Title Page

Title. Fibrosis, Biomarkers and Liver Biopsy in AAT Deficiency and Relation to Liver Z Protein Polymer Accumulation.

ClinicalTrials.gov Identifier: NCT02014415

Conflict of Interest: The authors received grant support from and maintain consulting relationships related to AATD with: NIH, Alpha-1 Foundation, Critical Path Institute, Arrowhead Inc., Takeda Inc., Vertex, Beam Therapeutics, BioMarin, Precision Biosciences, Convergence Inc., KorroBio.

Financial Support. Primary Sponsor: Alpha-1 Foundation with study data and materials available from the corresponding author with the approval of the study steering committee. Arrowhead Inc., Vertex Inc., Alnylam Inc.

[1]Anandini Suri: anandini.suri@gmail.com. Department of Pediatrics, Division of Pediatric Gastroenterology, Hepatology and Nutrition, Saint Louis University, St Louis, MO

[2]Zidong Zhang: zidong.zhang@health.slu.edu. AHEAD institute, Saint Louis University, MO, USA.

[3]Brent Neuschwander-Tetri: brent.tetri@health.slu.edu. Division of Gastroenterology, Saint Louis University, MO, USA

[4]David A Lomas: d.lomas@ucl.ac.uk. UCL Respiratory, Division of Medicine, University College London, London, UK.

[5]Nina Heyer-Chauhan: n.heyer-chauhan@ucl.ac.uk. UCL Respiratory, University College London, 5 University Street, London, UK

[6]Keith Burling: keith.burling@addenbrookes.nhs.uk. Core Biochemical Assay Laboratory, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

[7]Rohit Loomba: roloomba@health.ucsd.edu. Internal Medicine, University of California San Diego, CA, USA

[8]David A Brenner: rburinda@ucsd.edu Internal Medicine, University of California San Diego, CA, USA

[9]Rosemary Nagy: rosemary.nagy@health.slu.edu . Pediatric Clinical Trial Unit, Saint Louis University, MO, USA

[10]Andrew Wilson: awilson@bu.edu Internal Medicine, Boston University, MA, USA

[11]Danielle Carpenter: danielle.carpenter@health.slu.edu Pathology, Saint Louis University, MO, USA

[12]Keith Blomenkamp keith.blomenkamp@health.slu.edu Department of Pediatrics, Division of Pediatric Gastroenterology, Saint Louis University, St Louis, MO

[1]Jeffrey Teckman: Jeff.teckman@health.slu.edu Department of Pediatrics, Division of Pediatric Gastroenterology, Hepatology and Nutrition, Saint Louis University, St Louis, MO

Author Contributions.

Study design and subject recruitment: JT, KB, RN, AW, DB, RL.

Sample analysis: NH, DL, KB, KB, DC, JT.

Data analysis: JT, KB, AS, BNT, ZZ.

Manuscript preparation and editing: AS, ZZ, BNT, DL, NH, KB, RL, DB, RN, AW, DC, KB, JT.

Ethics approval statement:

Patient consent statement: Participant informed consent was obtained locally at each center in accordance with approvals from Institutional Review Boards at each institution.

Permission to reproduce material from other sources: No materials were reproduced from other sources.

Data request statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Contact Information.

Jeffrey Teckman

Jeff.teckman@health.slu.edu

Department of Pediatrics,

1465 South Grand Blvd

Saint Louis MO 63105

Phone: 314-577-5647

Fax: 314-268-2775

ORCID: 0000-0002-4870-2621 (Jeffrey Teckman)

Short Title: Biomarkers and Liver Biopsy in AAT Deficiency

Ethics: All procedures and subject consents are in accordance with the Declaration of Helsinki and approved by each local institutional board.

Lay summary: It is probably important for ZZ patients to have regular checkups, to have assessments of liver fibrosis and to avoid obesity, to optimize liver health.

List of Abbreviations.

AAT: Alpha 1 antitrypsin

AATD: Alpha 1 antitrypsin deficiency

US: united states

IRB: Institutional review board

AST: Aspartate amino transferase

ALT: Alanine amino transferase

CBC: complete blood count

AFP: Alpha feto protein

GGT: gamma glutamine transferase

NCEP ATP: National Cholesterol Education Panel Adult Treatment Panel

AUDIT: Alcohol Use Disorder Identification Test

MSD: Meso Scale Discovery

ELISA: Enzymes Linked Immunosorbent Assay

H&E: Hematoxylin and Eosin

PAS: Periodic acid Schiff

PAS D: Periodic acid Schiff with digestion

CLIA: Clinical laboratory Improvement Amendments

DC: data coordinating center

ANOVA: Analysis of Variance

IQR: Interquartile range

FEV1: Forced Expiratory Volume at first 1 min

ppFEV1%: Forced Expiratory Volume at first 1 min % precent predicted

APRI: AST Platelet ratio index

FIB 4: Fibrosis index 4

BMI: body mass index

NAI score: Necroinflammatory activity index score

Electronic word count for main body of manuscript: 3982

Number of figures and tables: three figures and three tables

Abstract.

Background & Aims: The course of adults with ZZ alpha-1-antitrypsin deficiency (AATD) liver disease is unpredictable. The utility of markers, including liver biopsy, is undefined. Methods: A prospective cohort, including protocol liver biopsies, was enrolled to address these questions.

Results: We enrolled 96 homozygous ZZ AATD adults prospectively at 3 US sites with standardized clinical evaluations, and protocol liver biopsies. Fibrosis was scored using Ishak (stage 0 – 6). 51% of the 96 subjects had Ishak score >1 fibrosis (49% Ishak 0-1, 36% Ishak 2-3, and 15% ≥4). Elevated AST more than ALT, high BMI, obesity, APRI, and elevated serum Z AAT polymer levels were associated with increased fibrosis. Steatosis did not correlate to Fibrosis. Increased fibrosis was associated with increased mutant Z polymer globular inclusions (p=0.002) and increased diffuse cytoplasmic Z polymer on biopsy (p=0.0029) in a direct relationship. Increased globule Z polymer was associated with increased serum AST (p=0.007) and increased periportal inflammation on histopathology (p=0.004), but there was no relationship of Z polymer hepatocellular accumulation with ALT, GGT, inflammation in other parts of the lobule, necrosis or steatosis. Serum Z polymer levels were directly correlated to hepatic Z protein polymer content. Lung function, smoking and alcohol consumption patterns were not associated with fibrosis.

Conclusion: In AATD high BMI, obesity and elevated AST are associated with increased fibrosis. Liver biopsy features are correlated to some serum tests. Serum Z AAT polymer levels could be a future biomarker to detect fibrosis early and is directly correlated to liver Z content.

