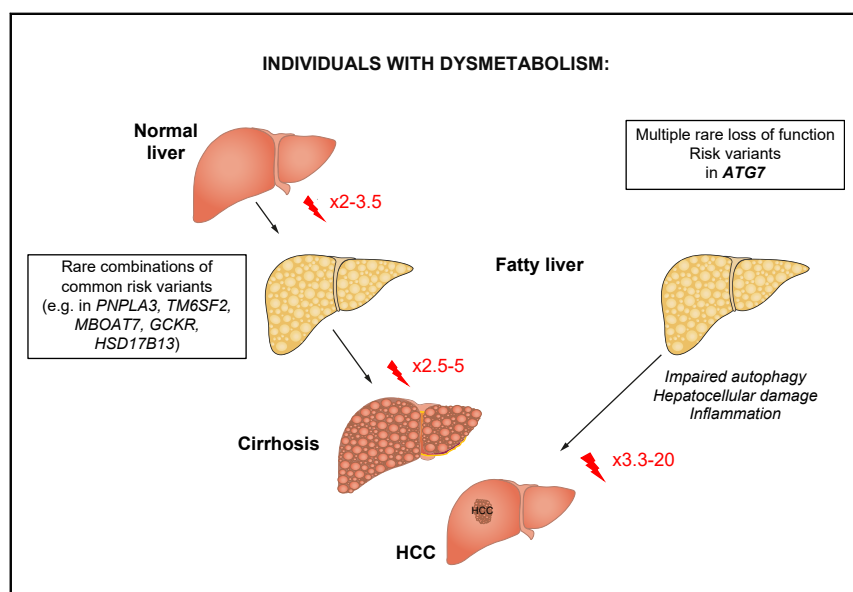


# Rare *ATG7* genetic variants predispose patients to severe fatty liver disease

## Graphical abstract



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## Lay summary

We found that rare mutations in a gene called autophagy-related 7 (*ATG7*) increase the risk of developing severe liver disease in individuals with dysmetabolism. These mutations cause an alteration in protein function and impairment of self-renewal of cellular content, leading to liver damage and inflammation.

## Highlights

- NAFLD is the leading cause of liver disorders and has a strong heritable component.
- Rare loss-of-function *ATG7* gene mutations increase the risk of severe liver disease in patients with NAFLD.
- *ATG7* mutations cause altered protein function and impairment of autophagy, leading to hepatocellular ballooning and inflammation.
- The most frequent variant is responsible for a meaningful fraction of predisposition to ballooning and hepatocellular carcinoma.

## Rare *ATG7* genetic variants predispose patients to severe fatty liver disease

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**Background & Aims:** Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disorders and has a strong heritable component. The aim of this study was to identify new loci that contribute to severe NAFLD by examining rare variants.

**Methods:** We performed whole-exome sequencing in individuals with NAFLD and advanced fibrosis or hepatocellular carcinoma (n = 301) and examined the enrichment of likely pathogenic rare variants vs. the general population. This was followed by validation at the gene level.

**Results:** In patients with severe NAFLD, we observed an enrichment of the p.P426L variant (rs143545741 C>T; OR 5.26, 95% CI 2.1-12.6; p = 0.003) of autophagy-related 7 (*ATG7*), which we characterized as a loss-of-function, vs. the general

population, and an enrichment in rare variants affecting the catalytic domain (OR 13.9; 95% CI 1.9-612; p = 0.002). In the UK Biobank cohort, loss-of-function *ATG7* variants increased the risk of cirrhosis and hepatocellular carcinoma (OR 3.30; 95% CI 1.1-7.5 and OR 12.30, 95% CI 2.6-36, respectively; p <0.001 for both). The low-frequency loss-of-function p.V471A variant (rs36117895 T>C) was also associated with severe NAFLD in the clinical cohort (OR 1.7; 95% CI 1.2-2.5; p = 0.003), predisposed to hepatocellular ballooning (p = 0.007) evolving to fibrosis in a Liver biopsy cohort (n = 2,268), and was associated with liver injury in the UK Biobank (aspartate aminotransferase levels, p <0.001), with a larger effect in severely obese individuals in whom it was linked to hepatocellular carcinoma (p = 0.009). *ATG7* protein localized to periportal hepatocytes, particularly in the presence of ballooning. In the Liver Transcriptomic cohort (n = 125), *ATG7* expression correlated with suppression of the TNF $\alpha$  pathway, which was conversely upregulated in p.V471A carriers.

**Conclusions:** We identified rare and low-frequency *ATG7* loss-of-function variants that promote NAFLD progression by impairing autophagy and facilitating ballooning and inflammation.

**Lay summary:** We found that rare mutations in a gene called autophagy-related 7 (*ATG7*) increase the risk of developing severe liver disease in individuals with dysmetabolism. These mutations cause an alteration in protein function and impairment of self-

Keywords: NAFLD; NASH; genetics; liver fibrosis; autophagy.

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renewal of cellular content, leading to liver damage and inflammation.

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) is most frequently associated with metabolic dysfunction and is the leading cause of liver-related mortality worldwide.<sup>1</sup> Fatty liver disease is a spectrum of conditions ranging from a mild liver fat accumulation to steatohepatitis characterized by ballooning degeneration of hepatocytes, inflammation and fibrogenesis, to life-threatening conditions at the end of the disease spectrum, namely advanced liver fibrosis and hepatocellular carcinoma (HCC). Fatty liver disease has a strong heritable component, and common germline variants regulating the hepatic remodeling, synthesis, and secretion of lipids account for a fraction of the risk.<sup>2</sup> The identification of these variants has enabled improvements in individual risk stratification and the identification of novel therapeutic targets.<sup>2,3</sup> However, a substantial fraction of NAFLD heritability remains unexplained.<sup>2,4</sup>

Within this context, we aimed to identify new inherited risk factors that contribute to NAFLD using whole-exome sequencing (WES) followed by the following strategy: we first examined a well-defined trait at the extreme of the NAFLD spectrum, namely advanced fibrosis and HCC, and next prioritized variants based on multiple *in silico* predictions before conducting the enrichment analysis of rare variants. The rationale for this approach was that rare variants with a large effect on protein function may contribute to variation in the susceptibility to develop severe NAFLD.

Indeed, most rare genetic variants have been proposed to be deleterious in humans with an effect size that is inversely related to their population frequency.<sup>5,6</sup> WES studies have identified a handful of genes<sup>6</sup> whose rare variants primarily associate with a complex trait. However, a) the large number of rare variants identified; b) the limited prioritization of these variants; c) the dilution of the effect size of these variants in poorly defined or mild phenotypes have so far limited the statistical power of WES studies. As a proof-of-principle, by using a candidate gene approach, we identified an enrichment of rare variants in *APOB* (apolipoprotein B) in individuals with severe NAFLD.<sup>7</sup>

Here, we first identified an enrichment of rare variants of autophagy-related 7 (*ATG7*) in patients with severe NAFLD. Strikingly, deletion of *ATG7* has previously been shown to facilitate liver injury due to defective autophagy and activation of inflammation.<sup>8-10</sup> We next validated the association of *ATG7* variants with advanced NAFLD in at-risk individuals and in a population-based cohort. Finally, we showed that *ATG7* is expressed in hepatocytes and linked to hepatocellular damage, specifically ballooning degeneration.

