ORIGINAL ARTICLE

Heterozygous carriage of the alpha1-antitrypsin Pi*Z variant increases the risk to develop liver cirrhosis

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ABSTRACT

Objective Homozygous alpha1-antitrypsin (AAT) deficiency increases the risk for developing cirrhosis, whereas the relevance of heterozygous carriage remains unclear. Hence, we evaluated the impact of the two most relevant AAT variants ('Pi*Z' and 'Pi*S'), present in up to 10% of Caucasians, on subjects with non-alcoholic fatty liver disease (NAFLD) or alcohol misuse.

Design We analysed multicentric case–control cohorts consisting of 1184 people with biopsy-proven NAFLD and of 2462 people with chronic alcohol misuse, both cohorts comprising cases with cirrhosis and controls without cirrhosis. Genotyping for the Pi*Z and Pi*S variants was performed.

Results The Pi*Z variant presented in 13.8% of patients with cirrhotic NAFLD but only in 2.4% of counterparts without liver fibrosis (p<0.0001). Accordingly, the Pi*Z variant increased the risk of NAFLD subjects to develop cirrhosis (adjusted OR=7.3 (95% CI 2.2 to 24.8)). Likewise, the Pi*Z variant presented in 6.2% of alcohol misusers with cirrhosis but only in 2.2% of alcohol misusers without significant liver injury (p<0.0001). Correspondingly, alcohol misusers carrying the Pi*Z variant were prone to develop cirrhosis (adjusted OR=5.8 (95% CI 2.9 to 11.7)). In contrast, the Pi*S variant was not associated with NAFLD-related cirrhosis and only borderline with alcohol-related cirrhosis (adjusted OR=1.47 (95% CI 0.99 to 2.19)).

Conclusion The Pi*Z variant is the hitherto strongest single nucleotide polymorphism-based risk factor for cirrhosis in NAFLD and alcohol misuse, whereas the Pi*S variant confers only a weak risk in alcohol misusers. As 2%–4% of Caucasians are Pi*Z carriers, this finding should be considered in genetic counselling of affected individuals.

Significance of this study

What is already known on this subject?

- Alpha1-antitrypsin (AAT) deficiency is among the most common genetic diseases.
- Homozygous carriers of the 'Pi*Z' variant of AAT are highly susceptible to develop liver cirrhosis due to 'gain-of-function' toxicity of the mutant protein in hepatocytes.
- Up to 10% of Caucasians carry an AAT, variant but the relevance of heterozygous carriage of the two most common AAT variants, 'Pi*Z' and 'Pi*S', for developing cirrhosis remains controversial.

What are the new findings?

- In two case–control cohorts with a total of 1184 individuals with non-alcoholic fatty liver disease (NAFLD) as well as in two case–control cohorts with a total of 2462 alcohol misusers, the presence of the Pi*Z variant was strongly associated with the development of cirrhosis (ORs of about 6–7).
- This is also the first study systematically investigating the role of the Pi*S variant revealing that it may represent a minor risk factor for developing cirrhosis in alcohol misusers.

INTRODUCTION

Cirrhosis is the end stage of all chronic liver diseases and is the result of a scarring process that is termed liver fibrosis. Together with its detrimental sequelae, cirrhosis constitutes a major and growing cause for

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- This study provides definitive evidence that the Pi*Z variant, but not the Pi*S variant, greatly contributes to progressive liver disease, especially in the setting of NAFLD or chronic alcohol misuse.
- As 2%–4% of Caucasians carry the Pi*Z variant, these findings should be considered in their genetic counselling.

morbidity and mortality worldwide.[1] Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are major causes of cirrhosis and liver-related mortality.[2 3] However, only a proportion of individuals with ALD or NAFLD will develop cirrhosis, and this heterogeneity is attributed to genetic risk factors.[4–6] Therefore, a better understanding of the underlying genetic risk factors is highly relevant. Both ALD and NAFLD share the same genetic risk factors. These comprise variants in the genes of patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*) and membrane-bound O-acyltransferase domain containing 7 (*MBOAT7*).[4 6]

These aforementioned genetic variants have been identified by genome-wide association studies that are well suited for analysis of common genetic variants. However, variants that cause monogenic disorders are often rare, and the genome-wide association approach fails to uncover them. A potential way to circumvent this problem is to directly address the biological role of genetic variants with a proven pathogenic potential. For this approach, mutations in alpha1-antitrypsin (AAT; *SERPINA1* gene) are attractive candidates. AAT is one of the most abundant serum proteins and is produced predominantly within hepatocytes.[7] The presence of AAT variants typically lead to decreased serum AAT concentrations giving rise to the systemic disease alpha1-antitrypsin deficiency (AATD). Severe AATD is characterised by very low AAT serum levels (10%–20% of normal) and constitutes a 'frequent rare disease' with an incidence between 1:2000 and 1:4000.[7–9] Accordingly, severe AATD is the third most common genetic disorder leading to death worldwide.[8 10] The lung and the liver are the most commonly affected organs, and their involvement accounts for the increased morbidity and mortality.[8 10]