Keywords: cirrhosis, liver disease, Aspartate amino transferase, Z polymer, obesity

Introduction

Homozygous ZZ alpha-1-antitrypsin (AAT) deficiency AATD) is a metabolic genetic disease, found in approximately 1 in 3,500 individuals in Europe and North America, which can cause liver disease in adults and children. Accumulation of AAT mutant Z protein in hepatocytes triggers an intracellular injury cascade leading to progressive liver injury in a subgroup of affected individuals.[1,2,3] The lifetime risk of cirrhosis may be 20-40%, but is unpredictable.[4,5] Liver biopsy is currently the gold standard to study fibrosis, although data relating liver biopsy and other clinical parameters is lacking. Many patients also develop emphysematous lung disease via mechanisms that appear not related to liver injury, although the overlap remains unclear. [6,3,7]

Efforts to document the natural history through patient registries have had some success.[3] In Sweden, there is a prospective cohort of more than 100 patients followed since birth which has provided useful insights about the natural history of the disease, although this cohort is genetically more homogenous than the mixed US or wider European populations, and has been followed inconsistently without uniform imaging or liver biopsy.[5,8,9] There has been an attempt to examine fibrosis in a subset of 52 of these individuals, but it only used serum studies and not liver biopsy[10]. Previous prospective cohorts to document liver disease in adults (Clark and colleagues) have been limited by either an over enrollment of lung disease patients without liver disease or non-standardized assessments lacking liver biopsy.[11-13] Recent exciting reports from the UK Biobank, Swedish registries and Strnad and colleagues have also added disease risk insight, but key questions still remain[14]. We also note that there are several reports which have looked at fibrosis risk in heterozygous, MZ, individuals, which is generally accepted as a negative modifier state of other liver diseases, but is known to be an entirely different liver condition than ZZ homozygotes and therefore of limited comparative value[15]. We hypothesized

that a prospective cohort with protocol liver biopsies at enrollment, and including a full range of liver disease severity would increase our understanding of liver disease in ZZ AATD.

Methods

Patient recruitment

Since 2014, we enrolled a cohort with the following inclusions: adults (age > 18 years), homozygous ZZ AATD, with or without lung disease, all genders, all races, all ethnic groups, and who agreed to protocol liver biopsies and 5 annual visits. We enrolled this prospective cohort at 3 US sites, Saint Louis University, Saint Louis MO, Boston University, Boston, MA and University of California San Diego, La Jolla, CA. The study was approved by IRB at all these institutions. Saint Louis University was the data coordinating site and specimen repository. At enrollment subjects underwent detailed evaluation including protocol liver biopsy. A “known severe” enrollment arm with the same inclusion criteria but without a liver biopsy was also available to patients with established cirrhosis for whom a repeat biopsy without benefit was not ethical. Collection of medical records from the known severe participants, including previous liver biopsy reports, allowed confirmation that all had cirrhosis with fibrosis at the Ishak 5 or 6 level. However, due to differences in the amount of tissue retained at local sites, the method of the local read, and limitations in data sharing it was not possible to give a more precise score. Therefore, and to optimize statistical power in the analysis, we analyzed data in the Ishak groupings described below. Verified clinical data relating to the status of portal hypertension in the known severe group were captured and reported as Child-Pugh Score, as below.

Exclusion criteria for all arms included a previous history of organ transplant, other identified liver disease, and advanced lung disease was excluded from liver biopsy (ppFEV1 < 40% predicted).

Data Collection

Medical history data were collected at enrollment and at four annual follow-up visits. Physical exams were performed by hepatologists at the site of enrollment and at follow up visits. Clinical laboratory tests including AST, ALT, CBC, AFP, and GGT were done as a part of the study at enrollment and at yearly visits. We collected data on common risk factors hypothesized to worsen liver disease including BMI, medical history of metabolic syndrome, hypertension, and lipid disorders. For the purpose of this study, we defined metabolic syndrome using components from NCEP ATP III definition [16] as three of the following: 1) obesity/overweight defined as BMI ≥ 25 kg/m2 or waist circumference > 35 inches in females; > 40 inches in males 2) hyperglycemia (Fasting glucose > 100mg/dl) or on treatment 3) dyslipidemia (TG >150 mg/dl) or treatment 4) hypertension. (Systolic BP > 130 mmHg, Diastolic > 85mmHg) or treatment. Pulmonary function tests were done at enrollment and at follow up visits yearly using the ATS guidelines.[17] History of smoking patterns, pack years, and lung exposures were documented.[1819] The Alcohol Use Disorders Identification test (AUDIT), was used to assess the alcohol consumption patterns.[20]

Circulating Z polymer determination

We measured the serum Z polymer levels (ug/ml) using 2C1 antibody in Meso Scale Discovery (MSD) ELISA-based assay 7 in all subjects at enrollment with sufficient samples, excepting those already on exogenous AAT intravenous protein augmentation for emphysema, as this interfered with the assay (n=55 available).[21,22]

Fibrosis assessment

Liver Biopsy

All participants in the biopsy group received enrollment ultrasound guided percutaneous liver biopsies using 16G BioPince™ instrument set to collect a 33mm core. Samples were sent for H&E, PAS with digestion (PASD), trichrome stain, and iron stain. We used the Ishak score (0 - 6) to assess the fibrosis and validated metrics to assess steatosis and inflammation, as shown.[23] We used the Brunt's steatosis score to grade the degree and type of steatosis on liver biopsy. All biopsies were interpreted in the local site CLIA process by a licensed pathologist with results available to the participants, and final confirmation and consensus reads were conducted at the data coordinating center (DC). Quantification of Z protein polymer in the liver samples was accomplished in 77 of the 96 participants representing all fibrosis groups using 2C1 anti-Z polymer antibody with Aperio Brightfield electronic scanning using a protocol previously validated in a separate set of ZZ liver samples (via Flagship Biosciences).[18,19] Signal windows were able to separately quantify the Z polymer signal in the globular inclusions which are known to be comprised of 100% Z protein aggregates in the polymerized conformation from diffuse cytoplasmic signal in the hepatocytes previously shown to represent Z polymer oligomers in a non-dilated endoplasmic reticulum pool.

Data Analysis

We divided the cohort for statistical analyses based on the degree of biopsy fibrosis as shown. The groups were chosen based on statistical tests to optimize power and clinical relevance in a data set with the number of participants available. Those who were cirrhotic on enrollment were analyzed as a part of the significant fibrosis group. Frequency of demographic and clinical

measures were compared using the Student's t-test and one-way Analysis of Variance (ANOVA) for continuous variables and Pearson's Chi-square for categorical variables. For cells in tables with fewer than 5 subjects, we used Fisher's test to assess the statistical significance of associations. The correlations between pathologic measures and clinical outcomes were assessed with McNair's test and quantified in Kappa coefficient. All tests were two-tailed with alpha of 0.05. All the data management and analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA).

