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Plasma Follistatin-Like Protein 1 is Elevated in Kawasaki Disease and May Predict Coronary Artery Aneurysm Formation

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Abstract

Objective—To determine if plasma levels of follistatin-like protein 1 (FSTL-1), a pro-inflammatory protein produced by mesenchymal tissue, including cardiac myocytes, correlate with the development of Kawasaki disease (KD) and coronary artery aneurysms (CAA).

Study design—FSTL-1 plasma levels were measured serially by ELISA in 48 patients with KD at time of diagnosis and, when available, 2 weeks, 6 weeks and 6 months following onset of disease. These were compared with 23 controls. Data were analyzed using Generalized Estimating Equations.

Results—Plasma FSTL-1 levels were elevated in patients with acute KD as compared with controls ($p=0.0086$). FSTL-1 levels remained significantly elevated at 2 weeks after disease onset but returned to control levels by 6 months. Seven patients with CAA had significantly higher FSTL-1 levels at the time of diagnosis than those who did not develop aneurysms ($p=0.0018$). Sensitivity and specificity for CAA at a specific FSTL-1 cutpoint (178ng/ml) was 85% and 71%.

Conclusions—Plasma levels of FSTL-1 are elevated in acute KD and may predict cardiac morbidity in this disease. These results suggest a possible role for FSTL-1 in the formation of coronary artery aneurysms.

Kawasaki disease (KD) is the major cause of acquired coronary artery aneurysms (CAA) in childhood [2]. The etiology of KD remains unknown. It is believed that a possible undefined infectious agent triggers systemic inflammation and vasculitis in predisposed individuals[3, 4]. The incidence of CAA has decreased due to treatment with IVIG, however up to 5% of treated patients still develop aneurysms, as compared with up to 25% of untreated patients[6–9]. Follistatin-like protein 1 (FSTL-1) is an extracellular glycoprotein which was originally cloned from an osteoblast cell line as a TGF- β inducible gene[12]. FSTL-1 is highly conserved across mammalian species. FSTL-1 is secreted by cells of the mesenchymal lineage, including cardiac myocytes, and suppression of FSTL-1 expression by siRNA treatment leads to increased cardiomyocyte apoptosis[13]. FSTL-1 has also been

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shown to have a role in promoting revascularization of skeletal muscle after ischemic injury[14]. FSTL-1 expression has been found to be elevated in patients with heart failure but returns to normal levels upon recovery[15].

We have previously shown that FSTL-1 can have proinflammatory effects. Overexpression of FSTL-1 leads to exacerbation of arthritis in mice while its neutralization ameliorates arthritis[16]. Furthermore, overexpression of FSTL-1 in monocytes leads to upregulation of IL-1 β , IL-6, and TNF- α [17]. The current study was designed to determine if plasma levels of FSTL-1 correlate with the development of KD and development of CAA.

METHODS

Banked frozen plasma samples were obtained from patients with typical KD whose diagnosis was made through established clinical criteria[19]. These samples were obtained through the pediatric cardiology clinics and inpatient services at Cincinnati Children's Hospital Medical Center and from Children's Memorial Hospital in Chicago. Serial samples from 48 individual patients were obtained, when available, at acute presentation (prior to IVIG), at 2 weeks, 6 weeks, and 6 months following presentation. One of these patients developed CAA. Six patients in this group did not have an acute sample but had samples collected at later time points. Six additional acute samples from patients with KD who were known to have subsequently developed CAA were obtained from Children's Memorial Hospital. In total, plasma samples from 54 patients with KD were analyzed. Coronary artery aneurysms were identified by echocardiogram according to the American Heart Association guidelines of Dajani et al. There was a greater proportion of Hispanic and Asian patients in the aneurysm group as compared with the other groups. However, there was no significant inter-ethnic difference in FSTL-1 concentration within the aneurysm group itself. Control plasma samples were obtained from 23 children of similar age and sex with no history of inflammatory disease who underwent surgical procedures including hernia repair, tonsillectomy and adenoidectomy, and simple ophthalmologic procedures. One additional control sample was excluded from the analysis because its FSTL-1 value measured above the limit of detection. Assigning a random very high value to this sample did not alter the statistical significance of the results and, because a value could not accurately be assigned to it, this sample was excluded from the analysis. Use of samples was approved by the Institutional Review Board at the University of Pittsburgh. FSTL-1 ELISA assays were performed at the Children's Hospital of Pittsburgh.

