primer</td>
<td>5’ – AACTTCTCTCTCCTTTGCTTTCACA – 3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5’ – TCTACAGTGCCCTTTATCCCTCC – 3’</td>
</tr>
<tr>
<td>VIC</td>
<td>5’ – TTCCTGCTTCATGCC – 3’</td>
</tr>
<tr>
<td>FAM</td>
<td>5’ – CCTGCTTCATCC – 3’</td>
</tr>
</tbody>
</table>

The 96-well plates were used. In 84 wells 2 µl of genomic DNA was disposed. In four wells 2 µl of buffer alone without DNA were pipetted (i.e. the negative control wells), in the remaining 8 wells (i.e. positive control wells) only genomic DNA was disposed. After addition of the PCR mix, the plate was covered with the PCR plate sealer and briefly centrifuged in the plate centrifuge.
The PCR amplification conditions were as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-read run</td>
<td>2 min</td>
<td>60 ° C</td>
<td>1 cycles</td>
</tr>
<tr>
<td>DNA denaturation</td>
<td>10 min</td>
<td>95 ° C</td>
<td>1 cycles</td>
</tr>
<tr>
<td>Amplification phase I</td>
<td>15 sec</td>
<td>95 ° C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Amplification phase II</td>
<td>1 min</td>
<td>60 ° C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Post-read run</td>
<td>1 min</td>
<td>60 ° C</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

The results were analysed using the allelic discrimination software (SDS version 2.0.5) as shown in example in Figure 4. To ensure genotyping quality, we included negative controls and DNA samples with known PNPLA3 genotypes as internal controls.
Figure 4. Allelic discrimination plot. The diagram presents the results of SNP genotyping with a solution-phase hybridization reaction including two dye-labelled probes and 5'-nuclease and fluorescence detection in the 7300 Real-Time PCR system (see Methods). Red and blue dots represent homozygous carriers of the variant, heterozygous individuals are marked in green.
3.6 Statistical analysis

Allele and genotype frequencies of the adiponutrin (PNPA3) SNP p.I148M were tested for consistency with Hardy-Weinberg equilibrium (HWE) using an exact test (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Median liver stiffness values among carriers of the [CC], [GC] and [GG] genotypes were compared by Kruskal-Wallis non-parametric analysis of variance (ANOVA) and Mann-Whitney U tests. The relationship between liver stiffness and the adiponutrin variant as well as other potentially profibrogenic factors (age, BMI, gender, hepatitis C virus (HCV) genotype) was assessed by univariate and multivariate linear regression analysis. Only variables that were significant in the univariate analysis were included in the multivariate model.

The association between the SNP p.I148M and liver cirrhosis, as defined by liver stiffness ≥ 13.0 kPa (Friedrich-Rust M 2008), was analyzed in contingency tables. Allele frequency differences were assessed by \( \chi^2 \) test and genotype differences by Armitrage’s trend test (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). The effects of the adiponutrin SNP and the other potential profibrogenic factors on the development of cirrhosis were further estimated by univariate and multivariate logistic regression analysis. The population attributable fraction (PAF) was calculated using PARC software (http://www.miner.rochester.edu/cpm/education/ match/productspubs.html) (Grünhage F 2007).

Unless stated specifically, all statistical analyses was performed with SPSS 18.0 (SPSS, Munich, Germany) and two-sided P values < 0.05 were regarded as significant. Phenotypic quantitative data were expressed as medians and ranges and analysed using Mann-Whitney U tests. Frequencies of qualitative phenotypes were analysed
Patients and methods

using contingency table statistics. Elastography results were analysed for correlation with fibrosis stages applying Spearman $\rho$ tests.
4. Results

4.1 Fibrosis staging with elastography is in line with liver histology

In total, 229 individuals underwent both transient elastography and percutaneous liver biopsy (Table 3). No major complications of the latter procedure were recorded. As reported by others (Pinzani 2008, Castéra L 2010c), a direct comparison of liver stiffness and histological fibrosis stages showed a close correlation between both methods (Spearman’s ρ = 0.743, P < 0.01). Further regression analysis demonstrated that in our overall cohort, liver stiffness values were not affected by liver inflammation as assessed by serum ALT activities (P > 0.05).

