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Increased Frequency of Heterozygous Alpha-1 Antitrypsin Deficiency in Liver Explants from Non-Alcoholic Steatohepatitis Patients

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1. ABBREVIATIONS:

A1AT: alpha-1 antitrypsin

A1ATD: alpha-1 antitrypsin deficiency

BMI: body mass index

ESLD: end-stage liver disease

FFPE: formalin-fixed paraffin-embedded

HCV: hepatitis C virus

NASH: non-alcoholic steatohepatitis

PASD: periodic acid-Schiff-diastase

QC: quality control

RT-PCR: real-time PCR

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ABSTRACT

Cirrhotic explanted livers occasionally have unexpected PASD (periodic acid-Schiff-diastase) positive globules within the hepatocyte cytoplasm. It is often unclear whether

this finding is a non-specific consequence of cirrhosis or indicative of underlying A1ATD (alpha-1 antitrypsin deficiency) contributing to the cirrhosis. In this study, explanted livers were retrospectively evaluated for histopathology (including PASD status with confirmatory A1AT immunohistochemistry), and chart review provided etiology of liver failure and general clinical parameters. RT-PCR (real-time PCR) was used to detect A1AT genotype (*SERPINA1* S and Z alleles) by melting curve analysis on liver explant tissue from selected cases. Of 196 explanted livers, 21 (11%) had PASD+ globules. PASD+ globules were significantly enriched in patients with a clinical diagnosis of NASH (non-alcoholic steatohepatitis, 47%) compared to other causes such as chronic ethanol use (16%) and chronic hepatitis C virus infection (6.7%) (P 0.001). Immunohistochemistry confirmed all PASD+ globules were A1AT positive, with 20 of 21 cases demonstrating diffuse A1AT staining. In an expanded NASH cohort, 42% (14/33) of explants had PASD+ globules, and 92% of these PASD+ cases were homozygous (n=1) or heterozygous (n=11) for the *SERPINA1 Z* allele, corresponding to nearly 40% of all NASH patients. Overall, the Z allele was present in 10% of all liver explants tested, with 85% of PASD+ cases genotyping homozygous (n=2) or heterozygous (n=20), far in excess of the estimated 2% in the general population. Conclusion: These results indicate PASD+ A1AT globules (with confirmatory genotyping showing at least one Z allele) are commonly observed in NASH, suggesting a synergistic relationship towards liver fibrosis. In addition, the high frequency of *SERPINA1 Z* alleles in liver transplantation patients supports the utility of pre-transplant genotyping.

INTRODUCTION

Alpha-1 antitrypsin deficiency (A1ATD) is an autosomal recessive genetic disease with incomplete penetrance that can lead to progressive pulmonary and liver disease. The diagnosis of A1ATD is most commonly established by the demonstration of low serum A1AT (alpha-1 antitrypsin) concentration and/or isoelectric focusing of serum proteins (1). A1AT serum deficiency is caused by mutations in the *SERPINA1* gene which lead to protein misfolding or transcript instability and is sometimes accompanied by A1AT polymerization within hepatocytes (2). The *SERPINA1* S and Z (NM_000295.4: c.863A>T, p.E288V and c.1096G>A, p.E366K, respectively) alleles account for 95% of A1AT deficiency alleles (1, 2). If polymerization occurs, the A1AT glycoprotein accumulates in the endoplasmic reticulum of hepatocytes to form cytoplasmic globules that may be visualized on routine histology and highlighted by PASD (periodic acid-Schiff-diastase) stain. This accumulated protein is thought to be the primary cause of liver injury in A1ATD. (3)