For detection of human FSTL-1 in plasma, Nunc Immunomodule MaxiSorp F8 Framed ELISA plates were coated with 5 μ g/ml polyclonal anti-FSTL1 (AF1694; R&D Systems, Minneapolis, MN) in phosphate buffered saline (PBS) and incubated at 4 $^{\circ}$ C overnight. Plates were then washed with PBS/0.05% Tween 20 and blocked for one hour with bovine serum albumin (BSA) buffer (1% BSA and 5% sucrose in PBS). Plates were washed again, and human plasma samples diluted 1:10 in Serum Dilution Buffer (20mM Tris, 150mM NaCl 0.1 % BSA, 0.05% Tween 20; pH 7.3) were added and incubated for one hour. After washing, 2.5 μ g/ml biotinylated monoclonal anti-FSTL1 (MAB1694; R&D systems) was added for 1 hour. Plates were washed again and incubated with Streptavidin-HRP conjugate at 0.25 μ g/ml for 20 minutes. BD OptEIA TMB Substrate Reagent was added, and plates were incubated for an additional hour, following which development was stopped with addition of 1M H₂SO₄. Plate absorbance was read on a microplate reader with dual measurement of 450nm and 570nm reference level. A titration of purified FSTL-1 was used to generate a standard curve from which plasma concentration of samples was calculated.

Statistical Analysis

Statistical analysis was performed using STATA version 11. A series of comparisons between study groups were made using t-tests and Generalized Estimating Equations (GEE) to account for the repeated measures for comparisons of FSTL-1 levels over time. Among KD patients, n=48, excluding the 6 patients specifically selected for aneurysm (to avoid statistical oversampling), mean FSTL-1 levels were compared over acute, 2 weeks, 6 weeks and 6 month time points, using GEE with an independent correlation structure and robust variance estimates. Mean baseline FSTL-1 levels were then compared between controls, KD patients without aneurysms who had samples available at the acute time point (n=41) and KD patients with aneurysms (n=7). All reported p-values are 2-sided and considered significant at $p < 0.05$, and are corrected for multiple comparisons using the Bonferroni method. The two KD patient groups were combined in order to calculate sensitivity and specificity for aneurysm development by FSTL-1 concentration cutpoint, and a Receiver Operating Curve (ROC) was constructed.

RESULTS

Patient demographics are summarized in the Table. As shown in Figure 1, the mean FSTL-1 level in acute KD was significantly elevated compared to controls ($161.7 \pm \text{SEM } 7.0 \text{ ng/ml}$ vs. $128.3 \pm \text{SEM } 6.1 \text{ ng/ml}$, $p = 0.0086$). Mean FSTL-1 level was still significantly elevated at 2 weeks ($150.3 \pm \text{SEM } 5.6 \text{ ng/ml}$, $p=0.0424$) but by 6 weeks was no longer significantly different from controls ($141.9 \pm \text{SEM } 5.2 \text{ ng/ml}$). Mean FSTL-1 levels declined further to $119.6 \pm 5.2 \text{ ng/ml}$ at 6 months, which was not significantly different from normal controls, but was significantly different from the acute time point ($p < 0.0001$). Using a paired t-test, comparison of patients with both an initial (acute) time point and a recovery (6 month post presentation) time point was statistically significant, $p=0.012$. In patients with KD without aneurysms, there was a positive correlation between FSTL-1 level and height of fever ($p = 0.02$).

As shown in Figure 2, the mean FSTL-1 level in patients who developed aneurysms was significantly higher than in patients who did not ($219.2 \pm \text{SEM } 19.4 \text{ ng/ml}$ versus $161.2 \pm \text{SEM } 7.2 \text{ ng/ml}$; $p = 0.0018$). Patients who developed CAA had significantly higher levels of FSTL-1 at presentation than did patients without CAA at all stages of disease. FSTL-1 level had a high sensitivity and specificity as a biomarker for aneurysm development. The ROC analysis for FSTL-1 levels at non-aneurysm and aneurysm status had an area under the curve of 0.8223, (95% CI 0.6863, 0.9583) (Figure 3). Using the ROC, a threshold of 178 ng/ml yielded a sensitivity of 85%, with specificity of 71%.