The nomenclature of AAT variants is based on the mobility of the resulting protein in the electric field.[7 10] The most relevant variant is termed 'Pi*Z' (rs28929474), which has a prevalence of up to 8% in Northern Europe.[7 9] The somewhat less severe variant, termed 'Pi*S' (rs17580), is more common in Southern Europe, with a prevalence of up to 20%.[7] In total, approximately 120 million people worldwide carry at least one Pi*Z or Pi*S allele and up to 10% of Caucasians carry an AAT variant.[7 9] 'Pi*MM' describes the normal genotype, and 'Pi*ZZ' (ie, the homozygous Pi*Z carriage) constitutes the classic severe AATD form.[7 8] While presence of the Pi*ZZ genotype precipitates the development of liver disease,[7 11] the impact of heterozygous Pi*Z carriage (Pi*MZ genotype) was suggested by several smaller studies but remains controversial.[10 12–18] In particular, only few studies performed AAT genotyping of the entire cohort,[13 17 18] while others did phenotyping based on serum analyses or histological presence of AAT inclusions[14–16 19–25] (online supplementary table 1). Even less well studied is the impact of heterozygous or homozygous Pi*S carriage (Pi*MS or Pi*SS genotype, respectively) on liver disease.

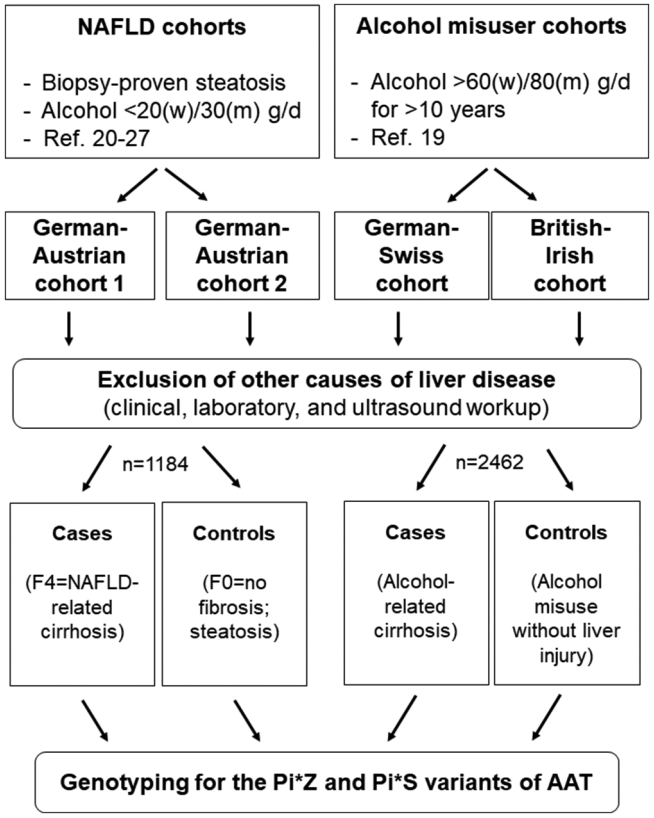

Figure 1 Overview of analysed patient cohorts. AAT, alpha1-antitrypsin; NAFLD, non-alcoholic fatty liver disease.

Led by the a priori hypothesis that the presence of heterozygous AAT variants may spur the liver disease development in susceptible individuals, we evaluated the impact of heterozygous Pi*Z or Pi*S carriage in the two most relevant settings of chronic liver disease. Hence, we analysed well-characterised case–control cohorts of patients with biopsy-proven NAFLD and chronic alcohol misuse.

METHODS

Cohorts of NAFLD subjects

A total of 1184 cases and controls of self-reported European descent were recruited from multiple sites in Germany and Austria. Of these, 643 subjects comprise the first German–Austrian cohort ('GER-AUS 1' cohort: cases=68, controls=362) and 541 subjects comprise the second German–Austrian cohort ('GER-AUS 2' cohort: cases=26, controls=256) (figure 1). In both cohorts, *cases* were defined as patients with biopsy-proven NAFLD-related cirrhosis (stage F4), whereas *controls* had histological NAFLD without evidence of liver fibrosis (stage F0).

In all subjects, presence of NAFLD was diagnosed using liver biopsy. The presence of fibrosis was assessed histologically according to Kleiner classification (stage F0: no fibrosis; stage F1: perisinusoidal fibrosis to portal/periportal fibrosis; stage F2: perisinusoidal and portal/periportal fibrosis; stage F3: bridging fibrosis; and stage F4: cirrhosis). The liver biopsies were read by experienced pathologists in a blinded fashion and prior to AAT genotyping. The presence of concomitant liver disease was excluded through a comprehensive workup: exclusion criteria were laboratory or clinical evidence of autoimmune,

viral (serology for hepatitis B and C as well as HIV) or hereditary causes (serum ceruloplasmin and ferritin indicating the presence of Wilson disease and hereditary hemochromatosis, respectively) of liver disease and clinically relevant alcohol consumption (self-reported consumption of >30 g/day for men and >20 g/day for women). Moreover, subjects with a current or previous use of steatosis-inducing drugs (eg, amiodarone and methotrexate) were also excluded. Further details were reported previously.[26–32] Liver samples were obtained percutaneously in patients undergoing liver biopsy for suspected NAFLD/non-alcoholic steatohepatitis (NASH) or intraoperatively in patients in whom an intraoperative liver biopsy was indicated on clinical grounds such as during scheduled liver resection, exclusion of liver malignancy during major oncological surgery or assessment of liver histology during bariatric surgery. All patients gave their written informed consent for liver biopsy and genetic testing. All subjects consented to inclusion in the study.