Liver transplantation is the last treatment option for patients suffering from ESLD (end stage liver disease) due to cirrhosis. Liver explants removed from patients with ESLD due to cirrhosis may contain PASD positive cytoplasmic globules within hepatocytes. These globules have been previously divided into type 1 and type 2 globules. Type 1 globules are located in the periportal region and are associated with cirrhosis; type 2

globules are in a centrilobular region and are generally not associated with cirrhosis. A1AT globules in A1ATD are characteristically type 1 PASD globules (4). The PASD stain has been shown to be a good screening stain to identify hepatocyte A1AT globules; however, A1AT immunohistochemical staining is still useful to help confirm that the globule is A1AT (5). Type 1 PASD positive globules may be unexpectedly encountered in liver explants that were not performed for A1ATD. In fact, in the majority of cirrhotic livers with incidentally discovered PASD+ globules, there is no documented history of A1ATD (6). Prior studies estimate that diffuse cirrhotic type PASD+ globules occur in approximately 10% of patients with ESLD (6), an observation that raises concerns about the specificity of type 1 PASD + globules in the setting of liver explanation.

The contribution of A1ATD to the development of ESLD, particularly MZ heterozygosity, is an evolving area of research (7-11). Multiple studies have identified increased rates of Z allele heterozygosity in advanced liver disease (10, 12); however, not all studies have shown consistent results (13). Furthermore, The majority of liver transplants performed for documented A1ATD (~88%) occur in older adults (peak age range of 50-64 years), leading some authors to speculate that the principle phenotype of A1ATD may be an age-dependent degenerative disease (14). However, it is unclear how comorbidities, such as NASH (non-alcoholic steatohepatitis), might play a role in an age-dependent degenerative disease model of A1ATD.

Individuals with unexplained chronic liver disease should be tested for A1ATD and those found to be positive for a mutant A1ATD allele should be followed with annual imaging and laboratory evaluations. Family members of affected individuals should be provided with genetic counseling (15). The significance and management of unexpected PASD+ globules in adult liver explants is less clear. The identification of A1AT globules in the explanted liver may not be a priority for clinical management because the transplant is curative. The ramifications on family members, however, should be considered, regardless of whether the patient is cured. In this study, we evaluated the frequency of PASD+ globules in liver explants with A1AT

immunohistochemical confirmation, noted patient characteristics, and assessed the presence of the *SERPINA1* gene Z and S alleles using a novel genetic assay developed and validated within our clinical lab (16).

EXPERIMENTAL PROCEDURES

Cirrhotic livers removed at the time of orthotopic transplantation for ESLD during a 3-year period (Jan 1, 2013 - Dec 31, 2015) from a single institution were retrospectively reviewed after full institutional review board approval. No organs from executed prisoners were used in this study. For each case, liver pathology, including degree of cirrhosis, presence of cytoplasmic globules within hepatocytes, and positivity of the globules on PASD staining, were reviewed by two pathologists (MW, GC). PASD staining was performed on an automated platform (Artisan Link; Dako, Carpinteria, CA). All cases were stained with PASD, and all cases positive for PASD globules had confirmatory A1AT immunohistochemistry performed. The latter immunostaining was performed on an automated platform (Bond III; Leica, Buffalo Grove, IL) using a polyclonal anti-A1AT antibody (1:750, Cell Marque, Rocklin, CA) and developed with a polymer-based detection kit (Bond Polymer Refine; Leica) for chromogen development on the slide. A read-out of A1AT the immunohistochemical assays was performed by two pathologists (MW, GC) who were blinded to the histologic and clinical diagnosis.

General clinical parameters, including age, gender, etiology of ESLD, and BMI (body mass index) at time of transplantation were extracted from retrospective chart review for each patient. Initial diagnostic liver biopsies were generally unavailable for evaluation; therefore, histopathologic features of these biopsies were not included in the demographic dataset. Serum A1AT concentrations from any time point in the patient's care were collected, if available.

In a second round of case reviews following initial observations of the described cohort, all liver explants with NASH as the underlying etiology from 2006-2012 (from the same institution as the primary explant cohort) were similarly assessed. A1AT immunohistochemistry was performed retrospectively on all NASH cases, both PASD+ and PASD-. We used the Student's t-test to compare means of continuous variables and we used Fisher's exact test to compare categorical data. A p-value < 0.05 was considered significant.

