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Genetic variations in bile acid homeostasis are not overrepresented in alcoholic cirrhosis compared to patients with heavy alcohol abuse and absent liver disease

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Increased serum bile salt levels have been associated to a single-nucleotide polymorphism in the bile salt export pump (BSEP; *ABCB11*) in several acquired cholestatic liver diseases but there is little evidence in alcoholic liver disease (ALD). Furthermore, a crosstalk between vitamin D and bile acid synthesis has recently been discovered. Whether this crosstalk has an influence on the course of ALD is unclear to date. Our aim was to analyse the role of genetic polymorphisms in BSEP and the vitamin D receptor gene (*NR1I1*) on the emergence of cirrhosis in patients with ALD. Therefore, 511 alcoholic patients (131 with cirrhosis and 380 without cirrhosis) underwent *ABCB11* genotyping (rs2287622). Of these, 321 (131 with cirrhosis and 190 without cirrhosis) were also tested for *NR1I1* polymorphisms (bat-haplotype: *BsmI* rs1544410, *ApaI* rs7975232 and *TaqI* rs731236). Frequencies of *ABCB11* and *NR1I1* genotypes and haplotypes were compared between alcoholic patients with and without cirrhosis and correlated to serum bile salt, bilirubin and aspartate aminotransferase levels in those with cirrhosis. Frequencies of *ABCB11* and *NR1I1* genotypes and haplotypes did not differ between the two subgroups and no significant association between genotypes/haplotypes and liver function tests could be determined for neither polymorphism. We conclude that *ABCB11* and *NR1I1* polymorphisms are obviously not associated with development of cirrhosis in patients with ALD.

Introduction

Alcohol has been identified as the third leading global risk factor for disease and disability (1) and represents a major cause of liver cirrhosis (2). The fact that only up to 20% of alcoholics develops cirrhosis (3) suggests that other factors such as age (4), sex (4,5), genetics (6,7) and environmental influences (6) affect the course of alcoholic liver disease (ALD) (8).

The mechanisms underlying alcohol-induced liver damage are still largely unknown (9). However, the pathogenesis involves multiple cell types and noxious factors such as oxidative stress and proinflammatory cytokines, especially tumour necrosis factor-alpha, which are directly involved in the pathogenesis of alcoholic hepatitis (6,10,11) and at the same time implicated in the downregulation of hepatobiliary transport systems in both inflammatory and obstructive cholestasis (12). Besides changes in transporter gene expression, ethanol also functionally impairs bile salt secretion by hepatocellular alterations in membrane fluidity and microtubule formation (13–15). As expected from these experimental studies in rodents, a single small heterogenous human study in 11 human subjects with inflammatory cholestasis (7/11 with alcoholic hepatitis) demonstrated the downregulation of major transport systems in liver biopsy tissue as an acquired component of cholestasis (16). Other studies underlining the importance of hepatobiliary transport systems in alcoholic hepatitis have shown that the severity of cholestatic alcoholic hepatitis is associated with bile salt retention (17), which was suggested as an even more relevant prognostic indicator of outcome than jaundice (18). Functionally, bile salt accumulation contributes to ethanol-induced hepatotoxicity since hydrophobic bile salts aggravate steatosis, lipid peroxidation and cytolysis in rats fed an ethanol-containing liquid diet (19). Endogenous bile salts could therefore, in part, mediate ethanol-induced hepatotoxicity. It has even been proposed that elevation of total serum bile salt concentrations may actually correlate better with histological progression of ALD than do standard biochemistries (13).

A possible interplay of bile acid homeostasis and vitamin D has been suggested recently. At least in mice, vitamin D has been involved in the activation of the intestinal hormone fibroblast growth factor 19 (FGF19) and subsequent receptor-mediated downregulation of hepatic cytochrome P450 7A1 (CYP7A1), the rate-limiting enzyme in bile acid synthesis (20). Decreased 25 OH-vitamin D levels are a common phenomenon in patients with alcoholism and have been described in several studies even in the absence of advanced liver disease (21). Furthermore, chronic ethanol consumption leads to the induction of the renal 1,25 $(OH)_2$-vitamin D3-24-hydroxylase (CYP24A1) which is responsible for the inactivation of the active form of vitamin D3 (22). Considering the well-described anti-inflammatory, antiproliferative and immunomodulatory activities of vitamin D (23), hypovitaminosis D may play a relevant role in the progression of ALD. Therefore, the recently described crosstalk between vitamin D and bile acid synthesis renders the vitamin D receptor (VDR) another attractive target for genetic analysis in ALD.

Genetic case–control studies analysing single-nucleotide polymorphisms (SNP) of genes related to oxidative stress detected either no association with ALD or produced

conflicting results (6,7). Genetic variants in genes implicated in vitamin D homeostasis and bile trafficking have not been studied as yet, although the evidence detailed above renders this hypothesis attractive. Thus, we set out to characterise the role of frequent genetic polymorphisms in the bile salt export pump (BSEP; *ABCB11*), the major driving force for bile flow, and the VDR (*NR1I1*) haplotype on the emergence of cirrhosis in patients with chronic ALD.

