KAWASAKI DISEASE

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KAWASAKI DISEASE

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EDITORIAL
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Editorial: Kawasaki Disease

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Keywords: Kawasaki disease, Tomisaku Kawasaki, vasculitis, infant, coronary artery aneurysm, infant, children

Editorial on the Research Topic

Kawasaki Disease

On June 5, 2020, the esteemed Dr. Tomisaku Kawasaki, the pediatrician who first described mucocutaneous lymph node syndrome (later named in his honor) passed away at the age of 95 (1). For 53 years from the time, he published the first 50 cases of this syndrome, he inspired and encouraged researchers around the world to study this elusive illness (2). Since many questions remain regarding Kawasaki disease (KD) in terms of epidemiology, pathology, immunology, cause, and cardiovascular injury, Frontiers in Pediatrics offered us a collection of articles related to KD as a Research Topic. This issue of 13 articles concerning KD in Frontiers in Pediatrics demonstrates the commitment from clinical researchers around the globe to better understand this elusive illness.

This issue includes, for the first time, an explanation of the presentation and outcome of infants with KD and adjunctive therapies for children suffering from KD in a region of the world (Latin America) where little has been previously published about KD. Two comprehensive articles concerning infantile KD cases warn that patients younger than 3–6 months have a higher rate of coronary artery aneurysms. Garrido-Garcia et al., found that these very young infants were particularly at high risk for KD shock and giant coronary artery aneurysms. Moreno et al. found that the admission diagnosis of infants <6 months old ultimately diagnosed with KD was in error in more than 60% of these infants. Fortuna-Reyna et al. detailed for the first time additional therapies administered throughout Latin America for IVIG-resistant KD and aneurysms, revealing a limitation in adjunctive therapies in this part of the world. These works come from the Latin American KD Network (REKAMLATINA- Red de la Enfermedad de Kawasaki en America Latina) (3), inspired by dedicated Latin American physicians that have taken to heart Dr. Kawasaki's call to study this illness. A case report entitled by Saez-de-Ocariz et al. from Mexico highlighted that at times there is a complexity in differentiating KD from other vasculitides.

The work presented also demonstrates a collaborative network of researchers from the US, Europe, and the UK addressing the complexity of the diagnosis of KD with biomarkers, as written in the article by Zandstra et al.. Ching et al. from the Hawaii University investigated a novel biomarker related to cardiovascular dysregulation, and reported the possibility of a novel factor predicting the complication of coronary artery. The risk of hemolysis due to high levels of IVIG therapy, especially in obese children, is addressed in the article by Van Anh et al., which sheds lights on the risk/benefit ratio of IVIG in the context of the obesity epidemic.

Extensive body of work also illustrates the impact of research from Asian countries affected by KD, including Taiwan, China, and Japan. Ming-Huey Guo et al. is an original research article reporting a potentially novel mechanism of macrophage polarization control. The authors found alterations in markers that respond to CpG site methylation in patients with KD. Further, Liu et al. from China, showed swelling of extremities and polymorphous rash, as well as laboratory

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values, linked to IVIG-resistance. Okubo et al. is a multi-center study from Japan investigated differences in treatment selection and prognosis of the coronary artery outcomes before and after 2012 when the Japanese guidelines shifted toward the utilization of combined immuno-globulin and glucocorticoid therapy. Ito et al. examined the rate of coronary artery aneurysms and IVIG resistance in children administered 30 vs. 50 mg/kg/days during the acute phase of KD. Oshima et al. cautioned about the management of water and electrolytes in the acute phase of severe KD cases. Akimoto et al. discusses the complexities of surgically managing giant aneurysms.

This international effort underscores the importance that we continue to work collectively to understand KD better and honor Dr. Kawasaki's work. We wish to inspire the whole medical community with Dr. Kawasaki's words, "Be Strict for the Medical Study, Be Warm for the Medical Practice."

AUTHOR CONTRIBUTIONS

MA reviewed the articles submitted from Asian countries and edited them, unless he disclosed a conflict of interest as a co-author. IM managed and advised the article collection comprehensively. AT reviewed the submitted articles from Europe and the Americas, and edited them, unless she disclosed a conflict of interest as a co-author. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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The Impact of Changes in Clinical Guideline on Practice Patterns and Healthcare Utilizations for Kawasaki Disease in Japan

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Objective: Previous studies showed the efficacy of glucocorticoids on prevention of coronary artery lesions (CAL) among Kawasaki disease (KD) patients, and clinical guideline for KD in Japan was changed regarding glucocorticoid use in 2012. However, little is known regarding how the guideline change had impacts on healthcare utilizations and clinical outcomes.

Methods: We conducted a retrospective observational study using national inpatient database in Japan among KD patients aged under 18 years during 2010–2015. Recent trends in practice patterns were analyzed, and we divided the hospitals into four groups based on glucocorticoid use: (1) consistently using hospital, (2) started using hospital, (3) stopped using hospital, and (4) never using hospital. Then, we compared healthcare utilizations and risks of coronary artery lesions before and after the guideline change.

Results: We identified 24,517 inpatients with KD. From 2010 to 2014, use of glucocorticoid increased from 8.9 to 17.4% of KD inpatients. All types of hospitals showed reduction in coronary artery lesions, but the reduction was the most prominent in hospitals that started using glucocorticoid therapy after clinical guideline change in 2012 (adjusted OR, 0.22; 95%CI, 0.07–0.68). Also, Glucocorticoid consistently using hospitals, started using hospitals, and never using hospitals showed reductions in hospitalization costs, whereas hospitals that stopped using glucocorticoids after clinical guideline change had elevated healthcare costs as opposed to natural trends observed in other groups. Guideline complying hospitals had the greatest reductions in healthcare costs.

Conclusions: The early stage glucocorticoid use could be a cost-saving strategy for treatment for KD patients without increasing risks of CAL.

Keywords: coronary artery aneurysm, Kawasaki disease, Diagnosis Procedure Combination inpatient database, healthcare utilization, clinical guideline, practice pattern

INTRODUCTION

Kawasaki disease (KD) is an acute systemic vasculitis among children (1–5). KD is one of the most common cause of acquired heart disease during childhood in developed countries, and the devastating sequelae are coronary artery lesions (CAL) (1–5). An early suppression of vessel inflammation is thought to be important to prevent development of CAL (1–5).

The standard initial treatments are high-dose intravenous immune-globulin (IVIG) and aspirin (ASA) for children with KD in the acute phase (2, 5–7). Most of the previous studies indicated that 15–20% of KD patients failed to respond to the initial IVIG and ASA treatments, and the non-responders were more likely to develop CAL than IVIG responders, indicating the further requirement for alternative treatment strategies (8–14). In Japan, randomized controlled studies were conducted, which showed the effectiveness of corticosteroids added to IVIG and ASA in acute phase among only high-risk patients using the scoring systems (15, 16). Based on these results, the 2012 Clinical Guideline for Medical Treatment of Acute Stage Kawasaki Disease in Japan describes recommendation for adding corticosteroid to the IVIG and ASA in acute phase to potential high-risk patients or non-responders by the clinical scoring systems (2). Thus, currently many pediatricians in Japan are expected to apply early initial anti-inflammatory therapy (glucocorticoids to the conventional treatment) for KD patients with high clinical scores (4). However, no previous study has investigated the effects of change in the guideline on the trends in clinical practice patterns and healthcare utilizations at a national level.

Therefore, our study investigated the recent changes in clinical practice patterns and healthcare utilizations among children hospitalized with KD, using a national inpatient database in Japan. Furthermore, we also ascertained how the change in KD guidelines impacted on health care costs and risks of CAL among KD patients at hospital levels.

METHODS

Study Population and Participation

We extracted hospital discharge records of children under 18 years of age hospitalized with KD using the Diagnosis Procedure Combination (DPC) database, a national inpatient database, between July 1, 2010 and March 31, 2015. The details of the DPC database have been described elsewhere (17). In short, the data in the DPC was obtained from more than 1,000 hospitals, and it covers ∼55% of data among all inpatients who were admitted to acute-care hospitals in Japan. The DPC data has hospital discharge records and administrative claims information for about seven million inpatients per year. The database contains information on the following: the patient's primary diagnosis; pre-existing comorbidities on admission; complications and comorbidities during hospitalization; the patients' demographic information such as age, sex, and body weight; procedures and treatments. We imputed missing values of body weight based on ideal body weight as defined by WHO recommendations (18). We obtained the approval for the present study from the Institutional Review Board at The University of Tokyo. The Board waived the requirement for informed consent because of the anonymous nature of the data.

Hospitalizations with KD were identified using the International Classification of Diseases, Tenth Revision code (ICD-10 code, M30.3) as the main diagnosis at admission. We included KD patients who were aged <18 years and those who were treated with initial IVIG for their first-time admissions in order to maximize the diagnostic accuracy and prevent misclassifications of KD.

Measurements of Variables

Patient demographic consisted of age, sex, weight in kg, month and year of admission, ambulance use, types of hospitals (academic or non-academic), dose of IVIG, use of glucocorticoids, cyclosporine A, infliximab, ulinastatin, plasmapheresis, and the need for intensive care.

The outcomes of interest were total hospitalization and drug costs, development of CAL, readmission, total length of hospital stay in days, total IVIG dose, and additional treatment. Development of CAL was identified using an ICD-10 code (I25.4: aneurysm of coronary vessels) and use of antithrombotic drugs (warfarin, ticlopidine, and clopidogrel), assuming that the ICD-10 code reflected all diagnoses of CAL and that antithrombotic use was for medium- or large-sized CAL.

Statistical Analyses

We investigated the yearly trends in treatment patterns, risk of CAL and readmission, total hospitalization costs, and length of hospital stay (LOS) from fiscal year 2010 to 2014. To evaluate the trends, we constructed multivariable mixed effects logistic and linear regression models that had fiscal year of admission as an independent categorical variable. All models included fixed effects for patient characteristics and random effects for hospitals to account for clustering.

To capture the secular trends in glucocorticoid use within 2 days of initiation of IVIG for Kawasaki disease, we calculated rate of glucocorticoid use in the initial phase. The rates were calculated based on the number of patients who received glucocorticoids in the initial phase as the numerator and the number of patients who were hospitalized with KD as the denominator. A Poisson regression model was used with the natural log of the number of hospitalized patients with KD by months as an offset, and the scale parameter was added to the model to allow the variance to be proportional rather than equal to the mean to account for the over-dispersion (19). We applied a log-linear spline regression model because we assumed that the rate of glucocorticoid use in the initial phase started increasing on January 2012 (3 months before the first randomized controlled study for the effects of glucocorticoid on prevention of CAL was published) and leveled off after March 2013 (3 months after the change in KD guideline).

To investigate the impacts of change in clinical practice guideline on risks of CAL and hospitalization costs at hospital levels, we divided the hospitals into four groups based on glucocorticoid use within 2 days of initiation of IVIG. As

glucocorticoid use increased from January 2012 to March 2013 (**Figure 1**), we considered hospitals that used glucocorticoids during the acute phase of KD before and after the period as consistently using hospitals. Hospitals that never used glucocorticoids during the acute phase before and after the period were considered as never using hospitals. We considered hospitals that never used glucocorticoids during the acute phase before and started using glucocorticoids after the period as started using hospitals. Hospitals that used glucocorticoids during the acute phase before the period and stopped using glucocorticoids after the period were considered as stopped using hospitals. To avoid the potential misclassification of the four types of hospitals, we included patients who were admitted to hospitals that had at least ten patients with KD per year over the 5-year period. After stratifying the hospitals into four categories, we compared total hospitalization and drug costs, risk of CAL, LOS, total mean dose of IVIG, and use of additional treatment (glucocorticoid use more than 3 days after initial IVIG use, cyclosporine A, infliximab, ulinastatin, plasmapheresis) before January 2012 and after March 2013 using mixed effects models. We also used patient characteristics as fixed effects and hospitals as random effects to account for random variations between the hospitals.

The differences in outcomes of interest were estimated with 95% confidence intervals (95%CIs). For the mixed effects linear regression models, log-transformation was required to ensure the error term normality assumption. As the doses of IVIG were missing for 2,345 patients, the analyses relevant to IVIG dose were conducted with the remaining 22,172 patients. We set statistical significance at two-sided $P < 0.05$ for all of the analyses. We used STATA software version 14.1 for all data analyses (StataCorp LP, TX, USA).

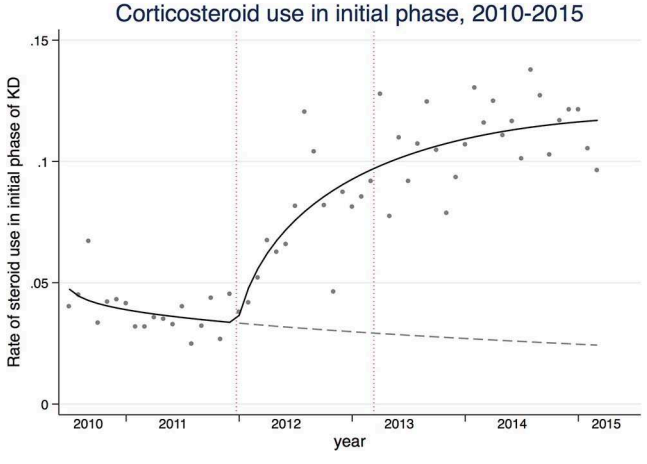

FIGURE 1 | The solid line represents the rates of glucocorticoid use in the acute phase of Kawasaki disease. The gray dashed line represents the counterfactual that would have been observed if the trend were the same before study reports of the efficacy of glucocorticoids and subsequent guideline change. The dashed vertical lines are 3 months before the official publications of the studies (January, 2012) and 3 months after the guideline change (March, 2013).

RESULTS

We observed a total of 24,517 children hospitalized with KD over the study period. **Table 1** shows the summary of the baseline patient characteristics, treatment patterns, and healthcare utilizations, stratified by fiscal years of admissions. In the mixed effects model analyses, glucocorticoid use for both initial and all phases of KD showed an upward trend ($P_{trend} < 0.001$). The spline model showed an increasing trend in glucocorticoid use during the initial phase that started in January 2012 and leveled off after March 2013 (**Figure 1**). An upward trend in infliximab use was observed ($P_{trend} < 0.001$), whereas little changes were observed in dose of IVIG, use of cyclosporine A, ulinastatin, and plasmapheresis. Diagnoses of CALs defined by both ICD-10 and antithrombotic therapy decreased from 4.5 and 1.0% in 2010 to 2.4 and 0.7% in 2014, respectively. Total hospitalization costs and LOS also showed decreasing trends over the study period ($P_{trend} < 0.001$).

We identified 91 hospitals that had at least 10 patients per year during 2010–2014, which included 38 consistently using hospitals, 17 started using hospitals, seven stopped using hospitals, and 29 never using hospitals (**Table 2**). **Table 3** shows the total hospitalization costs and risks of CAL stratified by hospital types based on glucocorticoid use in the initial phase of KD. Total hospitalization costs comparing before January 2012 to after 2013 March were reduced in the always using hospital, stopped using hospital, and never using hospitals. The most prominent reduction was observed in started using hospitals (adjusted difference, −20,295 JPY; 95%CI, −28,865 to −13,448). In contrast, only stopped using hospitals did not show reduction of hospitalization costs (adjusted difference, −10,198 JPY; 95%CI, −15,731 to −4,479). The odds of CAL development were decreased in always using and never using hospitals. The most prominent reduction in odds of CAL was observed in started using hospitals (adjusted OR, 0.22; 95%CI, 0.07–0.68).

Table 4 shows the changes in total drug costs, LOS, IVIG dose, and additional treatment use. Reduction in total drug costs were observed in the always using, started using, and never using hospitals, whereas an elevated drug cost was found in stopped using hospitals. All hospitals showed decreases in LOS, and the difference was substantial on stopped using hospitals. Only stopped using hospitals showed an increase in average total IVIG dose (adjusted difference, 0.18 mg/kg; 95%CI, 0.01–0.36).

DISCUSSION

In the present study, we observed increasing trends in initial phase glucocorticoid use for KD patients, after publications that proved the efficacy of glucocorticoid use for preventing CAL and subsequent KD treatment guideline change. We observed the reduction in hospitalization costs among hospitals that consistently used glucocorticoids, started using after the change in guideline, and never used glucocorticoids. On the other hand, hospitals that stopped using glucocorticoids did not show decreasing trends in hospitalization costs. To our best knowledge, this is the first study that assessed the clinical practice patterns for

TABLE 1 | Baseline characteristics of children hospitalized with KD by fiscal year of admission and trend analyses for hospital utilizations and adjunctive treatment.

		Fiscal year of admission					
	Total	**2010**	**2011**	**2012**	**2013**	**2014**	
Total Admissions, *n*	24,517	3,369	4,699	4,700	4,893	6,856	
Age (years), mean (SD)	2.53 (2.0)	2.45 (3.9)	2.46 (4.0)	2.48 (4.1)	2.55 (4.1)	2.65 (4.2)	
Male, *n* (%)	14,146 (57.7)	1,980 (58.8)	2,724 (58.0)	2,690 (57.2)	2,806 (57.3)	3,946 (57.6)	
Ambulance, *n* (%)	508 (2.1)	63 (1.9)	74(1.6)	86 (1.8)	145 (3.0)	140 (2.0)	
Academic, *n* (%)	4,283 (17.5)	588 (17.5)	738(15.7)	835 (17.8)	997 (20.4)	1,125 (16.4)	
Season							
Spring, *n* (%)	5,747 (23.4)	400 (11.9)	1,237(26.3)	1,234 (26.3)	1,324 (27.1)	1,552 (22.6)	
Summer, *n* (%)	6,255 (25.5)	839 (24.9)	1,196(25.5)	1,222 (26.0)	1,249 (25.5)	1,749 (25.5)	
Fall, *n* (%)	5,473 (22.3)	907 (26.9)	981 (20.9)	999 (21.3)	1,020 (20.8)	1,566 (22.8)	
Winter, *n* (%)	7,042 (28.7)	1,223(36.3)	1,285 (27.3)	1,245 (26.5)	1,300 (26.6)	1,989 (29.0)	
	Total	**2010**	**2011**	**2012**	**2013**	**2014**	P_{trend}
IVIG (g/kg), mean (log-SD)	2.32 (0.01)	2.27 (0.01)	2.23 (0.01)	2.37 (0.01)	2.32 (0.01)	2.32 (0.01)	0.37
Glucocorticoid use							
All phases, *n* (%)	3,410 (13.9)	299 (8.9)	405 (8.6)	647 (13.8)	869 (17.8)	1,190 (17.4)	<0.001
Initial phase, *n* (%)	2,007 (8.2)	141 (4.2)	175 (3.7)	381 (8.1)	516 (10.5)	794 (11.6)	<0.001
Other Treatment							
Cyclosporine A, *n* (%)	223 (0.9)	23 (0.7)	29 (0.6)	48 (1.0)	59 (1.2)	64 (0.9)	0.11
Infliximab, *n* (%)	105 (0.5)	5 (0.1)	13 (0.2)	8 (0.2)	27 (0.6)	52 (0.8)	<0.001
Ulinastatin, *n* (%)	955 (3.9)	83 (2.5)	163 (3.5)	202 (4.3)	227 (4.6)	280 (4.1)	0.12
Plasmapheresis, *n* (%)	97 (0.4)	6 (0.2)	11 (0.2)	19 (0.4)	30 (0.6)	31 (0.5)	0.17
Intensive care, *n* (%)	181 (0.7)	18 (0.5)	29 (0.6)	34 (0.7)	50 (1.0)	50 (0.7)	0.59
Outcomes							
Readmission, *n* (%)	397 (1.6)	45 (1.3)	79 (1.7)	70 (1.5)	88 (1.8)	115 (1.7)	0.62
CAL (ICD-10), *n* (%)	725 (3.0)	153 (4.5)	142 (3.0)	137 (2.9)	128 (2.6)	165 (2.4)	<0.001
CAL (drug), *n* (%)	178 (0.7)	34 (1.0)	22 (0.5)	30 (0.6)	44 (0.9)	48 (0.7)	0.043
Cost, JPY	300,619	314,387	310,060	302,964	298,179	288,056	<0.001
LOS, days	10.8	11.0	10.8	10.8	10.8	10.3	<0.001

SD, standard deviation; CAL (ICD-10), diagnoses of coronary artery lesion based on ICD-10; CAL (drug), diagnoses of CAL based on clopidogrel, ticlopidine, or warfarin use; JPY, Japanese yen; LOS, length of stay.

TABLE 2 | Selection and timing of steroid use at hospital levels.

	Before January 2012, hospital (%)		
	Non-initial use or never use	**Initial use**	**Total**
	46	**45**	**91**
After March 2013, hospital (%)			
Non-initial use or never use	29 (31.9)	7 (7.7)	36
Initial use	17 (18.7)	38 (41.8)	55

KD and the impacts of KD guideline change on risks of CAL and healthcare costs at hospital levels.

Recommendations, regarding to adjunctive therapy for primary treatment with IVIG and ASA and additional therapy in the IVIG-resistant case, are slightly different between the clinical guideline in Japan and that in the US (2, 5). First, the risk stratification scores have been proven useful to identify Japanese children in Japan with KD (10, 11), whereas the scores showed low sensitivity and poor negative predictive value outside of Japan (20). Second, recommendations of pulsed methylprednisolone for adjunctive therapy for primary treatment are different between the guidelines in Japan and the US (2, 5). Third, the clinical guideline in Japan describes ulinastatin and plasmapheresis as one of treatment options for additional treatment in the IVIV-resistant cases (2).

An increasing trend in glucocorticoid use was observed over the study period. The trend could be explained by the impacts of previous randomized controlled studies that identified efficacy of glucocorticoids in initial treatment of KD and subsequent change in Japanese KD guideline during 2012 (4, 15, 16). In fact, our study showed that the upward trend started 3 months before publishing the previous studies and leveled off after change in Japanese KD guideline. Correspondingly, the risks of CALs showed a decreasing trend. However, we believe that the decreasing trend in CALs could be multifactorial such as upward trends in glucocorticoid use and other aggressive anti-inflammatory treatment, and earlier administrations of IVIG. Indeed, a nationwide survey of KD in Japan reported that the proportions of IVIG administrations within 5 days

TABLE 3 | Differences in total costs and proportions of CAL between before 12/2011 and after 4/2013 stratified by use of steroid use defined at hospital levels.

Hospital characteristics	Always use	Started use	Stopped use	Never use
Number of patients, *N*	4,029	1,544	624	2,483
Number of hospitals, *N*	38	17	7	29
Total hospitalization cost				
Before 12/2011, mean JPY (log-SD)	301,374 (0.5)	289,294 (0.5)	304,882 (0.6)	286,081 (0.6)
After 4/2013, mean JPY (log-SD)	290,259 (0.5)	266,495 (0.5)	321,142 (0.6)	279,568 (0.5)
Adjusted Difference, JPY (95%CI)	−11,270 (−15,546, −6,887)	−20,295 (−26,865, −13,448)	7,926 (−5,838, 22,856)	−10,198 (−15,731, −4,479)
CAL				
Before 12/2011, *N* (%)	59 (3.5)	11 (1.9)	11 (3.0)	33 (3.2)
After 4/2013, *N* (%)	44 (1.9)	5 (0.5)	4 (1.5)	27 (1.9)
Adjusted OR (95%CI)	0.54 (0.35, 0.82)	0.22 (0.07, 0.68)	0.52 (0.15, 1.77)	0.50 (0.29, 0.86)

JPY, Japanese-Yen; Log-SD, log-transformed standard deviation; CAL, coronary artery lesion; CI, confidence interval; OR, odds ratio.

TABLE 4 | Differences in drug costs, mean intravenous immunoglobulin (IVIG) dose, length of hospital stay (LOS), proportions of additional treatment between before 12/2011 and after 4/2013 stratified by use of steroid use defined at hospital levels.

Hospital characteristics	Always use	Started use	Stopped use	Never use
Total drug cost, JPY				
Before 12/2011, mean (log-SD)	284,637 (0.5)	278,098 (0.4)	274,191 (0.6)	275,328 (0.5)
After 4/2013, mean (log-SD)	273,282 (0.5)	265,580 (0.5)	303,979 (0.5)	269,850 (0.5)
Adjusted Difference, (95%CI)	−9,115 (−13,271, −5,238)	−16,092 (−22,140, −9,803)	12,172 (109, 25,151)	−8,688 (−13,737, −3,474)
Total LOS in days				
Before 12/2011, mean (log-SD)	10.7 (0.5)	11.8 (0.4)	11.5 (0.4)	10.7 (0.5)
After 4/2013, mean (log-SD)	10.2 (0.5)	11.6 (0.4)	9.3 (0.4)	10.3 (0.4)
Adjusted Difference, (95%CI)	−0.35 (−0.60, −0.09)	−0.30 (−0.72, 0.13)	−2.37 (−2.92, −1.79)	−0.71 (−0.97, −0.46)
Total IVIG dose per kg				
Before 12/2011, mean (log-SD)	2.37 (0.01)	2.46 (0.02)	2.35 (0.03)	2.33 (0.02)
After 4/2013, mean (log-SD)	2.37 (0.01)	2.36 (0.02)	2.55 (0.02)	2.34 (0.01)
Adjusted Difference, mean (95%CI)	0.02 (−0.04, 0.09)	−0.03 (−0.09, 0.02)	0.18 (0.01, 0.36)	−0.01 (−0.09, 0.073)
Additional treatment				
Before 12/2011, *N* (%)	60 (3.6)	23 (4.0)	25 (6.8)	64 (6.1)
After 4/2013, *N* (%)	101 (4.3)	41 (4.2)	14 (5.4)	118 (8.2)
Adjusted OR (95%CI)	1.31 (0.93, 1.86)	1.06 (0.62, 1.81)	0.79 (0.39, 1.62)	1.23 (0.83, 1.84)

Additional treatment includes use of glucocorticoid use more than 3 days after initial IVIG, cyclosporine A, infliximab, methotrexate, ulinastatin, and plasma exchange; JPY, Japanese-Yen; Log-SD, log-transformed standard deviation; CAL, coronary artery lesion; CI, confidence interval; OR, odds ratio.

increased from 67.7% in 2010 to 69.2% in 2014 (4). Furthermore, slightly upward trends in use of infliximab and ulinastatin were observed in our study, probably because of the guideline recommendations for early aggressive anti-inflammatory therapy and the study results that reported the effectiveness of infliximab and ulinastatin (4, 21–24). We believe that these factors may have contributed to the change in treatment strategy and reduction in risks of CAL, rather than a single effect of glucocorticoid use in the initial phase.

The effects of changes in treatment strategies at hospital levels on the risk of CAL and healthcare costs are uncertain, and finding a cost-saving strategy with improving prognosis is extremely important considering the increasing healthcare expenditure in high-income countries (25). We observed decreasing trends in risks of CAL and healthcare costs among hospitals that used glucocorticoids consistently and those that never used them. As we cited above, we believe that the decreasing trends in the risks of CAL reflected several factors such as increasing use of glucocorticoid, earlier administrations, and elevated doses of IVIG. Furthermore, decreasing trends in healthcare costs in consistently and never using hospitals could be explained by natural trends in healthcare costs among pediatric inpatients in Japan, rather than effects of change in the clinical guideline for KD. In fact, downward trends in total hospitalization costs were already observed for other pediatric disorders in Japan, such as immune thrombocytopenia and respiratory infections (26–28).

Complying with the changes in guideline in terms of glucocorticoid use in the initial phase of KD could be the most effective strategy to save healthcare costs for KD inpatients. Indeed, we observed the greatest reduction in healthcare costs (−20,295 JPY per KD inpatients) among hospitals that started using glucocorticoids compared with consistently using and never using hospitals. The greatest reduction in total hospitalization costs in the started using hospitals could reflect decreases in drug costs and slightly decreased LOS. On the other hand, stopped using hospitals showed slightly elevated healthcare costs, which contradicted to natural trends in reduction of healthcare costs. Considering for the increase in IVIG dose, we believe that the stopping the use of initial phase glucocorticoid use may have increased the cases of non-response to initial IVIG, which resulted in elevated total IVIG dose and subsequent increases in hospitalization costs.

We acknowledge several limitations to our study. The number of KD inpatients and subsequent development of CAL could have been underestimated because of possible underreporting and/or misclassification of ICD-10 code. However, we maximized diagnostic accuracy of KD and CAL by restricting to patients who received IVIG and identified patients with antithrombotic treatment. As a result, our estimates of CAL were mostly similar to the findings from the previously reported national survey in Japan. The detailed clinical presentation, laboratory data, and patient information for the follow-up of KD at outpatient visits were unavailable in the DPC database. Due to these limitations, we were unable to reliably evaluate and compare the difference of pre-treatment severity scores among KD patients, and we could not compare patients between hospitals with different treatment strategies. In addition, we were not able to detect cases of CAL in KD patients that were detected during outpatient follow-up periods. The strength of this study was the use of the DPC database, which is the only national inpatient database currently available in Japan. Using the DPC database, we calculated the robust estimates of the recent clinical practice patterns in KD and the impact of change in KD guideline at hospital levels throughout Japan.

In summary, this study added novel insights into strategies of KD treatment by comparing the different treatment strategies. Glucocorticoid treatment in the initial phase of KD could be a cost-saving strategy with improving clinical outcomes. We believe that our investigations showed valuable information to improve prognosis for CAL and save healthcare expenditure at hospital levels.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

YO and NMi designed the data collection instruments, coordinated data, drafted the initial manuscript, and performed the initial analyses. KF, HY, MM, TK, HM, and NMo supervised data collection, revised the manuscript, and approved the final manuscript as submitted. Each author listed on the manuscript has seen and approved the submission of this version of the manuscript and takes full responsibility for its contents.

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Hemolysis From Intravenous Immunoglobulin in Obese Patients With Kawasaki Disease

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Objective: We assessed the risk of IVIG-associated hemolytic anemia in patients with acute Kawasaki disease (KD) and evaluated the risk of weight-based dosing in our obese patients.

Methods: IVIG-associated hemolytic anemia was assessed in acute KD patients treated with IVIG at Rady Children's Hospital-San Diego. Patients in whom hemolytic anemia was suspected had a decrease in z-score of their hemoglobin (zHgb) at least two standard deviations below the cohort's mean change in zHgb from baseline to 2 weeks post-IVIG treatment. These patients were further evaluated for spherocytosis, blood type, need for transfusion, red cell distribution width, reticulocytosis, and direct Coombs test. Body mass index was calculated.

Results: Of the 30 IVIG-resistant KD patients who received a second dose of IVIG, 2 (6.7%) developed hemolytic anemia after a total of 4 g/kg of IVIG dosed on actual body weight, or a mean of 4.6 g/kg of IVIG based on lean body mass. Compared to 496 non-obese KD patients who received a single dose of IVIG with no cases of hemolytic anemia, two (5.6%) of 36 obese KD patients developed hemolytic anemia after a single dose of IVIG (2 g/kg) dosed on actual body weight, or a mean of 2.7 g/kg IVIG based on lean body mass.

Conclusions: In addition to following patients carefully for hemolytic anemia after a second dose of IVIG, physicians should consider IVIG dosing based on lean body mass for obese patients.

Keywords: Kawasaki disease (KD), intravenous immunoglobulin (IVIG), hemolytic anemia, obesity, lean body mass

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INTRODUCTION

Kawasaki disease (KD) is an acute vasculitis in children that if left untreated can lead to coronary artery aneurysms in 25% of patients (1). If treated with intravenous immunoglobulin (IVIG), the rate of coronary artery aneurysms decreases to 5% (2). The recommended treatment for patients with acute KD is a single high-dose (2 g/kg) IVIG infusion within 10 days of illness onset (3). Common adverse effects of IVIG in acute KD patients include mild effects such as headache and mild anemia acutely following treatment, as well as more serious effects such as thrombosis, anaphylaxis or hemolysis requiring transfusion (4, 5). The complication of antibody-mediated hemolytic anemia is increased in patients receiving a high cumulative dose of IVIG,

and thus increases in patients given a second dose of IVIG for treatment-resistant KD (6–9). The risk is also higher in patients with non-O blood group. Cases of IVIG-related hemolytic anemia have been reported in the treatment of various other conditions including immune-mediated thrombocytopenia, Guillain-Barre syndrome, and myasthenia gravis (10). The risk of hemolysis appears to be higher in pediatric patients treated with two doses of 2 g/kg of IVIG for KD than in pediatric and adult patients treated for chronic inflammatory or immunodeficiency disorders (9). Rates of hemolytic anemia in patients with KD related to IVIG treatment have varied from 0.06 to 16% after a single dose of IVIG, and as high as 44% in KD patients treated with a second IVIG infusion (5, 7, 9–11). The concern for hemolytic anemia was confirmed in a post-marketing surveillance study in which 39% of IVIG-associated hemolytic events in pediatric patients were in children with KD (10). Given this concern, we assessed the occurrence of suspected IVIG-associated hemolytic anemia in KD patients treated at our center, with a focus on comparing rates of hemolytic anemia in obese and non-obese KD patients.

MATERIALS AND METHODS

IVIG-associated hemolytic anemia was assessed utilizing the Rady Children's Hospital-San Diego (RCHSD) Pharmacy Clarity reporting system, Research Electronic Data Capture (REDCap) KD database, and medical records of the acute KD patients treated at RCHSD between January 1, 2010 and January 31, 2018. The following clinical data were prospectively collected for the 661 acute KD patients treated with IVIG at RCHSD: patient demographics, illness day at diagnosis (illness day 1 = first day of fever), response to IVIG therapy, and coronary artery status (Z scores, echocardiographic measurements of the internal diameter normalized for body surface area of the proximal right coronary artery and left anterior descending coronary artery). Weight, height and age were used to calculate body mass index (BMI), and a BMI of >95% for age was considered obese (12). We retrospectively recorded details of the IVIG infusion including lot number and concentration of IVIG administered and time and duration of IVIG infusion. All patients received Gammagard (Baxter) product as either the 5 or 10% solution. Fever was defined as rectal or oral temperature $\geq$38.0°C; patients with persistent or recrudescent fever $\geq$36 h after completion of IVIG were classified as IVIG-resistant (3). Patients in whom hemolytic anemia was suspected had a decrease in zHgb ([Observed hemoglobin-Mean hemoglobin for age]/Standard deviation for age) of at least two standard deviations below the cohort's mean change in zHgb from the acute to the subacute phase (median = 14 days post-IVIG treatment, IQR = 12–16.5). These patients were further evaluated for spherocytosis, blood type, need for a blood transfusion, red cell distribution width, reticulocytosis, and direct Coombs test when available. Due to the retrospective nature of this study, the direct Coombs test, serum haptoglobin, reticulocyte count, and indirect bilirubin levels were unavailable for definitive identification of hemolytic anemia in many patients. Fisher's exact test was used to compare the rates of IVIG-associated hemolytic anemia in obese and non-obese patients.

The study protocol was reviewed and approved by the University of California San Diego's Institutional Review Board. Written informed consent was obtained from a parent or legal guardian, and assent, when appropriate, was obtained from the patient.

RESULTS

Of the 661 acute KD patients treated with IVIG (2 g/kg), 99 received infliximab initially as either part of a clinical trial or because of significant coronary artery damage or KD shock (13). Of the remaining 562 IVIG-treated KD patients who were initially treated with single dose of IVIG, 78 (13.9%) were IVIG-resistant. Of these IVIG-resistant KD patients, 9 (11.5%) received a second IVIG infusion (2 g/kg), 48 (61.5%) received infliximab (5–10 mg/kg), and 21 (26.9%) received both a second dose of IVIG (2 g/kg) and infliximab (**Figure 1**). Hemolytic anemia was defined as a drop in zHgb of at least −3.17 (2 SD below the mean drop in zHgb of −0.024 in this study population) between the acute and subacute phase and the presence of at least polychromasia and anisocytosis on the peripheral smear. Of the 30 IVIG-resistant acute KD patients who received a second IVIG infusion, two (6.7%) developed hemolytic anemia.

The first patient developed hemolytic anemia after receiving 76.4 g (4 g/kg) of IVIG. This patient was admitted with a baseline hemoglobin level of 11.4 g/dl (zHgb of −1.125), which dropped to 10.7 g/dl (zHgb of −2) following the first IVIG infusion, and 7.7 g/dl (zHgb of −5.75, change in zHgb of −4.625 from the pre-IVIG value) after the second IVIG infusion. This patient had a BMI of 16.2 kg/m^2 (68.8% for age). Spherocytosis and anisocytosis were present on the blood smear. Blood type was not determined, and direct Coombs test was not performed as the patient did not require a blood transfusion and the hemoglobin increased to 11.8 g/dl within a month after treatment.

The second patient developed hemolytic anemia after receiving 90.4 g (4 g/kg) of IVIG. This patient was admitted with a baseline hemoglobin level of 11.3 g/dl (zHgb of −2), which dropped to 10.6 g/dl (zHgb of −2.17) following the first IVIG infusion, and 6.1 g/dl (zHgb of −7.78, change in zHgb of −5.78 from the pre-IVIG value) after the second IVIG infusion. This patient had a BMI of 13.4 kg/m^2 (4.7% for age). Spherocytosis, anisocytosis, and polychromasia were noted on the blood smear consistent with hemolytic anemia. The reticulocyte count was elevated to 7.1%. This patient was blood type A and was transfused 15 cc/kg of O- blood with prompt improvement in clinical symptoms of fatigue and dizziness as well as an increase in hemoglobin to 11.7 g/dl. Direct Coombs test was not performed and therefore unavailable for analysis. Given recrudescence of fever, this patient then received a dose of infliximab (10 mg/kg) without worsening of the anemia. Of the 48 KD patients who received only infliximab for IVIG-resistance, none developed hemolytic anemia.

As compared to KD patients who received two doses of IVIG, only two of the 532 (0.4%) KD patients who received a single dose of IVIG developed hemolytic anemia and both met the definition for obesity with a BMI at or above the 95th percentile for age (12). Of the 532 KD patients who received a single dose of IVIG, 36 (6.6%) were obese. Thus, the rate of hemolytic anemia in KD

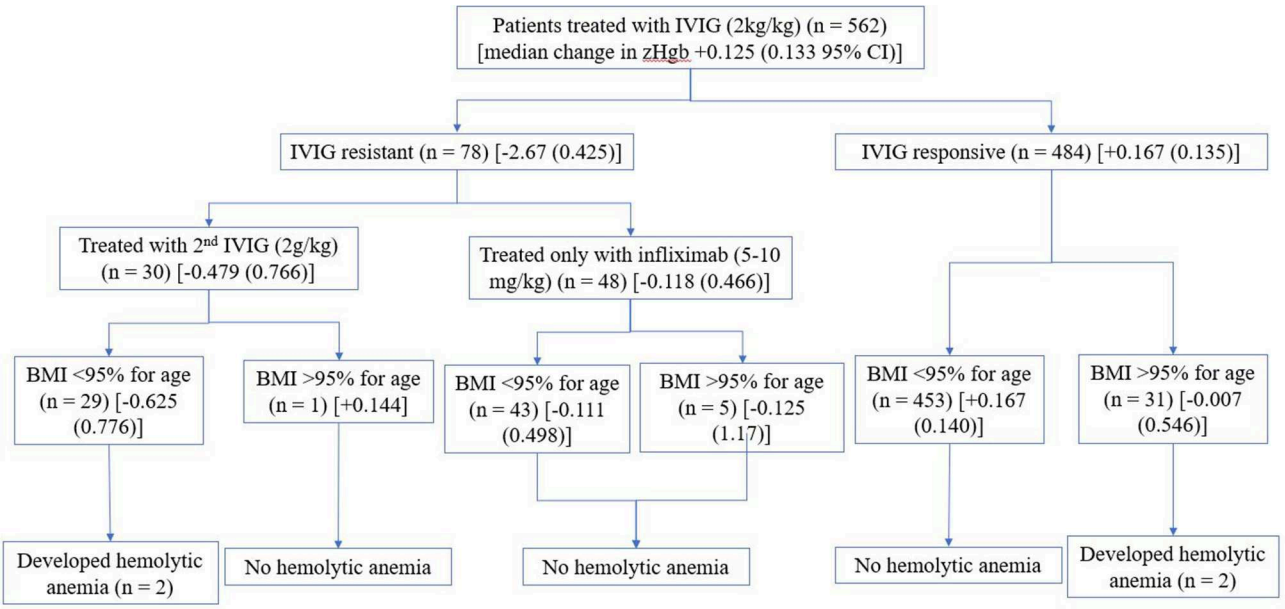

FIGURE 1 | Summary of cohort [median change in zHgb (95% confidence interval)].

patients receiving a single dose of IVIG was significantly higher in our obese KD patients (2/36, 5.6%) as compared to non-obese KD patients (0/496, 0%; $p = 0.0045$, although confounded by small numbers). These 36 obese patients had a median change in zHgb of −0.125 (0.49 95% CI) between the acute and subacute phase as compared to a median change in zHgb of +0.167 (0.135 95% CI) in the 496 non-obese patients ($p = 0.23$).

The first obese patient developed hemolytic anemia after receiving 68 g (2 g/kg) of IVIG. This patient was admitted with a baseline hemoglobin of 12.5 (zHgb of −0.22), which dropped to 9.1 (zHgb of −4, change in zHgb of −3.78) 2 days after a single dose of IVIG (2 g/kg). At the age of 6 years and 10 months, this patient met the definition of obese with a weight of 34 kg (98.5% for age), a height of 127 cm (86.9% for age), and thus a BMI of 21.1 kg/m^2 (98.3% for age). Moderate anisocytosis was seen on the peripheral blood smear. The red blood cell distribution width was 17.5%, consistent with hemolytic anemia. Blood type was not determined, and direct Coombs test was not performed as the patient was not symptomatic. The hemoglobin increased to 13.9 (zHgb of 1.33) 3 weeks after receiving IVIG.

The second obese patient developed hemolytic anemia after receiving 134 g (2 g/kg) of IVIG. This patient was admitted with a baseline hemoglobin of 13.6 (zHgb of 0.56), which dropped to 9.3 (zHgb of −4.22, change in zHgb of −4.78) 11 days after a single dose of IVIG (2 g/kg). This was associated with clinical symptoms of light-headedness and fatigue. At the age of 11 years, this patient met the definition of obese with a weight of 67 kg (99.2% for age), a height of 149.1 cm (77.4% for age), and thus a BMI of 31 kg/m^2 (99.1% for age) (12). Moderate polychromasia and anisocytosis were seen on the peripheral blood smear. The reticulocyte count and red blood cell distribution width were elevated at 11.5 and 22%, respectively, consistent with hemolytic anemia. This patients' blood type was A+. Direct Coombs test was not performed. Screening of the lot of the IVIG product administered to this patient was conducted by the RCHSD Blood Bank for titers of anti-A and -B antibodies. A manual tube method with 3-5% of reagent red blood cells (Quotient Biodiagnostics) showed the following: A1 cells 3+, A2 cells 2+, and B Cells 1+, suggesting the presence of high titer of anti-A antibodies. Subgroups of A blood types, including blood type A2, have been associated with anti-A1 antibodies which can react with A1 cells (14). The hemoglobin normalized without transfusion and was 12.7 g/dl 6 weeks after treatment for KD.

DISCUSSION

We report four cases of hemolytic anemia following IVIG therapy for acute KD. Two of these patients had hemolytic anemia after two doses of IVIG (6.7% rate of hemolytic anemia) while two obese patients developed hemolytic anemia after a single dose of IVIG (5.6% rate of hemolytic anemia in obese patients). None of the 48 patients treated with infliximab for IVIG-resistant KD developed hemolytic anemia. The addition of infliximab prior to treatment with IVIG has been shown to shorten fever duration, more rapidly reduce inflammation and eliminate the risk of transfusion reactions if given before IVIG, but has shown no significant difference in adverse events, such as hemolytic anemia, following IVIG (13).

IVIG causes hemolysis either via isohemagglutinins in the IVIG product administered, or by enhanced erythrocyte sequestration via activation of the complement pathway by the IgG complexes, leading to erythrophagocytosis and a reduction in hemoglobin (10, 15). Hemolysis has only been reported in patients with blood group O in exceptional cases while the

frequency of hemolytic events in patients with blood group AB exceeded the frequency of patients with blood group A or B (10). Patients with blood group AB had a ratio of cases to population distribution of 2.6–9 compared to 1.8–2.8 for blood group A, 0.3–0.7 for blood group B, and 0.05–0.08 for blood group O (10). Of the four patients with hemolytic anemia in our study, two were blood type A while the other two were unknown. As with previous reported cases of hemolytic anemia, our patients had spherocytosis, polychromasia and an elevated reticulocyte count, and the two patients who received a second dose of IVIG had an abrupt drop in hemoglobin concentration following the second infusion (6–9). One patient had an A+ blood type and a high level of anti-A antibody in the lot of IVIG with which he was treated. Isoagglutinin reduction measures such as anti-A donor screening and anti-A/anti-B specific immunoaffinity chromatography in IVIG manufacturing have been predicted to reduce the risk of hemolysis in patients with non-O blood groups, especially those with blood group AB (16).

While the rate of hemolytic anemia after IVIG correlates with increasing doses of IVIG, little is known about the risk of hemolytic anemia in obese patients as dosing is based on absolute weight. A study of cases of IVIG-associated hemolysis over 10 years found that IVIG-associated hemolysis tends to occur in patients who received doses exceeding 2 g/kg body weight (60% of cases), as well as in patients with comorbidities such as hypertension or anemia (10). Childhood obesity is associated with comorbid metabolic risk factors such as hypertension, type 2 diabetes, and abnormal lipid profiles (17). Obese children are at a three-fold higher risk for hypertension than non-obese children (18). The two obese patients who developed hemolytic anemia received 68 g of IVIG based on a weight of 34 kg and 134 g of IVIG based on a weight of 67 kg. Had the 2 g/kg dose of IVIG been based on estimated lean body mass of 26.6 and 46.4 kg, they would have received 53.2 and 92.8 g of IVIG. Instead, based on lean body mass, these two obese patients received 2.6 and 2.9 g/kg of IVIG, respectively (19).

The two non-obese patients who developed hemolytic anemia after a second dose of IVIG each received an equivalent of 4.7 and 4.4 g/kg of IVIG respectively, based on lean body mass. The 496 non-obese patients who received a single dose of 2 g/kg of IVIG received on average 2.3 g/kg ($SD = 0.12$) of IVIG based on lean body mass. The 36 obese patients who received a single dose of IVIG received on average 2.6 g/kg ($SD = 0.15$) of IVIG based on lean body mass. As IVIG is distributed primarily in the vascular space because it is a relatively polar molecule, plasma concentrations of IVIG, and consequently anti-A antibodies, are higher for obese as compared to lean patients if IVIG is dosed on actual body weight (20, 21). There have been several other case reports of thrombotic and hemolytic complications following IVIG treatment in obese patients with primary immunodeficiency disorders, possibly due to large doses of IVIG (22, 23). Other studies have also shown that the correlation of serum immunoglobulin levels is strongest with doses based on ideal or adjusted body weight rather than actual body weight in obese patients (21, 24). While several studies have discussed the cost-saving benefits of weight-adjusted dosing of IVIG in obese patients (25, 26), only a limited number have reported on tolerability or safety related outcomes. One retrospective multicenter study of 297 adult patients treated with IVIG for any indication, the most common being neuroimmunological disorders, showed no increase in 30-day hospital readmission or length of stay with ideal body weight-based dosing of IVIG compared with total body weight dosing (27). Another study also showed that an individualized IVIG treatment protocol using a dosing weight calculated from the patient's ideal body weight is clinically non-inferior to standard dosing for chronic inflammatory demyelinating polyneuropathy, and is 10–25% more cost-effective (28). Thus, this raises the issue as to whether obese KD patients should be dosed based on estimated lean body mass rather than actual body weight.

This study had several strengths and weaknesses. This study included a large cohort of acute KD patients evaluated for hemolytic anemia post-IVIG. In addition, the use of either infliximab or second IVIG for IVIG-resistant KD allowed comparison of rates of hemolytic anemia following these two treatments. This is also the first study to compare the rates of hemolytic anemia in obese and non-obese populations following IVIG treatment for Kawasaki disease. Limitations included small number of cases of hemolytic anemia limiting the ability to perform statistical analyses, missing laboratory data for assessment of hemolytic anemia, and the ability to only assess one of the four IVIG aliquots administered for anti-A/anti-B antibodies. Thus, we may have underestimated the rate of IVIG-associated hemolytic anemia. The KIDCARE trial, a nationwide study comparing a second dose of IVIG vs. infliximab for IVIG-resistant KD will provide prospectively collected, standardized data on the prevalence of hemolytic anemia following second IVIG infusion (NCT #03065244) (29). Physicians should be aware of hemolytic anemia as a potential treatment complication, particularly in KD patients treated with a second infusion of IVIG and need to consider lean body mass dosing for obese patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study protocol was reviewed and approved by the University of California San Diego's Institutional Review Board. Written informed consent was obtained from a parent or legal guardian, and assent, when appropriate, was obtained from the patient.

AUTHOR'S NOTE

The data were presented at the NIH Short-Term Research Training Grant annual conference at the University of California, San Diego, in La Jolla, California.

AUTHOR CONTRIBUTIONS

K-VV contributed to the conception and design of the work, data collection, data analysis and interpretation, and writing of the manuscript. SS contributed to the conception and design, data collection, data analysis, and interpretation, and drafting

of the manuscript. AT was responsible for the conception and design of the project, supervision of the work, and provided critical revision of the manuscript. All authors contributed to the final manuscript.

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Epigenetic Regulation of Macrophage Marker Expression Profiles in Kawasaki Disease

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Kawasaki disease (KD) is a common systemic vasculitides in children younger than 5 years of age. Activated macrophages are key drivers of vascular inflammation in KD. The aim of this study was to examine differences in M1 and M2 macrophage marker expression in patients with KD. Blood samples were obtained from 18 healthy controls and 18 patients with KD at 24 h prior and 21 days after to intravenous immunoglobulin therapy. GeneChip Human Transcriptome Array 2.0 and Illumina HumanMethylation450 BeadChip were used to examined the mRNA expression and corresponding CpG site methylation ratios of 10 M1 surface markers and 15 M2 surface markers. Of the markers examined 2 M1 markers (TLR2, IL2RA) and 8 M2 markers (ARG1, CCR2, TLR1, TLR8, TLR5, MS4A6A, CD36, and MS4A4A) showed increased mRNA expression in the acute phase of KD which decreased after IVIG therapy ($P < 0.05$). Corresponding CpG sites in the promoter regions these markers were hypomethylated in the acute phase of KD and significantly increased after IVIG therapy. In conclusion, both M1 and M2 markers showed increased mRNA expression in the acute phase of KD. CpG site methylation may be one of the mechanisms governing macrophage polarization in KD.

