UNIVERSITY OF CALIFORNIA, SAN DIEGO

Biomarker Protein Panel for Diagnosis of Kawasaki Disease

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Yuichiro Zeno Sato is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011

DEDICATION

Dedicated to family, friends, and teachers.

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A paper on plasma N-terminal pro-B-type natriuretic peptide, soluble ST2, and serum cardiac troponin I concentrations in acute Kawasaki disease (KD), convalescent KD, and febrile and healthy controls will be published by International Journal of Cardiology in 2011. An abstract of this paper was published by Pediatric Academic Society/Society for Pediatric Research. 2010 May: E-PAS20103704.72. I was the primary investigator and first author of the paper and abstract. Co-authors included Delaram P. Molkara, MD, Lori B. Daniels, MD, MAS, Adriana H. Tremoulet, MD, MAS, Chisato Shimizu, MD, John T. Kanegaye, MD, Brookie M. Best, PharmD, MAS, James V. Snider, PhD, Jeffrey R. Frazer, MD, Alan Maisel, MD, and Jane C. Burns, MD.

Figures of RBM biomarkers will be submitted for publication in 2011. Co-authors will include Janusz Dutkowski, PhD and Jane C. Burns, MD.

ABSTRACT OF THE THESIS

Biomarker Protein Panel for Diagnosis of Kawasaki Disease

by

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Protein biomarkers are traceable substances that can be used as tools in assessing the conditions of the human body. Using biomarkers related to the pathophysiology of Kawasaki disease (KD), a diagnostic tool can be formulated. I evaluated markers of cardiovascular injuries (n=8) and inflammation (n=90). Among cardiovascular

biomarkers, plasma N-terminal pro-B-type natriuretic peptide, soluble ST2, midregional pro-atrial natriuretic peptide, and procalcitonin concentrations were elevated in acute KD vs. febrile control (FC). However, a large overlap in protein levels and average values of area under the receiver operating characteristics (0.68-0.77) suggest that no single biomarker has the sensitivity and specificity to identify KD.

Combining results from laboratory tests and inflammatory markers, 14 analytes were found to have significant difference in concentration distributions among KD and FC. An unsupervised clustering analysis using these laboratory test results (erythrocyte sedimentation rate, C-reactive protein, absolute neutrophil count, γ-glutamyl transpeptisdase, and alanine amino transferase) and inflammatory markers (Inter-cellular adhesion molecule 1, alpha-1antitrypsin, macrophage inflammatory protein-1α, CD40, fibrinogen, matrix metalloproteinase-3, serum glutamic oxaloacetic transaminase, tissue inhibitor of metalloproteinases 1, and vascular endothelial growth factor) revealed an 84% performance at differentiating KD from FC, suggesting that a group of biomarkers is more reliable at diagnosing KD than a single marker.

INTRODUCTION

Background on KD

Kawasaki disease (KD) is the leading causes of acquired heart disease in children in the United States [1]. An immunologic reaction to an environmental agent in genetically susceptible children is proposed to cause the clinical symptoms [2-4]. However, a causative agent has not yet been discovered, thus restricting the possibilities for prevention and immunization. At present, patients with persistent fever are diagnosed using a constellation of clinical signs that include bilateral conjunctival injection, changes of lips and oral cavity, changes of the peripheral extremities, polymorphous rash, and cervical lymphadenopathy (Table 1) [5]. However, these features can be shared by other pediatric illnesses. The diagnosis of KD is supported by laboratory findings that suggest high levels of systemic inflammation in combination with four of five of the clinical criteria, or three of five criteria with at least one dilated coronary artery (Table 1) [5].

Without prompt treatment, 25% of patients will develop irreversible heart complications, including coronary artery aneurysms [6]. Treatment with intravenous immunoglobulin (IVIG) is effective in reducing the cardiovascular sequelae of KD to 5% if administered within the first ten days after disease onset [7]. Patients who experienced coronary artery damage are at risk of myocardial infarction and congestive heart failure later in life [8].

Between 15 to 30% of KD patients do not meet complete clinical criteria [9, 10]. These patients, defined as “incomplete KD”, are at risk for misdiagnosis and delayed treatment. Therefore, a diagnostic test is urgently needed for early detection of KD.

Epidemiology of KD

KD was first reported in Japan by Dr. Tomisaku Kawasaki in 1967 [11]. Since then, KD has been reported worldwide and in children of all ethnic groups, although previous studies indicated higher incidences in children of Asian and Pacific Islander decent[12]. A recent study conducted in San Diego County (California) revealed that Filipino-Americans had higher coronary artery aneurysm rate than KD patients of non-Filipino and non-Asian decent [13]. The estimated hospitalization rate for the United States due to KD is 9 to 18 per 100,000 children under the age of five [14, 15]. At the Rady Children's Hospital in San Diego, there has been an increase in KD cases from 63 patients in 2004 to 91 patients in 2010. In comparison, nearly 12,000 new cases of KD are diagnosed each year in Japan, and its incident rate is continuously increasing [16].

Biomarkers of KD

Biomarkers are measurable proteins in the blood that assess the disease state of the human body. Injury to endothelial cells and myocardium, and activation of the immune system are hallmarks of KD. Therefore, I tested a panel of biomarkers that are associated with these features. N-terminal pro-B-type natriuretic peptide (NT-proBNP), midregional pro-atrial natriuretic peptide (MR-proANP), and soluble ST2 (sST2) are markers for cardiomyocyte stress, while cardiac troponin I (cTnI) reflects cardiomyocyte necrosis. Midregional pro-adrenomedullin (MR-proADM), procalcitonin (PCT), C-terminal pro-vasopressin (copeptin), and C-terminal pro-endothelin-1 (CT-proET-1) are markers associated with cardiovascular inflammation and homeostasis. Markers of inflammation were also studied, including pentraxin-3 (PTX3) and neopterin. Samples

were sent to Rules-Based Medicine (RBM) for Multi-Analyte Profile of 88 inflammatory markers, such as interleukin and matrix metalloproteinase.

Previous studies on KD biomarkers

Previous studies investigated protein biomarkers as tools for diagnosing acute KD, including brain natriuretic peptide (BNP) and vascular endothelial growth factor (VEGF). However, a large overlap in the protein levels among KD and control groups suggest that no single biomarker has the sensitivity or specificity to reliably identify this disease. Hence, I propose that a combination of biomarkers that represent different facets of KD pathophysiology is more viable than a single marker.

Previous studies included controls that shared no clinical features with KD patients. As the purpose of the diagnostic test is to differentiate between children with KD vs. other rash-fever illnesses, it is important that the control group present with some clinical features that are also seen in KD, such as scarlet fever. These children are determined not to have KD based on history, physical exam, and laboratory evaluation and serve as the febrile controls (FC) for the study.

MATERIALS AND METHODS

Biomarkers for the diagnosis of KD were studied in the process depicted in Figure 1. The Human Research Protection Program of the University of California, San Diego approved this research protocol and written informed consent was obtained from the parents of all subjects.

Patients

KD samples were from consecutive, unselected KD subjects for whom both plasma and serum samples were available. All KD subjects fulfilled American Heart Association diagnostic criteria for KD (Table 1). Acute KD samples were obtained prior to treatment with IVIG. Convalescent samples were obtained at 26-73 days after the onset of fever when the erythrocyte sedimentation rate (ESR) and platelet count had returned to normal, and a subset of subject had additional samples (late convalescent) obtained 1-2 years after disease onset.

Controls

FC subjects were previously healthy children recruited from the Emergency Department at Rady Children's Hospital San Diego and had ≥ 3 days of fever and at least one of the clinical signs of KD: rash, conjunctival injection, cervical lymphadenopathy, erythematous oral mucosa, and erythematous or edematous hands or feet. Healthy control (HC) subjects were children undergoing minor elective surgery for polydactyly.

Basic Clinical and Laboratory Data

We recorded age, sex, illness day at patient evaluation (first calendar day of fever= illness day 1), and laboratory data, including C-reactive protein (CRP), ESR, white blood count (WBC), percent polymorphonuclear leukocytes, percent bands, absolute neutrophil count, hemoglobin, platelet count, γ-glutamyl transpeptisdase (GGT), and alanine amino transferase (ALT). We normalized the hemoglobin concentration for age to allow valid comparisons across the age spectrum of our subjects. For KD subjects only, we recorded response to IVIG and echocardiographic data. IVIG-resistance was defined as persistent or recrudescent fever ($T \geq 38^{o}C$) at least 24 hours after completion of the IVIG infusion (2 g/kg). Aneurysms were defined as a focal region of the coronary artery 1.5 times the diameter of the adjacent segment.

Protein Biomarkers

Table 2 shows the diagnoses of FC used for the biomarker study.

Group 1 Biomarkers

EDTA plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP), sodium-citrate plasma soluble ST2 (sST2), and serum cardiac troponin I (cTnI) concentrations were determined for the following subjects: 55 acute KD (30 of whom had paired convalescent samples), 54 age-similar FC, and 50 age-similar HC. cTnI levels were also determined for 17 KD subjects who had late convalescent serum obtained.