Materials and methods

Patients and study protocol

One hundred thirty-one Caucasian patients of central European descent with alcoholic liver cirrhosis from a previously reported cohort (24–26) were recruited and analysed (cirrhosis group). For those previous studies (24–26), consecutive alcoholic patients of whom sufficient clinical data were available and who reflected past and/or present heavy alcohol consumption as defined by at least 60 g/day for women and 80 g/day for men for >10 years were recruited between 2000 and 2009 in the participating centers. For confirming eligibility of patients, present alcohol consumption was quantified through interrogation during a face-to-face interview. All patients received a diagnosis of alcohol dependence (per Diagnostic and Statistical Manual of Mental Disorders-IV criteria) by the consensus of two clinical psychiatrists. All patients underwent careful clinical examination, standard laboratory testing and abdominal ultrasound. Alcoholic liver cirrhosis was detected through a liver biopsy or evidenced by unequivocal clinical and/or laboratory results including (i) standard blood tests (coagulation tests, serum albumin concentration and platelet count), (ii) cirrhosis-related complications, including encephalopathy or ascites, (iii) abdominal ultrasound and/or computed tomography and (iv) detection of esophageal varices via upper gastrointestinal endoscopy as described (24). Other causes of chronic liver disease were excluded in all patients. Testing for hepatitis B surface antigen, anti-hepatitis B core antibody and third-generation hepatitis C antibody enzyme-linked immunosorbent assay was negative. To rule out hereditary hemochromatosis, serum levels of ferritin and transferrin saturation were determined, and neither clinical nor serological signs of autoimmune liver disease were detectable (24).

Alcoholics with normal appearance of the liver on ultrasound and normal liver enzyme levels were recruited as controls for the mentioned previous studies. Of that cohort, 380 patients with sufficient available DNA were used as a control for our study.

The study was approved by all the relevant local ethics committees, and all the patients provided written informed consent prior to inclusion.

DNA isolation, storage and genotyping of ABCB11 and *NR1I1* polymorphisms

DNA was isolated from whole blood/peripheral blood mononuclear cells using the QIAamp DNA Minikit (Qiagen, Hilden, Germany) and stored at −80°C. Genotyping of the SNP *ABCB11* c.1331T > G (p.V444A, rs2287622) as well as of *NR1I1* polymorphisms forming the bat-haplotype (rs1544410, rs7975232 and rs731236) (27) was performed using standard TaqMan technology. Fluorogenic 5′-nuclease (TaqMan) assays on a ABIPRISM 7700 sequence detection system was used for SNP determination. Probe solution (0.25 µl) and 2.5 µl 2× Universal PCR Master-Mix (Applied Biosystems) were brought to 5 µl with 10 ng genomic DNA. All genotyping data were analysed with the SDS 2.3 software.

Haplotype analysis

For haplotype reconstruction, the freely available Haploview software (Broad Institute of MIT and Harvard, Massachusetts, USA; http://www.broadinstitute.org/mpg/haploview) was used.

Quantification of serum bile salt levels

Serum bile salts were quantified using a commercially available enzymatic assay (Bile Acids Procedure 450, Trinity Biotech, Bray, Ireland) with a linear range of serum bile salt concentrations between 0 and 200 µmol/l (28). Blood was routinely drawn in a fasting state in the morning between 8 and 10 A.M. to avoid falsely high bile salt levels.

Serum liver function tests

Quantification of bilirubin and aspartate aminotransferase (AST) levels has been performed using standard autoanalyser techniques.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 (The Predictive Analytics Company, Chicago, IL, USA). To evaluate the significance of the differences in allelic and genotypic frequencies between alcoholic patients with and without cirrhosis, the χ^2 test was used. The association of serum markers (bile salts, bilirubin and AST) to genotypes and respective haplotypes was investigated using the Spearman Rho correlation. Effects of age and gender on bile salt level and emergence of cirrhosis were analysed using univariate and multiple logistic regression. Odds ratios with 95% confidence intervals were computed using logistic regression (Supplement 1, available at *Mutagenesis* Online). The needed number of patients within the two different groups of alcoholic patients was estimated under the assumption of similar allelic frequencies as in a previous Caucasian cohort study in hepatitis C infected patients where the minor allele frequency for BSEP (minor allele: c.1331C) was 73.2% in cirrhotics and 60.3% in controls (29). The frequency of *NR1I1* bAt[CCA]-haplotype in cirrhotic hepatitis C infected patients was 63.5 and 48.6% in controls (30). Given these figures and our number of patients and controls, we expected a power of 0.73 for BSEP ($\alpha = 0.05$) and 0.94 for CCA haplotype ($\alpha = 0.05$) when designing this study.

Results

Patient characteristics

A total of 511 patients addicted to alcohol, with and without cirrhosis, were included in this genetic cohort study for *ABCB11* genotyping. Additionally, *NR1I1* genotyping was performed for the 321 patients of the same cohorts with sufficient remaining DNA. Cohort characteristics are shown in Table I. Frequencies of the determined genotypes and haplotypes in cirrhotic patients versus those without cirrhosis were analysed. Furthermore, genotypes and haplotypes were correlated to liver function tests in the cirrhosis group, where individual data were available. By definition, all patients in the control group had normal liver function tests.

