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Low Serum Fetuin-A as a Biomarker to Predict Pneumococcal Necrotizing Pneumonia and Hemolytic Uremic Syndrome in Children

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Abstract: *Streptococcus pneumoniae*, a neuraminidase-producing pathogen, can cause invasive pneumococcal disease (IPD) with or without hemolytic uremic syndrome (HUS) in humans.

We aimed to identify serum sialoglycoproteins that are targeted by neuraminidases in severe pneumococcal infection. We hypothesized that serum sialoglycoprotein such as fetuin-A can serve as a biomarker to predict IPD or HUS.

We constructed serum sialoglycoprotein profiles before and after pneumococcal neuraminidase treatment using liquid chromatography-tandem mass spectrometry (LC-MS/MS), a proteomic approach. An observational study was conducted using clinical data and serum samples from pediatric patients with pneumococcal infection to verify the predictive role of fetuin-A in IPD. Serum fetuin-A levels were determined by enzyme-linked immunosorbent assay.

The most abundant serum sialoglycoproteins identified by LC-MS/MS after neuraminidase treatment and peanut lectin capture were immunoglobulins, apolipoproteins, fibrinogens, keratins, complement system proteins, and fetuin-A. Serum fetuin-A levels in the HUS patients were significantly lower (207 ± 80 mg/L, $P < 0.001$) than in patients with lobar pneumonia (610 ± 190 mg/L) as well as the healthy controls (630 ± 250 mg/L). In comparing HUS with necrotizing pneumonia and lobar pneumonia, the ROC area under the curve was 0.842; a cutoff value of 298 mg/L yielded sensitivity of 92.9% (95% CI: 68.5–98.7%) and specificity of 71.9% (95% CI: 54.6–84.4%).

This observational study with validation cohorts of patients with HUS, complicated pneumonia, and lobar pneumonia demonstrates the high performance of low serum fetuin-A levels as a biomarker to predict severe IPD and HUS in children.

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Abbreviations: AGP = alpha-1-acid glycoprotein, ANOVA = analysis of variance, apt = activated partial thromboplastin time, AUC = area under the ROC curve, CI = confidence interval, CP = complicated pneumonia, CRP = C-reactive protein, CSF = cerebrospinal fluid, DIC = disseminated intravascular coagulopathy, ELISA = enzyme-linked immuno-sorbent assay, HUS = hemolytic uremic syndrome, IPD = invasive pneumococcal disease, LC-MS/MS = liquid chromatography-tandem mass spectrometry, LP = lobar pneumonia, NA = not available, NC = normal controls, NPV = negative predictive value, PCT = procalcitonin, PNA = peanut lectin, PT = prothrombin time, RBC = red blood cells, ROC = receiver operator characteristic, TA = Thomsen–Friedenrich antigen (T-antigen), WBC = white blood cells.

INTRODUCTION

Streptococcus pneumoniae can colonize the upper respiratory tract of humans and subsequently cause mucosal infections such as sinusitis, otitis media, and pneumonia, and also invasive pneumococcal disease (IPD) including complicated pneumonia (empyema and necrotizing pneumonia), bacteremia, and meningitis.[1] One of the most severe complications of IPD is hemolytic uremic syndrome (HUS) which mainly occurs in children and is associated with hemolytic anemia, thrombocytopenia, and acute renal failure (HUS triad).[2] In a recent study, we examined the distribution of 3 neuraminidase genes (*nanA*, *nanB*, and *nanC*) in pneumococcal isolates derived from HUS patients and those without. *S. pneumoniae* intrinsically carried *nana* and *nanB*, while relative to 89% of the HUS isolates that harbored *nanC*, only 42% of the IPD isolates carried the gene.[3,4] We thus speculated that NanC might contribute to the risk of developing HUS in an additive manner in the presence of NanA and NanB by increasing the overall activity of pneumococcal neuraminidases, and so was associated with the occurrence of HUS following pneumococcal infection.[3]

Serum sialoglycoproteins are a rich source of sialic acids for *S. pneumoniae*. Sialoglycoproteins C-reactive protein (CRP), alpha-1-acid glycoprotein (AGP), and fibrinogen are acute phase proteins that are currently being used as biomarkers for presence of disease, response to therapy, and ultimate recovery.[5–8] In this study, we constructed a complete serum sialoglycoprotein profile after neuraminidase treatment using state-of-the-art proteomic instrument. We aimed to identify serum sialoglycoproteins that potentially could be the target of neuraminidases in severe pneumococcal infection. We sought to test the hypothesis that serum sialoglycoprotein fetuin-A (also known as the α2-Heremans-Schmid glycoprotein; *Ahsg*) can be used as a biomarker to predict severe pneumococcal infection.