Results

Cohort Demographics

A total of 96 participants were enrolled in the study, but one did not participate in data collection thereafter leaving 95, 82 in the biopsy arm, 14 in the known severe group. The mean age at diagnosis of AATD was 45.9 ± 17.7 years, and mean age at enrollment was 54.6 ± 14.1 years. 49.5% (47/95) were males, and 94.8 % were Caucasian, which is consistent with the known North-West European genetic origin of this disease. Only 6.3% of subjects had their initial diagnostic AATD testing done due to liver disease-related symptoms, but these participants were significantly more likely to have advanced liver disease (Table 1, p=0.0113). Of the 82 liver biopsies, 93% had hepatocellular inclusion "globules" by PASD stain characteristic of this disease identifiable by light microscopy, but 100% had AAT protein accumulation in liver by immunohistochemistry.

Liver Disease burden in the cohort

Figure 1 shows the distribution of degree of fibrosis in the cohort. Using the Ishak Fibrosis score, the frequency distribution of fibrosis was grade 0-19.8%, 1 - 29.2%, 2 - 21.9%, 3 - 8.3%; ≥4 - 20.8%, respectively. In the known severe group 1 participant was Childs-Pugh A, 11 Childs-Pugh B, and 2 Childs-Pugh C. In the biopsy group some participants with Ishak ≥3 had data and findings consistent with a Childs-Pugh Score (1 score A, 9 score B). For initial analysis, given the limited number of participants with some features we wished to compare to fibrosis, the cohort was divided into two fibrosis groups: Minimal fibrosis group (Ishak 0 - 1) and the significant fibrosis group (Ishak >1). This is an approach similar to that reported for the cohort of Clark and colleagues and enables some key comparisons to that cohort which was lacking in more advanced fibrosis[11]. At enrollment, 49% (47/96) of subjects had minimal fibrosis, and 51% (49/96) had significant fibrosis (Table 1). Known risk factors for advanced fibrosis in other liver diseases, including age and male sex was not significantly associated with increased fibrosis in this cohort. Neonatal cholestasis is one of the most common liver presentations of AATD and the most common presentation in childhood. Our previous reports have shown that while neonatal cholestasis is associated with increased risk of portal hypertension in childhood, that the association is not strong and neonatal cholestasis is not seen in all patients who subsequently go on to portal hypertension. 6.3% of subjects in this adult cohort reported a history of neonatal cholestasis, but this was not associated with increased fibrosis in this cohort. Lung function, alcohol intake, and smoking patterns were similar in the two fibrosis groups (Table 1). Mean AUDIT score of the cohort was 2.3 ± 3.3 indicating that our cohort did not have high-risk alcohol consumption patterns at enrollment. In fact, those with more advanced liver disease had quit drinking alcohol. 67% of our cohort were never-smokers (Table 1).

Lung Disease burden in the cohort

AATD can also cause lung disease, although there is persistent debate as to any clinical relationship to liver disease.[3] Lung function testing was performed at enrollment on 52 subjects. 62% (31/52) of subjects had normal lung function (ppFEV1>80% predicted), which is more representative of ZZ individuals as a whole, than the predominantly lung affected cohort reported by Clark and colleagues. Mean ppFEV1 predicted of the cohort was 82.0 ±22.6. The mean ppFEV1 predicted was significantly lower in those who were past smokers than those that were not (70.2 ± 23.1 vs 86.3 ± 21.0; p=0.0212) (Figure 2 and Supplementary Table 1). 36.5% (35/96) of our cohort was on protein replacement therapy for lung disease and they were older and had lower mean ppFEV1 than those who were not on therapy. (58.9 ± 10.2 years vs 52.2 ±15.4 years; p=0.0119; and 65.5% ±19.7% vs 95.0% ±15.0%; p <0.0001) (Table 1 and supplementary table 1). Childhood asthma is thought to be associated with AATD. Our cohort reported 15% history of childhood asthma, which is higher than the 5-7% prevalence typically documented in the US. No significant association of any lung parameter to liver fibrosis was found, despite investigation with a wide range of statistical tests and alternative fibrosis groupings (not shown).[3]

Association of Clinical Liver Disease Factors and Fibrosis

For many parameters of particular interest, the frequency in the cohort allowed for further division into three fibrosis groups of Ishak 0 - 1, Ishak 2 - 3 and Ishak $\geq$ 4, but with preservation of statistical power. We felt studying the data in this way would be more informative to clinicians and for the design of future drug trials, as we might better document progression of disease or factors related to progression. These three fibrosis groups were similar in age and gender distribution. Clinical signs of advanced liver disease detected during follow up were, as would be expected, significantly associated with Ishak $\geq$4 compared to Ishak 2-3; variceal bleed ($p<0.0004$), splenomegaly ($p<0.0001$), portal hypertension ($p<0.01$), ascites ($p<0.04$).

Biochemical markers of liver injury ALT, AST and GGT were associated with degree of fibrosis. However, mean values of ALT, AST and GGT increased to clinically relevant values only in those with Ishak ≥ 4 (Table 2, For Ishak ≥ 4 Mean ALT 40.4 U/L, Mean AST 48.8 U/L, Mean GGT 67.2 U/L). Increased mean APRI and FIB 4 scores were also significantly associated with degree of fibrosis. APRI <0.5 and FIB 4 <1.3 had a NPV of 60% and 55% respectively to predict the degree of fibrosis (Supplementary Figure 1a, b; p<0.0001).

Metabolic parameters and liver disease in ZZ AATD

We examined the role of BMI and metabolic syndrome, as data from the Clark cohort, and others, has suggested these might be cofactors in AATD liver disease progression, and it has been suggested that weight loss might be tested as an intervention.[11] The mean enrollment BMI of the cohort was 27.8 ± 6.4 kg/m^2 . BMI at enrollment was higher in those with significant fibrosis (29.2 ± 6.5 kg/m^2 vs 26.4 ± 6.1 kg/m^2; p=0.0384). BMI at enrollment was associated with degree of fibrosis. (26.4 ± 6.1 Ishak 0-1, vs 27.5 ± 4.8 Ishak 2-3; and 31.7 ± 7.9kg/m^2 Ishak ≥4; p= 0.0083). Prevalence of obesity (defined as BMI > 29) was also associated with degree of fibrosis. (14.9% Ishak 0-1, vs 31% Ishak 2-3; and 50% Ishak ≥4; p= 0.0107). The prevalence of metabolic syndrome in our cohort was 11.5%, which is less than the prevalence often quoted for the US population as a whole. Metabolic syndrome was associated with an increased degree of fibrosis, and none of the subjects in the minimal fibrosis group had metabolic syndrome (p=0.0058) (Table 2). The individual components of metabolic syndrome of hypertension and dyslipidemia were not associated with the degree of fibrosis (Table 2). We also assessed the degree of steatosis on liver biopsy and studied its association with fibrosis. Brunt's

macrovesicular steatosis score did not correlate with the degree of fibrosis (Table 3, Supplementary 2).