In order to determine the A1AT genotype of the archived samples in this retrospective analysis, our group validated a RT-PCR (real-time PCR) method using FFPE (formalin-fixed paraffin-embedded) liver tissue (16). Briefly, genomic DNA was extracted from FFPE blocks liver tissue from all PASD+ cases and all PASD- NASH cases. RT-PCR was employed to amplify *SERPINA1* to detect the S and Z alleles (NM_000295.4: c.863A>T, p.E288V and c.1096G>A, p.E366K, respectively) using allele-specific fluorescence resonance energy transfer (FRET) probes and melting curve analysis. Validation samples had mean melting temperatures for the Z (61.2°C, SD=0.34) and S (55.4°C, SD=0.30) alleles that were clearly separated from the non-Z (54.7°C, SD=0.19) and non-S (48.6°C, SD=0.28) alleles.

RESULTS

During the 2013-2015 study period, 196 (male: female, 133:63) liver explants were removed at our institution during liver transplantation for ESLD. The etiologies of ESLD in the liver explants included: HCV (hepatitis C virus)-induced cirrhosis (n=90); chronic ethanol use-related cirrhosis (n=43); NASH-related cirrhosis (n=15); and a mixture of other diagnoses including (among others) primary sclerosing cholangitis and autoimmune hepatitis (n=61) (Figure 1). A subset of patients (n=13) had two etiologies clinically considered to contribute to ESLD.

Over the course of three years, 11% (21/196) of all liver explants in the primary cohort removed for ESLD had PASD+ hepatocyte globules. All PASD+ cases were also immunohistochemically positive for A1AT. Of these, 20 (96%) were strongly and diffusely reactive for A1AT, while a single weakly PASD+ case (4.0%) was only focally positive. In general, the PASD/A1AT IHC+ globules tended to be more conspicuous in hepatocytes at the periphery of cirrhotic nodules (Figure 2).

Among the 196 cases from all etiologies in the 3-year primary cohort (Figure 1), PASD+ globules were identified significantly more often in explants performed for NASH than for other causes (P 0.001): 47% of NASH; 16% of chronic alcohol abuse; 6.7% of HCV chronic infection; and 6.6% of other etiologies (1 case of A1ATD, 2 cases of autoimmune hepatitis, and 1 case of primary sclerosing cholangitis). Within the secondary expanded NASH cohort (n=33), the overall rate of PASD+ globules were similarly increased (42%; 14/33). All PASD+ NASH cases were A1AT positive and all PASD- NASH cases were A1AT negative.

The mean BMI for all cases at the time of the liver transplant in the primary cohort was 27 kg/m^2. The mean BMI in PASD+ cases was 32 kg/m^2, while the mean BMI in PASD- cases (27 kg/m^2) was significantly less (P 0.001, table 1). PASD+ NASH cases had a lower BMI on average (mean BMI 31 kg/m^2) than PASD- NASH cases (mean BMI 33 kg/m^2), although this difference was not statistically significance (P 0.15). The increase

in BMI in patients with PASD+ globules, however, may represent ascites, as the majority of patients with PASD+ globules had ascites (62%; 13/21).

Of the patients with PASD+ globules at time of explant, only a single patient had a clinical work-up for A1ATD. This was the primary driver for the development of an in-house PCR based assay to genotype the *SERPINA1* gene from FFPE (Figure 3). Eighty-five percent of cases with strong PASD+ globules (n=26, including those cases from the primary and secondary expanded NASH cohorts) were homozygous (n=2) or heterozygous (n=20) for the *SERPINA1 Z* allele. The one case weakly PASD+ with only focal A1AT staining was negative for the *SERPINA1 Z* allele. The S allele was not detected in any PASD+ globule cases (table 2). Among the strong PASD+ cases, 1 sample failed QC (quality control parameters) and was excluded in the analysis. In patients who underwent liver transplantation for NASH, 92% of cases with PASD+ globules were homozygous (n=1) or heterozygous (n=11) for the Z allele. In contrast, among PASD- liver explants performed for NASH, no Z alleles were detected. The PASD- NASH cases had a high percentage of QC failure (12/19 cases). No Z allele was detected among the 7 PASD- NASH cases which passed QC or among the 12 PASD- NASH cases which failed QC. Only samples that produced a threshold cycle (Ct) less than 32.0 were considered acceptable for genotype determination and inclusion in the analyses. A single case in the PASD- NASH group had a heterozygous S allele detected (table 3).