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METHODS

Protein Expression, Purification, and Human Serum Sialoglycoproteins Profiling

Recombinant NanA, NanB, and NanC proteins of *S. pneumoniae* strain CGSP14 were expressed in *E. coli* BL21 (DE3) and purified by Ni^{2+} affinity chromatography using Nickel-Chelating Resin (Invitrogen, Carlsbad, CA).[9,10] To identify serum sialoglycoproteins that are targeted by pneumococcal neuraminidases, human serum was treated with NanA, NanB, and NanC and asialoglycoproteins were captured by peanut lectin (PNA) conjugated agarose column (Vector Laboratories, Burlingame, CA) and the eluted sample was analyzed by Western blot hybridization.[11,12] And protein samples were submitted to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.[13] See Supplemental Content, which illustrates detailed methods.

Fetuin RBC Protection Assay and ELISA

Red blood cells (RBC) were treated with NanA, NanB, or NanC and incubated for 1 to 2 hours at 37°C. Labeling was done with fluorescein-PNA and flow cytometric analysis (FACScan, Becton Dickinson, San Jose, CA, USA) was performed. Different concentrations of bovine fetuin (Sigma, St. Louis, MO) were added to the above assay to analyze the protective role of fetuin. Serum fetuin-A levels from patients and controls were determined by a sandwich enzyme-linked immuno-sorbent assay (ELISA) (Human fetuin-A ELISA kit, R&D Systems, Minneapolis, MN). See Supplemental Content, which illustrates detailed methods.

Patients

An observational study was conducted using the clinical data and serum samples from patients with pneumococcal infections treated in Chang Gung Children's Hospital, a tertiary care medical center in Taiwan, from 2010 to 2013. The study was approved by Institutional Review Board of Chang Gung Memorial Hospital (IRB-98-3451B). Medical records of the patients were reviewed. Information abstracted included demographic data, available clinical and laboratory characteristics, and outcome. We defined IPD as isolation of *S. pneumoniae* from normally sterile sites such as blood, cerebrospinal fluid (CSF), or pleural fluid. Patients with alveolar infiltration in segmental or lobar distribution shown in chest radiographs were considered to have lobar pneumonia. As part of our routine practice, urine pneumococcal antigen was screened in all patients with suspected bacterial pneumonia using a commercial kit (BinaxNOW, Alere, Waltham, MA). Complicated pneumonia includes necrotizing pneumonia and/or empyema. We defined necrotizing pneumonia as the presence of small lucencies or pneumatoceles on a chest radiograph and as cavities of nonenhancement on a contrast-enhanced CT image. Empyema was defined as the presence of 1 major criterion or 2 minor criteria; the definition of major and minor criteria was according to a previous study by Hardie et al.[14] HUS was defined using the Centers for Disease Control and Prevention definition.[15] Coagulation tests were done at the time of HUS diagnosis to rule out disseminated intravascular coagulopathy (DIC). Coagulation parameters prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen were analyzed in some patients, wherever indicated, using Sysmex CA-1500 System from Sysmex Corporation (Kobe, Japan).[16] Normal references for age were obtained from the Nelson Textbook of Pediatrics.[17] The presence of normal fibrinogen levels in the diagnostic criteria for HUS was used to rule out DIC. TA activation in blood was tested by the peanut (*Arachis hypogaea*) lectin agglutination method.[18] All the HUS patients in this study were positive for TA activation. We used serum from healthy children who came to our hospital for immunization as controls. An informed consent was obtained from these children. IPD patients without HUS had no records indicating acute renal failure and TA activation.

Statistical Analysis

Analysis of variance (ANOVA) test was used for comparison of multiple independent groups, followed by the Scheffé test when results were found to be significant by ANOVA. The values of P less than 0.05 were considered statistically significant. The receiver operator characteristic (ROC) curve analysis was generated in SPSS 17.0 (SPSS Inc, Chicago, IL). ROC curve statistics were applied to determine cut-off values, area under the ROC curve (AUC), specificity, sensitivity, and predictive values.