NT-proBNP concentration was measured with a chromatographic immunoassay (*Status*First CHF NT-proBNP test devices, Nanogen, San Diego, CA; 99% for reference

value for healthy adults=125 pg/mL), in combination with the DXpress Reader (Nanogen, San Diego, CA). sST2 levels were determined using the Presage sST2 assay kit (Critical Diagnostics, New York, NY; 99% for reference value for healthy adults= 50.2 ng/mL). cTnI was measured using the Verisens human cTnI assay (Nanosphere, Northbrook, IL; 99% for reference value for healthy adults =0.0045 ng/mL).

Group 2 Biomarkers

EDTA plasma C-terminal pro-vasopressin (copeptin), C-terminal pro-endothelin-1 (CT-proET-1), midregional pro-atrial natriuretic peptide (MR-proANP), midregional pro-adrenomedullin (MR-proADM), and procalcitonin (PCT) concentrations were determined for a subset of acute KD (n= 23), convalescent KD (n=10), and HC (n=14) cohorts from group 1 for whom there were sufficient plasma and suitable sex, age-matched (± 8 months) FC (n=23). Copeptin, CT-proET-1, MR-proANP, MR-proADM, and PCT concentrations were measured with the KRYPTOR System (Brahms AG, Hennigsdorf/Berlin, Germany).

Group 3 Biomarkers

EDTA plasma pentraxin 3 (PTX3) were measured in 4 age, sex (± 1 year) matched KD and FC. Neopterin (EDTA plasma) concentrations were measured in 10 independent and age-sex (±6 months) matched acute KD and FC. Neopterin and PTX3 were measured by neopterin and PTX3 ELISA kits, respectively (Brahms AG, Hennigsdorf/Berlin, Germany).

Group 4 Biomarkers

A total of 88 protein analytes (plasma sodium-citrate; Supplemental Table 1) in inflammatory pathways were measured using the Luminex antibody-coated bead system (Human Map, version 1.6, Rules Based Medicine Inc.). Protein concentrations were determined for 28 age-sex paired (± 7 months) acute KD and FC, respectively

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) software, and presented as medians and interquartile range. Mann-Whitney U test was used for non-parametric data. Paired data for acute and convalescent KD were analyzed using a Wilcoxon signed rank test. Correlations between continuous variables were performed using Spearman's test. Categorical data were analyzed with Fisher's exact test. The p values were not adjusted for multiple testing and values <0.05 were considered significant. Area under the receiver operating characteristics (AUC-ROC) curve was used to evaluate biomarkers' performance to differentiate KD from FC subjects (not calculated for group 3 and 4). Additional analyses were performed for biomarkers in group 4 using unsupervised clustering (heatmap) in collaboration with Dr. Janusz Dutkowski at University of California, San Diego.

RESULTS

Clinical Characteristics of the Study Population

Patient characteristics for the KD and FC groups are shown in Table 3 and 4. Overall, KD patients had a higher C-reactive protein (CRP) level, ESR, white blood cell count (WBC), absolute neutrophil count (ANC), platelet count (PLTS), alanine amino transferase (ALT), and γ-glutamyl transferase (GGT), and lower age-adjusted hemoglobin levels.

Biomarker Analyses

Group 1Biomarkers

Plasma NT-proBNP was significantly elevated in acute KD subjects compared to convalescent KD and control groups ($p<0.0001$) (Figure 2A, Table 5). NT-proBNP concentrations positively correlated with sST2, ALT, and GGT levels (Table 6). Concentrations of NT-proBNP negatively correlated with age (Table 6), consistent with the observation that NT-proBNP concentrations are higher in infants and young children [17]. Six KD subjects had markedly elevated NT-proBNP levels (>1,000 pg/mL) associated with elevated levels of sST2 (5 subjects) and cTnI (4 subjects). For the majority of KD subjects, NT-proBNP concentrations declined by the convalescent phase (26 of 30) (Figure 3A, Table 5). AUC-ROC values for NT-proBNP is 0.75 (Figure 4).

Plasma sST2 concentrations were significantly elevated in acute KD compared to convalescent KD and both control groups ($p<0.002$) (Figure 3B, Table 5) and negatively correlated with illness day suggesting that concentrations were highest in the earliest

stages of the illness (Table 6). sST2 concentrations correlated strongly with ALT and GGT. For the majority of KD subjects, sST2 concentrations declined by the convalescent phase (29 of 30) (Figure 3B, Table 5). AUC-ROC value for sST2 is 0.68 (Figure 4).

There were no significant difference in serum cTnI concentrations between acute KD and FC (Table 5). The levels of cTnI were increased in the convalescent as compared to the acute stage of KD ($p=0.0003$, $n=30$) and normalized in all 17 subjects with late convalescent samples (Figure 3C, Table 5). Elevated cTnI concentrations (>0.0045 ng/mL) were observed in 16 (29%) and 17 (31%) subjects in the acute KD and FC groups, respectively. For the KD subjects, there was a weak correlation of cTnI concentration with NT-proBNP (Table 6) and no significant correlation with the other biomarkers or laboratory tests. AUC-ROC value for cTnI is 0.51.

Group 2 Biomarkers

Plasma concentrations of MR-proANP, CT-proET-1, MR-proADM, and PCT were higher in acute KD subjects than in HC ($p<0.005$), but only MR-proANP and PCT levels were higher in acute KD than in FC (Table 7, Figure 5). Copeptin concentrations did not differ between acute and convalescent KD subjects, and FC and HC. MR-proANP, PCT, and MR-proADM levels were higher in acute vs. convalescent KD. (Table 7)

MR-proANP negatively correlated with age and illness day, and strongly correlated with ALT and GGT levels (Table 8). In comparison, PCT negatively correlated with illness day and PLT, and strongly correlated with ALT and GGT. MR-proANP and PCT correlated with NT-proBNP, sST2, MR-proANP, CT-proET, and MR-proADM.

The AUC-ROC values for these biomarkers were as follows: 0.77 for MR-proANP (Figure 6), 0.76 for PCT (Figure 6), 0.63 for CT-proET, 0.64 for MR-proADM, and 0.50 for copeptin.

Group 3 Biomarkers

Plasma PTX3 levels were not significantly different (p=0.89) between acute KD (Median = 12.8, IQR=5.9-19.4 ng/mL) vs. FC (Median=14.6, IQR=4.3-22.3 ng/mL). **N**eopterin concentrations were not significantly different (p= 0.44) between acute KD (Median=16.7, IQR=13.7-22.0 nmol/L) and FC (Median = 18.1, IQR=15.5-24.3 nmol/L).

Group 4 Biomarkers

Twenty-six protein concentrations were significantly different in acute KD vs. FC (Table 9). Combined with data from laboratory test results, 14 biomarkers were identified to have distinctive concentration distributions between KD vs. FC (Figure 7). These markers included alpha-1antitrypsin, macrophage inflammatory protein-1 alpha (MIP-1 alpha), matrix metalloproteinase-3 (MMP-3), CRP, fibrinogen, intercellular adhesion molecule 1 (ICAM-1), tissue inhibitor of metalloproteinases 1 (TIMP-1), GGT, ESR, ALT, serum glutamic oxaloacetic transaminase (SGOT), CD40, vascular endothelial growth factor (VEGF), and ANC.

Using unsupervised learning, these 14 biomarkers successfully clustered 20 KD (71%) and 27 FC (96%) into separate groups (Figure 8). However, 8 KD subjects were falsely identified as FC (29%). Taken together, this panel of biomarkers had an average

of 84% performance to differentiate KD from FC, demonstrating better performance than a single marker.

DISCUSSION

Plasma NT-proBNP, sST2, MR-proANP, and PCT concentrations were elevated in acute KD vs. FC. AUC values for these biomarkers ranged from 0.68 to 0.77, revealing that no single marker could reliably identify acute KD. In comparison, a group of biomarkers (10 protein analytes from RBM assay, and 4 from laboratory tests) had an 84% performance at differentiating KD from FC. NT-proBNP, sST2, MR-proANP, and PCT positively correlated with markers of inflammation, oxidative stress (GGT), and hepatocyte injuries (ALT), and negatively correlated with illness days, suggesting elevated levels in early stages of KD.