The prevalence of Z alleles in the primary cohort of all etiologies of ESLD was 8.0% (16/196), corresponding to 76% (16/21) of PASD+ cases. Among the 16 cases with a Z allele detected in the primary all etiologies of ESLD cohort, one was homozygous and 15 were heterozygous. Hepatocellular carcinoma was identified in the liver explant specimen in 2 of the 15 cases with Z allele heterozygosity among the primary all etiologies of ESLD cohort.

DISCUSSION

Unexpected PASD+ globules, in the context of advanced liver disease, are a specific finding which indicate the presence of a mutant A1AT allele. In our cohort, all but one PASD+ case had strong and diffuse A1AT immunohistochemical staining and 85% of PASD+ cases demonstrated at least one *SERPIN1A1 Z* allele on genotyping. Furthermore, explants performed for a clinical diagnosis of ESLD due to NASH were highly enriched for the *SERPIN1A1 Z* allele, suggesting a synergistic relationship between the MZ genotype and advanced fibrosis in NASH. Only a single case within our cohort had minimal clinical evaluation for A1ATD after PASD+ globules were reported in the explanted liver. This highlights a potential gap, at least in the study institution, in the follow up of potentially important information for family members. Anecdotally, due to this study's demonstration that PASD+ globules are specific to a mutant A1AT genotype and the increased overall rate of *SERPIN1A1 Z* alleles in the liver transplantation population, the study institution has increased clinical work up following an unexpected PASD+ result as well as evaluates A1AT phenotype on all patients starting an evaluation for liver transplantation.

The rate of unexpected PASD+ globules in the primary cohort in this study (11%) closely matches a prior study in which 10% of ESLD cases had PASD+ globules (6). Worldwide, 3.7% of the population has a variant A1AT genotype (2), therefore the frequency of PASD+ globules in our ESLD cohort is nearly 3 times that of non-MM

patients. This suggests the presence of A1AT globules is not simply reflective of the expected polymorphic variants of A1AT present in the population, but represents a possible association between ESLD and A1AT globules.

PCR testing in this study showed that PASD+ globules in the cirrhotic explants was highly correlated with the presence of at least one Z Allele; 85% of PASD+ cases were either homozygous or, more commonly, heterozygous for the Z allele. It is perhaps not surprising that A1AT globules accumulate in patients with heterozygosity for the *SERPINA1* gene Z allele, as this mutation leads to A1AT protein misfolding and intrahepatic polymerization (2). Among adults presenting with chronic liver failure, prior studies have demonstrated a higher percentage of MZ heterozygotes (8.4%) than expected by chance in the general population (2% of the population of North America carries a single Z allele) (12). In our retrospective study, at least 10% of all cases reviewed (including all cases 2013-2015 and all NASH cases 2006-2013) showed at least heterozygosity for the Z allele. Recently, Scheafer et al similarly found the MZ prevalence in liver transplantation was 9.7% while the MS prevalence was not significantly different than the general population (10). This data clearly demonstrates that liver transplantation patients are enriched for the Z allele compared to the general population.

Furthermore, in this study, nearly 50% of ESLD cases clinically attributed to NASH had PASD+ globules, and in all but 1 of these PASD+ cases the patient carried at least one Z allele. Remarkably, nearly 40% of ESLD patients who carried a clinical diagnosis of NASH were at least heterozygous for the Z allele, far in excess of the predicted rate of 2% in the general population and the 8-10% previously showed in advanced liver disease patients. (12) Since our genotypic assay only tests for the Z and S alleles, it is possible that the few cases with PASD+ A1AT globules that did not contain a Z allele may have a less common pathogenic *SERPINA1* allele that results in the accumulation of A1AT protein, such as the M_{malton} *SERPINA1* allele. (2)