Keywords: Kawasaki disease, macrophage, methylation, transcriptome array, methylation array

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INTRODUCTION

Kawasaki disease (KD) is one of the most common systemic vasculitides in children younger than 5 years of age. First described by Dr. Tomisaki Kawasaki in 1967 (1), characteristic symptoms include fever for more than 5 days and at least 4 out of five signs of mucocutaneous inflammation including: oral mucosal redness or inflammation, non-suppurative conjunctivitis, lymphadenopathy, swelling of the hands or feet or a polymorphous rash (2). If inadequately treated, patients with KD have a high risk of developing coronary artery aneurysms, sometimes resulting in lifelong anticoagulation and an increased risk of myocardial infarction and other cardiovascular complications later in life (3). Intravenous immunoglobulin therapy (IVIG) in particular, if given within the first 10 days after disease onset, has been found to significantly reduce the risk of coronary artery lesions to <5%. However, up to 10–20% of patients with KD do not response to initial IVIG therapy (2), and have an increased risk of developing coronary artery aneurysms (3). The search for alternative therapies is therefore greatly needed, particularly for patients who are IVIG resistant.

Although the precise immunopathogenesis of KD is unclear, histopathology of the coronary arteries has revealed clues to the immunologic sequence of events that lead to coronary artery dilatation and destruction in patients with KD. Edema of the tunica media layer of the coronary arteries begins around the 6th to 8th day after disease onset. This is then followed by increased infiltration of lymphocytes, monocytes, and neutrophils by the 10th day of disease, which then infiltrates all layers of the coronary artery. This panarteritis is characterized by the formation of proliferative granulomatous inflammation consisting mostly of monocytes and macrophages (4, 5). Macrophages in particular are key drivers of vascular inflammation in KD, producing inflammatory cytokines such as Tumor necrosis factor-α(TNF-α), vascular endothelial growth factor, and proteases such as matrix metalloproteinase-2 (MMP-2) and MMP9 which may destroy elastin fibers and structural components within the arterial wall (6). This in turn results in the development of coronary artery aneurysms beginning at about the 12th day after disease onset. These findings point to the pivotal role aberrantly activated macrophages play in the formation of coronary artery aneurysms in KD.

Macrophages are considered an important component of innate immunity and may initiate inflammation and subsequently adaptive immune responses. Recently it has been found that activated macrophages can also be divided into M1 and M2 subsets, which mirror the Th1 and Th2 polarization seen in T-helper cells (7). Polarization of macrophages into either the M1 or M2 phenotype is highly dependent on the local tissue environment. Classically activated macrophages, also known as M1 macrophages are induced by either IFN-γ alone or in combination with microbial stimuli such as lipopolysaccharides (LPS) or cytokines including tumor necrosis factor (TNF). M1 macrophages produce effector molecules such as reactive oxygen and nitrogen intermediates and also inflammatory cytokines including IL-1β, TNF, and IL-6, thereby amplifying the inflammatory response. They are also capable of inducing Th1 cells and thereby increasing immune responses against intracellular pathogens and increasing tumor resistance (8). In contrast, alternatively activated macrophages, or M2 macrophages, are induced by IL-4, IL-13, IL-10, or immune complexes, produce low levels of inflammatory cytokines, are capable of inducing Th2 cells and are considered immune-modulatory in nature (7).

To date, there have been no studies that specifically examine the role of macrophage M1/M2 polarization in patients with KD. Results from the limited number of existing studies regarding macrophage polarization in other forms of autoimmune vasculitis reveal that both M1 and M2 activation may be involved (8). As an example, in giant cell arteritis, vasculitis of the temporal arteries contain both M1 (inducible nitric oxide positive) and M2 (CD163 positive) macrophages (9, 10). In light of the paucity of research regarding the role of macrophage polarization and the development of KD, the aim of this study was to examine differences in M1 and M2 macrophage marker expression in the patients with acute and resolving KD.

MATERIALS AND METHODS

Subject Recruitment

Initially, a total of 18 patients with KD and 18 healthy controls were enrolled in this study for Human Transcriptome array analysis, and another 12 healthy controls and 12 patients with KD were enrolled for Human Methylation Beadchip analysis. We then enrolled an additional 30 healthy controls and 30 patients with KD for confirmation of initial screening results with RT-PCR, 10 of which were analyzed by pyrosequencing.

Patients were diagnosed with Kawasaki disease if they fulfilled the criteria proposed by the American Heart Association, namely fever which persists for more than 5 days and at least four of the following five clinical criteria: oral mucosal involvement (such as injected or fissured lips, strawberry tongue), bilateral non-exudative conjunctival injection, cervical lymph node enlargement of over 1.5 cm in diameter, changes in the peripheral extremities (edema of the hands and feet, erythema over the plantar, or palmar area in the acute phase or periungual desquamation in the convalescent phase), and a polymorphous rash (2). Patients with Kawasaki disease were then given at least one dose of IVIG (2 g/kg/dose) over a 12 h period after diagnosis in accordance with current clinical guidelines (2). This study has been approved by the Chang Gung Medical Foundation Institutional Review Board (IRB No.:101-0680A3), and conforms to the principles outlined in the Declaration of Helsinki. Informed consent was obtained from the parents or guardians of all patients included in this study.

DNA and RNA Extraction From Peripheral Blood Samples

Blood samples from the KD group were obtained at two time points: the first blood draw was performed within 24 h prior to the initial dose of IVIG therapy during the acute phase of KD (KD1), and the second blood draw was performed at least 21 days after initial IVIG therapy during the convalescent phase (KD2). All blood samples were centrifuged and the buffy coat containing white blood cells was then removed for further experiments. Total RNA was then extracted from white blood cells according to manufacturer's instructions (mirVana^{TM} miRNA Isolation Kit, Catalog number: AM1560, Life Technologies, Carlsbad, CA). Total RNA samples were checked for quality and quantity and all RNA samples were confirmed to have a RIN (RNA integral number) of $\geq$7 as previously described (11). Total genomic DNA was extracted according to manufacturer's instructions (Gentra extraction kit). After quantification of all DNA samples was performed by using NanoDrop ND-1000, 500 ng of genomic DNA was taken from each sample for further bisulfite conversion using Zymo EZ DNA Methylation kit (Zymo Research) and stored at 20°C until further use (12).

Gene Expression Level Profiling With Human Transcriptome Array

For unbiased results, 18 mRNA samples each from the healthy control group (HC group), KD1 group (KD patients 24 h prior to IVIG therapy) and the KD2 group (KD patients 21 days after IVIG therapy) were combined into three pooled

RNA libraries each containing six RNA samples. We then used GeneChip® Human Transcriptome Array 2.0 (HTA 2.0, Affymetrix, Santa Clara) to determine gene expression profiles of macrophage surface markers which are commonly expressed on M1 and M2 macrophages after literature review (13–16). Prior to hybridization to the HTA 2.0 microarray chips, all RNA samples were prepared according to manufacturer instructions with the WT PLUS Reagent kit. The hybridized HTA 2.0 microarray chips then underwent quality control inspection. All chips passed quality control examination and were then analyzed using commercial software specific for microarray data analysis (Partek, St. Louis).

DNA Methylation of M1 and M2 Macrophage Surface Markers

DNA samples taken from the 12 healthy controls, and the 12 Kawasaki disease patients at two time points, 24 h prior to IVIG therapy (KD1) and 21 days after IVIG therapy (KD2) were then used to examine DNA methylation patterns of M1 and M2 macrophage surface markers. Two hundred nanograms of bisulfite-converted genomic DNA were then applied to the Illumina HumanMethylation450 (M450K) BeadChip assay as described in previously (11, 17). Cytosine methylation percentages (β values) were then calculated for each CpG marker within in the promoter region of all M1 and M2 markers included.

Confirmation of mRNA Expression With Reverse Transcription Polymerase Chain Reaction (RT-PCR)

We then selected the following markers for RT-PCR confirmation of mRNA expression levels: IL1R1, TLR2, TLR4, IL1T2, ARG1, and TLR5. All mRNA samples were first transformed into cDNA according to manufacturer instructions (cDNA-High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Cat. 4368813). Samples were then prepared by adding 2.5 ng/μL of cDNA with 0.2 μL each of forward and reverse primers (10 μM) and 5 μL of SYBR Green PCR Master Mix (ABI, Cat. 4367659) before performing quantitative RT-PCR on the LightCycler R 480 Real-Time PCR System (Roche Molecular Systems, Inc. Indiana, USA). 18S was used as an internal control. IL1R1—Forward (F) AAAGATGACAGCAAGACACCTG, Reverse (R):GTTTGCAATCCTTACCACGCAA; TLR2—(F): GAGTTCTCCCAGTGTTTGGTGT (R):CACACCATCAGAAC CCTGTC; IL1R2—(F):ATGACACCCACATAGAGAGCG (R):GAAGAGCGAAACCCACAGAGT; ARG1—(F):ACTTA AAGAACAAGAGTGTGATGTG (R) CGCTTGCTTTTC CCACAGAC; TLR5—(F):TGCTACTGACAACGTGGCTT (R):CCAGGAAAGCTGGGCAACTA; TLR4—(F):ATGCCAGG ATGATGTCTGCC (R):TGGATTTCACACCTCCACGC; 18S—(F):GTAACCCGTTGAACCCCATT (R):CCATCCAATCG GTAGTAGCG. We then calculated the relative quantification of mRNA expression levels by using the comparative threshold cycle (CT) method (i.e., by comparing the RT-PCR cycle number required to reach target fluorescence) by using the following equation: $2^{-(\Delta CTtarget-\Delta CTcalibrator)}$, also known as $2^{-\Delta\Delta CT}$.

Pyrosequecing of Selected CpG Sites

To confirm the percentage of methylation of selected CpG, further pyrosequencing was performed in a separate cohort. First 0.5 μg of genomic DNA underwent bisulfite modification according to manufacturer instructions (EZ DNA methylation kit, Zymo Research) and with eluted in Tris buffer (10 mM) for a final volume of 20 μl. Polymerase chain reaction (PCR) with a reaction mixture containing bisulfite-converted DNA, PyroMark PCR Master Mix (Qiagen), and biotinylated forward and reverse primers of the selected CpG sites. Primers used are as follows: IL1R1 (cg05886087) F:TTTTTGGAAATAGATT GTTTAAGAATGAGA, R:ATAATAATAACTTCCTTCCTTCT AATACAC; TLR2 (cg06618866) F: AGGTTGGGTAGAAGAAG AGAGT, R: AACACTTAACTTTCCCTATAATTACCA; IL1R2 (cg20340242) F: ATTGTAGTTTGGGGGTGT, R: TTCACC TCCAACAAAAACTCA; ARG1 (cg01699630) F: TTAGGGTTG GAAGGGATGTGATA, R:AACCAAAAAATAACAATCAA CAATCTTAC; TLR5 (cg05858079) F:AGGGTTTTAGAGTTTT TTTAGGGTAGTA, Reverse:AATAACTTATCCCCAATCACTT TC; TLR4 (cg13730105) F:GAGGGAAAGTTTAGAGGAGTT, R:ACCACCCATTCACAAAACCACTAC. We then calculated the percentage of methylation at for all CpG sites by comparing relative peak height differences at each site (18).

Statistical Analysis

Statistical analysis was performed using SPSS version 22.0 (SPSS, Inc., Chicago, USA). Data from HTA 2.0 microarray was compared using one-way ANOVA and Bonferroni Correction was used to account for multiple testing. Beta-values from the M450K Array were first transformed into M-values by using the following equation: M-value = log2(beta-value / 1- beta-value) (19). M-values of selected CpG sites were then compared with student's *t*-test for the comparison of healthy controls and patients with KD; and paired *t*-test was used to compare data from KD patients at 2 time points, 24 h prior to IVIG therapy and 21 days after IVIG therapy. Likewise, for data obtained from RT-PCR and pyrosequencing, students *t*-test was used to compare data from healthy controls and KD patients, and paired *t*-test was used to compare data from patients with KD from 2 different time points. Data are presented as mean ± standard error and a $p < 0.05$ was considered as statistically significant. The datasets analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

M1 Marker and M2 mRNA Expression in KD Patients and Controls Using HTA 2.0 Microarray Analysis

In this study we examined the mRNA expression of 10 M1 surface markers (IL1R1, TLR2, TLR4, SOCS3, CCR7, IL2RA, IL15A, IL7R, CD68, CD86) in three groups, healthy control (HC), acute KD (KD1) and in KD patients 21 days after IVIG therapy (KD2) (**Figure 1**). We found that of the 10 M1 surface markers examined, 2 markers TLR2, and IL2RA had significantly elevated mRNA expression in the acute phase of KD (mRNA

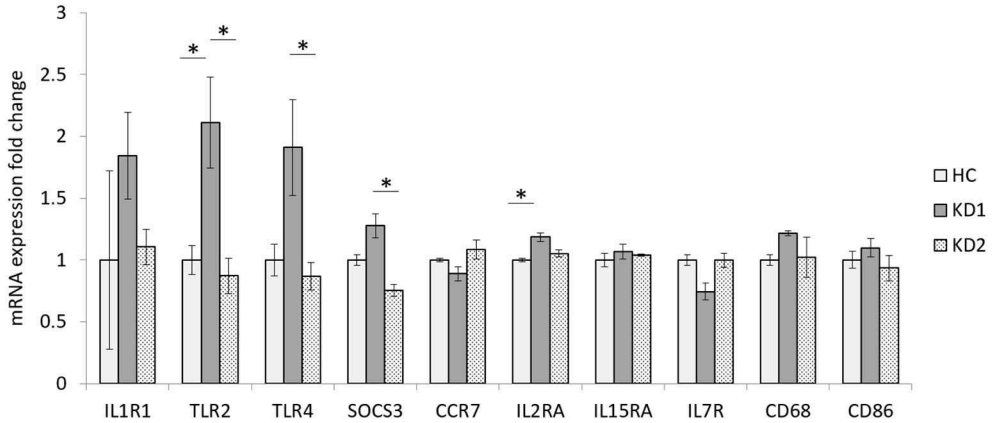

FIGURE 1 | Comparison of M1 macrophage marker mRNA expression fold change by between healthy controls and patients with KD. HC, Healthy controls (18 patients); KD1, acute Kawasaki disease 24 h prior to IVIG therapy (18 patients); KD2, resolving Kawasaki disease 21 days after IVIG therapy (18 patients). An asterisk denotes a $p < 0.05$. Data are expressed as mean ± standard error.

expression fold change HC vs. KD1 1 ± 0.118 vs. 2.113 ± 0.367, $p = 0.023$; 1 ± 0.013 vs. 1.185 ± 0.034, $p = 0.011$, presented as mean ± standard error, respectively). IVIG therapy resulted in a significant decrease in expression of three genes including TLR2, TLR4, and SOCS3 (KD1 vs. KD2 2.113 ± 0.367 vs. 0.873 ± 0.142, $p = 0.009$; 1.911 ± 0.388 vs. 0.868 ± 0.113, $p = 0.021$, 1.276 ± 0.095 vs. 0.755 ± 0.048, $p = 0.032$ presented as mean ± standard error, respectively).

We also examined the mRNA expression of the 15 M2 cell surface markers (CD163, MRC1, CXCR2, IL1R2, ARG1, CCR2, TLR1, TLR5, TLR8, CXCR4, MS4A6A, CD36, MS4A4A, CXCR1, and FCER2) in both KD patients and healthy controls (**Figure 2**). Of the 15 M2 cell surface markers examined, 8 markers (ARG1, CCR2, TLR1, TLR8, TLR5, MS4A6A, CD36, and MS4A4A) had significantly increased mRNA expression in acute phase KD (HC vs. KD1 1 ± 0.036 vs. 4.046 ± 1.185, $p < 0.029$; 1 ± 0.069 vs. 2.491 ± 0.171, $p = 0.001$; 1 ± 0.134 vs. 2.364 ± 0.423, $p = 0.013$; 1 ± 0.126 vs. 2.390 ± 0.360, $p = 0.011$; 1 ± 0.120 vs. 3.178 ± 0.459, $p < 0.002$; 1 ± 0.099 vs. 1.799 ± 0.143, $p = 0.011$; 1 ± 0.029 vs. 1.616 ± 0.197, $p = 0.045$; 1 ± 0.070 vs. 2.261 ± 0.257, $p = 0.003$ presented as mean ± standard error, respectively), which were then significantly decreased after IVIG therapy (KD1 vs. KD2 4.046 ± 1.185 vs. 0.914 ± 0.003, $p = 0.001$; 2.491 ± 0.172 vs. 0.828 ± 0.121, $p < 0.001$; 2.363 ± 0.423 vs. 0.854 ± 0.094, $p = 0.005$; 2.390 ± 0.360 vs. 0.885 ± 0.131, $p = 0.005$; 3.178 ± 0.459 vs. 0.872 ± 0.106, $p < 0.001$; 1.799 ± 0.143 vs. 0.916 ± 0.117, $p = 0.005$; 1.616 ± 0.197 vs. 0.879 ± 0.057, $p = 0.013$; 2.261 ± 0.257 vs. 0.929 ± 0.027, $p = 0.002$; presented as mean ± standard error, respectively). One marker, CXCR2 showed a statistically significant decrease in mRNA expression after IVIG therapy (KD1 vs. KD2 1.528 ± 0.151 vs. 0.834 ± 0.158, $p = 0.038$). Two markers, CXCR4 and FCER2, had decreased mRNA expression in the acute phase of KD (HC vs. KD1 1 ± 0.078 vs. 0.609 ± 0.071, $p = 0.006$; 1 ± 0.059 vs. 0.703 ± 0.033, $p = 0.004$ presented as mean ± standard error, respectively) which increased after IVIG therapy (KD1 vs. KD2 0.609 ± 0.071 vs. 1.057 ± 0.011, $p = 0.003$; 0.703 ± 0.033 vs. 0.912 ± 0.012, $p = 0.022$ presented as mean ± standard error, respectively).

Confirmation of mRNA Expression Levels Using RT-PCR

We then chose three M1 markers (IL1R1, TLR2, TLR4) and three M2 markers (IL1R1, ARG1, TLR5) with the highest fold change between acute KD (KD1) and healthy controls (HC), for RT-PCR confirmation of mRNA expression levels in a separate cohort of 30 KD patients and 30 healthy controls (**Figure 3**). For the three M1 markers selected, TLR2 and TLR4 mRNA expression levels were higher on average in the acute KD group (HC vs. KD1 1 ± 0.143 vs. 1.073 ± 0.204, $p = 0.771$, 1 ± 0.128 vs. 1.452 ± 0.504 $p = 0.388$ presented as mean ± standard error, respectively), which increased after IVIG therapy (KD1 vs. KD2 1.073 ± 0.204 vs. 1.184 ± 0.262, $p = 0.737$, 1.452 ± 0.504 vs. 1.554 ± 0464, $p = 0.878$ presented as mean ± standard error, respectively), although these changes were not statistically significant. IL1R1 on the other hand, was lower in the acute phase of KD (KD1) (HC vs. KD1 1 ± 0.649 vs. 0.655 ± 0.190, $p = 0.612$ presented as mean ± standard error), and increased after IVIG therapy (KD1 vs. KD2 0.655 ± 0.190 vs. 0.789 ± 0.344 $p = 0.739$ presented as mean ± standard error), but these changes were again not statistically significant.

For the three M2 markers selected, IL1R2 and ARG1 showed higher average expression in acute KD (KD1) (HC vs. KD1 1 ± 0.108 vs. 1.607 ± 0.375, $p = 0.129$, 1 ± 0.207 vs. 1.098 ± 0.235, $p = 0.754$ presented as mean ± standard error, respectively), which decreased after IVIG therapy (KD1 vs. KD2 1.607 ± 0.375 vs. 1.238 ± 0.223, $p = 0.334$, 1.098 ± 0.235 vs. 0.895 ± 0.155, $p = 0.319$ presented as mean ± standard error, respectively), although these changes were not statistically significant. TLR5 showed lower expression in the acute phase of KD (HC vs. KD1 1 ± 0.416 vs. 1.098 ± 0.252, $p = 0.685$, presented as mean ± standard error), which increased slightly after IVIG therapy (KD1 vs. KD2 1.098 ± 0.252 vs. 0.847 ± 0.272, $p = 0.881$, presented as mean ± standard error), but was also not statistically significant.

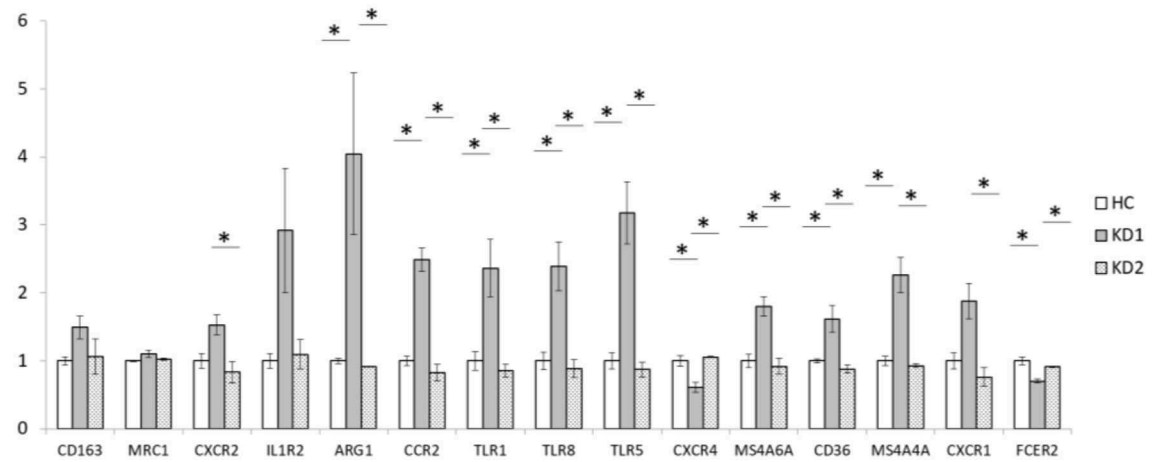

FIGURE 2 | Comparison of M2 macrophage marker mRNA expression fold change by between healthy controls and patients with KD. HC, Healthy controls (18 patients); KD1, acute Kawasaki disease 24 h prior to IVIG therapy (18 patients); KD2, resolving Kawasaki disease 21 days after IVIG therapy (18 patients). An asterisk denotes a $p < 0.05$. Data are expressed as mean ± standard error.

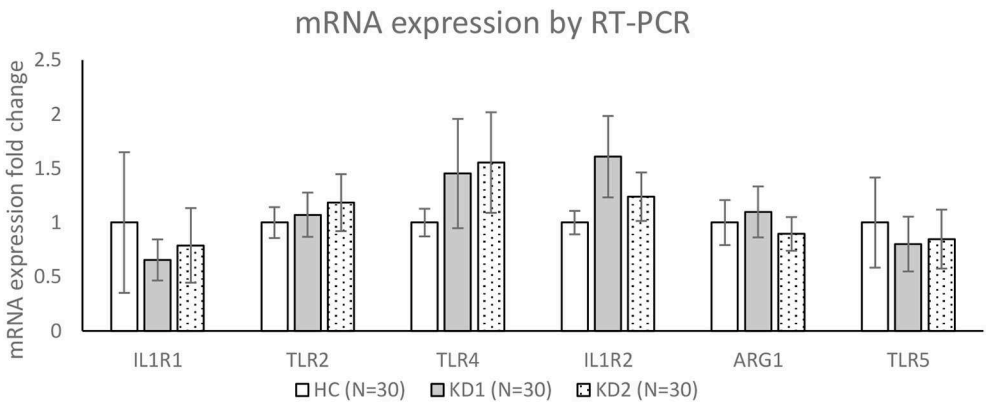

FIGURE 3 | Confirmation of mRNA expression levels by RT-PCR. HC, Healthy controls (30 patients); KD1, acute Kawasaki disease 24 h prior to IVIG therapy (30 patients); KD2, resolving Kawasaki disease 21 days after IVIG therapy (30 patients). Data are expressed as mean ± standard error.

Methylation Levels of Corresponding M1 and M2 Marker CpG Sites

We then used Infinium HumanMethylation 450 BeadChip (Illumina) to examine the CpG site methylation trends of the 10 M1 and 15 M2 surface markers included in this study (full results in **Tables 1**, **2**). Because hypomethylation of CpG sites in the promoter region of genes leads to increased gene mRNA expression (20), we tried to identify corresponding CpG sites which were hypomethylated in genes with increased mRNA expression.

Of the three M1 markers with the highest fold change in mRNA expression on HTA 2.0, we identified three corresponding CpG sites the highest negative beta-value fold change between KD1 and HC groups, indicating that these sites were likely to be hypomethylated. Selected CpG sites included cg05886087 (KD1 vs. HC beta-value fold change −1.217, $p < 0.001$) within the IL1R1 gene, cg06618866 (KD1 vs. HC beta-value fold change −1.0313, $p < 0.001$) within the TLR2 gene, cg 13730105 (KD1 vs. HC beta-value fold change −1.154, $p < 0.001$) within the TLR4 gene. Likewise, for the M2 markers three corresponding CpG sites if the highest negative beta-value fold change were selected including cg20340242 (KD1 vs. HC beta-value fold change −1.191, $p < 0.001$) within the IL1R2 gene, cg01699630 (KD1 vs. HC beta-value fold change −0.1271, $p < 0.001$) within the ARG1 gene, cg05858079 (KD1 vs. HC beta-value fold change −1.011, $p = 0.025$).

The six selected CpG sites were then confirmed with a separate cohort of 10 healthy controls, and 10 patients with KD by pyrosequencing. Four of the CpG sites selected corresponding to the M1 markers IL1R1 (cg05886087), TLR4 (cg13730105), and the M2 markers IL1R2 (cg20340242) and ARG1 (cg01699630) were significantly hypomethylated in the acute KD group (HC vs. KD1, 0.607 ± 0.0322 vs. 0.451 ± 0.026, $p = 0.001$; 0.340 ± 0.023 vs. 0.226 ± 0.019, $p = 0.002$; 0.405 ± 0.025 vs. 0.274 ±

TABLE 1 | Comparison of CpG site methylation fold changes of M1 macrophage markers in healthy controls and patients with Kawasaki Disease.

Gene symbol	Target ID	Fold-Change (KD1 vs. HC)	*p*-value (KD1 vs. HC)	Fold-Change (KD2 vs. HC)	*p*-value (KD2 vs. HC)	Fold-Change (KD2 vs. KD1)	*p*-value (KD2 vs. KD1)
IL1R1	cg05886087	−1.21732	0.000006*	−1.05198	0.041622*	1.15716	0.000431*
	cg06880612	−1.10287	0.000006*	−1.04512	0.000028*	1.05526	0.001306*
	cg06943668	−1.00203	0.981921	−1.01157	0.016226*	−1.00952	0.020868*
	cg09363443	−1.04316	0.000003*	−1.02102	0.009032*	1.02168	0.026471*
	cg19754707	−1.01321	0.005930*	−1.02287	0.000020*	−1.00954	0.019204*
TLR2	cg02345613	−1.00936	0.001359*	−1.01118	0.081380	−1.0018	0.775385
	cg03523945	−1.01976	0.000002*	−1.00686	0.058977	1.01281	0.021652*
	cg03610073	−1.0096	0.142018	−1.03862	0.003742*	−1.02875	0.009883*
	cg06405222	−1.00235	0.978750	−1.02161	0.000003*	−1.01922	6.44E-05*
	cg06618866	−1.0313	0.000083*	−1.02138	0.013046*	1.00971	0.3621
	cg15852258	1.00144	0.701207	1.01416	0.000083*	1.0127	0.001127*
	cg16547110	1.0028	0.484426	−1.00836	0.026797*	−1.01118	0.000831*
	cg17916835	−1.01637	0.012428*	−1.01335	0.051072	1.00299	0.381223
	cg19037167	−1.02192	0.000062*	−1.01232	0.020365*	1.00948	0.021885*
TLR4	cg04061482	1.00672	0.766085	−1.0013	0.958727	−1.00803	0.031436*
	cg05429895	−1.01334	3.51E-07*	1.0092	0.008932*	1.02266	2.55E-06*
	cg13730105	−1.15383	0.000035*	−1.0344	0.125541	1.11546	0.000666*
SOCS3	cg01897823	1.00161	0.174351	1.0103	0.000612*	1.00868	0.005838*
	cg03752138	−1.04133	0.000013*	−1.08298	1.61E-10*	−1.04	9.92E-06*
	cg04548563	−1.00384	0.157371	1.00241	0.243839	1.00626	0.000491*
	cg04610187	−1.07261	0.000012*	−1.09513	3.61E-07*	−1.02099	0.011633*
	cg10368834	−1.01261	0.500669	−1.01755	0.313913	−1.00488	0.159929
	cg12999453	1.00176	0.756747	1.00261	0.327255	1.00085	0.491749
	cg14253096	−1.00833	0.263096	−1.00123	0.900064	1.00708	0.434305
	cg14496305	−1.02192	0.028009*	−1.0291	0.000009*	−1.00703	0.000825*
	cg14721618	1.00797	0.028757*	−1.03565	4.00E-11*	−1.0439	1.03E-07*
	cg15502888	1.00195	0.173635	1.01376	0.000056*	1.01178	0.000944*
	cg16253629	−1.00525	0.363686	−1.00862	0.133850	−1.00335	0.619161
	cg18538958	−1.01177	0.116957	−1.04835	0.000455*	−1.03616	0.004421*
	cg18855780	1.00358	0.054970	−1.00029	0.496837	−1.00387	0.011058*
	cg21500342	−1.01037	0.001759*	−1.00208	0.502114	1.00827	0.023568*
	cg22749855	1.04158	0.000056*	−1.03244	0.003358*	−1.07537	5.71E-06*
	cg23985214	−1.00508	0.111600	1.00529	0.070625	1.0104	0.000407*
	cg26747885	1.01678	0.001820*	−1.01665	0.000177*	−1.0337	9E-06*
CCR7	cg07248223	1.19679	0.000020*	1.01481	0.484177	−1.17932	0.000231*
	cg08724510	−1.00393	0.420426	−1.01055	0.246296	−1.00659	0.678295
	cg13270626	−1.00996	0.010916*	−1.01008	0.086185	−1.00011	0.578922
	cg13504059	1.04995	0.005605*	−1.00974	0.577696	−1.06018	0.001253*
	cg16047279	1.22107	0.000005*	1.03122	0.180002	−1.18411	0.000176*
	cg17067993	−1.00135	0.830882	1.01779	0.000009*	1.01916	6.75E-05*
	cg26960939	1.12657	0.000234*	1.00808	0.677247	−1.11754	0.000322*
IL2RA	cg10486505	−1.00264	0.267540	1.00034	0.969791	1.00298	0.404473
	cg11733245	1.01058	0.003400*	1.02302	0.000089*	1.01231	0.0232*
	cg16949914	1.04095	0.001556*	1.01301	0.333223	−1.02758	0.028752*
	cg26105232	−1.22018	0.000002*	−1.05966	0.042674*	1.15148	0.000197*
	cg26316423	1.03938	0.008769*	−1.00981	0.516062	−1.04957	0.000267*
	cg27131821	1.00412	0.159932	1.01061	0.000098*	1.00645	0.031687*
IL15RA	cg00508950	−1.00036	0.989579	−1.00867	0.392564	−1.00831	0.282137
	cg01718738	−1.01164	0.066812	−1.05996	7.07E-09*	−1.04776	2.66E-06*
	cg03691156	−1.00144	0.280415	−1.00355	0.000505*	−1.0021	0.009006*

(Continued)

TABLE 1 | Continued

Gene symbol	Target ID	Fold-Change (KD1 vs. HC)	*p*-value (KD1 vs. HC)	Fold-Change (KD2 vs. HC)	*p*-value (KD2 vs. HC)	Fold-Change (KD2 vs. KD1)	*p*-value (KD2 vs. KD1)
	cg05144147	−1.00041	0.777642	−1.01095	0.000867*	−1.01054	0.002767*
	cg08676905	−1.0012	0.508908	1.0071	0.017166*	1.00831	0.002231*
	cg09290866	−1.04138	0.000096*	−1.02537	0.028776*	1.01561	0.18332
	cg10686550	−1.01486	0.000056*	−1.00163	0.580957	1.01321	0.007886*
	cg13730379	−1.02743	0.000268*	−1.10803	5.56E-12*	−1.07845	1.67E-07*
	cg15071696	−1.00745	0.000153*	−1.01708	0.000065*	−1.00956	0.077432
	cg19979643	−1.00974	0.001077*	1.0096	0.001914*	1.01944	4.11E-05*
	cg24348573	−1.09086	0.000021*	−1.03349	0.046193*	1.05552	0.006376*
	cg24677732	−1.00015	0.690520	−1.00477	0.001312*	−1.00463	0.001144*
IL7R	cg01804183	1.10724	0.000005*	−1.00733	0.648630	−1.11535	1.11E-05*
	cg04312209	1.1607	0.000017*	1.06724	0.013245*	−1.08758	0.000639*
	cg27582180	−1.0079	0.027828*	−1.01512	0.002740*	−1.00717	0.128131
CD68	cg01934632	−1.00606	0.018303*	−1.00528	0.030173*	1.00077	0.780858
	cg02346901	−1.02463	0.000072*	−1.03887	0.000001*	−1.0139	0.112221
	cg02613380	−1.00316	0.322148	−1.00648	0.157876	−1.0033	0.380751
	cg04510815	−1.0012	0.230227	−1.05302	0.000001*	−1.05175	0.000165*
	cg05209483	−1.17957	0.000002*	−1.06637	0.008743*	1.10615	0.000196*
	cg05270696	−1.00707	0.234327	−1.054	1.15E-09*	−1.0466	9.25E-07*
	cg05652328	−1.02215	0.010914*	−1.02657	0.011688*	−1.00432	0.647877
	cg06852395	−1.03391	0.006797*	1.01227	0.219162	1.0466	6.35E-05*
	cg07421515	−1.02056	0.008227*	1.01715	0.023434*	1.03806	5.47E-05*
	cg10587621	−1.03019	0.000017*	1.0162	0.115484	1.04688	0.000478*
	cg13749033	−1.0266	0.000005*	−1.04487	4.77E-08*	−1.01779	0.008606*
	cg15095917	−1.17028	0.000003*	−1.06479	0.005198*	1.09907	0.00052*
	cg16056611	1.00386	0.119835	1.00356	0.522221	−1.0003	0.677193
	cg16547235	−1.00794	0.001642*	−1.02074	0.000032*	−1.0127	0.040127*
	cg18900669	−1.11258	0.000012*	−1.0362	0.040849*	1.07371	0.001219*
	cg21808406	−1.15626	0.000027*	−1.03643	0.163403	1.11562	0.000321*
	cg22259724	1.00184	0.699657	−1.05907	1.18E-08*	−1.06102	2.5E-06*
	cg24219033	−1.00705	0.127193	−1.10006	1.93E-09*	−1.09236	6.89E-06*
	cg27010959	−1.00357	0.073877	1.00212	0.524067	1.0057	0.080075
CD86	cg01021483	1.00741	0.925748	−1.00276	0.457571	−1.01018	0.040495*
	cg01436254	−1.02563	0.031212*	1.00976	0.445134	1.03563	0.000256*
	cg01878435	−1.01772	0.003025*	−1.00637	0.236206	1.01127	0.024484*
	cg04387658	−1.0469	0.000221*	−1.05514	0.000120*	−1.00788	0.332995
	cg04880737	−1.03367	0.000270*	−1.0287	0.140235	1.00483	0.639097
	cg06327732	−1.01266	0.035835*	−1.09175	3.86E-08*	−1.07809	9.93E-05*
	cg07108581	−1.00402	0.571690	−1.01501	0.036759*	−1.01095	0.053199
	cg11874272	1.00599	0.580171	−1.02365	0.023441*	−1.02978	0.00443*
	cg13069531	−1.09587	0.000010*	−1.02491	0.118781	1.06923	0.000181*
	cg13617155	−1.07336	3.95E-08*	−1.01786	0.081353	1.05452	5.63E-05*
	cg16331599	−1.03419	0.000019*	1.03103	0.017603*	1.06628	0.000159*

HC, Healthy controls (12 patients), KD1, acute Kawasaki disease 24 h prior to IVIG therapy (12 patients), KD2, resolving Kawasaki disease 21 days after IVIG therapy (12 patients). An asterisk denotes a $p < 0.05$. Data are expressed as mean ± standard error.

0.028, $p = 0.003$; 0.686 ± 0.036 vs. 0.499 ± 0.030, $p = 0.001$; beta values presented as mean ± standard error.), and became hypermethylated after IVIG therapy (KD1 vs. KD2 0.451 ± 0.026 vs. 0.652 ± 0.031, $p < 0.001$; 0.226 ± 0.019 vs. 0.344 ± 0.022, $p < 0.001$; 0.274 ± 0.028 vs. 0.434 ± 0.027, $p < 0.001$; 0.499 ± 0.030 vs. 0.720 ± 0.039, $p < 0.001$; beta values presented as mean ± standard error.) The CpG site selected for the M1 marker TLR2 (cg06618866) showed significant hypermethylation after IVIG therapy (KD1 vs. KD2 0.057 ± 0.033 vs. 0.074 ± 0.004, $p < 0.001$). There were no significant changes in methylation of the Cpg site

TABLE 2 | Comparison of CpG site methylation fold changes of M2 macrophage markers in healthy controls and patients with Kawasaki Disease.

Gene symbol	Target ID	Fold–Change (KD1 vs. HC)	*p*–value (KD1 vs. HC)	Fold–Change (KD2 vs. HC)	*p*–value (KD2 vs. HC)	Fold–Change (KD2 vs. KD1)	*p*–value (KD2 vs. KD1)
CD163	cg05939324	−1.00564	0.607439	−1.03034	8.19E-09*	−1.02457	0.000004*
	cg07264679	−1.02182	0.001080*	−1.00376	0.442244	1.018	0.004770*
CXCR2	cg00002473	−1.0051	0.169105	−1.12953	3.17E-12*	−1.1238	5.84E-08*
	cg03464560	−1.01415	0.042228*	1.01414	0.035118*	1.02849	0.000457*
	cg06547715	−1.21994	0.000003*	−1.08806	0.004337*	1.1212	0.001580*
	cg10591797	−1.04278	0.000003*	−1.02701	0.002627*	1.01535	0.013146*
	cg13739417	−1.02656	1.51E-07*	−1.00984	0.038776*	1.01656	0.000003*
	cg14150666	−1.09239	0.000017*	−1.05641	0.004698*	1.03405	0.056927
	cg17081998	−1.02885	0.000021*	−1.01866	0.000020*	1.01	0.049850*
	cg19225688	−1.15693	0.000002*	−1.1111	0.000010*	1.04125	0.081946
	cg25941354	−1.14501	0.000001*	−1.0989	0.000003*	1.04196	0.026826*
IL1R2	cg01716667	1.0002	0.774800	1.00303	0.302792	1.00283	0.439499
	cg01820934	−1.05541	0.000496*	−1.04066	0.000002*	1.01417	0.656054
	cg08879111	1.03957	0.021655*	−1.00696	0.617709	−1.04681	0.016664*
	cg12910851	1.05778	0.000246*	−1.0293	0.082031	−1.08877	0.000187*
	cg14325025	−1.1719	0.000002*	−1.05687	0.008913*	1.10884	0.000907*
	cg15400471	−1.00467	0.364754	−1.04807	1.24E-09*	−1.0432	0.000009*
	cg17142183	−1.1585	0.000002*	−1.06719	0.004062*	1.08556	0.007689*
	cg20340242	−1.1908	0.000011*	−1.06805	0.004795*	1.11493	0.002271*
	cg20470722	1.00181	0.531958	1.00174	0.426552	−1.00007	0.968432
	cg20856504	−1.01286	0.022020*	−1.02197	0.000400*	−1.00899	0.047466*
	cg21603144	−1.16457	0.000003*	−1.06695	0.004560*	1.09149	0.002574*
	cg25920665	−1.00515	0.163200	−1.08262	8.02E-14*	−1.07707	2.07E-08*
ARG1	cg01699630	−1.27067	0.000006*	−1.07096	0.013553*	1.18648	0.000784*
	cg02862362	−1.0219	0.000423*	−1.05761	5.22E-09*	−1.03494	0.000836*
	cg04639555	−1.01709	0.048098*	−1.09702	4.20E-08*	−1.07859	0.000009*
	cg06975018	−1.21034	0.000006*	−1.10044	0.001201*	1.09987	0.012810*
	cg16178743	−1.02082	0.000970*	−1.04491	0.000001*	−1.0236	0.001477*
CCR2	cg03928384	1.03628	0.000361*	−1.00716	0.535037	−1.0437	0.025890*
	cg04110105	−1.02183	0.013246*	1.05256	0.000014*	1.07554	0.000001*
	cg06585307	−1.01249	0.001057*	−1.00794	0.115795	1.00451	0.318164
	cg07824265	1.00619	0.845400	1.00272	0.597202	−1.00346	0.660749
TLR1	cg02016764	1.01476	0.192395	1.03396	0.146065	1.01892	0.552275
	cg08757862	−1.01801	0.000255*	−1.01152	0.017786	1.00642	0.146685
	cg09316306	−1.05901	4.44E-08*	1.00289	0.840879	1.06207	0.000025*
	cg22839308	−1.01643	0.000005*	1.00323	0.336331	1.01972	0.000031*
TLR8	cg00741717	−1.03908	0.004612*	−1.04154	0.001406*	−1.00236	0.868754
	cg07759587	−1.18112	0.000183*	−1.07489	0.005605*	1.09883	0.007118*
	cg13153942	−1.04969	0.000121*	−1.17152	2.16E-08*	−1.11606	0.000323*
TLR5	cg00255925	1.00094	0.724266	−1.02269	0.000009*	−1.02364	0.000001*
	cg01181681	1.05831	0.050795	−1.02191	0.226850	−1.0815	0.002321*
	cg03702975	1.0162	0.025828*	−1.00228	0.701607	−1.01852	0.014849*
	cg04219417	1.04379	0.007797*	−1.02969	0.072505	−1.07478	0.000045*
	cg05696109	−1.00085	0.899629	1.0164	0.000280*	1.01726	0.000233*
	cg05858079	−1.01157	0.025045*	−1.03379	0.000113*	−1.02197	0.009294*
	cg07015886	1.05173	0.018117*	−1.04237	0.007657*	−1.09629	0.000078*
	cg07538512	−1.00577	0.008527*	1.00509	0.282785	1.01089	0.004198*
	cg07574686	−1.00294	0.547926	−1.00489	0.589048	−1.00194	0.844872
	cg09025215	1.04164	0.002594*	−1.00674	0.685264	−1.04867	0.001783*
	cg12275981	1.02507	0.001353*	1.00879	0.257961	−1.01615	0.083901

(Continued)

TABLE 2 | Continued

Gene symbol	Target ID	Fold–Change (KD1 vs. HC)	*p*–value (KD1 vs. HC)	Fold–Change (KD2 vs. HC)	*p*–value (KD2 vs. HC)	Fold–Change (KD2 vs. KD1)	*p*–value (KD2 vs. KD1)
	cg12900151	−1.01145	0.561033	−1.07805	0.004476*	−1.06584	0.000014*
	cg13557530	−1.00724	0.036950*	−1.06435	7.88E-09*	−1.0567	0.000035*
	cg14015211	−1.00305	0.288593	−1.02824	1.15E-07*	−1.02511	4.64E-07*
	cg14228103	1.02911	0.649290	−1.04962	0.242205	−1.08018	0.000703*
	cg17599809	1.00068	0.204633	1.00472	0.012943*	1.00403	0.003094*
CXCR4	cg01150411	1.00506	0.249898	−1.03548	6.45E-09*	−1.04072	0.000001*
	cg01679081	−1.01258	0.003439*	−1.00938	0.131749	1.00317	0.879754
	cg02367708	−1.00685	0.120859	1.05452	0.000005*	1.06175	2.83E-08*
	cg02902079	1.00481	0.005133*	1.01141	0.000008*	1.00658	0.002594*
	cg04513185	1.00501	0.602533	−1.01922	0.001923*	−1.02433	0.002504*
	cg05303524	−1.00251	0.240073	1.01704	0.000026*	1.01959	0.000039*
	cg06679534	−1.00207	0.009328*	1.00728	0.000080*	1.00937	0.000001*
	cg07784959	1.03481	0.001492*	1.00939	0.405797	−1.02519	0.034825*
	cg10020290	−1.01579	0.103705	−1.03207	0.122123	−1.01602	0.904041
	cg10718991	−1.00136	0.204748	1.00433	0.264240	1.0057	0.059655
	cg12311057	−1.00072	0.923893	1.02168	0.000089*	1.02241	0.000095*
	cg12595667	−1.094	0.000188*	1.015	0.213481	1.1104	0.000055*
	cg13075444	1.00835	0.890689	1.01556	0.211114	1.00715	0.179608
	cg14341558	−1.00002	0.891365	−1.00541	0.008769*	−1.00539	0.001941*
	cg15629460	−1.01642	0.002006*	−1.00823	0.223973	1.00812	0.006788*
	cg16675708	−1.00436	0.075055	1.00814	0.012377*	1.01253	0.003664*
	cg17398233	−1.00147	0.261895	1.02067	2.47E-07*	1.02216	0.000010*
	cg18443412	−1.01099	0.023076*	1.00747	0.312259	1.01854	0.009974*
	cg19238531	1.00042	0.509786	1.0132	0.000001*	1.01278	0.000010*
	cg20366284	1.01161	0.002974*	1.02284	0.001200*	1.0111	0.078279
	cg20823742	−1.00807	0.025481*	−1.00004	0.998787	1.00802	0.062154
	cg20824211	−1.00134	0.908299	1.00446	0.114488	1.00581	0.036624*
	cg22376465	−1.00422	0.299725	1.05887	1.43E-08*	1.06334	3.19E-07*
	cg22923827	−1.00025	0.853380	1.02835	0.000002*	1.02861	0.000013*
	cg23374992	−1.12467	0.000058*	−1.03645	0.024458*	1.08512	0.002011*
	cg23885472	1.00005	0.609237	−1.04216	1.55E-12*	−1.04222	3.97E-09*
	cg26191576	−1.02438	0.004549*	−1.02045	0.016970*	1.00385	0.230726
	cg26425669	−1.00038	0.970585	1.01703	0.000070*	1.01742	0.000935*
	cg26548542	−1.00298	0.640367	−1.00034	0.947957	1.00263	0.706639
MS4A6A	cg00673646	−1.028	0.000001*	−1.01275	0.016401*	1.01506	0.020049*
	cg03055440	−1.0639	0.000177*	−1.04838	0.000674*	1.01481	0.107192
	cg04353769	−1.22143	0.000003*	−1.09082	0.003478*	1.11973	0.001009*
	cg05893353	−1.03021	0.000357*	−1.0289	0.000546*	1.00128	0.552353
	cg05995607	−1.13031	2.05E-07*	−1.06293	0.001182*	1.06339	0.000064*
	cg06881914	−1.18836	0.000007*	−1.07923	0.002601*	1.10111	0.002903*
	cg09552517	−1.00065	0.835430	−1.00237	0.102849	−1.00172	0.344976
	cg20284999	−1.00459	0.138725	−1.02688	0.000441*	−1.02219	0.010529*
	cg26250585	−1.23688	0.000002*	−1.08552	0.001559*	1.13943	0.000222*
MS4A4A	cg01229998	−1.03957	0.001109*	−1.03609	0.000101*	1.00336	0.459733
	cg02893799	−1.02222	0.000912*	1.00069	0.920470	1.02292	0.000049*
	cg03841312	−1.02138	0.094573	1.00158	0.758436	1.02299	0.087823
	cg08564601	−1.11027	2.86E-07*	−1.05303	0.000514*	1.05435	0.001913*
	cg12604169	−1.00689	0.179323	−1.09988	1.91E-11*	−1.09235	2.35E-07*
	cg13350775	−1.00083	0.753622	−1.03844	0.000004*	−1.03758	0.000091*
	cg16952963	−1.01145	0.499234	1.02103	0.284685	1.03272	0.019107*

(Continued)

TABLE 2 | Continued

Gene symbol	Target ID	Fold–Change (KD1 vs. HC)	*p*–value (KD1 vs. HC)	Fold–Change (KD2 vs. HC)	*p*–value (KD2 vs. HC)	Fold–Change (KD2 vs. KD1)	*p*–value (KD2 vs. KD1)
	cg18025430	−1.09764	0.000003*	1.00365	0.809193	1.10164	0.000019*
	cg23754934	−1.06136	0.003444*	−1.06635	0.000279*	−1.0047	0.711363
	cg27008678	1.00064	0.759894	−1.09093	0.000001*	−1.09163	0.000002*
CD36	cg05917188	1.02088	0.000404*	−1.07051	3.81E-07*	−1.09287	1.11E-07*
	cg10207609	−1.03718	0.000611*	−1.02136	0.109615	1.01549	0.101425
	cg14093018	−1.00448	0.332432	−1.03382	0.000835*	−1.02921	0.009032*
	cg14479884	−1.06917	0.000012*	−1.05796	0.000423*	1.0106	0.091037
	cg15383705	1.0018	0.585782	−1.01006	0.059004	−1.01188	0.014507*
	cg18433146	−1.00851	0.103165	−1.0955	9.88E-09*	−1.08625	0.000001*
	cg18508525	−1.01533	0.000764*	−1.00922	0.058274	1.00605	0.089320
	cg19096849	−1.0014	0.731414	−1.04094	3.84E-07*	−1.03948	0.000063*
	cg21055948	−1.00666	0.076670	−1.05859	1.56E-10*	−1.05159	3.65E-07*
	cg25783969	−1.00568	0.140078	−1.03079	0.000008*	−1.02497	0.000296*
	cg27625491	1.01064	0.044850*	−1.04251	0.000001*	−1.0536	0.000072*
CXCR1	cg00832199	−1.04132	0.000014*	1.00947	0.122855	1.05118	0.000056*
	cg01218945	−1.00254	0.085168	−1.00439	0.058763	−1.00184	0.467406
	cg06683602	−1.08885	0.000663*	−1.0358	0.128723	1.05122	0.011153*
	cg07016356	−1.01573	0.033727*	1.01907	0.019301*	1.0351	0.000039*
	cg09294937	1.00932	0.044589*	−1.00548	0.452138	−1.01485	0.028151*
	cg13048967	−1.10816	0.000013*	−1.01322	0.247233	1.0937	0.000082*
	cg13519373	−1.04173	0.000103*	1.00969	0.113034	1.05182	0.000027*
	cg14702787	1.00427	0.259337	−1.04983	3.98E-08*	−1.05432	0.000008*
	cg15426604	1.00342	0.680212	−1.00075	0.986117	−1.00418	0.733233
	cg15768138	−1.23706	0.000001*	−1.06298	0.019749*	1.16376	0.000040*
	cg15908708	−1.1178	0.000002*	−1.00354	0.722836	1.11385	0.000035*
	cg18467756	−1.04023	0.000322*	−1.07416	0.000001*	−1.03262	0.000725*
	cg21004129	−1.08522	0.000055*	−1.02151	0.139107	1.06237	0.001292*
FCER2	cg02796568	1.00445	0.295506	−1.00339	0.423123	−1.00785	0.032055*
	cg03221619	1.0228	0.032891*	−1.02625	0.016817*	−1.04964	0.000054*
	cg05489904	1.00099	0.850242	−1.02234	0.023137*	−1.02335	0.011843*
	cg05641903	−1.16318	0.000025*	−1.04813	0.038301*	1.10977	0.002059*
	cg09773499	−1.04717	0.000005*	−1.01668	0.070251	1.02999	0.000100*
	cg10488777	−1.00702	0.015982*	−1.00947	0.039725*	−1.00243	0.705201
	cg12261095	−1.07231	0.000001*	−1.07899	2.80E-08*	−1.00623	0.778167
	cg12387247	−1.04274	0.000491*	−1.0523	0.000635*	−1.00917	0.535602
	cg20234640	−1.00111	0.778083	−1.03487	0.007825*	−1.03372	0.010365*
	cg26040211	1.0026	0.260376	1.00999	0.061587*	1.00737	0.287632

HC, Healthy controls (12 patients), KD1, acute Kawasaki disease 24 h prior to IVIG therapy (12 patients), KD2, resolving Kawasaki disease 21 days after IVIG therapy (12 patients). An asterisk denotes a $p < 0.05$. Data are expressed as mean ± standard error.

selected for the M2 marker TLR5, cg05858079 (**Figure 4**). These results suggest that changes in gene methylation may be one of the many mechanisms governing M1 and M2 polarization in patients with KD.