Circulating serum Z polymer levels in AATD liver and lung disease

In AATD, liver disease is caused by the accumulation of the mutant Z protein in hepatocytes, which triggers an intracellular injury cascade, hepatocellular death, regeneration and fibrosis. Some of the mutant Z protein attains a unique, “polymerized” conformation, which is especially toxic. Tiny amounts of the Z polymer (<1% total serum Z AAT) are found in the serum of patients with ZZ AATD. Mean circulating serum Z polymer levels of the cohort were 11.9 ± 7.1 µg/ml. Higher mean circulating Z polymer levels were associated with increased degree of fibrosis (9.7 ± 6.8 µg/ml; Ishak 0-1, 12.1 ± 4.3 µg/ml; Ishak 2-3, 16.1 ± 8.1 µg/ml; Ishak ≥ 4; p= 0.0194). The ratio of circulating Z polymer to total AAT was also higher in those with significant fibrosis (Table 2). Higher circulating Z polymer levels were also associated with features of inflammation on the biopsy of periportal and portal inflammation, and disease activity as indicated by NAI score (Supplementary table 3). Regression model output showed that increase in circulating Z polymer levels was associated with increase in AST, and not associated with changes in ALT or GGT (β = 0.1510; p= 0.0292, Supplementary table 4). Changes in circulating Z polymer levels are also associated with reduction in lung function (β = -0.5541; p= 0.0354). Circulating Z polymer levels were not affected by smoking, gender, or BMI, and were not significantly correlated to age.

Other Biochemical and histological characteristics of the cohort

While increased inflammation is well described in previous reports of ZZ liver, it is usually characterized as modest. The role of inflammation is controversial. Is it part of the injury

cascade, or is it a secondary or bystander effect of the lack of the full level of the anti-inflammatory action of wild type AAT in serum? Here we observed that portal inflammation (70.7% prevalent) and lobar inflammation (61% prevalent) were the most common histological features seen in biopsies in our cohort, other than AAT protein accumulation, although the degree of inflammation was usually mild. NAI score that depicts grade of chronic hepatitis or disease activity was low (mean score 2.4± 1.98) but correlated with the stage of fibrosis (1.55±1.44 in the minimal fibrosis group vs 3.69 ± 1.96 in advanced fibrosis group; $p < 0.0001$, Table 3). In those with significant fibrosis; majority (83.3% vs 10.6 %; $p <0.0001$; Supplementary table 2) had higher NAI score (≥3) Periportal and portal inflammation correlated to degree of fibrosis (Table 3), and were significantly more frequent in biopsies with significant fibrosis (Supplementary table 2; p: <0.0001, 0.0026, respectively).

Biochemical markers for chronic liver disease studied include low albumin, high AST, ALT, GGT, total and direct bilirubin, low platelet counts, high INR and alkaline phosphatase. Although mean levels were associated with significantly increased fibrosis (Supplementary table 5), they would not be regarded as particularly important elevations to most clinicians. We studied elevations of AST, ALT and GGT (ALT> 35, AST > 35, GGT> 40 U/L) and its relationship to inflammation and fibrosis (supplementary table 3). AST > 35 U/L was associated with increased chronic hepatitis activity as indicated by NAI score, increased periportal and lobar inflammation, and increased steatosis. ALT > 35 U/L, however, was not associated with inflammation on biopsy except portal inflammation (p=0.0496; Supplementary Table 3).

Hepatic Z polymer protein content correlates with fibrosis and non-invasive markers of injury.

Finally, we used published techniques to label intrahepatic accumulations of the toxic Z protein polymer with 2C1 antibody immunohistochemistry and quantified with previously validated electronic scanning. Previous work has shown that the Z protein in the polymer confirmation is present in both non-dilated ER (a diffuse cytoplasmic stain pattern), and as the “globule” hepatocellular inclusions characteristic of this disease, which are known to be composed of 100% Z protein polymer (Figure 3)[1, 3]. We found that increased fibrosis was significantly associated with increased biopsy area of mutant Z polymer “globular” inclusions (p=0.002) and increased diffuse cytoplasmic Z polymer area (p=0.0029). Univariate analysis revealed increased serum Z polymer was highly significantly associated with increased biopsy area of mutant Z polymer “globular” inclusions (p=0.0001), but also increased diffuse cytoplasmic Z polymer area to a lesser degree(p=0.014). Higher serum AST was significantly associated with increased globular inclusion area (p=0.0064), but not associated with diffuse cytoplasmic stain area. ALT had no significant relationship to liver Z polymer accumulation.

Discussion

Our study is a unique multicenter, prospective cohort study of adults with a full range of ZZ AATD liver involvement, employing protocol liver biopsies, unlike any other previous study of this disease. We were especially focused on gathering data on the relationship of liver biopsy findings and hepatic content of the toxic Z protein polymer, to fibrosis and to other

parameters[25,26].[24] We were able to study a wide spectrum of liver disease characteristics in ZZ patients, including advanced and end stage liver disease. About half of our cohort had increased liver fibrosis which will increase the utility compared to other cohorts which reported very few advanced cases.[5,11] Previous adult prospective cohorts described liver disease mainly in the non-cirrhotic group, or in lung disease patients with only minimal fibrosis.[11]

We found that the common serum tests of AST, ALT, GGT, APRI and Fib4 are correlated with increased fibrosis but are so variable that single measures are not very useful until fibrosis is advanced. AST being somewhat an exception as it appears more sensitive and is strongly correlated to both hepatic content of the toxic Z protein polymer and to liver fibrosis. Other biopsy features were variable in their value. Inflammation was modest but even small increases in periportal inflammation were associated with multiple indicators of worse disease. Interestingly, despite the apparent role for obesity, steatosis on the biopsy, a feature known to be common in AATD liver, was not related to fibrosis, other markers of injury or serum Z polymer.

We documented a strong link between increased serum Z polymer level, AST, fibrosis and other markers of more severe disease. Perhaps most surprising is the strong and direct proportional relationship between serum Z polymer and liver Z polymer accumulation as globules. In future clinical trials of liver interventions to reduce liver Z polymer related injury, it might not be necessary to do liver biopsies if serum Z polymer levels track so closely with liver content of the accumulated toxic protein polymers.

