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Plasminogen activator inhibitor is significantly elevated in liver transplant recipients with decompensated NASH cirrhosis

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ABSTRACT

Background Non-alcoholic fatty liver disease is a prohaemostatic state with abnormal primary, secondary and tertiary haemostasis. Plasminogen activator inhibitor (PAI)-1 is the best-established marker for prohaemostasis in non-alcoholic fatty liver disease. While epidemiological studies demonstrate decompensated non-alcoholic steatohepatitis (NASH) cirrhosis patients have increased rates of venous thromboembolism, including portal vein thrombosis, mechanistic studies have focused exclusively on patients without or with compensated cirrhosis. We aimed to characterizecharacterise PAI-1 levels in decompensated NASH cirrhosis.

Methods PAI-1 level was measured in consecutive adult liver transplant recipients immediately prior to liver transplantation. Multivariable models were constructed using linear regression to assess factors related to PAI-1 level.

Results Forty-six subjects with mean age 57 (IQR 53–62) years and Model for Endstage Liver Disease (MELD) score of 34 (IQR 30–40) were enrolled. Baseline characteristics were similar between NASH (n=10) and non-NASH (n=36) subjects except for rates of diabetes and hyperlipidaemia. Mean PAI-1 level was greater in NASH (53.9, 95% CI 33.3 to 74.5 mg/mL) when compared with non-NASH (36.1, 95% CI 28.7 to 43.5), p=0.040. NASH remained independently predictive of PAI-1 level prior to transplant on adjusted multivariable modelling (β 40.13, 95% CI 14.41 to 65.86, p=0.003). Conclusions: PAI-1 level is significantly elevated in decompensated NASH cirrhosis independent of other pro-haemostatic factors. This may explain the greater rates of venous thromboembolism in decompensated NASH cirrhosis. Future study focusing on prevention of venous thromboembolism in this population is paramount to improve patient-oriented outcomes given the high morbidity and mortality of venous thromboembolism and the significant impact it has on transplant candidacy.

Summary box

What is already known about this subject?

► It is known that plasminogen activator inhibitor (PAI)-1 is the best-established marker for prohaemostasis in non-alcoholic fatty liver disease.

What are the new findings?

► PAI-1 level is significantly elevated in decompensated non-alcoholic steatohepatitis (NASH) cirrhosis independent of other prohaemostatic factors. This may explain the greater rates of venous thromboembolism in decompensated NASH cirrhosis.

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

► Understanding the role of PAI-1 levels in venous thromboembolism events in this population is vital to prevent such events and improve patient-oriented outcomes given the high morbidity and mortality of venous thromboembolism and the significant impact it has on transplant candidacy.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in the US affecting 100 million US adults.[1] In parallel with the worsening obesity epidemic, NAFLD rates are expected to double by 2025.[2–4] Non-alcoholic steatohepatitis (NASH) is the more severe variant of NAFLD and progresses to cirrhosis in upwards of one in every five patients.[5] Twenty-five million US adults have NASH; five million have NASH cirrhosis.[6–8] Progression of NASH cirrhosis to endstage liver disease (ESLD) is common and NASH cirrhosis is projected to be the leading reason for liver transplantation by 2025. This is already true for women.[9]

Beyond the vast need for lifesaving liver transplantation, there are many extrahepatic manifestations of NAFLD and NASH including those attributable to the unique prohaemostatic environment.[10] Patients with NAFLD and NASH are at increased risk for venous thromboembolism (VTE).[11–13] Chronic inflammation from hepatic steatosis culminates in activation of the coagulation system and abnormalities in all three phases

of haemostasis.[8 14] Haemostasis can be broken down into primary, secondary and tertiary components. Increased platelet activation (primary haemostasis) has been observed in NASH as have elevated levels of vonWillebrand factor.[14–16] Hypercoagulability (secondary haemostasis) manifests through increased levels of Factor VIII and fibrinogen.[8 14] Levels of anticoagulants antithrombin and protein C are also decreased, tipping the haemostatic balance towards clotting. Plasminogen activator inhibitor one (PAI-1) is elevated in NASH while tissue activating factor antigen and tissue plasminogen activator are decreased. This leads to a chronic state of hypofibrinolysis (tertiary haemostasis) or clot breakdown.[17 18]