RESULTS

NanA, NanB, and NanC Cleaved Fetuin-A and Other Sialoglycoproteins in Human Serum

Activity and specificity of neuraminidases NanA, NanB, and NanC were confirmed by ex vivo and in vitro assays (see Figures S1 and S2, Supplemental Content). We confirmed the presence of asialoglycoproteins in the eluate from the PNA-agarose precipitation by Western blot hybridization. When the western blot was probed with streptavidin-HRP, multiple proteins were detected in the NanA-, NanB-, and NanC-treated serum samples. However NanB- and NanC- treated samples showed a similar protein pattern, indicating similar sialoglycoproteins specificity (see Figure S3, Supplemental Content). LC/MS analysis for proteins eluted from the PNA agarose column resulted in identification of 15, 48, and 28 proteins in the untreated, NanA- and NanC-treated samples, respectively (see Table S1, Supplemental Content). Immunoglobulins, apolipoproteins, fibrinogens, keratins, and complement system proteins were predominantly desialylated by NanA and NanC. Eight proteins were found to be common in both untreated and neuraminidase-treated samples. All the 28 proteins identified in NanC-treated samples were also identified in NanA-treated samples, all the remaining 20 proteins were unique to NanA. This data demonstrated that α2-3 sialyl linkages present on 28 glycoproteins can be targeted by both NanA and NanC, while the remaining glycoproteins contain α2-6 sialyl linkages that can be targeted by NanA only. Fetuin-A glycoprotein was positively identified in the peptide sequence information obtained from LC/MS (see Table S2, Supplemental Content). Western blot hybridization on samples after PNA-agarose precipitation with an anti-Fetuin A antibody revealed a band of about 55 kDa in NanA-, NanB-, and NanC-treated serum. This band was absent in the untreated serum (see Figure S3, Supplemental Content). Western blot analysis also revealed a higher intensity band in NanA-treated serum when compared with NanB- and NanC-treated serum. This might be due to the presence of more α2-6 sialyl linkages when compared with α2-3 sialyl linkages on fetuin-A; due to activity of NanA on both α2-6 and α2-3 sialyl linkages and subsequent exposure of multiple Galβ1-3GalNAc residues on fetuin-A, proteins containing more residues of Galβ1-3GalNAc residues are

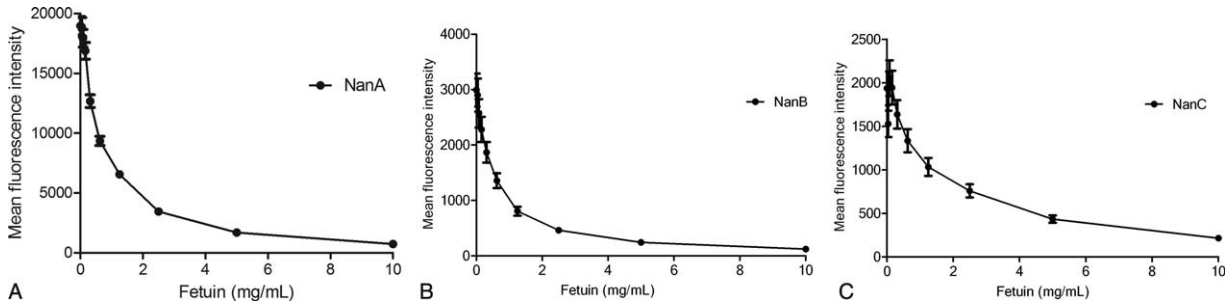

FIGURE 1. Fetuin inhibits T antigen exposure on RBC by NanA, NanB, and NanC. TA exposure was inhibited by bovine fetuin in a dose-dependent manner in the presence of NanA (A), NanB (B), and NanC (C). MFI represents the mean fluorescence intensity values. Fluorescein-PNA was used to detect TA on red blood cells.

selectively precipitated by PNA-agarose. Presence of fetuin-A in neuraminidases-treated and the untreated serum was confirmed by Western blot hybridization with an anti-fetuin-A antibody. NanA-treated samples showed a lower fetuin-A band likely due to desialylation of both α2-6 and α2-3 sialyl linkages when compared with the untreated samples.

Fetuin Protects RBC From Desialylation and Subsequent TA Exposure

Inhibition of TA exposure on RBC by bovine fetuin was analyzed in the presence of NanA, NanB, and NanC. TA exposure was inhibited by bovine fetuin in a dose-dependent manner; 50% inhibition in TA exposure was observed in the presence of fetuin at 2.59 mg/mL for NanA, 2 mg/mL for NanB, and 1.2 mg/mL for NanC, respectively (Figure 1).