Biomarkers are emerging as valuable tools in assisting with disease prognosis and risk stratification for a variety of cardiovascular conditions. The natriuretic peptides are released in the setting of myocardial strain and are well-established for aiding in the diagnosis, prognostication, and monitoring of heart failure patients.[18] Previous studies have measured plasma BNP and NT-proBNP levels in acute and convalescent KD subjects and found results similar to those reported here.[19-24] As expected, a strong correlation was found between the concentrations of NT-proBNP and MR-proANP, which are released by the cardiomyocytes of the ventricle and atria, respectively. [25]

Unexpectedly, a subset of the FC (n=4) discharged from the Emergency Department with self-limited febrile illnesses had NT-proBNP levels >1,000 pg/mL with sST2 levels 27-93 ng/mL and cTnI levels 0.0003-0.019 ng/mL, suggesting myocardial stress. The diagnoses in these 4 subjects were staphylococcal scalded skin syndrome (n=1), adenovirus (n=1), and viral syndrome (n=2). Currently, I am mentoring an

undergraduate student to write a case report on these FC subjects to the Pediatric Infectious Disease Journal.

sST2, a member of the IL-1 receptor family and a decoy receptor for IL-33, is released by cardiomyocytes and fibroblasts exposed to biomechanical stress.[26] Although elevated levels of sST2 are powerfully predictive of adverse events across a broad spectrum of cardiovascular conditions including heart failure and acute myocardial infarction, the mechanism by which sST2 mediates these effects is incompletely understood.[26-30] sST2 may have a direct role in fibrosis or remodeling following myocardial injury.[31] The prognostic significance of sST2 levels in acute KD is unknown.

cTnI, a measure of cardiomyocyte injury or death, was elevated in a third of both KD subjects and FC. A previous study that may have used a less sensitive assay did not detect elevated cTnI concentrations in children with acute KD.[32] Acute phase concentrations of cTnI did not correlate with markers of systemic inflammation or oxidative stress suggesting that other factors lead to myocardial necrosis or cardiomyocyte damage.[32] This was surprising as it would be logical to think that all of the effects on the myocardium were a result of systemic inflammation. However, this relationship did not hold for cTnI. The highly sensitive assay may detect variations in levels that are not physiologically significant with respect to myocardial function, though they seem to reflect disease severity to some degree since 4 of the 6 individuals with elevated NT-proBNP also had elevated cTnI. The elevation of cTnI in most KD subjects at the convalescent time point was also unexpected and may indicate that cardiomyocyte injury persists after systemic indicators of inflammation have returned to normal. The

fact that levels of cTnI returned to normal in all subjects who had samples measured 1-2 years after disease onset suggests that the previous elevations were related to cardiomyocyte injury or death.

Our data and previous reports [33] demonstrate a 10-fold elevation of serum concentrations of PCT in acute KD compared to healthy controls. However, the ability of PCT levels to predict coronary artery aneurysms has been questioned [34]. MR-proADM is a stable, surrogate marker for adrenomedullin (ADM), a multifunctional peptide hormone with cardiovascular effects (vasodilatation, increased myocardial contractility) and antimicrobial peptide activity.[35, 36] MR-proADM is secreted by a wide variety of tissues, but the source of the elevated levels in our KD subjects is unknown. Like PCT, ADM is a downstream product of the IL-1 and TNFα signaling pathways and is therefore a marker of acute inflammation. Monocyte ADM transcription and plasma levels of the mature ADM peptide are increased in acute KD. [37, 38] The strong correlation of MR-proADM with GGT in our subjects suggests a link to oxidative stress.

Concentrations of copeptin, a stable fragment of the precursor of arginine vasopressin, were not significantly elevated in our KD patients, despite that fact that hyponatremia has been reported in KD patients.[39, 40] This suggests that the mechanism of hyponatremia in KD is not mediated through the AVP pathway. Pro-ET-1 concentrations were only modestly elevated in acute KD, in comparison to HC. Pro-ET-1 is a precursor to ET-1, a neurohormone involved in ventricular and vessel fibrosis. [41]

KD is a self-limited vasculitis, which may explain the relationship among the biomarkers seen in Figure 8. Because of the inflammatory nature of the disease, markers of inflammation (CRP and ESR) and markers of inflammatory response (MIP-1 alpha)

are elevated in patients [5]. Levels of fibrinogen are elevated during systemic inflammation, which increases the sedimentation rate of red blood cells. Thus, explaining the close relationship between ESR and fibrinogen. Rise in immune activation due to inflammation is supported by elevated ANC and CD40 levels. CD40 is expressed on endothelial cells and vascular smooth muscle cells, and its interaction with CD40 ligand results in various immune and inflammatory responses. [42]

VEGF is involved in vascular permeability and development of coronary artery lesion in KD [43]. This molecule is expressed in the endothelium and smooth muscle cells [44], and stimulation or destruction of the cells by inflammation induces its release. ICAM-1 is a cell surface adhesion molecule on the endothelial cells [45]. Elevated circulation of this adhesion molecule is reported in systemic vasculitis [46]. Alpha 1 antitrypsin is secreted by the hepatocytes and may play a role in the resolution of inflammation by modulating neutrophil elastase involved in tissue injuries [47, 48].

Remodeling of the extracellular matrix is a feature of vascular lesion in KD, and it involves the interaction between matrix metalloproteinases (MMPs) and its inhibitor (TIMPs). [49] MMP-9 has been reported in coronary lesion of the left coronary arteries, and it is regulated by TIMP-1[49]. Elevated concentrations of TIMP-1 was seen between KD and FC (Table 9, Figure 8), and could explain why the MMP-9 were undetectable in the RBM assay (data not shown). MMP-3 was reported to be involved in coronary artery aneurysm formation in KD patients [50]. Only five out of 28 KD subjects studied in group 4 biomarkers developed coronary artery aneurysms, which could explain the large overlap in protein levels between KD and FC.

GGT catabolizes extracellular glutathione, the main thiol intracellular antioxidant in mammalian cells. Membrane-bound GGT is released into the serum from hepatocytes and the elevated levels in acute KD have been attributed to hepatobiliary inflammation, with the highest levels seen in association with hydrops of the gallbladder.[51] Results presented here, however, suggest that elevated GGT and ALT concentrations may be at least in part related to oxidative stress during the acute illness, with concentrations positively correlating with the biomarkers of cardiomyocyte strain. SGOT, like ALT, is a marker of liver inflammation, but can be elevated in diseases affecting other tissues, which might explain the similar protein levels between KD and FC.

Biomarker panel for the diagnosis of KD is an ongoing project. In collaboration with Dr. Dutkowski, we used supervised learning algorithms (decision trees and random forests) with the 14 biomarkers in group 4 to create a series of concentration cutoffs that would differentiate KD from FC (data not shown). At present, I am working with Dr. Dutkowski and Dr. Bruce Ling (Stanford University, California) to validate our findings in group 4 biomarkers using independent KD and FC subjects (n=44). Further investigations were performed correlating echocardiography data with NT-proBNP, sST2, and cTnI. NT-proBNP concentrations positively correlated with the internal diameter of the coronary arteries (RCA/LAD Z_{worst}) and MV peak A wave, and negatively correlated with mitral valve inflow E:A ratio. Both NT-proBNP and sST2 negatively correlated with deceleration time, suggesting that as myocardial strain worsened, diastolic dysfunction became more pronounced. These findings were submitted as an article to the International Journal of Cardiology, and an abstract was published by the Pediatric Academic Society (E-PAS20103704.72.).

For future studies, I propose analyzing other potential KD biomarkers, multi-diagnostic analysis of NT-proBNP, sST2, MR-proANP, and PCT, and combining cardiovascular markers with inflammatory markers. I recognize several limitations to our study. The volumes of plasma and serum samples were limited in this population of young infants and children, so not all measurements were performed on all patients. Although this is the largest study of biomarkers in acute KD, the sample size was still small and thus the power to detect differences between groups was limited.

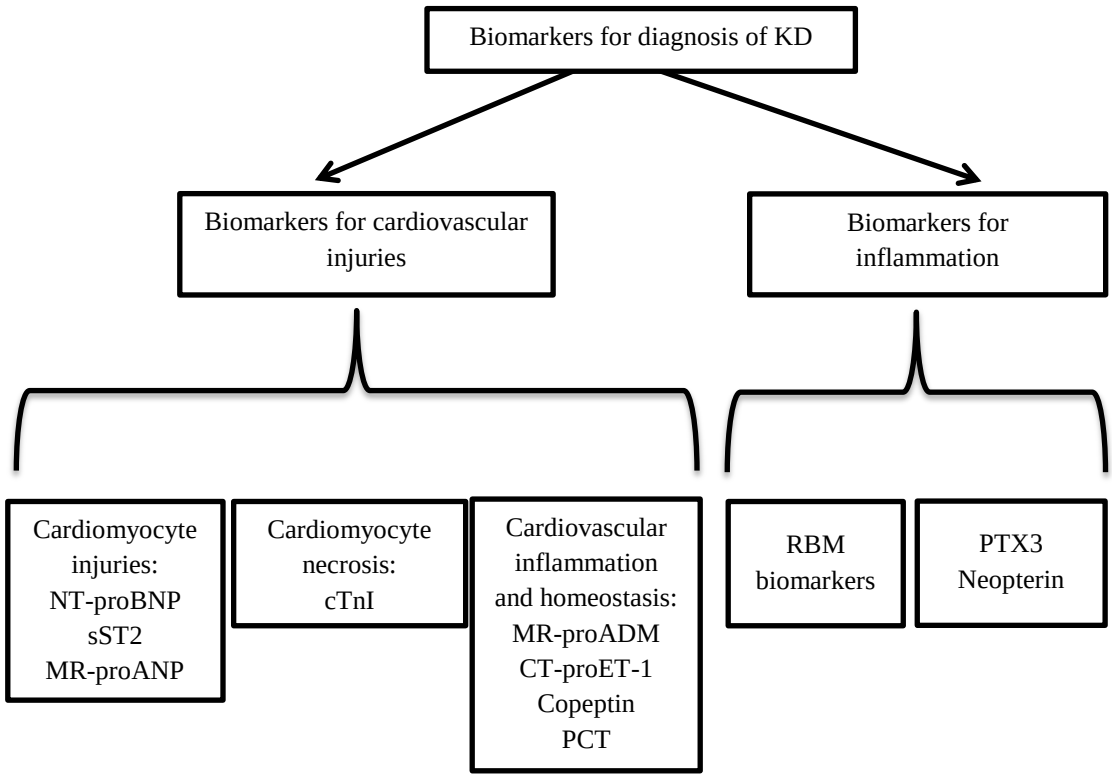

Figure1: Study of protein biomarkers for diagnosis of Kawasaki disease (KD)