Previous epidemiology research has pointed to a synergistic effect between homozygous A1ATD and other factors in the progression to advanced liver disease. Bowlus et al. demonstrated obesity as a predisposing risk factor for advanced liver disease in the subset of homozygous A1ATD patients who develop cirrhosis (8) and Propst et al. showed that individuals with ZZ or Z genotypes who developed chronic liver disease had high rates of concurrent viral hepatitis (7). The role of Z allele heterozygosity in chronic liver disease, and NASH in particular, is more controversial. Schaefer et al. found 9.7% of orthotropic liver transplants in their cohort to be MZ and that MZ was associated with more advanced liver disease (10). Graziadei et al. found 8.2% of their transplant population in the late 1990's was MZ; of this, 26.9% carried a diagnosis of cryptogenic cirrhosis. (12) Valenti et al. showed that more non-MM genotype patients were diagnosed with non-alcoholic fatty acid disease (10.8% compared to 3.5% MM), but ultimately did not find an association with liver damage. (9) Czaja et al. found that NASH patients had a greater frequency of A1ATD phenotype, than those with chronic hepatitis C infection (20% vs. 7%). (17). One additional study from Europe has also reported that the presence of A1AT heterozygosity is significantly higher in NASH patients than the general population. (18) Several other studies have pointed out associations between A1AT heterozygosity and chronic liver disease, including in alcoholic and cryptogenic chronic liver disease. (19-23). Not all studies, however, have shown an increase in the frequency of a heterozygous Z allele in chronic liver disease (13).

The results from this study suggest a relationship between heterozygosity for the *SERPIN1A1* Z allele and those conditions (primarily NASH, but to a lesser extent ethanol associated liver damage) where PASD+ is overrepresented in our cohort. In A1AT heterozygotes, it is possible that parenchymal inflammation and injury induced by conditions such as NASH result in both the A1AT globule accumulation and suppression of disposal mechanisms such as autophagy that have been shown to be important in the cellular handling of mutant Z protein. (24, 25) The function of alpha 1 antitrypsin in playing its anti-inflammatory role may be subsequently decreased. (26) This may allow the increased secretion of chemokines and cytokines such as tumor

necrosis factor and interleukin-6 that occurs in obese patients to remain unchecked (27). These mediators along with the increased free fatty acids and other proteins associated with metabolic dysregulation may lead to liver injury, NASH, and higher propensity for progression to cirrhosis. NASH is currently the second leading cause of liver disease among adults awaiting liver transplantation in the United States. With the advent of improved therapies for HCV infection (28), it is expected that ESLD patients with NASH will soon outnumber those with HCV in the US (29). Currently, there is a paucity of reliable markers to predict the risk of progression to advanced liver disease in patients with NASH. The major risk factors predictive of disease progression are diabetes and obesity, but even these have not been described in all longitudinal studies (30). Given the increasing prevalence of NASH, identification of additional biomarkers that independently predict risk of progression to ESLD might provide improved prognostic information and suggest changes in management, such as smoking cessation and evaluation of family members.

Limitations of the current study include its retrospective nature, overlapping clinical etiologies of ESLD, and our inability to independently validate the underlying etiology of ESLD in many cases. For most cases, only the cirrhotic explanted liver was available for pathologic review. In addition, serologic testing for A1AT concentration and phenotype in advanced cirrhosis and following liver transplantation are not accurate and in our cohort were mostly not available. DNA was successfully extracted from FFPE liver tissue stored up to 10 years after surgical removal. While only a single PASD+ case failed RT-PCR amplification, there was a high rate of QC failure among PASD- NASH case, which precluding melting-curve analysis and genotype determination in the slight majority of PASD- NASH cases. The higher number of QC failures among PASD- NASH explant samples is likely due to the older age of these specimens compared to samples that passed QC (Ct <32.0). A majority of the PASD- negative samples (83%, 10/12) that failed QC were over 5 years old. Recent work by Guyard *et al.* demonstrated that, compared with DNA immediately extracted from FFPE tissue, archived samples stored for a mean of 5.4 years had 53% lower DNA quantity and only 11% of the original DNA could be amplified by real-time PCR (31). Although the QC failures could

introduce bias, the fact that no Z alleles were detected in the PASD- samples that had amplifiable DNA is reassuring. An additional limitation in this analysis is the lack of histologic and genotype data of the full cohort of liver explants performed for all etiologies of ESLD, in order to reduce the potential for bias in the NASH-only subset analysis.