We also not only confirm previous findings from Clark that metabolic syndrome is a significant risk factor for worse liver fibrosis in AATD, but since we captured a wider range of disease we were able to drill down further on this finding. We see that it is the BMI factor, not hypertension

or dyslipidemia which is the most powerful influence on liver fibrosis in AATD. We propose that weight loss advice to maintain BMI <29 kg/m^2 could be a useful intervention to prevent fibrosis, although further study will be needed to test this intervention. These data showing the importance of increasing fibrosis strongly support a recommendation that individuals with any detected increase in fibrosis should be monitored on a regular, probably at least annual, basis for rapid progression of liver disease. This group would also be ideal for enrollment into many of the new drug trials which are now being opened for this disease.

It also seems wise to strongly advise all AATD “lung disease” patients to be followed regularly for the development of liver fibrosis. Previous studies have shown, and our data confirm, that liver enzymes may have limited value in identity patients with liver injury in ZZ AATD.[27,28,29]

In our study, elevated AST, rather than ALT, was strongly associated with fibrosis and with increased liver Z polymer accumulation. AST is cytoplasmic and a mitochondrial enzyme in the hepatocyte, while ALT is mostly cytoplasmic.[30,31] Mitochondrial injury is an important mechanism of liver injury in ZZ AATD.[3,32] We hypothesize that AST is a surrogate for mitochondrial injury. This can also be related to the utility we show of the APRI. We propose that since serum Z polymer is directly correlated to intrahepatic Z polymer, that the more intrahepatic Z polymer, the more mitochondrial injury and the higher the AST. We note that previous data we reported in children found that GGT was a powerful clinical predictor, but that was not found to be as powerful in these adult subjects.

Our study, despite provocative and new, does have significant limitations. First, is a rather modest “n.” This limited somewhat the statistical tests which could be applied, for example analysis of Kaplan-Meier curves, given the number of events, did not add any clarity to the interpretation of the results. Furthermore, we could only measure the serum Z polymer in

participants who were not on exogenous alpha-1 intravenous protein replacement for lung disease. While this was not the majority of participants, and since we did not see a relationship between lung disease severity and liver disease severity, it seems likely that this situation did not affect the conclusions, but it must be noted.

In conclusion, increased fibrosis is prevalent in adults with ZZ AATD. Clinical signs of liver disease and elevations of liver enzymes are often delayed until fibrosis is significant. Early detection of increasing fibrosis, which might require a liver biopsy to fully clarify, and would then more strongly encourage close follow up, is necessary. Obesity could be a modifiable risk factor to prevent progression of fibrosis. Liver enzymes have limited value in identifying early fibrosis. Serum Z polymer levels appear to be a promising, non-invasive, disease specific biomarker. Further study, including 5-year follow up data and correlation to biopsy is needed to determine use in clinical care and study enrollments.

References

1. Lindblad D, Blomenkamp K, Teckman J. Alpha-1-antitrypsin mutant Z protein content in individual hepatocytes correlates with cell death in a mouse model. *Hepatology*. Oct 2007;46(4):1228-35. doi:10.1002/hep.21822
2. Teckman JH, Blomenkamp KS. Pathophysiology of Alpha-1 Antitrypsin Deficiency Liver Disease. *Methods Mol Biol*. 2017;1639:1-8. doi:10.1007/978-1-4939-7163-3_1
3. Greene CM, Marciniak SJ, Teckman J, et al. alpha1-Antitrypsin deficiency. *Nat Rev Dis Primers*. Jul 28 2016;2:16051. doi:10.1038/nrdp.2016.51
4. Bowlus CL, Willner I, Zern MA, et al. Factors associated with advanced liver disease in adults with alpha1-antitrypsin deficiency. *Clin Gastroenterol Hepatol*. Apr 2005;3(4):390-6. doi:10.1016/s1542-3565(05)00082-0
5. Tanash HA, Piitulainen E. Liver disease in adults with severe alpha-1-antitrypsin deficiency. *J Gastroenterol*. Jun 2019;54(6):541-548. doi:10.1007/s00535-019-01548-y
6. Hamesch K, Mandorfer M, Pereira VM, et al. Liver Fibrosis and Metabolic Alterations in Adults With alpha-1-antitrypsin Deficiency Caused by the Pi*ZZ Mutation. *Gastroenterology*. 09 2019;157(3):705-719.e18. doi:10.1053/j.gastro.2019.05.013