An exploratory analysis of the patients who underwent liver biopsy (Table 3) in comparison to the remaining patients (Table 1) showed a significantly higher prevalence of cirrhosis and deteriorated liver function, as indicated by significantly higher bilirubin and INR levels as well as lower serum albumin and platelets concentrations; the biopsied patients were significantly older and comprised more women as compared to the other patients (all P < 0.05).
### Table 3

**Clinical characteristics of the individuals scheduled for liver biopsy**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Subject characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (male / female)</td>
<td>229 (122 / 107)*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53 (20 - 78)*</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>24.4 (16.1 - 38.8)</td>
</tr>
<tr>
<td>Alcohol consumption (g/24 h)§</td>
<td>0 (0 - 40) / mean ± SD: 5.2 ± 10.7</td>
</tr>
<tr>
<td>Overt cirrhosis</td>
<td>114 (49.8%)*</td>
</tr>
</tbody>
</table>

**Liver biopsy**

<table>
<thead>
<tr>
<th>Specimen length (mm)</th>
<th>22 (7 - 79)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage of fibrosis</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20 (8.7%)</td>
</tr>
<tr>
<td>1</td>
<td>50 (21.8%)</td>
</tr>
<tr>
<td>2</td>
<td>24 (10.5%)</td>
</tr>
<tr>
<td>3</td>
<td>18 (7.9%)</td>
</tr>
<tr>
<td>4</td>
<td>117 (51.1%)</td>
</tr>
</tbody>
</table>

**Transient elastography**

<table>
<thead>
<tr>
<th>Liver stiffness (kPa)</th>
<th>11.3 (2.2 - 75.0)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 13.0 kPa (N)</td>
<td>108*</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.9 (0.2 - 6.0)*</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.8 (0.5 - 5.1)</td>
</tr>
<tr>
<td>INR</td>
<td>1.1 (0.9 - 2.1)*</td>
</tr>
<tr>
<td>MELD</td>
<td>8 (6 - 31)*</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>41 (3 - 56)*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>48 (5 - 341)</td>
</tr>
<tr>
<td>Gamma-GT (U/L)</td>
<td>80 (13 - 2597)*</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>98 (60 - 195)</td>
</tr>
<tr>
<td>Platelets (×10^9/l)</td>
<td>177 (23 - 477)*</td>
</tr>
</tbody>
</table>

*Values are given as medians (ranges), unless stated otherwise.

* P < 0.05 in exploratory comparison to the remaining patients who underwent elastography only (Mann-Whitney U tests or contingency table statistics). § during the 12 months prior to transient elastography. Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; gamma-GT, γ-glutamyl transferase; INR, international normalised ratio; MELD, model of end-stage liver disease.
4.2 The adiponutrin variant p.I148M increases fibrosis levels

Overall, genotyping success rate was 100%. In total, the frequencies of the [C] allele (p.I148) and the [G] allele (p.148M) were 73.5% and 26.5%, respectively. The genotypes [CC], [GC] and [GG] genotypes were carried by 485, 351 and 63 individuals, respectively. All allele and genotypes frequencies were consistent with HWE (Figure 5) and in line with frequencies deposited in the Entrez SNP database (http://www.ncbi.nlm.nih.gov/snp). Liver stiffness was successfully determined in 899 of 1,000 recruited patients, i.e. 10% of patients were excluded from the study due to unreliable or failed elastography (see Methods). The most common reasons were massive and/or perihepatic ascites or obesity (BMI > 30 kg/m², increased waist circumference).
Figure 5. De Finetti diagram with Hardy-Weinberg parabola for the PNPLA3 p.I148M polymorphism. The diagram illustrates genotype and allele frequencies in cases (individuals with liver stiffness ≥ 13.0 kPa, blue line) and "controls" (stiffness < 13.0 kPa, black line). Frequencies of the [C] allele are shown at the intersection between the vertical dotted line and the bottom perpendicular. The frequencies of [GG] and [CC] homozygotes are depicted on the left and right diagonal axes, respectively, whereas the frequency of [GC] heterozygous PNPLA3 patients is plotted on the vertical axis on the left. Genotype frequencies in cases and controls plot on the diagram's parabola, i.e. they are in HWE (both $P > 0.05$). The distinctive intersections of the blue and black vertical dotted lines with the bottom perpendicular illustrate that the risk allele frequency is significantly ($P < 0.05$) higher in cases as compared to controls ($P < 0.05$).
Results