Produced by adipose cells, elevated PAI-1 is the best-described marker for prohaemostasis in NAFLD and NASH. PAI-1 independently promotes thrombotic risk and may accelerate liver disease progression due to local tissue ischaemia stemming from intrahepatic thrombi, a theory coined by Ian Wanless as *parenchymal extinction*.[19 20] Clinically, the presence of at least one thrombotic risk factor is associated with a nearly twofold fibrosis stage increase in NASH, further supporting the notion of thrombosis and disease progression.[18]

While epidemiological studies clearly demonstrate that decompensated NASH cirrhosis patients have increased rates of VTE, including deep vein thrombosis (DVT), pulmonary embolism (PE) and portal vein thrombosis (PVT).[12 13 21] Mechanistic studies have ignored this population at greatest risk and instead focused exclusively on patients without or with compensated cirrhosis. As DVT, PE and PVT have significantly increased morbidity and mortality rates and the occurrence of each greatly impacts not only liver transplantation candidacy but also post-transplant survival, a better understanding of the prohaemostatic environment in liver transplant candidates including those candidates with NASH who are at greatest risk for VTE, is imperative to improve patient-centred outcomes.[22–24] For these reasons, we aimed to characterise PAI-1 levels in decompensated NASH cirrhosis in order to further study the prohaemostatic environment of this common condition.

EXPERIMENTAL PROCEDURES

Consecutive adult liver transplant recipients were enrolled at a single US-based tertiary care academic centre from 2011 to 2018. Status 1a recipients were excluded. Baseline demographics, aetiology and severity of liver disease as well as standard laboratories were captured. Plasma samples were prospectively collected and obtained on day 0 immediately prior to and again on day 5 following liver transplantation and placed into a biobank.

PAI-1 measurement

PAI-1 level was determined by ELISA from the samples collected in the biobank. The assay began by coating a 96-well multiwell plate with PAI-1 antihuman monoclonal antibody overnight at 4°C. The sample was then added to the coated wells. PAI-1 present in the sample or standard binded to the PAI-1 antihuman monoclonal antibody. Next, a biotin-conjugated anti-human PAI-1 polyclonal antibody was added and it binded to the PAI-1 captured. The plate was then incubated for 2 hours at room temperature (18°C to 25°C). After incubation, the plate was washed (three times) with wash buffer (phosphate-bufered saline with 0.5% Tween 20). Streptovidin-Horseradish Peroxidase Conjugate anti-human PAI-1 antibody was added to the plate. Next, it was incubated for 1 hour at room temperature. Following incubation, the wells were washed (five times) with wash buffer. Penultimate, a colour producing substrate TMB (tetramethyl-benzidine) substrate solution, was added to the wells. The plate was incubated for 10 min at room temperature and in the dark. A blue colour formed based on an enzymatic reaction. The intensity of the colour was directly proportional to the amount of human PAI-1 present in the standards. Finally, stop solution (1 M phosphoric acid) was added to the whole plate. The samples turned yellow indicating the end of the enzymatic reaction. To measure the fluorescent output signal, we used a microplate reader (BIO-TEK EL311). Seven serially diluted PAI-1 standards were included in the plate to generate a standard curve, which then was used to calculate the concentration of the unknown samples.

Statistical analysis

Subjects with decompensated NASH cirrhosis were compared with those without-NASH across multiple important baseline demographics, aetiology and severity of liver disease and laboratories, including PAI-1 levels. Standard univariate analysis was performed for categorical and continuous variables as appropriate using student t-test, χ^2, Fisher-exact test and Wilcoxon sign rank test. Multivariable models were constructed using linear regression to assess factors related to PAI-1 level. Final variables included in the model included age in years, body mass index (BMI) (kg/m^2), diabetes, hyperlipidaemia, NASH and MELD score. Variables were entered into the model for a p value <0.1 or if the variable had previously been shown to be clinically significant. SAS V.9.4 (Cary, North Carolina) was used for all statistical analyses. A p value of <0.05 was considered statistically significant. No data imputation was performed. No transplants for prisoners were included in the analysis. The Penn State Health Sciences Research Institutional Review Board approved this study.