Sialoglycoproteins and Fetuin-A Levels as a Biomarker to Predict HUS and Complicated Pneumonia

Blood samples were collected at acute stage of the infection in the patients, usually between day 2 and day 12 after admission. The clinical characteristics of the patients are shown in Table 1. The age of HUS patients was 4.05 ± 4.8 years, similar to 5.3 ± 0.6 years of normal controls. *S. pneumoniae* urine antigen test was positive in 93% of patients with HUS and 80% of those with complicated pneumonia. Sixty-four percent and 55% of HUS and complicated pneumonia patients, respectively, were *S. pneumoniae* culture positive (blood, CSF, or pleural fluid) (Table 1). Patients with only lobar pneumonia were all negative in culture. Length of hospitalization in HUS patients (28.6 ± 12.8 days) was significantly longer, when compared with lobar pneumonia group ($P < 0.001$) (Table 1). The length of stay in hospital after determination of fetuin-A was 21.2 ± 11.1 days. As shown in Table 2, CRP levels were significantly higher in patients with HUS and patients with complicated pneumonia, relative to patients with lobar pneumonia and the normal reference (≤1 mg/L). Procalcitonin (PCT) was significantly higher in patients with HUS and patients with complicated pneumonia, compared with the normal reference (≤0.1 μg/L). AGP was significantly higher in patients with HUS and those with complicated pneumonia, compared with normal controls and the normal reference (360–1460 mg/L). Fibrinogen levels were significantly higher in patients with HUS and patients with complicated pneumonia, compared with the normal reference (125–400 mg/dL). aPTT was >40 seconds among 50% of the HUS patients and 10% of patients with complicated pneumonia. Since the proteomic analysis shows that coagulation pathway proteins fibrinogen and factor XII were targeted by pneumococcal neuraminidases,

TABLE 1. Clinical Characteristics and Results of Laboratory Tests in Patients With HUS, Complicated Pneumonia (CP) and Lobar Pneumonia (LP) and Normal Controls (NC)

Characteristics	IPD With HUS* (n = 14)	Necrotizing Pneumonia and/or Empyema† (n = 20)	Lobar Pneumonia (n = 12)	Normal controls (n = 20)
Age (yr)	4.1 ± 4.8	3.4 ± 2.8	5.80 ± 3.5	5.3 ± 0.6
Male (%)	6 (42.9)	13 (65)	8 (66.7)	12 (60)
WBC (/mm^3), mean (range)	10,857 (3600–26,600)	11,150 (2300–47,400)	10,941 (4100–21700)	NA
Culture confirmed‡ (% positive)	9 (64%)	11 (55%)	0	NA
Pneumococcal antigen in urine (% positive)	13 (93%)	16 (80%)	5 (42%)	NA
Hospital stay (days)	28.6 ± 12.8§	20.6 ± 13.6‖	7.25 ± 2.5	NA

HUS = hemolytic uremic syndrome; IPD = invasive pneumococcal disease; NA = not available or not applicable; WBC = white blood cells.
* Two cases with pneumococcal meningitis.
† CP includes necrotizing pneumonia and/or empyema. One case with pneumococcal meningitis.
‡ From blood or other sterile site specimen.
§ $P = 0.001$ (HUS vs LP).
‖ $P = 0.011$ (CP vs LP).

TABLE 2. Biological Markers During Acute Phase in Patients With HUS, Complicated Pneumonia (CP) and Lobar Pneumonia (LP) and Normal Controls (NC)

Markers	IPD With HUS (n = 14)	Necrotizing Pneumonia and/or Empyema (n = 20)	Lobar Pneumonia (n = 12)	Normal Controls (n = 20)	Normal Reference Values [Sources]	*P* value				
						HUS *vs* CP	HUS vs LP	HUS vs NC	CP vs LP	CP vs NC
CRP (mg/L)	319.3 ± 84.9	239.5 ± 133	126.9 ± 92.9	NA	<1.1 [17]	0.114	0.001	0.001	0.015	0.001
Procalcitonin (μg/L)	27.2 ± 26.2	15.1 ± 18.3	NA	NA	<0.1 [7]	0.233	NA	NA	NA	NA
AGP (mg/L)	4015 ± 2357	1780.1 ± 856.5	NA	886 ± 378.7	360–1460 [24]	0.110	NA	0.001	0.209	0.087
Fibrinogen (mg/dL)	712 ± 188.8	606.6 ± 232.3	NA	NA	125–400 [17]	0.240	NA	NA	NA	NA
aPTT (s)	47.1 ± 16.1	38.5 ± 11.7	NA	NA	25–35 [17]	0.089	NA	NA	NA	NA
Fetuin (mg/L)	207 ± 80	390 ± 220	610 ± 190	630 ± 250	220–700 [26]	0.097	0.001	0.001	0.038	0.001