Figure 6 shows that median liver stiffness differed significantly (P = 0.017 by non-parametric ANOVA) among the three groups. Whereas carriers of the genotype [CC] presented with median liver stiffness of 6.3 kPa (range 2.3 - 75.0), [GC] and [GG] carriers displayed 6.8 kPa (range 2.2 - 75.0) and 7.5 kPa (range 2.2 - 75.0), respectively. Hence, carriers of the [G] allele were predisposed to develop more advanced liver fibrosis.

Figure 6. Box-and-Whisker plots illustrating liver fibrosis as phenotyped by transient elastography (liver stiffness) in carriers of distinct adiponutrin genotypes (PNPLA3 p.I148M). Due to the skewed distribution of stiffness values ranging up to 75.0 kPa (see Figure 7), all values were log transformed. Liver stiffness values differ significantly (P = 0.017, non-parametric ANOVA) between carriers of distinct adiponutrin genotypes. In particular, patients carrying [GC] or [GG] genotypes, previously reported to represent risk factors for fatty liver (Romeo S 2008) and alcoholic liver disease (Tian C 2010, Sticke F 2011), display significantly (*, P = 0.039) more advanced liver fibrosis than patients with genotype [CC].
Figure 7 illustrates the distribution of liver stiffness in our cohort, stratified for carriers of the adiponutrin risk allele [G] (red bars) and non-carriers of this allele (green bars), and demonstrates that risk genotypes were more prevalent among individuals with increased liver stiffness.

Figure 7. Histogram illustrating the distribution of liver stiffness values stratified for adiponutrin variants. Red bars represent patients with at-risk adiponutrin genotypes [GC and GG] and individuals with the low risk genotype [CC] are presented with green bars. For each liver stiffness value (rounded to the closest integer), the absolute numbers of patients (N) are shown. Carriers of the risk allele prevail among individuals with higher liver stiffness values, whereas the low risk genotype is more prevalent in patients with mild fibrosis.
Linear regression analysis demonstrated that the adiponutrin variant (P < 0.01), age (P < 0.001) and alcohol consumption (P < 0.01) significantly increased liver stiffness in our cohort. No association was found between liver fibrosis and other potential profibrogenic factors (see Methods). Subsequent multivariate analysis identified age (P < 0.001), alcohol consumption (P < 0.01) and the adiponutrin variant (P < 0.01) as independent risk factors for enhanced liver fibrosis. However restricting the analysis to the subgroup of 229 patients who underwent liver biopsy, we did not detect a statistically significant association between variant adiponutrin and histological fibrosis stages (P > 0.05).