## Patients and methods

### Study cohorts

The study cohorts and design are presented in Fig. S1. The discovery EPIDEMIC cohort included 72 Italian patients with NAFLD-HCC, who were enrolled during 2010-2015. The EPIDEMIC validation cohort comprised a further 129 patients with advanced NAFLD including 59 Italian patients with severe

fibrosis (stage F3-F4) and 70 patients with NAFLD-HCC from the UK.<sup>7</sup> The Validation cohort included an additional 100 Italian patients with severe fibrosis (PERSPECTIVE cohort). Secondary causes of fatty liver disease were excluded by history, including at-risk alcohol intake ( $\geq 30/20$  g/day in M/F), use of drugs known to precipitate steatosis, other viral, autoimmune, and genetic liver disorders. Severe NAFLD was defined in the presence of advanced fibrosis (histological stage F3-F4 or clinically overt cirrhosis) or HCC.<sup>11</sup> We also considered a local ethnically matched control group of comparable sex distribution including 50 healthy Italian blood donors without NAFLD or metabolic abnormalities.<sup>7,12</sup> Clinical features of these individuals (clinical cohort) are presented in Table S1. Single variants allele frequency in the Discovery cohort as well as the mutational burden at the *ATG7* locus were compared to those of a sample representative of the European population (Fig. S1A-B): we considered non-Finnish Europeans (NFEs) studied by WES from the genome aggregation consortium (gnomAD,  $n = 56,885$ ).<sup>13</sup> As a further control group for the burden test analysis, we included 404 healthy NFEs from the 1000 genomes project with individual-level genotype data, whose genetic data were processed by the same pipeline of cases.<sup>14</sup> Evaluation of family members is reported in the supplementary methods.

The European NAFLD Liver biopsy cohort included 1,946 European individuals undergoing a liver biopsy for suspected non-alcoholic steatohepatitis.<sup>3,15</sup> Clinical features of the patients stratified by *ATG7* p.V471A and p.P426L genotypes are reported in Table S2.

The Liver-Bible-2021 cohort is a Milan cohort, where healthy individuals with at least 3 features of metabolic syndrome were phenotyped non-invasively for the presence of liver damage (by vibration controlled transient elastography) and for metabolic traits.<sup>16</sup> Cohort composition and characterization is presented in the supplementary methods and Table S3. Fatty liver was defined as a controlled attenuation parameter  $>275$  dB/m,<sup>17</sup> and severe NAFLD as a FAST (Fibroscan-AST) score  $>0.35$ .<sup>18</sup>

The UK Biobank (UKBB) is a large-scale study including more than 500,000 participants (age 40-69 years), recruited between 2006-2010 from 22 assessment centers across the UK; clinical features are presented in Table S4.<sup>19</sup>

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, was approved by the Ethical committees of the involved Institutions and was performed according to the recommendations of the hospitals involved. Informed consent was obtained from each patient.

Clinical cohorts genotyping, transcriptomic analysis in the Transcriptomic cohort,<sup>20</sup> liver histology and immunohistochemical analysis and cellular models are described in the supplementary methods.

### Whole-exome sequencing, variant calling, prioritization and statistical analysis

The bioinformatic pipeline for variant calling and annotation and quality control have previously been described and are reported in detail in the supplementary methods.<sup>7</sup> The variant prioritization pipeline is summarized in Fig. S1 and described in the supplementary methods. Our purpose was to increase the study power by reducing the number of variants tested (and therefore the denominator when correcting for multiplicity of testing), by selecting variants with a higher likelihood of being pathogenic. To this end, we focused on rare missense/splice variants robustly

predicted *in silico* to damage protein function and on those in genes with a very low likelihood of carrying damaging variants in the population. As a proof-of-principle, study power gain conferred by the need to correct for a lower multiplicity of testing and thus a less stringent alpha level (applying Bonferroni correction) is shown in Fig. S2B.

Genotype to phenotype correlations were assessed by generalized linear models using multivariate binomial logistic, ordinal logistic or linear modelling when appropriate. Burden test analysis is described in the supplementary methods.

Statistical analysis was performed using R version 4.0.3.

## Results

### Enrichment of *ATG7* rs143545741 p.P426L in severe NAFLD

To identify novel genetic determinants of severe NAFLD, we first developed a prioritization pipeline, based on frequency, predicted impact on protein function, and genic intolerance prediction, which is described in the supplementary methods and in Fig. S1. This approach increased the study power to detect rare variants with a large effect size by reducing the burden of multiple hypothesis testing (Fig. S2B). We detected 27 variants enriched in the Discovery cohort (whose features are reported in Table S1) compared to the general population (gnomAD-NFE, supplementary dataset A; adjusted  $p < 0.05$ ). Among those variants only 2 were also present in the Validation cohort (Table 1), and only *ATG7* rs143545741 C>T, encoding for the p.P426L amino acid substitution, was nominally associated with an increased risk of severe NAFLD in this group ( $p = 0.034$ , OR 4.33, Table 1). Considering both the discovery and validation sets, the p.P426L variant was associated with a moderate-large increase in the risk of severe NAFLD (OR 5.26, 95% CI 2.06–12.61;  $p = 0.0031$ ). However, in family pedigrees, 2 young women (daughters of probands with severe NAFLD) with no risk factors for liver disease carried the p.P426L variant and did not have evidence of liver damage (supplementary results).

### Increased burden of *ATG7* C-terminal domain variants in severe NAFLD

To evaluate the overall impact of *ATG7* variants on the risk of severe NAFLD, we next examined the enrichment of missense functional variants, compared to the general population (NFEs included in the 1000 genomes cohort, for whom individual data are available, and local controls, who were processed by the same bioinformatic pipeline;  $n = 454$ ). The gnomAD database could not be used for this specific analysis due to the lack of individual-level genotype data. The burden test for enrichment (sequence kernel association test [SKAT]) of variants in severe NAFLD vs. controls is presented in Table 2, while the list of variants is provided in Table S5 and Fig. 1. We detected a consistent enrichment in *ATG7* variants for all case cohorts vs. controls (Fig. 1 and Table 2;  $p < 0.05$ ). Mutations clustering at the “Apg7 homology domain” within the *ATG7* C-terminal catalytic domain were consistently enriched in all clinical cohorts (Table 2 and Fig. 2,  $p < 0.05$  using both SKAT and cohort allelic sum test [CAST]). Overall, carriage of at least 1 rare variant in the *ATG7* C-terminal domain was associated with a 13.9 increase in the risk of advanced NAFLD (95% CI 1.9–611.5,  $p = 0.002$  by CAST). The association remained significant after removing the rs143545741 p.P426L variant (OR  $\infty$ ; 95% CI 1.0– $\infty$ ;  $p = 0.025$  by CAST;  $p = 0.012$  by SKAT), indicating an independent contribution of the other missense variants located in this protein domain.

**Table 1. Variants enriched in individuals with severe NAFLD in the Discovery cohort observed in the Validation cohort.**

Variant	2:g.32740353C>T	3:g.11389502C>T
dbSNP	rs61757638	rs143545741
Gene	<i>BIRC6</i>	<i>ATG7</i>
Amino acid change	A3622V	P426L
<b>Discovery</b>		
Cases (n = 72)		
Alleles	3/144	2/144
MAF	0.0208	0.0139
Controls (n = 56,935)		
Alleles	347/113340	196/112822
MAF	0.0031	0.0017
OR (95% CI)	6.93 (1.86–20.39)	8.09 (1.41–29.46)
p value	0.0103	0.0267
<b>Validation</b>		
Cases (n = 229)		
Alleles	2/458	3/458
MAF	0.0044	0.0066
Controls (n = 123,456)		
Alleles	672/246912	375/246912
MAF	0.0027	0.0015
OR (95% CI)	1.61 (0.28–5.97)	4.33 (1.17–13.07)
p value	0.3548	0.0339
<b>Overall</b>		
Cases (n = 301)		
Alleles	5/602	5/602
MAF	0.0083	0.0083
Controls (n = 180,391)		
Alleles	1019/359580	571/359359
MAF	0.0028	0.0016
OR (95% CI)	2.95 (1.16–7.08)	5.26 (2.06–12.70)
p value	0.0300	0.0031

*ATG7*, autophagy-related 7; *BIRC6*, baculoviral-inhibitor of apoptosis repeat containing 6; HCC, hepatocellular carcinoma; MAF, minor allele frequency; OR, odds ratio. Frequencies were compared to the general population ( $n = 56,885$  gnomAD non-Finnish Europeans plus 50 local ethnically matched healthy individuals from the same geographical area) for the Discovery cohort and to European individuals without liver disease included in UK Biobank for the Validation cohort ( $n = 123,456$ ). Only variants represented in both the Discovery and Validation cohort are reported. Of patients positive for the *ATG7* p.P426L variant, in the Discovery cohort 2/2 had cirrhosis and HCC, while in the Validation cohort 3/3 had advanced fibrosis without HCC.