First German–Austrian NAFLD cohort ('GER-AUS 1')

A total of 643 patients of self-reported European descent with biopsy-proven NAFLD were examined. Three hundred and fifty-nine German subjects were recruited during routine NAFLD workup at Leipzig, Frankfurt, Homburg, Kiel and Hamburg. Two hundred and eighty-four Austrian subjects were recruited from the liver outpatient clinics at Oberndorf and Salzburg Hospitals through routine diagnostic work-up for suspected NAFLD/NASH. Portions of this NAFLD cohort have been described previously.[26–32]

Second German–Austrian NAFLD cohort ('GER-AUS 2')

A total of 541 patients of self-reported European descent with biopsy-proven NAFLD were recruited independently from the first NAFLD cohort. Three hundred and eight German subjects were recruited during routine NAFLD workup at tertiary referral centres in Homburg, Würzburg, Mainz, Hannover, Heidelberg, Cologne, Hamburg and Freiburg within the framework of the multicentric NAFLD Clinical Study Group. Details of this cohort were presented elsewhere.[33] Two hundred and thirty-three Austrian subjects were recruited during routine NAFLD workup at Salzburg and Oberndorf. Portions of this NAFLD cohort have been described previously.[31]

Cohorts of alcohol misusers

A total of 2462 cases and controls were recruited from multiple centres. Of these, 1798 subjects were of self-reported German/Swiss descent ('German–Swiss' cohort: cases=934, controls=864) whereas 664 subjects were of self-reported British/Irish descent ('British–Irish' cohort: cases=317, controls=347) (figure 1). For both cohorts, *cases* (n=1251) were defined as patients with alcohol-related cirrhosis (defined histologically or based on clinical, laboratory, endoscopic and imaging criteria) on a background of past and/or present alcohol consumption of at least 60 g/day for women and 80 g/day for men for more than 10 years, after exclusion of other common causes of cirrhosis. *Controls* (n=1211) had similar alcohol exposure but no evidence of advanced liver disease (based on clinical, laboratory, endoscopic and imaging criteria as well as non-invasive assessment of liver fibrosis or liver histology). In all alcohol misusers, the relationship between the presence of cirrhosis and the observed Pi*Z and Pi*S genotypes was analysed separately. In both cohorts of alcohol misusers, the presence of concomitant liver disease was excluded by a comprehensive workup (see below for each cohort).

German–Swiss alcohol misuser cohort

A total of 1798 alcohol misusers of self-reported German or Swiss descent were examined. *Cases* with alcohol-related cirrhosis (n=934) were recruited from academic hepatology centres at various sites in Germany and Switzerland (Dresden, Leipzig, Bonn, Regensburg, Erlangen, Luebeck, Homburg, Hamburg, Bern and Lausanne). *Controls* without evident liver disease (n=864) were recruited at psychiatry centres specialised in addiction medicine in Germany (Regensburg, Munich and Mannheim).

All patients underwent clinical examination, laboratory testing and abdominal ultrasound. Past and present alcohol consumption was quantified through a face-to-face interview. Concomitant liver disease was excluded by the following workup: chronic infection with hepatitis B and C was excluded serologically in all subjects. Serum levels of ferritin and transferrin saturation were determined to rule out hereditary haemochromatosis, and neither clinical nor serological signs of autoimmune liver disease were present. Further details including details of recruitment protocols were reported previously.[34] All patients gave written informed consent, and the study received approval from the ethics committees of all participating centres.

British–Irish alcohol misuser cohort

Six hundred and sixty-four alcohol misusers of self-reported British or Irish descent were recruited from the Department of Hepatology at the Royal Free Hospital, London. The cohort consisted of 317 *cases* with alcohol-related cirrhosis (n=317) and 347 *controls* without evident liver disease (n=347).

All patients had a history of prolonged alcohol misuse and fulfilled Diagnostic and Statistical Manual of Mental Disorders, 4th Edition criteria for a diagnosis of alcohol dependence. All patients were examined by two experienced, senior clinicians for signs of liver injury. All underwent abdominal ultrasound and/or abdominal CT/MRI as indicated; all underwent routine upper gastrointestinal (GI) endoscopy; histological examination was undertaken, whenever possible, of liver biopsy material obtained by percutaneous, ultrasound-guided or transjugular routes or else of explant or postmortem liver tissue. Patients were excluded if they had any other potential cause of liver injury by the following workup: serological testing was undertaken for antibodies to hepatitis A–E, cytomegalovirus, Epstein-Barr virus, herpes simplex and varicella; mitochondrial, nuclear, smooth muscle and liver kidney autoantibodies; iron, total iron binding capacity and ferritin; copper and caeruloplasmin; AAT; and tissue transglutaminase. Further details were reported previously.[34] In contrast to the German–Swiss cohort, alcohol misusers with body mass index (BMI) >30 kg/m^2 or diabetes mellitus were excluded. Patients with AAT serum levels below laboratory reference range were genotyped (UK reference laboratory in Sheffield), and they were excluded if they had the Pi*ZZ genotype. Further details are stated elsewhere.[34] The study protocol was approved by the institutional review board, and all included subjects provided written informed consent.

Genotyping

Genotyping for the presence of the two most relevant AAT mutations, the Pi*Z variant (rs28929474, also known as p.E342K or Glu342Lys) and the Pi*S variant (rs17580, also known as p.E264V or Glu288Val) was performed. For the NAFLD and alcohol misuser cohorts, genomic DNA from all cases and controls was genotyped using TaqMan chemistry (Applied Biosystems) as described before.[34] Moreover, genotyping was performed for the *PNPLA3* variant rs738409 (hcv7241), the

TM6SF2 variant rs58542926 (hcv89463510) and the *MBOAT7* variant rs641738 (hcv8716820). All process data were logged and administered using a database-driven LIMS.[35]

Data sharing

Patient level data, technical appendix and statistical code are available from the corresponding author and can be shared on request. An informed consent for data sharing was not obtained from study participants, but the presented data are anonymised and the risk of identification is low.