4.3 Carriers of variant adiponutrin are at high risk of developing liver cirrhosis

To further ascertain our results, we compared allele and genotypes frequencies between patients with and without liver cirrhosis using contingency tables. Table 4 and Figure 5 show that there is a significant association between the adiponutrin variant and liver stiffness values that indicate the presence of cirrhosis (i.e., ≥ 13.0 kPa) (Friedrich-Rust M 2008). Indeed, both homo- and heterozygous carriers of the risk allele [G] showed an increased risk of developing liver stiffness above the threshold for cirrhosis (OR = 1.57, 95% CI = 1.14 - 2.15, P = 0.005). Accordingly, Table 4 shows that carriers of the p.I148M risk genotypes [GC] and [GG] were significantly (P = 0.002) overrepresented among cases (55%) with respect to controls (43%).
Table 4
Distribution of alleles and genotypes for PNPLA3 p.I148M and association tests

<table>
<thead>
<tr>
<th>PNPLA3 p.I148M allele / genotype</th>
<th>Counts of alleles / genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (2N = 1396)</td>
</tr>
<tr>
<td>C</td>
<td>1049 (0.75)</td>
</tr>
<tr>
<td>G</td>
<td>347 (0.25)</td>
</tr>
<tr>
<td>CC</td>
<td>394 (0.57)</td>
</tr>
<tr>
<td>GC</td>
<td>261 (0.37)</td>
</tr>
<tr>
<td>GG</td>
<td>43 (0.06)</td>
</tr>
</tbody>
</table>

Association tests

<table>
<thead>
<tr>
<th></th>
<th>( \chi^2 )</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele frequency difference test</td>
<td>8.96</td>
<td>0.002</td>
</tr>
<tr>
<td>Armitrage’s trend test</td>
<td>8.98</td>
<td>0.002</td>
</tr>
</tbody>
</table>

OR statistics

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>[G] ↔ [C]</td>
<td>1.445</td>
<td>1.135 - 1.840</td>
</tr>
<tr>
<td>[GG] ↔ [GC]</td>
<td>1.493</td>
<td>1.073 - 2.078</td>
</tr>
<tr>
<td>[GG] ↔ [GC + CC]</td>
<td>1.683</td>
<td>0.966 – 2.933</td>
</tr>
<tr>
<td>[GC + GG] ↔ [CC]</td>
<td>1.567</td>
<td>1.143 - 2.148</td>
</tr>
</tbody>
</table>

Patients with cirrhosis as defined by liver stiffness values ≥ 13.0 kPa were regarded as cases. [G] represents the fibrosis risk allele.

Abbreviations: CI, confidence interval; I, isoleucine; M, methionine; OR, odds ratio; p, protein (amino acid number); PNPLA3, adiponutrin.
Table 5A summarizes the results of univariate logistic regression analysis, demonstrating that the p.I148M variant, age and alcohol consumption, but neither BMI nor gender significantly affect the development of liver cirrhosis. Irrespective of the known associations between HCV genotypes 2 and 3 and non-alcoholic fatty liver disease (Cheung O 2008), fibrosis levels in the subgroup of patients with chronic HCV infection were not affected by HCV genotype. The inclusion of the adiponutrin variant, age and alcohol consumption in multivariate regression analysis (Table 5B) confirmed independent effects for the adiponutrin variant (OR = 1.86, P = 0.001) age (OR = 1.03, P < 0.001) and alcohol (OR = 1.001, P = 0.001) on liver cirrhosis.

The phenotypic characterization of liver fibrosis by elastography allowed us to perform a sensitivity analysis, as depicted in Figure 8. For this analysis, we calculated the OR of developing advanced fibrosis in homo- and heterozygous carriers of the [G] allele in comparison to [CC] homozygotes. At first we divided the study cohort into two subgroups, using different elastography cut-offs from 3.0 to 75.0 kPa. Individuals with liver stiffness values below the given cut-off were assigned as "controls", whereas carriers of the higher values were regarded as cases. Figure 8 illustrates that the [G] allele increases the risk of liver fibrosis for cut-offs between 12.0 and 40.0 kPa (all P < 0.05), resulting in OR between 1.5 and 2.2. On the other hand for liver stiffness cut-offs < 12.0 kPa and > 40.0 kPa, no significant risk could be detected.
### Table 5
Risk factors for developing liver cirrhosis