Enrichment in functional low-frequency and in rare variants of *ATG7* in patients with severe NAFLD ( $n = 301$ ) vs. external controls included in the gnomAD NFE database was also confirmed by ProxECAT, considering the possible bias due to the use of external controls sequenced by a different platform (Table S6;  $p < 0.05$  irrespective of the threshold to filter rare variants). This integrated approach confirmed the absence of bias due to different sequencing platforms used in the experiments and the resulting association from previous burden tests.

To highlight other clinically relevant *ATG7* variants, enrichment of specific variants occurring more than once in the clinical cohort of patients with severe NAFLD ( $n = 301$ ) was assessed against the European population (gnomAD-NFE,  $n = 56,885$ ). Besides the p.P426L, we found an enrichment in the low-frequency variant rs36117895 T>C (minor allele frequency [MAF] = 0.060 vs. 0.035) encoding for p.V471A (Table S2; OR 1.7; 95% CI 1.2–2.5;  $p = 0.003$ ).

### *ATG7* p.P426L and p.V471A variants behave as loss-of-function in hepatocytes *in vitro*

We next investigated the functional impact of *ATG7* variants identified in the clinical cohort on lipid levels in human



Table 2. Enrichment in *ATG7* variants in patients with severe NAFLD vs. the general population.

Cohort	<i>ATG7</i> gene									
	All variants					Rare variants (MAF <0.005)				
	n variants	Cases (%)	Controls (%)	SKAT <i>p</i>	n variants	Cases (%)	Controls (%)	SKAT <i>p</i>	CAST OR (95% CI)	CAST <i>p</i>
EPIDEMIC discovery (n = 72)	7	0.24	0.09	0.001	6	0.06	0.01	0.006	6.6 (1.2–36.2)	0.015
EPIDEMIC validation (n = 129)	7	0.11	0.09	0.040	6	0.04	0.01	0.009	4.5 (1.0–23.1)	0.029
EPIDEMIC overall (n = 201)	9	0.15	0.09	0.007	8	0.05	0.01	0.002	5.3 (1.4–23.7)	0.004
PERSPECTIVE (n = 100)	8	0.14	0.09	0.142	7	0.03	0.01	0.069	3.5 (0.5–20.9)	0.115
ALL (n = 301)	12	0.15	0.09	0.029	11	0.04	0.01	0.005	4.7 (1.4–20)	0.008
<b><i>ATG7</i> C-terminal domain ("Apg7 homology domain" – CD01486)</b>										
Cohort	Rare variants (MAF <0.005)									
	n variants	Cases (%)	Controls (%)	SKAT <i>p</i>	n variants	Cases (%)	Controls (%)	SKAT <i>p</i>	CAST OR (95% CI)	CAST <i>p</i>
	n variants	Cases (%)	Controls (%)	SKAT <i>p</i>	n variants	Cases (%)	Controls (%)	SKAT <i>p</i>	CAST OR (95% CI)	CAST <i>p</i>
Discovery (n = 72)	3	0.22	0.09	0.002	2	0.04	0.002	0.006	19.5 (1.5–1029.2)	0.009
Validation (n = 129)	2	0.10	0.09	0.039	1	0.02	0.002	0.019	10.7 (1.0–565.5)	0.036
EPIDEMIC (n = 201)	3	0.14	0.09	0.006	2	0.03	0.002	0.003	13.9 (1.7–640.4)	0.004
PERSPECTIVE (n = 100)	5	0.14	0.09	0.045	4	0.03	0.002	0.011	13.9 (1.1–733.7)	0.020
ALL (n = 301)	6	0.14	0.09	0.027	5	0.03	0.002	0.004	13.9 (1.9–611.5)	0.002

CAST, cohort allelic sum test; MAF, minor allele frequency; OR, odds ratio; SKAT, sequence kernel association test. (n = 404 from 1000 genomes project NFE plus n = 50 local controls).

hepatoma cells. Downregulation of *ATG7* using small-interfering RNA (Fig. S3A) resulted in increased fat content in both HepG2 and HepaRG cells ( $p < 0.05$  for both, Fig. S3B and S3C). Importantly, *ATG7* silencing increased intracellular lipid accumulation in primary human hepatocyte cultures, in 2D and 3D culture, with a larger effect size compared to immortalized cells (Fig. 2A and Fig. 2B, respectively).

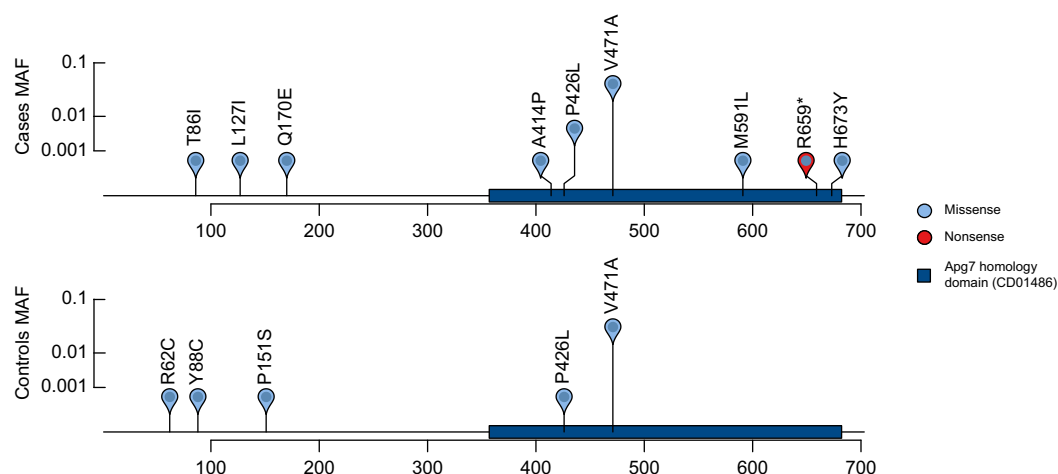
To corroborate previous results, *ATG7* wild-type, p.P426L and p.V471A were transiently overexpressed in HepaRG cultured with oleic acid. Overexpression of the p.P426L *ATG7* variant did not affect protein stability, whereas p.V471A showed a non-significant trend for lower protein levels compared to the wild-type (Fig. S3D). The impact of additional variants identified in the clinical cohort on *ATG7* protein stability is reported in the supplementary results. Overexpression of wild-type *ATG7* reduced intracellular neutral lipid levels ( $p < 0.05$  Fig. 2C and 2D), whereas either the p.P426L or the p.V471A mutants reduced the ability of *ATG7* to decrease the intracellular neutral lipid content ( $p < 0.05$ ; Fig. 2C and 2D).