NT-proBNP = N-terminal pro-B-type natriuretic peptide, sST2 = soluble ST2, MR-proANP = midregional pro-atrial natriuretic peptide, cTnI = cardiac troponin I, MR-proADM = midregional pro-adrenomedullin, CT-proET-1 = C-terminal pro-endothelin-1, copeptin = C-terminal pro-vasopressin, PCT = procalcitonin, RBM = Rules-Based Medicine, PTX3 = pentraxin-3

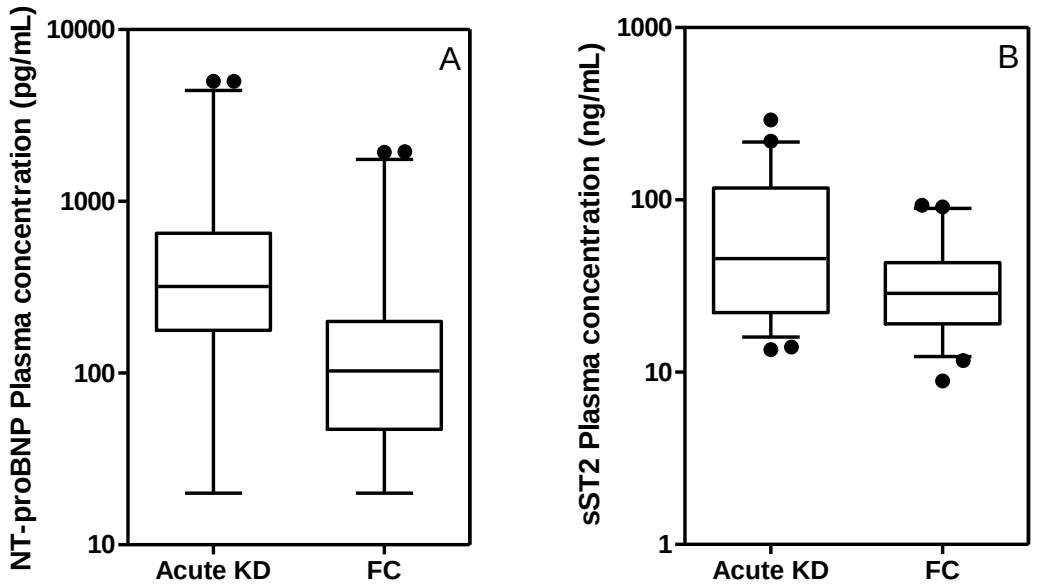

Figure 2: Plasma concentrations of N-terminal pro-B-type natriuretic peptide (NT-proBNP) and soluble ST2 (sST2). **A)** Comparisons of NT-proBNP concentrations $p<0.0001$ for acute KD vs. FC). **B)** Comparisons of sST2 concentrations ($p=0.002$ for acute KD vs. FC). Box plot represents median (bar) with interquartile range (box), and T-bars show 5^{th}-95^{th} percentile. Data presented in a logarithmic scale. Outlying values are represented by black dots.

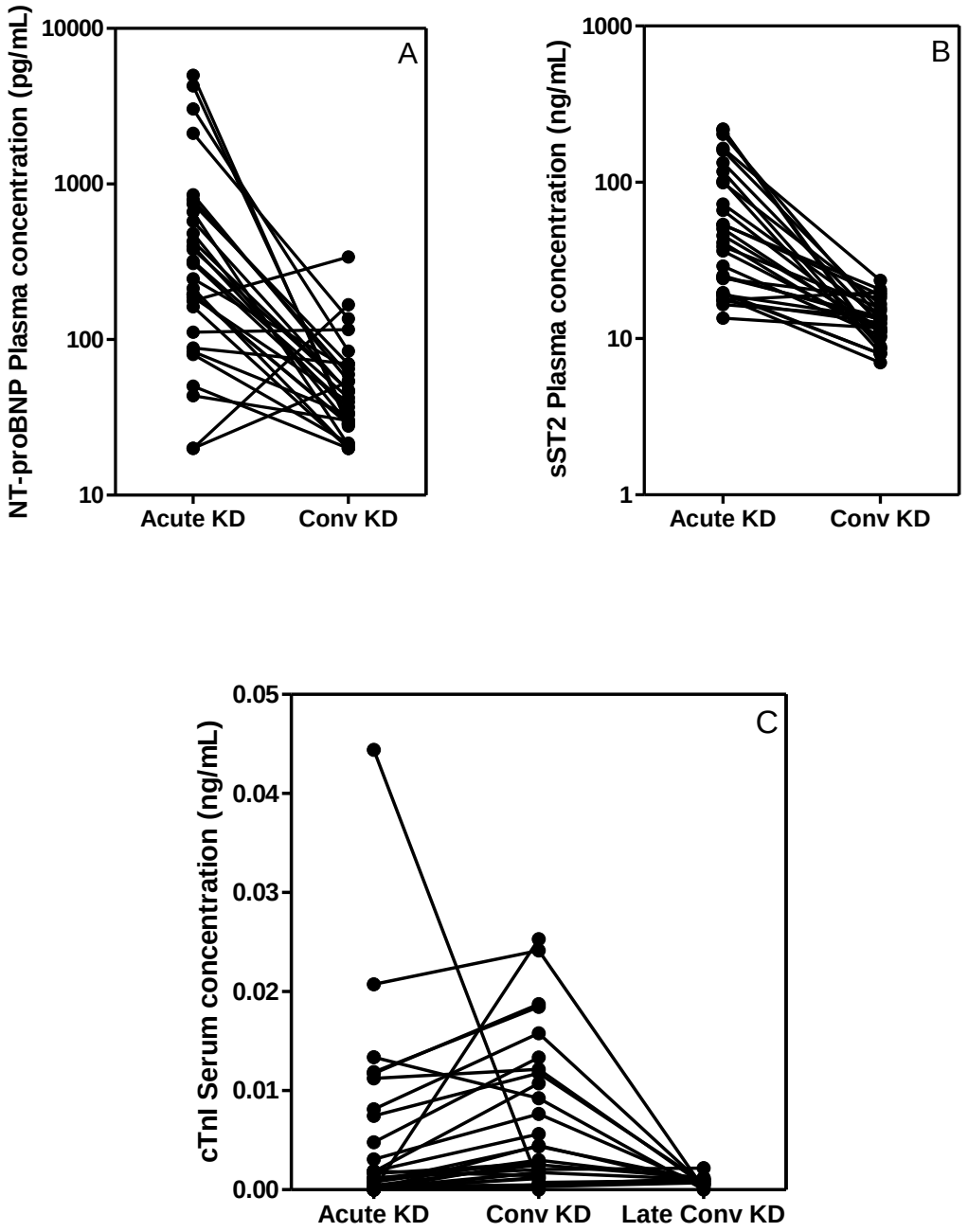

Figure 3: Acute Kawasaki disease (KD) versus convalescent KD. A) Concentrations of N-terminal pro-B-type natriuretic peptide (NT-proBNP), n=30 and B) soluble ST2 (sST2), n=30 in paired acute and convalescent (conv.) Kawasaki disease (KD) plasma samples plotted on a logarithmic scale. C) Cardiac troponin I (cTnI) concentrations in serial acute, convalescent (conv.), and late conv. (> 1yr.) serum samples from KD subjects plotted on a linear scale.