Genotype and allelic distribution of the ABCB11 c.1331T > G polymorphism according to fibrosis stage in alcoholic patients

We determined the genotype and allelic frequency of the *ABCB11* c.1331T > G SNP in the cohort of alcoholics with cirrhosis ($n = 131$) in comparison to the complete control cohort of patients without cirrhosis ($n = 380$) (Table II). The *ABCB11* genotype distribution was similar to other studies in Caucasian European patients with intrahepatic cholestasis of pregnancy (31) and chronic hepatitis C infection (29). No difference between the two cohorts could be detected for either genotype (data not shown) or allelic frequency (Figure 1).

Influence of the ABCB11 c.1331T > G polymorphism on liver function tests and serum bile salt levels in alcoholic cirrhosis patients

The *ABCB11* c.1331T > G polymorphism was also correlated with bilirubin and AST levels—markers for cholestasis and liver cell damage—as well as with bile salt levels in the subgroup of alcoholic cirrhosis patients (bilirubin $n = 105$, AST $n = 105$ and bile salts $n = 32$). Carriers of the C allele showed a slight tendency towards higher bile salt levels compared to carriers of the T allele, although without statistical significance (median bile salt level for the C allele 58 versus 42 µmol/l for the T allele; $P = 0.413$) (Figure 2). A similar trend was observed for the CC genotype compared to *ABCB11* c.1331TC or TT (data not shown). For both *ABCB11* c.1331 alleles and respective genotypes, no relevant difference could be observed for either serum bilirubin or AST levels (data not shown). In line with this finding, no significant correlation of *ABCB11* genotype to histological inflammatory activity was detectable in this study.

Table I. Cohort characteristics

	Cohort with cirrhosis for *ABCB11* and *NR1I1* genotyping (n = 131)
Gender (male/female) (n = 122)	102/20
Median age (range) (n = 119)	57 (26–77)
Alcohol intake (g/d) mean value (n = 27)	159 ± 102
AST (U/l) mean value (n = 105)	104 ± 312
Bilirubin (mg/dl) mean value (n = 105)	30 ± 41
Bile salts (μmol/l) mean value (n = 32)	68 ± 60
	Cohort without cirrhosis for *ABCB11* genotyping (n = 380)
Gender (male/female) (n = 323)	249/74
Median age (range) (n = 323)	44 (26–74)
Alcohol intake (g/d) mean value (n = 42)	348 ± 235
	Cohort without cirrhosis for *NR1I1* genotyping (n = 190)
Gender (male/female) (n = 152)	127/25
Median age (range) (n = 152)	41 (27–74)
Alcohol intake (g/d) mean value (n = 37)	362 ± 234

Table II. Allelic and genotypic frequencies of *ABCB11* SNP in alcoholic patients with and without cirrhosis

Genotype SNP	Alcoholic patients with cirrhosis, n (%)	Alcoholic patients without cirrhosis, n (%)
BSEP (rs2287622)	131 (100)	380 (100)
CC	52 (39.7)	139 (36.6)
TT	21 (16.0)	66 (17.4)
CT	58 (44.3)	175 (46.0)
Frequency C Allele	162 (61.8)	453 (59.6)
Frequency T Allele	100 (38.2)	307 (40.4)

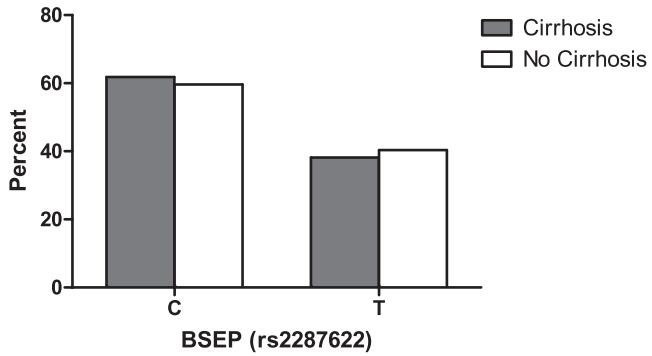

Fig. 1. Comparison of allelic frequencies of *ABCB11* c.1331T > G between alcoholic patients with and without cirrhosis. χ^2 test revealed no significant differences in the distribution of alleles between the groups of patients with and without cirrhosis (P = 0.559).

Genotype and haplotype distribution of common NR1I1 SNPs according to fibrosis stage in alcoholic patients

In analogy to the *ABCB11* study, *NR1I1* genotyping was performed for alcoholics with cirrhosis (n = 131) in order to investigate the genotype and allelic frequency in our cohort and compared to a control group of alcoholics without cirrhosis (n = 190 with sufficient DNA) (Table III). Again, the *NR1I1* genotype distribution was comparable to previously published data in Caucasian European patients with chronic hepatitis C infection (30). No significant difference in the distribution of genotypes (Table III) and haplotypes (Table IV) between cirrhosis patients and controls was found (Figure 3).