To address the functional consequence of the p.V471A variant on protein activity, we engineered HepG2 hepatocytes (which were heterozygous for the p.V471A, hereafter called *ATG7* V471A<sup>+/-</sup>) to generate 2 syngeneic independent cell clones, carrying the p.V471A in homozygosity (thereafter termed *ATG7* V471A<sup>+/+</sup>) or *ATG7* gene deletion (thereafter termed *ATG7*<sup>-/-</sup>), using CRISPR-Cas9 (Fig. S4). Despite the unaltered transcript abundance (Fig. S5A), *ATG7* V471A<sup>+/+</sup> hepatocytes displayed reduced *ATG7* protein levels, similar to the *ATG7*<sup>-/-</sup> clone (Fig. S5B). We next investigated the autophagic flux by inducing autophagy and blocking the lysosomal degradation by chloroquine treatment. *ATG7* catalyzes the conversion of microtubule-associated protein 1A/1B-light chain 3 by phosphatidylethanolamine conjugation (LC3B-I to LC3B-II) resulting in reduced levels of the autophagosome cargo protein p62. We observed a reduction of LC3B-II conversion in V471A<sup>+/+</sup> vs. V471A<sup>+/-</sup> cells ( $p < 0.05$ ; Fig. 2E and 2F), whereas pathway activation was almost completely suppressed in *ATG7*<sup>-/-</sup> cells (Fig. 2E and 2F). In keeping, p62 levels were higher in *ATG7* V471A<sup>+/+</sup> and *ATG7*<sup>-/-</sup> cells compared to reference V471A<sup>+/-</sup> cells (Fig. 2E and 2F). Finally, like *ATG7*<sup>-/-</sup> cells, *ATG7* V471A<sup>+/+</sup> cells also showed a higher susceptibility to accumulate intracellular lipid droplets compared to *ATG7* V471A<sup>+/-</sup> hepatocytes after exposure to fatty acids ( $p < 0.001$ ; Fig. S5C–D).

Taken together, these data suggest that the p.V471A is a hypomorphic variant, namely it results in an unstable protein, leading to an impairment of the autophagic flux that in turn facilitates lipid accumulation in hepatocytes.

### Loss-of-function *ATG7* variants predispose to liver disease in the population

Missense variants in the active domain of *ATG7* are very likely to lead to protein loss-of-function (LoF). To confirm the association of rare LoF variants with severe liver disease at a population level, we examined rare (MAF <0.005) LoF variants in unrelated Europeans from the UKBB. LoF were defined as variation causing stop gain, frameshift, splice acceptor, or splice donor variants (Table S5). The impact of LoF variants on liver disease in the UKBB is reported in Table 3. We observed a progressive enrichment of these variants with severity of liver disease going from a 55% enrichment in chronic liver disease (SKAT-*p* = 0.025) to a more than 12-fold enrichment in HCC (SKAT-*p* = 0.001). To test



**Fig. 1. Nonsense and missense variants in the ATG7 main isoform identified by their amino acid change in cases and control groups according to their protein sequence localization.** Scheme of the variants retrieved in patients with advanced NAFLD ( $n = 301$ ) and in the general population ( $n = 404$  from 1,000 genomes project NFE plus  $n = 50$  local controls), which were evaluated at burden test analysis. Missense variants are indicated in yellow, while nonsense (truncating \*) variants are in red. The height of the variant symbols in the lollipop graph reflects their frequency. The Apg7 homology domain is indicated in blue. ATG7, autophagy-related 7; CD, conserved domain number (NCBI database); MAF, minor allele frequency; NAFLD, non-alcoholic fatty liver disease; NFE, non-Finnish European.

if excess in body weight may interact with these rare variants in determining liver disease, we stratified the cohort based on BMI. In obese individuals ( $BMI \geq 30$ ) there was a similar progressive enrichment of rare LoF variants throughout the spectrum of liver disease that was approximately 2-fold larger for any given class of liver disease, with a more than 23-fold enrichment for HCC. In addition, carriage of ATG7 variants was associated with increased risk of HCC ( $p < 0.05$ ) and of liver-related phenotypes irrespective of the reported alcohol intake (Table S7).

#### ATG7 p.V471A is associated with liver injury and disease through hepatocellular ballooning

In the Liver-Bible-2021 cohort, carriage of either p.P426L or p.V471A variants was associated with higher aspartate aminotransferase (AST), alanine aminotransferase (ALT), and risk of fatty liver and non-invasively assessed severe NAFLD (Table S8;  $p < 0.05$  for all). However, it was not associated with metabolic features.

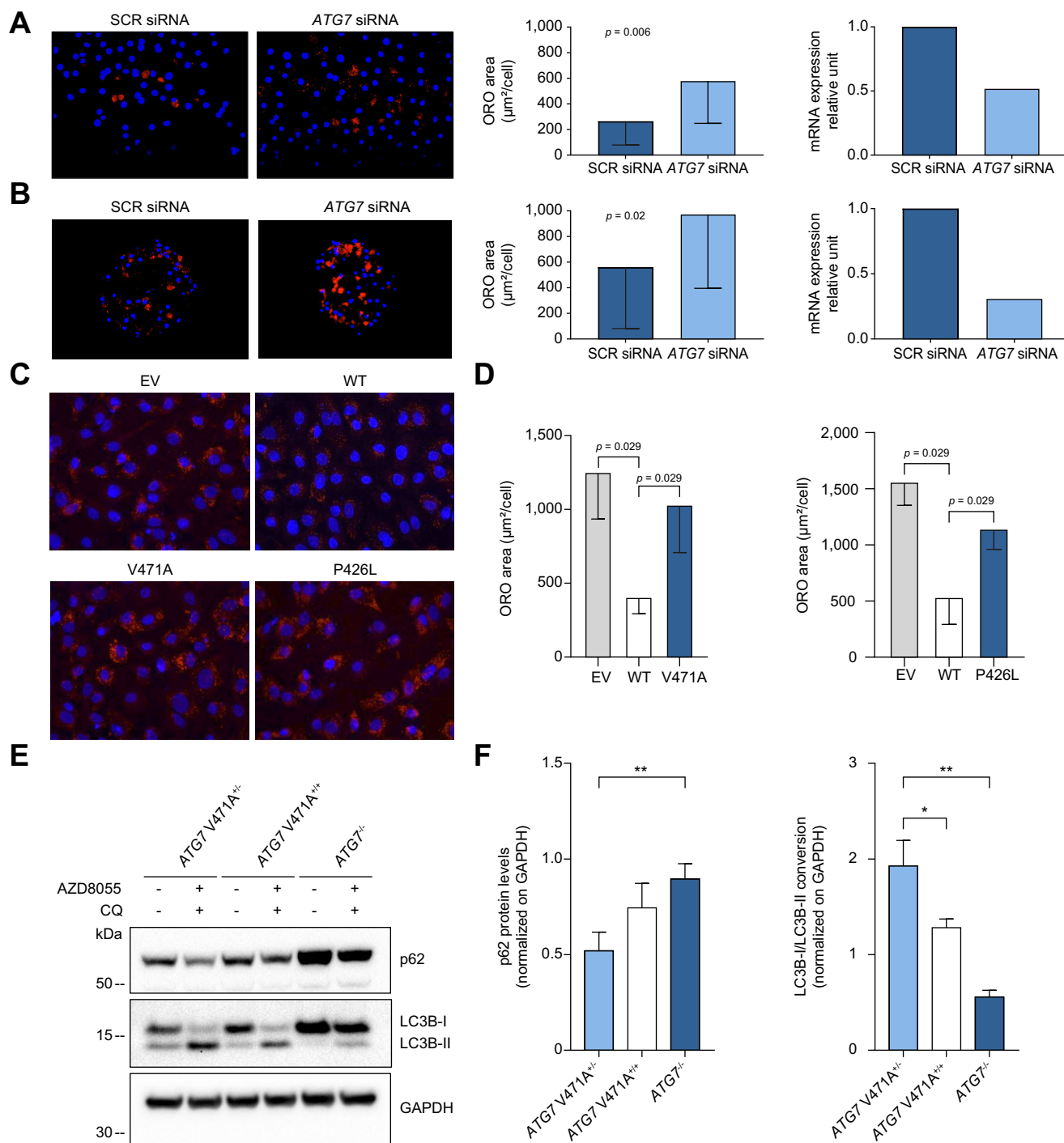
In the overall UKBB, the rs36117895 p.V471A variant was associated with AST levels ( $p = 7.7 \times 10^{-4}$ ; Table S4). After stratification for BMI there was an association between HCC and the p.V471A variant in those with  $BMI \geq 35$  (OR 2.31; 95% CI 1.2–4.4;  $p = 0.009$ , Fig. S6), corresponding to the largest impact on AST levels (Fig. S6).