RESULTS

Study cohort

A total of 46 subjects with mean age 57 (IQR 53–62) years and MELD score of 34 (IQR 30–40) met the criteria for inclusion in our study analysis as shown in table 1.

Eighty per cent of the cohort was men. Ten subjects had NASH cirrhosis and 36 were non-NASH (11 chronic hepatitis C, 10 hepatitis C with alcohol-associated liver

Table 1 Baseline comparison of NASH vs non-NASH liver transplant recipients

	NASH (n=10)	Non-NASH (n=36)	P value
Age, years	56.5 (15.2)	56.8 (8.3)	0.939
Male sex	70 (7)	83 (30)	0.974
BMI, mean kg/m^2	33.8 (7.9)	29.8 (5.6)	0.16
Laboratories			
Creatinine (mg/dL)	1.8 (0.9)	1.1 (0.5)	0.003
INR	3.3 (1.1)	2.2 (1.3	0.014
MELD score	37.0 (3.0)	33.0 (7.0)	0.088
Sodium (mEq/L)	132.9 (12.0)	138.0 (5.9)	0.069
Total bilirubin (mg/dL)	18.2 (13.5)	9.3 (9.4)	0.022
Portal hypertension			
Ascites	60 (6)	42 (15)	0.69
Gastro-oesophageal varices	60 (6)	44 (16)	0.736
Hepatic encephalopathy	70 (7)	42 (15)	0.651
Metabolic risk factors			
Diabetes	5 (50)	5 (14)	0.001
Hyperlipidaemia	3 (30)	1 (3)	<0.001
Hypertension	5 (50)	9 (25)	0.2
PAI-1 (mg/mL)	53.9 (26.8)	36.1 (21.3)	0.04

Categorical variables are presented as % (n); continuous variables are presented as mean (standard deviation).
In general, NASH and non-NASH recipients except for a greater frequency of metabolic risk factors in the NASH group and greater PAI-1 levels.
BMI, body mass index; INR, international normalised ratio; MELD, Model for End Stage Liver Disease; NASH, non-alcoholic steatohepatitis; PAI, plasminogen activator inhibitor.

disease, 7 alcohol-associated liver disease, 5 autoimmune biliary disease, 2 cryptogenic without metabolic risk factors, 1 autoimmune hepatitis). When comparing NASH to non-NASH recipients, baseline demographics between the groups were in general similar with several exceptions. NASH recipients had higher MELD scores (37.0±3.0 vs 33.0±7.0, p=0.088), although this was not statistically significant (table 1). NASH recipients also had greater rates of diabetes (50% vs 14% diabetes p=<0.001) and hyperlipidaemia (30% vs 3%, p<0.001). NASH recipients were also more likely to require renal replacement therapy (40% vs 14%, p<0.001). Importantly, perioperative coagulation management was similar between the two groups with respect for the need for intraoperative product administration. Packed red blood cells (6.8 vs 5.7, p=0.611), fresh frozen plasma (4.3 vs 7.2, p=0.184), cryoprecipitate (2.5 vs 2.2, p=0.663) and platelets (2.0 vs 1.8, p=0.792) were transfused at similar frequencies between NASH and non-NASH recipients.

Pretransplant day 0 analysis

Mean PAI-1 level was significantly higher in subjects with NASH cirrhosis (53.9, 95% CI 33.3 mg/mL to 74.5 mg/mL) when compared with non-NASH (36.1, 95% CI 28.7 to 43.5), p=0.040 as depicted in figure 1 and described in table 2.

On adjusted multivariable analysis, NASH cirrhosis remained independently predictive of PAI-1 level (40.13, 95% CI 14.41 to 65.86, p=0.003). No other variable in the model was significant on adjusted analysis including age, BMI, diabetes, hyperlipidaemia or severity of liver disease as measured by MELD score (table 2).