7. Teckman JH. Emerging Concepts and Human Trials in Alpha-1-Antitrypsin Deficiency Liver Disease. *Semin Liver Dis*. May 2017;37(2):152-158. doi:10.1055/s-0037-1602586
8. Mostafavi B, Diaz S, Tanash HA, Piitulainen E. Liver function in alpha-1-antitrypsin deficient individuals at 37 to 40 years of age. Observational Study. *Medicine (Baltimore)*. Mar 2017;96(12):e6180. doi:10.1097/MD.0000000000006180
9. Teckman J, Rosenthal P, Hawthorne K, et al. Longitudinal Outcomes in Young Patients with Alpha-1-Antitrypsin Deficiency with Native Liver Reveal that Neonatal Cholestasis is a Poor Predictor of Future Portal Hypertension. *J Pediatr*. Dec 2020;227:81-86 e4. doi:10.1016/j.jpeds.2020.07.031
10. Janciauskiene S, Wallmark A, Piitulainen E, Kohnlein T, Welte T, Sveger T. Performance of enhanced liver fibrosis plasma markers in asymptomatic individuals with ZZ alpha1-antitrypsin deficiency. *Eur J Gastroenterol Hepatol*. Aug 2011;23(8):716-20. doi:10.1097/MEG.0b013e328347daaf
11. Clark VC, Marek G, Liu C, et al. Clinical and histologic features of adults with alpha-1 antitrypsin deficiency in a non-cirrhotic cohort. *J Hepatol*. 12 2018;69(6):1357-1364. doi:10.1016/j.jhep.2018.08.005
12. Schneider CV, Hamesch K, Gross A, et al. Liver Phenotypes of European Adults Heterozygous or Homozygous for Pi *Z Variant of AAT (Pi *MZ vs Pi *ZZ genotype) and Noncarriers. *Gastroenterology*. Aug 2020;159(2):534-548 e11. doi:10.1053/j.gastro.2020.04.058
13. Fromme M, Schneider CV, Pereira V, et al. Hepatobiliary phenotypes of adults with alpha-1 antitrypsin deficiency. *Gut*. Feb 2022;71(2):415-423. doi:10.1136/gutjnl-2020-323729
14. Fromme M, Schneider CV, Trautwein C, Brunetti-Pierri N, Strnad P. Alpha-1 antitrypsin deficiency: A re-surfacing adult liver disorder. *J Hepatol*. Apr 2022;76(4):946-958. doi:10.1016/j.jhep.2021.11.022
15. Chen VL, Burkholder DA, Moran IJ, et al. Hepatic decompensation is accelerated in patients with cirrhosis and alpha-1 antitrypsin Pi *MZ genotype. *JHEP Rep*. Jun 2022;4(6):100483. doi:10.1016/j.jhepr.2022.100483
16. Huang PL. A comprehensive definition for metabolic syndrome. *Dis Model Mech*. 2009;2(5-6):231-7. doi:10.1242/dmm.001180
17. Graham BL, Steenbruggen I, Miller MR, et al. Standardization of Spirometry 2019 Update. An Official American Thoracic Society and European Respiratory Society Technical Statement. *Am J Respir Crit Care Med*. Oct 15 2019;200(8):e70-e88. doi:10.1164/rccm.201908-1590ST
18. Accessed 08/29, 2019. https://www.cdc.gov/nchs/nhis/tobacco/tobacco_glossary.htm
19. Pleasants RA, Rivera MP, Tilley SL, Bhatt SP. Both Duration and Pack-Years of Tobacco Smoking Should Be Used for Clinical Practice and Research. *Ann Am Thorac Soc*. Jul 2020;17(7):804-806. doi:10.1513/AnnalsATS.202002-133VP
20. Crabb DW, Im GY, Szabo G, Mellinger JL, Lucey MR. Diagnosis and Treatment of Alcohol-Associated Liver Diseases: 2019 Practice Guidance From the American Association for the Study of Liver Diseases. *Hepatology*. Jan 2020;71(1):306-333. doi:10.1002/hep.30866
21. Tan L, Dickens JA, Demeo DL, et al. Circulating polymers in α1-antitrypsin deficiency. *Eur Respir J*. May 2014;43(5):1501-4. doi:10.1183/09031936.00111213
22. Elliott PR, Bilton D, Lomas DA. Lung polymers in Z alpha1-antitrypsin deficiency-related emphysema. *Am J Respir Cell Mol Biol*. May 1998;18(5):670-4.
23. Goodman ZD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *J Hepatol*. Oct 2007;47(4):598-607. doi:10.1016/j.jhep.2007.07.006
24. Teckman JH, Rosenthal P, Abel R, et al. Baseline Analysis of a Young alpha-1-Antitrypsin Deficiency Liver Disease Cohort Reveals Frequent Portal Hypertension. *Journal of pediatric gastroenterology and nutrition*. Jul 2015;61(1):94-101. doi:10.1097/MPG.0000000000000753
25. Sterling RK, Wegelin JA, Smith PG, et al. Similar progression of fibrosis between HIV/HCV-infected and HCV-infected patients: Analysis of paired liver biopsy samples. *Clin Gastroenterol Hepatol*. Dec 2010;8(12):1070-6. doi:10.1016/j.cgh.2010.08.004

26. Ryder SD, Irving WL, Jones DA, Neal KR, Underwood JC, Group THCS. Progression of hepatic fibrosis in patients with hepatitis C: a prospective repeat liver biopsy study. *Gut*. Mar 2004;53(3):451-5. doi:10.1136/gut.2003.021691
27. Clark VC, Dhanasekaran R, Brantly M, Rouhani F, Schreck P, Nelson DR. Liver test results do not identify liver disease in adults with α(1)-antitrypsin deficiency. *Clin Gastroenterol Hepatol*. Nov 2012;10(11):1278-83. doi:10.1016/j.cgh.2012.07.007
28. American Thoracic Society/European Respiratory Society Statement: Standards for the Diagnosis and Management of Individuals with Alpha-1 Antitrypsin Deficiency. *Am J Respir Crit Care Med*. Oct 1 2003;168(7):818-900.
29. Neuschwander-Tetri BA, Unalp A, Creer MH, Network NSCR. Influence of local reference populations on upper limits of normal for serum alanine aminotransferase levels. *Arch Intern Med*. Mar 24 2008;168(6):663-6. doi:10.1001/archinternmed.2007.131
30. Lala V ZM, Minter DA. Liver Function Tests. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing;. https://www.ncbi.nlm.nih.gov/books/NBK482489/
31. Giannini EG, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. *CMAJ*. Feb 01 2005;172(3):367-79. doi:10.1503/cmaj.1040752
32. Teckman JH, An JK, Blomenkamp K, Schmidt B, Perlmutter D. Mitochondrial autophagy and injury in the liver in alpha 1-antitrypsin deficiency. *Am J Physiol Gastrointest Liver Physiol*. May 2004;286(5):G851-62. doi:10.1152/ajpgi.00175.2003

Legends for Figures

Figure 1. Proportion of the cohort with Ishak fibrosis stages as shown.

Figure 2: Relationship of lung function at enrollment to age at enrollment shown by plotting enrollment ppFEV1 versus age. Decreased ppFEV1 is significantly associated with increased age but ppFEV1 is not significantly associated with increased fibrosis (p=0.8).

Figure 3: Representative photomicrographs at low and higher power of liver from participants with Ishak 0, 2, and 4 fibrosis (fib), as shown, stained with H&E, Periodic Acid-Schiff with Digestion (DPAS), and 2C1 anti-polymer antibody “IHC”.

Table 1: Demographics of the cohort

	Overall (N=96)	**Ishak score- 0-1 (N=47)**	**Ishak Score >1 (N=49)**	**p value**
Gender	%	%	%	
Female	50.0	53.2	46.9	0.5402
Male	50.0	46.8	53.1	
Race (reported)				1.0000
White	94.8	95.7	93.9	
Other/Multiracial	5.2	4.3	6.1	
Mean Age at enrollment (years)	54.6 (14.1)	55.4 (12.6)	54.0 (15.4)	0.6436
Mean Age at diagnosis (years)	45.9 (17.7)	45.6 (15.6)	46.1 (19.2)	0.9151
Reason for AATD testing				0.0113
Liver disease	6.3	2.1	10.2	
Lung disease	34.4	38.3	30.6	
Family testing	25.0	36.2	14.3	
Other reasons	22.9	19.2	26.5	
More than one reason	7.3	0.0	14.3	
Unknown	4.2	4.3	4.1	
Previous medical history				
Neonatal cholestasis	6.3	8.5	4.1	0.5989
Childhood asthma	14.6	17.0	12.2	0.4261
Lung Function status	N = 52	N = 24	N = 28	
Mean ppFVC (SD)	95.6 (15.5)	100.0 (14.5)	91.8 (15.6)	0.0558
Mean ppFEV1 (SD)	82.0 (22.6)	81.8 (24.8)	82.1 (20.9)	0.9605