DISCUSSION

In the first portion of our study we utilized HTA 2.0 microarray analysis to identify M1 and M2 markers with the highest degree of mRNA expression fold-change. Of the 10 M1 markers examined in our study, only two had significantly higher mRNA expression in the acute phase of KD which decreased after IVIG therapy. In contrast, a higher percentage of M2 surface markers, eight out of the 15 surveyed showed significantly increased mRNA expression in the acute phase of KD which decreased after IVIG therapy. Markers with the highest degree of mRNA fold change include the M1 markers TLR2, TLR4, and IL1R1, and the M2 markers TLR5, IL1R2, and ARG1. Expression levels of the six markers with the highest mRNA expression fold changes were then confirmed in a separate cohort of 30 patients with KD and 30 healthy controls by RT-PCR. Of the six selected markers, only IL1R2 and ARG1, both M2 markers were found to have

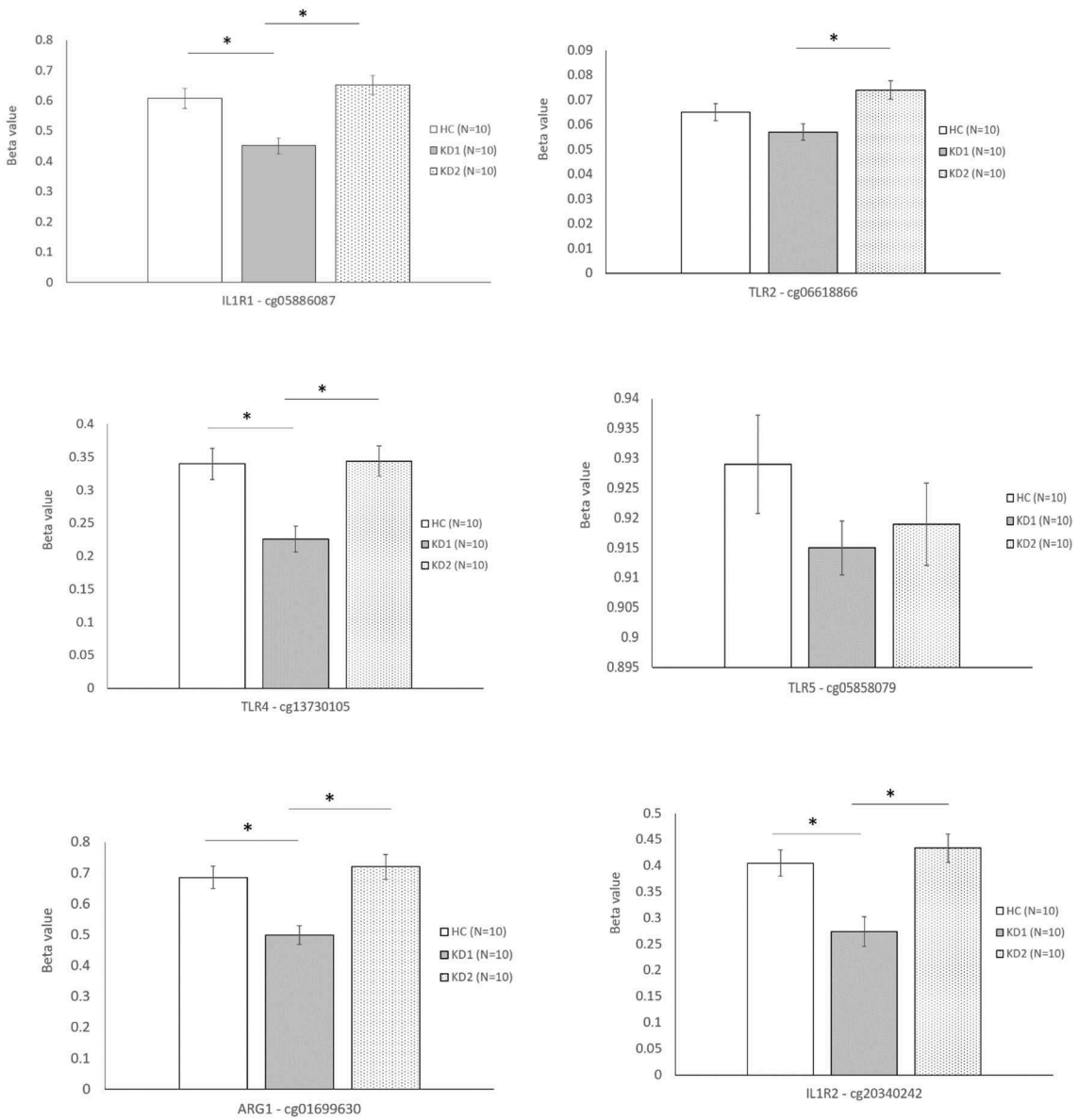

FIGURE 4 | Beta values of selected markers after pyrosequencing. HC, Healthy controls (10 patients); KD1, acute Kawasaki disease 24 h prior to IVIG therapy (10 patients); KD2, resolving Kawasaki disease 21 days after IVIG therapy (10 patients). An asterisk denotes a $p < 0.05$. Data are expressed as mean ± standard error.

elevated mRNA expression levels in the acute phase of KD which decreased after IVIG therapy, although these results were not statistically significant.

Because CpG site hypomethylation is one of the many mechanisms which regulate gene expression, we then examined the methylation data of corresponding M1 and M2 marker gene CpG sites included on M450K array. Six CpG sites with the highest degree of hypomethylation fold-change corresponding to the three M1 (IL1R1, TLR2, TLR4) and three M2 markers (IL1R2, TLR5, ARG1) with the highest mRNA expression fold change on HTA 2.0 were selected for confirmation. Pyrosequencing of the six CpG sites selected was performed in a separate cohort of 10 KD patients and 10 healthy controls. Four of the CpG sites selected corresponding to the M1 markers IL1R1 (cg05886087), TLR4 (cg13730105), and the M2 markers IL1R2 (cg20340242) and ARG1 (cg01699630) were significantly hypomethylated in the acute KD group and were then hypermethylated after IVIG therapy, which mirrored the increased mRNA expression of these genes on HTA 2.0 microarray. This suggests that CpG site methylation may be one of the many mechanisms which regulate M1 and M2 macrophage polarization in KD.

In our previous research, we have found that both Th-1 and Th2 subset activation are involved in the acute phase of KD. Th1 associated cytokines including IL-2 IL-6, TNF-α, and IFN-γ were significantly increased in the acute phase of KD and

decrease after IVIG therapy (21–23). In addition, lower levels of TNF-α prior to IVIG therapy was associated with a lower risk of IVIG resistance, and higher levels of IL-6 levels after IVIG therapy was associated with a higher risk of coronary artery lesions (21). Similarly, Th2 associated cytokines including IL-4, IL-5, and IL-10 are elevated in the acute phase of KD (21, 23, 24), although patients with higher IL-5 and eosinophil counts after IVIG therapy were less likely to develop coronary artery lesions (24). The results of these studies suggest that while both Th1 and Th2 activity are associated with the acute phase of KD, higher levels of Th1 activity may increase the risk of coronary artery lesions, while increased Th2 activity may protect against them. Because M1 and M2 macrophage activation increases Th1 and Th2 immunity respectively, we initially hypothesized that both M1 and M2 macrophage activity would increase in the acute phase of KD.

Monocyte derived macrophages can be to activated into either M1 or M2 macrophages according to the tissue microenvironment. As mentioned earlier, stimuli such as IFN-γ, TNF-α or LPS promotes macrophage polarization toward the M1 phenotype, and IL-13 and IL-4 promotes macrophage polarization into the M2 phenotype. In addition, there appears to be plasticity among the M1/M2 phenotypes; once a macrophage is activated into the M1 phenotype, it can still be repolarized into the M2 phenotype according to dynamic changes in the tissue microenvironment, and vice versa (25). In this study we also found that although both M1 and M2 markers are elevated in the acute phase of KD, a higher percentage of M2 markers were elevated.

Both M1 and M2 marker mRNA expression decreased after IVIG therapy. Although there are no other studies to date that specifically examine M1 and M2 macrophage activation in KD, one study of 28 patients with KD showed that CD14+ CD16+ monocytes were elevated in the acute phase of KD (26). CD14+ and CD16+ monocytes are considered to be "intermediate-type" monocytes which can differentiate into both M1 and M2 macrophages, and are highly associated with IL-10 production (27). Likewise, both M1 and M2 macrophages appear to be implicated in the development of other autoimmune vasculitis. As an example, in a murine model of systemic lupus erythematosus (SLE), M1 macrophages are recruited to the kidney early after kidney injury and create a pro-inflammatory environment that aids with the clearance of damaged or apoptotic cells (28). In addition, M2b macrophages, an immune-regulatory M2 macrophage subtype, can be induced by incompletely phagocytosed immune complexes in lupus patients, and are associated with the development of lupus in both mouse models and in patients with SLE glomerulonephritis (29). Although the most direct way of examining macrophage polarization would be to perform immunohistochemical staining of the coronary arteries, specimens can only be obtained from patients who have died of KD. Therefore, we can only hypothesize that both M1 and M2 macrophages are activated in the acute phase of KD. It is possible that M2 macrophages, due to their role in tissue healing and repair are responsible for the resolution of KD, although further functional studies are required.

Of the 10 M1 macrophage markers and 15 M2 macrophage markers examined in this study, we found that the M1 markers TLR2, TLR4, and IL1R1 and the M2 markers TLR5, IL1R2, and ARG1 had the highest levels of mRNA expression in the acute phase of KD. Previous research has found that toll-like receptors, particularly toll-like receptor 2 (TLR2) is associated with the development of KD. In human patients with KD, increased expression of TLR2 positive CD14 monocytes occurs in the acute phase of KD (22), and is down regulated after IVIG therapy (30), and can successfully predict risk of coronary artery lesions and IVIG resistance (31). In mouse models of KD, mice which were TLR2 or MyD88 deficient failed to develop KD after induction by Lactobacillus casei cell-wall extract (LCCWE), suggesting that TLR2 signaling through the MyD88 pathway is crucial for the development of KD (32). In addition to TLR2, we also found that TLR4 and TLR5 had high levels of mRNA expression in the acute phase of KD. TLR2, TLR4, and TLR5 are capable of recognizing pathogen associated molecular patterns (PAMPs) including lipoteichoic acid, lipopolysaccharides, and flaggellin on bacteria and aids the phagocytosis of pathogens and further signal transduction and amplification of the immune response through the MyD88 pathway (33). These results lend credence to the hypothesis that bacterial infections may pose a possible trigger for the development of KD (34).

Both IL1R1 and IL1R2 belong to the interleukin-1 receptor family. IL1R1 which is expressed on M1 macrophages, acts as a receptor of the cytokines IL-1a, IL-1b, and IL-1 receptor antagonist (IL-1RA). Conversely, IL1R2 which is expressed on M2 macrophages, acts as a decoy receptor for IL-1a, IL-1b, and IL-1RA thereby inhibiting the further signal transduction of these cytokines (35). The IL-1 pathway is a crucial part of the development of KD, as evidenced by one study which examined the whole blood transcriptional profiles of 146 subjects with KD, and found that all of the top five pathways upregulated in KD included IL-1 signaling molecules such as IL1R1 and IL1R2 (36). IL-1α and IL-1β signaling is also essential to the development of a mouse model of KD (37), and treatment with IL-R antagonist (Anakinra) has been shown to inhibit arterial aneurysm formations in mouse models (38).

Furthermore, we found that the M2 marker ARG1 showed significant increase of mRNA expression in the acute phase of KD. ARG1 diverts arginine away from the inflammatory iNOS (inducible nitric oxide synthase) pathway, thereby modulating inflammatory responses (39). Although there have been no studies which specifically examine ARG1 expression in KD, there have been many studies that show a link between enhanced iNOS expression in the acute phase of KD and the development of coronary artery disease (40, 41) perhaps suggesting that increased ARG1 expression in acute KD may be associated with protection against coronary artery lesions.

Finally, we found that four CpG sites corresponding to the M1 markers IL1R1 (cg05886087), TLR4 (cg13730105), and the M2 markers IL1R2 (cg20340242) and ARG1 (cg01699630) were significantly hypomethylated in the acute KD group and were then hypermethylated after IVIG therapy. Previous studies have found that epigenetic regulation of gene expression particularly through DNA methyltransferases (DNMT) may

be one of the ways which regulate M1 and M2 macrophage polarization. DNMTs are enzymes that bind methyl groups to DNA strands, and include DNMT1, which maintains DNA methylation by adding methyl groups to hemimethylated strands of DNA during replication; and DNMT3a and DNMT3b, which add methyl groups to previously unmethylated DNA (i.e., *de novo* methylation). There is evidence that increased expression of DNMT1 promotes polarization toward the M1 macrophage phenotype possibly through regulation of the PPAR-γ pathway (42). In a mouse model of obesity, dietary supplementations of saturated fatty acids significantly increased DNMT1 expression and macrophages and increased methylation of the promoter region of PPAR-γ. This led to a decrease in PPAR-γ expression, a transcriptional factor that promotes macrophage polarization toward the alternatively activated M2 phenotype, therefore suppressing M2 macrophage polarization. These findings were corroborated in another study, where macrophage specific overexpression of DNMT1 in a transgenic mouse model of atherosclerosis led to decreased PPAR-γ expression and increased inflammatory macrophage activation and increased atherosclerosis. The researchers also found that DNMT1 expression was negatively correlated to PPAR-γ expression in the peripheral monocytes of human subjects with atherosclerosis (43, 44). Conversely, M2 macrophages have higher levels of expression of DNMT3a and DNMT3a1 which may be associated with AMP-activated protein kinase (AMPK) signaling (42, 45).

Because DNA and RNA samples were extracted from whole white blood cells (including neutrophils, lymphocytes and monocytes), the heterogeneity of cells used is one of the limitations of our study. In our study we found that acute Kawasaki disease (KD1) was associated with a higher percentage of neutrophils (KD1 vs. HC 55.28 ± 2.79% vs. 31.05 ± 1.95%), and a lower percentage of lymphocytes (KD1 vs. HC 33.62 ± 2.56% vs. 59.75 ± 2.14%) when compared to the healthy controls. The ratios of neutrophils and lymphocytes normalized and were comparable to healthy controls 21 days after IVIG therapy (KD2), and the percentage of monocytes was similar in all three groups (HC 5.6 ± 0.50%, KD1 5.8 ± 0.46%, KD2 4.8 ± 0.37%). In our study we found that a much higher percentage of M2 related markers showed increased mRNA expression in the acute phase of KD, but decreased after IVIG therapy, particularly IL1R2, TLR5, and ARG1. While TLR5 is also expressed on neutrophils, it is more highly expressed in monocytes (46). Likewise, while IL1R2 is also expressed on B cells and T cells, it is more densely expressed on monocytes (47, 48).

In conclusion, this is the first study to specifically examine the role of M1 and M2 macrophage polarization in patients with KD. We found that both M1 and M2 markers showed increased expression in the acute phase of KD which decreased after IVIG therapy, with the M1 markers TLR2, TLR4, and IL1R1 and the M2 markers TLR5, IL1R2, and ARG1 showing the highest levels of mRNA expression. We also found that the corresponding promoter CpG sites of these markers were hypomethylated in the acute phase of KD, and hypermethylated after IVIG therapy, which suggest that CpG site methylation may be one of the mechanisms governing macrophage polarization in KD. Further functional studies are needed to confirm the role of macrophage polarization in the development of KD, which may prove to be a novel therapeutic target in the future.

DATA AVAILABILITY STATEMENT

The data analyzed in this study can be found in https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109430 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109351.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Chang Gung Medical Foundation Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MG participated in the study design, analyzed data, and wrote the main manuscript. L-SC, Y-HH, and F-SW participated in data collection and analysis. H-CK was responsible for study designs and edited the main manuscript. All authors have reviewed the manuscript prior to publication.

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frontiers in Pediatrics

CASE REPORT
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A Teenager With Rash and Fever: Juvenile Systemic Lupus Erythematosus or Kawasaki Disease?

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Rationale: Kawasaki disease (KD) is an acute vasculitis of small and medium vessels; whereas systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease. Their presentation is varied and not always straightforward, leading to misdiagnosis. There have been case reports of lupus onset mimicking KD and KD presenting as lupus-like. Coexistence of both diseases is also possible.

Patient concerns: We present three adolescents, one with fever, rash, arthritis, nephritis, lymphopenia, and coronary aneurysms, a second patient with rash, fever, aseptic meningitis, and seizures, and a third patient with fever, rash, and pleural effusion.

Diagnoses: The first patient was finally diagnosed with SLE and KD, the second patient initially diagnosed as KD but eventually SLE and the third patient was diagnosed at onset as lupus but finally diagnosed as KD.

Interventions: The first patient was treated with IVIG, corticosteroids, aspirin, coumadin and mycophenolate mofetil. The second patient was treated with IVIG, corticosteroids and methotrexate and the third patient with IVIG, aspirin and corticosteroids.

Lessons: Both diseases may mimic each other's clinical presentation. KD in adolescence presents with atypical signs, incomplete presentation, and develop coronary complications more commonly. An adolescent with fever and rash should include KD and SLE in the differential diagnosis.

Keywords: Kawasaki disease, juvenile systemic lupus erythematosus, intravenous immunoglobulins, adolescent, atypical Kawasaki disease

INTRODUCTION

Kawasaki disease (KD) and systemic lupus erythematosus (SLE) are immune mediated diseases characterized by varied clinical features that may include vasculitis (1–3). Vasculitis in lupus is most commonly due to the local deposition of immune complexes, but some patients have an inflammatory vasculopathy in the absence of local immune complex deposition (3). SLE can present coronary arteritis with aneurysm formation (4). We present three patients with overlapping

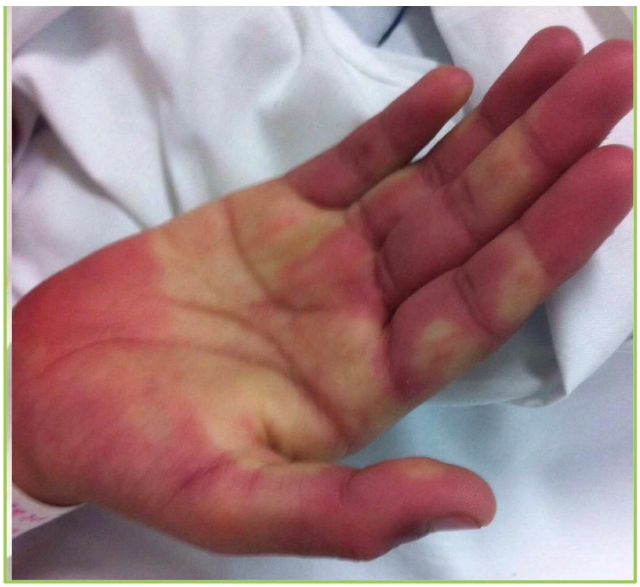

FIGURE 1 | Erythema in palms accompanied by intense Raynaud's phenomenon.

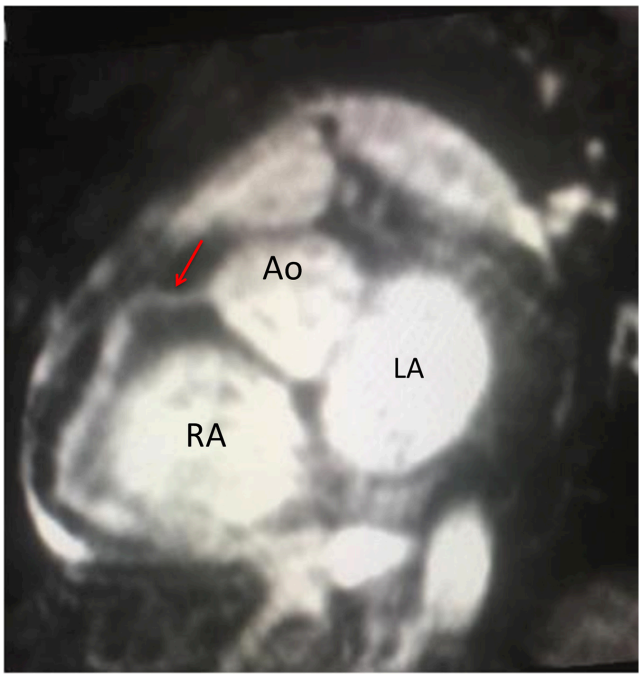

FIGURE 2 | Magnetic resonance coronary angiography in a Whole-Heart iPAT sequence in a short axis view. Red Arrow: normal proximal right coronary artery 3 mm (z-score + 054), with dilated mid right coronary artery 6 mm (z-score + 7.35) and dilated distal right coronary artery 6 mm (z-score + 8.07). Ao, aorta; RA, Right atrium; LA, Left atrium (Courtesy of Dr. Roberto Cano).

features of KD and SLE. All patients and/or parents provided informed consent for publication of the cases.

Case 1

A 16-year-old-male presented with a history of fever, weakness, headache with photophobia, abdominal pain, vomiting, and axillar lymphadenopathy. On physical examination he had persistent fever, conjunctival injection, malar erythema, erythematous and cracked lips, bilateral parotid enlargement, cervical lymphadenopathy and a diffuse photosensitive rash. Sicca symptoms were not present. KD was diagnosed, and intravenous immunoglobulins were started at 2 g/kg in addition to aspirin. The echocardiogram was within normal limits. After treatment, he was afebrile for 24 h, after which he presented seizures and neurological deterioration. Cranial computed tomography revealed changes suggestive of aseptic meningitis. A skin biopsy demonstrated an atrophic epidermis, necrotic keratinocytes, hydropic degeneration of the basal layer, basal membrane thickening and periadnexal and perivascular lymphocytic infiltration. Anti-Ro and IgM anti-β2-glycoprotein-1 antibodies were positive, antinuclear antibodies were negative. The diagnosis of systemic lupus erythematosus was made based on the presence of seizures, malar rash, photosensitivity and, positive anti-β2-glycoprotein-1 and anti-Ro antibodies. Corticosteroids, hydroxychloroquine, and methotrexate were started. He has been followed for more than 2 years, and the corticosteroids have been tapered with good evolution.

Abbreviations: KD, Kawasaki disease; SLE, systemic lupus erythematosus; IVIG, intravenous immunoglobulins; AST-Aspartate aminotransferase, ALT-Alanine transaminase.

TABLE 1 | Kawasaki disease classification criteria (AHA 2017 Guidelines).

Fever for at least 5 days in the presence of ≥ principal clinical features
Erythema and cracking of lips, strawberry tongue, and/or erythema of oral and pharingeal mucosa.
Bilateral bulbar conjunctival injection.
Erythema and edema of hands and feet in acute phase and/or peringueal desquamation in subacute phase.
Cervical lymphadenopathy ≥ 1.5 cm diameter.
A careful history may reveal that ≥1 principal clinical features were present during the illness but resolved by the time of presentation. Patients who lack full clinical features of classic KD are often evaluated for incomplete KD. If coronary artery abnormalities are detected, the diagnosis of KD is considered confirmed in most cases.
Other clinical findings: myocarditis, pericarditis, valvular regurgitation, gallbladder hydrops, aseptic meningitis, desquamating rash in the groin, anterior uveítis, erythema at the BCG inoculation site.

Case 2

A 12-year-old male was transferred to our hospital with the diagnosis of lupus. He had a history of 20 days of fever, arthralgias, alopecia, a thoracic and abdominal rash, vomiting, oral ulcers, pleural effusion, pancytopenia, lymphopenia, and positive antinuclear antibodies (1:320). On physical examination the patient had palmoplantar erythema with desquamation and perineal erythema. Work-up showed a complete blood cell count within normal limits,

TABLE 2 | Definitions of SLE classification criteria.

ACR 1997	SLICC 2012	EULAR/ACR 2019
	Clinical criteria	
1. Malar rash	1. Acute cutaneous lupus	1. Acute cutaneous lupus (malar rash or generalized maculopapular rash observed by a clinician)
2. Discoid rash	2. Chronic cutaneous lupus	2. Subacute cutaneous or discoid lupus
3. Photosensitivity		3. Fever
4. Oral or nasal ulcerations	3. Oral or nasopharyngeal ulcerations	4. Oral ulcers
	4. Nonscarring alopecia	5. Nonscarring alopecia
5. Nonerosive arthritis: Involving two or more joints, characterized by tenderness, swelling or effusion	5. Synovitis involving two or more joints	6. Joint involvement
6. Pleuritis or pericarditis	6. Serositis	7. Acute pericarditis
		8. Pleural or pericardial effusion
7. Renal disorders: persistent proteinuria or cellular casts	7. Renal disorders	9. Proteinuria >0.5 g/24 h
		10. Class II or V lupus nephritis on renal biopsy according to ISN/RPS 2003 classification
		11. Class III or IV lupus nephritis on renal biopsy according to international Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003
8. Neurologic disorder: seizures or psychosis	8. Neurologic disorder	12. Delirium
		13. Psychosis
		14. Seizure
9. Hematologic disorders: • Hemolytic anemia or • Leukopenia or • Lymphopenia or • Thrombocytopenia	9. Hemolytic anemia	15. Leucopenia 16. Thrombocytopenia 17. Autoimmune hemolysis
	10. Leucopenia or lymphopenia	
10. Immunologic disorder: 1. anti-DNA antibody to native DNA or 2. anti-Sm antibody or 3. Positive antiphospholipid antibodies: 1) IgG or IgM anticardiolipin 2) positive lupus anticoagulant (LA) or 3) false positive test for syphilis	11. Thrombocytopenia	18. Antinuclear antibodies (ANA)
11. Positive antinuclear antibody by IFT or an equivalent assay	*Immunological criteria*	19. Low C3 OR low C4
	1. ANA level above laboratory reference range	
	2. Anti-dsANA antibody level above laboratory reference range	20. Low C3 AND low C4
	3. Anti-Sm antibody	
	4. Antiphospholipid antibody positive, by any of the following: -medium or high titer anti-cardiolipin -positive test for anti-beta-2glycoprotein	21. Anti-dsDNA antibodies OR anti-Smith (Sm) antibodies
	5. Low complement	
	6. Direct Coombs test in the absence of hemolytic anemia	22. Positive antiphospholipid antibodies

(Continued)

increased AST (70 UI/l), ALT (59 UI/l) and bilirrubin (total 8.1 mg/dl, direct 5.5 mg/dl), and negative anti-dsDNA and anti-Sm antibodies. The echocardiogram revealed cardiomegaly and pericardial effusion. The presence of fever, palmoplantar erythema with desquamation, perineal erythema, elevated transaminases, gallbladder hydrops and pericardial effusion led to a diagnosis of incomplete KD and intravenous immunoglobulins, aspirin and corticosteroids were administered. On follow up, cardiac and liver abnormalities resolved.

Case 3

An 11 year-old-female presented with a history of cervical adenopathy, followed 2 months later by left knee arthritis, malar rash, photosensitivity, dark urine and fever. On physical examination malar rash and intense Raynaud's phenomenon were noted (**Figure 1**). The diagnosis of lupus was made based on acute cutaneous lupus - malar erythema and photosensitivity -, arthritis, renal disease - cylindruria and proteinuria -, autoimmune hemolytic anemia, lymphopenia, positive antinuclear antibodies and anti-dsDNA antibodies. During her hospitalization fever continued and she presented erythematous crusted lips and a generalized rash with palmoplantar erythema. Intravenous immunoglobulins were administered with a presumptive diagnosis of Parvovirus-B19 infection. Methylprednisolone pulses were started, and improvement was observed. She was discharged with hydroxycloroquine, prednisone and mycophenolate mofetil. She presented periungueal desquamation while at home. One month later, she was readmitted to the hospital due to headache, seizures and persistent hypertension. Echocardiogram and heart MRI revealed large ectasia of the main left coronary artery (z-score + 6.12), large ectasia of the circumflex artery (z-score + 5.19), with normal proximal right coronary artery and large ectasia of the mid right coronary artery (z-score + 7.35) with mild mitral regurgitation (**Figure 2**).

TABLE 3 | Cases with overlapping features of KD and SLE.

References	Gender	Age	KD	SLE		Treatment	Final diagnosis
Laxer et al. (5)	Female	10 m-5 yo	Fever (7 days), pruritic erythematous maculopapular rash, erythema of the palms and soles, bilateral noneudative conjunctivitis, rige posterior cervical lymph node, dry fissured lips, edema of her hands and feet., peeling of the skin over her fingers and toes	3 years later Fever, anorexia, photosensitivity, facial rash, livedo reticularis, painless palatal ulcer, generalized lymphadenopathy	Hemoglobin 8.3 g/dl, Leukocytes 4,000, ANA 1:640, Anti DNA positive, Rheumatoid Factor 40 UI (+). C3, C4 Markedly reduced. Immune Complexes 1,350 mcg/ml. Urianalysis: proteinuria and hematuria.	Aspirin 75 mg for 8 weeks. 3 years later. PDN 2 mg kg day.	KD and SLE
Marchetto et al. (7)	Male	15 yo	Fever, cheilitis, strawberry tongue, bilateral non exudative conjunctivitis with hemorrhages in the left eye and diffuse maculopapular rash, hands and feet with periungueal digital peeling	Butterfly rash on his face, arthralgia, muscle weakness, headache	ANA, antineurtrophil cytoplasmatic antibody, anti-DNA were negative. Positive anticardiolipin autoantibodies.	IVIG and acetyl salicylic acid. Recurrent KD Methylprednisolone an a second cycle of IVIG	KD
Diniz et al. (6)	Female	13 yo	Fever (7 days), bilateral bulbar nonexudative conjunctivitis, erythema of the oral an pharyngeal mucosa, cervical lymphadenopathy (2cc), erythema of Palms an diffuse maculopapular rash	Irritability, myalgia and arthritis (edema and tenderness in elbows and proximal interphalangeal joints in both hands an ankles),	Hemoglobin 9.7 gr/dl Urianalysis: Proteinuria 0.57 g/24 h. Leukocytes 3,000, Erythrocytes 1,000 Positive ANA 1:320, anti-dsDNA 516, anti-Ro. Negative anticardiolipin C3 42, C4 5	IVIG (2 g/kg do), and aspirin 80 mg/kg day Three pulses of intravenous methylprednisolone. PDN 30 mg/d Chloroquine Diphosphate, Azathioprine, aspirin 100 mg/d.	KD and SLE
Diniz et al. (6)	Female	4 yo	Fever (12 days), bilateral bulbar nonexudative conjunctivitis, cheilitis and strawberry tongue, cervidal lymphadenopathy (1.5cc), erythema of Palms, diffuse maculopapular rash, desquamation of the fingers and toes and in periungual region.	1 year later Irritability, Acute swelling of the eyelids, hands and feet, hypertension and pericarditis	Hemoglobin 7.4 g/dl, Leukocytes 3,800, Lymphocytes 874 Urinalysis: Leukocyturia Erythrocyturia Proteinuria g/24 h, C3 71 C4, <010 ANA 1:320 Anti-dsDNA 654.	IVIG (2 g/kgdo), and aspirin 80 mg/kg day 1 year later Three pulses of Intravenous methylprednisolone Cyclophosphamide Chloroquine Diphosphate	KD and SLE
Agarwal et al. (8)	Female	9 yo	Fever (Intermittent) Bilateral conjunctival erythema ECHO mild dilatation of the LMCA, and diffuse ectasia of the LAD, mild mitral regurgitation suggestive of carditis.	Abdominal pain arthralgias (ankles, wrists, right knee) weakness of lower extremities aphtous ulcer under the tongue	Hemoglobin 11.3 g/dL Leukocytes 3,100 ANA 1;2560 Positive Coombs Anti- dsDNA >200	Ethosuximide (discontinued) Intravenous Methylrednisolone pulse therapy (30 mg/kg day) for 3 days. Oral Steroids Methotrexate Hydroxychloroquine Aspirin (81 mg/day)	SLE

(Continued)

TABLE 3 | Continued

References	Gender	Age	KD	SLE		Treatment	Final diagnosis
Agarwal et al. (8)	Female	6 yo	Fever Conjunctivitis non- exudative Cervical Adenopathy Rash 2 days later Recurrence of fever 2 day later Recurrence of Fever Sandpaper-like rash Cervical Lymphadenopathy ECHO dilated LMCA	Arthralgias (Ankle and Knee) Abdominal PAIN 4 days later Sinovitis of her wrists and knees.	Hemoglobin 9 g/dL ANA 1:640 Myeloperoxidase antibodies 28 mg/dL. 4 days later Hemoglobin 9.7 g/dL Platelet Count 530 k/ml Low C3 complement 64 mg/dL. Normal C4 complement ANA 1:2560 Myeloperoxidas and proteinase 3 antibodies negative. Antibodies-DSdna >200 Positive Combs Positive ENA-RNP	Intravenous Gammaglobulin 2 g/kg Aspirin 2 days later Intravenous Gammaglobulin 2 g/kg Aspirin 2 day later Intravenous Methylrednisolone pulse therapy (30 mg/kg day) for 3 days. Oral Steroids Hydroxicloroquine Aspirin 81 mg (daily) Methotrexate	SLE
Agarwal et al. (8)	Male (Family history for Lupus and Sarcoidosis)	13 yo	Eczema Fever (intermittent) Pruritic Rash Chill Bilaterally Injected Sclera Cervical Lymphadenopathy Bullous pemphigoid rash to the extremities Non pitting edema of lower extremities ECHO showed dilatation of the LMCA, LAD, and RCA without pericardial effusion, mild tricúspide insufficiency.	Joint pains Swelling of his hands and feet Palatal ulcers Synovitis of the small joints (hands, elbows, and knees)	Hemoglobin 4.9 g/dL. ANA 1:1280 Positive Coombs Antibodies-dsDNA >200 Positive anticardiolipin IgM, anti-Sm, anti-RNP, anti-SSA and SSB, β2-glycoprotein-1 antibodies. C3 20 mg/dL C4 < 2 mg/dL	Intravenous methylprednisolone pulse therapy (30 mg/kg day) for 3 days. Rituximab (750 mg/m^2) on day 3 of steroid pulse, and a second dose given 2 weeks after Oral PDN Oral Enalapril Hydroxychloroquine Aspirin 81 mg/day 2 months later Mofetil mycophelolate	SLE
Argarwal et al. (8)	Female (Family history was notable for mother deceased due to complications of Rheumatoid Arthritis, SLE, Sjogren's syndrome, and dialysis'dependent end'stage renal disease).	13 yo	Fever Raynaud's phenomenon Bilateral pruritic red rash on her lower extremities Periorbital Edema ECHO demonstrated dilatation of the LMCA, LAD, and RCA, with perivascular echogenic brightness around the coronary branches. Borderline Leith ventricular hypertrophy and small circumferential pericardial effusion.	Headaches, swelling of both legs, bilateral synovitis of the elbows	Hemoglobin 6 g/dL BUN 33 mg/dL Cr 1.67 mg/dL Urinalysis hematuria an proteinuria >300 mg/dL. ANA 1:2560 Positive Coombs Antibodies-dsDNA >200 Positive RNP Positive anti-Sm antiRo antibodies. C3 17 mg/dL C4 2 mg/dL.	Intravenous Methylrednisolone pulse therapy (2 mg/kg day) for 3 days. Oral enalapril. Oral PDN Furosemide Hydroxychloroquine Mofetil mycophenolate	SLE

(Continued)

TABLE 3 | Continued

References	Gender	Age	KD	SLE		Treatment	Final diagnosis
Zhang et al. (9)	Male	13 yo	Fever, rash, non-exudative conjunctivitis, cervical lymphadenopathy, arthralgia. ECHO showed coronary artery dilation (LCA 5.4 mm, RCA 6.9 mm)	Erythema, hepatosplenomegaly	Positive ANA and dsDNA antibodies. Hypocomplementemia. Positive Coombs. Leukopenia.	Intravenous methylprednisolone.	SLE (and KD?)
Case 1	Male	16 yo	Fever (1 month), painful cervical lymph nodes, rash on the trunk and extremities, conjunctival injection, cracked lips, oral mucosa erythematous	Malar erythema, Seizures and deterioration of neurological, Aseptic meningitis	Positive.β2-Anti- Glycoprotein-1 IgM type 44.02. anti Ro (+) antibodies.	IVIG (2 g/kgdo), and aspirin 80 mg/kg day later Methotrexate Hydroxychloroquine 400 mg/day. PDN 10 mg/day. Acenocumarine 2 mg/day	SLE
Case 2	Male	12 yo	Fever Palmoplantar erythema, desquamation hands and feet Perineal erythema, Gallbladder hydrops	Pleural and pericardial effusions, oral ulcers	Pancytopenia, Positive ANA	Methylprednisolone pulses IVIG	KD
Case 3	Female	11 yo	Fever, generalized rash, cervical lymphadenopathy, palmoplantar erythema, erythematous lips, desquamation hands	Malar rash, Raynaud's phenomenon, livedo reticularis	Positive ANA, anti-dsDNA, anti-Ro, anti-β2-glycoprotein-1, proteinuria Coombs positive hemolytic anemia	Methylprednisolone pulses IVIG Mofetil mycophenolate	SLE and KD

IVIG, intravenous immunoglobulins; PDN, Prednisone; ANA, antinuclear antibodies.
ECHO, LMCA, Left main coronary artery; LAD, proximal left Anterior descending coronary artery; RCA, proximal right coronary arteria.

DISCUSSION

Clinical criteria are used to diagnose KD with the presence of fever and principal clinical features involving the mouth, eyes, skin, hands and feet and cervical lymphadenopathy (**Table 1**). SLE is a complex autoimmune disease with variable clinical features. In the absence of SLE diagnostic criteria, SLE classification criteria are often used by clinicians to help identify some of the salient clinical features when making the diagnosis. Children who fulfill the ACR criteria, SLICC criteria or the new EULAR/ACR criteria are considered to have definitive SLE (**Table 2**). Of note is that in the recent EULAR/ACR criteria, fever is considered a criterion suggestive of SLE.

KD and SLE share several clinical manifestations: both diseases can present with fever, lymphadenopathy, arthritis or arthralgia, ocular and mucosal manifestations, rash and multisystemic involvement. However, the coexistence of both or misdiagnosis among them has seldom been reported (5–9). There are two previously reported cases of lupus-onset mimicking Kawasaki disease and vice versa and another three reported cases of the coexistence of both diseases (5–9) (**Table 3**).

The first patient was diagnosed with SLE and KD in an almost concurrent presentation, since she presented diagnostic criteria for both diseases. It can be discussed whether this case could only correspond to lupus with carditis, as the ones reported by Agarwal et al., however it is important to note that none of the four patients described by this author completed diagnostic criteria for KD (8). Recently, Zhang et al. (9) report a 13 year-old male who presented fever, rash, non-exudative conjunctivitis with cervical lymphadenopathy and an echocardiogram presenting coronary artery dilation. He was eventually diagnosed as SLE since he presented autoimmune hemolytic anemia, positive ANA, dsDNA and hypocomplementemia (9). As can be seen from previous reports (**Table 3**), both diseases can present simultaneously or with years of difference (5, 6, 9).

Coronary arteritis is not an exclusive feature of KD as other diseases like lupus and other vasculitis present this complication. In fact, coronary artery lesions have been documented in asymptomatic patients with microscopic polyangiitis, polyarteritis nodosa, and Wegener granulomatosis with MRI (10). Children with systemic onset juvenile idiopathic arthritis may present coronary artery dilation on echocardiograms similar to that observed for children with KD (11).

In our second patient the initial clinical picture made KD a diagnostic possibility; the skin biopsy was useful, as features were unequivocal for lupus. Parotitis was an unusual manifestation and can be present in both KD and lupus (12, 13). The third case was initially diagnosed as SLE, but eventually the clinical picture - despite atypical features such as pleural effusion, the response to treatment and the current health status under no medication, are more compatible with atypical KD (14).

Both KD and SLE share common features in terms of mechanisms of vascular inflammation and both may present with coronary artery dilatation. The two of them have been associated with the presence of anti-peroxiredoxin antibodies and the elevation of IL-17 (15, 16).

At this point, with the previously reported cases and our own it can be said that both diseases may mimic each other's clinical presentation. Interestingly, the majority of the patients that often present with the clinical challenge were tweens and teenagers (an unusual age for KD). KD in adolescence presents with atypical signs, incomplete presentation, and develop coronary complications more commonly (17). An adolescent with fever and rash should include KD and SLE in the differential diagnosis. As always in medicine, an accurate diagnosis is necessary to give appropriate treatment and reduce complications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Signed informed consent was obtained from the parents and the patients.

AUTHOR CONTRIBUTIONS

MY-N, MS, and MP-H conceptualized and designed the study, reviewed, and revised the manuscript. MS and MY-N carried out the initial analyses and drafted the initial manuscript. FR-L and MG-G critically reviewed the manuscript. MP-H, EV-M, and MG-D recollected the data. All the authors were responsible for the treatment of the patient and read and approved the final manuscript.

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Risk Factors of Intravenous Immunoglobulin Resistance in Children With Kawasaki Disease: A Meta-Analysis of Case-Control Studies

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Previous studies have shown that children with Kawasaki disease (KD) who fail to respond to intravenous immunoglobulin (IVIG) therapy are at higher risk of developing coronary artery lesions (CALs). We aimed to conduct a meta-analysis to uncover the risk factors associated with IVIG resistance in children with KD. PubMed, Embase, and Cochrane Library databases were searched up to 31st October 2019, and 23 case-control studies were finally eligible, enrolling 2,053 patients of IVIG resistance and 16,635 patients of IVIG sensitivity. Potential factors were comprehensively analyzed by using stata15 software with a standard meta-analysis procedure and consequently found that in addition to patients with polymorphous rash or swelling of extremities symptoms had a tendency to be non-responders, IVIG resistance was more likely to occur in patients with severe anemia, hypoalbuminemia, decreased baseline platelet count, and elevated levels of erythrocyte sedimentation rate (ESR), total bilirubin, alanine aminotransferase (ALT) and neutrophils percentage. Particularly, male sex, hyponatraemia, increased aspartate aminotransferase (AST), and C-reactive protein (CRP) were confirmed as the risk factors favor IVIG resistance in Mongoloids from Asia countries, but not in Caucasians from non-Asia regions. In summary, we report several risk factors relevant to IVIG resistance in children with KD, which may provide guidance for the prediction of IVIG resistance. But a proposing of an optimal prediction system with high specificity and sensitivity needs further studies because of confounding factors.

Keywords: Kawasaki disease, intravenous immunoglobulin resistance, risk factors, meta-analysis, children

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INTRODUCTION

Kawasaki disease (KD) is an acute medium-sized vasculitis of unknown etiology, characterized by persistent fever and five typical clinical manifestations, including swelling of extremities, polymorphous rash, cervical lymphadenopathy, oral lesions, and bilateral conjunctivitis (1). It predominantly affects children younger than 5 years old and results in coronary artery lesions (CALs) such as ectasias or aneurysms in 25% of untreated patients (2), considering one of the most common cause of acquired heart disease in children in

many countries (3). Timely treatment with intravenous immunoglobulin (IVIG) and oral aspirin has reduced the prevalence of CALs from 25% to about 4% (4). However, 10%–20% of patients with KD fail to respond or develop recrudescent fever 36–48 h after the first dose of IVIG (5), which are termed as IVIG resistance. Previous studies have shown that resistant patients are at higher risk of developing CALs (5, 6), and addition use of glucocorticoid or cyclosporine have been confirmed effectively reduce the incidence of CALs in children predicted with IVIG resistance before treatment according to different randomized clinical trails (7, 8). Therefore, it is of importance to predict targeted patients who will be IVIG non-responders so that they can benefit from the more aggressive therapy regarding CALs prevention.

The pathological development of KD is a systemic inflammatory process, during which the severity degree of inflammation is reflected in the duration of fever, more severe or clearer diagnostic clinical manifestations, and higherly activated laboratory parameters such as hemoglobin, baseline platelet count, percentage of neutrophils, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), total bilirubin, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum sodium and other inflammatory biomarkers, which once have been reported to differ remarkably between IVIG non-responders and responders before IVIG infusion. But these factors have no clear consensus on predicting IVIG resistance so far and the conflicting data may derived from ethnic and genetic backgrounds (9, 10).

This study was designed to perform a meta-analysis to identified risk factors associated with IVIG resistance in patients with KD. It may be helpful for prediction of IVIG non-responsiveness.

MATERIALS AND METHODS

This meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-analyses Statement (11).

Database Search

We searched databases including PubMed, Embase and Cochrane Library up to 31st October 2019 by using Medical Subject Headings (MeSH) terms or Emtree thesaurus terms combined with keywords, the search strategy was ["Mucocutaneous lymph node syndrome" OR "Kawasaki disease"] AND ["IVIG resistance" OR "IVIG non-responsiveness" OR "IVIG unresponsiveness"]. The language was restricted to English and a manual search was conducted using reference lists of original articles for further articles of interest.

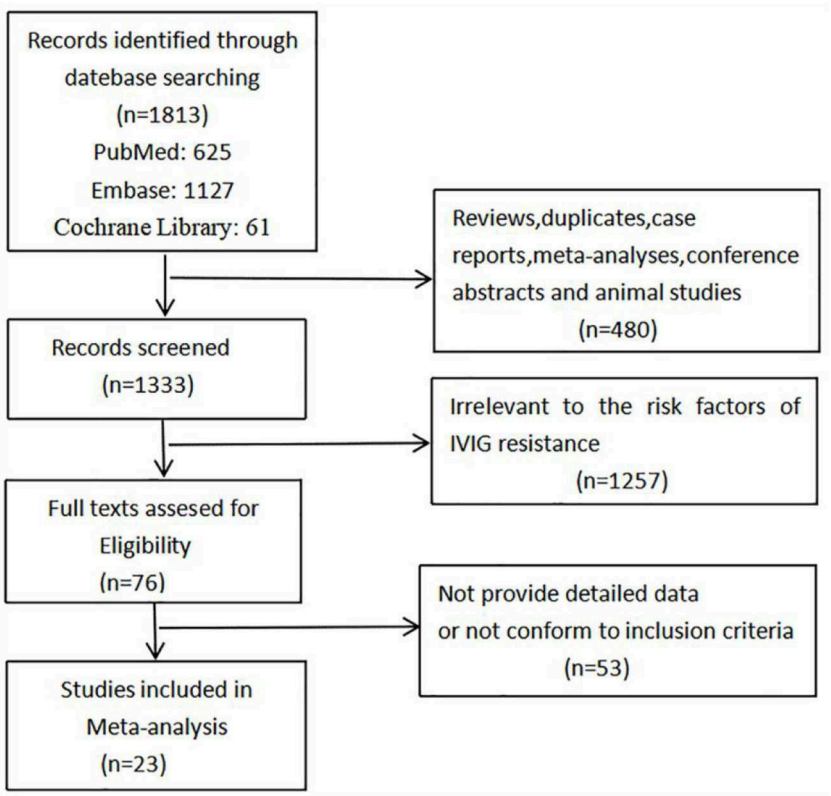

FIGURE 1 | Flow chart of studies selection process.

Inclusion and Exclusion Criteria

Criteria for Inclusion

①Case-control study or cohort study; ②All subjects were children (aged 0–18 years) diagnosed with KD according to Japanese diagnostic criteria (12) or the 2017 American Heart Association common standards (4) and received IVIG infusion in the cumulative dose of 2 g/kg plus oral aspirin for the initial therapy; ③Odds ratio (OR) and 95% confidence interval (CI) provided for categorical variables in the original data or mean and standard deviation provided for continuous variables, and all the data provided were measured at the time of admission; ④Clear description of statistical methods and correct statistical analyses.

Criteria for Exclusion

①Animal studies; ②Reviews, duplicates, case reports, meta-analyses, conference abstracts or unpublished literatures; ③OR and 95% CI were not provided for categorical variables or mean and standard deviation were not provided directly or indirectly for continuous variables.

Data Extraction and Quality Assessment

The data were independently gathered by 2 investigators on the basis of a predefined standard form. The data extracted from the studies included such details as the first author, publication year, region, sample size, clinical symptoms and laboratory indicators of cases and controls. We assessed quality of every included study with respect to cases and controls selection, comparability, and exposure using the Newcastle-Ottawa Quality Assessment Scale (NOS) for case-control or cohort studies (**Supplementary Material**). which has a total score of 9 stars, a study that awards ≥7 stars is considered high methodological quality, ≤3 as low quality, and 3–7 as moderate.

Statistical Analysis

Data analysis was performed by using Stata15 software. Cochrane Q test and I^2 statistic were calculated to assess heterogeneity across studies. If the studies were shown to be homogeneous with $P \geq 0.10$ and $I^2 < 50\%$, the fixed effects model was selected, otherwise, the random effects model was applied (13). The pooled effects were presented with OR and corresponding 2-tailed 95% CI for dichotomous variables, or weighted mean difference (WMD) and corresponding 2-tailed 95% CI for continuous variables. The significance of the pooled effects were determined by the Z-test, all *P*-values were 2-tailed and a $P < 0.05$ was considered statistical significantly. Sensitivity analysis was conducted by omitting a single study involved in the meta-analysis in turn to identify the potential influence of each individual data on the pooled effects and to confirm that our results were not driven by any single study. Publication bias was estimated via funnel plot and Egger's test (14), a $P > 0.05$ was considered no significantly publication bias.

TABLE 1 | Characteristics of included studies in the meta-analysis.