AGP = α-1-acid glycoprotein; aPTT = activated partial thromboplastin time; CRP = C-reactive protein; HUS = hemolytic uremic syndrome; IPD = invasive pneumococcal disease; NA = not available. Statistically significant *P* values are in bold.

we examined the effect of recombinant NanA, NanB, and NanC on aPTT. As a result, we found that only NanA could prolong aPTT in a dose-dependent manner (Figure 2).

After determining the fetuin-A levels by ELISA, we found that fetuin-A level in the HUS patients was significantly lower (207 ± 80 mg/L), relative to patients with lobar pneumonia (610 ± 190 mg/L) and the healthy controls (630 ± 250 mg/L) (Figure 3). Fetuin-A level in patients with complicated pneumonia was also lower (390 ± 220 mg/L), when compared with patients with lobar pneumonia ($P = 0.038$). From a 5-year-old patient with pneumococcal meningitis and brain abscess, 3 consecutive blood samples were collected. We found that fetuin-A level during acute stage of infection dropped to 236 mg/L, 4 weeks later it increased to 609 mg/L, and 6 weeks later, when the patient finished complete antibiotic therapy, to 742 mg/L (see Figure S4, Supplemental Content). This suggests that response to therapy and recovery from IPD could be monitored by measuring serum fetuin-A.

ROC curves for fetuin-A level were constructed by comparing patients with HUS and those with complicated pneumonia and lobar pneumonia, as well as patients with HUS and complicated pneumonia and those with lobar pneumonia (Figure 4). These curves were used to identify various cutoff values with differing sensitivities and specificities. In comparing HUS with complicated pneumonia and lobar pneumonia, the ROC area under the curve (AUC) was 0.842; a cutoff value of 298 mg/L yielded sensitivity of 92.9% (95% confidence interval, CI: 68.5–98.7%), specificity of 71.9% (95% CI: 54.6–84.4%), negative predictive value (NPV) of 95.8% (95% CI: 79.8–99.2%), and negative likelihood ratio of 0.36 (95% CI: 0.04–0.36). With a cutoff value of 298 mg/L, 5 HUS patients with negative *S. pneumoniae* culture and 1 HUS patient with negative urine antigen test would have been classified as positive for pneumococcus. In discriminating HUS and complicated pneumonia and those with lobar pneumonia, the ROC curve had an AUC of 0.873. For this analysis, a cutoff value of 340 mg/L gave a sensitivity of 73.5% (95% CI: 56.9–85.4%), specificity of 100% (95% CI: 75.8–100%), NPV of 57.1% (95% CI: 36.5–75.5%), and negative likelihood ratio of 0.27 (95% CI: 0.10–0.68). The results suggest that physicians should watchfully observe patients with IPD for the possibility of developing HUS when their serum fetuin-A level dropped below 298 mg/L, and that a level less than 340 mg/L would be an indicator for complicated pneumonia with or without HUS in patients with suspected pneumococcal infection.

DISCUSSION

Neuraminidase-producing organisms like *S. pneumoniae* acquire sialic acid by cleaving host sialoglycoconjugates and use it as carbon and nitrogen source.[10] During pneumococcal infection, cleavage of sialyl linkages to expose TA in host cells is due to a higher neuraminidase activity from NanA, NanB, or NanC or an additive effect of the three.[3] Previously, *S. pneumoniae* serotype 2 (strain R6) and serotype 4 (strain TIGR4) were used to confirm α2-3 sialyl linkages specificity of the NanB and NanC.[19,20] This study used a serotype 14 strain to confirm substrate specificity of pneumococcal neuraminidases both in vitro and ex vivo; NanA showed activity toward both α2-3 and α2-6 sialyl linkages, while both NanB and NanC cleaved only α2-3 sialyl linkages. Brittan et al[21] reported that NanA and NanB mutants of *S. pneumoniae* are deficient in adherence to 3 epithelial cell lines, as well as to primary nasopharyngeal cells. In the respiratory tract, distribution of