Mean ppFEV1/FVC (SD)	86.0 (21.7)	82.8 (25.4)	88.8 (17.9)	0.3189
Mean AUDIT score (SD)	2.3 (3.3)	2.1 (2.5)	2.5 (3.9)	0.6001
Smoking status (%)				0.7194
Non-Smoker	67.7	66.0	69.4	
Past Smoker	32.3	34.0	30.6	
Smoking burden	N = 96	N = 46	N = 46	
Mean pack years (SD)	1388.5 (3156.6)	1633.7 (3541.3)	1352.9 (2780.6)	0.6673
Protein replacement therapy (%)	36.5	42.6	30.6	0.2243
Oxygen therapy (%)	11.5	10.6	12.2	1.0000

Fig 1. Liver biopsy-based fibrosis in the cohort

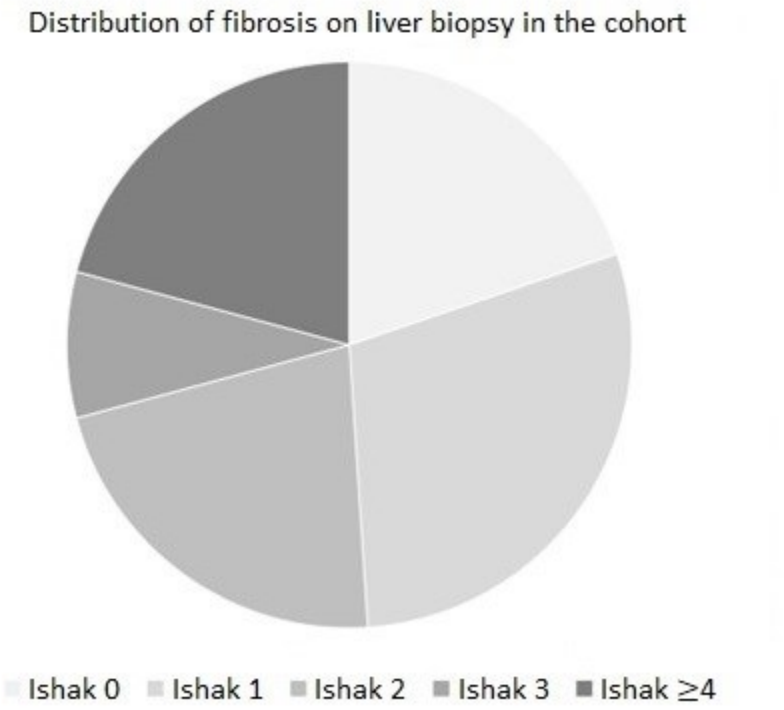

Figure 2: Relationship between lung function and age at enrollment

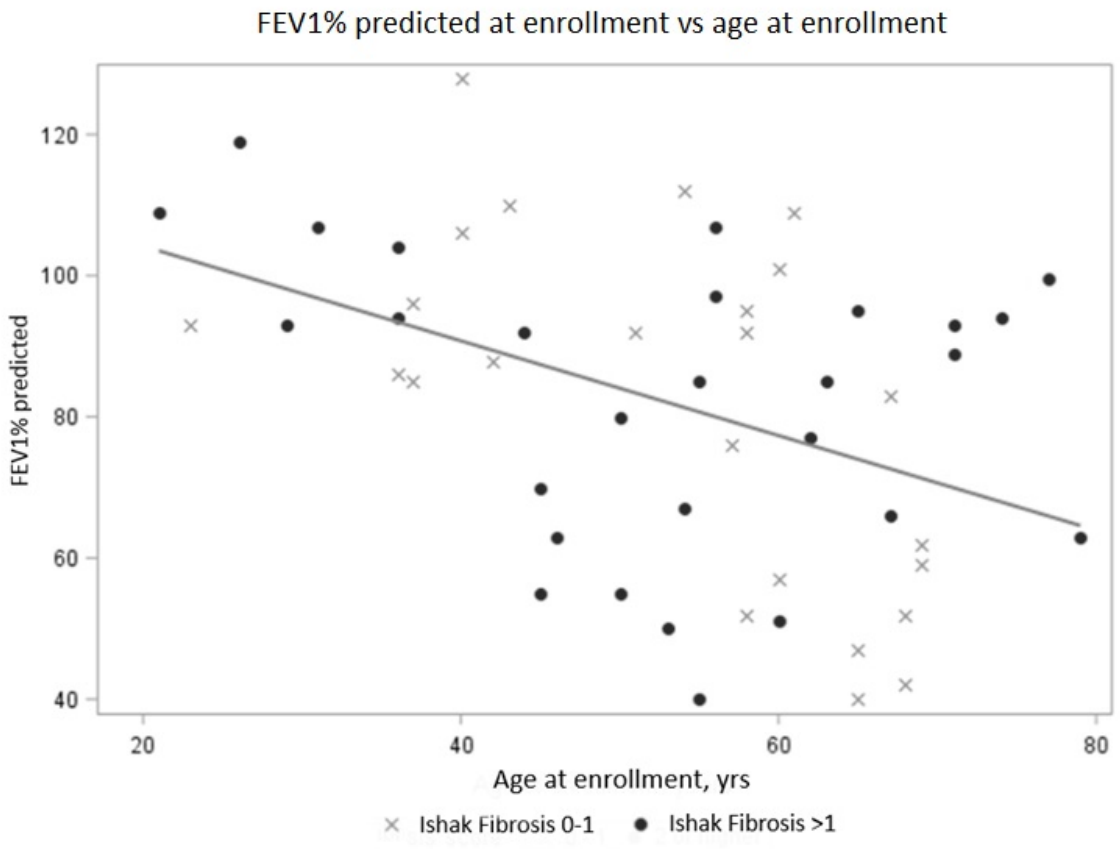

Fig 2: Relationship of lung function at enrollment to age at enrollment shown by plotting enrollment ppFEV1 versus age. Decreased ppFEV1 is significantly associated with increased age but ppFEV1 is not significantly associated with increased fibrosis (p=0.8).