References	Region	Gender (male/female)	Number of patients (*n*)		Resistant rate (%)
			IVIG-resistant	IVIG-sensitive	
Arane et al. (15)	Israel	239/43	52	230	18.44
Chantasiriwan et al. (16)	Thailand	141/76	26	191	11.98
Egami et al. (17)	Japan	184/136	41	279	14.7
Fu et al. (18)	China	746/431	211	966	17.93
Gamez-Gonzalez et al. (19)	Japan	244/175	101	318	24.11
Kim et al. (20)	South Korea	401/302	118	585	16.79
Kim et al. (21)	South Korea	–/–	524	4627	10.17
Kim et al. (22)	South Korea	84/51	22	113	16.3
Kuo et al. (23)	Chinese Taiwan	93/38	20	111	15.27
Loomba et al. (24)	USA	105/77	58	124	31.87
Nakagama et al. (25)	Japan	109/62	54	117	31.58
Park et al. (26)	South Korea	161/148	30	279	9.71
Sanchez-Manubens et al. (27)	Spain	238/161	67	332	16.79
Sano et al. (28)	Japan	59/53	22	90	19.64
Sato et al. (29)	Japan	62/43	21	84	20
Sleeper et al. (30)	USA	125/73	27	171	13.64
Tan et al. (31)	China	3,269/2,009	348	4,929	6.59
Tang et al. (32)	China	584/326	46	864	5.05
Yang et al. (33)	China	827/533	78	1282	5.74
Lin et al. (34)	Chinese Taiwan	107/74	22	159	13.84
Lee et al. (35)	South Korea	47/44	11	80	12.09
Bar-Meir et al. (36)	Israel	202/110	42	270	13.46
Kobayashi et al. (37)	Japan	231/315	112	434	20.51

TABLE 2 | Quality assessment of included studies by NOS.

References	Selection	Comparability	Exposure	NOS scores
Arane et al. (15)	★★★	★★	★★	7
Bar-Meir et al. (36)	★★★★	★★	★★★	9
Chantasiriwan et al. (16)	★★★★	★★	★★	8
Egami et al. (17)	★★★	★★	★★	7
Fu et al. (18)	★★★	★★	★★	7
Gamez-Gonzalez et al. (19)	★★★	★★	★★	8
Kim et al. (20)	★★★	★★	★★	7
Kim et al. (21)	★★★★	★★	★★	8
Kim et al. (22)	★★★	★★	★★	7
Kobayashi et al. (37)	★★★★	★★	★★	8
Kuo et al. (23)	★★★★	★★	★★★	9
Lee et al. (35)	★★★	★★	★★	7
Lin et al. (34)	★★★	★★	★★★	8
Loomba et al. (24)	★★★	★★	★★	7
Nakagama et al. (25)	★★★	★★	★★	7
Park et al. (26)	★★★★	★★	★★	8
Sanchez-Manubens et al. (27)	★★★	★★	★★★	7
Sano et al. (28)	★★★	★★	★★★	8
Sato et al. (29)	★★★	★★	★★	7
Sleeper et al. (30)	★★★★	★★	★★★	9
Tan et al. (31)	★★★	★★	★★	8
Tang et al. (32)	★★★	★★	★★	7
Yang et al. (33)	★★★★	★★	★★	8

RESULTS

Search Results

Of 1,813 articles initially searched, 480 reviews, duplicates, case reports, meta-analyses, conference abstracts and animal studies were firstly removed. After screening the titles and abstracts, 1,257 studies that irrelevant to the risk factors of IVIG resistance were excluded, and another 53 studies that did not provide detailed origin data or not in accordance with our inclusion criteria were excluded after assessing the full text. Finally, 23 studies were found to conform to our specific inclusion criteria and were included consequently in our meta-analysis. The studies selection process is shown in **Figure 1**.

Study Characteristics and Quality Assessment

All the 23 included studies (15–37) were case-control studies, the general characteristics were summarized in **Table 1**. A total of 18,688 patients were enrolled, of which 2,053 were assigned in the IVIG-resistant group and 16,635 in the IVIG-sensitive group. These studies were conducted in different ethnic populations and different regions, including Mongoloids from Asia (Japan, China, Chinese Taiwan, South Korea, Thailand) and Caucasians from non-Asia (Spain, Israel, USA). The quality assessment found that all the included studies awarded ≥7 stars **(Table 2)**, indicating that the studies are of high methodological quality and persuasive.

Risk Factors

A total of 13 clinical and laboratory indicators were found associated with IVIG resistance, the results were summarized in **Table 3**. Our meta-analysis revealed that male patients were more likely to be IVIG non-responders (OR = 1.19, 95% CI: 1.01 to 1.42, $P = 0.043$) than females, and there was an increase in the association of both swelling of extremities (OR = 1.25, 95% CI: 1.01 to 1.54, $P = 0.040$) and polymorphous rash (OR = 1.56, 95% CI: 1.20 to 2.02, $P < 0.001$) with the odds of IVIG resistance. In terms of laboratory parameters, IVIG non-responders had significantly lower hemoglobin (**Figure 2**, WMD = −0.24, 95% CI: −0.33 to −0.14, $P < 0.001$), baseline platelet count (**Figure 3**, WMD = −32.4, 95% CI: −45.22 to −19.59, $P < 0.001$), albumin (**Figure 4**, WMD = −0.26, 95% CI: 0.33 to −0.20, $P < 0.001$) and serum sodium (WMD = −1.24, 95% CI = −1.63 to −0.85, $P < 0.001$) values than responders, while percentage of neutrophils (**Figure 5**, WMD = 7.49, 95% CI: 6.13 to 8.85, $P < 0.001$), ESR (**Figure 6**, WMD = 3.70, 95% CI: 0.42 to 6.97, $P = 0.018$), CRP (WMD = 2.21, 95% CI = 1.54–2.89, $P < 0.001$), total bilirubin (WMD = 0.49, 95% CI: 0.42 to 0.57, $P < 0.001$), AST(WMD = 42.27, 95% CI: 25.54 to 59.00, $P < 0.001$), and ALT (WMD = 39.76, 95% CI: 26.65 to 52.87, $P < 0.001$) values were significantly higher than responders.

We also identified several factors that were not relevant to IVIG resistance, including WBC (WMD = 0.33, 95% CI: −0.04 to 0.70, $P = 0.078$), age (WMD = 0.39, 95% CI: −0.90 to 1.68, $P = 0.554$), conjunctivitis (OR = 0.85, 95% CI: 0.62 to 1.15, $P = 0.277$), oral lesions (OR = 0.76, 95% CI: 0.56 to 1.03, $P = 0.081$) and cervical lymphadenopathy (OR = 0.99, 95% CI: 0.70 to 1.42, $P = 0.974$).

Subgroup Analyses

Subgroups were selected based on different ethnic populations from different regions (Asian or non-Asian). All the high-risk factors with significant heterogeneity and more than 10 studies enrolled were analyzed, including male, baseline platelet count, percentage of neutrophils, CRP, albumin, ALT, AST, and serum sodium. It turned out that there were significant ethnicity-specific and region-specific differences in factors of male sex, elevation of CRP and AST, and decreased serum sodium, but not in baseline platelet count, percentage of neutrophils, albumin and ALT.

The summary WMDs for baseline platelet count were −21.87 (95% CI: −41.32, −2.42) and −35.73 (95% CI −68.47, −3.00) in Asian and non-Asian populations (P difference = 0.151). The WMDs for neutrophils percentage were 7.67 (95% CI: 6.22, 9.12) and 5.00 (95% CI: 0.21, 9.79) for the studies in Asian and non-Asian populations (P difference = 0.085). The WMDs for albumin and ALT were −0.25 (95% CI: −0.33, −0.18) vs. −0.29 (95% CI: −0.39, −0.18), and 45.98 (95% CI: 29.41, 62.56) vs. 21.30 (95% CI: 5.83, 36.77), respectively, for the

TABLE 3 | Meta-analysis of risk factors for IVIG resistant KD patients.

Factors	Studies number	Heterogeneity		Pooled effect		Egger's test (*P*)
		I^2 (%)	*P*	OR/WMD (95% CI)	*P*	
Male	20	36.60	0.052	1.19 (1.01, 1.42)	0.043	0.922
Swelling of extremities	7	36.40	0.138	1.25 (1.01, 1.54)	0.040	0.901
polymorphous rash	7	2.10	0.409	1.56 (1.20, 2.02)	<0.001	0.646
Hemoglobin (g/dL)	11	0.00	0.607	−0.24 (−0.33, −0.14)	<0.001	0.748
PLT count ($\times 10^9$/L)	18	49.40	0.009	−32.40 (−45.22, −19.59)	<0.001	0.431
Neutrophils (%)	14	52.90	0.010	7.49 (6.13, 8.85)	<0.001	0.804
ESR (mm/h)	11	14.90	0.302	3.70 (0.42, 6.97)	0.018	0.260
CRP (mg/L)	20	64.70	0.000	2.21 (1.54, 2.89)	<0.001	0.148
Total bilirubin (mg/dL)	12	30.10	0.152	0.49 (0.42, 0.57)	<0.001	0.095
Albumin (g/L)	19	68.10	0.000	−0.26 (−0.33, −0.20)	<0.001	0.094
ALT	18	66.30	0.000	39.76 (26.65, 52.87)	<0.001	0.714
AST	19	84.10	0.000	42.27 (25.54, 59.00)	<0.001	0.630
Sodium (mmol/L)	16	68.70	0.000	−1.24 (−1.63, −0.85)	<0.001	0.549

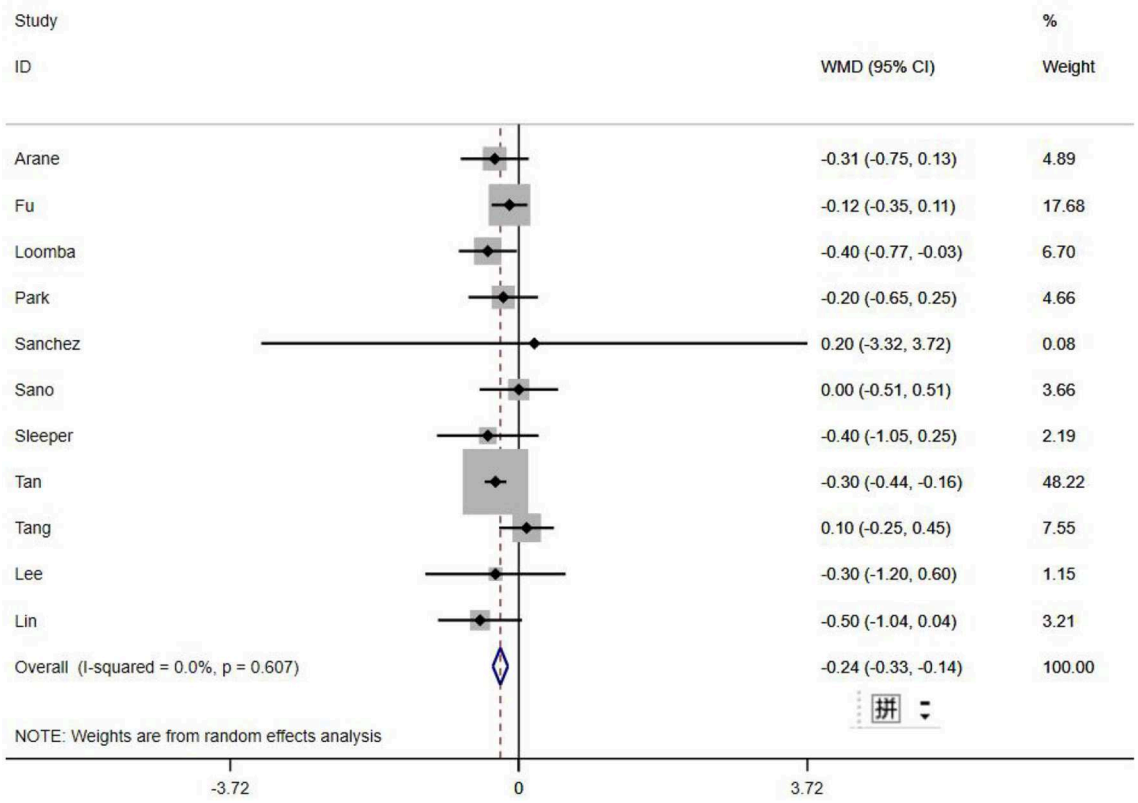

FIGURE 2 | Pooled weighted mean difference for IVIG resistance by hemoglobin (g/dL).

studies in Asian and non-Asian populations, with P difference = 0.430 and 0.183.

It is noteworthy that Asian male patients were more likely to be non-responders ($P = 0.027$), but there was no significant difference between responders and non-responders in non-Asian patients ($P = 0.507$). As well as male sex, Asian patients with higher CRP, AST and lower serum sodium were more likely to be IVIG non-responders (All $P < 0.001\%$), and no significant differences were observed between responders and non-responders in Caucasians from non-Asia regions (All $P > 0.05$) (**Figures 7–9**).

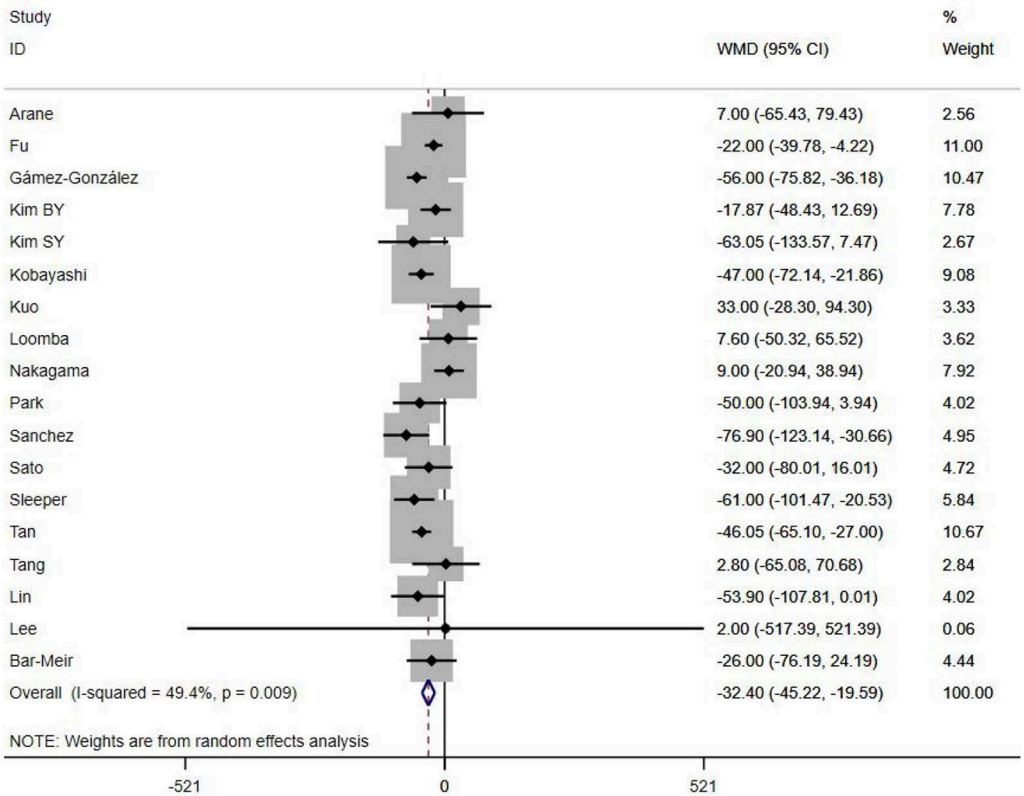

FIGURE 3 | Pooled weighted mean difference for IVIG resistance by baseline platelet count ($\times 10^9$/L).

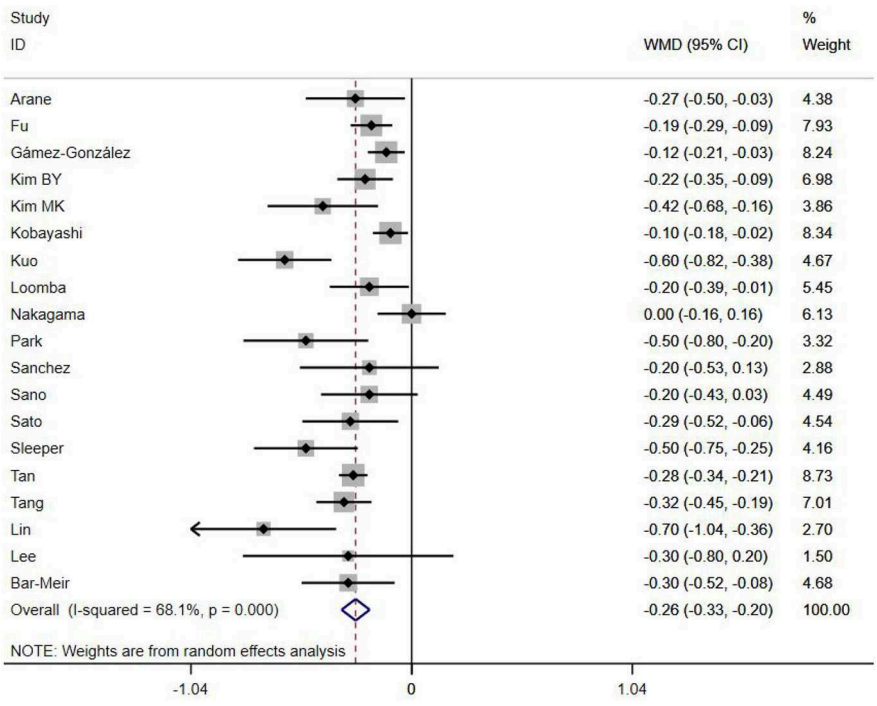

FIGURE 4 | Pooled weighted mean difference for IVIG resistance by albumin (g/L).

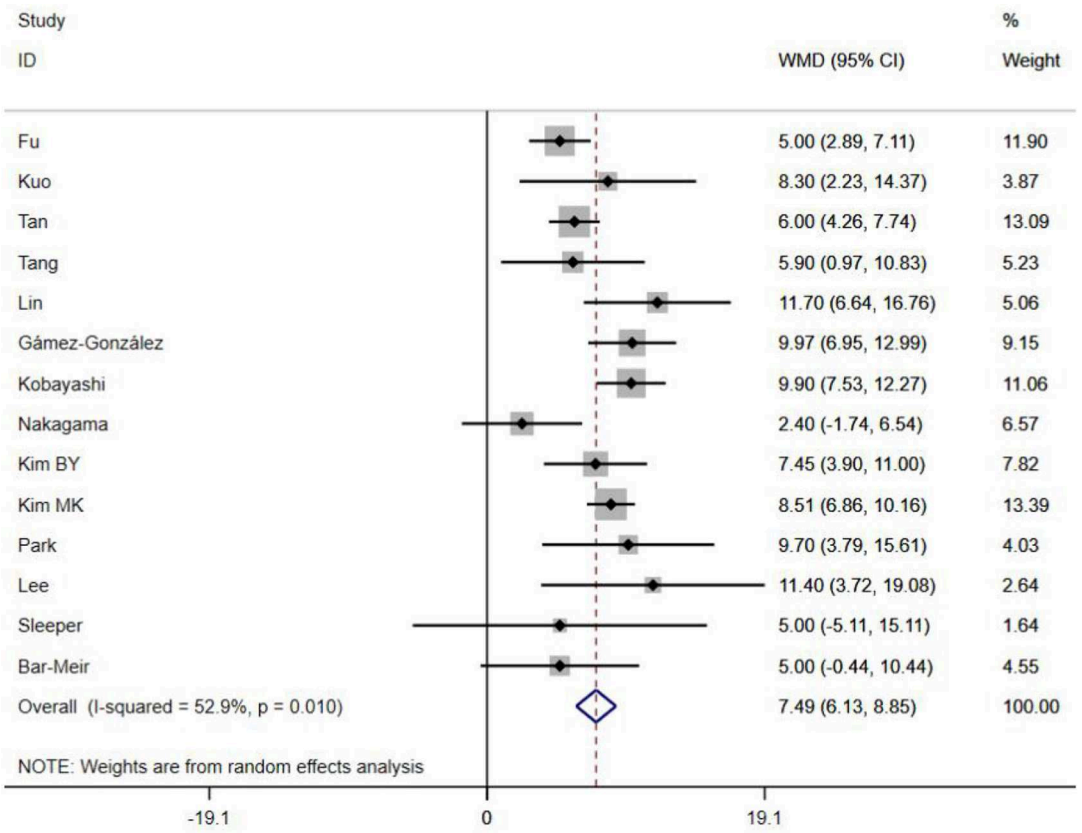

FIGURE 5 | Pooled weighted mean difference for IVIG resistance by percentage of neutrophils (%).

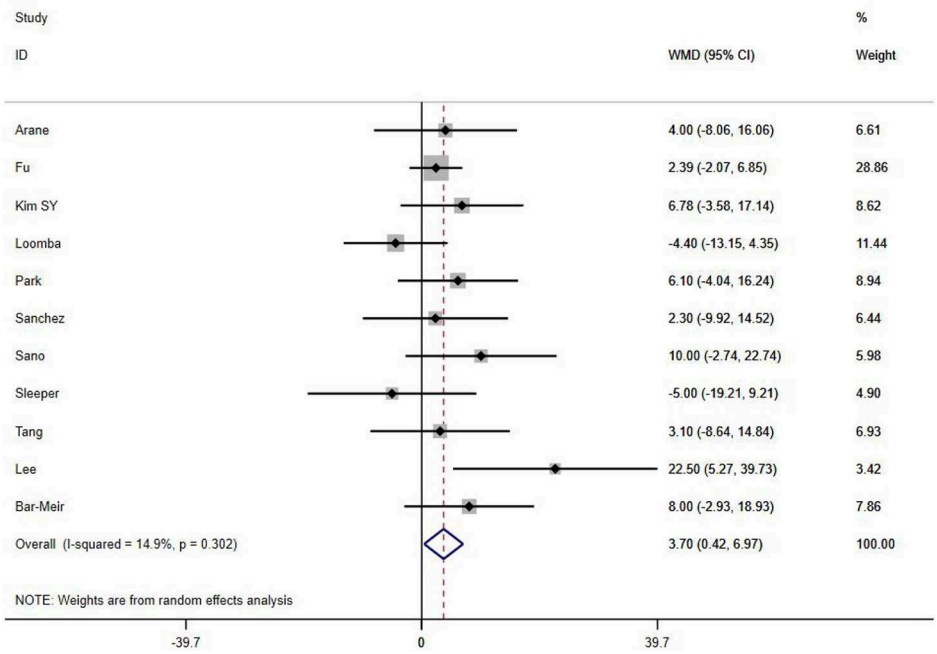

FIGURE 6 | Pooled weighted mean difference for IVIG resistance by ESR (mm/h).

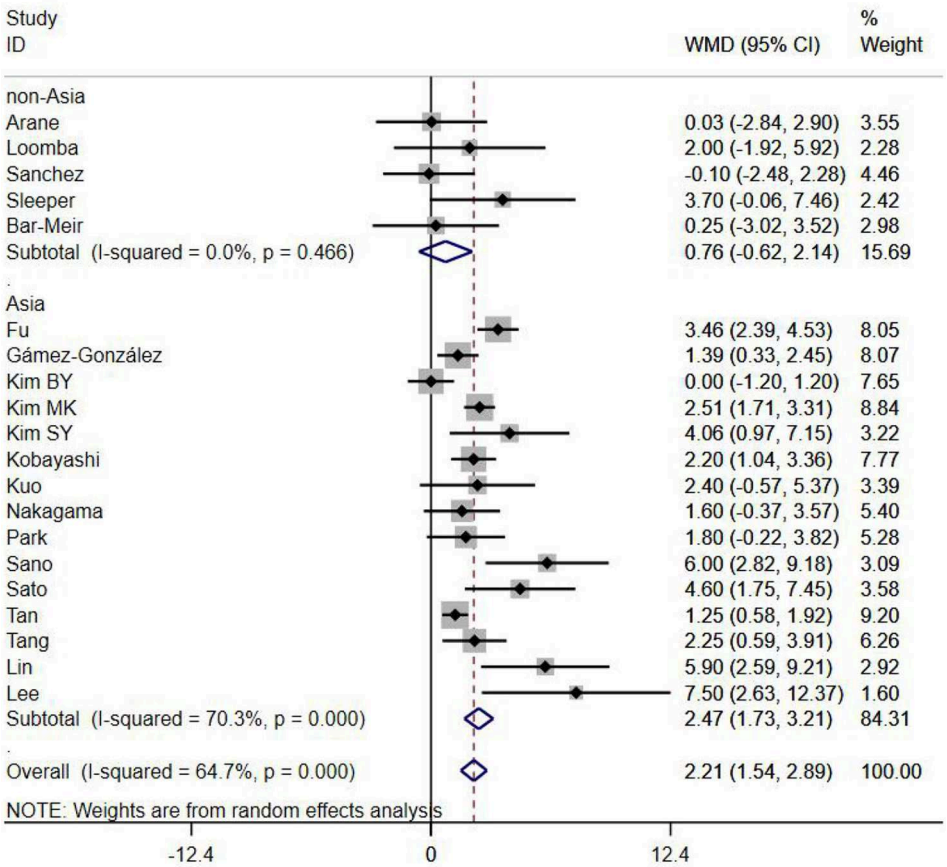

FIGURE 7 | Subgroup analysis for IVIG resistance by CRP (mg/L).

Sensitivity Analysis

If there is significant heterogeneity among studies, addition or reduction of any one study may lead to remarkable change in results, so we used sensitivity analysis to verify the reliability of the meta-analysis findings. In addition to the discovery that Tan's study (31) had a great impact on pooled effect in the meta-analysis of ESR as shown in **Figure 10** (The ESR data of this study was omitted in this meta-analysis), there was no significant change in the pooled OR and WMD after every single study was omitted, this indicated that our results were convinced because of good stability and not driving by any single study.

Estimation of Publication Bias

Publication bias was estimated via funnel plot and Egger's test. All the funnel plots showed generally symmetrical (e.g., the funnel plot of male sex shown in **Figure 11**) and all the *p*-value of Egger's test >0.05 **(Table 3)**, which mean no significant publication bias was found in the meta-analyses of each risk factor.

DISCUSSION

KD is an infection-related immune-mediated systemic inflammation, although the clinical phenotype of KD varies across individuals, the pathogenesis is basically definite. After an infection of unknown agents, immune cells (especially T cells) are activated. Then the hyperactivated immune cells produce massive cytokines, leading to a cytokine imbalance associated with further endothelial cell injury (38), CALs may begin to develop and progress in the early phase of the inflammation, so early prediction of IVIG resistance through clinical manifestations and laboratory parameters is indeed a wise method for the severely affected patients who need early intensive treatment.

In the present meta-analysis, we included 23 studies from Japan, South Korea, China, Chinese Taiwan, Thailand, Spain, Israel and the United States of America, to analyze the relationships between non-responsiveness and clinical or laboratory indicators that are associated with inflammation. Also, we have discussed the indicators between different ethnicities based on various genetic backgrounds. The results showed that in addition to patients with polymorphous rash or swelling of extremities symptoms had a tendency to be non-responders, IVIG resistance was more likely to occur in patients with severe anemia, hypoalbuminemia, decreased baseline platelet count, and increased ESR, total bilirubin, ALT and neutrophils percentage. In particular, male sex, hyponatraemia, elevated levels of AST and CRP were confirmed as the risk factors favor IVIG resistance in Mongoloid patients from Asia countries,

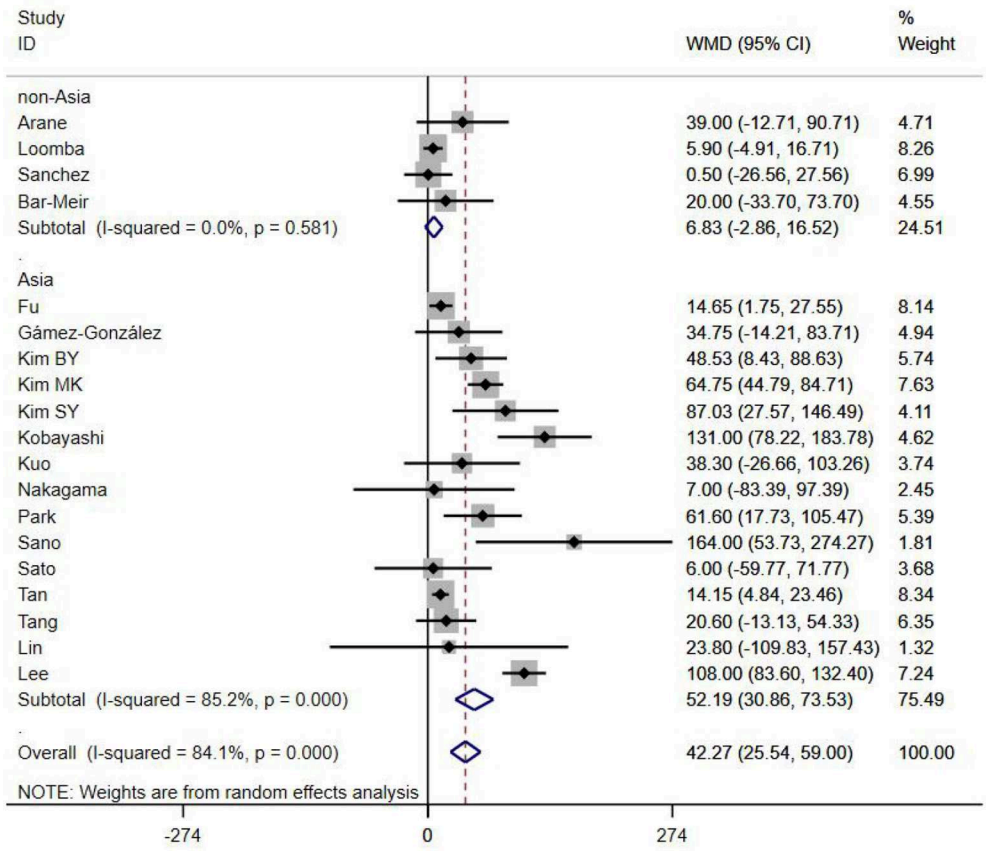

FIGURE 8 | Subgroup analysis for IVIG resistance by AST (IU/L).

but not in Caucasians from non-Asia regions. No significant difference was found in WBC count, age, conjunctivitis, oral lesions and cervical lymphadenopathy between responders and non-responders. Subgroup analyses showed that some factors differed by ethnicity. The difference of gender, CRP, AST, and serum sodium between Asian and non-Asian patients were significant. Male Asian patients with increased CRP, AST and decreased serum sodium were more likely to be IVIG non-responders, while the same phenomenons were not observed in Caucasians from non-Asia regions.

Changes in these indicators can be explained by the pathophysiology of KD. KD inflammatory reaction changes the redox state of serum, with elevated inducible nitric oxide synthase (39), which would alter the homeostasis of red blood cells (RBCs) and result in a type of premature in these cells that lead to anemia and thrombus. RBC aging, inflammation and thrombus inevitably result in increased ESR. Intensive inflammation and immune reaction lead to serious vascular permeability and liver damage, resulting in albumin leakage and transaminases elevation. It is confirmed that cytokines such as plasma IL-6 and tumor necrosis factor-α (TNF-α) participate in inflammation of KD in the acute phase and were markedly increased in IVIG non-responders compared with responders (40–42), and the release of ADH is promoted by IL-6 and TNF-αduring inflammation (43), so the probable pathophysiological for hyponatremia may be relevant to inappropriate release of ADH. Overall, anemia, decreased serum albumin and sodium, elevated levels of neutrophils percentage and acute phase reactants such as ESR and CRP largely represent a more severe degree of inflammation and intensive immune response.

There are several limitations of this study should be noted. Firstly, the presence of confounding factors may reduce the accuracy of prediction and treatment guidance. The intensity of KD inflammation gradually increases in the acute stage and reach the peak, then decreases and enters to the convalescent stage (44), and the immune reaction before the peak may be responsible for tissue cell injury while immune reaction after the peak may be responsible for tissue cell repair (38), so inflammatory indices change throughout this process over time. Obviously, fever duration is a confounding factor predicting IVIG resistance. Besides, some laboratory values varied according to age, personal immunity and organ or tissues involvement in individuals. Secondly, the definition of IVIG resistance is not completely uniform in all the included studies, the observation periods after IVIG are different (24, 36, or 36–48 h). Thirdly, there are some differences in the infusion modalities of IVIG, some patients treated with IVIG 2 g/kg as a single infusion, while others received IVIG 1 g/kg for 2 days or 400 mg/kg for

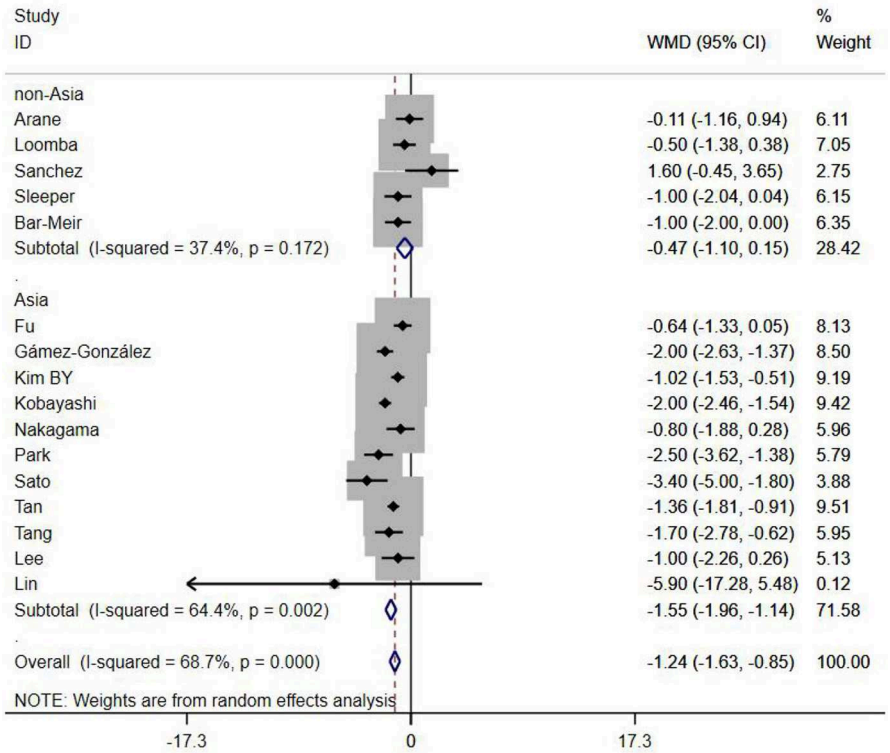

FIGURE 9 | Subgroup analysis for IVIG resistance by sodium (mmol/L).

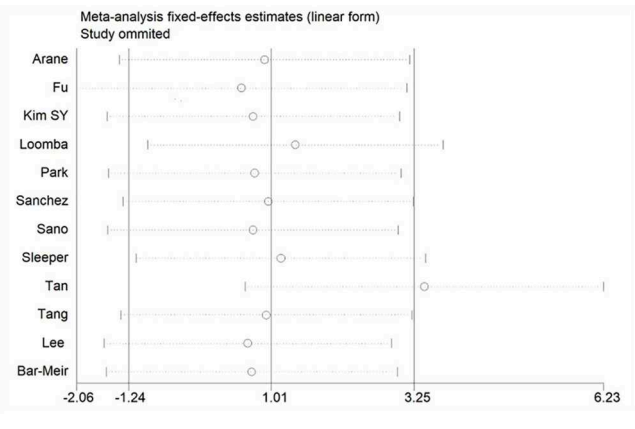

FIGURE 10 | Sensitivity analysis of ESR.

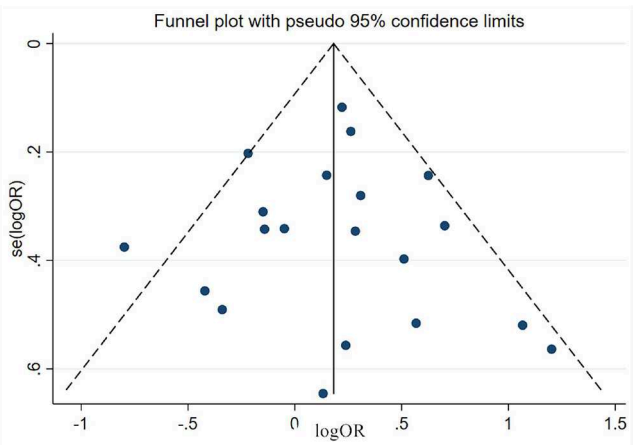

FIGURE 11 | The funnel plot of male factor.

5 consecutive days, this may lead to slightly different sensitivity to IVIG due to dose-response effects. As well as limitations, our meta-analysis also has significant aspects. We included various studies involving different ethnic populations from all over the world to ensure the applicability of our findings and to investigate a wide range of risk factors for IVIG resistance, and we had a sufficient sample size to carry out Egger's test for most factors and found no apparent publication bias.

In conclusion, some parameters were demonstrated associated with IVIG resistance, and clinicians should aware an increased likelihood that the patient may fail to respond to initial IVIG therapy when such factors present, but further studies are needed because of confounding factors in data analysis.

DATA AVAILABILITY STATEMENT

All datasets analyzed for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

GL and ZD designed the study. GL contributed to literatures search, data collection, statistical analysis, and drafting the manuscript. SW contributed to literature search and data collection. ZD performed manuscript review. All authors have read and approved the content of the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00187/full#supplementary-material

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BRIEF RESEARCH REPORT
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Aspirin Dose and Treatment Outcomes in Kawasaki Disease: A Historical Control Study in Japan

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Aspirin has been used as a concomitant drug in the treatment of Kawasaki disease (KD). In recent years, there has been discussion concerning whether high-dose aspirin is appropriate for treatment in the acute phase of KD. We retrospectively investigated the incidence of coronary artery abnormalities (CAAs) and the antipyretic effect of 30 to 50 mg/kg/day aspirin, the minimum and the maximum approved doses in Japan. This was a single-center, non-randomized, retrospective, historical cohort study. Patients were routinely treated with 50 mg/kg/day aspirin (50-mg Group) between 2007 and April 2014, and with 30 mg/kg/day aspirin (30-mg Group) between May 2014 and 2016. All patients were given initial and, if necessary, subsequent intravenous immunoglobulin (IVIG) 2.0 g/kg. The primary endpoint was incidence of CAAs defined as a CA diameter with a Z score $\geq$2.5 at treatment week 4. The secondary endpoint was incidence of further treatment. Incidences were compared using inverse probability weighting analysis adjusting for age, sex, and risk scores. In 587 patients, there was no significant difference in incidence of CAAs (odds ratio in 30-mg Group 0.769, 95% confidence interval (CI): 0.537–1.101, $p = 0.151$). Risk of further treatment after the first IVIG in the 30-mg Group was significantly higher than that in the 50-mg Group (odds ratio 1.379, 95% CI: 1.051–1.811, $p = 0.021$). Although this study has some limitations, the findings suggest that aspirin 50 mg/kg/day may have no significant effect on improving incidence of CAAs compared with 30 mg/kg/day but may have a lower rate of further treatment.

Keywords: aspirin, treatment, coronary artery, Kawasaki disease (KD), immunoglobulin

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INTRODUCTION

Kawasaki disease (KD), which is an acute vasculitis in young children and causes coronary artery abnormalities (CAAs) such as coronary artery aneurysm and dilation, is the leading cause of acquired heart disease in children in developed countries (1, 2). In the five decades since the initial recognition of KD, the standard treatment is a combination of intravenous immunoglobulin (IVIG) and acetylsalicylic acid (ASA) (3). High-dose (80–100 mg/kg) and medium-dose (30–50 mg/kg) ASA have been recommended as standard treatment during the acute febrile phase by the American Heart Association (AHA) and Japanese Society of Pediatric Cardiology and Cardiac Surgery (JSPCCS) (4, 5).

ASA was first confirmed to be effective for KD in the latter half of the 1970s in Japan. That prospective study compared ASA, flurbiprofen, and prednisolone plus dipyridamole (6). At 1 month after onset, ASA could not prevent the incidence of CAAs, but at 1 year after onset, only 1% of ASA-treated patients had CAAs. These results were far better compared with 12% in the flurbiprofen-treated group and 9% in the prednisolone plus dipyridamole-treated group, indicating the superiority of ASA (4). Mortality dropped from 1.7 to 0.2–0.3% after the ASA treatment was adopted (7). A US study prior to the spread of immunoglobulin therapy has also shown that ASA suppresses the development of CAAs (8).

In the 1990s, IVIG became the mainstay of treatment, and when the development of CAAs was the final prognosis, the impact of ASA became less pronounced (9, 10). In recent years, some retrospective studies have raised the question of whether high-dose and medium-dose ASA would be appropriate for KD treatment in terms of risk-benefit (11–14). Starting treatment with high or medium doses of ASA (≥30 mg/kg/day) was found not to prevent CAAs (11–14).

In Japan, the Pharmaceuticals and Medical Devices Agency has approved ASA 30–50 mg/kg/day for KD. However, no studies have investigated ASA dose and outcomes in KD within the recent IVIG era in Japan. For 11 years now we have treated KD patients with a unified treatment protocol of IVIG and ASA as first-line therapy. Between January 2007 and April 2014, patients received 50 mg/kg/day ASA. After May 2014, patients were given 30 mg/kg/day ASA. In this study, we retrospectively investigated differences in the incidence of CAAs as well as the antipyretic effect between historical control 50 mg/kg/day and 30 mg/kg/day ASA at a single center in Japan.

METHODS

This single-center, non-randomized, retrospective, historical cohort study had a target population that comprised 587 children between 0 and 12 years of age diagnosed with complete or incomplete KD between 2007 and 2016 at Tokyo Women's Medical University Yachiyo Medical Center, Chiba, Japan. Between January 2007 and April 2014, patients received 50 mg/kg/day ASA. Between May 2014 and September 2016, patients were given 30 mg/kg/day ASA.

Inclusion criteria were children with a diagnosis of KD who were treated with IVIG and ASA. Given that the diagnosis of KD cannot be confirmed definitively, patients were eligible if the clinical suspicion of KD was such that treatment with IVIG was deemed clinically indicated.

Exclusion criteria were as follows: patients for whom a final diagnosis of KD was clearly ruled out; patients who had preexisting CAAs before treatment diagnosed at bedside according to the Japanese Ministry of Health, Labour and Welfare criteria (15), which are based on absolute values (≥3 mm in children <5 years old, ≥4 mm in children ≥5 years old); patients who had received any treatment for KD at other medical hospital before arriving at our institution; patients who were enrolled in a clinical study and had received cyclosporine with IVIG plus aspirin as first-line therapy (16).

Research Ethics Board approval was granted at Tokyo Women's Medical University (number 3636-R2). Only anonymous retrospective data were obtained for this study, and the requirement for patient consent was waived by the Research Ethics Board.

Patients received initial IVIG 2.0 g/kg for 24 h and ASA. An additional dose of IVIG 2.0 g/kg was administered to patients who were non-responders, defined as body temperature ≥37.5°C at 48 h after initiation of primary therapy, and those who had KD relapse, defined as a return of fever (≥37.5°C) without another likely source after a ≥48-h afebrile period from the initiation of primary therapy. Patients whose body temperature did not drop to below 37.5°C at 24 h after the initiation of the second dose of IVIG received cyclosporine or/and a third dose of IVIG as third-line therapy.

Between January 2007 and April 2014, patients received 50 mg/kg/day ASA (ASA 50-mg Group). Between May 2014 and September 2016, patients were given 30 mg/kg/day ASA (ASA 30-mg Group). At 48 h after defervescence, aspirin dose was reduced to 5 mg/kg/day in all patients. Low-dose ASA was continued for 4 to 6 weeks.

The primary endpoint was the incidence of CAAs at treatment week 4. CAAs were diagnosed according to Z scores in Japanese children—echocardiographic measurements of the internal diameter normalized by body surface area—of coronary artery dimensions for the following three segments: proximal right coronary artery, left main coronary artery, and proximal left anterior descending artery (17). The maximum Z score was defined by the largest Z score in the three segments. CAAs were defined as present when the maximum Z score was ≥2.5, according to 2017 AHA guidelines. The value of the Z score was retrospectively calculated using absolute diameters (mm) in the echo reports at 1 month after onset.

The secondary endpoint was the incidence of second- and third-line therapy in the two treatment groups.

Adverse events were extracted from elevated liver enzymes, anemia, and bleeding. The incidences of elevated liver enzymes in ASA 50-mg Group and ASA 30-mg Group were compared. Elevated liver enzymes was defined as aspartate aminotransferase >50 U/L and alanine aminotransferase >50 U/L in the convalescent phase of KD. Differences in hemoglobin levels before and after the first IVIG dose were compared between the two groups. The incidence of severe bleeding or digestive symptoms suggesting gastritis or gastric ulcer was also compared between the two groups.

Statistical Analysis

We used a non-inferiority design to assess whether the risk ratio of CAAs between treatment groups would remain within the 95% confidence interval (CI) of a predefined clinically acceptable margin. In primary endpoint and secondary endpoint, data were adjusted by inverse probability weighting method for age, sex, and risk scores, and logistic regression was performed. A regression coefficient test was performed using the Wald test, and a $p < 0.05$ was considered significant.

TABLE 1 | Patient characteristics.

Characteristic	ASA 50-mg Group $n = 414$	ASA 30-mg Group $n = 173$	*p*-value
Age, years	2.6 (1.9)	2.7(2.1)	0.628*[a]
Age <1 year at diagnosis, %	22.2%	24.2%	0.680*[b]
Male sex, %	57.6%	54.0%	0.376*[b]
Risk score (Kobayashi)	3.5 (2.3)	3.6 (2.6)	0.837*[a]
Day of illness at first IVIG treatment, days	5.2 (1.6)	5.1 (1.3)	0.339*[a]
Hemoglobin, g/dL	11.3 (1.2)	11.6 (1.0)	0.830*[a]
White blood cell count, $\times 10^3/\mu L$	13.9 (4.4)	13.9 (4.6)	0.851*[a]
Neutrophils ≥80% at diagnosis	23.7%	23.7%	0.423*[c]
Platelet count, ×104/μL	33.2 (10.2)	32.1 (9.3)	0.053*[c]
C-reactive protein, mg/dL	7.5 (4.6)	8.3 (4.7)	0.373*[c]
Albumin, g/dL	3.9 (0.3)	3.9 (0.4)	0.842*[c]
Na, mEq/L	134 (2.6)	134 (2.9)	0.410*[c]
Aspartate aminotransferase, U/L	104 (215)	121 (240)	0.183*[c]

Data are shown as the mean (SD) or percentage.
*[a] *Welch's t-test.*
*[b] *Pearson's χ^2 test.*
*[c] *t-test, two-sided.*

Items under patient characteristics were assessed using Welch's *t*-test, Pearson's χ^2 test, or a two-sided *t*-test. The incidence of adverse events was assessed using Fisher's exact test. Two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

A total of 655 patients were identified and screened for eligibility. Of them, 68 were excluded for the following reasons: 8 patients had CAAs before treatment; 39 patients had received any treatment for KD at other hospitals before referral to our institution; and 21 patients were enrolled in a clinical study and received cyclosporine with IVIG plus ASA as first-line therapy. The 587 remaining patients were included in the final analysis. Among them, 414 patients were routinely prescribed 50 mg/kg/day ASA between January 2007 and April 2014 (ASA 50-mg Group). The remaining 173 patients were routinely prescribed 30 mg/kg/day ASA between May 2014 and September 2016 (ASA 30-mg Group).

(**Table 1**) presents the characteristics of the study population. Both groups were similar in age, sex, risk scores (Kobayashi score for predicting resistance to IVIG) (18), number of days of fever before the first IVIG dose, C-reactive protein before treatment, and hemoglobin levels.

Our primary outcome, the presence of CAAs, was observed in 70 patients (12%) in total. Of these, number of patients with small CAAs (z score ≥2.5 but <5), medium-sized CAAs, and large-sized CAAs in each treatment group are shown in (**Table 2A**). (**Table 2B**) presents the proportion of patients with CAAs according to treatment groups as well as the unadjusted and adjusted risk ratios. The incidence of CAAs was not significantly different between the ASA 50-mg Group and the ASA 30-mg Group.

For the secondary endpoint, requiring further treatment after the first IVIG dose was observed in 143 patients (24%) in total. The risk was significantly higher in the ASA 30-mg Group than in the ASA 50-mg Group (**Tables 3A,B**).

For safety outcomes, the incidences of elevated liver enzymes at convalescent phase were similar between the two groups (**Table 4A**). There was no significant difference between the two groups in the reduction of hemoglobin before and after the first IVIG (**Table 4B**). No severe hemorrhagic complications nor digestive symptoms were observed in either group.

DISCUSSION

The results of our study suggest that using 30 mg/kg/day ASA, which is the lower limit of the approved dose for acute KD in Japan, is not inferior to 50 mg/kg/day ASA, which is the upper limit dose, in preventing CAAs. However, we observed a significant effect of ASA dose on treatment resistance.

Single IVIG 2 g/kg/day is the current standard treatment and is recommended as class I in the AHA and JSPCCS guidelines (4, 5). During this study period, the impact of high-dose ASA on treatment has decreased (9). Recently, controversies have arisen regarding the optimal dose of ASA to be used. In 2004, the first query regarding high-dose ASA treatment was proposed. Hsieh et al., a Taiwanese group, originally performed IVIG treatment without using ASA in the acute phase, and retrospectively aggregated the antipyretic rate and the incidence of CAAs (11). Their study concluded that 94% of patients had defervescence within 3 days of IVIG treatment, and 14% had CAAs, consistent with various reports with IVIG plus ASA. In 2016, Kim et al. studied a large sample of 8,456 children from a retrospective survey of 116 hospitals in South Korea. They reported that compared with low-dose ASA (3–5 mg/kg/day), medium or higher-dose ASA (>30 mg/kg/day) for treatment of acute KD was associated with a higher risk of CAAs after adjustment for cofounders (14). In 2018, a Canadian group reported the results of a multicenter retrospective study (12). They compared the incidence of CAAs with a coronary artery Z score of ≥2.5 between high-dose aspirin (80–100 mg/kg/day) and low-dose aspirin (3–5 mg/kg/day) groups. Non-inferiority for suppression of CAAs in the low-dose group was shown. Our study in a Japanese cohort suggested non-inferiority in the ASA 30-mg Group vs. the ASA 50-mg Group. This result is consistent with a study by a Canadian group, although we used an aspirin dose difference of 20 mg/kg/day in this study.

Ogata et al. reported that 40% of KD patients had CAA with Z score ≥2.5, whereas our results indicated that 12% of patients had CAAs with Z score ≥2.5, which was much lower than the previous report. Ogata et al. collected the maximal internal diameter (mm) of the left anterior descending coronary artery and the right coronary artery within 12 weeks after onset (19). We only collected data at 1 month after onset. This may account for the low incidence of CAAs in this study.

TABLE 2 | Coronary artery outcomes.

(A) Numbers and percentages of patients with CAAs.

	ASA 50-mg Group *n* = 414 number (%)	**ASA 30-mg Group *n* = 173 number (%)**
All	52 (12.6)	18 (10.4)
Small (Z ≥2.5, Z <5.0)	50 (12.1)	14 (8.1)
Medium (Z ≥5.0, Z <10.0)	1 (0.2)	3 (1.7)
Large (Z ≥10.0)	1 (0.2)	1 (0.6)

(B) Unadjusted and adjusted odds ratios.