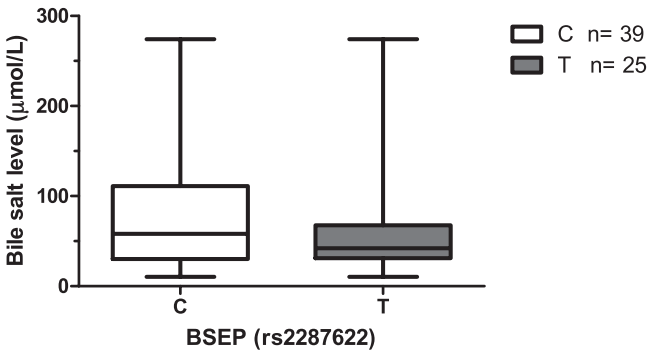

Fig. 2. Correlation of *ABCB11* c.1331T > G with serum bile salt levels. In alcoholic patients with cirrhosis, bile salt levels were correlated to *ABCB11* c.1331T and C alleles using Spearman Rho correlation. The C allele showed a trend towards higher bile salt levels compared to the T allele (median bile salt level for the C allele 58 versus 42 μmol/l for the T allele; P = 0.413).

Influence of NR1I1 genotype and haplotype on liver function tests and serum bile salt levels in alcoholic cirrhosis patients

Subsequently, *NR1I1* genotypes and haplotypes were correlated with bilirubin, AST and bile salt levels in the subgroup of alcoholic cirrhosis patients (bilirubin n = 105, AST n = 105 and bile salts n = 32). For the bAt[CCA]-haplotype, a slight trend towards higher bile salt levels compared to all other *NR1I1* haplotypes could be observed (median bile salt level for the bAt[CCA]-haplotype 58 versus 38 μmol/l for all other *NR1I1* haplotypes; P = 0.216) (Figure 4A). However, no such trend could be detected for the individual *NR1I1* SNPs (Figure 4B–D). Similarly, no correlation between *NR1I1* genotypes/haplotype and serum bilirubin or AST levels was observed (data not shown). In support, no significant correlation of the bAt[CCA]-haplotype to histological inflammatory activity was detectable.

Discussion

The present study investigated the genetic influence of the *ABCB11* c.1331T > G polymorphism and the common *NR1I1* bAt[CCA]-haplotype on the presence of liver cirrhosis in alcoholic patients. There are minor differences in the baseline characteristics between the two cohorts. As expected from the literature, median age in the cirrhosis group is ~10 years higher than in the control cohort since development of cirrhosis typically

Table III. Allelic and genotypic frequencies of *NR1I1* SNPs in alcoholic patients with and without cirrhosis

Genotype SNP	Alcoholic patients with cirrhosis, *n* (%)	Alcoholic patients without cirrhosis, *n* (%)
Bsm I (rs1544410)	124 (100)	187 (100)
CC	47 (37.9)	65 (34.8)
TT	21 (16.9)	34 (18.2)
CT	56 (45.2)	88 (47.0)
Frequency C Allele	150 (60.5)	218 (58.3)
Frequency T Allele	98 (39.5)	156 (41.7)
Apa I (rs7975232)	131 (100)	189 (100)
AA	38 (29.0)	52 (27.5)
CC	33 (25.2)	51 (27.0)
CA	60 (45.8)	86 (45.5)
Frequency A Allele	136 (51.9)	190 (50.3)
Frequency C Allele	126 (48.1)	188 (49.7)
Taq I (rs731236)	130 (100)	189 (100)
AA	47 (36.2)	65 (34.4)
GG	22 (16.9)	31 (16.4)
AG	61 (46.9)	93 (49.2)
Frequency A Allele	155 (59.6)	223 (59.0)
Frequency G Allele	105 (40.4)	155 (41.0)

Table IV. *NR1I1*-haplotype frequencies in alcoholic patients with and without cirrhosis

NR1I1-haplotype (Bsm_Apa_Taq)	Haplotype distribution in the cohort with cirrhosis, *n* (%)	Haplotype distribution in the cohort without cirrhosis, *n* (%)
Haplotype (Bsm_Apa_Taq)	248 (100)	372 (100)
TAG (BaT)	94 (37.9)	149 (40.0)
CCA (bAt)	121 (48.8)	183 (49.2)
CAA (bat)	27 (10.9)	33 (8.9)
CAG (baT)	2 (0.8)	1 (0.3)
TAA (Bat)	2 (0.8)	4 (1.1)
TCG (BAT)	2 (0.8)	2 (0.5)

develops with this interval during chronic liver damage. Given the absence of any clinicochemical or imaging pathologies in the non-cirrhotic controls, the likelihood of chronic liver damage and subsequent development of cirrhosis is very low. Nevertheless, the unknown long-term outcome of non-cirrhotic controls is a given limitation of this study. Differences in alcohol intake are not relevant for our findings since mean intake in the non-cirrhosis group even exceeds the cirrhosis group.

Despite a slight trend towards bile salt retention in alcoholic cirrhosis patients carrying the *ABCB11* c.1331T > G polymorphism and the common *NR1I1* bAt[CCA]-haplotype, no increased frequency could be observed in the present study compared to our control cohort without liver damage. Similarly, earlier genetic studies analysing SNP in oxidative stress-related genes did not show a clear association with ALD (6,7). The only robust genetic rsik factor for progressive ALD so far is an SNP in the gene coding for patatin-like phospholipase domain-containing protein 3 (PNPLA3; rs738409), which has been established quite recently (7,26,32).