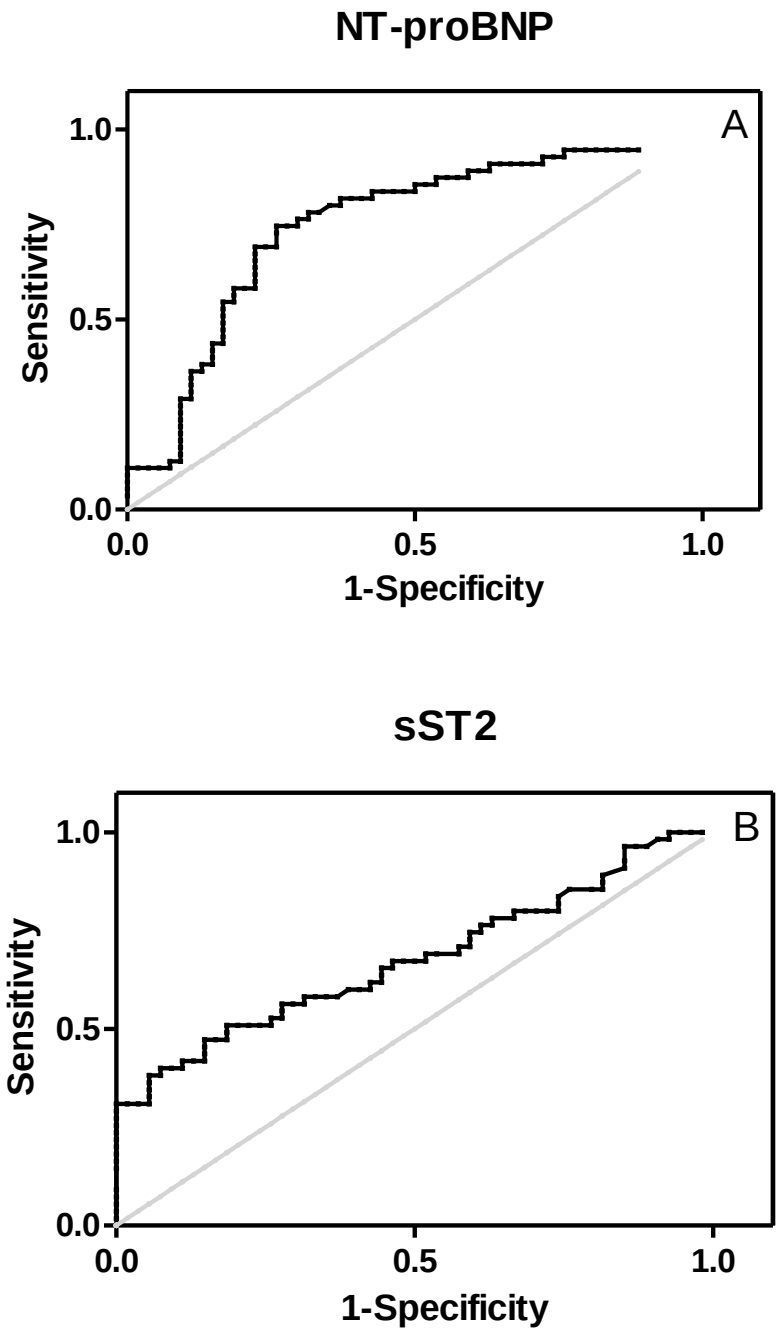

Figure 4: Receiver operation characteristics (ROC) curve of: A) N-terminal pro-B-type natriuretic peptide (NT-proBNP), AUC = 0.75, and B) Soluble ST2 (sST2), AUC = 0.68.

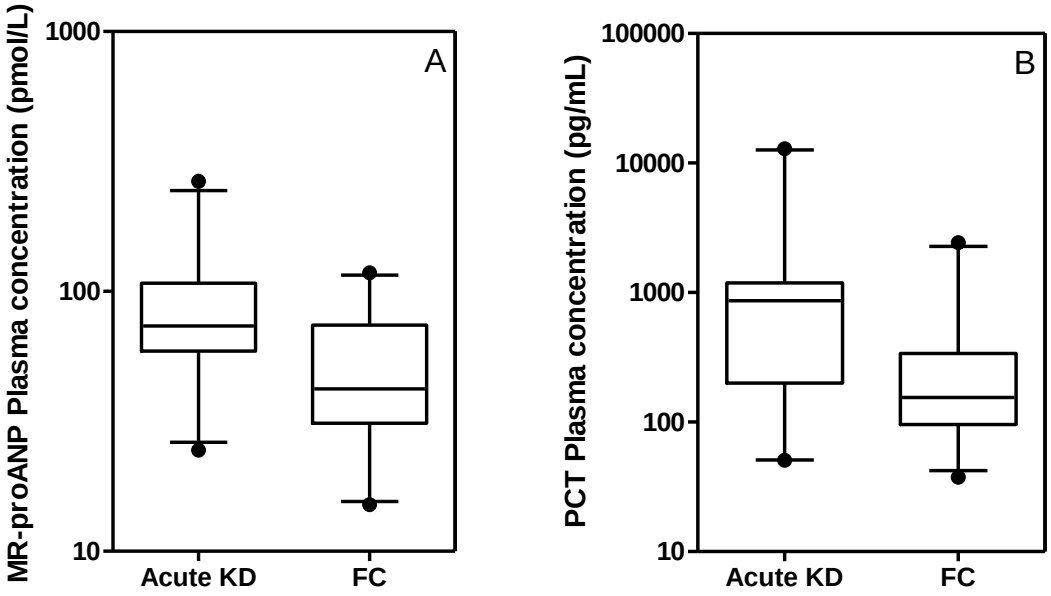

Figure 5: Plasma (EDTA) concentrations of: A**)** Midregional pro-atrial natriuretic peptide (MR-proANP), p=0.002, acute Kawasaki disease (KD) vs. febrile control (FC), and B**)** Procalcitonin (PCT), p=0.002, acute KD vs. FC. Box plot represents median (bar) with interquartile range (box), and T-bars show 5^{th}-95^{th} percentile. Data presented in a logarithmic scale. Outlying values are represented by black dots.

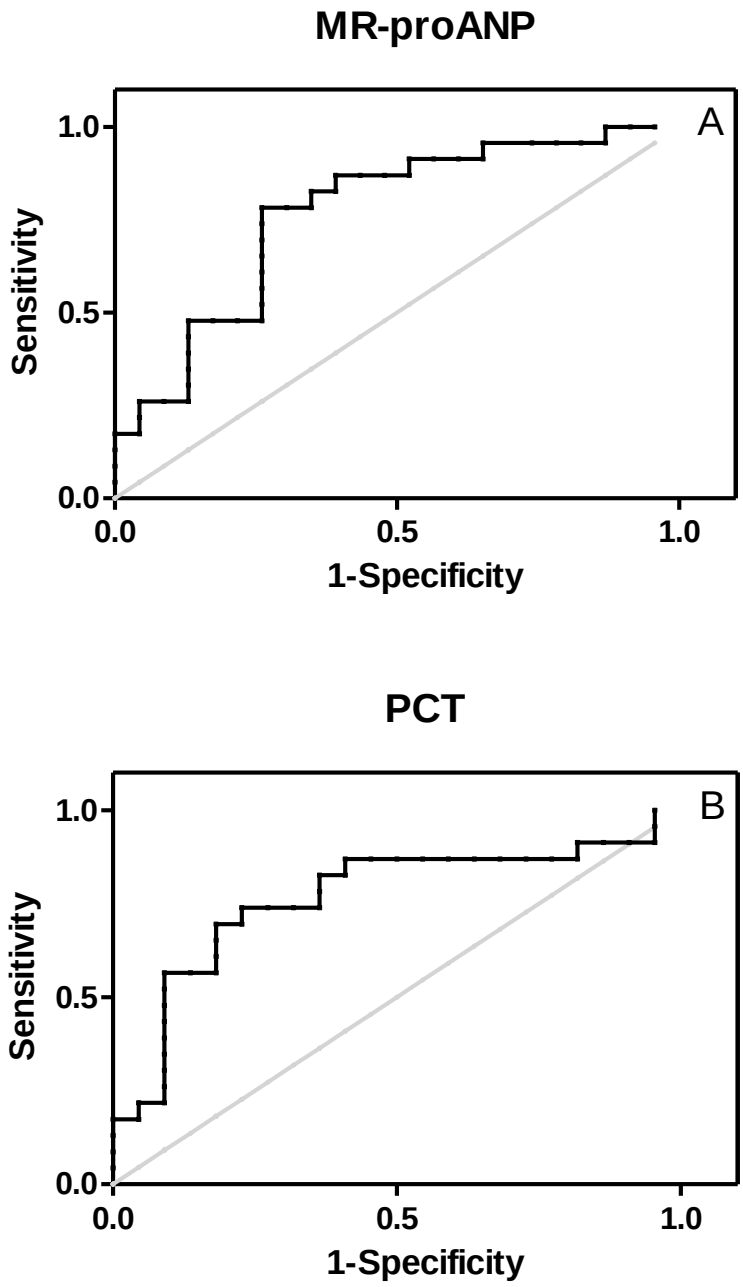

Figure 6: Receiver operation characteristics (ROC) curve of: A) Midregional pro-atrial natriuretic peptide (MR-proANP), n=23, AUC = 0.77, and B) Procalcitonin (PCT), n=23, AUC = 0.76.