	Unadjusted		**Adjusted***	
	***p*-value**	**Odds ratio (95% CI)**	***p*-value**	**Odds ratio (95% CI)**
ASA 30-mg Group	0.346	0.760 (0.430–1.345)	0.151	0.769 (0.537–1.101)
Age <12 months	0.006	2.148 (1.243–3.711)	0.003	1.844 (1.237–2.747)
Sex=M	0.935	0.979 (0.592–1.619)	0.455	0.872 (0.609–1.249)
Risk score (Kobayashi)	0.966	0.979 (0.376–2.553)	0.074	1.897 (0.939–3.832)

*****Inverse probability weighting analysis.*

TABLE 3 | Patients needing second IVIG and third-line treatment.

(A) Numbers of patients needing second IVIG and third-line treatment

	ASA 50-mg Group *n* = 414 number (%)	**ASA 30-mg Group *n* = 173 number (%)**
Second IVIG	95 (22.9)	48 (27.7)
Third-line treatment	21 (5.0)	13 (7.5)

(B) Incidence of patients needing second IVIG and third-line treatment.

		Unadjusted		**Adjusted***	
		***p*-value**	**Odds ratio (95% CI)**	***p*-value**	**Odds ratio (95% CI)**
Second IVIG	ASA 30-mg Group	0.112	1.405 (0.923–2.139)	0.021	1.379 (1.051–1.811)
	Age <12 months	0.006	0.457 (0.263–0.795)	0.003	0.579 (0.402–0.835)
	Sex = M	0.066	1.456 (0.975-2.175)	0.079	1.281 (0.971-1.689)
	Risk score (Kobayashi)	<0.001	9.427 (4.105–21.647)	<0.001	9.453 (5.345–16.718)
Third-line treatment	ASA 30-mg Group	0.264	1.534 (0.725–3.246)	0.106	1.512 (0.916–2.495)
	Age <12 months	0.288	0.554 (0.186–1.647)	0.597	0.832 (0.422–1.642)
	Sex=M	0.140	1.780 (0.827-3.830)	0.041	1.716 (1.023-2.878)
	Risk score (Kobayashi)	<0.001	135.399 (17.170–1067.709)	<0.001	256.728 (58.648–1123.812)

*****Inverse probability weighting analysis.*

The incidence of additional treatment in the ASA 30-mg Group was significantly higher than in the ASA 50-mg Group; the difference in incidence was 5%. Studies in Canada and South Korea have shown different incidences of 3 and 6%, respectively (12, 14). In this study, it is important to mention the high IVIG resistance rate. In our institution, non-responders were defined as those with body temperature ≥37.5°C at 48 h after initiation of primary therapy, which is the standard time point in Japan. This time point is different from that of the US, which may explain the high IVIG resistance rate. Akagi et al. and Lee et al. reported that high-dose ASA could shorten the duration of fever in their studies in a Japanese and Korean cohort, respectively (20, 21). Specifically, the duration of fever and the frequency of further treatments are not the same, but our results do not conflict with these previous reports. If the additional treatment is IVIG, the cost of medical care rises and in

TABLE 4A | Safety outcomes.

(A). Hepatic dysfunction.

		ASA 50-mg/kg/day Group *n* =414, mean ± SD	ASA 30-mg/kg/day Group n=173, mean ± SD	*p*-value
Incidence of patients with hepatic dysfunction *[a], %		15%	17%	0.078*[b]
Liver enzymes at convalescent phase	Aspartate aminotransferase	61.6 ± 82.1	54.2 ± 60.5	0.228*[c]
	Alanine aminotransferase	43.7 ± 66.8	33.7 ± 52.7	0.057*[c]

*[a] *Defined as aspartate aminotransferase >50 U/L and alanine aminotransferase >50 U/L at convalescent phase.*
*[b] *Pearson's* χ^2 *test.*
*[c] *Welch's t-test.*

TABLE 4B |

(B). Hemoglobin.

	ASA 50-mg Group, mean (SD)/percentage	ASA 30-mg Group, mean (SD)/percentage	*p*-value
Pre IVIG, g/dL	11.3 ± 1.2	11.6 ± 1.0	0.045*[a]
Post IVIG, g/dL	10.9 ± 1.2	11.1 ± 1.4	0.833*[a]
Difference (post-pre), g/dL	−0.4 ± 1.1	−0.5 ± 1.5	$p = 0.895$*[a]

*[a] *Welch's t-test.*

Japan, the facilities go into deficit. In addition, the hospitalization period is extended and the patient burden increases. Thus, 50 mg/kg/day ASA would be superior to 30 mg/kg/day ASA in terms of medical costs.

Regarding adverse events, Kuo et al. reported a decrease in hemoglobin after IVIG treatment in patients who received high-dose ASA (13). In our Japanese cohort, however, anemia did not worsen in the ASA 50-mg Group, although hemoglobin levels decreased in both groups as previously reported (22). Japanese individuals have limited hepatic metabolism of aspirin, and liver dysfunction is more frequent at the high dose of 80–100 mg/kg/day ASA. Therefore, the high dose is not approved in Japan. The incidence of liver dysfunction was similar between the two groups. There were no severe bleeding events either. In this study period, we had no patients with digestive symptoms. According to Huang et al., digestive symptoms were the most common side effects of ASA therapy (23).

There are several limitations of this study. The first limitation is selection bias in study patients. Patients who were enrolled in a clinical study (16) were excluded from the ASA 30-mg Group. The odds ratio of the ASA 30-mg Group vs. that of the ASA 50-mg Group in the incidence of CAAs was 0.769 (<1.0), whereas that in the incidence of treatment resistance was 1.379 (>1.0). Usually, the two odds ratios would be proportional. Exclusion of patients with severe disease who were enrolled in a clinical study could have influenced the primary endpoint. Second, we excluded patients who had preexisting CAAs before treatment, which was diagnosed according to the Japanese criteria (15). CAAs are underdiagnosed according to the Japanese criteria, and this study may have included patients with CAAs before treatment defined by the AHA guidelines, which diagnose CAA based on a Z score ≥2.5. Third, the aspirin dose difference between the two groups is not large. The government has approved 30–50 mg/kg/day for KD and this observational retrospective study was performed using the study design within this dose range. The 4th limitation is that this is a retrospective and single-center study.

A prospective study is currently underway in Taiwan comparing IVIG plus ASA low-dose and high-dose initial treatment (24). We are eagerly awaiting the results of that study.

In conclusion, the suppression of the development of CAAs, which is the ultimate goal of treatment for KD, might not be inferior with 30 mg/kg/day ASA compared with 50 mg/kg/day in Japanese patients. However, the administration of ASA 50 mg/kg/day could decrease the need for additional treatment without increasing adverse events.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Board, Tokyo Women's Medical University (3636-R2). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HH and TM designed the study. HH reviewed the research and monitored ethical issues. YI analyzed data and wrote the manuscript. JT, KY, TH, and KA did critical review for the manuscript.

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Elevated Levels of Pentraxin 3 Correlate With Neutrophilia and Coronary Artery Dilation During Acute Kawasaki Disease

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Kawasaki disease (KD) is the leading cause of acquired pediatric heart disease in the developed world as 25–30% of untreated patients and at least 5% of treated patients will develop irreversible coronary artery lesions (CAL). Pentraxin-3 (PTX-3) has been well-studied in inflammatory diseases, particularly in cardiovascular diseases associated with vascular endothelial dysfunction. We hypothesized that PTX-3 plays an important role in the development of KD-associated CAL and investigated the circulating levels of PTX-3 in the serum of KD patients. Children with acute KD were followed from diagnosis through normalization of the clinical parameters of inflammation (convalescent phase). Serum samples were obtained and echocardiograms were conducted at several phases of the illness: acute [prior to intravenous immunoglobulin (IVIG) treatment], sub-acute (5–10 days after IVIG treatment), and convalescent (1–4 months after KD diagnosis). Seventy children were included in the final cohort of the study, of whom 26 (37%) presented with CAL and 18 (26%) developed IVIG resistance. The patients included in this study came from diverse ethnic backgrounds, mostly with mixed ancestry/ ethnicity. Significantly increased PTX-3 levels were observed during the acute phase of KD compared to the sub-acute and the convalescent phases. The PTX-3 levels during acute KD were significantly higher among KD patients with CAL compared to patients with normal coronary arteries (NCA). Also, the PTX-3 levels were significantly higher in patients with IVIG resistance. Furthermore, the PTX-3 levels were significantly higher in IVIG-resistant KD patients with CAL as compared to the NCA group. Moreover, the PTX-3 levels were significantly correlated to coronary artery z-score during acute KD and to neutrophil counts throughout KD progression regardless of coronary artery z-score. Elevated PTX-3 levels correlated to elevated neutrophil counts, a known source of PTX-3 in acute inflammation and an important player in the development of KD vasculitis. We, therefore, suggest PTX-3 as a novel factor in the development of KD-associated CAL and propose neutrophil-derived PTX-3 as contributing to KD vascular dysfunction.

Keywords: Kawasaki disease, pentraxin 3, coronary artery lesions, coronary artery dilatation, coronary artery aneurysm, IVIG resistance

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INTRODUCTION

Kawasaki disease (KD) is the leading cause of acquired pediatric heart disease in the developed world, presenting in young children as acute, febrile, self-limiting, systemic vasculitis (1, 2). The clinical and epidemiological features of KD have suggested an infectious cause (3). However, the etiology of the disease remains unknown. Clinically, KD manifests with prolonged high fever, a typical rash, conjunctivitis, and lymphadenopathy (4). The vasculitis in KD particularly affects the coronary arteries. If not recognized and treated within the first 7–10 days of illness, there is a 25% chance of lasting damage to the coronary arteries, often with aneurysm formation, which can lead to death due to coronary artery thrombosis or rupture (5). The case fatality rate for KD in Japan is $<0.01\%$ (6). Even with prompt recognition and recommended treatment—intravenous immunoglobulin (IVIG) and high-dose aspirin—30% of KD children develop transient coronary artery lesions (CAL), and about 5% have persistent CAL or aneurysms after recovery from the illness. Persistent coronary damage from KD can cause serious complications, including death (7–10).

A specific diagnostic test for timely and accurate identification of KD would be a boon to clinicians, facilitating early treatment and reduction of the risk for development of coronary involvement. Previous studies have demonstrated the risk for CAL in KD patients who present with higher baseline coronary artery dimensions, exhibit IVIG resistance (persistent fever for more than 24 h after initial IVIG infusion), or have a delay in diagnosis causing a delay in treatment (11–15). Several algorithms have been developed in Japan to predict IVIG resistance and subsequent coronary damage; these include the Kobayashi (16), Egami (17), Sano (18), and Harada (19) scoring systems. These incorporate demographic, clinical, and laboratory parameters. While these scoring systems are useful in the Japanese population, their efficacy is lost in populations outside of Japan (4, 20). As a result, clinicians in other parts of the world do not uniformly use these Japanese scoring systems. Son and colleagues described the great need for improved methods/scoring systems to identify KD patients at risk for coronary aneurysms, demonstrating the high predictive value of a maximum z-score of ≥ 2.0 (20).

Current theories of KD pathogenesis include (i) infection with classic immune responses to an as-yet identified pathogen(s), (ii) an autoantibody or T-cell driven autoimmune response triggered by an antigen, i.e., molecular mimicry, and (iii) an autoinflammatory response, which includes innate immune responses that cause systemic inflammation as well as damage to the coronary arterial wall (3). Previous studies have focused on cytokines and chemokines during acute KD. However, these proteins are relatively non-specific and are elevated in many inflammatory processes (21, 22).

PTX-3 is a member of the pentraxin protein family, a class of soluble pattern recognition receptors (PRR), which includes C-reactive protein (CRP), a widely used laboratory parameter of clinical inflammation, and serum amyloid P component (23). This family of proteins plays integral roles in complement activation, amplification, and regulation (24). PTX-3 is thought to have a role in several processes in the cardiovascular system, including inflammation, angiogenesis, tumorigenesis, and cell adhesion (25). PTX-3 is a soluble PRR associated with the local activation of the innate immune system and inflammation. Studies of adult coronary artery disease have identified PTX-3 as a predictor of all-cause mortality, cardiac death, and cardiac events (26). High levels of immune and endothelial cell-derived PTX-3 have been associated with coronary artery dysfunction and adverse outcomes, with correlations to CRP and MMP9 levels (27). PTX-3 is also associated with vascular endothelial dysfunction and morphological alterations through impairment of the nitric oxide pathway (28). From these studies, we hypothesized that PTX-3 might play a role in KD pathogenesis and could be employed as a diagnostic or prognostic marker of KD. We investigated the circulating PTX-3 levels in the serum of KD patients with and without CAL. Here we report elevated levels of PTX-3 during the acute phase of KD, particularly in association with CAL. These data suggest a role for PTX-3 in KD pathogenesis, specifically the vascular damage that leads to CAL in the most severe cases of KD.

MATERIALS AND METHODS

Study Population and Laboratory Evaluation

All children recruited in this study were admitted to the KMCWC in Honolulu, Hawai'i, between October 2013 and November 2018. The children were evaluated by experienced clinicians at KMCWC and fulfilled the diagnostic criteria for KD as endorsed by the American Heart Association (AHA). All patients were treated, per AHA guidelines, with IVIG and high-dose acetylsalicylic acid as primary treatment. IVIG resistance was defined as patients having a persistent or recurrent fever for more than 36 h after completion of the initial IVIG treatment. Serum was separated within 24 h of blood collection and stored at -80°C until further analysis. The serum samples for research were collected from a total of 70 patients at three phases: 62 patients at the time of KD diagnosis/disease onset and prior to IVIG treatment (acute phase), 65 patients at 1 to 2 weeks following IVIG treatment (sub-acute phase), and 64 patients at 3 weeks to 4 months following disease onset (convalescent phase). There were 53 patients whose samples were obtained at all three phases. Convalescence was defined by the normalization of the clinical laboratory features of KD such as elevated erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, and white blood cell (WBC) counts. In addition to these parameters, echocardiogram measurements and additional clinical laboratory data [i.e., ESR, CRP, complete blood count with differential hemoglobin (Hgb), hematocrit (Hct), red blood cell (RBC) count, WBC count, absolute lymphocyte count (ALC), absolute neutrophil count (ANC), absolute eosinophil count, absolute basophil count, absolute monocyte count (MO), and platelet (PLT) count] were evaluated at each phase of the disease. The echocardiogram measurements were collected for all patients at each phase of the disease. However, not all clinical laboratory

parameters were captured at each phase of the disease in all patients.

Echocardiogram Measurements

A complete echocardiogram was conducted as part of the standard diagnostic evaluation of KD for all patients at each phase of the disease. The inner diameter of the right coronary artery (RCA) and the left anterior descending coronary artery (LAD) was measured in the modified parasternal views, and the z-score of the coronary artery diameters was calculated based on the patient's body surface area using the Boston dataset for standards (29). CAL was defined as having either an RCA or LAD z-score $\geq$2.5 at any phase of KD (30).

Protein Expression Analysis

Circulating levels of PTX-3 were measured in the patient's serum samples by MILLIPLEX MAP Human Cardiovascular Disease Multiplex Assay (Cat. No. HCVD4MAG-67K; MilliporeSigma, Burlington, MA, USA) following the manufacturer's instructions. The patient's samples were tested in duplicate. Plates were analyzed on a Luminex 200 system (Luminex Corp., Austin, TX, USA) and data were analyzed using xPONENT 3.1 (Luminex Corp.) and Milliplex Analyst 5.1 Software (MilliporeSigma).

Statistical Analysis

The patient's clinical and demographic characteristics were summarized by descriptive analysis such as median and interquartile range (IQR) for continuous variables and frequency and percentage for categorical variables. Repeated-measures analysis of variance (ANOVA) was used to characterize how clinical laboratory parameters and PTX-3 levels change over the course of KD pathogenesis (i.e., acute, sub-acute, and convalescence) (within-factor) and CAL (between-factor), accounting for within-subject dependence. Model diagnostics were explored (e.g., *Q*–*Q* plot and residual plots) and the outcome variables were transformed by natural logarithm to satisfy the model assumption (normality and constant variance). With the final model, Tukey's *post-hoc* test was conducted to compare the different phases. Spearman's correlation was computed to assess bivariate associations between PTX-3 and coronary artery z-score or clinical laboratory variables at each phase of KD. Fisher's exact test was used to evaluate the IVIG response group effect and computed odds ratio. Repeated-measures ANOVA, Spearman's correlations, and Fisher's exact test were conducted using GraphPad Prism version 8.4.0 (GraphPad Software, San Diego, CA, USA). To assess the association between immune factors and laboratory variables over the clinical course of KD, repeated-measures correlations were calculated using the package (31) in R version 3.5.3. Repeated-measures correlation assesses the association between paired variables measured multiple times within subjects. A heat map was generated to depict repeated-measures and Spearman's correlation. The number in each cell in the heat map indicates the repeated-measures correlation between the column and the row variables. The cells in shades of red show positive correlations and the cells in shades of blue show negative correlations.

RESULTS

Clinicoepidemiological Characteristics of KD Patients

A total of 222 patients were eligible and 70 patients were enrolled in this study. The patient population consists of an almost equal number of boys (n = 34, 49%) and girls (n =36, 51%), with 86% (n =60) under 5 years of age (**Table 1**). The study population includes a variety of self-reported whole or partial ethnic backgrounds, with the majority of patients from Asian ancestry (n =58, 83%), and half of the patients reported two or more ethnicities (up to five) (n =35, 50%) (**Table 1**). Most patients (n = 68, 97%) received primary treatment within the first 10 days of onset of fever; IVIG resistance was observed in 18 patients (26%) (**Table 1**).

This study included 26 (37%) patients who presented with CAL (**Table 1**). Among patients with CAL, maximal coronary artery z-scores occurred most frequently during the acute phase [n = 15; 3.96 (3.27–4.35)], with fewer during the sub-acute phase [n = 11; 4.38 (3.81–7.40)], and none at the convalescent phase. Clinicoepidemiological characteristics were reported among patients with normal coronary arteries (NCA) and CAL. We did not observe any significant differences in clinicoepidemiological characteristics among the NCA and the CAL groups, with the exception of IVIG resistance placing children at a higher risk for CAL (OR = 7.8; 95% CI 2.38–23.54, $p < 0.001$). However, in our study population, males accounted for more of the CAL patients (n = 16, 62%) as compared to females (n = 10, 38%), and White children were less susceptible to CAL (n = 7, 27%) as compared to non-White children (n = 19; 73%).

Elevated Levels of PTX-3 During Acute KD

The KD patients were evaluated for circulating levels of PTX-3 throughout the clinical course of the disease. This analysis revealed a significant increase in PTX-3 levels in the acute (5.87 $\pm$ 1.76 pg/ml, 0.08–84.53) as compared to the sub-acute (0.58 $\pm$ 0.10 pg/ml, 0.05–3.84) and the convalescent (0.54 $\pm$ 0.82 pg/ml, 0.04–7.18) KD phases (**Figure 1**, **Table 2**). The CRP levels and the WBC and the ANC counts peaked in the acute phase and returned to normal levels in the convalescent phase. The ESR levels were elevated in the acute phase, peaked in the sub-acute phase, and returned to normal in the convalescent phase. Furthermore, red blood cell parameters such as RBC, Hgb, and Hct and MO count significantly decreased in the acute phase and peaked in the convalescent phase. The ALC and PLT counts peaked in the sub-acute phase. The lymphocyte count remained elevated in the convalescent phase (**Table 2**, **Supplementary Figure 1**).

Increased Coronary Artery Z-Score Is Associated With Elevated Levels of PTX3 During Acute KD

We analyzed the PTX-3 levels in KD patients who developed CAL and compared them to KD patients with NCA. The KD patients who presented with a coronary artery z-score $\geq$2.5 in either LAD or RCA at any phase of the disease were assigned to the CAL group (n = 26), and all other KD patients were included

TABLE 1 | Patients' clinicoepidemiological characteristics.

Variables	All ($n = 70$)		Normal coronary arteries ($n = 44$)		Coronary artery lesions ($n = 26$)	
Gender						
Male	$n = 34$	(49%)	$n = 18$	(41%)	$n = 16$	(62%)
Female	$n = 36$	(51%)	$n = 26$	(59%)	$n = 10$	(38%)
Age in years, median (IQR)	2.63 (1.00–3.90)		2.25 (0.83–3.46)		3.21 (1.33–4.63)	
<1	$n = 17$	(24%)	$n = 12$	(27%)	$n = 5$	(19%)
1–5	$n = 43$	(61%)	$n = 28$	(64%)	$n = 15$	(58%)
>5	$n = 10$	(14%)	$n = 4$	(9%)	$n = 6$	(23%)
Ethnicity[a]						
Asian	$n = 58$	(83%)	$n = 37$	(84%)	$n = 21$	(81%)
NHOPI	$n = 30$	(43%)	$n = 19$	(43%)	$n = 11$	(42%)
White	$n = 29$	(41%)	$n = 22$	(50%)	$n = 7$	(27%)
Hispanic or Latino	$n = 5$	(7%)	$n = 3$	(7%)	$n = 2$	(8%)
AI/AN	$n = 2$	(3%)	$n = 1$	(2%)	$n = 1$	(4%)
Black or African American	$n = 2$	(3%)	$n = 2$	(5%)	$n = 0$	(0%)
Other	$n = 1$	(1%)	$n = 1$	(2%)	$n = 0$	(0%)
Two or more ethnicities	$n = 35$	(50%)	$n = 22$	(50%)	$n = 13$	(50%)
Clinical features						
Duration of fever in days, median (IQR)	5.00 (4.00–7.00)		5.00 (4.00–6.00)		6.00 (5.00–8.00)	
Interval between fever onset and treatment administration in days, median (IQR)	5.00 (4.00–6.00)		4.00 (3.00–6.00)		5.00 (4.00–8.00)	
IVIG treatment within 10 days of fever onset	$n = 68$	(97%)	$n = 43$	(98%)	$n = 25$	(96%)
IVIG resistance	$n = 18$	(26%)	$n = 5$	(11%)	$n = 13$	(50%)
CAL z-score ≥2.50[b]	$n = 26$	(37%)	$n = 0$	(0%)	$n = 26$	(100%)
CAL z-score[b], median (IQR)	4.23 (3.37–4.7)		1.48 (1.12–1.87)		4.16 (3.35–4.70)	

SEM, standard error mean; IQR, interquartile range; AI/AN, American Indian/American native; NHOPI, Native Hawaiian or Pacific Islander; Other, other not specified.
[a]*Can have multiple ethnicities.*
[b]*Number of children whose z-score of the right coronary artery or the left anterior descending artery is equal or >2.5 at either acute or sub-acute visits.*

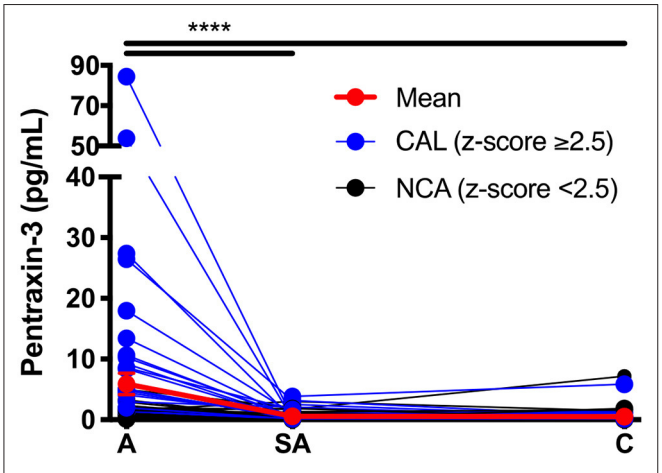

FIGURE 1 | Circulating levels of PTX-3 throughout Kawasaki disease (KD) progression. Repeated-measures ANOVA was used to compare the PTX-3 levels between the three phases of KD. The black lines track the KD patients with normal coronary artery ($n = 44$), the blue lines track the KD patients with coronary artery lesion ($n = 26$), and the red line tracks the mean ± SEM of all the patient serum samples ($n = 70$) evaluated at each phase of the disease. A, acute ($n = 62$); SA, sub-acute ($n = 65$); C, convalescent ($n = 64$); SEM, standard error mean. ***$p \leq 0.001$.

in the NCA group ($n = 44$). During the acute phase, the PTX-3 levels (14.28 ± 4.44 pg/ml, 1.37–84.53) in the CAL group were significantly higher as compared to the NCA group (1.22 ± 0.21 pg/ml, 0.08–16.01) (**Table 3**). There was no significant difference in the PTX-3 levels between the CAL and the NCA groups at the sub-acute and the convalescent KD phases (**Figure 2A**, **Table 3**). The PTX-3 levels during acute KD in the NCA group remained significantly elevated as compared to the same group's convalescent phase (**Table 3**). When we compared the PTX-3 levels with the maximal coronary artery z-score at each phase of KD (largest coronary artery diameter of either LAD or RCA), we observed a significant correlation between the PTX-3 levels and the CA z-score during the acute KD phase ($r_s = 0.529$, 95% CI 0.304–0.698). However, this relationship was lost at the sub-acute and the convalescent KD phases (**Figures 2B–D**).

Subsequently, we compared the clinical laboratory parameters in CAL *vs.* NCA groups. None of the parameters evaluated revealed any significant differences between these two patient groups (**Table 3**, **Supplementary Figure 2**). The group stratification, however, did reveal some trends among the clinical laboratory parameters. The mean ESR, CRP, and ANC levels were higher in the CAL group as compared to the NCA group in the acute phase. Mean Hgb, Hct, RBC, and WBC levels were

TABLE 2 | PTX-3, coronary artery z-scores, and clinical assessment of inflammation throughout Kawasaki disease (KD) progression.

	Acute ($n = 70$)		***n***	**Sub-acute ($n = 69$)**		***n***	***P*[a]**	**Convalescent ($n = 66$)**		***n***	***P*[b]**
PTX-3 (pg/ml)	5.87 ± 1.76	(0.08–84.53)	62	0.58 ± 0.10	(0.05–3.84)	65	***	0.54 ± 0.82	(0.04–7.18)	64	***
Right coronary artery z-score	1.63 ± 0.38	(−2.35–20.83)	70	1.55 ± 0.43	(−2.40–24.03)	69	ns	1.02 ± 0.47	(−1.92–2.25)	66	**
Left anterior descending coronary artery z-score	2.40 ± 0.50	(−3.00–26.24)	70	2.20 ± 0.69	(−3.00–36.30)	69	*	1.62 ± 0.74	(−2.25–37.23)	66	***
CA z-score MAX	2.75 ± 0.49	(−2.35–26.24)	70	2.72 ± 0.68	(−2.34–36.30)	69	ns	2.03 ± 0.72	(−1.00–37.23)	66	***
Erythrocyte sedimentation rate (mm/h)	78.38 ± 3.40	(28.02–120.0)	68	85.32 ± 3.85	(6.00–120.0)	66	ns	14.39 ± 1.69	(1.00–84.00)	66	***
C-reactive protein (mg/L)	110 ± 10.16	(84.39–427.0)	69	7.45 ± 1.23	(0.00–42.60)	66	***	1.14 ± 0.28	(0.00–11.40)	60	***
Hemoglobin (g/dl)	10.97 ± 0.13	(1.11–13.50)	70	10.71 ± 0.13	(8.30–13.30)	67	ns	12.16 ± 0.12	(8.90–14.80)	66	***
Hematocrit (g/dl)	32.60 ± 0.36	(3.00–38.40)	69	32.16 ± 0.37	(25.00–38.50)	67	ns	36.21 ± 0.32	(29.20–43.20)	66	***
Red blood cell (10^{12}/L)	4.09 ± 0.05	(0.41–4.99)	69	3.99 ± 0.05	(2.89–4.90)	66	ns	4.55 ± 0.05	(3.69–5.35)	66	***
White blood cell (10^9/L)	14.97 ± 0.75	(6.24–42.00)	70	11.66 ± 0.52	(4.20–25.60)	67	***	9.00 ± 0.31	(3.50–17.50)	66	***
Absolute lymphocyte count (10^9/L)	3.11 ± 0.23	(1.86–8.40)	63	5.70 ± 0.27	(1.89–11.10)	65	***	5.19 ± 0.27	(1.41–11.55)	64	***
Absolute neutrophil count (10^9/L)	11.39 ± 1.10	(8.83–63.20)	64	4.90 ± 0.47	(0.67–17.66)	63	***	3.01 ± 0.21	(0.74–8.78)	65	***
Absolute eosinophil count (10^9/L)	0.40 ± 0.05	(0.35–1.38)	49	0.44 ± 0.05	(0.01–1.66)	57	ns	0.38 ± 0.05	(0.03–2.28)	61	ns
Absolute basophil count (10^9/L)	0.07 ± 0.01	(0.08–0.41)	29	0.11 ± 0.02	(0.02–0.37)	22	ns	0.08 ± 0.01	(0.02–0.20)	40	ns
Monocyte (10^9/L)	0.76 ± 0.07	(0.54–2.80)	61	0.75 ± 0.06	(0.10–2.02)	63	ns	2.39 ± 0.39	(0.07–14.00)	65	**
Platelet (10^9/L)	383 ± 15.79	(132.1–699.0)	70	633 ± 22.57	(302.0–1,365)	67	***	380 ± 13.9	(207.0–743.0)	66	ns

Mean ± SEM (range) evaluated for the indicated variable of all the patient serum samples (n = 70) at the acute, sub-acute, and convalescent phases of the disease. Repeated-measures ANOVA was used to compare the coronary artery z-scores and the clinical laboratory parameters between the three phases of KD. p-value comparing the effect of the variable at each phase.

[a]*p-value between acute and sub-acute phases.*

[b]*p-value between acute and convalescent phases.*

****$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$.*

SEM, standard error mean; CA, coronary artery; CA z-score MAX, largest coronary artery diameter between RCA and LAD z-scores; ns, not significant ($p > 0.05$).

TABLE 3 | PTX-3, coronary artery z-scores, and clinical assessment of inflammation throughout Kawasaki disease (KD) progression in patients with normal coronary arteries (NCA) and in patients who develop coronary artery lesions (CAL).

	NCA (z-score < 2.5) (n = 44)										CAL (z-score ≥ 2.5) (n = 26)										NCA *vs.* CAL
	Acute (n = 44)		n	Sub-acute (n = 44)		n	P^a	Convalescent (n = 41)		n	P^b	Acute (n = 26)		n	Sub-acute (n = 26)		n	P^a	Convalescent (n = 28)		n	P^b	P
PTX-3 (pg/ml)	1.22 ± 0.21	(0.08–16.01)	38	0.48 ± 0.11	(0.05–3.03)	40	***	0.46 ± 0.18	(0.04–7.18)	42	***	14.28 ± 4.44	(1.37–84.53)	21	0.73 ± 0.19	(0.10–3.84)	26	***	0.67 ± 0.23	(0.08–5.85)	25	***	***c, nsd,e
Right coronary artery z-score	0.66 ± 0.16	(−2.35–2.19)	44	0.47 ± 0.16	(−2.40–1.84)	44	ns	0.17 ± 0.14	(−1.92–2.23)	41	ns	3.27 ± 0.9	(−0.50–20.83)	26	3.39 ± 1.04	(−0.35–24.03)	26	ns	2.39 ± 1.19	(−1.25–25.77)	26	ns	*c,d,nse
Left anterior descending coronary artery z-score	0.89 ± 0.17	(−3.00–2.50)	44	0.37 ± 0.17	(−3.00–2.44)	44	ns	0.26 ± 0.16	(−2.25–2.17)	41	ns	4.95 ± 1.18	(−0.60–26.24)	26	5.31 ± 1.69	(−0.09–36.30)	26	ns	3.84 ± 1.87	(−1.01–37.23)	26	***	***c,d, nse
CA z-score MAX	1.18 ± 0.15	(−2.35–2.5)	44	0.89 ± 0.14	(−2.34–2.44)	44	ns	0.69 ± 0.11	(−1.00–2.23)	41	***	5.42 ± 1.13	(1.73–26.24)	26	5.82 ± 1.65	(0.85–36.30)	26	ns	4.21 ± 1.84	(0.48–37.23)	26	***	***c,d,e
Erythrocyte sedimentation rate (mm/h)	75.89 ± 4.26	(14.00 – 120.0)	44	84.35 ± 4.92	(6.00–120.0)	40	ns	13.2 ± 1.8	(1.00–59.00)	41	***	82.96 ± 5.63	(19.00–120.0)	24	86.81 ± 6.32	(20.00–120.0)	26	ns	16.36 ± 3.36	(1.00–84.00)	25	***	nsc,d,e
C-reactive protein (mg/L)	107.6 ± 12.8	(1.80–427.5)	44	7.26 ± 1.6	(0.00–42.60)	41	***	0.94 ± 0.31	(0.00–8.50)	36	***	114.7 ± 16.9	(7.40–310.7)	25	7.76 ± 1.94	(0.30–37.5)	25	***	1.45 ± 0.54	(0.00–11.40)	24	***	nsc,d,e
Hemoglobin (g/dl)	11.13 ± 0.16	(8.80–13.50)	44	10.91 ± 0.18	(8.60–13.30)	41	ns	12.14 ± 0.17	(8.90–14.80)	41	***	10.71 ± 0.23	(8.20–12.50)	26	10.41 ± 0.2	(8.30–12.00)	26	ns	12.19 ± 0.18	(10.50–13.60)	25	***	nsc,d,e
Hematocrit (g/dl)	33.15 ± 0.42	(27.10–38.40)	44	32.69 ± 0.47	(27.30–38.50)	41	ns	36.2 ± 0.45	(29.20–43.10)	41	***	31.64 ± 0.64	(25.60–36.30)	25	31.33 ± 0.59	(25.00–36.90)	26	ns	36.23 ± 0.44	(31.00–40.40)	25	***	nsc,d,e
Red blood cell (10^{12}/L)	4.17 ± 0.06	(3.23–4.99)	44	4.08 ± 0.06	(3.14–4.90)	41	ns	4.52 ± 0.06	(3.69–5.35)	41	***	3.95 ± 0.09	(3.09–4.55)	25	3.85 ± 0.09	(2.89–4.49)	25	ns	4.6 ± 0.07	(3.87–5.14)	25	***	nsc,d,e
White blood cell (10^9/L)	15.59 ± 0.97	(7.80–42.00)	44	12.25 ± 0.69	(4.20–25.60)	41	**	9.2 ± 0.41	(5.20–17.50)	41	***	13.92 ± 1.15	(3.10–28.10)	26	10.73 ± 0.77	(5.00–21.00)	26	ns	8.67 ± 0.45	(3.50–13.60)	25	***	nsc,d,e
Absolute lymphocyte count (10^9/L)	3.39 ± 0.28	(0.39–7.17)	40	5.93 ± 0.35	(1.89–11.10)	40	***	5.41 ± 0.35	(2.14–11.55)	40	***	2.62 ± 0.4	(0.44–8.40)	23	5.32 ± 0.41	(2.32–8.69)	25	***	4.83 ± 0.4	(1.41–8.36)	24	***	nsc,d,e
Absolute neutrophil count (10^9/L)	10.76 ± 1.02	(1.64–35.70)	40	5.13 ± 0.66	(0.84–17.66)	38	***	2.91 ± 0.28	(0.74–8.78)	40	***	12.43 ± 2.43	(1.09–63.20)	24	4.56 ± 0.66	(0.67–13.23)	25	***	3.17 ± 0.3	(0.78–7.07)	25	***	nsc,d,e
Absolute eosinophil count (10^9/L)	0.47 ± 0.07	(0.03–1.38)	31	0.5 ± 0.07	(0.07–1.66)	35	ns	0.42 ± 0.07	(0.03–2.28)	37	ns	0.28 ± 0.06	(0.01–0.91)	18	0.35 ± 0.06	(0.01–1.21)	22	ns	0.31 ± 0.04	(0.04–0.92)	24	ns	nsc,d,e
Absolute basophil count (10/L)	0.07 ± 0.02	(0.02–0.41)	21	0.13 ± 0.03	(0.02–0.37)	13	ns	0.08 ± 0.01	(0.02–0.20)	24	ns	0.07 ± 0.02	(0.03–0.18)	8	0.07 ± 0.01	(0.03–0.10)	9	ns	0.08 ± 0.01	(0.03–0.19)	16	ns	nsc,d,e
Monocyte (10^9/L)	0.78 ± 0.08	(0.12–2.45)	39	0.85 ± 0.08	(0.11–2.02)	39	ns	2.55 ± 0.5	(0.15–14.00)	40	ns	0.72 ± 0.12	(0.11–2.80)	22	0.58 ± 0.06	(0.10–1.09)	24	ns	2.15 ± 0.62	(0.07–13.00)	25	ns	nsc,d,e
PLT (10^9/L)	380 ± 16.9	(150.0–650.0)	44	642.9 ± 23.4	(381.0–1,014)	41	***	380.9 ± 15.9	(228.0–743.0)	41	ns	388.1 ± 31.9	(68.00–699.0)	26	616.9 ± 45.5	(302.0–1,365)	26	***	379.8 ± 26.2	(207.0–737.0)	25	ns	nsc,d,e

Mean ± SEM (range) evaluated for the indicated variable of all the patient serum samples (n = 70) at the acute, sub-acute, and convalescent phases of the disease stratified by NCA and CAL. Repeated-measures ANOVA was used to compare the coronary artery z-scores and the clinical laboratory parameters between the three phases of KD. p-value comparing the effect of the variable at each phase.

[a]*p-value between acute and sub-acute phases.*

[b]*p-value between acute and convalescent phases.*

[c]*p-value comparing the acute NCA and the acute coronary artery lesion groups.*

[d]*p-value comparing the sub-acute NCA and the sub-acute coronary artery lesion groups.*

[e]*p-value comparing the convalescent NCA and the convalescent coronary artery lesion groups.*

$^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

SEM, standard error mean; CA, coronary artery; CA z-score MAX, largest coronary artery diameter between the right coronary artery and the left anterior descending coronary artery z-scores; ns, not significant (p > 0.05).

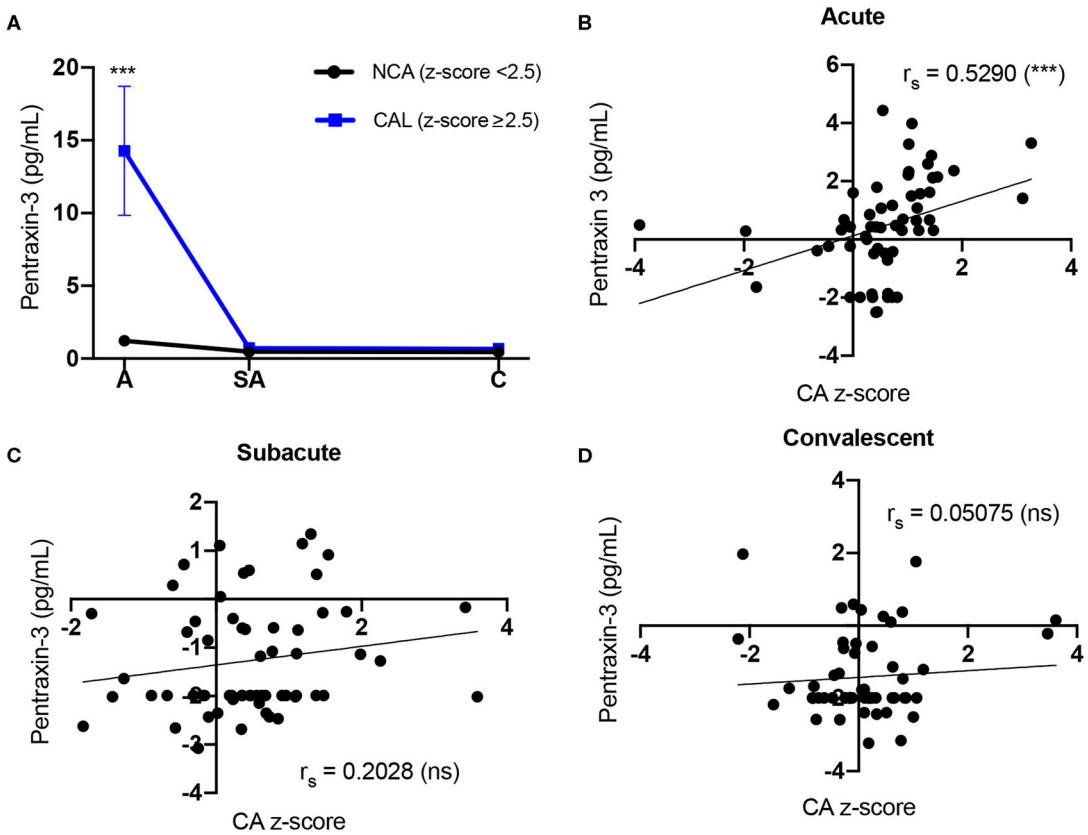

FIGURE 2 | Comparison of Kawasaki disease (KD) patients' PTX-3 levels and coronary artery z-score. **(A)** Circulating PTX-3 levels in KD patients who develop coronary artery lesions (CAL) (z-score ≥ 2.5) and patients with normal coronary artery (NCA) (z-score < 2.5). Repeated-measures ANOVA was used to evaluate group differences (i.e., CAL and NCA groups) of PTX-3 levels at the three phases of KD. Each graph represents the mean ± SEM for all the KD patients in the NCA (black line; $n = 44$, $A = 39$, SA = 40, $C = 40$) or CAL (blue line; $n = 26$, A = 23, SA = 25, C = 24) groups. A, acute; SA, sub-acute; C, convalescent; SEM, standard error mean. **(B–D)** Spearman correlation between KD patients' circulating levels of PTX-3 and coronary artery z-score at the acute **(B)**, sub-acute **(C)**, and convalescent **(D)** phases of KD. The black line represents the best-fit linear regression between circulating PTX3 levels and coronary artery z-score. The variables were not normally distributed and thus were transformed by natural logarithm for analysis and plotting to reduce variance and satisfy model assumptions. r_s, Spearman correlation analysis *r* value; A, acute ($n = 62$); SA, sub-acute ($n = 65$); C, convalescent ($n = 64$); CA, coronary artery; SEM, standard error mean; ns, not significant ($p > 0.05$). ***$p \leq 0.001$.

lower in the CAL group as compared to the NCA group in the acute phase (**Table 3**, **Supplementary Figure 2**).

PTX-3 Levels Are Highly Correlated to Neutrophil Counts in KD

To evaluate the role of PTX-3 in the inflammatory process of KD, we correlated PTX-3 to the clinical markers of inflammation. Longitudinal correlations between PTX-3 and clinical laboratory parameters by repeated-measures correlations (r_{rm}) demonstrated a significant, strong positive correlation between PTX-3 and ESR, CRP, WBC, and ANC and significant, strong negative correlations with Hct, ALC, and PLT (**Figure 3**). At the individual phases of KD, we observed weaker Spearman correlations (r_s) between PTX-3 and the clinical laboratory parameters (**Figure 3**). Moderately positive r_s correlations were observed between PTX-3 and CRP and ANC during the acute phase, weak positive r_s correlations were observed between PTX-3 and ANC, Hgb, and MO during the sub-acute phase, and no r_s correlations were observed in the convalescent phase (**Figure 3**).

PTX-3 Levels and Clinical Laboratory Parameters of Inflammation Have Strong Positive Correlations in KD Patients With CAL

To further investigate the1 differences in PTX-3 levels between KD patients with and without CAL, we stratified the data between the CAL and the NCA patient groups and then conducted a correlation analysis between PTX-3 and the clinical laboratory parameters of inflammation (**Figure 4**). Stratifying the KD patients by coronary artery z-score to evaluate correlations between PTX-3 levels and thee clinical laboratory parameters of inflammation revealed differences in the repeated-measures correlation (r_{rm}) and Spearman's correlation (r_s) analyses between the two groups. Overall, the r_{rm} and r_s correlations were stronger in the CAL group. Similar to the un-stratified analysis, the r_{rm} analysis in both the NCA and the CAL groups showed a significant, strong positive correlation between PTX-3 and ESR, CRP, WBC, and ANC and significant, strong negative correlations with ALC and PLT (**Figure 4**). In the CAL group,

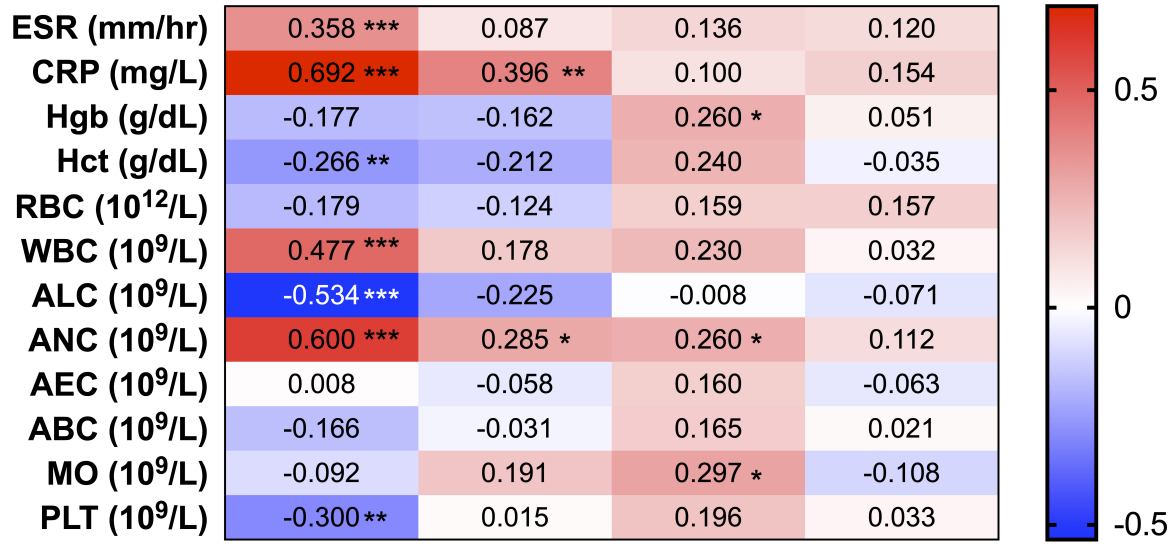

FIGURE 3 | Correlations between PTX-3 and clinical measures of inflammation (i.e., C-reactive protein, erythrocyte sedimentation rate, and complete blood count). The number in each cell in the heat map indicates the repeated-measures correlation analysis (r_{rm}) between PTX-3 and the clinical laboratory parameters of inflammation at all phases of Kawasaki disease (KD) and Spearman's correlation (r_s) analysis between PTX-3 and the clinical laboratory parameters of inflammation at the acute (A r_s), sub-acute (SA r_s), and convalescent (C r_s) phases of KD. The red cells show positive correlations and the blue cells show negative correlations. $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

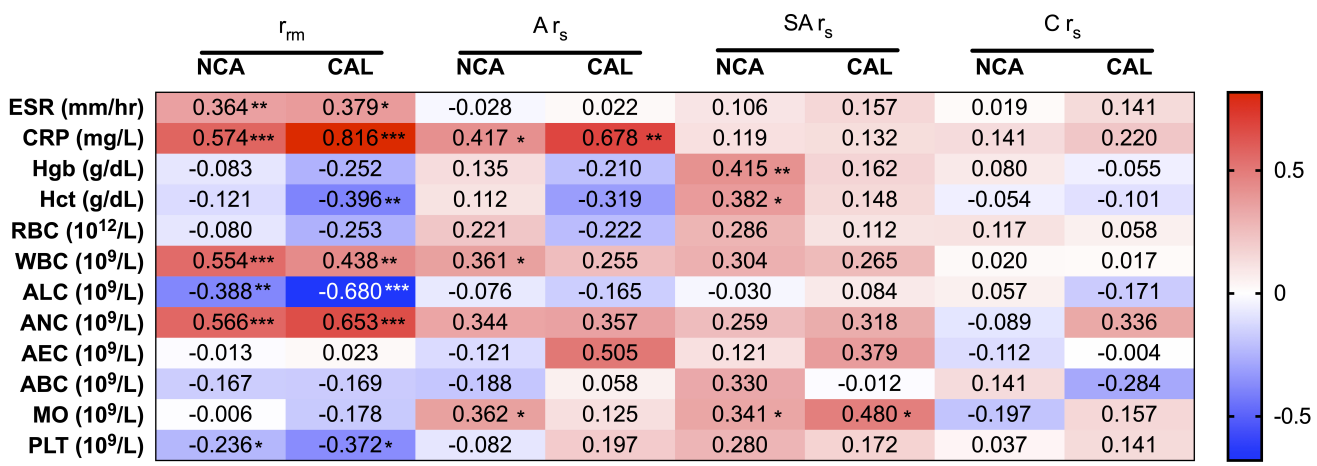

FIGURE 4 | Correlations between PTX-3 and the clinical laboratory parameters of inflammation (i.e., C-reactive protein, erythrocyte sedimentation rate, and complete blood count) stratified by normal coronary artery (z-score < 2.5) and coronary artery lesion (z-score ≥ 2.5) groups. The number in each cell in the heat map indicates the repeated-measures correlation (r_{rm}) analysis between PTX-3 and the clinical assessment of inflammation at all phases of Kawasaki disease (KD) and Spearman's correlation (r_s) analysis between PTX-3 and the clinical laboratory parameters of inflammation at the acute (A r_s), sub-acute (SA r_s), and convalescent (C r_s) phases of KD. The red cells show positive correlations and the blue cells show negative correlations. $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

there was a strong negative r_{rm} between PTX-3 and Hct, which was not observed in the NCA group (**Figure 4**). During the acute phase, r_s analysis between PTX-3 and CRP remained significantly positive in both groups. Analysis of the acute phase NCA group by r_s revealed weak positive relationships between PTX-3 and ALC and MO. Similarly, analysis of the sub-acute phase revealed positive r_s between PTX-3 and MO in both the NCA and the CAL groups. In the NCA group, sub-acute phase r_s analysis between PTX-3 and Hgb and Hct showed weak positive relationships. Furthermore, there were no r_s between PTX-3 and the clinical parameters of inflammation in the convalescent phase for the NCA and the CAL groups (**Figure 4**).

Elevated Levels of PTX-3 and CAL Is Associated With IVIG Resistance

IVIG resistance is a well-known risk factor for CAL. Our study population included 18 (26%) IVIG-resistant patients (**Table 1**), of whom 13 (72%) also exhibited CAL. Fisher's exact test confirmed that IVIG resistance was a risk factor for CAL (OR = 7.8; 95% CI 2.38–23.54, $p < 0.001$) in this study. Comparisons of the coronary artery z-scores between IVIG-responsive and IVIG-resistant patients demonstrated significantly larger coronary artery z-scores throughout KD progression among IVIG-resistant patients as compared to IVIG-responsive patients (**Figure 5A**, **Table 4**). These differences were more pronounced by further stratification of IVIG resistance among KD patients with NCA and CAL. The coronary artery z-scores among IVIG-resistant KD patients with CAL were significantly larger than those of KD patients responsive to IVIG treatment at all phases of the disease (**Figure 5B**, **Table 4**). There were no statistically significant differences in the coronary artery z-score among IVIG-resistant groups with NCA or CAL.