The major driving force for bile flow represents the BSEP located in the canalicular membrane of hepatocytes (33). Several studies have analysed the role of SNPs and mutations in hepatobiliary transport systems. Low BSEP protein expression levels have been associated with the non-synonymous c.1331C allele (p.444A) of *ABCB11* (34) and elevated serum bile salt levels have been found in carriers of the 'cholestatic' c.1331CC genotype in independent studies (29,31). The particular pathogenetic role of bile salt retention in ALD clearly correlates with histological inflammatory scores and histological progression (13,17).

Although largely negative, this study contains pathogenetic information for disease progression in ALD, at least in the differentiation from other forms of chronic liver disease. As such, polymorphisms in *ABCB11* encoding for BSEP have been identified as risk factors for intrahepatic cholestasis of pregnancy (31). Further evidence exists for a role of BSEP in hereditary forms of cholestasis (33) as well as drug-induced cholestasis (35). However, this polymorphism does not exclusively affect cholestatic liver disease since the c.1331C allele (p.444A) of *ABCB11* has recently also been associated with the progression to cirrhosis in chronic viral hepatitis C (HCV) patients (29). The same trend towards increased serum bile salt levels observed for patients with intrahepatic cholestasis of pregnancy (31) and chronic viral hepatitis C (29) carrying the c.1331C allele (p.444A) of *ABCB11* in independent studies can be detected in the present cohort. The fact that no correlation of *ABCB11* c.1331C with advanced fibrosis has been observed in another large cohort of patients with non-alcoholic fatty liver disease (29) allows the conclusion that bile salt retention may not play a central role in the disease progression to cirrhosis in neither alcoholic nor non-alcoholic fatty liver disease which are histologically indiscernible. Nevertheless, at least in ALD, the pivotal role of bile salt retention in the pathogenesis of acute liver injury and its prognosis remains unchallenged.

Hypovitaminosis D is a common feature of alcoholism (21). The well-described anti-inflammatory, antiproliferative and immunomodulatory activities of vitamin D and a recently described crosstalk between vitamin D and bile acid synthesis (20) render the VDR (*NR1I1*) a second attractive target gene in ALD. In fact, genetic variations in the VDR (*NR1I1*) gene have been characterised as important modulators of multiple diseases, including hepatic disorders such as primary biliary cirrhosis and autoimmune hepatitis (36,37). Recently, our group has identified a significant association between the *NR1I1* bAt[CCA]-haplotype and fibrosis progression rate as well as development of cirrhosis in HCV patients (30). VDR (*NR1I1*) haplotypes are also significantly associated with the occurrence of hepatocellular carcinoma in patients with liver cirrhosis (38). Of note, this relationship is even more specific for patients with an alcoholic aetiology. From these data, it appeared attractive to hypothesise a pathogenetic role of VDR (*NR1I1*) haplotypes in ALD. However, the present study does not support this assumption since no increased frequency could be observed in alcoholic cirrhosis patients compared to a control cohort without liver damage.

Certainly, it would have been attractive to study the influence of *NR1I1* SNPs on vitamin D levels but unfortunately blood samples were no longer available. However, another recent study of our group in HCV-infected patients showed no correlation between VDR (*NR1I1*) polymorphism and 25-OH vitamin D levels (30).

Based on the assumption of a significant difference in previous genetic studies investigating *ABCB11* and VDR genotypes/haplotypes in HCV patients, the present study was a priori adequately powered. Given the minimal difference in minor allele frequencies for both genes even a 10-fold number of patients would be marginal to detect significant differences with then questionable relevance. In conclusion,