While *SERPINA1* Z-allele carriers are generally not thought to have a discernable clinical phenotype, this study suggests an obvious candidate. Interestingly, Chu et al. have suggested that liver disease in A1ATD might be better understood as an age-dependent degenerative disease (14). Our study identified a liver transplantation population enriched for the Z allele, with a relatively low number of liver explants in MZ patients with hepatocellular carcinoma. Heterozygosity for the Z allele has been shown to increase the risk of hepatocellular carcinoma (32-34); however, more recent reports have questioned this relationship (35). While our cohort suggests a phenotype of liver disease in Z allele heterozygotes of more advanced liver fibrosis when combined with certain comorbidities (NASH), it did not show a large increase in hepatocellular carcinoma in the Z allele heterozygotes. Additional studies evaluating families who are Z-allele carriers may lend insight into subtler phenotypes of MZ patients.

A1ATD evaluation has been recommended in cases of unexplained liver disease (15); however, our results suggest that A1ATD evaluation may be appropriate in all individuals with progressive chronic liver disease. Furthermore, our observation that almost no patient had clinical information or follow-up after the demonstration of PASD+ globules in explanted livers highlights the need for evaluation of the patient and family, even after liver transplantation. Family members of affected individuals are at 50% risk for inheriting the Z allele, and there may be a role for screening or early intervention to reduce the likelihood of ESLD in family members. At a minimum, the strong correlation between PASD+ globules and heterozygosity for A1ATD in liver explants warrants clinical follow-up.

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TABLES

	PASD+ (n=21)		**PASD- (n=175)**		**p-value**
Age, years	54.4		55.5		0.64
BMI, kg/m^2	31.6		26.9		0.001
Female, n (%)	9 (42.9%)		54 (30.9%)		
Ethnicity, n (%)	White Caucasian	18 (85.7%)	White Caucasian	133 (76.0%)	
	Asian	0	Asian	13 (7.4%)	
	Mexican	2 (9.5%)	Mexican	8 (4.6%)	
	American Indian	1 (4.8%)	American Indian	8 (4.6%)	
	African American	0	African American	6 (3.4%)	
	Middle Eastern	0	Middle Eastern	4 (2.3%)	
	Other	0	Other	3 (1.7%)	

Table 1: Demographic and clinical data for patients with liver explant specimens containing PASD+ and PASD- hepatocyte globules in the primary cohort.

	Strong PASD+
Z or ZZ Allele (QC pass)	22/26 (85%)
S or SS Allele (QC pass)	0/26 (0%)
QC Fail/Total	1/27

Table 2: Z and S allele frequency among cases with strong PASD (periodic acid-Schiff-diastase) positive hepatocyte globule staining in both the primary and expanded NASH cohorts. A single case failed QC (quality control) on two repetitions of the assay and was therefore not included in the analysis of Z or S allele frequency.

	PASD+ NASH	**PASD- NASH**
Z or ZZ Allele (QC pass)	12/13 (92%)	0/7 (0%)

S or SS Allele (QC pass)	0/13 (0%)	1/7 (14%)
QC Fail/Total runs	1/14	12/19

Table 3: Z and S allele frequency in NASH (non-alcoholic steatohepatitis) cases with and without PASD (periodic acid-Schiff-diastase) positive hepatocyte globule staining. A single PASD positive case and twelve PASD negative cases had 2 QC (quality control) run failures and were therefore not included in the analysis of Z or S allele frequency.