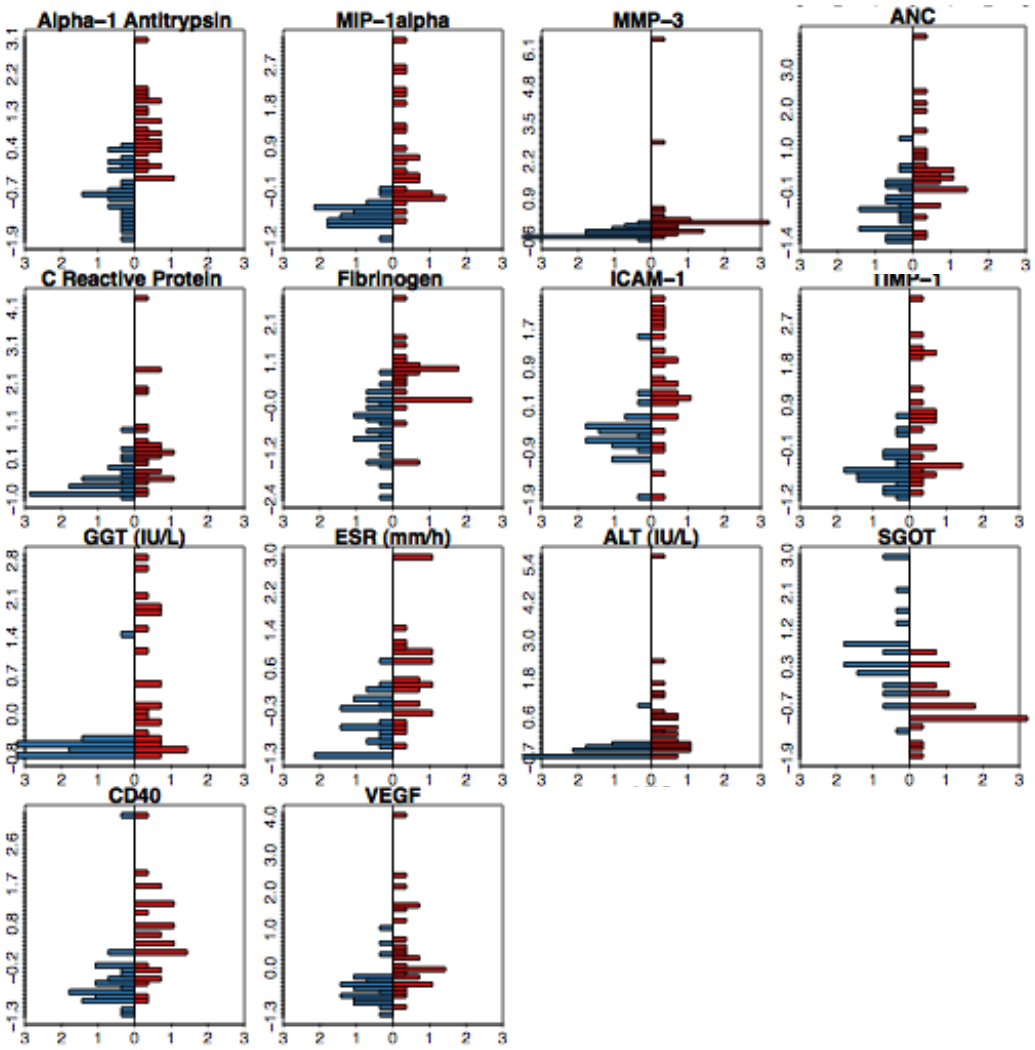

Figure 7: Histogram of 14 biomarkers in group 4 that had significantly different concentration distributions between Kawasaki disease (KD) and febrile controls (FC)

Red = KD; Blue = FC; x-axis = arbitrary number of patients with similar protein concentrations; y-axis = standardized protein concentrations

MIP-1 alpha = macrophage inflammatory protein-1α, MMP-3 = matrix metalloproteinase-3, ANC = absolute neutrophil count, ICAM-1 = Inter-cellular adhesion molecule 1, TIMP-1 = tissue inhibitor of metalloproteinases 1, GGT = γ-glutamyl transpeptisdase, ESR = erythrocyte sedimentation rate, ALT = alanine amino transferase, SGOT = serum glutamic oxaloacetic transaminase, VEGF = vascular endothelial growth factor

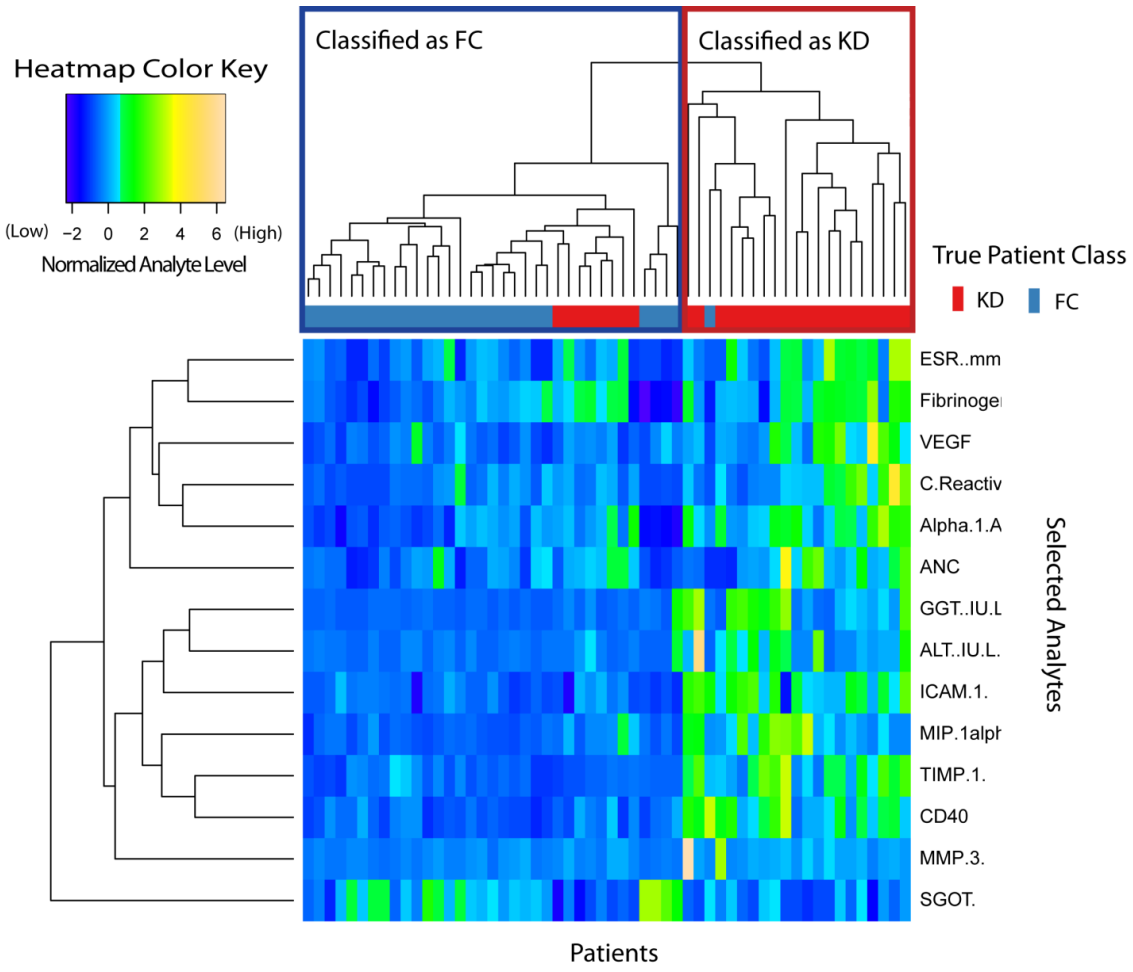

Figure 8: Heatmap of unsupervised clustering of Kawasaki disease (KD) and febrile control (FC) subjects based on results of 14 analytes.

ESR = erythrocyte sedimentation rate, VEGF = vascular endothelial growth factor, C.Reactiv = C-reactive protein, Alpha. 1. A = alpha 1 antitrypsin, ANC = absolute neutrophil count, GGT = γ-glutamyl transpeptisdase, ALT = alanine amino transferase, ICAM-1 = Inter-cellular adhesion molecule 1, MIP.1.alp = macrophage inflammatory protein-1α, TIMP-1 = tissue inhibitor of metalloproteinases 1, MMP-3 = matrix metalloproteinase, SGOT = serum glutamic oxaloacetic transaminase

Table 1: Diagnostic criteria for Kawasaki disease (KD) (5)

Patients were labeled with complete diagnostic criteria when they had at least 3 days of fever, but not more than 10 days, and met 4 of 5 standard diagnostic criteria, or 3 of 5 criteria with at least one dilated coronary artery

1. Bilateral conjunctival injection
2. Changes of lips and oral cavity
3. Changes of the peripheral extremities
4. Polymorphous rash
5. Cervical lymphadenopathy

Table 2: Diagnoses of febrile controls (FC)

Biomarker groups	1 (n=54)	2 (n=23)	3: PTX3 (n=4)	3: Neopterin (n=10)	4 (n=28)
Bacterial infections (total n)	8	4	1	2	4
Scarlet fever	3	1	0	1	3
Staphylococcal scalded skin syndrome	2	1	0	1	0
Streptococcal pharyngitis	3	2	0	0	1
Pyelonephritis	0	0	0	0	0
Unknown abscess organism	0	0	1	0	0
Viral infections (total n)	46	19	3	8	24
Measles	1	1	0	0	0
Culture-proven adenovirus	11	6	0	4	7
Viral syndrome*	34	12	3	4	16
Epstein-Barr virus	0	0	0	0	1
Enterovirus	0	0	0	0	0

*Viral syndrome defined as self-limited, minor febrile illness with negative throat and rectal viral cultures

Table 3: Clinical and laboratory characteristics of acute Kawasaki disease (KD) and febrile control (FC) subjects measured for biomarkers in groups 1 to 3.