DISCUSSION

Development of CAA is the major cause of morbidity and mortality associated with KD. Efforts to define clinical or serological risk factors for the development of CAA have been described over the last 30 years. These risk factors have included non-coronary cardiac abnormalities[20],[21], incomplete clinical presentation at very young age, higher body temperature during certain phases of disease[22], and resistance to IVIG therapy at older ages[23, 24]. Clinical scoring mechanisms to predict development of CAA have been developed that utilize neutrophil and band counts, hemoglobin concentrations, platelet counts, and temperature on the day after infusion of IVIG[25]. Lab markers such as high white blood cell count, thrombocytopenia, anemia, C-reactive protein levels, age of presentation and male sex have been identified as risk factors for development of aneurysm formation[25, 26].

Lin et al investigated other serologic markers for development of CAA including IL-6, TNF- α and soluble IL-2 receptor[27]. This evaluation included multiple monthly blood samples and showed that elevated levels of IL-6 and IL-8 in the first week of illness could predict development of CAA. The findings reported here indicate that elevated FSTL-1 levels correlate with increased risk of development of CAA in KD, suggesting that a simple blood test at the time of diagnosis might be highly sensitive and specific in identifying patients at high risk.

Limitations to our study include the relatively small numbers of patients in the CAA group, as well as the overrepresentation of Hispanic patients in this group. There were no Asians or Hispanics in the control population. Additionally, all of our patients met the criteria for complete KD, and we cannot generalize the findings to patients with atypical or incomplete KD. Additional prospective studies with a larger numbers of patients with CAA would provide further validation of our findings.

Despite these limitations, other studies on FSTL-1 and cardiac disease suggest a rationale for our conclusions. FSTL-1 expression has been shown to be increased in the heart in the setting of cardiac injury and may play a protective role against hypoxic injury to myocytes[13]. FSTL-1 was also found to be elevated in patients with heart failure, and FSTL-1 staining was seen in myocytes and vascular endothelial cells of capillaries, small vessels, and smooth muscle cells of larger vessels in these patients[15].

Although the mechanism by which FSTL-1 might promote CAA is speculative, possible effects on endothelial nitric oxide synthase (eNOS) expression are worth consideration. Alterations in eNOS expression have been found to be associated with aneurysm formation both in mouse and human models of aneurysmal disease. Aged eNOS knockout mice have decreased aneurysm formation in comparison to wild type mice[28] suggesting that eNOS activity contributes to aneurysm formation. Polymorphisms in the eNOS gene have been shown to predispose humans to develop abdominal aortic aneurysms[29], and alterations in nitric oxide production has been shown to cause development of cerebral aneurysms in rats[30]. Finally, histopathologic analysis of coronary artery aneurysms in KD demonstrates decreased eNOS staining, among other features of coronary artery senescence[31]. FSTL-1 overexpression was recently shown to improve the revascularization of ischemic limbs in wild type mice, to enhance endothelial cell differentiation and migration, and to lead to phosphorylation and activation of eNOS[14]. Furthermore, FSTL-1 overexpression did not lead to revascularization in mice deficient in eNOS. These actions of FSTL-1 on vascular endothelium through the Akt-eNOS signaling pathway thus suggests a mechanism by which elevated expression of FSTL-1 might contribute to aneurysm formation in KD.

Our findings suggest that high levels of FSTL-1, which is produced by cardiac myocytes, might be a biomarker for development of CAA in KD.