Statistical analyses

Continuous variables were displayed as mean±SD and were compared by an unpaired, two-tailed t-test. Categorical variables were reported as absolute (n) and relative (%) frequencies, and contingency tables were analysed with χ^2 test or Fisher's exact test, where appropriate. Mantel-Haenzsel linear-by-linear test for trend was used to assess the relationship between advanced fibrosis stage and observed Pi*Z and Pi*S risk genotypes in NAFLD subjects. Associations between clinical outcome parameters (ie, presence of cirrhosis) and potential risk factors (ie, genotype) were assessed by binary logistic regression analysis in order to calculate ORs. ORs were given with their corresponding 95% CIs (in brackets). Multivariable logistic regression as well as ordinal and nominal regression was used to test for independent associations. Nominal two-sided p values were reported for all tests and were corrected for multiple testing by Bonferroni correction, wherever appropriate. Fixed-effect model meta-analysis was performed using the inverse variance-weighted method to summarise Pi*Z and Pi*S effect sizes in different cohorts. For this, the program META (V.1.6.0) was used. Differences were considered to be statistically significant when p<0.05. Statistical analyses were performed using SPSS V.23, and the graphs were created with Prism 5 (GraphPad, La Jolla, California, USA).

RESULTS

Risk of NAFLD subjects carrying the Pi*Z or Pi*S variant for developing cirrhosis

First, we genotyped a German–Austrian cohort of 643 patients with biopsy-proven NAFLD for the presence of the Pi*Z and the Pi*S variants (figure 1). Subjects with NAFLD-related cirrhosis were significantly older, more likely to be female, had a higher BMI and were more likely to be diabetic than their counterparts without liver fibrosis (table 1).

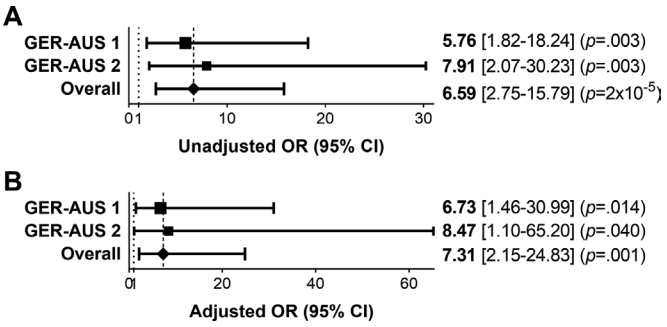

Figure 2 Risk of developing cirrhosis in biopsy-proven non-alcoholic fatty liver disease (NAFLD) patients heterozygous for the alpha1-antitrypsin Pi*Z variant. An univariable (A) and a multivariable (B) analysis in 378 patients with NAFLD from Germany and Austria ('GER-AUS 1') as well as additional 282 patients with NAFLD from Germany and Austria ('GER-AUS 2'). The term 'overall' depicts the meta-analysis of both cohorts. The odds for developing cirrhosis (stage F4) versus no fibrosis (stage F0) are displayed. The multivariable analysis was adjusted for sex, age, body mass index and the presence of diabetes mellitus.

No patient had the homozygous Pi*SS or Pi*ZZ genotype or the compound heterozygous Pi*SZ genotype. A comparison of carriers of the heterozygous Pi*MZ and Pi*MS genotype with non-carriers revealed that Pi*MZ carriers, but not Pi*MS carriers, displayed a significant shift towards higher fibrosis stages (p=0.001 and p=0.320, respectively; online supplementary table 2). In contrast to fibrosis, the frequencies of both genotypes did not obviously vary among the subjects with different NAFLD activity scores (online supplementary table 3).

Noteworthy, the Pi*MZ genotype was over-represented in NAFLD patients with cirrhosis versus their counterparts without fibrosis (13.2% vs 2.5%, p<0.0001; table 1). However, the Pi*MS genotype was detected at comparable frequencies in NAFLD patients with cirrhosis versus no fibrosis (4.4% vs 3.9%, p=0.740; table 1).

The Pi*Z variant constituted a strong risk factor for developing NAFLD-related cirrhosis (stage F4) versus no fibrosis (stage F0)

Table 1 Characteristics of the first non-alcoholic fatty liver disease (NAFLD) cohort

	Total n=643	Controls (stage F0) n=362 (56.3%)	Cases (stage F4) n=68 (10.6%)	Significance (p values)
Characteristics				
Age (years)	49±14	46±13	61±13	<0.0001
Sex (women)	322 (50)	165 (46)	41 (60)	0.026
BMI (kg/m^2)	37±12	36±12	28±7	<0.0001
Diabetes mellitus (yes/no/NA, % yes)	188/403/52 (32)	84/265/13 (24)	24/14/30 (63)	<0.0001
Frequency of AAT genotypes				
Pi*MZ genotype	36 (5.6)	9 (2.5)	9 (13.2)	<0.0001
Pi*ZZ genotype	0 (0)	0 (0)	0 (0)	NA
Pi*MS genotype	29 (4.5)	14 (3.9)	3 (4.4)	0.740*
Pi*SS genotype	0 (0)	0 (0)	0 (0)	NA
Pi*SZ genotype	0 (0)	0 (0)	0 (0)	NA