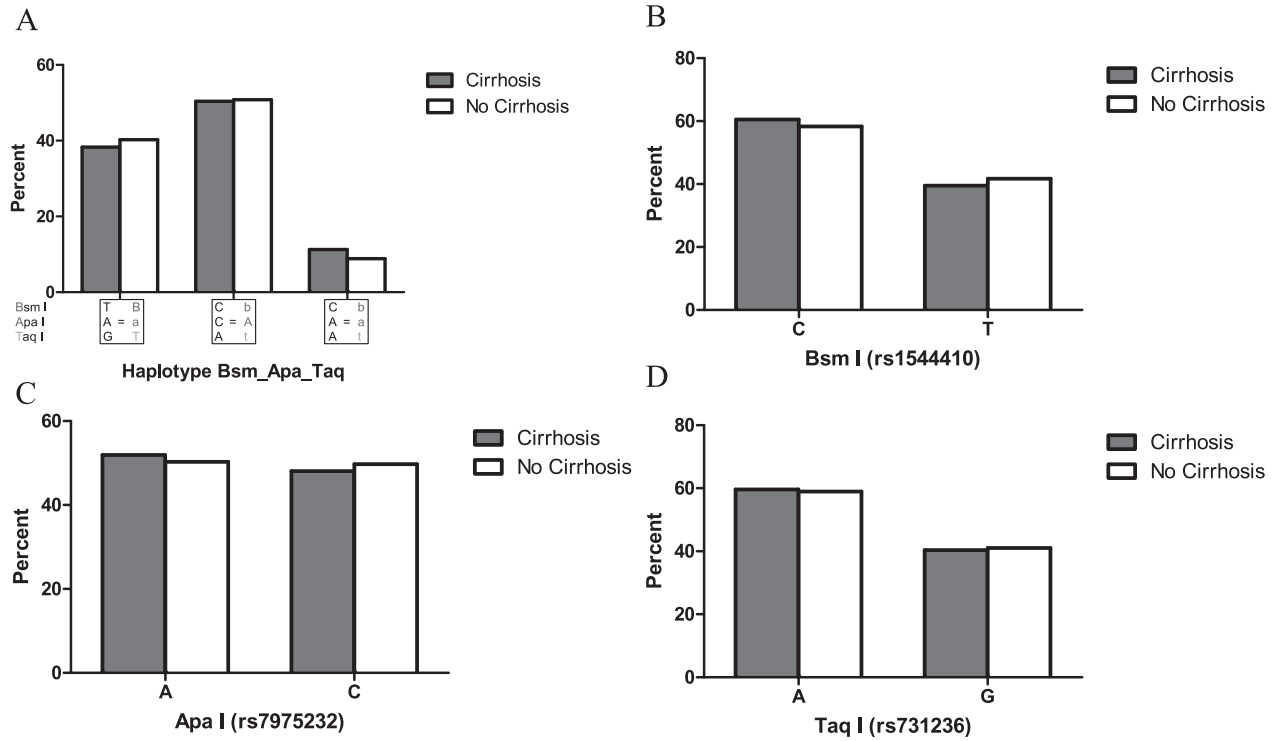

Fig. 3. Comparison of *NR1I1* allelic haplotype and individual allele frequencies between alcoholics with and without cirrhosis. (**A**) χ^2 test revealed no significant differences in the distribution of the three most frequent haplotypes between the two cohorts ($P = 0.630$). (**B–D**) Neither of the individual *NR1I1* allele frequencies significantly differed between cirrhosis patients and controls ($P = 0.618$ for BsmI, $P = 0.689$ for ApaI and $P = 0.935$ for TaqI).

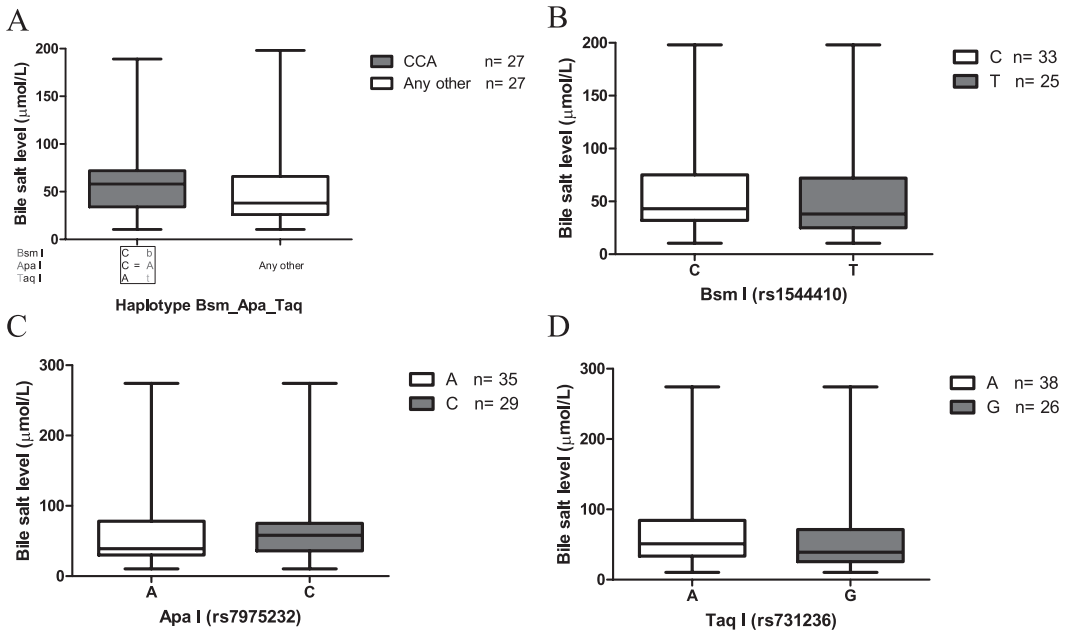

Fig. 4. Correlation of *NR1I1* allelic haplotype and individual alleles with serum bile salt levels. (**A**) In alcoholic patients with cirrhosis, bile salt levels were correlated to *NR1I1* haplotypes using Spearman Rho correlation. The bAt[CCA]-haplotype showed a slight trend towards higher bile salt levels compared to other *NR1I1* haplotypes (median bile salt level for the bAt[CCA]-haplotype 58 versus 38 µmol/l for all other *NR1I1* haplotypes; $P = 0.216$). (**B–D**) No correlation between the alleles of individual *NR1I1* SNPs and serum bile salt levels could be detected ($P = 0.223$ for BsmI, $P = 0.303$ for ApaI and $P = 0.265$ for TaqI).