Acknowledgments

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List of abbreviations

FSTL-1	Follistatin like protein 1
KD	Kawasaki Disease

CAA Coronary artery aneurysms

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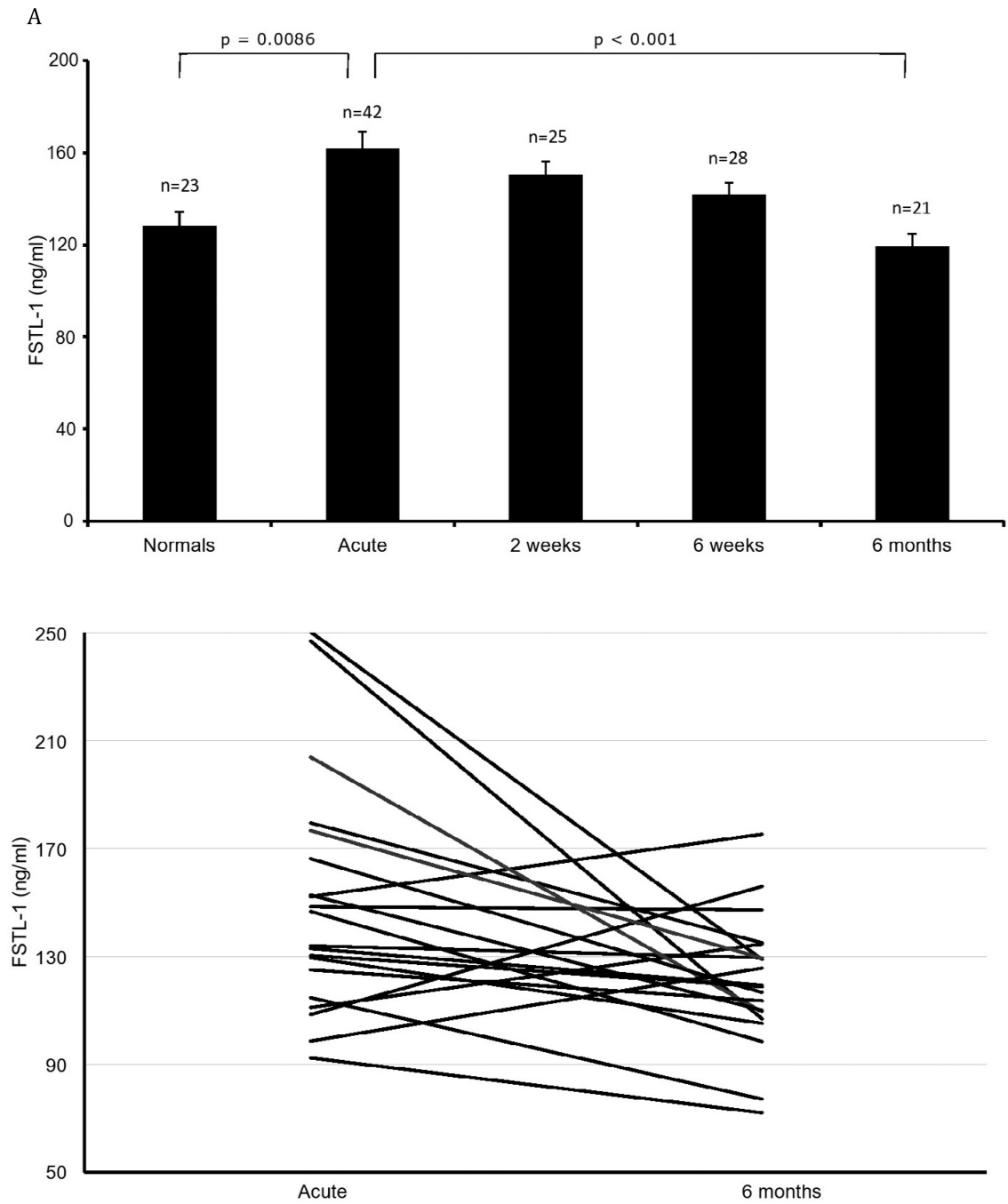


FIGURE 1.
FSTL-1 plasma levels in KD. Each bar represents the mean \pm SEM. n=number of patients.