Cases were defined as NAFLD patients with histological cirrhosis (stage F4), whereas controls were defined as NAFLD patients without histological evidence of fibrosis (stage F0). Quantitative measures are shown as mean±SD or as an absolute count (n) and relative frequency (%).
*Fisher's exact test.
AAT, alpha1-antitrypsin; BMI, body mass index; NA, not available/applicable; Pi*MZ, heterozygous for the Pi*Z variant; Pi*ZZ, homozygous for the Pi*Z variant; Pi*MS, heterozygous for the Pi*S variant; Pi*SS, homozygous for the Pi*S variant; Pi*SZ, compound heterozygous for the Pi*S and the Pi*Z variant.

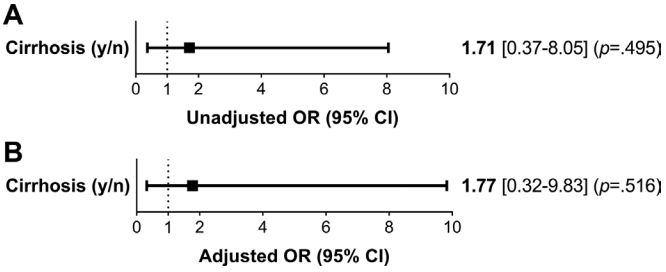

Figure 3 Risk of developing cirrhosis in biopsy-proven non-alcoholic fatty liver disease (NAFLD) individuals heterozygous for the alpha1-antitrypsin Pi*S variant. An univariable (A) and a multivariable (B) analysis was performed in the first cohort of 378 patients with NAFLD from Germany and Austria. The multivariable analysis was adjusted for sex, age, body mass index and the presence of diabetes mellitus. The odds for developing liver cirrhosis (stage F4) versus no fibrosis (stage F0) are displayed. y, yes; n; no.

in the univariate analysis (unadjusted OR=5.76 (95% CI 1.82 to 18.24), p=0.003; figure 2A). This association remained significant after multivariable adjustment for sex, age, BMI, and diabetes (OR=6.73 (95% CI 1.46 to 30.99), p=0.014; figure 2B). After further adjusting for the well-established NAFLD risk variants in the genes *PNPLA3*, *TM6SF2* and *MBOAT7*, the adjusted OR did not change numerically but was no more statistically significant (OR=5.24 (95% CI 0.83 to 32.97); p=0.077), most likely due to a decreased number of individuals with available data (n=348). Moreover, we performed an ordinal regression analysis considering all covariates (sex, age, BMI, diabetes, *PNPLA3*, *TM6SF2* and *MBOAT7*). This analysis also revealed that Pi*Z carriage associates with higher fibrosis stages (OR=2.33 (95% CI 1.14 to 4.77), p=0.021). The nominal regression of each fibrosis stage (F1–F4) against no fibrosis, considering all covariates, is shown in online supplementary table 4 as well as online supplementary figure 1. Apart from fibrosis stage 3 with only a limited number of individuals, all other fibrosis stages displayed at least a trend towards higher Pi*Z carriage.

In contrast to the Pi*Z variant, no link with cirrhosis development was found for the Pi*S variant, neither in the univariate analysis (OR=1.71 (95% CI 0.37 to 8.05), p=0.495; figure 3A), nor after adjusting for sex, age, BMI and diabetes (adjusted OR=1.77 (95% CI 0.32 to 9.83), p=0.516; figure 3B). Further adjustment for the above-mentioned variants in *PNPLA3*, *TM6SF2* and *MBOAT7* did not substantially change the findings (OR=1.14 (95% CI 0.15 to 8.39); p=0.901).

To validate the finding that the Pi*Z variant is a strong risk factor for cirrhosis development in NAFLD, we analysed an independent cohort of 541 German–Austrian patients with biopsy-proven NAFLD. Cases with NAFLD-related cirrhosis were significantly older and were more likely to be diabetic than their counterparts with NAFLD without liver fibrosis (table 2).

Similar to the first NAFLD cohort, heterozygous Pi*Z carriers displayed a significant shift towards higher fibrosis stages (p=0.008; online supplementary table 5). Likewise, heterozygous Pi*Z carriers were over-represented in NAFLD patients with cirrhosis versus their counterparts without fibrosis (15.4% vs 2.3%, p=0.007; table 2). As seen in the first NAFLD cohort, the Pi*Z variant constituted a strong risk factor for developing NAFLD-related cirrhosis (stage F4) versus no fibrosis (stage F0) in the univariate analysis (unadjusted OR=7.91 (95% CI 2.07 to 30.23), p=0.003; figure 2A). This association remained significant after multivariable adjustment for the previously described covariates sex, age, BMI and diabetes (OR=8.47 (95% CI 1.10 to 65.20), p=0.040; figure 2B). A meta-analysis for both cohorts revealed a highly significant association between the Pi*Z variant and NAFLD-related cirrhosis in both the univariable and the multivariable analysis (unadjusted OR=6.59 (95% CI 2.75 to 15.79), p=<0.0001; adjusted OR=7.31 (95% CI 2.15 to 24.83), p=0.001; figure 2).

Taken together, these data indicate that carriage of the Pi*Z variant, but not the Pi*S variant, constitutes a strong risk factor to develop NAFLD-related cirrhosis.