Next, we evaluated the IVIG response group effects on the circulating levels of PTX-3 (**Figure 5C**, **Table 4**). The PTX-3 levels were significantly elevated during acute KD in IVIG-resistant patients as compared to those IVIG-responsive patients (**Figure 5D**, **Table 4**). There were no statistically significant differences in the PTX-3 levels among IVIG-responsive and IVIG-resistant KD patients at the sub-acute and the convalescent phases of the disease. The PTX-3 levels were further stratified in the NCA and the CAL groups among the IVIG-resistant groups. The PTX-3 levels during the acute phase were significantly higher in KD patients who were IVIG resistant with CAL as compared to those in KD patients who were IVIG resistant with NCA and in KD patients responsive to IVIG treatment. However, there were no significant differences between KD patients resistant to IVIG with NCA and KD patients responsive to IVIG treatment ($p = 0.99$) (**Figure 5E**, **Table 4**). An investigation of the effect of IVIG response in NCA and CAL patients on ANC did not reveal any statistically significant differences (**Figures 5F,G**, **Table 4**).

DISCUSSION

PTX-3 is produced in response to proinflammatory signals and microbial stimulation and by a wide variety of immune and endothelial cells with roles in the regulation of inflammation and complement activation as well as in vascular inflammation and endothelial cell dysfunction (23, 32). Elevated levels of PTX-3 have been found in septic shock (33, 34), chronic kidney disease (35), stroke (36), and a variety of cardiovascular diseases (25, 37, 38). In younger populations, elevated levels of PTX-3 have been associated with neonatal sepsis (39), severe pediatric microbial infections (40, 41), and autoimmune diseases, such as childhood-onset systemic lupus erythematosus (42), juvenile idiopathic arthritis (43), and asthma (44). In this study, we found significantly elevated circulating levels of PTX-3 in a cohort of patients with acute KD as compared to the PTX-3 levels in the same patients upon resolution of the disease, i.e., convalescent

TABLE 4 | Coronary artery z-scores, PTX-3 levels, and absolute neutrophil counts (ANC) throughout Kawasaki disease (KD) progression.

		Intravenous immunoglobulin (IVIG) susceptible ($n = 52$)			IVIG resistant ($n = 18$)			P^a	IVIG resistant, NCA ($n = 5$)			IVIG resistant CAL ($n = 13$)			P^b	P^c
CA z-score	Acute	$n = 70$	1.87 ± 0.25	(−2.35–7.63)	52	5.32 ± 1.66	(1.42–26.24)	18	*	$n = 70$	1.81 ± 0.15	(1.42–2.25)	5	6.66 ± 2.2	(1.73–26.24)	13	*	ns
	Sub-acute	$n = 70$	1.53 ± 0.27	(−2.34–9.57)	52	6.17 ± 2.37	(0.83–36.30)	18	**	$n = 70$	1.55 ± 0.3	(0.83–2.34)	5	7.94 ± 3.18	(1.43–36.30)	13	***	ns
	Convalescent	$n = 70$	1.11 ± 0.23	(−1.00–10.09)	52	5.38 ± 2.52	(0.00–37.23)	18	**	$n = 70$	1.18 ± 0.15	(0.71–1.66)	5	7 ± 3.42	(0.00–37.23)	13	**	ns
	CA z-score MAX	$n = 70$	2.21 ± 0.27	(−1.00–10.09)	52	6.76 ± 2.41	(1.42–37.23)	18	*	$n = 70$	1.87 ± 0.16	(1.42–2.34)	5	8.64 ± 3.21	(2.16–37.23)	13	**	ns
PTX-3 (pg/ml)	Acute	$n = 62$	4.08 ± 1.46	(0.08–53.92)	40	10.29 ± 4.9	(0.14–84.53)	16	*	$n = 62$	1.59 ± 0.66	(0.14–3.24)	5	13.92 ± 6.73	(1.37–84.53)	11	**	*
	Sub-acute	$n = 65$	1.17 ± 0.43	(0.05–19.10)	47	0.99 ± 0.39	(0.09–7.23)	18	ns	$n = 65$	0.18 ± 0.06	(0.09–0.43)	5	1.28 ± 0.51	(0.13–7.23)	13	ns	ns
	Convalescent	$n = 64$	0.62 ± 0.19	(0.04–7.18)	48	0.28 ± 0.07	(0.08–1.17)	16	ns	$n = 64$	0.12 ± 0.02	(0.08–0.17)	5	0.35 ± 0.1	(0.14–1.17)	11	ns	ns
Absolute neutrophil count (10^9/L)	Acute	$n = 64$	11.31 ± 1.32	(1.64–63.2)	48	11.63 ± 2	(1.09–35.7)	16	ns	$n = 64$	17.76 ± 4.98	(6.32–35.70)	5	8.84 ± 1.32	(1.09–16.04)	11	ns	ns
	Sub-acute	$n = 64$	4.75 ± 0.49	(0.84–15.53)	48	5.34 ± 1.23	(0.67–17.66)	16	ns	$n = 64$	8.32 ± 3.71	(1.56–17.66)	4	4.35 ± 1.07	(0.67–13.23)	12	ns	ns
	Convalescent	$n = 64$	2.87 ± 0.22	(0.74–7.55)	48	3.39 ± 0.5	(0.78–8.78)	15	ns	$n = 64$	4.46 ± 1.21	(1.96–8.78)	5	2.94 ± 0.48	(0.78–7.07)	12	ns	ns

Mean ± SEM (range) evaluated for the indicated variable of all the patient serum samples ($n = 70$) evaluated at the acute, sub-acute, and convalescent phases of the disease in IVIG responsive, IVIG resistant, IVIG resistant with normal coronary artery (NCA), and IVIG resistant with coronary artery lesion (CAL). Repeated-measures ANOVA was used to compare the coronary artery z-scores, circulating PTX-3 levels, and absolute neutrophil counts among IVIG response groups between the three phases of KD. p-value comparing the effect of the IVIG response groups for coronary artery z-score, PTX-3 levels, and ANC.

[a]p-value between IVIG-responsive and IVIG-resistant KD patients.

[b]p-value between IVIG-responsive and IVIG-resistant with CAL KD patients.

[c]p-value between IVIG-resistant with NCA and IVIG-resistant with CAL KD patients.

**** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$.*

SEM, standard error mean; CA, coronary artery; CA z-score MAX, largest coronary artery diameter the right coronary artery and the left anterior descending coronary artery z-scores; ns, not significant ($p > 0.05$).

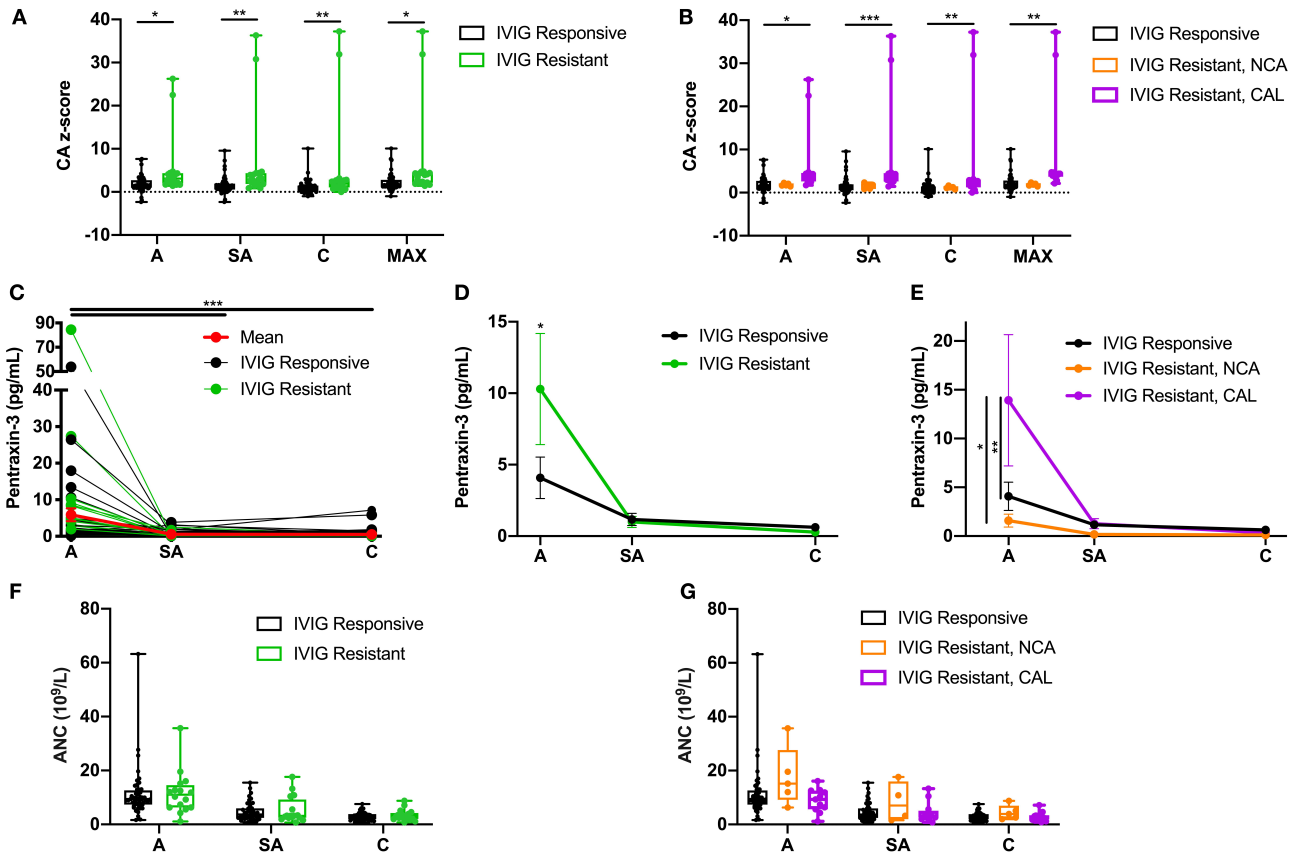

FIGURE 5 | Effects of intravenous immunoglobulin (IVIG) response on coronary artery z-scores, circulating PTX-3 levels, and absolute neutrophil count (ANC). **(A)** Coronary artery z-score among Kawasaki disease (KD) patients responsive to IVIG treatment (black; $n = 52$) and resistant to IVIG treatment (green; $n = 18$) throughout the disease progression. **(B)** Coronary artery z-score among KD patients responsive to IVIG treatment (black; $n = 52$) and resistant to IVIG treatment with normal coronary artery (NCA) (orange; $n = 5$) and IVIG resistant with coronary artery lesion (CAL) (purple; $n = 13$) throughout the disease progression. **(C)** Circulating PTX-3 levels in single patients throughout the KD progression: A, $n = 62$; SA, $n = 65$; and C, $n = 64$. The black lines track KD patients who were responsive to IVIG treatment ($n = 52$; A = 40, SA = 47, and C = 48), the green lines track KD patients who were resistant to IVIG treatment ($n = 18$, A = 16, SA = 18, and C = 16), and the red line track the mean ± SEM of all the patient serum samples ($n = 70$) evaluated at each phase of the disease. **(D)** Circulating levels of PTX-3 in KD patients responsive (black, $n = 52$) or resistant (green, $n = 18$) to IVIG treatment throughout the disease progression. **(E)** Circulating levels of PTX-3 in KD patients responsive to IVIG treatment ($n = 52$; A = 40, SA = 47, and C = 48), resistant to IVIG treatment with NCA ($n = 5$ for all three KD phases), and resistant to IVIG treatment with CAL ($n = 13$; A = 11, SA = 13, and C = 11). **(F)** ANC among KD patients responsive to IVIG treatment (black; $n = 52$, A = 48, SA = 48, and C = 48) and resistant to IVIG treatment (green; $n = 18$, A = 16, SA = 16, and C = 15) throughout the disease progression. **(G)** ANC among KD patients responsive to IVIG treatment (black; $n = 52$, A = 48, SA = 48, and C = 48), resistant to IVIG treatment with NCA (orange; $n = 5$, A = 5, SA = 4, and C = 5), and IVIG resistant with CAL (purple; $n = 13$, A = 11, SA = 12, and C = 12) throughout the disease progression. The box plots mean, 25, 75%, minimum, maximum, and individual points of all patients' ($n = 70$) maximal coronary artery z-score among the right coronary artery and left anterior descending coronary artery at the acute, sub-acute, and convalescent phases of KD, stratified by IVIG response (i.e., responsive or resistant) and coronary artery z-score (i.e., NCA or CAL). Repeated-measures ANOVA was used to compare the IVIG treatment response groups with NCA or CAL, with coronary artery z-scores, circulating PTX-3 levels, and ANC at the three phases of KD and the maximal coronary artery z-score throughout KD progression. C, convalescent; SEM, standard error mean; CA, coronary artery; MAX, largest coronary artery diameter between RCA and LAD z-scores; A, acute; SA, sub-acute. *p*-value comparing the effect of the IVIG treatment response groups at each phase. $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

phase. Furthermore, we observed a significant difference in PTX-3 levels in patients with CAL when compared to patients without CAL during acute KD.

PTX-3 in KD Is Correlated to Clinical Assessment of Inflammation

Consistent with previous studies of clinical laboratory data in KD progression (45–48), in our study, the levels of ESR, CRP, WBC, and ANC peaked in the acute phase, the platelet levels were highest in the sub-acute phase, and RBC, Hct, Hgb, and leukocyte levels were highest in the convalescent phase. Correlations between individual KD patient's clinical laboratory data and the PTX-3 levels throughout KD progression showed PTX-3 levels with positive correlations to ESR, CRP, WBC, and ANC levels and negative correlations with Hct, ALC, and PLT. During the acute phase, the PTX-3 levels were positively correlated to CRP and ANC. At the sub-acute phase, the PTX-3 levels were positively correlated to Hgb, ANC, and MO. No clinical laboratory parameters were correlated to the PTX-3 levels in the convalescent phase, consistent with PTX-3 as an

inflammatory modulator during acute KD pathogenesis. The ESR and the CRP levels are established inflammatory parameters (30). Strong repeated-measures correlation analysis between PTX-3 and these clinical inflammatory markers suggests a role for PTX-3 in monitoring KD disease progression and further supports the diagnostic and the prognostic potential of PTX-3 for KD diagnosis and risk scoring.

Neutrophils as a Source of PTX-3 During KD Progression

The positive repeated-measures correlation and Spearman's correlation in the acute phase between WBC, specifically neutrophils and PTX-3, suggest neutrophils as a possible source for PTX-3. Neutrophils have been shown to release PTX-3 when activated in cardiovascular disease and sepsis (33, 34, 39). Pathology studies of heart tissues from autopsies of children with KD has led to a model of KD vasculopathy that begins with an initial neutrophilic infiltration of the coronary artery, followed by infiltration of monocytes and macrophages, implicating these immune cells as key players in KD-associated CAL and vascular dysfunction (7, 8, 49, 50). Macrophages, dendritic cells, and endothelial cells express PTX-3 in response to lipopolysaccharides, interleukin (IL)-1 and -1β, and tumor necrosis factor (TNF)-α (51, 52). Similarly, PTX-3 is released from neutrophil granules in response to tissue damage (53). Interestingly, TNF-α (54) and IL-1β (55, 56) are recognized biomarkers in acute KD. Inflammatory biomarkers, immunological markers, and proteomic biomarkers have been demonstrated to be elevated in the blood of acute KD patients (22). In particular, elevated levels of the neutrophil-derived S100A12 protein, a pro-inflammatory ligand for the PRR receptor for advanced glycation end products (RAGE), were negatively regulated by soluble RAGE (57–60). Our data suggest that PTX-3 is among the neutrophil products that make up the inflammatory milieu in the coronary arteries of KD patients. Furthermore, previous studies of KD pathogenesis and PTX-3 expression suggest that neutrophils, in response to induction by IL-1β and TNF-α, induce PTX-3 production in the coronary artery during acute KD. Therefore, we propose that PTX-3 may play an important role in KD pathogenesis, particularly in vascular dysfunction leading to CAL.

PTX-3 in KD-Associated CAL

It has been suggested that PTX-3 plays dual roles, both protective and harmful, in the development and the progression of a cardiovascular disease (25, 28, 61). Previous studies have implicated a variety of stimuli to induce PTX-3 expression/release in immune and vascular endothelial cells (49, 50). Our analysis of the potential group effects in clinical laboratory parameters did not reveal any significant differences in the clinical laboratory parameters of KD patients with and without CAL. PTX-3 may have a different site-specific function, which could explain the pro-inflammatory and the anti-inflammatory roles of PTX-3 in cardiovascular diseases (62). PTX-3 decreases nitrogen oxide synthesis in endothelial cells, reducing cell proliferation and function and thus promoting endothelial dysfunction (63, 64). Similarly, PTX-3 inhibits angiogenesis through inhibition of fibroblast growth factor-2 that alters several functions including inflammation, tissue repair, and growth (65, 66). Furthermore, PTX-3 interacts with P-selectin, promoting lymphocyte recruitment, vascular inflammation, and endothelial dysfunction that can result in morphological alterations (28). Taken together, these observations and our data suggest the value of evaluating the PTX-3 levels in KD patients as other clinical assessments of inflammation fail to distinguish KD patients with CAL from those without CAL.

There have been several studies investigating PTX-3 in the context of coronary artery disease, with conflicting results. In nine studies, published between 2004 and 2017, among adult study populations conducted in Asia, Europe, and North America, there seems to be a consensus that patients with coronary disease and higher circulating PTX-3 levels had an increased risk of all-cause mortality, cardiac death, and cardiac events (26). In our study, we observed significantly higher PTX-3 levels in patients with CAL as compared to patients without CAL during the acute, but not in the sub-acute nor the convalescent, phase of KD. Comparisons between individual KD patient's coronary artery z-score to their PTX-3 levels revealed strong positive correlations between the clinical laboratory parameters in the acute phase, which were lost in the sub-acute and the convalescent phases of KD.

Stratification of KD patients by coronary artery z-scores in the CAL and the NCA groups revealed a stronger repeated-measures correlation between PTX-3 and the clinical assessments of inflammation in the CAL group. Previous studies have observed elevated sub-acute and convalescent WBC and ESR levels (46) and higher sub-acute PLT levels (67) associated with KD patients with CAL. Furthermore, elevated levels of PTX-3 in acute KD remained significant among both NCA and CAL groups. While the group stratification implicates the role of PTX-3 in the development of CAL, the protein is not exclusive to this process; rather, it is integral in the overall progression of KD. Thus, research in adult human coronary artery disease (26) in conjunction with our data implicates PTX-3 as a modulator of vascular dysfunction and remodeling and could have a specific role in KD-associated CAL. Therefore, we propose PTX-3 as a sensitive marker for coronary artery dilation in KD.

PTX-3 in KD-Associated IVIG Resistance

IVIG resistance is a risk factor for the development of CAL in KD (30). Fever status is an indicator of systemic inflammation. Patients resistant to IVIG treatment experience prolonged systemic inflammation, which includes inflammation of the coronary arteries, leading to an increased risk for CAL (68, 69). Among our patient population, the KD patients resistant to IVIG treatment were at a higher risk for CAL as compared to the KD patients who were responsive to IVIG treatment.

Researchers in Japan who evaluated the transcriptional regulation of infliximab therapy in IVIG-resistant KD patients identified high levels of PTX-3 transcript levels in IVIG-resistant KD patients (70). Therefore, we stratified our data on the

circulating levels of PTX-3 among IVIG-responsive and IVIG-resistant KD patients and confirmed that the PTX-3 levels were higher in the IVIG-resistant patients as compared to those in the IVIG-responsive patients. These differences in the circulating PTX-3 levels were more dramatic when further stratifying the IVIG resistance group by coronary artery z-score in the NCA and the CAL groups. The circulating PTX-3 levels in the IVIG-resistant patients with CAL were significantly elevated when compared to the IVIG-resistant patients with NCA. Furthermore, the PTX-3 levels in the IVIG-responsive group were similar to those in the IVIG-resistant patients with NCA. Collectively, these data suggest that the elevated levels of PTX-3 are indicative of enlarged coronary arteries rather than IVIG resistance.

Studies of clinical laboratory markers have described elevated CRP, liver enzyme level, WBC, and neutrophil counts to be associated with KD patients resistant to IVIG treatment (68). Our data implicate the role of PTX-3 in producing the heightened inflammatory environment in IVIG-resistant KD patients with CAL. Previous studies have suggested elevated neutrophil counts in IVIG resistance (17, 18, 71). However, we and others (72, 73) did not observe elevated neutrophil counts in either IVIG-resistant patients or IVIG-resistant patients with CAL as compared to those in IVIG-responsive patients. Perhaps our small and heterogeneous patient population may account for these differences in the neutrophil counts. Furthermore, most data on the positive correlation of IVIG resistance and neutrophil counts are from homogenous populations (16–18, 71).

Patient Population

In this study, we demonstrate the potential role of PTX-3 in KD pathogenesis and particularly in coronary dilation that is the most significant outcome of the disease. A strength of these findings is that they have been obtained in a population of mixed ethnicity. Ethnicity is a well-known risk factor for KD and KD-associated CAL. Much KD research has been conducted in ethnically homogenous study populations (i.e., Asian or White). Our study's geographic setting in Hawai'i has resulted in a heterogeneous study population, including a majority of mixed-race patients. Perhaps this allows the associations that we detect to have more robust disease implication rather than geographic or ethnic specificity.

Limitations and Future Directions

The limitations of this study include limited sample size, particularly within the CAL group, and some incomplete sampling of patients at a few time points. Convalescent phase samples were used as individual patient controls; however, the diagnosis of KD requires distinguishing KD patients from other febrile children admitted to the emergency room who present with similar symptoms (i.e., rash, inflammation of the mucous membranes, edema, conjunctivitis, and lymphadenopathy). Thus, additional studies investigating the specific role of PTX-3 in KD patients as compared to febrile and afebrile controls are warranted to evaluate the diagnostic potential of PTX-3. During normal physiological conditions, PTX-3 has been demonstrated as a non-specific systemic inflammatory protein known to have very low to undetectable levels in the circulation. The serum PTX-3 levels dramatically increase within 6–8 h of infection and inflammation (24). Thus, the non-specific role of PTX-3 in infection and inflammation might preclude the protein's utility as a general biomarker to distinguish KD patients from other febrile children. However, our results demonstrate a significant acute elevation of PTX-3 level among KD patients with CAL as compared to those with NCA. This data further suggests a specific role for PTX-3 in KD immunopathogenesis, particularly in CAL development. Future studies should focus on investigating the underlying mechanisms by which PTX-3 and other inflammatory proteins are involved in KD pathogenesis and CAL development.

CONCLUSIONS

Currently, KD diagnosis relies on the identification of specific symptoms by an experienced clinician. Without prompt diagnosis and administration of treatment, KD patients are at an increased risk for severe complications such as CAL and aneurysm formation, which may result in permanent coronary vasculopathy and life-long risk for cardiovascular diseases (9). Therefore, there is a pressing need for better, less subjective diagnostic and prognostic methods for the identification of children with KD and those children who are at a greatest risk for cardiovascular complications associated with the disease. The current findings demonstrate the potential role of PTX-3 in the inflammatory process of acute KD and, in particular, as a contributor to CAL.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the Kapi'olani Medical Center for Women and Children (KMCWC) Institutional Research and Ethics Committee (Western Consortium IRB Study No. 1140512). Informed consent was obtained from the parents or guardians of all patients prior to enrollment and specimen collection.

AUTHOR CONTRIBUTIONS

AB, MM, VN, and RS conceptualized and designed the study and interpreted the data. LC and VN conceptualized and designed the PTX-3 study. LC acquired the data, conducted an initial statistical analysis of the data, interpreted the data, and drafted the initial manuscript. VN supervised the data collection, interpreted the data, and drafted the initial manuscript. EL conducted a formal statistical analysis of the data. All authors reviewed and edited the final manuscript and have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00295/full#supplementary-material

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Presentation and Outcomes of Kawasaki Disease in Latin American Infants Younger Than 6 Months of Age: A Multinational Multicenter Study of the REKAMLATINA Network

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Objective: To characterize the clinical presentation and outcomes of Kawasaki disease (KD) in infants <6 months of age as compared to those ≥6 months in Latin America.

Methods: We evaluated 36 infants <6 months old and 940 infants ≥6 months old diagnosed with KD in Latin America. We compared differences in laboratory data, clinical presentation, treatment response, and coronary artery outcomes between the two cohorts.

Results: The majority (78.1%) of infants and children ≥6 months of age were initially diagnosed with KD, as compared to only 38.2% of infants <6 months. Clinical features of KD were more commonly observed in the older cohort: oral changes (92 vs.

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75%, $P = 0.0023$), extremity changes (74.6 vs. 57.1%, $P = 0.029$), and cervical lymphadenopathy (67.6 vs. 37.1%, $P = 0.0004$). Whether treated in the first 10 days of illness or after the 10th day, infants <6 months were at greater risk of developing a coronary artery aneurysm compared to KD patients ≥6 months treated at the same point in the course of illness [≤10 days (53.8 vs. 9.4%, $P = 0.00012$); >10 days (50 vs. 7.4%, $P = 0.043$)].

Conclusion: Our data show that despite treatment in the first 10 days of illness, infants <6 months of age in Latin America have a higher risk of developing a coronary artery aneurysm. Delay in the diagnosis leads to larger coronary artery aneurysms disproportionately in these infants. Thus, suspicion for KD should be high in this vulnerable population.

Keywords: Kawasaki disease, Latin America, infants, coronary artery abnormalities, delayed diagnosis

INTRODUCTION

Kawasaki disease (KD) is a medium-size vessel vasculitis of childhood with a clinical presentation that can be confused with other pediatric febrile illnesses. This can lead to a delay in diagnosis and treatment with intravenous immunoglobulin (IVIG), which in turn can lead to a higher rate of coronary artery abnormalities (CAA) and increased mortality and morbidity (1–6). Despite timely treatment with IVIG within the first 10 days of illness, up to 43.4% of infants <6 months in the United States have been reported to develop CAA (3).

Most of the epidemiological data for patients with KD <6 month of age are from regions outside of Latin America (LA) (2, 6–11). It is unknown whether the clinical presentation and outcomes are similar in infants <6 months in LA populations. Increasing collaborative academic and clinical efforts in Latin America to study the epidemiology and care of KD patients led to the development of a multinational network in LA entitled Red de Enfermedad de Kawasaki en America Latina (REKAMLATINA; Latin American KD Network) (8, 12). We aim to characterize the clinical presentation and outcomes of KD in infants <6 months old as compared to those ≥6 months of age in LA.

MATERIALS AND METHODS

Subjects and Clinical Data

All study data were obtained from review of the REKAMLATINA Research Electronic Data Capture (REDCap) database, housed at the University of California San Diego KD Research Center, which contains demographic, clinical and laboratory data from KD patients throughout LA. We reviewed retrospectively collected data from 36 KD subjects <6 months and 940 ≥6 months diagnosed and treated in 16 Latin American countries and 35 hospitals between January 1, 2009, to December 31, 2013.

In accordance with the American Heart Association (AHA) guidelines, complete KD presentation was defined as fever ≥ 5 days and 4 or more of the following clinical signs: erythema and cracking of lips, strawberry tongue, and/or erythema of oral and pharyngeal mucosa; bilateral bulbar conjunctival injection without exudate; polymorphous skin rash; changes such as edema, redness, and/or peeling of the hands or feet; and cervical lymphadenopathy (13). Incomplete KD was defined per the AHA guidelines as fever and fewer than 4 of the KD clinical criteria with either supportive laboratory or an echocardiographic abnormality. Illness day 1 is defined as the first day of fever. Specifics on the treatment course, including treatment with IVIG or antibiotics, was collected as was the presence of IVIG resistance defined as persistent fever ($T \geq 38.0^{\circ}C$ rectally or orally) > 36 h but less than 7 days after completion of the first IVIG infusion.

CAAs were classified as normal ($Z < 2.5$), dilated ($Z \geq 2.5$ to ≤ 4), aneurysmal ($Z > 4$ to ≤ 10), or giant aneurysm ($Z > 10$) as per the 2004 AHA KD Guidelines by the local site study investigator, given that these were the published guidelines at the time of acquisition of the data (3, 14). For a subset in whom CAA measurements, height and weight were available ($N = 481$), the internal diameter normalized for body surface area (Z-score) of the proximal right coronary artery (RCA) and left anterior descending artery (LAD) were calculated. Height and weight were verified using age-appropriate normal values for males and females and the body surface area was calculated using the Haycock formula. Z-score was calculated using the formula from Dallaire and Dahdah (15). The maximal Z-score of either the RCA or LAD (Z-max) was identified as the largest Z-score for the subjects within the first 6 weeks of illness. The study received Institutional Review Board approval at the University of California, San Diego as well as at each individual institution enrolling subjects in the REKAMLATINA database.

Statistical Methods

The primary comparisons were made between infants <6 months old and infants and children ≥6 months old. Differences in counts and categorical variables were compared using two-sided Fisher's exact test. Continuous variables were compared using the Mann-Whitney test. Statistical processing was performed using R version 3.1.0 (available at: http://www.R-project.org) and GraphPad Prism version 8.2.1 (available at: (https://www.graphpad.com).

TABLE 1 | Demographic and clinical characteristics of the study population[a].

Characteristic	<6 months old[b] (*N* = 36)	≥6 months old[b] (*N* = 940)	*p*-value
Age (months)	4.0 (3.0 to 5.0)	26.0 (16 to 48.0)	<0.0001*
Illness day at hospitalization (days)	4.0 (3.0 to 7.0)	6.0 (5.0 to 9.0)	0.00018*
Hospital stay (days)	8.0 (6.0 to 10.0)	5.0 (4.0 to 7.0)	<0.0001*
Male sex, *N* (%)	26 (72.2%)	572 (60.9%)	0.22
Race/Ethnicity, *N* (%)			
White	25 (69.4%)	427 (45.5%)	0.0059*
Asian	1 (2.8%)	4 (0.4%)	0.17
Afro-Latino	2 (5.6%)	28 (3.0%)	0.31
Indigenous	1 (2.8%)	40 (4.3%)	>0.99
Unknown	7 (19.4%)	439 (46.8%)	0.0010*
WBC ($x10^3$)	18.6 (15.7 to 23.2)	14.1 (10.9 to 18.0)	<0.0001*
Neutrophils	9.4 (7.0 to 12.5)	8.8 (5.9 to 12.1)	0.52
Hemoglobin z-score[c]	−1.4 (-2.2 to−0.29)	−1.2 (-2.3 to 0.17)	0.63
Platelets ($x10^3$)	439 (311 to 549)	400 (294 to 521)	0.52
ESR (mm/h)	56 (36 to 81)	46 (30 to 56)	0.21
CRP (mg/dl)	6.0 (1.9 to 9.7)	6.4 (2.4 to 12.1)	0.51
ALT (U/L)	22.0 (16.7 to 30.0)	40.0 (22.0 to 85.0)	0.00028*
GGT (U/L)	24.0 (18 to 61.0)	45.0 (22.5 to 120.8)	0.35
Albumin	3.1 (3.0 to 3.6)	3.3 (2.9 to 3.7)	0.94

[a]*All continuous values were expressed as median, with 25 to 75% IQR. Differences in continuous values were tested using Mann-Whitney test. Differences in count values were assessed using Fisher's exact test, two-sided.*

[b]*We had complete data except for each of the following variables: Illness day N = 961 (<6mo = 35, ≥6mo = 926), Hospital stay N = 955 (<6mo = 35, ≥6mo = 920), Race/Ethnicity N = 974 (<6mo = 36, ≥6mo = 938), WBC N = 939 (<6mo = 31, ≥6mo = 908), Neutrophils N = 894 (<6mo = 27, ≥6mo = 867), Hb Z score N = 947 (<6mo = 31, ≥6mo = 916), Platelets N = 938 (<6mo = 32, ≥6mo = 906), ESR N = 619 (<6mo = 10, ≥6mo = 609), CRP N = 829 (<6mo = 31, ≥6mo = 798), Albumin N = 597(<6mo = 22, ≥6mo = 575), ALT N = 791(<6mo = 21, ≥6mo = 770), GGT N = 157(<6mo = 5, ≥6mo = 152).*

[c]*Hemoglobin z-score is distance in SD units from the mean for age-adjusted hemoglobin values.*

**Indicates statistical significance (p < 0.05).*

TABLE 2 | Initial clinical diagnosis on admission.

Diagnosis[a]	<6 months old *N* (%)	≥6 months old *N* (%)	*p*-value and OR (95% CI)
Kawasaki disease	13 (38.2%)	711 (78.1%)	<0.0001*OR = 0.17 (0.084 to 0.35)
Scarlet fever	1 (2.9%)	36 (4.4%)	>0.99
Scalded skin syndrome	0 (0.0%)	9 (1.1%)	-
Sepsis/shock	9 (26.5%)	20 (2.4%)	<0.0001* OR = 14.5 (6.0 to 33.8)
Erythema multiforme/SJS	1 (2.9%)	13 (1.6%)	0.45
Urinary tract infection	5 (14.7%)	31 (3.8%)	0.011* OR = 4.4 (1.7 to 11.5)
Dengue	1 (2.9%)	20 (2.4%)	0.58
Adenovirus	0 (0.0%)	7 (0.9%)	-
Enterovirus	0 (0.0%)	6 (0.7%)	-
Other virus	2 (5.9%)	38 (4.6%)	0.67
Unspecified fever	9 (26.5%)	188 (22.6%)	0.68
Occult bacteremia	4 (11.4%)	19 (2.3%)	0.012* OR = 5.4 (1.9 to 15.7)

[a]*Some subjects were classified with more than one diagnosis or, in some other cases, a clinical diagnosis was not recorded on admission.*

**Indicates statistical significance (p < 0.05).*

RESULTS

Over a five-year period, we identified a total of 976 patients <13 years of age with KD in LA. Retrospectively collected demographic and clinical data are presented in **Table 1**. Of the 976 patients, 36 (3.7%) were <6 months of age. The remaining 940 patients (96.3%) were between 6 months and 12 years of age (**Table 1**). Most of the children <6 months (25 of 36, 69.4%) were described as white ($P = 0.0059$). Most subjects were male and there was no difference in sex by age cohort (72.2 vs. 60.9%, $P = 0.22$). As compared to subjects ≥6 months old, infants <6 months had an illness day at hospitalization of 4 days as compared to illness day 6 ($P = 0.00018$). The younger cohort had longer hospital stays on average (8 vs. 5 days, $P < 0.0001$).

Infants <6 months had a higher mean white blood cell count at diagnosis (18.6 vs. 14.1, $P < 0.0001$; **Table 1**). Other inflammatory markers such as neutrophils, ESR, and platelets were not significantly different between the two age groups. The younger cohort had lower levels of ALT, GGT, and albumin, but only the ALT difference was statistically significant between the age groups (22 vs. 40 U/L, $P = 0.00028$).

Infants <6 months were less likely to be initially diagnosed with KD (38.2 vs. 78.1%, OR = 0.17, 95% CI 0.084 to 0.35, $P < 0.0001$) and had an 11-fold increased risk of being diagnosed initially with sepsis or shock (26.5 vs. 2.4%, OR = 14.5, 95% CI 6.0 to 33.8, $P < 0.0001$) (**Table 2**). Furthermore, infants <6 months were 4 times more likely to be initially diagnosed with a urinary tract infection (14.7 vs. 3.8%, OR = 4.4, 95% CI 1.7 to 11.5, $P = 0.011$) and more likely to be suspected of occult bacteremia initially (11.4 vs. 2.3%, OR = 5.4, 95% CI 1.9 to 15.7, $P = 0.012$) as compared to older infants and children.

Table 3 describes clinical findings related to diagnostic criteria for KD. Oral changes, including erythematous oropharynx or lips or strawberry tongue, were less commonly noted in the younger patients (75 vs. 92%, OR = 0.26, 95% CI 0.12 to 0.58, $P = 0.0023$). Cervical lymphadenopathy was also noted less frequently in younger patients (37.1 vs. 67.6%, OR = 0.28, 95% CI 0.14 to 0.55, $P = 0.0004$), as were extremity changes (57.1 vs. 74.6%, OR = 0.45, 95% CI 0.23 to 0.88, $P = 0.029$). Subjects were classified as having complete vs incomplete KD, based on AHA 2017 guidelines (13). Overall, we found significantly more older infants and children presenting with complete KD (44.4 vs. 76.6%, OR = 0.24, 95% CI 0.13 to 0.47, $P < 0.0001$). The younger cohort had over 4-fold greater odds of being diagnosed as incomplete KD compared to their older counterparts (55.6 vs. 23.4%, OR = 4.1, 95% CI 2.1 to 8.0, $P < 0.0001$).

Table 4 describes IVIG therapy as well as use of antibiotics. The initial use of antibiotics and the treatment of patients ultimately diagnosed with KD with IVIG was high in both groups, though not statistically different between the cohorts.

TABLE 3 | Diagnostic criteria for KD.

Clinical Presentation	<6 months old *N* (%)	≥6 months old *N* (%)	*p*-value[d] and OR (95% CI)
Rash	30 (83.3%)	814 (87.2%)	0.45
Conjunctival injection	29 (80.6%)	812 (86.9%)	0.31
Oral changes[a]	27 (75%)	865 (92%)	0.0023* OR = 0.26 (0.12 to 0.58)
Cervical lymphadenopathy[a]	13 (37.1%)	627 (67.6%)	0.0004* OR = 0.28 (0.14 to 0.55)
Extremity changes	20 (57.1%)	694 (74.6%)	0.029* OR = 0.45 (0.23 to 0.88)
Complete KD[b], *N* (%)	16 (44.4%)	720 (76.6%)	<0.0001* OR = 0.24 (0.13 to 0.47)
Incomplete KD[c], *N* (%)	20 (55.6%)	220 (23.4%)	<0.0001* OR = 4.1 (2.1 to 8.0)

[a] *These clinical features are based on the American Heart Association criteria for KD. Oral changes include erythematous oropharynx or lips or strawberry tongue. Cervical lymphadenopathy is unilateral and is a lymph node at least 1.5 cm.*
[b] *Complete KD as defined by the AHA with at least 4 of the 5 clinical criteria.*
[c] *Incomplete KD by laboratory evaluation is defined the AHA with <4 clinical criteria and laboratory inflammation (ESR ≥ 40 mm/hr or CRP ≥ 3 mg/dl with ≥ 3 supplementary labs elevated: albumin levels of <3.0 g/dL, anemia for age, elevation of ALT level, >450,000 platelets per mm^3 after the seventh day, white blood cell count of >15,000 cells per mm^3, and >10 white blood cells per high-power field in the urine).*
[d] *Fisher's exact test was used to test differences in count data.*
**Indicates statistical significance (p < 0.05).*

TABLE 4 | IVIG therapy course, IVIG-resistance and rate of antibiotic use*.

	<6 months old	≥6 months old
Treated with IVIG, N (%)[a]	35/36 (97.2%)	879/937 (93.8%)
Treated with Antibiotics prior to KD diagnosis	26/35 (74.3%)	626/907 (69.0%)

[a] *All subjects who received IVIG were given a 2 g/kg dose.*
[b] *Fisher's exact test.*
**All p-values are >0.05 and thus not significant.*

Table 5 describes differences in the Z-max of the left anterior descending and right coronary arteries in young vs older KD patients by illness day. For children hospitalized in the first 10 days of illness, infants <6 months increased the aneurysm risk 6-fold compared to those subjects ≥6 months old (53.8 vs. 9.4%, OR = 11.25, 95% Cl 3.87 to 36.25, $P = 0.00012$). Infants <6 months hospitalized after day 10 of illness had a greater risk of having a giant coronary artery aneurysm as compared to older children (50 vs. 7.4%, OR = 12.50, 95% CI 1.62 to 84.97, $P = 0.043$). Infants <6 months had a higher baseline Z-score and Z-max compared to older patients, regardless of whether they were hospitalized in the first 10 days or after the 10th day of illness. When diagnosed within the first 10 days of illness, younger patients had a higher baseline Z-score and Z-max compared to older patients (2.37 vs. 0.77, $P = 0.0059$; 4.55 vs. 1.18, $P = 0.0022$), respectively. An even greater baseline Z-score and Z-max was observed for younger infants after the 10th day of illness (10.35 vs. 1.65, $P = 0.0064$; 8.04 vs. 1.86, $P = 0.0045$).

DISCUSSION

This is the first study to compare the clinical presentation, initial diagnosis and treatment, and coronary artery outcomes between infants <6 months compared to those 6 months and older throughout LA. As reflected in other recent studies, infants <6 months old with acute KD in LA are more likely to develop CAA than those older than 6 months old (2, 3, 6, 9, 16–18). In this study, based on calculated Z-max scores, 53.8% of infants <6 months with KD had an aneurysm or giant aneurysm compared to the older cohort 11.2%. By comparison, other studies have found ∼20% of infants <6 months developed an aneurysm or great aneurysm, whereas only 5% of infants ≥6 months had an aneurysm or great aneurysm (3). In the Rosenfeld et al. study the CAA present in infants <6 months was as high as 79%, compared with 44% of infants ≥6 months (6). Similarly to LA, a high prevalence of coronary artery abnormalities in infants <6 months has been observed in other parts of the globe. In a study from Chandigarh, India it was reported 35% of infants <6 months had coronary artery abnormalities and 65% in a study in Taipei, Taiwan (2, 18). While the increased rate in this cohort may be due to Z-scores only being available in the most severely affected patients, it does warrant assessing whether there is in fact a higher rate of aneurysms in infants in LA in a follow up cohort study. Furthermore, as per the recommendations of the 2017 AHA guidelines, these higher risk infants may benefit from adjunctive therapy.

A novel data-point captured in our database was "Initial Diagnosis." KD was the initial diagnosis in 74.2% of all subjects, but only in 38.2% of the infants <6 months, as sepsis and a urinary tract infection were the most common initial diagnoses. With nearly all patients being initially treated with antibiotics, it is important to continue to raise awareness that KD is not a diagnosis of exclusion and that in many cases antibiotics are not needed as KD alone is the leading diagnosis.

There are several strengths and limitations to our study. These data are from the largest international network and database of children with KD and provide the demographic and clinical data from an area of the world where little has been published widely about KD. That said, there were a larger proportion of infants and children ≥6 months as compared to those <6 months. For this specific retrospective database, illness day at diagnosis of KD was not available as some sites considered this to be protected health information. Instead illness day at hospitalization was recorded, keeping in mind that in some cases KD was not the initial diagnosis. In addition, Z-score could only be calculated in subjects in whom an accurate weight and height were available for calculating BSA.

CONCLUSIONS

In summary, our study shows that despite treatment in the first 10 days of illness, infants <6 months old in LA have a higher risk of developing a coronary artery aneurysm than older KD patients. Delay in the diagnosis leads to larger coronary artery aneurysms

TABLE 5 | Coronary artery abnormalities in KD subjects treated (All)[a,d,e].

Coronary artery	<6 months old *N* (%)	≥6 months old *N* (%)	*p*-value	OR (95% CI)
CLASSIFICATION BASED ON Z-MAX SCORE, EITHER LAD OR RCA				
Illness Day < = 10 days				
Normal[b]	5/13 (38.5%)	307/383 (80.2%)	0.0015*	0.15 (0.056 to 0.51)
Dilated Z-score ≥2.5 to ≤4	1/13 (7.7%)	33/383 (8.6%)	>0.9999	0.88 (0.080 to 5.14)
Aneurysmal Z > 4 to ≤ 10	7/13 (53.8%)	36/383 (9.4%)	0.00012*	11.25 (3.87 to 36.25)
Giant Aneurysm Z>10	0/13 (0.0%)	7/383 (1.8%)	>0.9999	-
First echo Z-score	2.37 (1.02 to 5.08)	0.77 (−0.03 to 1.72)	0.0059*	-
Z-max[c]	4.55 (1.09 to 5.95)	1.18 (0.19 to 2.07)	0.0022*	-
Illness Day > 10 days				
Normal[b]	0/4 (0.0%)	49/81 (60.5%)	0.03*	-
Dilated Z-score ≥2.5 to ≤4	0/4 (0.0%)	9/81 (11.1%)	>0.9999	-
Aneurysmal Z > 4 to ≤ 10	2/4 (50.0%)	17/81 (21.0%)	0.21	3.77 (0.55 to 24.87)
Giant Aneurysm Z>10	2/4 (50.0%)	6/81 (7.4%)	0.043*	12.50 (1.62 to 84.97)
First Echo Z-score	10.35 (5.73 to 14.50)	1.65 (0.31 to 4.31)	0.0064*	-
Z-max[c]	8.04 (5.32 to 15.38)	1.86 (0.54 to 4.38)	0.0045*	-

[a] *Data expressed as N (%) or median (IQR); Fisher's exact test, two sided.*

[b] *Subjects were classified as having normal (<2.5 standard deviation units [Z-score] from the mean, normalized for body surface area), dilated (Z-score ≥2.5 to ≤4), or aneurysmal (Z > 4 to ≤ 10 for giant aneurysm) coronary arteries on the basis of the maximal internal diameters of the right coronary artery (RCA) and left anterior descending artery (LAD) measured by echocardiography at the time of diagnosis and up to 8 weeks after onset of fever. Coronary artery abnormalities were classified as normal, dilated or aneurysmal (focal dilation of an arterial segment at least 1.5 times the diameter of the adjacent segment) based on the AHA 2004 KD Guidelines, as those were the published guidelines at the time of data collection.*

[c] *Z max defined as largest Z score of LAD or RCA in the first 6 weeks of illness.*

[d] *Unavailable CAA data for N = 495 (<6mo = 19, ≥6mo = 476).*

[e] *Available Z-score data on N = 489 (<6mo = 18, ≥6mo = 471), however, only Z-scores data on N = 481 (<6mo = 17, ≥6mo = 464) were used for CAA analysis. Subjects with Z-scores, but without a verified height and weight or illness day were excluded from the CAA analysis.*

**Indicates statistical significance (p < 0.05).*

disproportionately in these infants. In addition, the majority of infants ultimately diagnosed with KD are initially thought to have an infectious issue initially and thus treated with antibiotics. Thus, the suspicion for KD should be high in infants <6 months old given the likelihood of misdiagnosis and increased risk for coronary artery aneurysm formation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at the University of California, San Diego and by the ethics committee at each REKAMLATINA-2 Study participant site. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

AS, RU-G, and AT developed the concept for this project. EM, SG, EB, AP, and AT were responsible for data cleaning and analysis. AT provided mentorship to SG, EM, and AP throughout the study period. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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A Case of Kawasaki Disease Complicated With Cerebral Salt-Wasting Syndrome

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We report the case of a 3-years-old boy who developed severe hyponatremia and unconsciousness during an episode of Kawasaki disease (KD). He was diagnosed with cerebral salt-wasting syndrome (CSWS), which has not previously been reported as a complication of KD. He was diagnosed with KD with fever and four clinical signs and received intravenous immunoglobulin (IVIG) on the day after onset. Hyponatremia had been observed, and it worsened after IVIG. At first, syndrome of inappropriate antidiuretic hormone secretion (SIADH) was suspected, but his hyponatremia did not improve by restriction of water intake. The patient's consciousness level decreased along with the worsening hyponatremia. Electroencephalography revealed abnormal electrical discharge concordant with acute encephalopathy. Laboratory data showed hypouricemia with high fractional excretion of uric acid (FEUA), in addition to a negative balance of both Na and water. We diagnosed KD complicated with CSWS. The patient improved promptly with appropriate Na supplementation and water correction.

Keywords: Kawasaki disease, cerebral salt-wasting syndrome (CSWS), syndrome of inappropriate antidiuretic hormone secretion (SIADH), hyponatremia, fractional excretion of uric acid (FEUA)

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BACKGROUND

Cerebral salt-wasting syndrome (CSWS), a complication associated with central nervous system (CNS) diseases, is known to cause hyponatremia and dehydration due to excessive discharge of Na and free water (1). Hyponatremia is reportedly observed in 29–70 percent of Kawasaki disease (KD) patients (2). The mechanism of hyponatremia is associated with insufficient intake of Na and syndrome of inappropriate antidiuretic hormone secretion (SIADH) (2, 3). To our knowledge, there have been no reports of CSWS as a complication of KD. CSWS is usually induced by CNS disorders like encephalopathy. We report a case of KD complicated with CSWS following encephalopathy.

CASE REPORT

A 3-years-old boy was brought to our hospital by his parents because of fever, conjunctival congestion, and rash on the body, all of which appeared the previous day. He did not have any pertinent medical or family history.

At the outpatient clinic, his body temperature was high, and bilateral conjunctival congestion, reddened lips, bilateral cervical lymphadenopathy, and erythema all over the body were noted. Even though the duration of the fever was only 2 days, KD was strongly suspected due to the other symptoms present, and he was hospitalized for treatment.

On admission, his height was 96.7 cm (+0.9 *SD*), and his weight was 13.4 kg (−0.1 *SD*). Body temperature was 39.3°C, pulse rate 153/min, and blood pressure 106/64 mmHg. Other principal signs of KD such as erythema of palms and soles or redness at the BCG inoculation site were not observed. Thoracoabdominal physical findings were normal, and capillary refill time was <2 s.

Laboratory findings on days 2, 6, 8, or 9 and 20 are shown in **Table 1**. Leukocytes were normal, and C-reactive protein (CRP) was slightly elevated at 0.75 mg/dl. There was no abnormality in the coagulation and fibrinolysis system. Hepatic transaminase was normal, but serum Na was decreased to 132 mEq/L. There were no leukocytes in the urine, and rapid antigen tests were negative for group A beta-hemolytic streptococcus and adenovirus. Although white blood cell (WBC) and CRP values were not typical considering the diagnosis of KD, the five typical principal signs were recognized. Furthermore, his brain natriuretic peptide (BNP) elevated to as high as 68.9 pg/ml (normal range < 18.4) on day 6, his erythrocyte sedimentation rate (ESR) elevated to 77 mm/h on day 8, and his platelet count rose to 520,000/μl on day 20. These data retrospectively supported the diagnosis of KD.

Chest roentgenogram showed no heart enlargement or pleural effusion. There were no abnormalities in the electrocardiogram. The largest coronary artery inner diameters by echocardiogram were 1.8 mm (*Z* score – 0.96) for the left main trunk, 1.6 mm (*Z* score – 0.36) for the left anterior descending branch, 1.5 mm (*Z* score – 0.09) for the circumflex brunch, and 2.2 mm (*Z* score + 1.25) for the right coronary artery. Aortic and mitral valve regurgitation and pericardial effusion were not observed.