Characteristics	Group 1		Group 2		Group 3: pentraxin 3		Group 3: neopterin	
	Acute KD (n=55)	FC (n=54)	Acute KD (n=23)	FC (n=23)	Acute KD (n=4)	FC (n=4)	Acute KD (n=10)	FC (n=10)
Median age, yrs. (range)	2.8 (0.4-14.9)	2.4 (0.2-13.5)	3.7 (0.4-10.9)	3.6 (0.5-10.2)	2.3 (1.4-6.0)	2.5 (0.5-5.7)	4.0 (0.4-8.1)	3.8 (0.7-8.7)
Male, n (%)	35 (64)	31 (57)	14 (61)	14 (61)	3 (75)	3 (75)	8 (80)	8 (80)
Median Illness Day (range)[*]	6 (3-10)	4 (2-20)[‡]	6 (3-10)	5 (2-20)	6 (5-7)	4 (3-7)	6 (2-10)	5 (2-8)
Coronary artery status of subjects: n (%)	Normal: 35 (64) Dilated: 11 (20) Aneurysm: 9 (16)	NA	Normal: 14 (61) Dilated: 4 (17) Aneurysm: 5 (22)	NA	Normal: 0 (0) Dilated: 4 (100) Aneurysm: 0 (0)	NA	Normal: 6 (60) Dilated: 2 (20) Aneurysm: 2 (20)	NA
IVIG resistant: n (%)	17 (31)	NA	5 (22)	NA	0 (0)	NA	2 (20)	NA
CRP (mg/dL)[†]	8 (5-19)	2 (1-4)[‡]	14 (6-23)	3 (1-5)[‡]	13 (6-24)	8 (3-13)	12 (5-23)	3 (2-8)[‡]
ESR (mm/h)[†]	62 (44-78)	20 (15–38)[‡]	53 (35-75)	32 (14-46)[‡]	64 (60-83)	32 (21-44)[‡]	48 (28-67)	25 (15-44)
WBC ($x10^9$/L)[†]	14 (11-19)	9 (6-13)[‡]	16 (11-19)	9 (7-13)[‡]	13 (9-20)	8 (5-20)	12 (7-17)	10 (7-13)
% Polymorphonuclear leukocytes[†]	56 (46-66)	45 (31-63)[‡]	55 (47-67)	49 (34-63)	50 (37-50)	44 (38-66)	49 (46-63)	45 (35-59)
% Bands[†]	12 (8-21)	8 (4-15)	11 (8-19)	9 (4-16)	23 (19-29)	3 (1-7)[‡]	14 (1-24)	16 (7-22)
Absolute neutrophil count[†]	9520 (6519-13090)	4636 (2695-6790)[‡]	10308 (6500-15121)	4438 (3080-6790)[‡]	9811 (6018-11786)	4355 (2573-9435)	7460 (4473-11796)	5248 (4172-8355)
Age-adjusted Hgb, S.D. units[†]	-1.3 (-2.3- -0.5)	-0.4 (-1.3-0.9)[‡]	-1.2 (-2.1- -0.5)	0.25 (-1.3- 1.5)[‡]	-1.9 (-3.6- -1.8)	-1.4 (-2.8- -0.4)	-1.5 (-2.7- -1.2)	0.1 (-1.1-1.7)[‡]
Platelet count ($x10^9$/L)[†]	405 (321-465)	265 (213-349)[‡]	421 (327-512)	265 (219-361)[‡]	335 (232-457)	265 (190-308)	323 (208-418)	268 (233-330)
ALT (IU/L)[†]	45 (24-102)	24 (17-36)[‡]	34 (22-85)	22 (14-26)[‡]	15 (8-95)	24 (17-28)	49 (20-88)	16 (13-24)[‡]
GGT (IU/L)[†]	45 (19-150)	14 (12-17)[‡]	45 (19-110)	15 (11-17)[‡]	62 (13-125)	15 (8-19)	48 (20-82)	14 (10-21)[‡]

[*]First day of fever = Day 1; [†] Laboratory data are presented as median (interquartile range); [‡]Values significant ($P < 0.05$) vs. acute KD within same group. IVIG = intravenous immunoglobulin, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, WBC = white blood cell count, Hgb = hemoglobin concentration, ALT = alanine amino transferase, GGT = γ-glutamyl transferase, NA = not available.

Table 4: Clinical and laboratory characteristics of acute Kawasaki disease (KD) and febrile control (FC) subjects measured for biomarkers in group 4.

Characteristics	Acute KD (n=28)	FC (n=28)	*p*-Value
Median age, yrs. (range)	2.9 (0.4-11.0)	2.9 (0.3-10.9)	NS
Male, n (%)	18 (64)	18 (64)	NS
Median Illness Day (range)*	6(2-10)	6(2-14)	NS
Coronary artery status of subjects: n (%)	Normal: 17 (61) Dilated: 6 (21) Aneurysm: 5 (18)	NA	NA
IVIG resistant, n (%)	9 (32)	NA	NA
CRP (mg/dL)†	8 (4-16)	3 (2-5)	0.0001
ESR (mm/h)†	51 (35-73)	31(16-38)	0.0001
WBC ($x10^9$/L)†	13(11-16)	7(6-13)	0.0008
% Polymorphonuclear leukocytes†	54(46-67)	42(29-61)	NS
% Bands†	11 (2-18)	5 (0-14)	NS
Absolute neutrophil count†	8358 (6539-11033)	4317 (1879-6987)	0.0001
Age-adjusted Hgb, S.D. units†	-1.4 (-2.5- -0.6)	-0.1 (-1.0- -0.7)	0.001
Platelet count ($x10^9$/L)†	403 (322-453)	240 (198-349)	0.0004
ALT (IU/L)†	39 (25-88)	22 (14-26)	0.007
GGT (IU/L)†	41 (15-131)	15 (11-17)	0.002

*First day of fever = Day 1; † Laboratory data are presented as median (interquartile range)
IVIG = intravenous immunoglobulin, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, WBC = white blood cell count, Hgb = hemoglobin concentration, ALT = alanine amino transferase, GGT = γ-glutamyl transferase, NA = not available, NS = not significant.

Table 5: Comparison of biomarker levels in subjects with acute Kawasaki disease (KD), convalescent KD (conv. KD), febrile controls (FC), and healthy controls (HC) in group1

Biomarker Proteins	Acute KD (n=55)	FC (n=54)	Conv. KD (n= 30)	HC† (n=50)
NT-proBNP (pg/mL)	319.5 (117.3-651.6)	102.0* (46.1-197.3)	41.1* (28.7-66.2)	65.2* (45.0-112.3)
sST2 (ng/mL)	46.1 (22.7-114.8)	28.4* (19.0-43.1)	12.5* (9.9-15.3)	7.2* (4.3-9.7)
cTnI (ng/mL)	0.0014 (0.0003-0.0062)	0.0013 (0.0004-0.0056)	0.0030* (0.0014-0.0119)	0.0008* (0.0007-0.0012)

* Significant at $p \leq 0.005$ compared to acute KD by Mann Whitney U test and Wilcoxon signed rank test for paired acute and convalescent KD; † NT-proBNP, sST2, and cTnI levels of acute KD subjects were compared with n=20, n=30, and n=30 age-similar HC, respectively.

NT-proBNP = N-terminal pro-B-type natriuretic peptide; sST2 = soluble ST2; cTnI = cardiac troponin I.

Table 6: Correlation of protein biomarker concentrations and laboratory data from Kawasaki disease (KD) subjects in group1

Biomarkers	sST2 (ng/mL)	cTnI (ng/mL)	Age at Onset (yrs)	Illness Day	CRP (mg/dL)	ALT (IU/L)	GGT (IU/L)
NT-proBNP	-0.50†	0.27	-0.45*	-0.43*	0.39*	0.33	0.27
sST2	1.0†	NS	NS	-0.52†	NS	0.54†	0.58†

Values represent Spearman rank correlation coefficient, r. All values significant at $P < 0.05$, unless otherwise noted: * $P < 0.005$, † $P < 0.0001$. NS= not significant. cTnI did not significantly correlate with clinical laboratory data.