#### (A) Univariate analysis

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPLA3 p.I148M</td>
<td>1.567</td>
<td>143 - 2.148</td>
<td>0.005</td>
</tr>
<tr>
<td>Age</td>
<td>1.033</td>
<td>1.020 - 1.046</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.001</td>
<td>1.000 - 1.002</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI</td>
<td>0.996</td>
<td>0.963 - 1.064</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Gender</td>
<td>0.781</td>
<td>0.570 - 1.097</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>HCV genotype</td>
<td>0.982</td>
<td>0.797 - 1.210</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

#### (B) Multivariate analysis

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPLA3 p.I148M</td>
<td>1.860</td>
<td>1.295 - 2.673</td>
<td>0.001</td>
</tr>
<tr>
<td>Age</td>
<td>1.028</td>
<td>1.013 - 1.043</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.001</td>
<td>1.000 - 1.002</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; CI, confidence interval; HCV, hepatitis C virus; I, isoleucine; M, methionine; OR, odds ratio; p, protein (amino acid number); PNPLA3, adiponutrin.
A subgroup analysis of patients with viral hepatitis (chronic HBV and/or HCV infection) indicated that only homozygous [GG] carriers were at increased risk for developing advanced fibrosis \((P < 0.05)\) and liver stiffness values \(\geq 13.0\) kPa, indicating the presence of cirrhosis \((\text{OR} = 2.09, 95\% \text{ CI} 1.01 - 4.324, P < 0.05)\). On the other hand, in patients suffering from non-viral hepatitis, both [GG] homozygotes and [GC] heterozygotes showed more advanced liver fibrosis than patients with genotype [CC] \((P < 0.05)\) and were at risk of cirrhosis \((\text{OR} = 1.99, 95\% \text{ CI} 1.18 - 3.39, P < 0.05)\).

**Figure 8.** Odds ratios (OR) for developing advanced liver fibrosis among carriers of the [G] allele plotted against liver stiffness cut-off values between 3.0 and 75.0 kPa. According to a recent recommendation (Hosseinpoor AR 2010), OR is given on a log scale. For stiffness cut-offs between 12.0 and 40.0 kPa, the [G] allele of the adiponutrin variant p.I148M represents a significant risk factor for fibrosis.
4.4 Variant adiponutrin contributes to 16% of total cirrhosis risk

From the results of the association study (Table 4), we calculated the population attributable fraction (PAF), i.e. the proportion of the cirrhosis incidence in our population that is due to the adiponutrin risk allele. In other words, PAF corresponds to the fraction of cirrhosis cases that would be eliminated if exposure to the variant was absent. Based on OR = 1.56 (Table 4), the PAF for homo- and heterozygous carriers of the risk allele is 0.160, i.e. the p.I184M variant contributes 16.0% to the total cirrhosis risk.
5. Discussion

So far only a limited number of genes influencing liver fibrogenesis and no common hepatic fibrosis risk gene have been identified. Herein, we analyze the adiponutrin polymorphism p.I148M, previously identified as genetic risk factor for severe forms of NAFLD and ALD. Our results indicate that variant adiponutrin confers an increased risk for developing advanced liver fibrosis and cirrhosis in patients with chronic liver diseases.

Functional adiponutrin studies remain inconclusive. For example He et al. (He S 2010) demonstrated that the amino acid substitution at position 148 of the adiponutrin molecule affects its catalytic activity and that expression of the variant enzyme increases the cellular triglyceride content in cultured hepatocytes and mouse livers. Hence, carriers of the risk allele may display a genetic predisposition to lipotoxicity and hepatic inflammation, nourishing chronic liver injury (Sookoian S 2009). On the other hand, latest studies from two other groups (Chen W 2010, Basantani MK 2011) argue against an association between general PNPLA3 deficiency and NAFLD. Indeed, deletion of Pnpla3 in mice did neither affect triglyceride hydrolysis or glucose levels nor energy homeostasis and did not increase liver steatosis or inflammation (Basantani MK 2011). Likewise, no metabolic effects were found in Pnpla3 knockout mice fed a methacholine-deficient diet or additionally challenged with leptin deficiency (Chen W 2010). On the other hand, computer modelling implies that the substitution of isoleucine by methionin at amino acid position 148 alters substrate access to the catalytic serine residue and results in the loss of triglyceride hydrolysis activity (He S 2010). Such discrepancies
between genetic analyses in large cohorts, computer modelling and results in mouse models might be attributed to species-specific functions of adiponutrin. Therefore, further studies are needed before results from adiponutrin loss-of-function murine models can be directly extrapolated to humans carrying the PNPLA3 risk variant.