PAI-1 is significantly greater in patients with decompensated NASH cirrhosis at the time of liver transplantation

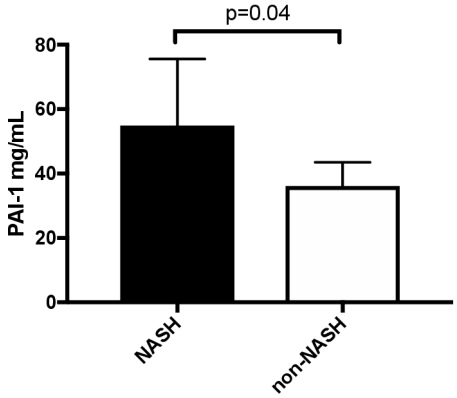

Figure 1 Mean PAI-1 (mg/mL) (+SD) of patients at the time of liver transplantation. N=10 with decompensated NASH cirrhosis and N=36 non-NASH. NASH, non-alcoholic steatohepatitis; PAI, plasminogen activator inhibitor.

Table 2 Multivariable modelling for predictors of PAI-1 level in liver transplant recipients

	β	95% CI	b	t	P
NASH	40.13	(14.41 to 65.86)	0.65	3.18	0.003
Age (years)	0.45	(–0.33 to 1.23)	0.19	1.17	0.249
Body mass index (kg/m^2)	–0.58	(–1.81 to 0.64)	–0.15	–0.97	0.340
Diabetes	–6.65	(–28.96 to15.65)	–0.13	–0.61	0.548
Hyperlipidaemia	–30.33	(–71.10 to 10.45)	0.14	–1.52	0.140
MELD score	0.81	(–0.32 to 1.95)	0.22	1.46	0.155

r^2=0.33 (46, p=0.040).
NASH was the only statistically significant predictor on multivariable modelling of PAI-1 level.
MELD, Model for End Stage Liver Disease; NASH, non-alcoholic steatohepatitis; NASH, nonalcoholic steatohepatitis.

Post-transplant day 5 analysis

While mean PAI-1 level on day 5 after liver transplant was similar between NASH and non-NASH (19.2, 95% CI 4.2 to 34.2 vs 26.3, 95% CI 8.1 to 44.1 mg/mL, p=0.273), PAI-1 level was reduced more significantly in recipients with NASH when compared with non-NASH as shown in figure 2 (–34.8, 95% CI –3.8 to –65.8 vs –9.2, 95% CI +13.6 to –32.1 mg/mL, p=0.009).

DISCUSSION

Previous studies have examined PAI levels in healthy controls and children. However, to our knowledge, this is the first study to investigate PAI-1 level in patients with decompensated NASH cirrhosis. Our findings combined with previous research identifying the mechanism of prohaemostasis in patients with NAFL, early stage NASH and well-compensated NASH cirrhosis advances our understanding of the haemostatic environment across all stages of NASH including ESLD. We have shown that PAI-1 levels are significantly higher in decompensated NASH cirrhosis subjects, independent of liver disease severity, obesity, metabolic risk factors and age, when compared with subjects without NASH cirrhosis. This study also showed that elevated PAI-1 levels in liver transplant recipients with NASH normalise within the first week following liver transplantation and are similar to levels for recipients without NASH cirrhosis.

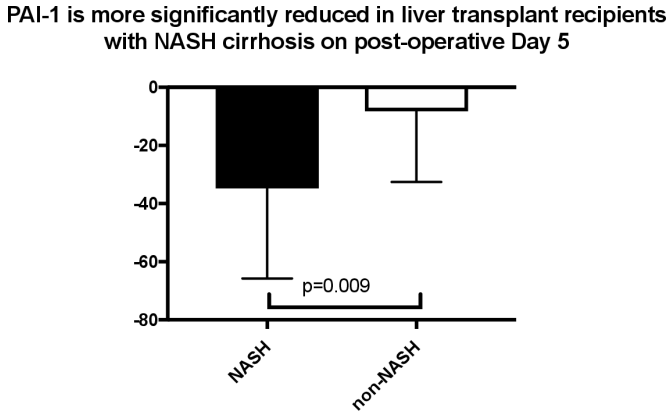

Figure 2 Mean PAI-1 (mg/mL) (+SD) of patients on the postoperative day 5. N=10 with decompensated NASH cirrhosis and N=36 Non-NASH. NASH, non-alcoholic steatohepatitis; PAI, plasminogen activator inhibitor.