Risk of alcohol misusers carrying the Pi*Z or Pi*S variant for developing cirrhosis

The association between the Pi*Z variant and the development of liver disease in patients with NAFLD prompted us to investigate the impact of the same two AAT variants, Pi*Z and Pi*S, in two well-characterised cohorts of alcohol misusers (figure 1), which in total comprised 2462 individuals (German–Swiss cohort, table 3, and British–Irish cohort, table 4). Alcohol misusers with cirrhosis were older than alcohol misusers without significant liver injury, and women were more likely than men to develop cirrhosis (tables 3 and 4). The German–Swiss cohort, in contrast to the British–Irish cohort, also included patients with a BMI >30 kg/m^2 and patients with diabetes mellitus. Both factors were significantly different in German–Swiss alcohol misusers with cirrhosis versus no significant liver injury (table 3).

Similar to the NAFLD cohort, the Pi*MZ genotype was over-represented among patients with alcohol-related cirrhosis compared with controls (German–Swiss: 6.2% vs 2.0%,

Table 2 Characteristics of the second non-alcoholic fatty liver disease (NAFLD) cohort

	Total n=541	Controls (stage F0) n=256 (47.3%)	Cases (stage F4) n=26 (4.8%)	Significance (p values)
Characteristics				
Age (years)	49±14	47±14	60±8	<0.0001
Sex (women)	251 (46)	110 (43)	15 (58)	0.150
BMI (kg/m^2)	33±10	33±10	31±8	0.366
Diabetes mellitus (yes/no/NA, % yes)	146/366/29 (27)	47/193/16 (18)	8/13/5 (31)	0.046
Frequency of Pi*Z allele				
Pi*Z heterozygous	22 (4.1)	6 (2.3)	4 (15.4)	0.007*
Pi*Z homozygous	2 (0.4)	1 (0.4)	1 (3.8)	0.155*

Cases were defined as NAFLD patients with histological cirrhosis (stage F4), whereas controls were defined as NAFLD patients without histological evidence of fibrosis (stage F0). Quantitative measures are shown as mean±SD or as an absolute count (n) and relative frequency (%).
*Fisher's exact test.
BMI, body mass index; NA, not available.

Table 3 Characteristics of German–Swiss cohort of alcohol misusers

	Total n=1798	Controls (no liver injury) n=864 (48.1%)	Cases (cirrhosis) n=934 (51.9%)	Significance (p values)
Characteristics				
Age (years)	51±11	45±10	56±10	<0.0001
Sex (women)	386 (22)	118 (14)	268 (29)	<0.0001
BMI (kg/m^2)	25.6±4.6	24.9±4.9	26.3±5.0	<0.0001
Diabetes mellitus (yes/no/NA, % yes)	209/1199/390 (15)	13/612/239 (2)	196/587/151 (25)	<0.0001
Frequency of AAT genotypes				
Pi*MZ genotype	75 (4.17)	17 (1.97)	58 (6.21)	<0.0001
Pi*ZZ genotype	4 (0.12)	0 (0)	4 (0.43)	0.073*
Pi*MS genotype†	120 (6.77)	47 (5.53)	73 (7.92)	0.046
Pi*SS genotype†	3 (0.17)	1 (0.06)	2 (0.11)	0.390*
Pi*SZ genotype†	6 (0.34)	0 (0)	6 (0.65)	0.019*

Cases were defined as alcohol misusers with cirrhosis whereas controls were defined as alcohol misusers without evidence of significant liver injury. Quantitative measures are shown as mean±SD or as an absolute count (n) and relative frequency (%).
*Fisher's exact test.
†1772 subjects (98.6% of all subjects) were genotyped for presence of the Pi*S variant.
AAT, alpha1-antitrypsin; BMI, body mass index; NA, not available; Pi*MZ, heterozygous for the Pi*Z variant; Pi*ZZ, homozygous for the Pi*Z variant; Pi*MS, heterozygous for the Pi*S variant; Pi*SS, homozygous for the Pi*S variant; Pi*SZ, compound heterozygous for the Pi*S and the Pi*Z variant.

p<0.0001, table 3; British–Irish: 6.0% vs 2.6%, p=0.029, table 4; both cohorts: 6.2% vs 2.2%, p<0.0001). In the German–Swiss cohort, the Pi*MS genotype was also over-represented among patients with alcohol-related cirrhosis compared with controls (7.9% vs 5.5%, p=0.046; table 3). In contrast, in the British–Irish cohort, the Pi*MS genotype was found at similar frequencies in patients with alcohol-related cirrhosis and controls (10.5% vs 8.2%; p=0.295; table 4). Notably, we also identified four subjects with previously unknown homozygous Pi*Z carriage (Pi*ZZ genotype) in the German–Swiss cohort as measurement of the AAT serum level was not part of the initial assessment. Eight patients were newly discovered as compound heterozygotes (Pi*SZ genotype). Eleven out of the 12 Pi*ZZ and Pi*SZ patients had already developed cirrhosis (tables 3 and 4). Five patients were homozygous for the Pi*S variant (Pi*SS genotype); three of them had cirrhosis (tables 3 and 4).