Here, we entertain that patients with chronic liver diseases who possess the risk variant of adiponutrin are more likely to develop progressive fibrosis and cirrhosis. Of note, our cohort encompasses patients suffering from a variety of common chronic liver diseases (Table 1), and subgroup analyses of patients with chronic viral hepatitis and non-viral liver diseases provided uniform association results. In contrast to the cohort published by Tian et al. (Tian C 2010), which encompassed patients with very high ethanol intake, we excluded patients with significant alcohol consumption (see Methods). Since PNPLA3 has been showed to affect liver fat contents in NAFLD and ALD (Kantartzis K 2009, Kotronen A 2009a, Sookoian S 2009, Romeo S 2010, Wagenknecht LE 2011), variant adiponutrin could promote liver injury and accelerate fibrosis only in the setting of additional cofactors, in particular alcohol (Poynard T 1997, Bellentani S 1999). Therefore, we performed uni- and multivariate analyses (see Results and Table 5), which indicated that both the gene variant and alcohol represent independent profibrogenic risk factors. Our findings support the hypothesis that the PNPLA3 variant p.I148M represents a general susceptibility factor for hepatic fibrosis in patients with chronic liver diseases.

Noteworthily, this is the first genetic study investigating liver fibrosis in which a large cohort of patients was phenotyped non-invasively by transient elastography. As pointed out by the International Fibrosis Group (McHutchison J 2006), liver biopsy represents a substantial barrier for clinical trials in fibrosis, and this group recommended
Discussion

that accurate, standardized, non-invasive tests would be helpful to optimize the design of antifibrotic studies. In our study, the correlation between liver stiffness and histological fibrosis stages was in line with the latest results by Wong et al. (Wong VW 2010), who demonstrated that neither inflammation grade nor NAFLD activity score affect the overall accuracy of transient elastography appreciably. Furthermore, the correlation even exceeded the correlation between fibrosis stages and collagen contents as assessed by morphometric image analysis (Goodman ZD 2009). The lack of association between adiponutrin and histological fibrosis stages in the subgroup of our patients who underwent liver biopsy might be related to several factors: Firstly, the risk allele [G] has a frequency of ~ 20% in the European population but causes only moderate fibrosis risk, as is to be expected for a complex multifactorial trait (Zondervan KT 2004). In our cohort, the p.I148M variant allele confers an OR of ~ 1.6 for developing liver cirrhosis (Table 5), which is close to the risk of 1.8 reported by Tian et al. (Tian C 2010) for patients with ALD. Accordingly, the inclusion of variant adiponutrin in a novel equation for predicting NAFLD and NASH in the general population does not substantially improve the area-under-the-curve (AUC) statistics in comparison to environmental factors (Kotronen A 2009b). Although fibrosis stages in patients who underwent liver biopsy (Table 3) were shifted towards cirrhosis as compared to the total study cohort (prevalence 51.1% versus 22.3%, Table 1), our group of 229 patients who underwent liver biopsy has limited statistical power, which strongly depends on allele frequency and OR, demonstrating further the usefulness of large non-invasively phenotyped cohorts for adequately powered association studies in liver fibrosis (Bataller R 2003).