To understand the mechanism underlying the association with liver disease, we next examined the impact of the p.V471A variant on histological liver damage in 2,268 patients from the NAFLD Liver biopsy cohort. The rs143545741 (p.P426L) variant was rare (MAF = 0.003,  $n = 14$  carriers) among Italians and absent in Finns (in line with public database data, not shown). Clinical features of patients from the Liver biopsy cohort stratified by rs36117895 p.V471A are shown in Table S2. We first assessed whether the impact of p.V471A on liver damage was accounted for by the association with a specific feature of liver damage. The p.V471A variant was not associated with steatosis or inflammation (Fig. 3A and 3B), but it was an independent predictor of ballooning ( $p = 0.007$ , Fig. 3C, Table S9 left panel, Bonferroni adjusted  $p = 0.028$ ) and explained a relevant proportion of the

susceptibility to severe ballooning (population attributable fraction = 0.09). In UKBB, ATG7 variants accounted for a meaningful fraction of HCC variability (population attributable fraction = 0.02). The effect of p.V471A on ballooning was also independent of hepatic fat accumulation (adjusted  $p = 0.009$ , Fig. 3, Table S9 right panel). The p.V471A variant was nominally associated with the presence of advanced fibrosis stage F3–F4 ( $p = 0.02$ , Table S2; adjusted  $p = 0.1$ , Fig. 2E). However, the impact of the p.V471A on severe fibrosis was consistent in those with the most severe steatosis grade S3 ( $n = 424$ ,  $p = 0.002$ ; Fig. 3F). Sensitivity analyses in PNPLA3 p.I148M carriers and patients with a high genetic risk of developing steatosis according to the polygenic risk score–hepatic fat content<sup>3,4</sup> are presented in Fig. S7 and Table S10. These showed a larger impact of the p.V471A variant in patients carrying PNPLA3 p.I148M or with higher genetic risk of NAFLD. Similarly, in UKBB individuals, the association between the p.V471A ATG7 variant and liver phenotypes was independent of the polygenic risk score–hepatic fat content. However, it was significant only in those with high scores ( $\geq 0.53$ ; adjusted OR 1.85; 95% CI 1.0–3.3;  $p = 0.022$ ).

#### Determinants of hepatic ATG7 mRNA and protein expression

To further understand the role of ATG7 in NAFLD, we examined the liver protein levels in a cohort of 22 patients with NAFLD by immunohistochemistry (age  $55 \pm$  years, 64% males, 71% with severe fibrosis). We observed a strong ATG7 expression in non-parenchymal cells (Fig. 4A). However, ATG7 was also present in periportal hepatocytes ( $p < 0.01$  vs. centrilobular hepatocytes) and staining was particularly intense around lipid droplets (Fig. 4B–D). Staining intensity in hepatocytes was qualitatively higher in patients with hepatocellular ballooning (Fig. 4E,  $p =$  n.s.), as well as in those with severe (grade S3) steatosis (Fig. 4F,  $p =$  n.s.). On the other hand, ATG7 staining diffusion correlated with steatosis severity (estimate  $0.51 \pm 0.21$ , adjusted  $p = 0.046$ ), with a tendency to be more restricted to periportal hepatocytes in carriers of PNPLA3 p.I148M (estimate  $0.50 \pm 0.23$ ; adjusted  $p = 0.11$ ).



**Fig. 2. ATG7 downregulation and overexpression influence intracellular lipid hepatocytes content and p.V471A and p.P426L result in a loss-of-function.** (A-B) Primary human hepatocytes were cultured in 2D (A) and 3D (B) and incubated with negative control (SCR) siRNA or ATG7 siRNA for downregulation. Intracellular neutral lipid content was visualized by ORO staining. ORO area was quantified per DAPI stained nuclei by Image J. Two representative images of ORO-stained sections were presented. Data are shown as average and  $\pm$  SD of 20 different sections. The  $p$  value was calculated by Mann-Whitney test. The ATG7 knockdown efficiency was evaluated by qRT-PCR. (C-D) Overexpression of recombinant wild-type-V5 and mutant ATG7-V5 in HepaRG cells. EV was used as negative control. Panel C shows representative images of the ORO staining. Panel D shows fluorescence intensity levels after ORO staining ( $n = 4$  for each group). Data are shown as mean and standard deviation;  $p$  values were calculated by using Mann-Whitney. (E-F) Western blot and relative quantification of the conversion of LC3B-I to LC3B-II, a process catalyzed by ATG7, and accumulation of the p62 autophagy-related cargo in ATG7 V471A<sup>+/+</sup>, V471A<sup>+/+</sup> and ATG7<sup>-/-</sup> HepG2 cells treated with (+) or without (-) AZD8055 and CQ, to promote autophagy and block lysosomal degradation, respectively. \* $p < 0.05$ , \*\* $p < 0.005$ . CQ, chloroquine; EV, empty vector; ORO, Oil Red-O; qRT-PCR, quantitative reverse-transcription PCR; RFU, relative fluorescence unit; RU, relative unit; siRNA, small-interfering RNA; WT, wild-type.

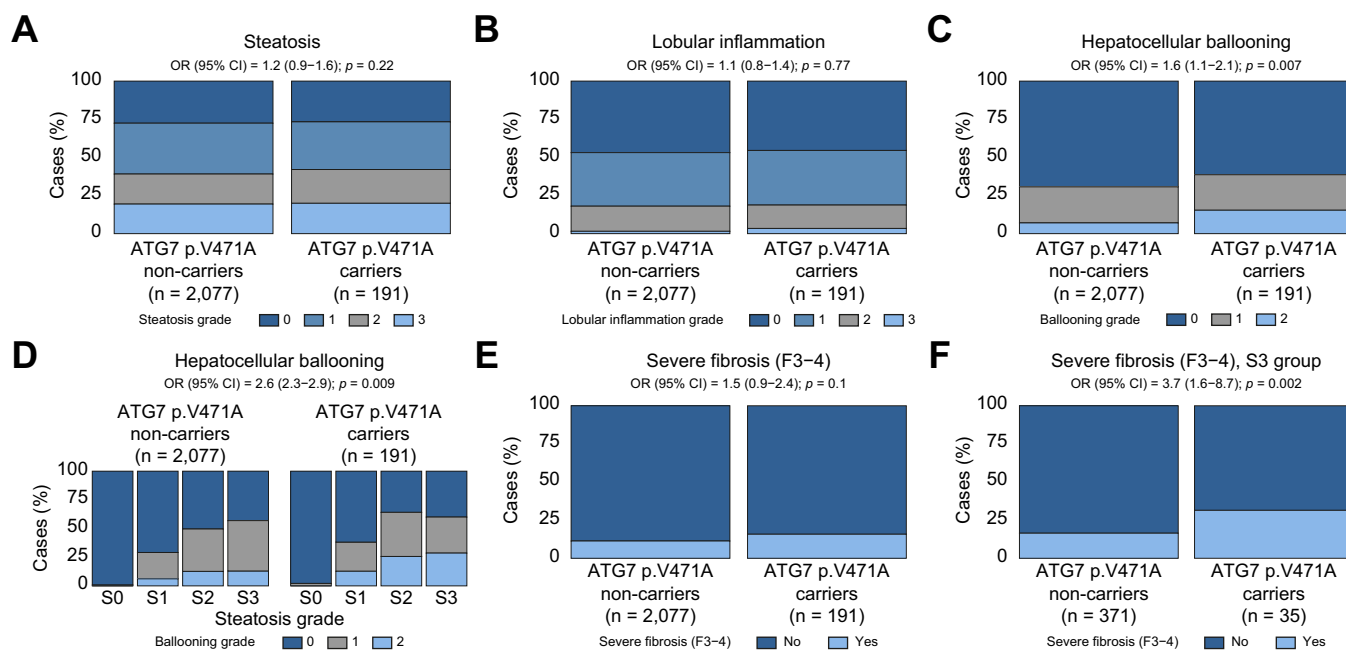
**Table 3. Analysis of *ATG7* rare LoF variants in the UK Biobank (n = 365,495).**