In line with these data, the presence of the Pi*Z variant was strongly associated with the risk of developing cirrhosis in alcohol misusers in the univariable analysis (German–Swiss: OR=5.35 (95% CI 2.53 to 11.34), p<0.0001; British–Irish: OR=4.88 (95% CI 1.42 to 16.76), p=0.012; meta-analysis: OR=5.22 (95% CI 2.75 to 9.91), p<0.0001; figure 4A). This association remained significant when the data were adjusted for sex, age, BMI and diabetes (German–Swiss: OR=6.14 (95% CI 2.63 to 14.31), p<0.0001; British–Irish: OR=5.11 (95% CI 1.47 to 17.81), p=0.010; meta-analysis: OR=5.79 (95% CI 2.87 to 11.68), p<0.0001; figure 4B). Moreover, this association remained significant after further adjusting for risk variants in the genes *PNPLA3*, *TM6SF2* and *MBOAT7*, which are well-established ALD modifiers (German–Swiss: OR=6.05 (95% CI 2.54 to 14.43), p<0.0001; British–Irish: OR=5.24 (95% CI 1.45 to 18.85), p=0.011; meta-analysis: OR=5.78 (95% CI 2.82 to 11.87), p<0.0001).

Table 4 Characteristics of British–Irish cohort of alcohol misusers

	Total n=664	Controls (no liver injury) n=347 (52.2%)	Cases (cirrhosis) n=317 (47.8%)	Significance (p values)
Characteristics				
Age (years)	51±11	49±10	53±10	<0.0001
Sex (women)	180 (27)	81 (23)	99 (31)	0.022
BMI (kg/m^2)~	24.7±2.5	24.7±2.3	24.6±2.6	0.901
Diabetes mellitus (yes/no/NA, % yes)~	–	–	–	NA
Frequency of AAT genotypes				
Pi*MZ genotype	28 (4.22)	9 (2.59)	19 (5.99)	0.029
Pi*ZZ genotype*	0 (0)	0 (0)	0 (0)	NA
Pi*MS genotype†	61 (9.30)	28 (8.16)	33 (10.54)	0.295
Pi*SS genotype†	2 (0.30)	1 (0.29)	1 (0.32)	0.499‡
Pi*SZ genotype†	2 (0.30)	1 (0.29)	1 (0.32)	0.499‡

Cases were defined as alcohol misusers with cirrhosis, whereas controls were defined as alcohol misusers without evidence of significant liver injury. Quantitative measures are shown as mean±SD or as an absolute count (n) and relative frequency (%).
*Patients with the Pi*ZZ genotype were excluded a priori.
†656 subjects (98.8% of all subjects) were genotyped for presence of the Pi*S variant.
‡Fisher's exact test.
AAT, alpha1-antitrypsin; BMI, body mass index; NA, not applicable; Pi*MZ, heterozygous for the Pi*Z variant; Pi*ZZ, homozygous for the Pi*Z variant; Pi*MS, heterozygous for the Pi*S variant; Pi*SS, homozygous for the Pi*S variant; Pi*SZ, compound heterozygous for the Pi*S and the Pi*Z variant.
~Subjects with diabetes and/or subjects with BMI >30 kg/m^2 have been excluded.

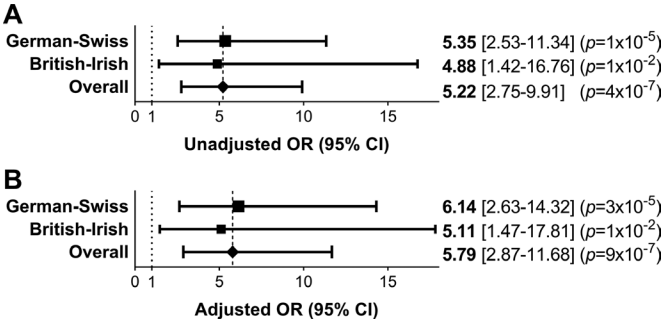

Figure 4 Analysis of the risk of developing cirrhosis in two cohorts of alcohol misusers heterozygous for the alpha1-antitrypsin Pi*Z variant. A univariable (A) and a multivariable (B) analysis was performed in 1191 alcohol misusers from Germany and Switzerland as well as 440 alcohol misusers from Great Britain and Ireland, both cohorts with and without cirrhosis. Adjustments were made for sex, age, body mass index and the presence of diabetes mellitus. The term 'overall' depicts the meta-analysis of both cohorts.

In contrast to the Pi*Z variant, a somewhat weaker association was observed between the Pi*S variant and the risk of developing cirrhosis in the univariable analysis (German–Swiss: OR=1.79 (95% CI 1.15 to 2.79), p=0.010; British–Irish: OR=1.10 (95% CI 0.60 to 2.01), p=0.750; meta-analysis: OR=1.51 (95% CI 1.06 to 2.15), p=0.024; figure 5A). After multivariable adjustment for sex, age, BMI and diabetes, this association remained significant in the German–Swiss cohort but lost its significance in the meta-analysis (German–Swiss: OR=1.70 (95% CI 1.01 to 2.87), p=0.047; British–Irish: OR=1.20 (95% CI 0.65 to 2.22), p=0.557; meta-analysis: OR=1.47 (95% CI 0.99 to 2.19), p=0.058; figure 5B). Moreover, after further adjustment for the variants in the *PNPLA3*, *TM6SF2* and *MBOAT7* genes, this association was lost in both cohorts (German–Swiss: OR=1.56 (95% CI 0.89 to 2.74), p=0.123; British–Irish: OR=1.21 (95% CI 0.62 to 2.36), p=0.570; meta-analysis: OR=1.40 (95% CI 0.91 to 2.16), p=0.122).

Altogether, these data indicate that carriage of the Pi*Z variant is a strong risk factor for developing cirrhosis in chronic alcohol misuse, whereas the Pi*S variant does not confer a major risk.