Intravenous immunoglobulin (IVIG) treatment and oral aspirin were started on day 2 under the diagnosis of KD (**Figure 1**). The fever did not resolve, and blood tests on day 4 indicated a high risk of IVIG non-responsiveness on Kobayashi's risk scale (4). A second IVIG was administered on the same day along with prednisolone. As serum Na was 126 mEq/L, water intake was restricted to 700 ml/day as a treatment for hyponatremia potentially due to SIADH. His KD signs including fever resolved gradually on day 5. We continued water restriction. However, as the hyponatremia worsened on day 6 (serum Na 123 mEq/L, **Table 1**), we considered a possibility of CSWS because urine volume was maintained at no <2 ml/kg/h and urine density was as high as 1.026.

His consciousness was estimated as Glasgow Coma Scale 12 (eye 3, verbal 4, and motor 5). Although dehydration was not clearly evaluated, the intake/output balance of water and Na was negative, urine Na was 140.1 mEq/L, plasma osmotic pressure was 263 mOsm/kg, and urine osmotic pressure was 641 mOsm/kg. In addition, renin–aldosterone did not increase. On

TABLE 1 | Laboratory data on day 2 (admission) and day 6*.

Hematology	**Day 2**	**Day 6**	**Day 8**	**Day 20**	**Units**
WBC	5,200	7,400	7,500	8,700	/μl
Neutrophil	78.1	81.8	91.0	70.2	%
RBC	5.08	4.24	4.16	4.16	$\times 10^6$/μl
Hb	13.4	11.4	10.9	11.2	g/dl
PLT	284	292	354	527	$\times 10^3$/μl
Coagulation	**Day 2**	**Day 6**			**Units**
PT	13.6	ND			s
APTT	40.8	ND			s
FDP	3.5	ND			μg/ml
Fibrinogen	472	ND			mg/dl
D-dimer	1.9	ND			μg/ml
Biochemistry	**Day 2**	**Day 6**	**Day 8**	**Day 20**	**Units**
T-bil	0.3	0.5	0.4	0.3	mg/dl
AST	39	45	63	80	U/L
ALT	17	13	13	15	U/L
LDH	281	272	291	346	U/L
CK	67	53	28	21	U/L
BUN	11.2	13.4	4.3	13.3	mg/dl
Cr	0.38	0.26	0.21	0.28	mg/dl
UA	ND	2.9 (day 7)	2.2 (day 9)	1.4	mg/dl
Na	132	123	126	133	mmol/L
K	4.9	4.5	3.7	4.1	mmol/L
Cl	96	90	94	97	mmol/L
TP	7.6	9.2	7.9	7.5	g/dl
Alb	4.5	3.2	3.1	3.8	g/dl
Ferritin	47.6	ND	ND	ND	ng/ml
s-Osm	ND	263	259	ND	mOsm
BNP	ND	68.9	26.0	ND	pg/dl
Immunology	**Day 2**	**Day 6**	**Day 8**	**Day 20**	**Units**
ESR	ND	ND	77	49	mm/h
CRP	0.75	0.08	0.12	0.01	mg/dl
IgG	830	4566	ND	ND	mg/dl
Rapid antigen test		**Day 2**			
Group A streptococcus		Negative			
Adenovirus		Negative			
Urine	**Day 2**	**Day 6**	**Day 9**	**Day 20**	
Color	Clear	Yellow	Yellow	Yellow	
pH	6.5	7.0	6.5	7.0	
Density	1.025	1.026	1.012	1.009	
Protein	−	±	−	−	
Sugar	1+	4+	2+	−	
Occult blood	−	−	−	−	
WBC	−	−	−	±	
Nitrites	−	−	+	−	
Ketone bodies	1+	2+	−	−	
u-Na	ND	140.1	153.2	29.2	
u-osm	ND	641	395	317	

**The day when Na value was the lowest.*
ND, no data.

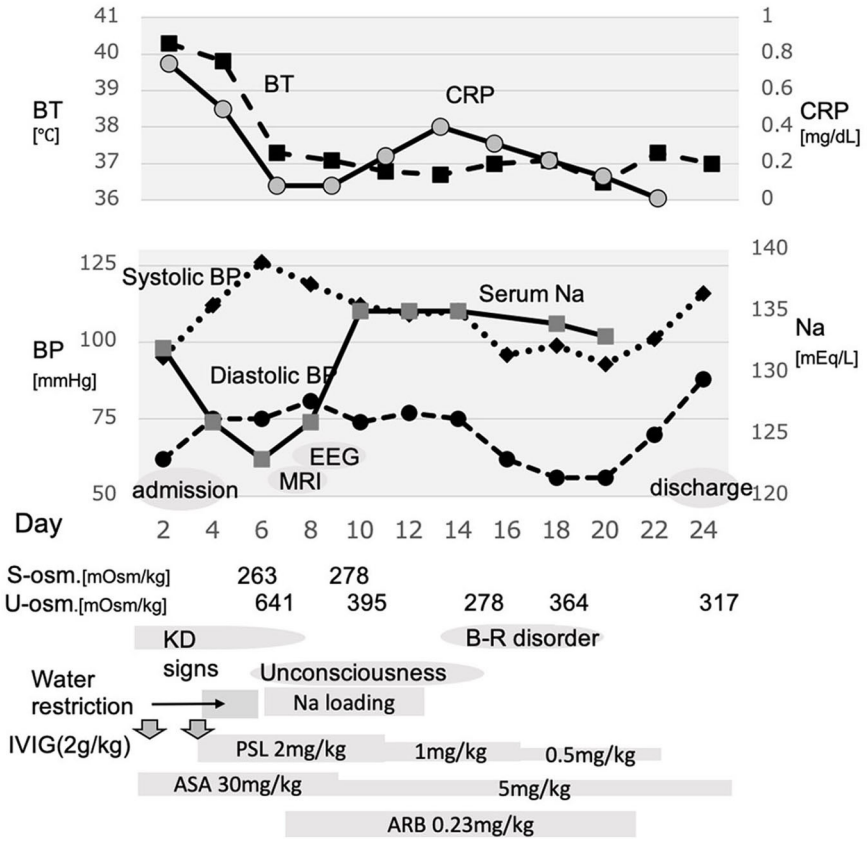


FIGURE 1 | Schema of the course of this case. After admission and treatment with immunoglobulin, moderate fever sustained. Additional immunoglobulin combined with prednisolone was administered, which relieved fever; however, the serum Na level began to decrease, and the consciousness level worsened. Water restriction resulted in worsened hyponatremia. Thereafter, Na loading was started and resulted in improved consciousness and bladder–rectal function. BT, body temperature; BP, blood pressure; s-osm, serum osmolality; u-osm, urine osmolality; EEG, electroencephalogram; MRI, magnetic resonance imaging; KD, Kawasaki disease; B-R disorder, bladder–rectal disorder; IVIG, intravenous immunoglobulin; PSL, prednisolone; ASA, acetylsalicylate; ARB, angiotensin receptor blocker.

day 6, in addition to the correction of water balance, hypertonic saline was started to correct Na.

Although the brain magnetic resonance imaging (MRI) did not show abnormalities on day 7, the electroencephalogram (EEG) showed diffuse slowing of the background activity, and acute encephalopathy was considered on day 8 (**Figure 2**). Since the patient's blood pressure had increased after starting prednisolone, oral administration of candesartan was added on day 8. Aspirin was reduced from day 9. Although the unconsciousness was prolonged with mild improvement, hyponatremia improved to 134 mEq/L on day 9. Urine output resumed to >4.5 ml/kg/h after day 9. Prednisolone was reduced from day 11. The patient was troubled with constipation, and an enema was administered on day 14, but he had poor stool discharge. On day 16, as the fecal impaction was remarkable, an enema with water-soluble contrast media (Gastrografin® oral/enema, Bayel Co., Ltd., Osaka, Japan) and manual evacuation were necessary. On day 16, the patient was fully conscious, and on day 20, the patient's urinary catheterization was no longer necessary, and he began to defecate normally. Candesartan and prednisolone were discontinued on day 20. A spinal MRI on day 22 was normal, and he discharged on day 24. No cardiac complications were noted during the course of hospitalization.

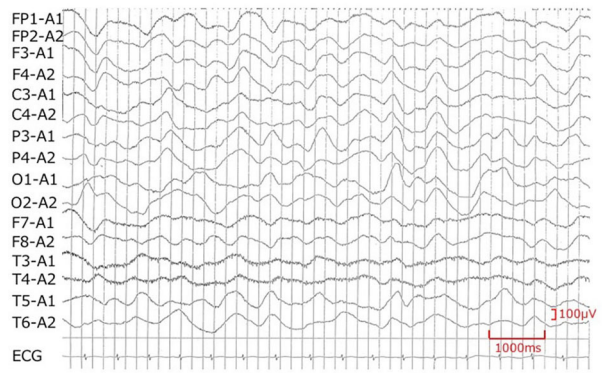


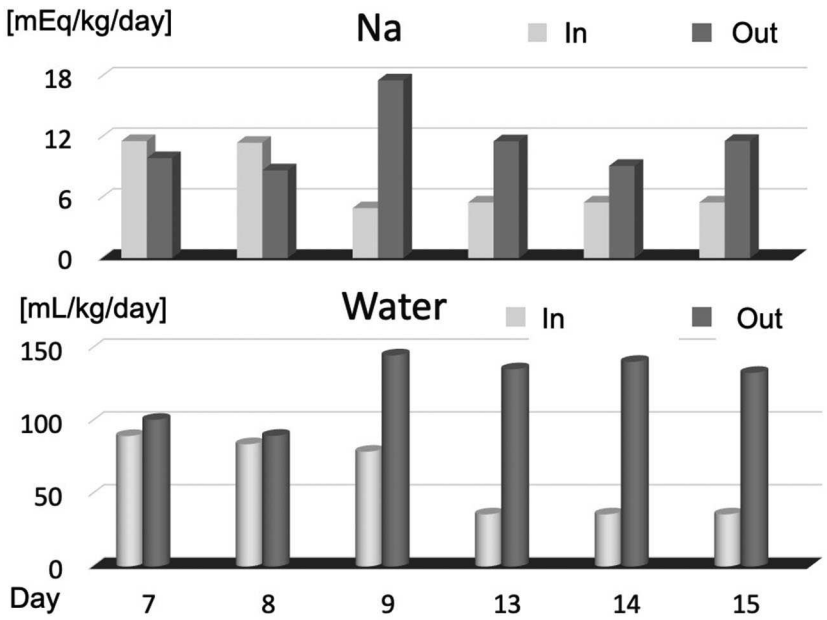
FIGURE 2 | Electroencephalogram recorded on day 8. Encephalopathy is suspected because of the diffuse slowing of the background activity.

DISCUSSION

CSWS is a syndrome based on CNS disorders in which inappropriate Na loss in urine causes a decrease in fluid volume and hyponatremia. It is known to occur in connection with head surgery, head trauma, or subarachnoid hemorrhage, but in recent years, CSWS associated with meningitis, encephalitis, and encephalopathy have been reported (5, 6). The underlying pathological condition is an increase in Na excretion in the renal tubules, and it is thought that the main site of the pathology is the proximal tubule (1). However, the mechanism by which Na excretion increases has not been clarified yet. It has been suggested that sympathetic nervous system activity involved in Na reabsorption in the proximal tubule and regulation of the renin–angiotensin system and diuretic factors such as atrial natriuretic peptide (ANP) and BNP are involved (7). CNS disorder is always involved in the development of CSWS, and according to the 21st Japanese Nationwide Surveillance of KD, CNS complications such as encephalitis and encephalopathy, including mild encephalopathy with a reversible splenial lesion (MERS), have a reported incidence of 0.09% in KD (8–11).

In our case, consciousness was impaired during treatment of KD, and although the brain MRI was normal, EEG showed generalized slowing of the background activity. Bladder and rectal disturbance was also recognized as a sign of encephalopathy. It is considered that encephalopathy caused suppression of the efferent sympathetic nervous system to the kidney, suppression of the renin–angiotensin system, and loss of Na and uric acid in the proximal tubules together with the decrease in extracellular fluid volume. Furthermore, humoral factors such as BNP may suppress the renin–angiotensin system and antagonize vasopressin in collecting ducts, possibly accelerating the development of CSWS.

When diagnosing CSWS, differentiation from SIADH is essential because distinctly different treatments are required for each condition. CSWS requires water correction and Na



s-Na [mEq/L]	125	126	134	133	135	135
u-Na [mEq/L]	140	146	153	110	83	113
s-UA [mg/dL]	2.9	ND	2.2	1.4	1.7	2.5
FEUA [%]	ND	ND	ND	16.5	13.5	13.3
Urine [ml/day] (ml/kg/hour)	747 (2.32)	800 (2.48)	1340 (4.16)	1412 (4.39)	1480 (4.60)	1380 (4.29)

FIGURE 3 | Na and water intake/output balance. After noting the Na loss on day 7, we checked serum Na, uric acid, FEUA, and intake/output balance of Na and water. On days 7 and 8, Na and water intake were increased, and serum Na normalized from day 9. After day 9, the output of Na and water was excessive, and the FEUA increased beyond the normal range (11%), which shows that this condition was CSWS. FEUA = [urinary uric acid (mg/dl) × serum creatinine (mg/dl)]/[serum uric acid (mg/dl) × urinary creatinine (mg/dl)] × 100 (%). UA, uric acid; FEUA, fractional excretion of uric acid; ND, no data.

loading, while SIADH requires water restriction, and an incorrect diagnosis may worsen either condition. Despite the differences of pathogenesis, with loss of Na and water in CSWS and dilution of serum by water retention in SIADH, both conditions exhibit hyponatremia and have similar laboratory findings. In both conditions, serum osmotic pressure decreases, and urine osmotic pressure increases, while urine Na elevates. Plasma antidiuretic hormone (ADH) levels may also elevate in CSWS; however, it usually takes a few days to get these results. The presence or absence of dehydration and the intake/output balance of Na and water are more useful in differentiating between CSWS and SIADH. Since CSWS patients lose both Na and water, their balances become negative; extracellular fluid decreases, and patients become dehydrated. In SIADH, intake/output balance of Na and water is equal or positive, and extracellular fluid increases due to the increase in ADH.

While the presence or absence of dehydration is an important index for diagnosing CSWS in the early stage of the disease, it is difficult to evaluate the amount of extracellular fluid in children. Measurement of extracellular fluid volume using radioisotope dilution is considered to be effective in terms of accuracy but is not practical in clinical use (12). Also, BUN/Cr, which is generally used as an indicator of dehydration, is not useful in CSWS because BUN does not increase (13). In practice, there is no other method than to evaluate body weight, blood pressure, skin turgor, hematocrit, and inferior vena cava (IVC) diameter. However, in many cases, these measurements are not conclusive unless the dehydration is severe. In our own case, blood pressure and weight were not decreased, and hematocrit was not increased, making it difficult to prove dehydration. However, the balance of Na and water was evaluated from day 7 because of the serious hyponatremia. Both Na and water showed a negative balance, and we diagnosed this situation as CSWS (**Figure 3**). In this case, urinary Na levels detected in the early stages of the disease were higher than 140 mEq/L, which was more indicative of CSWS than SIADH.

It is reported that changes in serum uric acid level and fractional excretion of uric acid (FEUA) are useful for differential diagnosis (1, 13). The definition of FEUA is "the percentage of urate which was filtered through the glomeruli then was excreted in urine." It is calculated by the formula shown in **Figure 3**, and the normal value is below 10–11% (1, 14, 15). Although the mechanism is unclear, serum uric acid levels are low and FEUA are high at the onset of both pathological conditions of SIADH and CSWS. Therefore, CSWS is characterized by continued hypouricemia and elevated FEUA (1). As shown in **Figure 3**, hypouricemia and elevated FEUA were observed concurrently with hyponatremia in our own case, and these continued after the improvement of hyponatremia, indicating that the condition was CSWS. Furthermore, when hyponatremia was recognized, water was restricted to about 700 ml/day as SIADH was presumed, and the progression of hyponatremia further confirmed the diagnosis of CSWS.

In recent years, there have been sporadic reports of conditions similar to CSWS without CNS disease, and the concept of the disease is changing to renal salt-wasting syndrome (1). Although further elucidation of the pathology is expected, CSWS should be considered when hyponatremia is recognized, even without CNS disease. Though we cannot precisely determine whether CSWS was induced by a neurological complication of KD or by a high-dose infusion of immunoglobulin (3, 16, 17), we should consider various possibilities of low concentration of Na and albumin or impaired renal function. When neurological complications and hyponatremia are present in KD, patients should be assessed for Na intake deficiency, SIADH, and CSWS. Whether or not dehydration is present, care should be taken while assessing the intake/output balance of water and Na, changes in uric acid, and changes in FEUA.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MO, JF, TNot, TNog, and MM were responsible for patient care. MO and MA prepared the draft manuscript. IM gave conceptual advices. All authors critically reviewed and approved the final manuscript.

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Kawasaki Disease in Infants in the First 3 Months of Age in a Mexican Population: A Cautionary Tale

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Background: Kawasaki disease (KD) is an acute febrile illness that largely affects young children before 5 years of age. Younger children with KD are reported to have a higher prevalence of coronary artery abnormalities. Little is known about infants in the first 3 months of age diagnosed with KD.

Methods: A retrospective study was conducted at the National Institute of Pediatrics in Mexico City from 1995 to 2019. Clinical features, laboratory results and cardiac outcomes were recorded. Infants in the first 3 months of age were compared with older patients. Wilcoxon-Mann-Whitney analysis was performed for continuous variables and Fisher's exact test for categorical variables.

Results: Six hundred and eighty-eight cases of KD were included in this study. Fourteen cases were diagnosed in the first three months of age. Heart failure and KD shock-syndrome was found in five cases (35.7%). Giant coronary artery aneurysms were found in six cases in the younger group (42.9%).

Conclusions: Diagnosis of KD in children younger than 3 months of age is rare. In most cases, an incomplete presentation contributed to a delay diagnosis, treatment, and complications. Younger patients with KD have an increased risk of presenting cardiac complications, including giant coronary artery aneurysms, shock, and death.

Keywords: Kawasaki disease, infants, cardiac complications, giant coronary artery aneurysms, Latinamerica

INTRODUCTION

Kawasaki disease (KD) is an acute febrile illness that largely affects young children with most cases diagnosed before 5 years of age. It is characterized by systemic vasculitis and it is complicated by coronary artery abnormalities (CAA).

Actually KD is considered the leading cause of acquired heart disease in children in developed countries (1). The highest incidence rate of KD in Japan is reported in children between 9 and 11 months of age (2). Younger children with KD are reported to have a higher prevalence of delayed diagnosis and therefore cardiac complications including CAA (3). In Mexico, as well as in other Latin-American countries, the incidence of the disease has not been reported.

The purpose of this study was to describe the clinical characteristics, laboratory results, treatments used and cardiac complications in patients in the first 3 months of age with KD and compared them with older children with KD in a Mexican population.

MATERIALS AND METHODS

A retrospective study was conducted at the National Institute of Pediatrics, which is an academic tertiary reference center in Mexico City, Mexico. The clinical records of patients under 18 years-old who were diagnosed in the acute phase of KD between August 1995 and August 2019 were retrospectively reviewed. The diagnosis of KD was made according the 2017 criteria of the American Heart Association (AHA). Those who had fever for 5 days and met more than four of the major criteria were classified as complete KD. As per the AHA guidelines, incomplete KD was defined as unexplained fever for 5 days and more, associated with two or three classical features of KD with coronary involvement (1).

Clinical and laboratory findings, in addition to echocardiographic measurements were recorded. Age, sex, weight, height, days of illness at admission, fever, presence of changes in lips, and oral cavity, changes in palms or soles, polymorphous exanthema, bulbar conjunctival injection, cervical lymphadenopathy, BCG reactivation, as well as gastrointestinal or neurological symptoms were recorded. We defined neurological manifestations if the patients presented with extreme irritability, somnolence, or encephalopathy, and seizures. Kawasaki disease shock syndrome was established if patients presented with cardiovascular collapse and hypotension during the acute phase of KD.

Z-score adjusted hemoglobin levels, white blood cells and platelets counts, neutrophil proportion, concentrations of C-reactive protein, erythrocyte sedimentation rate, albumin, sodium, and levels of bilirubin, aspartate aminotransferase and alanine aminotransferase were documented.

In all patients, an echocardiogram was performed at the time of diagnosis following the AHA recommendations. A pediatric cardiologist experienced in KD reviewed all echocardiograms corrected and adjusted the coronary dimensions by *z*-score body-surface area using the Haycock formula (4). Coronary artery dimensions were measured from the maximal diameter of the left main coronary artery, proximal left anterior descending artery, circumflex artery, and proximal right coronary artery and *z*-score was adjusted using the Dallaire's formula (5).

Treatment with intravenous immunoglobulin was used in patients with KD diagnosis. IVIG dose from 1995 to 1999 was 400 mg/kg/day for 5 days and after the year 2000 we used a single IVIG dose of 2 gr/kg.

Patients with incomplete records, without laboratory results or without echocardiography at diagnosis were excluded from the study.

Patients were divided in two groups by age at diagnosis. Group 1 children diagnosed with KD in the first 3 months of age and group 2 children with KD older than 3 months of age. Clinical manifestations, laboratory results, echocardiographic findings, treatment, and outcome were compared between these two groups.

Statistical analysis was performed using the SPSS program, version 21 (SPSS, Chicago IL, USA). Continuous variables were expressed as mean ± standard deviation and median; a Fisher's exact test was performed to compare categorical variables. Wilcoxon-Mann-Whitney test was used for normally distributed continuous variables; $p < 0.05$ were considered to be significant and the confidence interval was 95%.

The Institutional Research and Ethics Committee approved the study.

RESULTS

During the study period, 754 cases of KD were diagnosed at our institution; 66 cases were eliminated from the analysis because of incomplete data. We evaluated 688 cases of KD for this study. Fourteen of these cases were in the first 3 months of age (2.03%). Ten of these cases were male (66.6%). Median age at diagnosis in patients younger than 3 months of age was 2 months; the youngest patient with KD diagnosed at our institution started with fever at 15 days of age and was diagnosed 18 days later.

Time between the onset of fever and diagnosis of KD in children in the first 3 months of age was 16.86 ± 9.38 days compared to 8.66 ± 5.43 days in older patients ($p < 0.001$).

Regarding the classical signs of KD in patients younger than 3 months, in order of frequency were oral changes in 12 cases (85.7%) compared to 629 cases of older children (93.3%) ($p < 0.196$); exanthema in 11 cases (78.6%) compared to 577 (85.6%) ($p < 0.768$); changes in extremities in 10 cases (71.4%) compared to 492 (72.9%) ($p < 0.768$); conjunctival hyperemia was found in nine cases (64.2%) compared to 607 cases of older children (90.0%) ($p < 0.007$). The least frequent of the classical signs was cervical lymphadenopathy and was only found in five cases (35.7%) compared to 378 cases of older children (56.0%) ($p < 0.172$). Incomplete KD was diagnosed in six cases (42.9%) compared to 133 of older children (19.7%) ($p < 0.046$).

Other clinical signs found in our patients were BCG scar reactivation in six of our younger patients (42.9%) compared to 196 cases in older children (29.0%) ($p < 0.380$). Heart failure and Kawasaki disease shock-syndrome (KDSS) was diagnosed in five of the younger patients. Neurological manifestations were found in five cases (35.7%) compared to 83 cases of older children (12.3%) ($p < 0.001$). One patient developed peripheral vasculitis with distal gangrene of the right foot. The complete analysis of the clinical manifestations in patients in the first 3 months of age and the comparison to older children is shown in **Table 1**.

At diagnosis, patients in the first 3 months of age had statistically significant lower levels of albumin and higher leukocyte and platelet counts. The complete laboratory results are shown in **Table 2**.

In the initial echocardiographic evaluation, myocarditis was found in four cases in the younger group (28.6%) compared to 81 cases (12.0%) of the older group ($p < 0.061$); pericardial effusion was diagnosed in six cases of the younger patients (42.9%)

TABLE 1 | Comparison of complete clinical manifestations in patients younger and older than 3 months of age in the acute phase of Kawasaki disease in a population of Mexican children.

	KD $n = 688$				
Clinical manifestation	**First 3 months** $n = 14$		**>3 months** $n = 674$		***p-value***
	No	**%**	**No**	**%**	
Male	10	71.4	441	65.4	0.781
Days to diagnosis	**Median 16 Range (4–32)**		**Median 7 Range (3–41)**		**<0.001**
Oral changes	12	85.7	629	93.3	0.196
Exanthema	11	78.6	577	85.6	0.419
Changes in extremities	10	71.4	492	73.0	0.768
Conjunctival hyperemia	**9**	**64.2**	**607**	**90.0**	**<0.007**
Cervical lymphadenopathy	5	35.7	378	56.0	0.172
Incomplete KD	**6**	**42.9**	**133**	**19.7**	**<0.046**
BCG scar reactivation	6	42.9	196	29.0	0.380
KDSS	**5**	**35.7**	**37**	**5.5**	**<0.001**
Hospitalization days	Median 9 Range (1–23)		Median 5 Range (0–60)		0.116

KD, Kawasaki disease; BCG, Bacille Calmette-Guérin; KDSS, Kawasaki disease shock syndrome. The bold values were meant to highlight the variables with statistically significance.

TABLE 2 | Complete laboratory results in patients with Kawasaki disease younger and older than 3 months of age in a Mexican population.

	KD $n = 688$		
	First 3 months $n = 14$	**> 3 months** $n = 674$	
Laboratory result	**Median (Range)**	**Median (Range)**	***p-value***
Hemoglobin gr/dl	**9.15 (5.4–11.5)**	**11.7 (3.0–16.2)**	**< 0.000**
Hemoglobin *z*-score	−1.22 (−15.38–2.88)	−10.63 (−19.83–6.57)	0.304
Leukocyte count (mm^3)	**20,150 (7,500–43,200)**	**12,800 (5,300–48,400)**	**<0.004**
Platelet count (mm^3)	**512,000 (135,000–1,200,000)**	**347,000 (160–1,350,000)**	**<0.003**
ESR (mm/hr)	48 (12–63)	49 (2–95)	0.623
CRP (mg/l)	9.97 (2.71–16.0)	6.70 (0.05–32.0)	0.370
Sodium (mEq/lt)	135.5 (133–142)	136 (121–157)	0.699
Albumin (g/dl)	**2.27 (1.6–3.9)**	**3.10 (1.0–3.8)**	**0.011**
Total bilirubin (mg/dl)	0.70 (0.50–1.1)	0.62 (0.10–10.0)	0.717
Direct bilirubin (mg/dl)	0.20 (0.2–0.4)	0.16 (0.01–6.9)	0.175
Indirect bilirubin (mg/dl)	0.30 (0.3–0.8)	0.43 (0.01–5.3)	0.173
Alkaline phosphatase (IU/l)	128 (109–206)	195 (100–924)	0.113
GGT (IU/l)	120 (44–211)	47 (7–644)	0.466
LDH (IU/L)	120 (246–590)	46 (6–979)	0.192
AST (IU/l)	**35 (16–226)**	**42 (11–696)**	**<0.050**
ALT (IU/l)	**26 (9–115)**	**39 (6–681)**	**<0.022**

p-value < 0.05 indicates statistically significant.
ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein; GGT, gamma glutamyl transferase; LDH, Lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase. The bold values were meant to highlight the variables with statistically significance.

compared to 124 cases of the older group (18.4%) ($p < 0.019$). Pericarditis was diagnosed in six cases in patients in the first 3 months of age (42.9%) compared to 164 cases in the older group (24.3%) ($p < 0.091$). Coronary artery aneurysms were found in nine cases in the younger group (64.3%) compared to 195 cases in the older patients (28.9%) ($p < 0.005$). The comparison between the size of the coronary arteries and the adjusted *z*-scores in the 2 groups is shown in **Table 3**. In younger patients, giant coronary artery aneurysms (*z*-score > 10) were found in six cases (42.9%) compared to only 37 cases in the older group (5.5%) ($p < 0.000$) **Figure 1**.

Intravenous immunoglobulin (IVIG) was used in 11 cases in the younger group (78.6%) compared to 624 cases of the older group (92.6%) ($p < 0.062$). Steroids were used in nine cases of the younger group (64.3%) compared to 313 cases (46.4%) ($p < 0.167$). A second dose of IVIG was given to two patients in the younger group (14.2%) compared to 43 cases of the older group (6.4%). No additional treatment were used in the younger patients. Two patients died in the acute stage of KD; one per group. An autopsy was performed in both patients which showed giant coronary artery aneurysms, thrombosis and acute myocardial infarction. The patient in the older group, thrombosis was also demonstrated in peripheral arteries (axillar, renal, and femoral arteries).

DISCUSSION

KD is the most common acquired heart disease in developed countries (1). In Mexico as well in other Latin American countries, KD is underdiagnosed and there are only isolated reports of the disease (6). However, the number of cases of KD in Mexico has been increasing over the years[1] (7, 8).

KD is a disease of young children, with almost 80% of the cases diagnosed before 5 years of age (9). In the 23rd National Census

[1]Available online at: http://www.dgis.salud.gob.mx/contenidos/basededatos/BD_Cubos.html

TABLE 3 | Comparison of coronary artery size and z-score adjustments in patients with Kawasaki disease in the first three months of age in a Mexican population.

	KD *n* = 688				
Coronary size	**First 3 months *n* = 14**		**> 3 months *n* = 674**		***p-value***
	n	**Median (Range)**	***n***	**Median (Range)**	
z-score left mean coronary artery	14	3.58 (0.11–21.91)	674	1.05 (−1.83–29.89)	<0.002
z-score left anterior descending artery	8	7.08 (−0.32–19.31)	498	0.82 (−1.66–26.46)	<0.010
z-score circumflex artery	8	5.04 (0.28–13.05)	473	0.65 (−2.15–17.59)	<0.003
z-score proximal right coronary artery	14	17.26 (0.76–21.09)	674	0.66 (−1.41–40.0)	<0.002

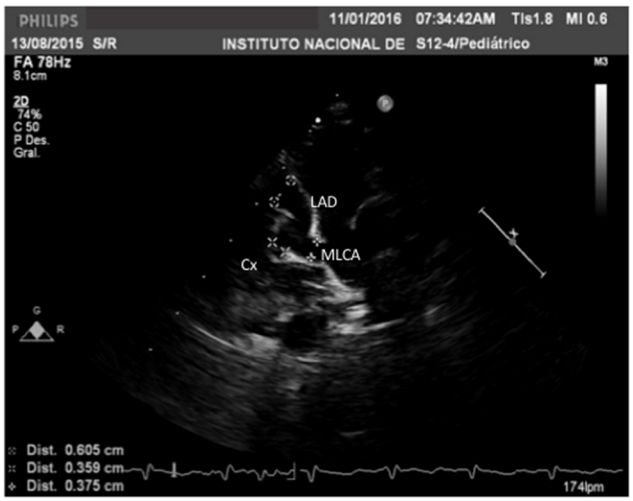

FIGURE 1 | Echocardiogram in a modified long-axis parasternal view in a 2-months-old boy with Kawasaki disease diagnosed after 18 days of the onset of fever. Weight 4.7 kg, height 55 cm, BSA 0.27 m^2. With a large coronary artery aneurysm in the main left coronary artery with z-score of +7.70; a giant coronary artery aneurysm in the left anterior descending artery with a *z*-score of +17.29 and a large aneurysm of the circumflex artery with a *z*-score of +8.99.

of Kawasaki disease in Japan, Makino et al., found that the most frequent age of presentation was between 9 and 11 months of age (2). In the last Korean survey of KD, the most frequent age of presentation was 29 months (10). In both countries, which is where the highest incidence of KD exists, cases are rare in very young infants. In Mexico, according to national data from 2000 to 2018, the most frequent age of presentation of KD was 12 months, and these patients represented 27% of the diagnosed KD cases in Mexico[1].

KD in very young children is a rare event. In a study in the San Diego area, between 2004 and 2013, Salgado et al. reported on 720 cases of the KD, in which 88 cases were in patients under the age of 6 months (3). Yoon et al. in Korea studied 239 cases of KD diagnosed between 2013 and 2015, and 26 cases were younger than 6 months of age (11). Singh et al. in a 20-years period study of KD in a single hospital in India, found 460 patients and only 17 cases were under 6 months of age (12). The largest study of KD in younger patients was performed in Korea by Lee et al., based on Korean National Surveys, 27,851 cases of KD were diagnosed and just 609 cases were children under the age of 3 months (2.18%) (13).

Our incidence of KD in infants in the first 3 months of age (2.03%) was similar to the large epidemiological studies in countries with a high incidence of KD.

As there is no accurate diagnostic marker for KD, diagnosis is made based on the combination of characteristic clinical findings. In very young children, a high incidence of incomplete and atypical KD has been reported (3, 11, 12). This clinical presentation often causes a delay in diagnosis and treatment and therefore infants with KD are at higher risk of developing severe cardiac complications as compared to older children (14). Maternal antibodies have been thought to be protective against KD supported by the rarity of KD before 3 months of age, contrasting with this theory are our findings and other multiples reports showing a severe presentation in this age group.

In our younger patients, classical clinical features of KD in order of frequency were oral changes (85.7%), exanthema (78.6%), changes in the extremities (71.4%), conjunctival hyperemia (64.2%), and cervical lymphadenopathy (36.7%). These data are similar with findings in younger patients with KD obtained in the San Diego area and in India (3, 12). Yoon et al. reported that conjunctival hyperemia was the most frequent clinical sign in patients under the age of 3 months which differed from our findings (11).

We found that six of our patients in the first 3 months of age had incomplete KD (42.9%), Salgado et al., found a 48.8% of incomplete presentation of the disease in patients under the age of 6 months (3). Hangai et al. based in the 22nd National Census of Kawasaki disease in Japan studied the presence of KD in newborns and found an incomplete presentation of the disease in 65% of the cases (15). Singh et al., in India studied 17 cases of KD in patients under 6 months of age and found that 88% of the cases had an incomplete presentation of the disease (12).

A BCG scar reactivation is an important clinical sign for diagnosis of KD in countries where the BCG vaccine is administered. Uehara et al., based in the 19th National Survey of Kawasaki disease in Japan, found that a BCG scar reactivation was present in 50% of patients with KD (16). This finding has also been reported by other authors like Singh, who found that a BCG scar reactivation was present in 11% of patients with KD younger than 6 months of age (12).

In a previous study performed in our hospital, Garrido-Garcia et al. found that a BCG scar reactivation occurred in 24.3% of 416 cases of KD, but this scar reactivation was increased to 32.3% in children younger than 12 months of age (17). In the present study, a BCG scar reactivation was found in six out

of 14 cases in the first 3 months of age (42.9%). Araki et al. found that BCG erythema in patients with KD is most closely associated with the interval from the BCG vaccination to onset of KD (18). Intriguingly, some studies have found a correlation between BCG scar reactivation and severity of the disease (more inflammation with increased white blood cells, platelet counts, and transaminases) while others have not (18–20). As proposed by many authors, in countries where BCG vaccine is mandatory (including Mexico), a BCG scar reactivation could be a very important clue to diagnose KD in very young patients (18–25).

Another difference in the clinical picture of KD in very young patients is the increased frequency of atypical presentations including facial palsy, seizures, myocarditis, and cardiogenic shock (12, 23). In our study group, the atypical manifestations of KD were present in 35.7% of the cases compared to 10.7% of older patients ($p < 0.019$). The most commonly atypical manifestations found in our study were KDSS and severe central nervous system manifestations.

The incomplete and atypical presentations of KD in younger patients often causes a delay in diagnosis of the disease. In our study, we found an important delay in diagnosis, with 16.86 ± 9.38 days in younger patients, compared to 8.66 ± 5.43 days in older patients. This finding contrasts with other studies in countries with a greater incidence of KD, in which the diagnosis of KD is made in less than 10 days despite the age of the patient. Therefore, it is important to remember the AHA recommendations that any infant with unexplained fever for 7 or more days have an evaluation for KD, including an echocardiogram. Delay in the diagnosis with uncontrolled inflammation for more days probably contributed to the higher incidence of KDSS and CAA in our younger patients.

Yoon et al. reported that diagnosis of KD was made at 5.8 ± 2.0 days in younger patients, compared to 5.7 ± 1.5 days in older children (11). Also, Salgado et al. did not find differences in the time to diagnosis of KD with a median of 6 days both in children under 6 months and in older patients (3).

Until now, there is no specific diagnostic test of KD, but laboratory results reflect the degree of inflammation that is very common in young patients with KD. In our study, we found statistical significant increased leukocyte and platelet counts. These laboratory findings had also been confirmed by other groups (3, 9–12).

As almost all studies in younger patients, we found an increased frequency of coronary artery aneurysms in patients in the first 3 months of age, with 64.3% of our cases compared to 28.9% in older children. However, the most severe complication was the development of giant coronary artery aneurysms (42.9% compared to 5.5% in older patients). These findings were also confirmed by other groups who described patients with a late diagnosis of KD developing myocardial ischemia, circulatory collapse, and even death caused by coronary artery aneurysms and thrombosis (26, 27). Hangai et al. reported an incidence of coronary abnormalities in 17% of neonatal KD and in 16% in patients under the age of 6 months (15). Salgado et al. found that the presence of coronary lesions was 43.4% in younger patients compared to 19.5% in patients above the age of 6 months (3). Singh et al., reported up to 35% of coronary lesions in patients under the age of 6 months (12). In Taiwan, Chang found that coronary aneurysms developed in 65% in patients under the age of 6 months, compared to 19% in older patients (25).

FIGURE 2 | Peripheral vasculitis with distal gangrene in the right foot of a 45-days-old girl with Kawasaki disease diagnosed after 21 days of the onset of fever.

KDSS was particularly frequent in our younger patients. Peripheral gangrene is a complication of KD that occurs almost exclusively in infants younger than 7 months (28). One of our patients presented with peripheral vasculitis with distal gangrene in the toes of the right foot **Figure 2**. These observations reinforce the notion that KD is more severe in very young patients.

Finally, regarding appropriate treatment, in our study IVIG was administered in only 78.5% of the cases compared to 92.6% in older children. This finding was related to a delay in diagnosis, since patients in our younger group who did not receive IVIG, were patients who were not diagnosed in the acute phase of the disease and whose fever and systemic inflammation had resolved. In our younger group we also found a higher incidence of IVIG resistance (14.2%) compared to the older group (6.4%). This finding could be explained by an increased and persistent inflammation in younger patients.

LIMITATIONS OF THE STUDY

Our study has strengths and limitations. To our knowledge, our series is the largest of KD in a single center in Latin America and is also the first to study KD in very young patients in the area. The most important limitation of the study is its retrospective design; therefore some date of our early patients are unknown. Data were obtained from a single institution, so it does not reflect the presentation of KD in the country. More studies have to be conducted in Mexico as well in other Latin American countries to establish the characteristics of infants with KD in Latin America.

CONCLUSIONS

KD diagnosis in very young patients is difficult. KD should be suspected in any patient ≤3 months of age, with persistent high fever, without etiology, even if only some or none of the classic manifestations of KD are present. It is also important to note that a BCG scar reactivation may be a useful sign for early diagnosis and timely treatment in this group of patients in countries where the BCG vaccine is part of their immunization program. Finally,

these patients have a higher rate of cardiovascular complications, atypical and incomplete presentations and KDSS as compared to older patients, and thus a high level of suspicion is critical.

WHAT'S KNOWN ON THIS SUBJECT

Kawasaki disease is the most common cause of acquired heart disease in developed countries; it is more common in children older than 1 year of age. An early diagnosis prevents the development of cardiac complications.

WHAT THIS STUDY ADDS

Kawasaki disease in young infants, is an uncommon presentation with greater risk to develop cardiac complications. To our knowledge, this study represents the largest series of Kawasaki disease in very young infants in a Latin American country.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee National Institute of Pediatrics. Mexico. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LG-G conceptualized and designed the study, performed the final data analysis, reviewed, and revised the final manuscript. JG-A designed the data collection instruments, collected data, carried out the initial analyses, and drafted the initial manuscript. AR-P designed the data collection instruments, collected data, carried out the initial analyses, and drafted the initial manuscript. AT reviewed the data analysis, reviewed, and revised the final manuscript. MY-N conceptualized and designed the study, reviewed, and revised the final manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Biomarkers for the Discrimination of Acute Kawasaki Disease From Infections in Childhood

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Background: Kawasaki disease (KD) is a vasculitis of early childhood mimicking several infectious diseases. Differentiation between KD and infectious diseases is essential as KD's most important complication—the development of coronary artery aneurysms (CAA)—can be largely avoided by timely treatment with intravenous immunoglobulins (IVIG). Currently, KD diagnosis is only based on clinical criteria. The aim of this study was to evaluate whether routine C-reactive protein (CRP) and additional inflammatory parameters myeloid-related protein 8/14 (MRP8/14 or S100A8/9) and human neutrophil-derived elastase (HNE) could distinguish KD from infectious diseases.

Methods and Results: The cross-sectional study included KD patients and children with proven infections as well as febrile controls. Patients were recruited between July 2006 and December 2018 in Europe and USA. MRP8/14, CRP, and HNE were assessed for their discriminatory ability by multiple logistic regression analysis with backward selection and receiver operator characteristic (ROC) curves. In the discovery cohort, the

combination of MRP8/14+CRP discriminated KD patients ($n = 48$) from patients with infection ($n = 105$), with area under the ROC curve (AUC) of 0.88. The HNE values did not improve discrimination. The first validation cohort confirmed the predictive value of MRP8/14+CRP to discriminate acute KD patients ($n = 26$) from those with infections ($n = 150$), with an AUC of 0.78. The second validation cohort of acute KD patients ($n = 25$) and febrile controls ($n = 50$) showed an AUC of 0.72, which improved to 0.84 when HNE was included.

Conclusion: When used in combination, the plasma markers MRP8/14, CRP, and HNE may assist in the discrimination of KD from both proven and suspected infection.

Keywords: kawasaki disease, infectious disease, vasculitis, coronary aneurysm, biomarker, bacterial infection, viral infection

INTRODUCTION

Kawasaki disease (KD) is a systemic vasculitis of early childhood occurring mainly in children under the age of 5 years. The origin of the disease is still unknown. The current paradigm is that KD is caused by an immunologic reaction elicited by an (infectious) trigger in genetically predisposed children. KD's most important complication is the development of coronary artery aneurysms (CAA), affecting 25% of the untreated patients (1). This makes KD the most common cause of acquired heart disease in developed countries in children. The risk of CAA development is decreased 5-fold when the patient is treated with high-dose intravenous immunoglobulin (IVIG) and oral aspirin within 10 days of disease onset (1).

No specific diagnostic laboratory test for KD is available to date and KD is diagnosed based on the presence of clinical criteria, including prolonged fever, rash, lymphadenopathy, conjunctival injection, and abnormalities of mucosae and extremities. However, KD can be easily misdiagnosed due to the symptomatology resembling several infections (1–3) and the fact that bacterial and viral pathogens are regularly found in KD patients (2). Additionally, atypical or incomplete KD may also hinder the diagnosis. Therefore, KD diagnosis and initiation of treatment are commonly delayed, with a resulting increased risk of CAA development.

A rapid blood test to distinguish KD from infection, which enables the diagnosis and early treatment initiation, would reduce the burden of KD-related heart disease. Up to now, studies on surrogate plasma markers for acute KD have not included adequate febrile infection controls (4–6). While previous studies have shown promise that KD patients can be discriminated from febrile controls using gene expression (5), findings based on proteins may be more easily translatable into tests with immediate clinical utility.

We selected plasma biomarkers shown to be increased in KD patients and combined these markers to investigate whether a multi-protein test would discriminate KD from infection. We focused on C-reactive protein (CRP) and two neutrophil-derived proteins, myeloid-related protein 8/14 (MRP8/14) and human neutrophil elastase (HNE). MRP8/14 (S100A8/9 or calprotectin), a cytoplasmic protein found in neutrophils, and to a much lesser extent in monocytes, belongs to a family of calcium-binding proteins (7). MRP8/14 is released into the extracellular space upon cell activation, operating as a damage-associated molecular pattern (DAMP). MRP8/14 is recognized as a biomarker for several inflammatory diseases, including inflammatory bowel disease, and rheumatoid arthritis (8–10). In acute KD patients, elevated MRP8/14 levels have been previously reported, but in studies with no febrile controls (4, 11, 12). HNE, a well-known marker of neutrophil activation, is a neutrophil-specific serine protease located in the neutrophil azurophilic granules. HNE has been shown to be increased in the acute phase of KD compared to afebrile healthy controls (13).

CRP, an acute inflammatory protein that is synthesized in hepatocytes, binds to polysaccharides on microorganisms, triggering the classical complement pathway and initiating an innate immune response. CRP is commonly used as a biomarker as it is elevated during both acute infection and inflammation (14, 15).

The aim of this study was to evaluate whether MRP8/14, CRP, or HNE may act as possible biomarker(s) to diagnose acute (including incomplete) KD in febrile children. We analyzed the MRP8/14 levels before and after treatment in paired samples available from KD patients and investigated whether MRP8/14, CRP, and/or HNE could be used as a marker for the prediction of responsiveness to IVIG treatment and/or of CAA development during acute KD.

METHODS

We have used three cohorts of KD patients: a discovery cohort and two independent validation cohorts. For comparison, we used prospective samples collected in independent studies from febrile children diagnosed with acute bacterial and viral infections as well as those suspected of infection.

KD Patients

Children (<18 years old) with acute KD based on the criteria of the American Heart Association (16) were recruited at the University Medical Center in Amsterdam [Amsterdam UMC, location Academic Medical Center (AMC)] and participating

hospitals in the Netherlands as approved by the Medical Ethical Board of the AMC (no. NL41023.018.12) (for the discovery cohort). Patients were recruited between July 2006 and December 2018. For our first validation cohort, KD samples were included from the ongoing UK Kawasaki study "*Genetic determinants of Kawasaki Disease for susceptibility and outcome*" (13/LO/0026). This study recruits acutely unwell children with KD during hospital admission in participating hospitals around the UK. Our second validation cohort included KD patients who met the American Heart Association criteria for complete or incomplete KD cared for at Rady Children's Hospital, San Diego, CA.

The medical records of KD patients were reviewed and the clinical details recorded. CAA were defined by worst ever *z* scores: CA dimensions as standard deviation units normalized for body surface area (17). CAA was defined as a coronary *z* score $\geq$2.5.

EDTA plasma samples of the KD discovery cohort were collected before and, if available, after IVIG treatment. All samples taken within 14 days from disease onset (fever) were selected for the analysis and only those within 2 days after the start of IVIG were included as "acute," except for the analysis of the paired samples of "acute disease" and convalescent after IVIG, from which the samples were taken during convalescence up to 1 year after the onset of disease. In the validation cohorts, samples from the "acute disease" were all samples before IVIG was administered.

Febrile Patients With Definite Infections

For the discovery and the first validation cohorts, we compared KD patients to children presenting with acute febrile illness caused by a bacterial or viral illness. All children with a bacterial infection had a microbiologically confirmed pathogen detected in a normally sterile site and a consistent corresponding syndrome, including sepsis, meningitis, osteomyelitis, or pneumonia. All children with a viral illness had a detected viral pathogen, confirmed with culture, molecular, and/or immunofluorescent testing, and a consistent corresponding syndrome, without hallmarks of bacterial illness. In the viral infection group, a CRP of <60 was used to define a set of patients with a confident viral infection, as described in (18).

Discovery cohort

EDTA plasma samples from children (<18 years old) were collected at the first available time after presentation (within 48 h), in European hospitals participating in the EUCLIDS Consortium (19) (EU-Childhood Life-Threatening Infectious Disease Study; www.euclids.eu) and in the GENDRES (GENetic, Vitamin D and RESpiratory Infections Research Network; www.gendres.org) (20). Patients were included between December 2009 and May 2014. Clinical details were recorded as part of the study, including gender, age at disease onset, hospitalization, and details about the type of disease and the invasive pathogen involved when cultured from sterile sites.

First Validation Cohort

Patients were included after local approval in the international study on febrile children (PERFORM; https://www.perform2020.org/). Overall, patients were included between July 2012 and December 2018. EDTA plasma samples were collected at presentation to hospital in the Emergency Department or ward. Clinical details were recorded as described above.

Febrile Patients With Suspected Infections

For the second validation cohort, we compared KD patients to children presenting with acute febrile illness with suspected infection.

Second Validation Cohort

Febrile control patients were recruited from the Emergency Department at Rady Children's Hospital, San Diego, CA. The study protocol was reviewed and accepted by the UCSD institutional review board. Parental consent was obtained, where appropriate. The inclusion criteria for the febrile controls were fever for at least 3 days, no use of steroids, and at least one clinical sign of KD. Review of the medical records at least 1 month after the onset of fever retrospectively confirmed that these patients all had a self-limited illness that required no anti-infective treatment and were deemed likely due to a non-specific viral illness. The majority of these patients had been initially referred to the hospital to rule out KD.

Healthy Controls

After informed consent, EDTA-anticoagulated blood samples from healthy volunteer donors were obtained *via* an internal system at Sanquin (the Dutch national blood bank) after consulting the Medical Ethical Committee from the Academic Medical Center Amsterdam. All procedures were conducted in accordance with the 1975 Declaration of Helsinki as revised in 2013, and local ethics approval were obtained within the PERFORM Study mentioned above. Median age was 37.8 (interquartile range, IQR = 37.8–58.7) years and 39.5% were male. No clear age-defined or gender-related effects were seen in the baseline values (13).

Analysis of Neutrophil Activation Markers

MRP8/14 was measured in a new enzyme-linked immunosorbent (ELISA) assay developed at Sanquin. All washes between the incubation steps were done with phosphate-buffered saline (PBS) 0.02% Tween-20 using Elx 405 (BioTek Instruments). Anti-human MRP8/14 monoclonal mouse IgG1 antibody, clone 27E10 (0.5 µg/ml in 0.1 M carbonate buffer, pH 9.6; BMA Biomedicals, Augst, Switzerland), was used to coat a Maxisorp Nunc-immunoplate (Thermo Scientific). After overnight static incubation at room temperature and wash, serum pooled from 30 healthy donors was added to give a calibration curve [diluted 2-fold from 8.5 ng/ml (*v*/*v*) in TTG (10 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.1% Tween-20, 0.2% gelatin, pH 7.4)]. As a positive control, the pooled sera was spiked with 10 µg/ml recombinant MRP8/14 (Hycult Biotech, Plymouth Meeting, PA, USA) and diluted to 0.1% (*v*/*v*). The samples, positive control, and calibration curve were incubated for 1 h. After washing, 500 ng/ml biotinylated mouse monoclonal IgG1 anti-MRP8/14 antibody, clone S36.48 (BMA Biomedicals), was added. After incubation for 1 h, further washing, and the addition of

streptavidin conjugated to poly-horseradish peroxidase [poly-HRP, 0.1% (*v*/*v*)] for 30 min followed by 100 μg/ml 3,3′,5,5′-tetramethylbenzidine (TMB; Merck Chemicals) in 0.11 M sodium acetate, pH 5.5, with 0.003% (*v*/*v*) H_2O_2, and 2 M H_2SO_4, absorbance was measured at 450/540 nm. The results are expressed in nanograms per milliliter by reference of the calibration curve.