Trait	p value			$\beta$ /OR (95% CI)	MAC case (%)	MAC control (%)
	SKAT	burden	SKAT-O			
CLD, yes	0.014	0.199	0.025	1.55 (0.72–2.9)	9 (0.25)	243 (0.17)
Cirrhosis, yes	0.001	0.008	0.002	3.30 (1.1–7.5)	5 (0.55)	247 (0.17)
SLD, yes	$2.4 \times 10^{-4}$	0.003	$3.4 \times 10^{-4}$	6.47 (2.1–15)	4 (0.88)	201 (0.16)
HCC, yes	$7.7 \times 10^{-4}$	0.006	0.001	12.30 (2.6–36)	2 (1.54)	250 (0.17)
ALT, IU/L	0.436	0.620	0.631	0.03 (-0.08–14)	-	-
AST, IU/L	0.250	0.285	0.395	0.07 (-0.05, -0.19)	-	-
<b>BMI &lt;30</b>						
CLD, yes	0.772	0.423	0.614	0.71 (0.15–2)	2 (0.10)	194 (0.17)
Cirrhosis, yes	0.366	0.859	0.543	1.73 (0.2–6.2)	1 (0.19)	195 (0.17)
SLD, yes	0.112	0.442	0.186	3.41 (0.39–12)	1 (0.37)	159 (0.17)
HCC, yes	>0.99	0.748	0.901	4.71 (0.04–33)	0 (0)	196 (0.17)
ALT, IU/L	0.731	0.842	0.892	-0.01 (-0.14–0.12)	-	-
AST, IU/L	0.671	0.965	0.851	0.003 (-0.13–0.14)	-	-
<b>BMI <math>\geq</math>30</b>						
CLD, yes	$2.6 \times 10^{-4}$	0.006	$5.1 \times 10^{-4}$	3.6 (1.5–7.3)	7 (0.48)	49 (0.15)
Cirrhosis, yes	$7.1 \times 10^{-5}$	$8.2 \times 10^{-4}$	$9.8 \times 10^{-5}$	8.96 (2.9–22)	4 (1.06)	52 (0.15)
SLD, yes	$5.2 \times 10^{-5}$	$7.3 \times 10^{-4}$	$8.7 \times 10^{-5}$	14.7 (3.9–39)	3 (1.62)	42 (0.15)
HCC, yes	$9.5 \times 10^{-5}$	0.002	0.001	23.4 (4.5–77)	2 (3.08)	54 (0.15)
ALT, IU/L	0.476	0.128	0.211	0.20 (-0.06–0.45)	-	-
AST, IU/L	0.246	0.030	0.051	0.29 (0.03–0.54)	-	-

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLD, chronic liver disease; HCC, hepatocellular carcinoma; MAC, minor allele counts; SLD, severe liver disease. The association of 33 rare (MAF <0.005) loss-of-function variants of *ATG7* and liver-related traits in UK Biobank was tested overall and within different BMI classes of non-obese (BMI <30) and obese (BMI  $\geq$ 30). Default beta weight was used for weighted kernel in SKAT. Odds ratios were calculated by Firth's penalized likelihood analysis in a gene-based collapsing model adjusted for age, gender, BMI and first 10 genomic principal components. No association was detected with hepatic fat content was detected in individuals where information was available, diabetes, hypertension, glucose, HbA1c and lipid levels, renal disease, and other gastroenteric tract cancers, including oesophagus, stomach, intestine, biliary tract and pancreatic cancer ( $p > 0.05$  at SKAT-O test for all).

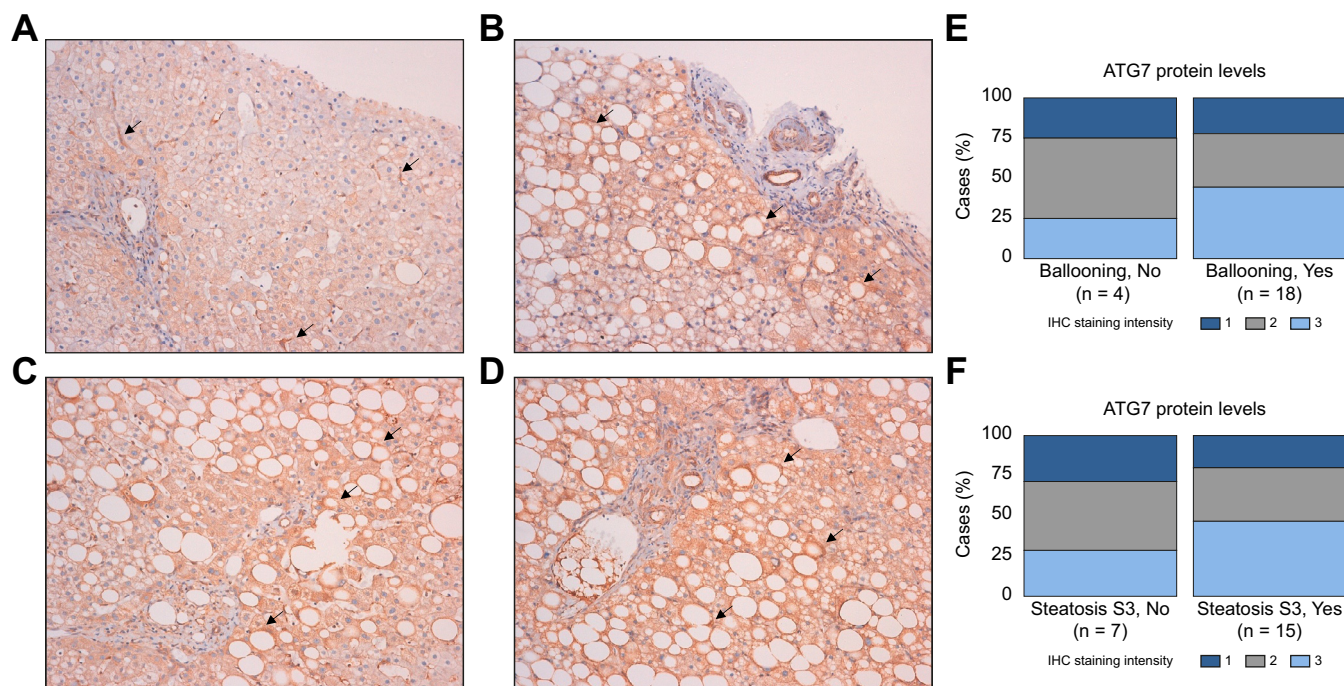
To investigate the molecular mechanisms underpinning increased susceptibility to hepatocellular damage in carriers of *ATG7* p.V471A, we examined its impact on the hepatic transcriptome (Transcriptomic cohort, supplementary dataset B).

First, in keeping with protein expression data, isoform profiling of *ATG7* expression revealed that the main *ATG7* transcript was overexpressed in patients with hepatocellular ballooning independently of age, sex, BMI, and *PNPLA3* p.I148M (Fig. S8,  $\beta = 0.55$



**Fig. 3. Association of p.V471A *ATG7* variant with liver damage in the NAFLD Liver biopsy cohort (n = 2,268).** (A–C) Impact on steatosis (A), lobular inflammation (B), and hepatocellular ballooning (C) according to the rs36117895 p.V471A variant carriage; (D) ballooning grade stratified by rs36117895 p.V471A variant carriage and by liver steatosis severity; (E,F) impact on severe fibrosis (stage F3–F4) in the overall cohort (E) and in patients with S3 steatosis (F). All statistical analyses were performed under an additive model by multivariate ordinal logistic or binomial logistic regression accounting for sex, age, BMI, T2D, and *PNPLA3* rs738409 C>G p.I148M, *TM6SF2* rs58542926 C>T p.E167K, *MBOAT7* rs641738 C>T, *GCKR* rs1260326 T>C p.P446L variant genotypes. Statistics reported in D were further adjusted for steatosis grade. *ATG7*, autophagy-related 7; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; T2D, type 2 diabetes.