VDR polymorphisms and the corresponding haplotype may not play a central role in cirrhosis development in patients with chronic alcohol consumption but this gene locus could contribute to the development of hepatocellular carcinoma as the most serious complication of end-stage ALD.

Taking into account the complexity of the pathogenesis of ALD and the fact that many genetic association studies have failed to show an association to disease progression (6,7) one could hypothesise that one SNP alone may not be enough to significantly influence the course of the disease.

Although the present study could not detect a significant genetic influence of the *ABCB11* p.V444A polymorphism and the common *NR1I1* bAt[CCA]-haplotype on the presence of liver cirrhosis in patients with chronic alcohol abuse, our findings do not preclude a relevant role of bile salts and vitamin D in acute ALD. Whereas treatment with hydrophilic bile acids such as ursodeoxycholic acid has beneficial effects to limit bile acid toxicity but does not represent a therapeutic standard, substitution of vitamin D may be discussed in patients with any stage of ALD to correct for hypovitaminosis.

Supplementary data

Supplement 1 is available at *Mutagenesis* Online.

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References

1. WHO (2011) *Global Status Report on Alcohol and Health*. World Health Organization, Geneva, Switzerland, p. 85.
2. Perz, J. F., Armstrong, G. L., Farrington, L. A., Hutin, Y. J. and Bell, B. P. (2006) The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J. Hepatol.*, **45**, 529–538.
3. Altamirano, J. and Bataller, R. (2011) Alcoholic liver disease: pathogenesis and new targets for therapy. *Nat. Rev. Gastroenterol. Hepatol.*, **8**, 491–501.
4. Raynard, B., Balian, A., Fallik, D., Capron, F., Bedossa, P., Chaput, J. C. and Naveau, S. (2002) Risk factors of fibrosis in alcohol-induced liver disease. *Hepatology*, **35**, 635–638.
5. Becker, U., Deis, A., Sørensen, T. I., Grønbaek, M., Borch-Johnsen, K., Müller, C. F., Schnohr, P. and Jensen, G. (1996) Prediction of risk of liver disease by alcohol intake, sex, and age: a prospective population study. *Hepatology*, **23**, 1025–1029.
6. Wilfred de Alwis, N. M. and Day, C. P. (2007) Genetics of alcoholic liver disease and nonalcoholic fatty liver disease. *Semin. Liver Dis.*, **27**, 44–54.
7. Stickel, F. and Hampe, J. (2012) Genetic determinants of alcoholic liver disease. *Gut*, **61**, 150–159.
8. Lucey, M. R., Mathurin, P. and Morgan, T. R. (2009) Alcoholic hepatitis. *N. Engl. J. Med.*, **360**, 2758–2769.
9. Miller, A. M., Horiguchi, N., Jeong, W. I., Radaeva, S. and Gao, B. (2011) Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines. *Alcohol Clin. Exp. Res*, **35**, 787–793.
10. Yin, M., Wheeler, M. D., Kono, H., Bradford, B. U., Gallucci, R. M., Luster, M. I. and Thurman, R. G. (1999) Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. *Gastroenterology*, **117**, 942–952.
11. McClain, C. J., Song, Z., Barve, S. S., Hill, D. B. and Deaciuc, I. (2004) Recent advances in alcoholic liver disease. IV. Dysregulated cytokine metabolism in alcoholic liver disease. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **287**, G497–G502.
12. Geier, A., Dietrich, C. G., Voigt, S., Kim, S. K., Gerloff, T., Kullak-Ublick, G. A., Lorenzen, J., Matern, S. and Gartung, C. (2003) Effects of proinflammatory cytokines on rat organic anion transporters during toxic liver injury and cholestasis. *Hepatology*, **38**, 345–354.
13. Tung, B. Y. and Carithers, R. L. (1999) Cholestasis and alcoholic liver disease. *Clin. Liver Dis.*, **3**, 585–601.
14. Mills, P. R., Meier, P. J., Smith, D. J., Ballatori, N., Boyer, J. L. and Gordon, E. R. (1987) The effect of changes in the fluid state of rat liver plasma membrane on the transport of taurocholate. *Hepatology*, **7**, 61–66.
15. Gregory, D. H., Vlahcevic, Z. R., Prugh, M. F. and Swell, L. (1978) Mechanism of secretion of biliary lipids: role of a microtubular system in hepatocellular transport of biliary lipids in the rat. *Gastroenterology*, **74**, 93–100.
16. Zollner, G., Fickert, P., Zenz, R. *et al.* (2001) Hepatobiliary transporter expression in percutaneous liver biopsies of patients with cholestatic liver diseases. *Hepatology*, **33**, 633–646.