NT-proBNP = N-terminal pro-B-type natriuretic peptide; sST2 = soluble ST2; cTnI = cardiac troponin I; Illness day = first day of fever is Day 1; CRP = C-reactive protein; ALT= alanine amino transferase; GGT = γ-glutamyl transferase;

Table 7: Comparison of biomarker levels in subjects with acute Kawasaki disease (KD), convalescent KD (conv. KD), febrile controls (FC), and healthy controls (HC) in group 2

Biomarker Proteins	Acute KD (n=23)	FC (n=23)	Conv. KD (n= 10)	HC (n=14)
MR-proANP (pmol/L)	73.6 (58.9-107.6)	42.2* (31.1-74.3)	33.1* (26.8-43.1)	46.4* (35.2-59.0)
Copeptin (pmol/L)	10.1 (5.7-13.4)	9.2 (4.2-15.1)	10.3 (7.7-16.4)	8.5 (7.5-13.4)
CT-proET-1 (pmol/L)	65.3 (50.2-80.2)	51.7 (45.0-71.7)	57.2 (47.9-80.7)	40.8* (27.0-54.6)
MR-proADM (pmol/L)	594.8 (344.6-915.8)	495.3 (391.1-561.5)	336.0* (254.7-397.2)	344.2* (310.6-367.9)
PCT (pg/mL)	863.3* (200.3-1187)	155.2* (95.6-338.9)	61.9* (40.2-90.8)	84.4* (55.9-131.2)

Values are presented as median (interquartile range). * Significant at $p \leq 0.005$ compared to acute KD by Mann Whitney U test and Wilcoxon signed rank test for paired acute and convalescent KD

MR-proANP = midregional pro-atrial natriuretic peptide; Copeptin = C-terminal pro-vasopressin; CT-proET-1 = C-terminal pro-endothelin-1; MR-proADM = midregional pro-adrenomedullin; PCT = procalcitonin

Table 8: Correlation of biomarkers and laboratory data from Kawasaki disease (KD) subjects in group 2

Biomarkers	NT-proBNP (pg/mL)	sST2 (ng/mL)	MR-proANP (pmol/L)	CT-proET (pmol/L)	MR-proADM (pmol/L)	Age at Onset (yrs)	Illness Day	PLT ($x10^3/mm^3$)	ALT (IU/L)	GGT (IU/L)
MR-proANP	0.87†	0.53	1.0†	0.48	0.68*	-0.50	-0.46	NS	0.59	0.53
PCT	0.71	0.67*	0.71*	0.47	0.87	NS	-0.39	-0.44	0.61*	0.60*

Values represent Spearman rank correlation coefficient, r. All values significant at $P < 0.05$, unless otherwise noted: * $P < 0.005$, † $P < 0.0001$. NS= not significant. No significant correlations with cardiac troponin I (cTnI) and C-terminal pro-vasopressin (copeptin).

MR-proANP = midregional pro-atrial natriuretic peptide; PCT = procalcitonin; NT-proBNP = N-terminal pro-B-type natriuretic peptide; sST2 = soluble ST2; CT-proET-1 = C-terminal pro-endothelin-1; MR-proADM = midregional pro-adrenomedullin; Illness day = first day of fever is Day 1; PLT = platelet count; ALT= alanine amino transferase; GGT = γ-glutamyl transferase

Table 9: Group 4 biomarkers significantly different in Kawasaki disease (KD) vs. febrile controls (FC)

Biomarkers	Acute KD (n=28)	FC (n=28)	*p*-Value
Alpha-1 Antitrypsin (mg/mL)	4.2 (3.7-5.0)	2.8 (2.4-3.5)	< 0.0001
Alpha-2 Macroglobulin (mg/mL)	0.86 (0.72-0.91)	0.97 (0.85-1.00)	0.004
Apolipoprotein A1 (mg/mL)	0.09 (0.06-0.12)	0.11 (0.08-0.14)	0.02
Cancer antigen 19-9	4.5 (3.1-16.0)	2.2 (1.2-3.6)	0.001
Calcitonin (pg/mL)	45 (21-142)	7 (3-27)	0.0002
CD40 (ng/mL)	1.2 (1.0-1.6)	0.8 (0.7-0.9)	< 0.0001
Complement 3 (mg/mL)	0.9 (0.8-1.1)	0.8 (0.6-0.9)	0.009
C Reactive Protein (ug/mL)	76 (38-111)	18 (2-43)	< 0.0001
EN-RAGE (ng/mL)	230 (84-376)	62 (25-113)	0.0005
Erythropoietin (pg/mL)	122 (13-256)	13 (13-22)	0.0007
Fibrinogen (mg/mL)	5.9 (4.9-6.2)	4.0 (3.1-4.7)	< 0.0001
Haptoglobin (mg/mL)	4 (2-5)	3 (1-4)	0.02
ICAM-1 (ng/mL)	270 (217-361)	175 (152-190)	< 0.0001
Interleukin-6 (pg/mL)	42 (28-120)	13 (6-27)	< 0.0001
Interleukin-8 (pg/mL)	35 (14-82)	15 (11-35)	0.03
Interleukin-16 (pg/mL)	732 (522-1002)	379 (284-642)	0.0005
MIP-1alpha (pg/mL)	89 (66-146)	44 (34-52)	< 0.0001
MIP-1beta (pg/mL)	298 (156-488)	153 (90-222)	0.003
Matrix metalloproteinase-3 (ng/mL)	3.2 (1.8-3.7)	1.1 (0.5-1.7)	< 0.0001
Prostatic acid phosphatase (ng/mL)	0.4 (0.2-0.7)	0.2 (0.1-0.3)	0.004
SGOT (ug/mL)	12 (11-13)	16 (14-18)	< 0.0001
Stem Cell Factor (pg/mL)	208 (166-252)	169 (117-210)	0.009
TIMP-1 (ng/mL)	168 (93-227)	86 (70-110)	0.0002
TNF RII (ng/mL)	12 (8-18)	7 (5-11)	0.0006
von Willebrand Factor (ug/mL)	52 (45-63)	44 (33-53)	0.01
VEGF (pg/mL)	623 (505-961)	449 (358-506)	< 0.0001

Values are presented as median (interquartile range), and significant at $p<0.05$ by Mann Whitney U test. ICAM-1= intercellular adhesion molecule 1; MIP-1 alpha = macrophage inflammatory protein-1 alpha; MIP-1 beta = macrophage inflammatory protein-1 beta; SGOT = serum glutamic oxaloacetic transaminase; TIMP-1 = tissue inhibitor of metalloproteinases 1; TNF RII = tumor necrosis factor receptor 2; VEGF = vascular endothelial growth factor

Supplemental Table 1: List of 88 Rules-Based Medicine (RBM) biomarkers

1. Adiponectin
2. Alpha-1-Antitrypsin
3. Alpha-2-Macroglobulin
4. Alpha-Fetoprotein
5. Apolipoprotein A-I
6. Apolipoprotein C-III
7. Apolipoprotein H
8. Apolipoprotein(a)
9. Beta-2-Microglobulin
10. Brain-Derived Neurotrophic Factor
11. C-Reactive Protein
12. Calcitonin
13. Cancer Antigen 125
14. Cancer Antigen 19-9
15. Carcinoembryonic Antigen
16. CD 40 antigen
17. CD40 Ligand
18. Complement C3
19. Creatine Kinase-MB
20. EN-RAGE
21. Endothelin-1
22. Eotaxin-1
23. Epidermal Growth Factor
24. Epithelial-Derived Neutrophil-Activating Protein 78
25. Erythropoietin
26. Factor VII
27. Fatty Acid-Binding Protein, heart
28. Ferritin
29. Fibrinogen
30. Fibroblast Growth Factor basic
31. Granulocyte Colony-Stimulating Factor
32. Granulocyte-Macrophage Colony-Stimulating Factor
33. Growth Hormone
34. Haptoglobin
35. Immunoglobulin A
36. Immunoglobulin E
37. Immunoglobulin M
38. Insulin
39. Insulin-like Growth Factor I
40. Intercellular Adhesion Molecule 1
41. Interferon gamma
42. Interleukin-1 alpha
43. Interleukin-1 beta
44. Interleukin-1 receptor antagonist
45. Interleukin-2
46. Interleukin-3
47. Interleukin-4
48. Interleukin-5
49. Interleukin-6
50. Interleukin-7
51. Interleukin-8
52. Interleukin-10
53. Interleukin-12 Subunit p40
54. Interleukin-12 Subunit p70
55. Interleukin-13
56. Interleukin-15
57. Interleukin-16
58. Leptin
59. Lymphotactin
60. Macrophage Inflammatory Protein-1 alpha
61. Macrophage Inflammatory Protein-1 beta
62. Macrophage-Derived Chemokine
63. Matrix Metalloproteinase-2
64. Matrix Metalloproteinase-3
65. Matrix Metalloproteinase-9
66. Monocyte Chemotactic Protein 1
67. Myeloperoxidase
68. Myoglobin
69. Plasminogen Activator Inhibitor 1
70. Pregnancy-Associated Plasma Protein A
71. Prostate-Specific Antigen, Free
72. Prostatic Acid Phosphatase
73. RANTES
74. Serum Amyloid P-Component
75. Serum Glutamic Oxaloacetic Transaminase
76. Sex Hormone-Binding Globulin
77. Stem Cell Factor
78. Thrombopoietin
79. Thyroid-Stimulating Hormone
80. Thyroxine-Binding Globulin
81. Tissue Factor
82. Tissue Inhibitor of Metalloproteinases 1
83. Tumor Necrosis Factor alpha
84. Tumor Necrosis Factor beta
85. Tumor Necrosis Factor Receptor-Like 2
86. Vascular Cell Adhesion Molecule-1
87. Vascular Endothelial Growth Factor
88. von Willebrand Fact

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