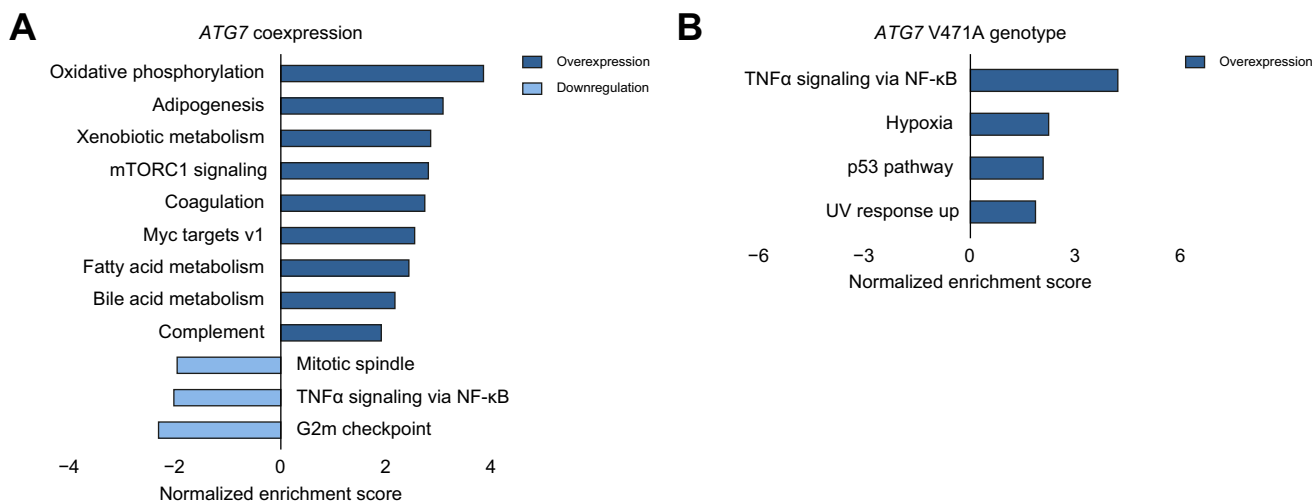


**Fig. 4. ATG7 protein expression in liver biopsies.** (A-D) Representative images of ATG7 immunohistochemical staining in liver biopsies; arrows highlight non-parenchymal cells (A) and lipid droplets (B-D). (E-F) ATG7 staining intensity distribution according to presence of hepatocellular ballooning (E) or S3 steatosis (F). ATG7, autophagy-related 7.

$\pm 0.19$ ,  $p = 0.004$ ). The association between ATG7 expression and ballooning was also independent of steatosis, lobular inflammation, and fibrosis ( $\beta = 0.49 \pm 0.20$ ,  $p = 0.015$ ).

To further explore ATG7's role in liver biology, co-expression analysis was performed. ATG7 expression level was positively correlated with that of genes involved in several metabolic pathways, mTORC1 signaling and coagulation. ATG7 expression levels were inversely correlated with genes involved in cell cycle progression and TNF $\alpha$  signaling (Fig. 5A, adjusted  $p < 0.05$ ). Conversely, gene set-enrichment analysis of nominally

associated transcripts revealed an overexpression in p.V471A carriers of the TNF $\alpha$  signaling, hypoxia, DNA damage, and p53 pathways (supplementary dataset C, Fig. 5B, false discovery rate-adjusted  $p < 0.05$ ). The impact of p.V471A on autophagy is shown in the supplementary results and Fig. S9A-B. The expression of genes belonging to this pathway and nominally associated with p.V471A variant carriage, including several genes involved in liver inflammation, is shown in Fig. S9C. Taken together, the dissociation of the impact of the p.V471A variant and ATG7 gene expression with activation of the TNF $\alpha$  pathway and autophagy



**Fig. 5. Transcriptomic analysis.** (A) Gene set-enrichment analysis was performed on pathways co-expressed with ATG7 in the Transcriptomic cohort and (B) on genes nominally associated with ATG7 p.V471A genotype in the Transcriptomic cohort ( $n = 125$ , at  $p < 0.05$  multivariate negative binomial regression for both). ATG7, autophagy-related 7.

genes together suggest that p.V471A acts as a LoF variant, inducing TNF $\alpha$ -dependent inflammation in carriers. By *in situ* hybridization analysis of liver tissue samples, we detected an association between the presence of the p.V471A variant and the number of TNF $\alpha$ -positive (non-parenchymal) cells (5-fold increase compared to lack of the *ATG7* variants;  $p = 0.05$ ; Fig. S10).

## Discussion

Aiming to circumvent the main limitation of current WES studies for complex traits, namely the difficulty in achieving an adequate statistical power,<sup>21</sup> as well as identifying new genetic determinants of severe NAFLD, we examined a cohort of individuals with severe NAFLD and developed an integrative data-mining approach to prioritize genetic variants contributing to this trait. To fulfil the selection criteria, genetic variants had to: a) be rare, b) cause protein sequence changes, as these are more likely to be deleterious,<sup>5</sup> c) be predicted to affect protein function with high confidence, and d) have a low rate of spontaneous variation in the general population. To increase the power of our study we specifically examined individuals at the extreme of a well-defined clinical spectrum, namely advanced fibrosis and HCC.

We first identified an association of the rare *ATG7* rs143545741 p.P426L variant with severe NAFLD. This rare variant conferred a large, greater than 7-fold, increase in the risk of severe liver disease in European individuals. To validate this first association, we considered the mutational burden at the *ATG7* locus highlighting the enrichment of rare protein-coding variants of *ATG7* in patients with severe NAFLD compared to the general population. In the clinical cohort, missense variants in the highly conserved catalytic domain were associated with a ~14-fold increased risk of severe liver disease, in the range of monogenic disorders. The phylogenetic conservation of the locus and high intolerance mutations of *ATG7* in the population, and the identification of an individual carrying a nonsense variant (R659\*) are consistent with the notion that these missense variants resulted in a LoF.

Next, we confirmed a higher risk of liver disease in carriers of LoF *ATG7* variants at a population level in unrelated Europeans from the UKBB. Consistent with a gene-environment interaction, the association effect size of *ATG7* variants was amplified by the presence of obesity, resulting in a ~23-fold higher risk of HCC in obese individuals. Taken together, these results suggest that excess body weight acts as a permissive factor exposing individuals to the deleterious effect of rare *ATG7* LoF variants on liver disease.

A fine-grained analysis of the role of specific *ATG7* variants highlighted a ~70% higher risk of severe NAFLD in carriers of the low-frequency rs36117895 p.V471A variant, which is comparable to that of the main NAFLD risk variants in *PNPLA3* and *TM6SF2*.<sup>2</sup> Of note, this variant was previously associated with a younger age of onset in Huntington's disease via its role in impairing autophagy.<sup>22</sup> The association of p.V471A with increased risk of liver injury (AST levels) was detectable in the UKBB, where the variant was also associated with an increased risk of HCC in severely obese individuals. In individuals with dysmetabolism, carriage of *ATG7* variants was associated with non-invasively assessed liver damage, including aminotransferase elevation, hepatic fat accumulation and liver damage, but not with metabolic traits (e.g. insulin resistance).

To characterize the mechanism underpinning the epidemiological association with liver damage, we next examined the

histological correlates of p.V471A carriage in the liver biopsy cohort. Although the p.V471A variant was not associated with steatosis, it was one of the main independent determinants of hepatocellular ballooning, particularly in those patients with more marked steatosis and in carriers of the *PNPLA3* p.I148M variant. These results are consistent with previous experimental data, suggesting that the combination of LoF of *ATG7* with *PNPLA3* p.I148M – a major modifier of hepatic lipid droplet remodeling – has a synergic impact on liver disease,<sup>23</sup> resulting in reduced lipo-autophagy in hepatocytes.<sup>24</sup> Data are also consistent with evidence from the family segregation analysis conducted in this study, showing that carriage of p.P426L did not confer a strong predisposition to NAFLD in the absence of metabolic triggers.