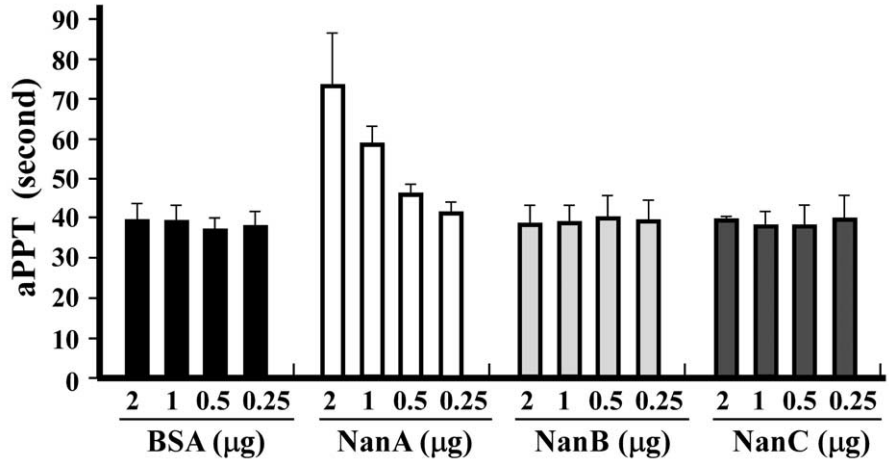

FIGURE 2. Neuraminidases effect on aPTT. Plasma samples from 3 healthy individuals were assayed for aPTT by adding actin FSL and $CaCl_2$ in a Sysmex CS-1500 analyser. Plasma samples were pretreated with $\leq 2\,\mu g$ of recombinant NanA, NanB, and NanC for 1 hour before initiating the reaction. Plasma samples treated with BSA ($\leq 2\,\mu g$), PBS, or imidazole (≤ 250 mM) were used as untreated controls.

α2-3 and α2-6 sialyl linkages varies depending on age, tissue, and cell types; α2-3 linkages are selectively present on goblet cells and secreted mucins and α2-6 linkages on ciliated respiratory epithelial cells.[11,22] Additional presence of NanC may therefore help pneumococci to acquire more sialic acid from the respiratory tract during colonization and infection.[3]

The most abundant serum sialoglycoproteins identified by liquid chromatography-tandem mass spectrometry after neuraminidase treatment and peanut lectin capture were immunoglobulins, apolipoproteins, fibrinogens, keratins, complement system proteins, and fetuin-A. Sialoglycoproteins AGP, fibrinogen, and CRP are currently being used as biomarkers in the clinically setting. AGP is one of the major acute phase proteins; its serum concentration increases during an acute-phase response, such as sepsis.[5,23,24] Our study indicates that the concentration of AGP was significantly ($P < 0.001$) elevated in all the HUS patients; however, AGP levels cannot differentiate the pneumococcal disease severity.

Patients with HUS usually have normal fibrinogen levels and normal or slightly elevated PT and aPTT.[25] However, our study shows that fibrinogen (mean, 712 mg/dL) and aPTT (mean, 47 seconds) were significantly elevated in IPD patients. Prolongation of aPTT was observed in only 50% of the HUS patients, which can be caused by abnormalities of coagulation factors in the intrinsic or common pathways of the clotting cascade or due to alteration of the progression through these pathways. We excluded fibrinogen deficiency as a possible cause as all HUS patients had increased fibrinogen levels. In this study, LC/MS data revealed that coagulation factors including high molecular weight kininogen, fibrinogen, and factor XII were targeted by pneumococcal neuraminidases, but in vitro experiment verified that only NanA could prolong aPTT.