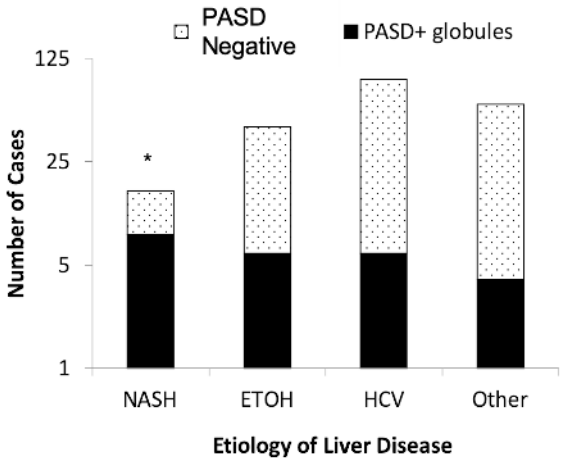
FIGURE LEGENDS

Figure 1:

The etiologies of ESLD (end-stage liver disease) in the liver explants were: 90 cases of hepatitis C virus-induced cirrhosis (HCV), 43 cases of chronic alcoholic disease-related cirrhosis (ETOH), 15 cases NASH-related cirrhosis (NASH), and 61 cases of a mixture of other diagnoses including, for example, primary sclerosing cholangitis and autoimmune hepatitis (Other). 13 cases had multiple etiologies. The * indicates increased frequency of PASD (periodic acid-Schiff-diastase) positivity in NASH cases (P 0.001, Fischer's exact test).

Figure 2:

A) An example of subtle cytoplasmic eosinophilic globules (see arrow) within the cytoplasm of cirrhotic hepatocytes on standard hematoxylin and eosin staining. C and D) Globules may be highlighted a bright magenta color within the hepatocyte cytoplasm with PAS-D stain. B) The confirmatory A1AT immunohistochemical staining strongly highlights prominent A1AT protein positivity in the cirrhotic hepatocytes.



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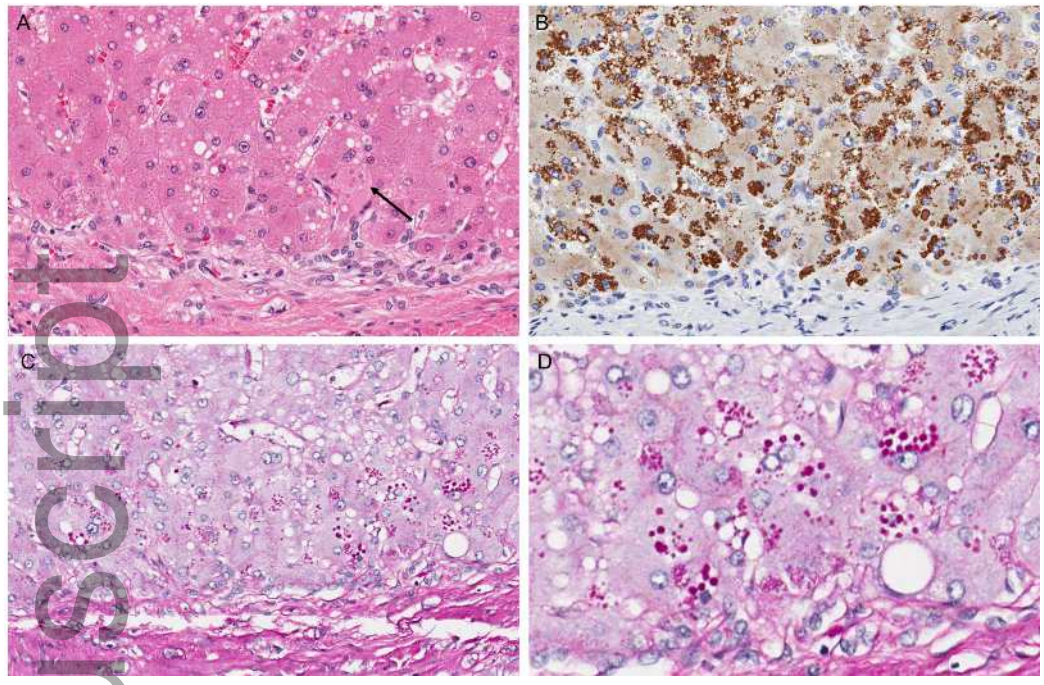

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