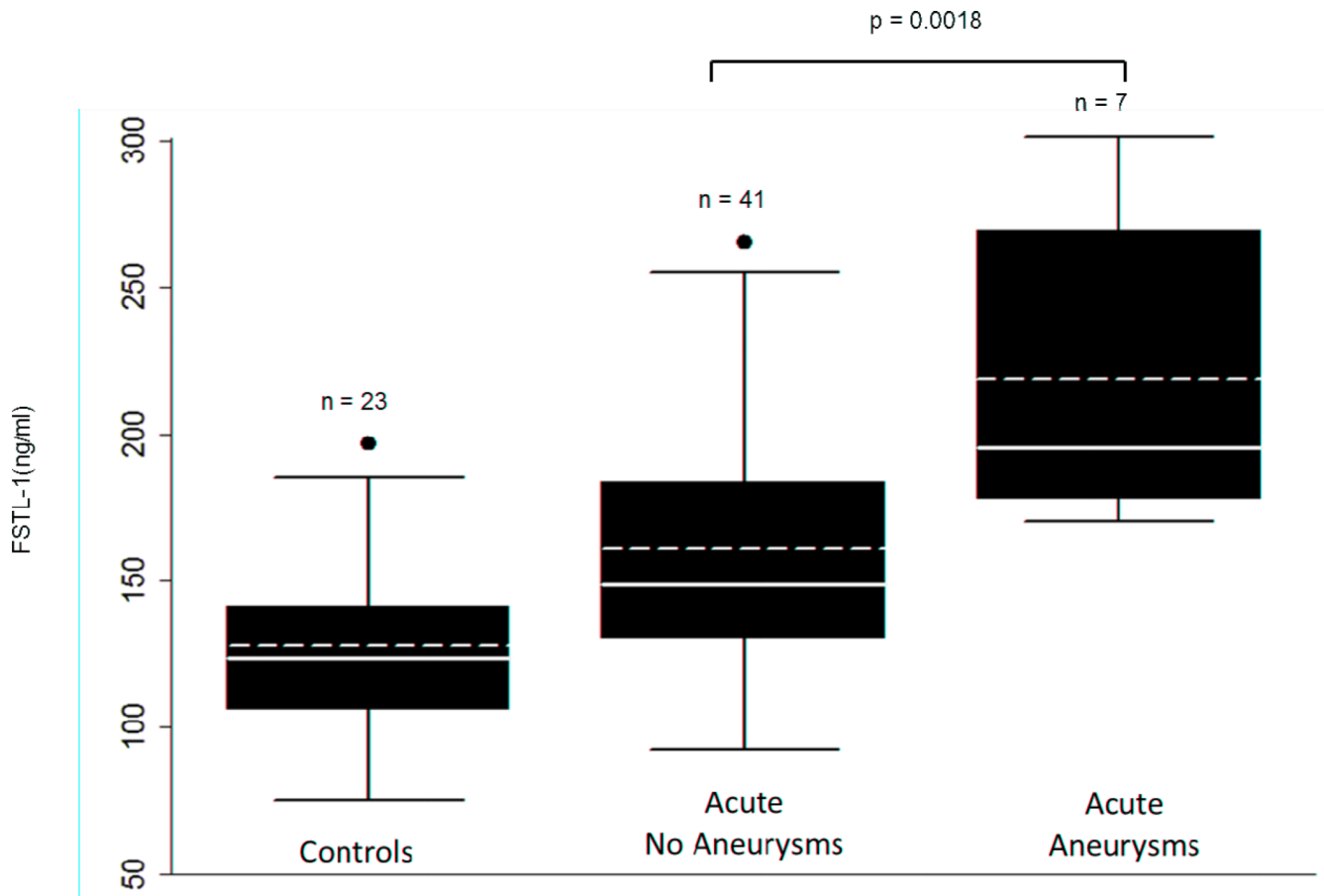


FIGURE 2.

Acute FSTL-1 plasma levels in patients with and without CA and controls. Solid line = median FSTL-1 level, dashed line = mean FSTL-1 level. n=number of patients.