Our understanding on the role of fetuin-A in infection remains preliminary. Fetuin-A is highly expressed in the liver and secreted into circulation.[26] Wigger et al reported that the serum concentration of fetuin-A (460 ± 240 mg/L) does not

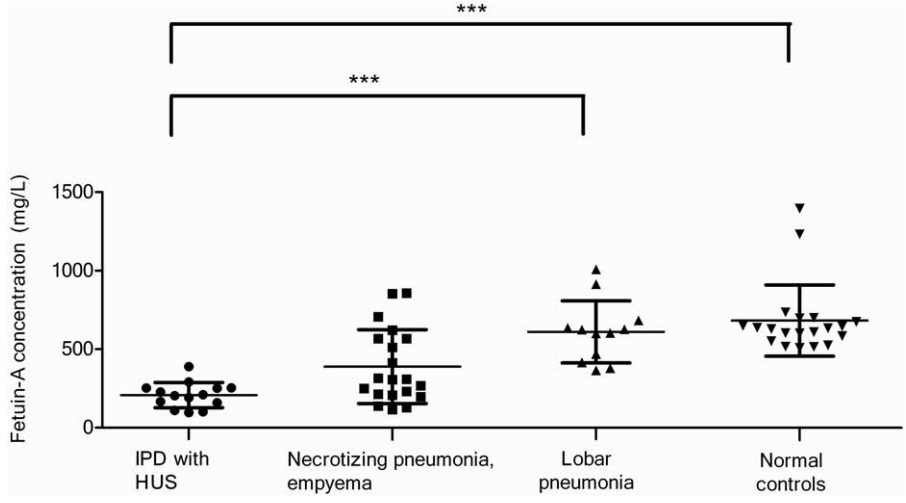

FIGURE 3. Fetuin levels in patients with HUS, necrotizing pneumonia with or without empyema and lobar pneumonia. Serum fetuin-A levels from patients and controls were determined by a sandwich ELISA. $^{***}P<0.001$.

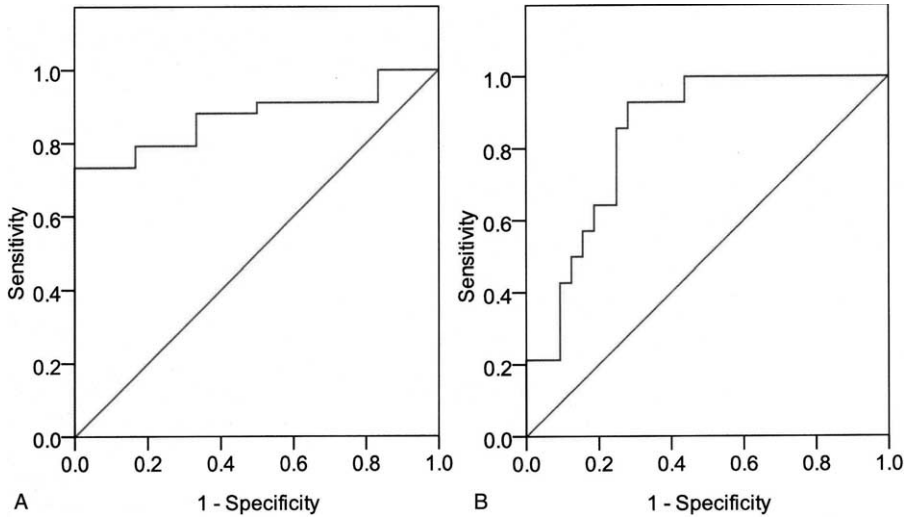

FIGURE 4. ROC curves for fetuin-A levels. A, Comparing HUS with necrotizing and lobar pneumonia the AUC was 0.842; cutoff value 298 mg/L, sensitivity 92.9%, and specificity 71.9%. B, Comparing HUS and necrotizing pneumonia with lobar pneumonia the AUC of 0.873, cutoff value 340 mg/L, sensitivity 73.5%, and specificity 100%.

vary with age and sex in a healthy population; however, its level would alter following infection, inflammation, and malignancy.[27,28] Recently, Kebapcilar et al described that baseline serum fetuin-A was 43% lower in *Helicobacter pylori*-infected patients than in *H. pylori*-negative subjects.[29] Li et al also reported that circulating fetuin-A levels were decreased in animal models of lethal endotoxemia and sepsis.[29,30] In HUS patients, pneumococcal neuraminidase cleaves *N*-acetylneuraminic acid (sialic acid) residues on RBC leading to the exposure of the Thomsen–Friedenrich antigen (T-antigen [TA]) on cells and allowing normally circulating anti-TA antibodies to react with the exposed TA to form immune complex and subsequently causes lysis of the cells.[2] We observed that fetuin protects RBC from desialylation and subsequent TA exposure; this might be due to a competitive inhibitory effect of bovine fetuin on the activity of NanA, NanB, and NanC on RBC. The bovine fetuin concentrations used to inhibit neuraminidases in vitro were 2 to 4 times greater than the concentration of fetuin-A in human serum; therefore, it is possible that other sialoglycoproteins in addition to fetuin-A could have some protective effect during pneumococcal infection, but this requires further studies to verify. As a major sialoglycoprotein in human serum, neuraminidase activity in blood in patients with IPD could be titrated by fetuin-A and the low concentration of fetuin-A or other sialoglycoproteins may indirectly reflect the severity of the infection.