Table 2: Factors associated with degree of fibrosis in our cohort

Parameter	**Overall (N=96)**	**Fibrosis 0-1 (N=47)**	**Fibrosis 2-3 (N=29)**	**Fibrosis ≥4 or Known-severe (N=20)**	**p value**
Age (years) at enrollment, Mean (SD)	54.7 (14.0)	55.4 (12.6)	53.9 (14.1)	54.3 (17.7)	0.8949
Sex (%)					
Female	50.0	53.2	41.4	55.0	0.5343
Male	50.0	46.8	58.6	45.0	
BMI (kg/m^2) at enrollment Mean (SD)	27.8 (6.4)	26.4 (6.1)	27.5 (4.8)	31.7 (7.9)	**0.0083**
Obesity (BMI >29 kg/m2) (%)	27.1	14.9	31.0	50.0	**0.0107**
Metabolic syndrome (%)	8.3	0.0	17.2	15.0	**0.0146**
AST(IU/L) Mean (SD)	31.1 (15.0)	25.6 (7.4)	28.1 (7.9)	48.8 (22.6)	**<.0001**
ALT(IU/L) Mean (SD)	29.3 (16.2)	23.3 (9.3)	31.9 (20.8)	40.2 (15.4)	**0.0002**
GGTP(IU/L) Mean (SD)	35.7 (30.8)	28.0 (22.7)	28.7 (15.6)	67.2 (46.0)	**<.0001**
AST/Platelet Ratio Index (APRI) Mean (SD)	0.49(0.72)	0.3 (0.1)	0.3 (0.1)	1.2 (1.4)	**<.0001**
FIB-4 score Mean (SD)	1.94 (2.28)	1.4 (0.7)	1.3 (0.6)	4.2 (4.3)	**<.0001**
Anti-Hypertension medication use (%)	19.8	21.28	24.14	10	0.4452
Anti- Hyperlipidemic drug use (%)	11.5	8.51	13.79	15	0.6684
Anti-Diabetic medication use (%)	1.0	0	3.45	0	0.3112
	Overall (N=55)	**Fibrosis 0-1 (N=27)**	**Fibrosis 2-3 (N=14)**	**Fibrosis ≥4 or Known-severe (N=14)**	
Circulating Z polymer ug/ml Mean (SD)	11.9 (7.1)	9.7 (6.8)	12.1 (4.3)	16.1 (8.1)	**0.0194**
percent total AAT as Z polymer Mean (SD)	4.3 (2.7)	3.5 (2.0)	3.4 (1.3)	6.8 (3.7)	**0.0002**

Figure 3. Liver Photomicrographs and Anti-Z Protein Polymer Immunohistochemistry

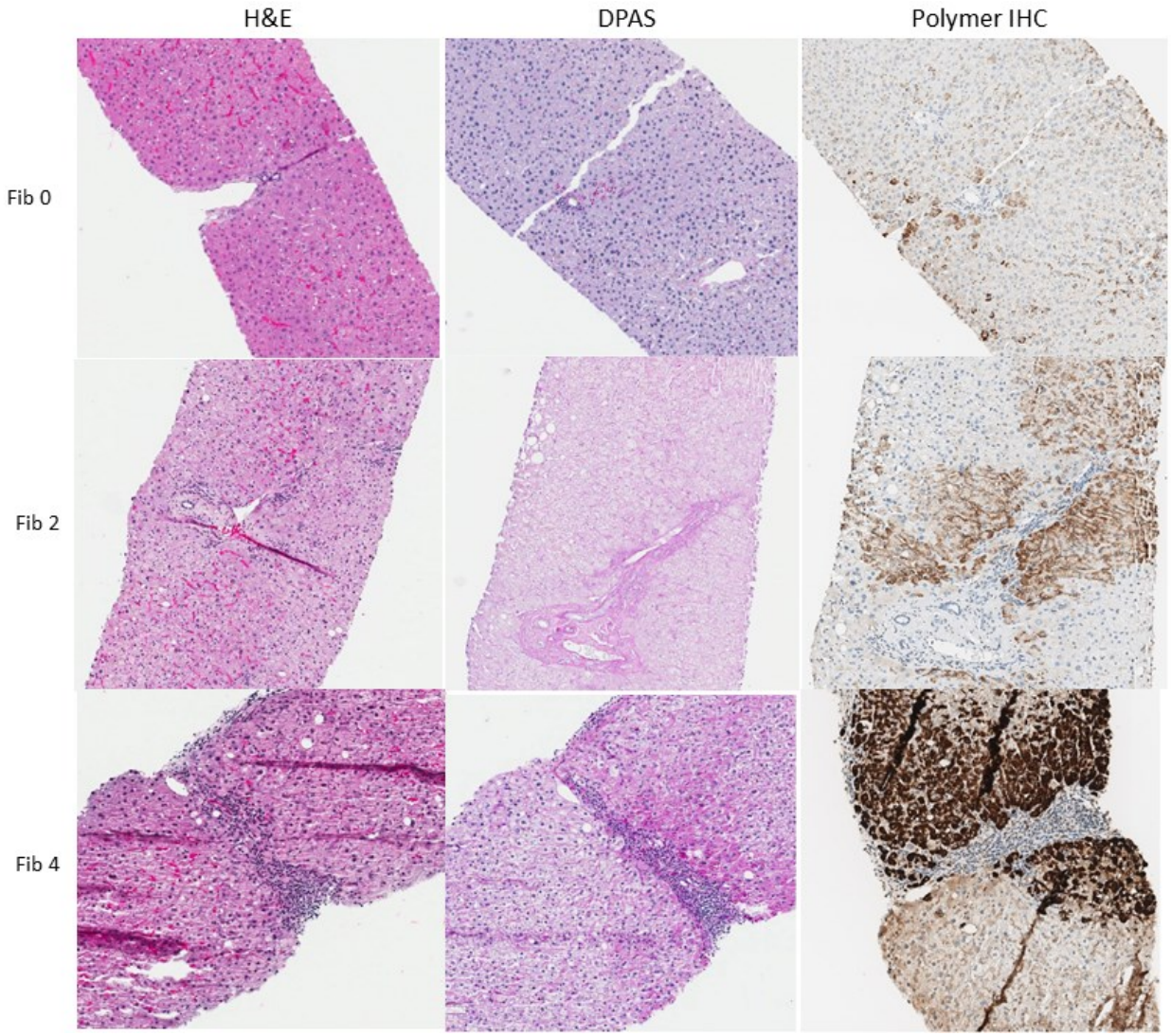

Table 3: Correlation of biopsy features and stage of fibrosis

Biopsy features	**Correlation to fibrosis stage**	**P value of correlation**
	N = 82	

Periportal inflammation	0.68776	<.0001
Lobar inflammation	0.20655	0.0626
Portal inflammation	0.60204	<.0001
Lobular necrosis	0.04013	0.7221
Necro inflammatory Activity Score	0.65245	<.0001
Brunt Macrovesicular Steatosis Score	0.06314	0.5731
Brunt Hepatocellular Ballooning Score	0.29827	0.0065
Microvesicular steatosis observed	0.08559	0.4445