Taken together, these data suggest that genetic variants in *ATG7* lead to impaired autophagy in hepatocytes and predispose to fatty liver disease progression in individuals with metabolic risk factors. On the other hand, initiation of autophagy and intact *ATG7* are required for hepatic stellate cell trans-differentiation and initiation of fibrogenesis,<sup>25</sup> which may limit the detrimental impact of *ATG7* LoF mutations in individuals without impairment in lipid droplet remodeling.

The overexpression of the main *ATG7* transcript and protein in individuals with more severe lipid accumulation and those progressing to ballooning degeneration also speaks in favor of a direct involvement of autophagy in the cell toxicity caused by lipids. Indeed, *ATG7* staining intensity tended to correlate with steatosis and to localize around large lipid droplets, in keeping with a role in macro-lipo-autophagy.<sup>26</sup> This is also consistent with reduced *ATG7* expression in perivenular hepatocytes, which are more susceptible to fat accumulation, especially in *PNPLA3* p.I148M carriers.

In line with this hypothesis and with a possible role of *ATG7* in lipid droplet catabolism,<sup>26</sup> *ATG7* knockdown resulted in lipid overload in human hepatoma cells, while *ATG7* overexpression rescued the intracellular lipid accumulation induced by oleic acid exposure. Conversely, overexpression of p.P426L and p.V471A mutants did not reverse the phenotype. Furthermore, we directly confirmed that the p.V471A variant results in a LoF of *ATG7* activity in hepatocytes, leading to a reduction in autophagy, the accumulation of p62 (SQSTM1), and of intracellular lipids.

The highly conserved C-terminal domain is involved in binding to the Ubiquitin-like proteins and contains the catalytic cysteine (Cys507) of *ATG7*,<sup>27</sup> an E1 enzyme involved in the regulation of autophagy. A growing body of evidence shows that autophagy regulates lipid metabolism in the liver, and that *ATG7* plays a key role in initiating the signaling cascade eventually leading to lipo-autophagy.<sup>26</sup> Autophagy also regulates the activity of other hepatic and extrahepatic cell types with a potential impact on liver disease progression. For example, adipose-specific *Atg7* knockdown ameliorated NAFLD progression in mice.<sup>28</sup> However, the human genetic data suggest that, overall, the effect of *ATG7* LoF is detrimental for the liver. In keeping with this, adult *Atg7* mouse knockout models displayed hepatic lipid accumulation due to reduced lipo-autophagy during fasting, resulting in liver damage. In hepatocytes, steatosis was accompanied by accumulation of p62/LC-III aggregates,<sup>29</sup> consistent with failure of autophagy,<sup>30</sup> that are typically associated with hepatocellular ballooning.<sup>26,31</sup> In both experimental models and in individuals with NAFLD, impairment in autophagy leads to hepatocellular ballooning characterized by accumulation of p62

ubiquitylated aggregates,<sup>32</sup> a process also involved in hepatic carcinogenesis,<sup>33,34</sup> which was promoted by the p.V471A variant and by *ATG7* deletion in human primary hepatocytes *in vitro*. Furthermore, hepatocyte-specific *Atg7* deletion initiates TNF $\alpha$ -dependent liver injury.<sup>35</sup>

Transcriptomic analysis provided an independent confirmation of the mechanism linking impaired *ATG7* activity with liver disease. Indeed, the expression levels of the autophagy genes upstream of *ATG7* were consistent with a strong induction of autophagy initiation in p.V471A variant carriers. Conversely, most of the genes downstream of *ATG7* were downregulated suggesting an interruption of the pathway at this level. In keeping with this result, while *ATG7* transcript levels correlated with fat oxidation<sup>36</sup> and suppression of TNF $\alpha$  signaling, this latter pathway was upregulated in p.V471A variant carriers, concomitantly with an increased frequency of non-parenchymal TNF $\alpha$ -positive cells. Overall, the data indicate that the p.V471A variant results in a LoF, which prevents *ATG7* from suppressing inflammation in response to an impaired lipo-autophagy and to lipotoxicity. The disease mechanism may encompass lysosomal permeabilization, caspase-8-dependent activation of the mitochondrial death pathway and TNF $\alpha$  release.<sup>35,37</sup>

The present study does not provide a comprehensive evaluation of the role of rare variants in NAFLD pathogenesis. Rather, due to current limitations in the size of cohort of patients with advanced disease, the study was designed to screen, by exploiting a heavy prioritization pipeline and an initial validation, single likely pathogenic variants associated with severe NAFLD, aiming to find robust candidates for subsequent evaluation. However, this was followed by extensive validation at the gene level and in multiple clinical and population-based cohorts, with functional studies. We found several other candidates in the first analyses, which will require further validation. A limitation of this study is that we could not demonstrate if the impact of *ATG7* LoF variants on liver disease progression is entirely mediated by predisposition to ballooning, or whether the variants have a direct carcinogenic effect. While previous studies in one experimental model have reported that full *Atg7* knockdown by deletion during adult life may inhibit hepatic carcinogenesis,<sup>38</sup> our data are consistent with the notion that heterozygous carriage of LoF variants favors liver disease development and is potentially carcinogenic. Finally, results may not be applicable to different ethnicities.

In conclusion, by several independent approaches we identified rare and low-frequency LoF genetic *ATG7* variants as modifiers of NAFLD progression in Europeans. The proposed mechanism involves the facilitation of hepatocellular ballooning degeneration in individuals with fatty liver and dysmetabolism. These findings highlight that examining well-characterized and extreme phenotypes together with prioritization of rare genetic variants can be used to identify novel candidate genetic determinants of human disease.

### Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; *ATG7*, autophagy-related 7; CAST, cohort allelic sum test; HCC, hepatocellular carcinoma; LoF, loss-of-function; MAF, minor allele frequency; NAFLD, non-alcoholic fatty liver disease; NFEs, non-Finnish Europeans; SKAT, sequence kernel association test; UKBB, UK Biobank; WES, whole-exome sequencing.

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### Conflict of interest

The authors declare that they have no conflict of interest relevant to the present study.

LV has received speaking fees from MSD, Gilead, AlfaSigma and AbbVie, served as a consultant for Gilead, Pfizer, AstraZeneca, Novo Nordisk, Intercept, Diatech Pharmacogenetics and Ionis Pharmaceuticals, and received research grants from Gilead. SR has served as a consultant for AstraZeneca, Celgene, Sanofi, Amgen, Akcea Therapeutics, Camp4, AMbys, Medacorp and Pfizer in the past 5 years, and received research grants from AstraZeneca, Sanofi and Amgen.

Please refer to the accompanying ICMJE disclosure forms for further details.

### Authors’ contributions

Conceptualization: LV, SR, GB, SP. Methodology: LV, SR, GB, SP, OJ. Investigation: GB, OJ, SP, EC, FM, PD, MM, CB, FT, AC, RMM, RDA, SP, LM, UVG, AF, JP, EB, ALF, HR, GS, MS, LS, AC, VV. Visualization: GB, OJ, FM, MM, AC, LV. Funding acquisition: LV, SR, DP, HR, EB, SP, LM. Project administration: LV. Supervision: LV, SR. Writing – original draft: GB, LV. Writing – review & editing: LV, SR, OJ, FM, RMM.

### Data availability statement

The ethical approval of the study does not allow to publicly share individual patients’ genetic data. All data, code, and materials used in the analysis are available upon reasonable request for collaborative studies regulated by materials/data transfer agreements (MTA/DTAs) to the corresponding author. The frequencies of genetic variants in the EPIDEMIC-2021 cohort are



reported in the open access Mendeley database (<https://doi.org/10.17632/gff2ffhmr.1>).

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### Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2022.03.031>.

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*Author names in bold designate shared co-first authorship*

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