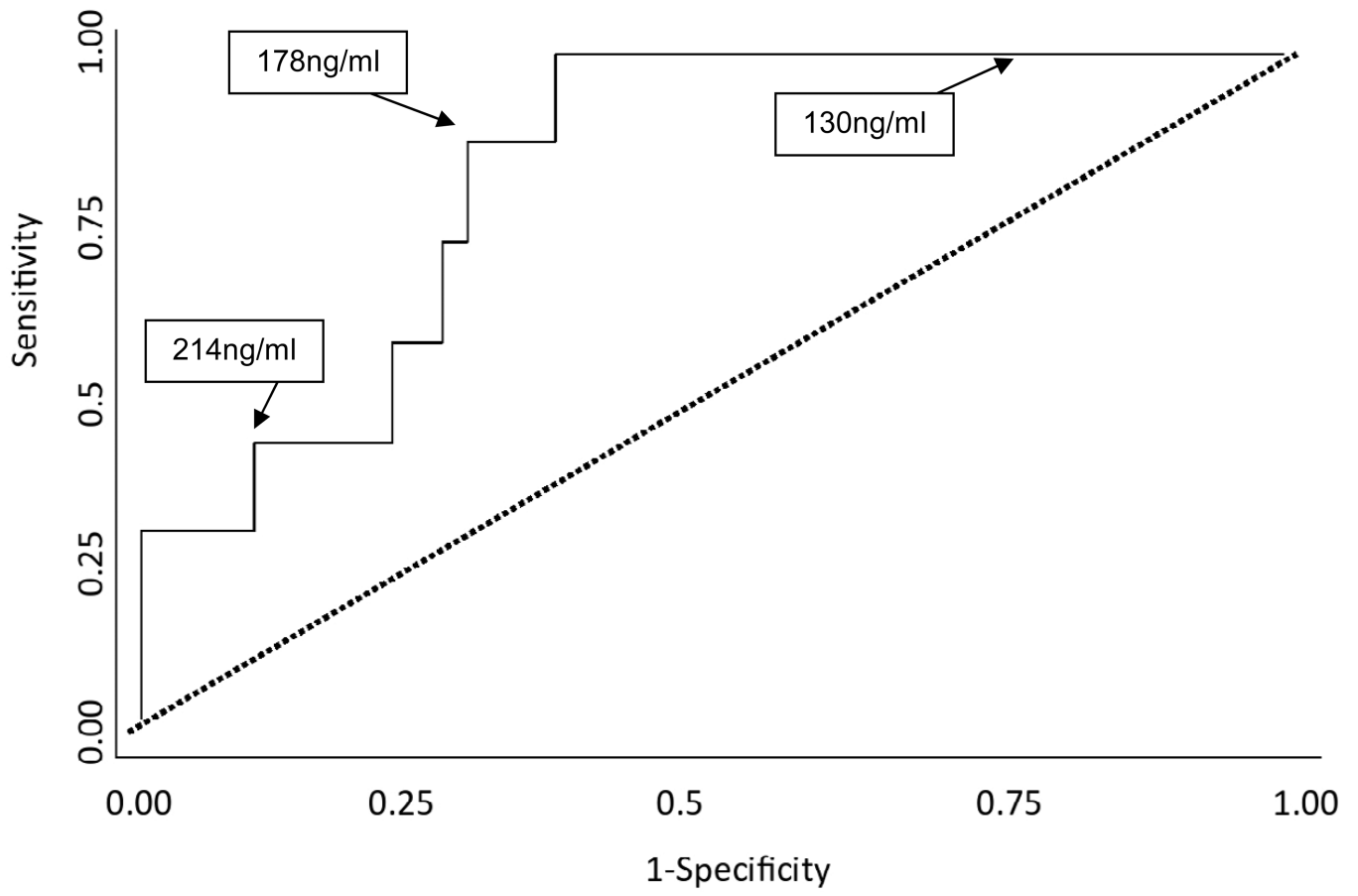


FIGURE 3. Receiver Operator Curve analysis shows an area under the curve of 0.8223, (95% CI 0.6863, 0.9583). Sensitivity of 85% and Specificity of 71% corresponded to 178 ng/ml.

TABLE 1

Baseline Patient Characteristics

Patient Characteristic	KD without aneurysm (n=47)	KD with aneurysm (n=7)	Control (n=23)
Age, months* (SD)	42.95 (32.7)	41.7 (51.3)	43.22 (25.2)
Sex[†]			
Male	29 (64%)	5	17 (74%)
Female	17 (36%)	2	6 (26%)
Unknown	1 (2%)	-	-
Race[‡]			
White	26	2	17
Black	17	1	6
Other/unknown	4 (1 Asian, 1 Hispanic, 2 unknown)	4 (1 Asian, 3 Hispanic)	-

* p value age: 0.9943 (Test of heterogeneity), age missing for 3 patients in KD without aneurysm.

[†] p value sex: 0.7065 (Exact test)

[‡] p value race: 0.0035 (Exact test)