17. Trinchet, J. C., Gerhardt, M. F., Balkau, B., Munz, C. and Poupon, R. E. (1994) Serum bile acids and cholestasis in alcoholic hepatitis. Relationship with usual liver tests and histological features. *J. Hepatol.*, **21**, 235–240.
18. Nissenbaum, M., Chedid, A., Mendenhall, C. and Gartside, P. (1990) Prognostic significance of cholestatic alcoholic hepatitis. VA Cooperative Study Group #119. *Dig. Dis. Sci.*, **35**, 891–896.
19. Montet, A. M., Oliva, L., Beaugé, F. and Montet, J. C. (2002) Bile salts modulate chronic ethanol-induced hepatotoxicity. *Alcohol Alcohol.*, **37**, 25–29.
20. Schmidt, D. R., Holmstrom, S. R., Fon Tacer, K., Bookout, A. L., Kliewer, S. A. and Mangelsdorf, D. J. (2010) Regulation of bile acid synthesis by fat-soluble vitamins A and D. *J. Biol. Chem.*, **285**, 14486–14494.
21. Malik, P., Gasser, R. W., Kemmler, G., Moncayo, R., Finkenstedt, G., Kurz, M. and Fleischhacker, W. W. (2009) Low bone mineral density and impaired bone metabolism in young alcoholic patients without liver cirrhosis: a cross-sectional study. *Alcohol Clin. Exp. Res.*, **33**, 375–381.
22. Shankar, K., Liu, X., Singhal, R., Chen, J. R., Nagarajan, S., Badger, T. M. and Ronis, M. J. (2008) Chronic ethanol consumption leads to disruption of vitamin D3 homeostasis associated with induction of renal 1,25 dihydroxyvitamin D3-24-hydroxylase (CYP24A1). *Endocrinology*, **149**, 1748–1756.
23. Nagpal, S., Na, S. and Rathnachalam, R. (2005) Noncalcemic actions of vitamin D receptor ligands. *Endocr. Rev.*, **26**, 662–687.
24. Stickel, F., Osterreicher, C. H., Halangk, J. *et al.* (2008) No role of matrixmetalloproteinase-3 genetic promoter polymorphism 1171 as a risk factor for cirrhosis in alcoholic liver disease. *Alcohol Clin. Exp. Res.*, **32**, 959–965.
25. Treutlein, J., Cichon, S., Ridinger, M. *et al.* (2009) Genome-wide association study of alcohol dependence. *Arch. Gen. Psychiatry*, **66**, 773–784.
26. Stickel, F., Buch, S., Lau, K. *et al.* (2011) Genetic variation in the PNPLA3 gene is associated with alcoholic liver injury in Caucasians. *Hepatology*, **53**, 86–95.
27. Uitterlinden, A. G., Fang, Y., Van Meurs, J. B., Pols, H. A. and Van Leeuwen, J. P. (2004) Genetics and biology of vitamin D receptor polymorphisms. *Gene*, **338**, 143–156.
28. Roda, A., Kricka, L. J., DeLuca, M. and Hofmann, A. (1982) Bioluminescence measurement of primary bile acids using immobilized 7 alpha-hydroxysteroid dehydrogenase: application to serum bile acids. *J. Lipid Res.*, **23**, 1354–1361.
29. Iwata, R. *et al.* (2011) A common polymorphism in the ABCB11 gene is associated with advanced fibrosis in hepatitis C but not in non-alcoholic fatty liver disease. *Clin. Sci. (Lond.)*, **120**, 287–296.
30. Baur, K., Baur, K., Stieger, B. *et al.* (2012) Combined effect of 25-OH vitamin D plasma levels and genetic vitamin D receptor (NR 1I1) variants on fibrosis progression rate in HCV patients. *Liver Int*, **32**, 635–643.
31. Meier, Y., Zodan, T., Lang, C., Zimmermann, R., Kullak-Ublick, G. A., Meier, P. J., Stieger, B. and Pauli-Magnus, C. (2008) Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump. *World J. Gastroenterol.*, **14**, 38–45.
32. Tian, C., Stokowski, R. P., Kershenobich, D., Ballinger, D. G. and Hinds, D. A. (2010) Variant in PNPLA3 is associated with alcoholic liver disease. *Nat. Genet.*, **42**, 21–23.
33. Stieger, B., Meier, Y. and Meier, P. J. (2007) The bile salt export pump. *Pflugers Arch.*, **453**, 611–620.
34. Meier, Y., Pauli-Magnus, C., Zanger, U. M. *et al.* (2006) Interindividual variability of canalicular ATP-binding-cassette (ABC)-transporter expression in human liver. *Hepatology*, **44**, 62–74.
35. Lang, C., Meier, Y., Stieger, B., Beuers, U., Lang, T., Kerb, R., Kullak-Ublick, G. A., Meier, P. J. and Pauli-Magnus, C. (2007) Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet. Genomics*, **17**, 47–60.
36. Valdivielso, J. M. and Fernandez, E. (2006) Vitamin D receptor polymorphisms and diseases. *Clin. Chim. Acta*, **371**, 1–12.
37. Vogel, A., Strassburg, C. P. and Manns, M. P. (2002) Genetic association of vitamin D receptor polymorphisms with primary biliary cirrhosis and autoimmune hepatitis. *Hepatology*, **35**, 126–131.
38. Falleti, E., Bitetto, D., Fabris, C. *et al.* (2010) Vitamin D receptor gene polymorphisms and hepatocellular carcinoma in alcoholic cirrhosis. *World J. Gastroenterol.*, **16**, 3016–3024.