NanA is anchored on cell surface and NanB and NanC are intracellular but can be secreted into bloodstream in the systemic phase of the infection.[31] In addition, all the 3 pneumococcal neuraminidases released following autolysis of pneumococci could reach the circulatory system and the overall activity of the 3 neuraminidases would predispose children with severe pulmonary infection to HUS.[3] Invasive pneumococcal infection is a common cause of HUS in Taiwan and the United States, and pneumococcal HUS is invariably associated with prolonged hospital stays.[32,33] Detection of TA activation using PNA was thought to be the most appropriate test to support the diagnosis of pneumococcal HUS.[34] A recent report indicated that TA activation was 83% sensitive and a positive direct Coombs test was only 58% sensitive.[34] Pneumococcal HUS is a well-characterized condition but continues to be under recognized. To improve the under-diagnosis and late detection of HUS, more specific laboratory tests are needed.[25] This study adds fetuin-A as a biomarker to predict severe IPD, such as HUS and complicated pneumonia. Recently, Copelovitch and Kaplan[25] modified the case definition for HUS to definite, probable, and possible by including a triad of HUS along with the evidence of IPD, positive Coombs test, and DIC. Based on the modified case definitions for HUS, among the 14 HUS cases identified in this study, 9 would be considered definitive and 5 possible for HUS. At a fetuin-A cut-off of 298 mg/L, sensitivity to predict HUS was 92.9%; at this cut-off value the 5 possible cases with negative *S. pneumoniae* culture would be considered a positive diagnosis for HUS. In addition, mean length of hospital stay after observing low levels of fetuin-A (207 ± 80 mg/L) was 24 days (18–55 days) in 79% of the HUS patients and in the remaining patients it was 10 days. Previous studies showed that majority of patients with complicated pneumococcal pneumonia, when complicated by HUS, would require surgical debridement and drainage.[3,32,33] This indicates that low fetuin-A measurement is of considerable diagnostic value, and therefore clinical laboratories should consider providing this testing for the diagnosis and treatment of severe pneumococcal infections.

Our study reconfirms the utility of PCT and CRP as biomarkers for the severity of pneumococcal pneumonia. Müller et al[7] recommended that blood cultures be drawn from patients with community-acquired pneumonia only when PCT levels are >0.25 μg/L to increase the likelihood for the diagnosis of bacteremic pneumonia. In this study in patients with complicated pneumonia with or without HUS, PCT levels were invariably high, so were the CRP levels. The rate of positive blood culture also appeared similar between the 2 groups of patients.

Fetuin-A levels (610 ± 190 mg/L) determined in healthy children using R&D Systems ELISA kits in the current study are higher than the levels (460 ± 240 mg/L) reported by other studies using Epitope Diagnostics ELISA kits (San Diego,

CA).[35] The results are similar to fetuin-A levels in adults with normal renal function (630 ± 120 mg/L) reported by Smith et al. Since commercial ELISA kits have divergent sensitivity and specificity, utility of fetuin-A levels in the diagnosis of HUS or complicated pneumonia would need to evaluate the different ELISA kits available to find the respective cut-off values.

One limitation of the study is the limited number of cases studied. A multicenter study involving more patients to compare serial measurements of fetuin-A in IPD patients with or without HUS is warranted. Furthermore, main technical limitation while using the commercial fetuin-A kits is the variation in antibody specificity for different glycosylated forms of plasma fetuin-A, which could lead to poor agreement among measurements by different commercial ELISA kits.[35]

In conclusion, by qualitative and quantitative analysis of serum fetuin-A in pneumococcal infections, we may identify complicated pneumonia with or without HUS caused by *S. pneumoniae*. Serial measurements of fetuin-A also have the potential to reflect patients' response to therapy and recovery from IPD. We recommend addition of fetuin-A to the panel of biomarkers currently used for severe IPD.

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