Secondly, the lack of association might be due to the technical limitations of transient elastography and liver biopsy. In our study, the median length of the biopsies
(Table 3) was adequate according to the German consensus guidelines (Schirmacher P 2004), but not all biopsies were > 15 - 25 mm (Bedossa P 2003). Although elastography appears to be a highly accurate method for the diagnosis of cirrhosis, liver abnormalities independent of fibrosis can influence stiffness (Castéra L 2010b), and obesity as well as other features of the metabolic syndrome contribute to elastography failure and unreliable results (Castéra L 2010a). Recently Castéra and Pinzani (Castéra L 2010c) pointed to the limitation of elastography for intermediate stages of liver fibrosis, a finding already reported in chronic viral hepatitis (Afdhal NH 2009).

In this setting, the sensitivity analysis (Figure 8) adds further value. Indeed, histological staging of liver fibrosis provides a semi-qualitative scale (F0 - F4), whereas elastography measurements represent continuous quantitative data with values ranging from 2.2 to 75.0 kPa (Table 1). Interestingly, this analysis revealed a significant association between the risk allele [G] and increased hepatic fibrosis for a wide range of liver stiffness cut-off values and a trend for all stiffness values above the normal range. The lack of association at values < 12.0 and > 40.0 kPa may underline the decreased accuracy of elastography for these fibrosis stages (Castéra L 2010c), but could also reflect the presence of additional determinants that affect initiation or decompensation of hepatic fibrosis. Additionally, the risk of developing liver cirrhosis in patients with chronic viral hepatitis was increased only for homozygous carriers of the risk allele, indicating that in this subgroup of patients the marked profibrogenic effect of the viral infection per se might override the dose effect of adiponutrin genotype depicted in Figure 6.

Although several diverse triggers can lead to hepatic fibrogenesis, this process always shares the same features: In case of chronic injury, progression from compensated to decompensated disease is inevitable (Bataller R, JCI 2005).
Progressive deterioration of hepatic function either leads to liver transplantation or patient’s death, in some cases hepatic malignancy develops. Interestingly, individuals with comparable risk profiles do not necessarily display the same risk of developing liver cirrhosis. Indeed, liver fibrogenesis is a complex (i.e. multifactorial) process governed by multiple genetic factors and their interactions with numerous environmental triggers. These genetic factors, which to date remain mostly unknown, have for long been suspected as critical determinants of chronic liver disease. With respect to the increasing mortality rates due to liver cirrhosis, prompt identification of risk variants is crucial for the development of novel antifibrotic drugs, planning of tailored preventive strategies as well as early identification of at-risk individuals. Our results show that the PNPLA3 p.I148M common polymorphism represents a serious threat for patients with chronic liver diseases. It can be anticipated that dedicated studies investigating regulators of adiponutrin expression could unravel further polymorphisms affecting liver fibrogenesis. This might lead to personalized antifibrotic strategies targeting adiponutrin-mediated pathways in genetically defined at-risk patients.

In summary, this study shows that variant adiponutrin is associated with progressive fibrosis and cirrhosis in patients with chronic liver diseases. Hence, we might consider genetic screening of individuals with chronic liver disease for the risk PNPLA3 variant p.148M. For these patients, careful and timely follow-up should be performed to detect rapid progression of liver fibrosis. Based on our results, this could be accomplished by evaluation of liver fibrosis with non-invasive elastography. As stated in our recent review (Krawczyk M 2010b), such results might soon be applied in clinical practice. As the costs of genotyping are decreasing dramatically, and currently do not exceed 0.00001 Euro per polymorphism per patient, even whole-genome sequencing
becomes available for patients. Hence, we envision prospective studies to validate the combination of genotyping risk variants and elastography for individualized prediction of cirrhosis risk.
6. ACKNOWLEDGEMENTS

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8. Curriculum Vitae

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2. **A genetic risk factor for developing fatty liver disease enhances liver fibrosis in a Fibroscan phenotyped cohort.** **European Society for Clinical Investigation, Bari, Italy, 2010.**

3. **Search for human gallstone genes.** **European Society for Clinical Investigation, Bari, Italy, 2010.**

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