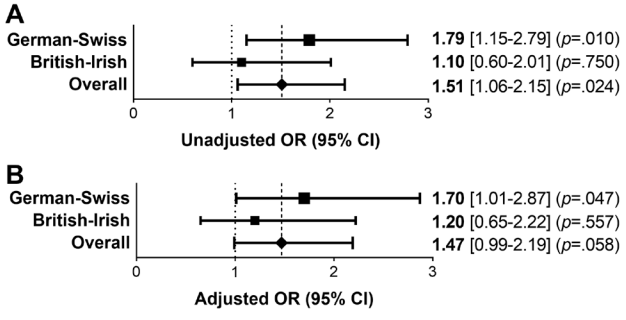

Figure 5 Analysis of the risk of developing cirrhosis in two cohorts of alcohol misusers heterozygous for the alpha1-antitrypsin Pi*S variant. A univariable (A) and a multivariable (B) analysis was performed in 1176 alcohol misusers from Germany and Switzerland as well as 437 alcohol misusers from Great Britain and Ireland, both cohorts with and without cirrhosis. Adjustments were made for sex, age, body mass index and the presence of diabetes mellitus. The term 'overall' depicts the meta-analysis of both cohorts.

DISCUSSION

In the present study, we evaluated the impact of the two most relevant AAT variants in two large and well-characterised cohorts of biopsy-proven NAFLD (both cohorts: n=1184) and chronic alcohol misuse (both cohorts: n=2462). We unambiguously found that the Pi*Z variant is a major risk factor for cirrhosis in the context of chronic metabolic injury such as NAFLD and chronic alcohol misuse. These findings are in line with previously published, smaller studies or studies pointing to an association with end-stage liver disease or cryptogenic cirrhosis in general.[12 14 15 17 18 21 22 24 25 36] These findings provide a definitive answer and end the controversy about the clinical relevance of heterozygous carriage of the Pi*Z variant in NAFLD and ALD (online supplementary table 1). Moreover, this study is the first report that has systematically investigated the role of the Pi*S variant providing evidence that this variant does not pose a major risk to develop alcoholic or NAFLD-associated cirrhosis. These observations are in agreement with the known pathogenic significance of both Pi*S and Pi*Z variants. Because Pi*S forms fewer polymers than Pi*Z, it is less retained within hepatocytes leading to a less prominent endoplasmic reticulum (ER) protein overload than Pi*Z.[37]

Our findings demonstrate that the Pi*Z variant confers an approximately six times higher odd to develop both NAFLD-association and ALD-associated cirrhosis. With a frequency of 2%–4% in Europe, the Pi*Z variant is relatively rare[7 9 10] but shows remarkable odds posing this variant as the hitherto strongest known genetic risk factor for NAFLD-related and ALD-related cirrhosis. In fact, the OR for developing cirrhosis of the Pi*Z variant exceed the ORs of other well-established genetic risk factors, such as the *PNPLA3* variant rs738409, the *MBOAT7* variant rs641738 and the *TM6SF2* variant rs58542926.[34 38–40] However, longitudinal analyses are needed to better characterise the contribution of the Pi*Z and Pi*S variants to development of NAFLD-related and ALD-related cirrhosis.

While future research needs to define the exact molecular mechanism how AAT variants predispose for NAFLD-related and ALD-related cirrhosis and what clinical modifiers are of particular importance, several factors are likely relevant. The cellular retention of mutated AAT (such as the Pi*Z variant) leads to a gain-of-function toxicity that appears to be proportional to the amount of retained protein.[10] Notably, AAT constitutes an established acute phase protein that is induced in patients suffering from liver injury.[41] Among other factors, ethanol was shown to stimulate AAT production in a human hepatoma cell line,[42] and thus, might aggravate gain-of-function toxicity in patients carrying AAT variants. Additionally, AAT mutations lower the threshold for an ER stress response,[43] which might be particularly relevant in NAFLD and ALD as both also challenge the ER.[10 44] Moreover, autophagy is crucial for the degradation of mutated AAT,[43] and both NAFLD and ALD have been shown to impair autophagic activity in hepatocytes.[45 46] Finally, an increased activity of neutrophil elastase, which constitutes the most established AAT substrate, has been implicated in the development of obesity and insulin resistance.[47] This pathway might be particularly important in subjects with AAT variants that display decreased serum AAT levels.[10]

The fact that the Pi*Z variant constitutes a clear and strong disease modifier should prompt its assessment in the workup of liver disease. In that respect, measuring serum AAT levels represents a relatively easy and cost-efficient method to rule out severe AATD (ie, homozygous Pi*ZZ genotype).[48] However, detecting heterozygous individuals (eg, Pi*MZ genotype) is

more challenging as their AAT serum levels are often within the reference range.[49] Further research is needed to define an optimal serum AAT cut-off level, especially in the context of chronic liver disease. Moreover, future studies should address the clinical relevance of heterozygous Pi*Z and Pi*S carriage in a prospective manner to delineate whether carriage of either AAT variant results in a clinical phenotype per se.

In summary, our study defines the Pi*Z variant as a strong single nucleotide polymorphism-based risk factor to develop NAFLD-associated and ALD-associated cirrhosis. This fact will help in genetic counselling of affected individuals. At the same time, our data advocate for a careful assessment of AAT serum levels in patients with chronic liver disease, especially in NAFLD and ALD.

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