These findings have important health implications in the care of liver transplant candidates before and immediately after liver transplantation. As there is a robust body of epidemiological evidence linking NASH to increased rates of PVT, DVT and PE, the findings that PAI-1 is significantly elevated in ESLD in NASH may offer further explanation for these clinically significant thrombotic events.[12 13 21] Whether or not PAI-1 level may be used as a biomarker for VTE risk remains unknown but offers an intriguing avenue for future study in this at-risk population. Additionally, as PAI-1 is produced by adipocytes, it is also unknown whether or not lifestyle intervention can lead to a reduction in PAI-1 through loss of adipocytes in the liver transplant population. Exercise training reduces PAI-1 level up to 37% in healthy persons or those with vascular disease.[25–27]

As NASH increases the risk of pretransplant PVT and this has been linked to post-transplant thrombosis, namely, hepatic artery thrombosis, which carries a significant risk of graft loss and death, the robust normalisation in PAI-1 level within the first week after liver transplantation in recipients with NASH also is worth noting and suggests that liver transplant for NASH may quickly improve or even resolve abnormal fibrinolysis by removing the fatty liver and placing a new liver in the recipient presumably without significant steatosis. Certainly, a dynamic clotting assessment with thromboelastography (TEG) in the immediate postoperative period would serve as a better assessment of the entire haemostatic system including that involved in fibrinolysis, however, TEG was unable to be performed in this study due to technical limitations with sample acquisition. We would also suggest this as an important area for further study to best determine the time frame in which the postoperative haemostatic environment changes following liver transplantation.

In addition to elevated PAI-1 levels in obese individuals, recent work suggests that PAI-1 plays a role in energy intake and expenditure.[28] Various peripheral organs such as the gut, endocrine pancreas and adipose tissue emit signals controlling energy balance. PAI-1 is also produced in gut epithelial and subepithelial cells, increasing the possibility

of unique roles in gastrointestinal function and energy balance.[29] And while we did not specifically measure PAI-1 in our proof of concept study, this is an intriguing line of study as to our knowledge, whether lifestyle alterations focusing on dietary regimens, physical activity and weight loss can lead to a reduction in PAI-1 through loss of adipocytes remains unknown in patients with NASH.

Our study has several other limitations. Specifically, it is based on a single-centre experience that was underpowered to discern post-transplantation outcomes including recipient and graft survival and post-transplantation clotting events including hepatic artery thrombosis and PVT. Larger studies are needed in the future to validate this finding, however, this serves as basis for further study and prospective investigation of PAI-1 as a biomarker of thrombosis risks in NASH cirrhosis. Our study also did not screen for inherited or acquired thrombophilia.[30] Additionally, the included cohort consisted exclusively of Child Pugh Class C disease and, therefore, may not be generalisable to earlier stage liver disease that is less decompensated. We also were unable to analyse differences in body composition, which is important because adipose tissue is a source of PAI-1.

In conclusion, our study adds further evidence that NAFLD and NASH are prohaemostatic states and validation of large-scale epidemiological data by demonstrating PAI-1 levels are significantly elevated in subjects with decompensated NASH cirrhosis. This finding may explain greater rates of VTE in patients with ESLD from NASH. Over the next 5 years, NASH cirrhosis is expected to become the leading indication for liver transplantation in the USA. A better understanding of the hypercoagulable environment in NASH is paramount to improve patient-oriented outcomes given the high morbidity and mortality of VTE in cirrhosis and it's significant impact on transplant candidacy.

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Contributors JS, DB, ZK designed research; JS, GR, BH-B performed research; JS analysed data; GR, JS wrote the paper. All authors approved the final version of this paper.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Penn State Health Milton S. Hershey Medical Center Penn State College of Medicine Human Subjects Protection Office Institutional Review Board IRB STUDY00008958.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data used in this study is deidentified and owned by the Penn State College of Medicine.

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