Plasma HNE is not present in blood alone, but in complex with α1-antitrypsin (α1AT). Therefore, plasma HNE was measured as HNE–α1AT complexes. CRP and HNE–α1AT complexes were determined by ELISAs, as described previously (21, 22).

Statistical Analysis

Data were assessed to determine normal distribution. Differences in the MRP8/14, CRP, and HNE values between the patient cohorts were plotted as the median + IQR. Log_{10}-transformed data were used with respect to linearity for further statistical analyses on the predictive values of the markers.

A logistic regression analysis with backward selection was used to examine the predictive value (odds ratio) of the candidate markers for acute KD vs. infections and for KD vs. bacterial and KD vs. viral infections.

Receiver operating characteristic (ROC) curves were plotted from the predictive markers derived from the logistic regression analysis. The area under the ROC curve (AUC) from both the individual candidate markers as well as a combination of candidate markers (the predicted probability) was used to check for discriminatory value. The best discriminatory cutoff value was calculated with Youden's J Statistic. The cutoff from the discovery cohort was applied to the validation cohorts and the corresponding sensitivity and specificity with 95% confidence intervals (CI) were calculated. Using predicted probabilities based on the discovery model, ROC curves in the validation cohorts were plotted and AUCs were compared to the AUC's from the discovery cohort.

For the analysis of the MRP8/14 levels pre- and post-IVIG treatment, a Wilcoxon signed-rank test was performed in paired samples. The predictive capacities of the markers for treatment response and CAA development in acute KD patients were calculated by a logistic regression with backward selection.

A $p < 0.05$ was considered to be statistically significant. SPSS version 24.1 (IBM, Armonk, NY) and R version 3.4.4 (The R Foundation for Statistical Computing, Vienna, Austria) were used for all statistical analyses.

RESULTS

Our discovery cohort consisted of 48 patients with acute KD (27 pre-IVIG treatment and 21 shortly after IVIG treatment had been administered, within 3 days). Of these acute patients, 10 were diagnosed with incomplete KD. In total, 105 patients were included with infections (40 patients with viral infections and 65 patients with bacterial infections). Our first validation cohort consisted of 26 patients with acute KD (pre-IVIG treatment) and 150 patients with infections (75 bacterial and 75 viral) diagnosed at different centers in Europe. The second validation cohort consisted of 50 KD patients (with paired measurements pre- and post-IVIG treatments, i.e., acute vs. convalescent disease) and 50 febrile control patients. The demographic and clinical characteristics of all patients are shown in **Table 1**. **Supplemental Table 1** provides a more detailed overview of the patients with definite (i.e., proven) infections in our discovery and first validation cohorts.

MRP8/14 and CRP Discriminate Between Acute KD and an Infection

First, we aimed to determine the differences in MRP8/14, CRP, and HNE in the discovery cohort (**Figures 1A–C**). MRP8/14, CRP, and HNE were significantly different between the KD and infectious patients. The results from our two validation cohorts are shown in **Supplemental Figure 1**. When assessing KD vs. bacterial infections only, the MRP8/14 levels were significantly elevated in KD. In the bacterial patients, a high- and low-MRP8/14 group could be distinguished. The patient group with the high MRP8/14 levels did not have a pathogen or diagnosis in common. No correlation was found between the MRP8/14 or HNE and absolute neutrophil counts (data not shown).

Multiple logistic regression analysis with backward selection on the discovery cohort showed that elevated levels of MRP8/14 (log_{10} OR = 7.7, 95% CI = 3.2–18.5) and CRP (log_{10} OR = 5.1, 95% CI = 1.7–14.7) were predictive for acute KD over an infection ($p < 0.001$). HNE was removed from the analysis during backward selection.

ROC curves were plotted for the predictive ability of MRP8/14 and CRP to discriminate between acute KD and an infection. The AUC for MRP8/14 was 0.86 (95% CI = 0.78–0.93, $p < 0.001$), with a sensitivity of 70% and a specificity of 91% and cutoff of 6,540 ng/ml. For CRP, the AUC was 0.76 (95% CI = 0.68–0.85, $p < 0.001$), with a sensitivity of 78% and a specificity of 67% and cutoff of 43.9 mg/l. The ROC curve of the predicted probability of KD when MRP8/14 and CRP were used together was 0.88 (95% CI = 0.82–0.95, $p < 0.001$), with a sensitivity of 85% and a specificity of 83% (**Figure 2A**). The optimal cutoff from the discovery cohort (predicted KD probability of 0.37 or higher) was applied to the first validation cohort and the corresponding sensitivity and specificity were determined, showing a sensitivity of 73% and a specificity of 77%. The ROC curve from the predicted probability of KD when both MRP8/14 and CRP were used in the first validation cohort showed an AUC of 0.81 (95% CI = 0.73–0.88) (**Figure 2B**).

The same optimal cutoff was also applied to our second validation cohort, with patients from the USA. Here, we found a sensitivity of 74% and a specificity of 62%. The ROC curve with both MRP8/14 and CRP showed an AUC of 0.72 (95% CI = 0.62–0.82) (**Figure 2C**).

Meta-Analysis of All Cohorts

In this study, we wanted to test the values of CRP, MRP8/14, and HNE in predicting KD over an infection. A summary of all the AUCs and the sensitivity and specificity values for MRP8/14+CRP in the different cohorts is shown in **Supplemental Table 2**. Three independent cohorts showed a strong predicted probability of KD over an infection when

TABLE 1 | Demographic and clinical characteristics of the patients in the acute Kawasaki disease, bacterial, and viral infections cohort and in the discovery and validation cohorts.

	Discovery cohort			**First validation cohort**			**Second validation cohort**		
	Kawasaki disease, *n* = 48 (27 pre-IVIG, 21 post-IVIG)	**Bacterial infections, *n* = 65**	**Viral infections, *n* = 40**	**Kawasaki disease, *n* = 26**	**Bacterial infections, *n* = 75**	**Viral infections, *n* = 75**	**Kawasaki disease, pre-IVIG, *n* = 50**	**Kawasaki disease, post-IVIG, *n* = 50**	**Febrile control, *n* = 50**
% Male (*n*)	64.4% (31)	47.7% (31)	45% (18)	69.2% (18)	54.7% (41)	57.3% (43)	46% (23)	46% (23)	48% (24)
Age at sample date (years, median +)	2.1 (1.1–4.5)	9 (3–14.3)	1.4 (0.2–3.6)	2.8 (1–3.8)	4.58 (0.8–7.5)	3 (0.8–6.9)	2.7 (1.6–5.5)	2.7 (1.6–5.5)	3.4 (1.2–5.2)
Days of fever before hospital admission (days, median +)	8 (6–11)	1 (0–2)	1 (0–2)	6 (5–8)	1 (1–5)	2 (1–5)	5 (4–7)	19 (17–21)	5 (4–7)
Days until IVIG treatment (days)	<10 days	Not applicable	Not applicable	<10 days	Not applicable	Not applicable	<10 days	Not applicable	Not applicable
% CAA (*n*)	CAA: 25% (12) Giant: 8.3% (4)	Not applicable	Not applicable	CAA: 15.4% (4) Giant: 0%	Not applicable	Not applicable	CAA: 24% (12) Giant: 0%	CAA: 24% (12) Giant: 0%	Not applicable
% Unresponsive to first IVIG course (*n*)	25% (12)	Not applicable	Not applicable	23% (6)	Not applicable	Not applicable	4% (4)	Not applicable	Not applicable
% Ethnicity	Caucasian: 68.6%; Asian: 4.2%; Other: 4.2%; Unknown: 23%	Caucasian: 80%; Asian: 1.5%; Other: 4.5%; Unknown: 14%	Caucasian: 62.5%; Asian: 12.5%; Hispanic: 12.5%; Other: 12.5%	Caucasian: 23%; Asian: 11.5%; African: 34.5%; Other: 15.5%; Unknown: 15.5%	Caucasian: 53.3%; Asian: 1.3%; Hispanic: 21.3%; Mixed: 1.3%; Other: 10.6%	Caucasian: 56%; Asian: 10.6%; Hispanic: 21.3%; Mixed: 1.3%; Other: 10.6%	Caucasian: 12%; Asian: 18%; Hispanic: 42%; Mixed: 28%	Caucasian: 12%; Asian: 18; Hispanic: 42%; Mixed: 28%	Caucasian: 20%; Asian: 8%; Hispanic: 42%; Mixed: 22%; Unknown: 8%

Giant aneurysm is defined by a z score >2.5.
CAA, coronary artery aneurysm.

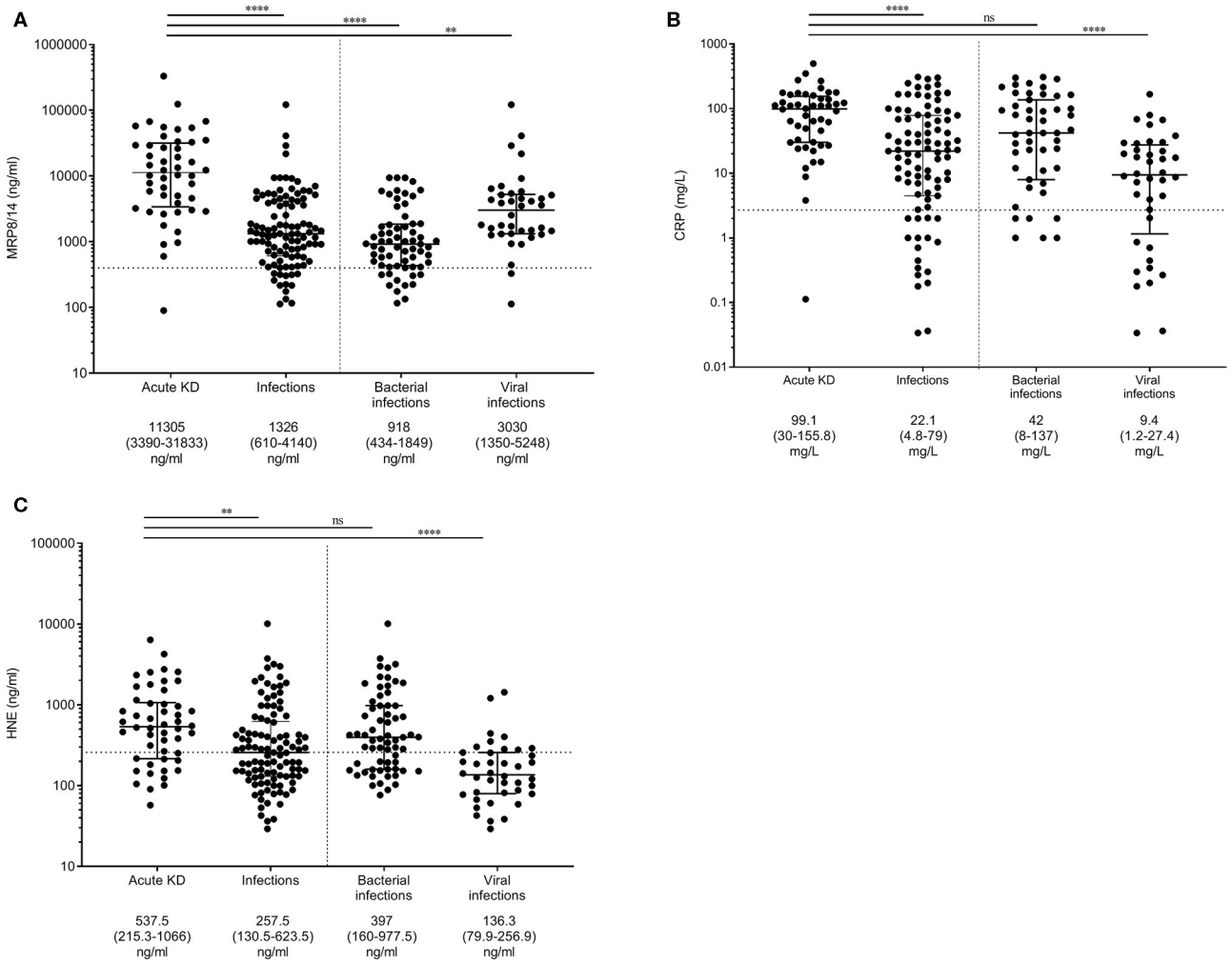

FIGURE 1 | Myeloid-related protein 8/14 (MRP8/14) **(A)**, C-reactive protein (CRP) **(B)**, and neutrophil-derived elastase (HNE) **(C)** levels in the discovery cohort. Patients with acute Kawasaki disease are compared to patients with infections and, more specifically, patients with either bacterial or viral infections. Each *dot* is a unique patient. *Dotted line* represents the 75% concentration in the healthy controls: 400 ng/ml MRP8/14, 2.7 mg/l CRP, and 37.3 ng/ml HNE. Median levels + interquartile ranges are listed *below the figure*. Differences were calculated with the Kruskal–Wallis test, followed by Dunn's multiple comparisons test. $^{**}p < 0.005$, $^{***}p < 0.001$; *ns*, not significant.

combining MRP8/14 and CRP. HNE was eliminated in the analysis after the logistic regression with backward selection in our discovery cohort. By analyzing all three cohorts together in a meta-analysis, we created a large cohort with a range of ethnicity. This meta-cohort was used to test our model and see whether revision or extension of the existing prediction model was necessary.

Our large cohort consisted of 124 acute KD patients and 305 febrile patients (proven infections combined with the "undefined" febrile controls). We re-estimated the effects of our current model and investigated a possible extension with HNE in this large cohort. Multiple logistic regression analysis showed that elevated levels of MRP8/14 ($\log_{10}$ OR = 5.5, 95% CI = 3.1–9.6), CRP ($\log_{10}$ OR = 2.4, 95% CI = 1.3–4.1), and HNE ($\log_{10}$ OR = 2.3, 95% CI = 1.3–4.0) were predictive of acute KD over an infection ($p < 0.001$). Although HNE was not included in the initial model, in our large cohort, we saw a significant effect of HNE in predicting KD over an infection. The discriminatory power of the model with MRP8/14, CRP, and HNE showed an AUC of 0.84 (95% CI = 0.80–0.88), with a sensitivity of 74% and a specificity of 83% (**Figure 3**).

MRP8/14 as a Marker for Treatment Response and CAA Development

Prediction of IVIG resistance and risk of developing CAA by biomarkers would greatly aid clinical decision making. In the discovery cohort and the second validation cohort, we compared the levels of MRP8/14 in paired samples and in those with and without CAA.

A total of 25 children had paired measurements pre-IVIG treatment and during convalescence [median of 49 days (IQR = 27–155 days) after disease onset]. The samples

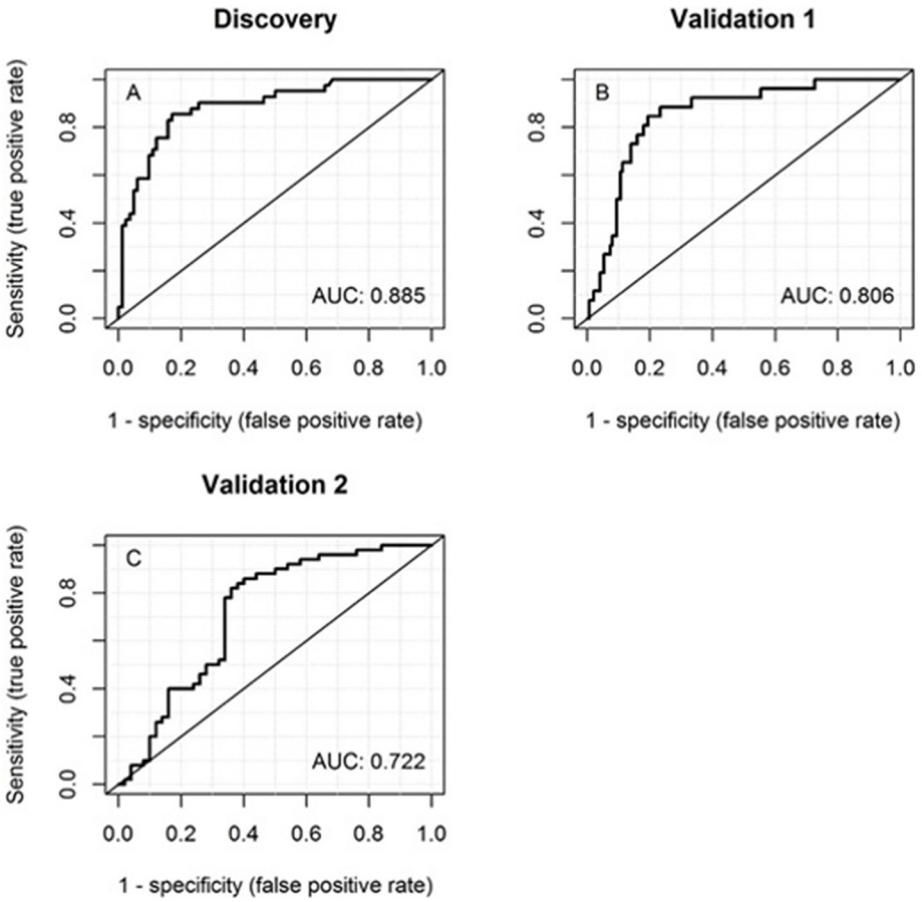

FIGURE 2 | Receiver operator characteristic (ROC) curves of the discovery cohort **(A)**, first validation cohort **(B)**, and the second validation cohort **(C)** of MRP8/14 and CRP together for discrimination between acute Kawasaki disease and infections. The corresponding areas under the ROC curve (AUCs) are listed *within the panels*.

collected prior to IVIG administration showed significantly higher levels of MRP8/14 (median = 10,400 ng/ml, IQR = 3,500–42,500 ng/ml) compared to the samples collected during post-IVIG convalescence (median = 1,600 ng/ml, IQR = 300–5,600 ng/ml, $p < 0.0001$) (**Figure 4A**).

In our second validation cohort, a total of 50 patients had paired measurements before IVIG treatment and during convalescence [median of 20 days (IQR = 17–22 days) after disease onset]. Again, the samples pre-IVIG treatment showed significantly higher levels of MRP8/14 (median = 8,398 ng/ml, IQR = 4,506–16,025 ng/ml) compared to the post-IVIG convalescence state (median = 836 ng/ml, IQR = 450.8–1,924 ng/ml, $p < 0.0001$) (**Figure 4B**). The same significant decrease was seen in CRP and HNE in both the discovery and validation cohorts (data not shown). A logistic regression analysis showed that neither MRP8/14 nor one of the other markers was predictive of clinical IVIG response.

As CAA are the most prevalent complications of KD, we combined all our KD samples and assessed whether the MRP8/14 values were elevated in patients with CAA, but no significant differences were seen (**Supplemental Figure 2**). Also, a logistic regression analysis showed that none of the markers was predictive of CAA development (data not shown).

DISCUSSION

To date, a rapid protein test to distinguish KD from infection is not available. Different studies have been conducted in the search for a biomarker, but have failed to find a single and reliable biomarker. This includes studies on a broad panel of routinely used parameters (23), antibody profiling using of *Escherichia coli* proteome microarrays, (24), or the identification of candidate biomarkers using multiplex cytokine and protein arrays (25). In this study, we have investigated inflammation-related plasma-derived biomarkers to identify pediatric patients with acute KD among their febrile peers with a proven or suspected infection. Our most important finding was that a combined measurement of MRP8/14 and CRP, and possible HNE, was indicative of KD, discriminating KD from probable infections in febrile children. When applying the optimal cutoffs to the validation cohorts, we observed an impaired specificity of the markers, with an

overestimation of KD. This might be a result of the differences in pathogens (**Supplemental Table 1**). The different case/control ratios in the three cohorts could possibly influence the estimated sensitivities, specificities, and the derived AUCs, but these effects are not systemic (26). In the discovery cohort, we included 21 KD patients in the acute group, up to 3 days after IVIG treatment. We split the KD group into pre-IVIG treatment and shortly after IVIG treatment and compared the levels of MRP8/14, CRP, and HNE in these two subgroups. There was no difference seen (data not shown), showing that these biomarkers are stable in the acute phase of KD up to 3 days after IVIG treatment. What we have noticed before is that HNE remains detectable in KD when measured at regular time points up to 3 months following the acute stage (13) and that CRP remains elevated in KD patients with CAA more than 5 years after disease onset (27). This indicates that low-grade inflammation may indeed be present subclinically much longer than previously thought.

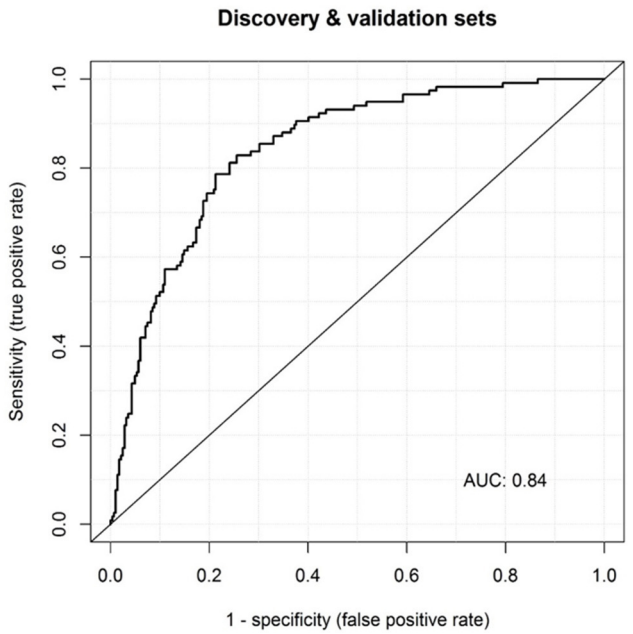

FIGURE 3 | Receiver operator characteristic (ROC) curves of MRP8/14, CRP, and HNE combined in the meta-analysis. The corresponding area under the ROC curve (AUC) is listed *within the panel*.

It is highly encouraging that candidate plasma markers for acute KD in febrile children have been found and validated against common bacterial and viral infections. Together with the clinical manifestations at presentation of these patients, the combined use of these markers will enable physicians to be more confident in their KD diagnosis. Although MRP8/14 is significantly lower after treatment with IVIG in the paired samples, its pretreatment levels could not predict clinical IVIG response in our discovery cohort and in the second validation cohort. We also did not find a correlation of the MRP8/14 (nor CRP and HNE) levels with CAA.

The most important consequence of a quick and correct KD diagnosis is the ability to promptly initiate IVIG treatment and, thereby, reduce the risk of CAA development. CRP is used, but is non-discriminatory. HNE has been used before in a previous study of our group by (13). A few studies have reported on the MRP8/14 levels in children with KD. Hirono et al. found increased MRP8/14 concentrations before IVIG as compared to post-IVIG in 45 IVIG-responding KD patients, whereas the levels did not decrease after IVIG in 16 IVIG non-responders (12). Four weeks after IVIG treatment, these patients still showed higher MRP8/14 concentrations compared to IVIG responders. Abe et al. found increased MRP8/14 levels in 32 KD patients before IVIG treatment compared to after IVIG treatment and compared to controls with fever of unknown origin (4), which may correlate with the epigenetic regulation of S100A genes (28). Viemann et al. found that the MRP8/14 concentrations dropped significantly within 24 h after IVIG treatment in 21 KD patients (11). After 1 month, the concentrations reached

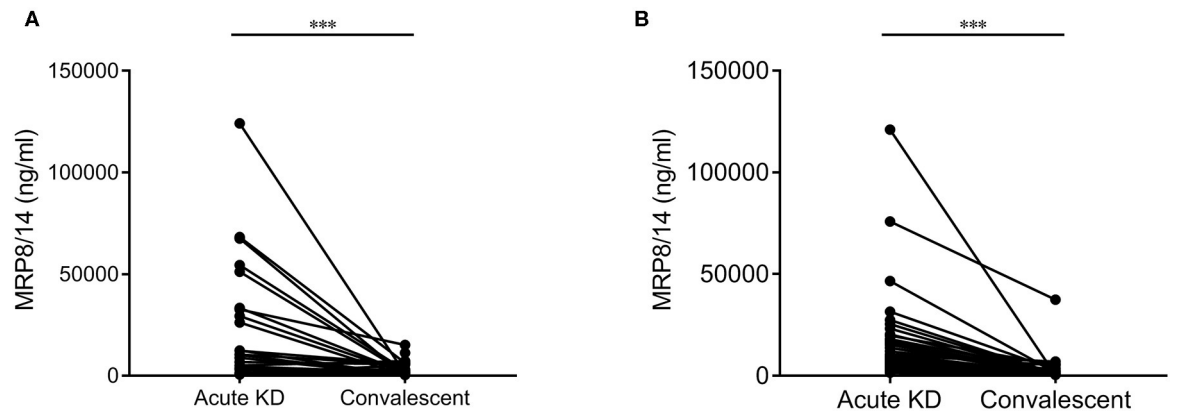

FIGURE 4 | Differences in the MRP8/14 levels in acute Kawasaki disease (KD) and convalescent in 25 paired samples from the discovery cohort **(A)** and 50 paired samples from the second validation cohort **(B)**. In the discovery cohort, one sample above 30,000 ng/ml MRP8/14 was excluded in this graph. Analyzed by a Wilcoxon matched-pairs test. ***$p < 0.0001$.

the values of their healthy controls. These authors found that the endothelium of the coronary arteries from the myocardial sections of three deceased KD patients were almost completely coated when stained with antibodies against MRP8 or MRP14 (S100A8 or S100A9). Recent investigation using a proteomic approach documented elevated levels of MRP8/14 decades after acute KD in patients with giant aneurysms (29). The variations in the correlations with CAA development may depend on technical differences in measuring CAA dimensions, but more likely will be determined by statistical power (or lack thereof) because of the small sample size of these studies, the non-prospective study design, and maybe also because of genetic differences in the cohorts tested. A conclusion as to whether the biomarker(s) really have predictive values will demand prospectively designed large studies to settle the diverse findings to date.

There are indications that MRP8/14 is a regulator of vascular inflammation (30) and is found to be elevated during different types of vasculitis (31, 32) and during vascular injury (33, 34). Furthermore, MRP8/14 has been shown to impair endothelial integrity (35), and high amounts of MRP8/14 in the coronary endothelium of KD patients are observed (11). Apart from acting as a DAMP, the special role of MRP8/14 during vascular inflammation could be a reason for its remarkable elevation during the acute vasculitis of KD. MRP8/14 is released from phagocytes after contact with the inflamed endothelium (36) and might also be released from the endothelium itself.

In our study, we have found that MRP8/14 and CRP, and possibly HNE, could be potentially used to help physicians diagnose KD in febrile children, enabling prompt treatment and lowering the risk of CAA formation. We were able to substantiate our findings in the validation cohorts, collected according to international diagnostic criteria. Additional prospective studies should now follow to more routinely perform our model of markers in pediatric patients with prolonged fever to further confirm our findings. The value of the combined tests may also prove to be helpful in the case of incomplete KD to decide whether IVIG treatment seems justified instead of further clinical observation waiting for microbiological results or the effect of antibiotics.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Board of the AMC (# NL41023.018.12). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JZ, AG, DS-B, CA, and SD collected data, performed data analyses, and wrote the initial manuscript. MWTT performed data analyses and reviewed and revised the final manuscript. NS, CS, AT, RG, and VW enrolled the patients, collected patient data, and reviewed and revised the final manuscript. RB, WZ, ME, DZ, MP, EU, HM, LS, EC, SP, MT, CF, SY, FM-T, JH, JB, and ML designed the study and reviewed and revised the final manuscript. TK coordinated and supervised data collection, designed the study, and reviewed and revised the final manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00355/full#supplementary-material

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Use of Adjunctive Therapy in Acute Kawasaki Disease in Latin America

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Objective: To characterize the use of adjunctive therapy in Kawasaki disease (KD) in Latin America.

Methods: The study included 1,418 patients from the Latin American KD Network (REKAMLATINA) treated for KD between January 1, 2009, and May 31, 2017.

Results: Of these patients, 1,152 received only a single dose of IVIG, and 266 received additional treatment. Age at onset was similar in both groups (median 2 vs. 2.2 years, respectively). The majority of patients were male (58 vs. 63.9%) and were hospitalized with the first 10 days of fever (85.1 vs. 84.2%). The most common adjunctive therapy administered was steroids for IVIG-resistance, followed by additional doses of IVIG. The use of biologics such as infliximab was limited. KD patients who received adjunctive therapy were more likely to have a lower platelet count and albumin level as well as a higher Z score of the coronary arteries.

Conclusion: This is the first report of adjunctive therapies for KD across Latin America. IVIG continues to be the initial and resistance treatment, however, steroids are also used and to a lesser extent, biological therapy such as infliximab. Future studies should address the barriers to therapy in children with acute KD throughout Latin America.

Keywords: Kawasaki disease, Latin America, steroids, infliximab, adjunctive therapy

INTRODUCTION

Kawasaki disease (KD) is an acute systemic vasculitis in children. Recent studies have implicated inflammatory cytokines and abnormalities in immune regulation as part of the pathophysiology in KD, creating new targets for adjunctive therapy (1–4). Publications about the epidemiology and available drugs and treatment schedules for children with acute KD in Latin America are scarce. One of the largest previous case summaries of children with KD in Mexico reported only 250 children in 32 years (5). In a study of cases in Central America (Guatemala, El Salvador, Honduras, Nicaragua, Costa Rica, and Panama) Ulloa-Gutierrez et al. found that from 2000 to 2010 there were only 11 reports from four countries, mostly consisting of single case reports and small series (6). Of all countries, Costa Rica contributed the highest number of cases (124 cases over 13 years). However, no cases were reported for Nicaragua or Guatemala. (6).

The Latin American KD Network (Red de la Enfermedad de Kawasaki en America Latina, REKAMLATINA) is a standardized registry where data from patients with acute KD throughout Latin American countries has been retrospectively and prospectively collected since January 2009. The network gathers information on demographics, clinical characteristics, laboratory evaluation, response to treatments, and outcomes. The initial and standard treatment for KD has been the use of high dose IV immunoglobulin (IVIG) in combination with aspirin (7). However, in some parts of Latin America, IVIG is not available for first line treatment of KD, and even when available, it may be difficult to acquire or administer early.

Although rates of IVIG resistance are unknown in Latin America, 10–20% of KD patients throughout the United States, Europe, and Asia have IVIG-resistant KD, increasing the risk of developing coronary artery abnormalities (CAA) (8). This population, in particular, may benefit from adjunctive therapy, as recently recommended in the revised 2017 American Heart Association KD guidelines (9). In addition, certain populations like infants with KD have a higher risk of developing CAA and thus may warrant additional adjunctive therapy (10, 11). A number of therapies exist for treating IVIG-resistant KD and those at high risk of CAA, including the second dose of IVIG, steroids, infliximab, or other immunomodulatory therapies (12–14). However, treatment options are limited in some regions of the world, including Latin America. Furthermore, as no study has determined which treatment is best for treating IVIG resistant KD or high-risk KD patients, the treatment choice is left to the treating physician. This study aimed to report the adjunctive therapies used to treat IVIG-resistant and high risk KD patients in Latin America.

MATERIALS AND METHODS

Subjects and Clinical Data

This analysis considers data from patients with KD in the REKAMLATINA registry between January 1, 2009, and May 31, 2017. This analysis included data from the first two multicenter studies of the network, REKAMLATINA-1, and REKAMLATINA-2. The latter study was retrospective and included patients who had a discharge diagnosis of classic or incomplete KD (according to the 2004's AHA diagnostic criteria), admitted from January 1, 2009, to December 31, 2013, at each of the participant referral pediatric or general hospitals. The REKAMLATINA-1 study enrolled KD patients prospectively, who were admitted at any of the participant centers from June 1, 2014, to May 31, 2017. Each patient was treated and followed according to the standard protocol in the referral hospital. The data collected included age at onset, gender, clinical criteria, administered medications, response to therapy, laboratory results, echocardiographic findings, and clinical outcomes.

The internal dimension of the coronary arteries was converted to Z-scores (standard deviations from the mean normalized for body surface area) (Dimensions as published by the American Heart Association). The maximal Z score (Zmax) of the left anterior descending artery (LAD) or right coronary artery (RCA) in the first 8 weeks was calculated.

Laboratories included white blood cell count (WBC), platelet count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), albumin, alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), and hemoglobin concentration normalized for age (zHgb). IVIG resistance was defined as fever ($\geq$38 C) more than 36 h, but not more than 7 days after completion of the IVIG without another cause. In assessing IVIG resistance, we included only patients who received IVIG within the first 10 days of fever onset. Patients were classified into 2 groups for analysis: (1) a single dose of IVIG or (2) other therapies, which included those treated with a combination of IVIG and adjunctive therapies, or steroids only.

Statistical Methods

Continuous variables were summarized as median [interquartile range (IQR), 25th−75th percentile].

Categorical variables were summarized as frequencies and percentages. Demographic, clinical laboratory data and

disease outcome among the two groups were compared using the Wilcoxon.

Mann-Whitney Test

All statistical analyses were conducted in Graph Pad Prism version 8.1.2 (available at: https://www.graphpad.com).

Ethics

The study received Institutional Review Board approval at the University of California San Diego as well, as at each institution enrolling subjects in the REKAMLATINA database.

RESULTS

A total of 1,855 patients diagnosed with KD in 18 Latin American countries were included in the Registry (**Table 1**), of which 437 patients were excluded due to a significant lack of demographic, laboratory, treatment, or echocardiographic data. Of the remaining 1,418 patients, 1,152 received only IVIG (81.2%), and 266 (18.8%) either IVIG in combination with adjunctive therapy or steroids alone (**Figure 1**). Age at onset was similar in both groups (median 2 vs. 2.2 years, respectively), as was the average days of the patients' illness at the time of hospitalization (median 7.2 vs. 7.3 days). The majority of patients were male (58 vs. 63.9%) and had been hospitalized in the first 10 days of illness (85.1 vs. 84.2%) (**Table 2**). There were no significant differences in the number of leukocytes, hemoglobin concentration adjusted for age, erythrocyte sedimentation rate, C-reactive protein levels, alanine aminotransferase, or gammaglutamyl transpeptidase at the time of diagnosis between the two groups. When compared to KD patients treated with a single dose of IVIG, those treated with adjunctive therapies had a lower platelet count (423 vs. 375, $P = <0.0001$) and lower albumin levels (3.3 vs. 3.1, $P = <0.0001$) (**Table 2**).

Of the patients included in this study, 1,415 (99.7%) were treated with IVIG, and 3 (0.2%) patients received only steroids (**Figure 1**). In total, 263 (18.6%) KD patients received more than a single medication for treatment of KD ($N = 198$, steroids; $N = 60$, additional IVIG; $N = 5$, infliximab). In 1,193 (84.3%) patients, the response to IVIG was reported. Of these, 69 (5.8%) KD patients were noted to be IVIG-resistant and the majority ($N = 60$, 87%) were treated with two doses of IVIG, with three of those patients getting a third dose of IVIG. In the remaining cases, patients were treated with either steroids ($N = 4$, 6.2%) or infliximab ($N = 5$, 7.2%) as rescue therapy. The IVIG response was unknown in 222 patients, 194 of whom received adjunctive therapy with steroids. Of these, 31 (16%) were <12 months old and 31 (16%) had CAA at the time of admission, which could have possibly led to the use of steroids. The rationale for giving only steroids without IVIG to three acute KD patients, specifically the availability of IVIG, is unknown.

With regards to the coronary artery, the Zmax of the coronary arteries was higher in KD patients treated with adjunctive therapy, especially those treated in the first 10 days of illness (0.73 vs. 5.07, $P < 0.0001$, **Table 2**).

TABLE 1 | Number of patients divided by each country (1,418 total patients).

Country	Number of patients contributed
Argentina	35
Brazil	31
Chile	82
Colombia	146
Costa Rica	215
Cuba	8
Dominican Republic	21
Ecuador	56
Guatemala	25
Honduras	27
Mexico	424
Panama	153
Paraguay	9
Peru	47
Puerto Rico	14
Salvador	92
Uruguay	31
Venezuela	2

DISCUSSION

This is the first multinational, multicenter study to report on the use of adjunctive therapies in children with KD from Latin America. While the majority of KD patients only received a single dose of IVIG, nearly 20% of patients were treated with adjunctive therapy after receiving an initial IVIG dose (8). This is consistent with what has been reported in the United States and Japan (15). Although the rationale for using adjunctive therapy was unknown in all these patients, the reasons for some adjunctive treatments were similar to those in other parts of the world, including IVIG-resistance, young age, and CAA. While steroids were the most reported adjunctive therapy, additional doses of IVIG were also administered relatively frequently. This is an interesting issue, given the worldwide shortage of IVIG and the reported risk of hemolytic anemia created by increasing doses of IVIG (16). In this study, we did not have sufficient data to assess whether the anemia seen in some KD patients was hemolytic and whether increasing doses of IVIG was associated with a higher risk of hemolytic anemia. However, a comparative effectiveness study is currently being conducted, examining the use of a second dose of IVIG vs. infliximab in IVIG-resistant KD patients, further evaluating the risk of hemolytic anemia in KD patients (16).

In this analysis, we had a low rate of IVIG-resistance, with only 5.8% of the total patients reported as such. This is most likely due to an underestimation of IVIG-resistance in Latin America, as the worldwide rate ranges from 9 to 20% (15, 17). In previous reports from Latin America, IVIG resistance was reported to be 9% (18) and in multiethnic studies from the United States, IVIG-resistance in Hispanic KD patients was found to be 15% (19). If we were to assume that 132 KD

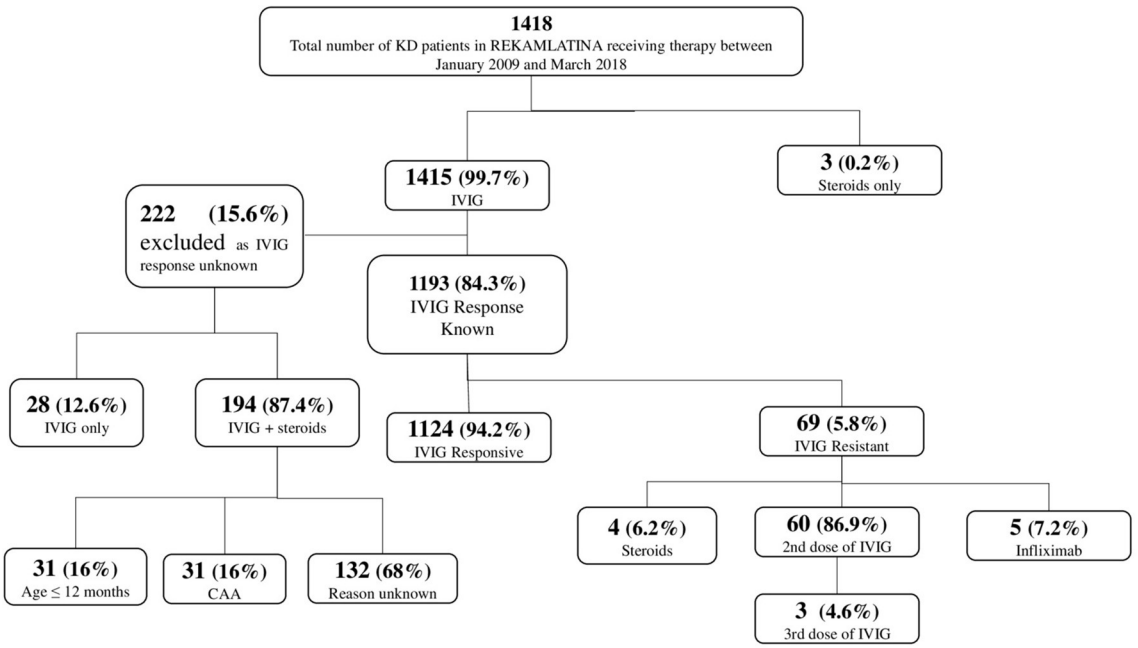

FIGURE 1 | Initial and subsequent treatments administered to 1,418 patients with acute Kawasaki disease in Latin America.

patients received adjunctive treatment with steroids because they had IVIG resistance, the IVIG-resistance rate would be 14.2% (201/1,415).

In this study, 14% of KD patients were treated with steroids as adjunctive therapy, a treatment that is commonly administered worldwide in KD patients. Dominguez et al. found that 7.7% of KD patients were treated with corticosteroids for resistance after a first dose of IVIG at the Children's Hospital of Colorado in the United States (20). Chen et al. reported 37.4% of Chinese KD patients were treated with steroids for resistance to the first dose of IVIG (21). In the most recent epidemiological survey of KD in Japan, 13% of KD patients were reported to have received steroids with initial IVIG given a high likelihood of IVIG-resistance (15).

In our study, only five KD patients were treated with infliximab in addition to IVIG. It may be that infliximab was not available in many centers or that there was limited experience in using the drug, thus, it was not the adjunctive therapy of choice. By comparison, the use of adjunctive infliximab therapy has ranged from 1.4% in Spain to 6.5% in the United States (20). In this study, there were also three patients, all of whom were diagnosed in the first 10 days of illness, who were treated only with steroids (methylprednisolone at 30 mg/kg/day). Although the reason these patients did not receive IVIG is unknown, it is difficult to acquire this drug in some regions of Latin America. This is either because it is not readily or quickly available, or because the cost is not covered by insurance, and might require the patient to pay for it themselves, which may not be feasible.

A lower albumin and platelet count were found in patients treated with adjunctive therapy. It has been reported that a low albumin and platelet count increases the risk for CAA, resistance, or recurrence in KD patients from other regions (9). The later median days of illness at the time of diagnosis in this cohort as compared to other countries may explain these lower albumin levels (22, 23). These patients were likely sicker, which warranted a higher rate of adjunctive therapies. Additionally, it is understandable that patients receiving adjunctive therapy had a higher Zmax, in line with the revised 2017 AHA KD guidelines, which recommend that high-risk KD patients receive more than a single dose of IVIG (9).

As this study is the largest data set to be analyzed for adjunctive treatment in the care of patients with acute KD in Latin America, it has both strengths and limitations. We analyzed KD patients who attended the main pediatric or referral countries in the region, which encompasses 18 countries. The previously mentioned lack of some data which resulted in the exclusion of patients, and the absence of consensus as to when or how to use adjunctive therapies are two limitations. There was also wide variability in reporting about patient responses to IVIG therapy, which limited our ability to calculate the exact IVIG-resistance rate. Furthermore, the lack of long-term follow-up data on patients meant that we were unable to study the impact of adjunctive therapies.

CONCLUSION

This is the first report from the Latin American KD Registry, focusing on the use of adjunctive therapies in treating patients with acute KD. This study indicates that a better understanding of treatment trends in Latin America could help improve the standard of care for KD patients in this region.

TABLE 2 | Comparison of demographic, clinical characteristics, and outcome among 1,418 patients with Kawasaki disease in Latin America stratified by treatment.

	IVIG alone (n = 1,152)	IVIG and adjunctive therapy (A) (n = 266)	P-value*
Age in yrs at onset	2 (1.2–3.8)	2.2 (1–4.4)	0.5029
Sex, Male	58%	63.9%	0.0810
Illness day at diagnosis	7.2 (5–8)	7.3(5–9)	0.75
Illness, day at hospitalization			
≤ 10 d	85.1%	84.2%	0.1799
>10 d	13%	15.7%	0.2765
White blood cell count K×103	14.2 (11–18.14)	14.5 (10.55–19.02)	0.8866
zHgb	−1.2 (−2.38 to 0.14)	−0.94 (−2.16 to 0.51)	0.0639
Platelet count K × 10^3	423 (298–516)	375 (262–453)	<0.0001
ESR mm/h	45 (30–56)	47.5 (28–54)	0.6247
CRP mg/dL	6.7 (2.47–12.2)	7 (2.3–15.8)	0.4800
ALT IU/L	41 (22–87.5)	42 (24–101.8)	0.3716
GGT IU/L	48.5 (20–137.3)	77 (26.2–176)	0.0644
Albumin mg/dL	3.3 (2.9–3.7)	3.1 (2.7–3.4)	<0.0001
Overall coronary artery outcome (Zmax)	0.8 (−0.09 to 2.05)	1.5 (0.57–4.3)	<0.0001
Coronary artery outcome if diagnosed ≤10 days (Zmax)	0.73 (−0.14 to 1.84)	5.07 (1.82–11.22)	<0.0001

ALT, Alanine aminotransferase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GGT, gamma glutamyl transpeptidase; zHgb, hemoglobin concentration normalized for age. Values are median (IQR) unless otherwise noted.

(A) Patients were treated with the following treatment combinations: two doses of IVIG, three doses of IVIG, steroids alone, steroids and IVIG, and infliximab.

**P-value Wilcoxon-Mann-Whitney test for continuous variables to compare the overall difference among the 2 groups.*

Data were available for the following number of patients in each variable (IVIG only; IVIG + adjunctive therapy): Age (n = 1,152; n = 266); Sex (n = 669; N = 170); Illness, day at hospitalization ≤10 d (n = 981; 319 n = 224) and >10 d (n = 150; n = 42); White blood cell count K × 10 3 (n = 1,130; n = 261); zHgb (n = 1,127; n = 320; 262); Platelet count K×103 (n = 1,113; n = 260); ESR (n = 715; n = 236); CRP (n = 1,022; n = 219); ALT (n = 969; n = 252); GGT (n = 316; n = 108); albumin (n = 773; n = 219); overall coronary artery outcome (n = 590; n = 86); coronary artery outcome among diagnosed ≤ 10 days (n = 502; n = 20).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study received Institutional Review Board approval at the University of California, San Diego as well as at each individual institution enrolling subjects in the REKAMLATINA database.

AUTHOR CONTRIBUTIONS

BF-R, AT, EB, and RU-G were responsible for data analysis and writing of the manuscript. The rest of the co-authors (principal investigators and coinvestigators) revised the manuscript and made intellectual contributions to its contents.

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CASE REPORT
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Coronary Revascularization of Giant Aneurysms in Children With Kawasaki Disease: A Report of Two Cases

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In recent years, >100 cases of coronary artery stenotic lesions due to Kawasaki disease were treated with coronary artery bypass grafting (CABG). Surgical indications include stenosis of >75%, myocardial infarction history, electrocardiographic changes, and ischemia, as detected by myocardial scintigraphy and electrocardiography, due to drugs or exercise. Some centers have reported good patency rates, even in infants. The advantages of CABG in younger patients are minimal loss of left ventricular function, early elimination of post-operative ischemia risk, and improved quality of life. However, the disadvantage of performing CABG in younger patients is the small coronary artery diameter and the thin vessel wall, which can lead to post-operative occlusion, especially when performed by inexperienced surgeons. The optimal timing of CABG varies by institution and case, which depends on the presence or absence of complications, such as left ventricular dysfunction or valve regurgitation, and surgeon's experience. Importantly, unlike adult surgery, childhood CABG needs to be kept open for the very longest possible period of time to determine the optimal conditions for surgery. We report two pediatric cases of giant coronary artery aneurysms diagnosed in infancy. During school age, the patients had a mild decline of left ventricular ejection function. In one case, there were no clinical symptoms because of the development of collateral vessels and limitation of exercise. Both patients underwent surgery with good results. The gastric gastroepiploic artery could be anastomosed owing to the development of collateral blood vessels, although it was obstructed. At 1 year after surgery, both patients had a good post-operative course without complications of anastomotic stenosis or myocardial damage due to aneurysm resection. If conditions are favorable, bypass surgery can be postponed to several years until the coronary arteries are sufficiently large to warrant a delay in coronary artery stenosis in cases of infantile Kawasaki disease.

Keywords: kawasaki disease, children, coronary artery bypass grafting, ischemic heart disease, collateral artery

INTRODUCTION

More than 40 years have passed since the first coronary artery bypass grafting (CABG) procedure in a child with Kawasaki disease (KD) was reported by Kitamura et al. (1). Improvements in treatment methods over time have reduced the number of cases involving giant coronary artery aneurysms to 0.2–0.3%. According to a nationwide survey conducted in Japan from 1999 to 2010, giant coronary artery aneurysm (CAL) rupture occurs within 1 month of KD onset, and there has been no death reported beyond 2 years (2). This suggests that treatment in the acute phase is important to avoid rupture, and the development of collateral circulation may help to avoid ischemic damage after the acute phase. In that survey, ∼20 patients underwent CABG to treat coronary artery stenosis. The median time of CABG from disease onset was 56 months (range, 3–117 months). The optimal timing of surgery also depends on the individual patient's age, condition (including factors such as ischemia and collateral circulation), and the surgeon's experience level with the procedure.

In this report, we describe two pediatric cases in which their conditions prompted us to delay surgeries until the children reached school age. Specifically, the first case required the removal of a calcified giant aneurysm on the left anterior descending artery (LAD). The second case involved a stenosis distal to the right coronary artery (RCA) and required further growth of the right gastroepiploic artery (GEA). Both cases had adequate collateral circulation development and cardiac function preservation. CABG in children can be postponed until after infancy when the coronary artery diameter and wall thickness have grown. In addition, if the surgeon is inexperienced with performing CABG in children, we recommend waiting until the children weighed 15 kg to reduce the risk of complications and provide stable blood flow for a long period of time, even until adulthood.

CASE PRESENTATION

Case 1

The first patient was a 10 year-old boy (141 cm, 36.5 kg). KD was diagnosed at the age of 9 months. Two courses of high-dose γ-globulin therapy (IVIG) and steroid pulse therapy were performed. From disease day 13 to day 17, the proximal LAD dilated to 8 mm, and the proximal RCA dilated to 6 mm. The patient was then transferred to our hospital. IVIG therapy was administered again on the day of admission; however, a thrombus was detected in the LAD aneurysm detected on the 22nd pathological day.

He had no symptoms, but ST elevation was detected in the electrocardiogram (ECG) in leads II, III, aVF, and V3–V5. We diagnosed this case as acute myocardial infarction (AMI). Intracoronary thrombolysis (ICT) with tissue plasminogen activator (t-PA) was performed on the LAD while the patient was intubated, and sedation was performed with phenobarbital and midazolam. After ICT, heparin, and warfarin were administered to avoid thrombus formation, and verapamil was administered to avoid rupture in order to control spastic changes in the vessel and to maintain blood pressure <100 mmHg. Fever continued intermittently, and IVIG therapy was given on days 28 and 35. Warfarin was used to maintain the international normalized ratio (INR) at 2.0–2.5 and sedative therapy was continued until day 45 to maintain the patient at rest. The diameter of the aneurysm on proximal RCA was maintained at 6 mm, similar to the size observed during the acute phase. Although the thrombus disappeared, the left CAL grew, reaching 28 mm on day 57.

The patient was discharged on day 81 and therapy was continued with aspirin, angiotensin II receptor blockers (ARB), and warfarin. Stress ECG, chest radiography, echocardiography, and magnetic resonance coronary angiography (MRCA) were performed during outpatient visits to monitor for the development of stenosis or thrombus. After 3 months, his coronary angiography (CAG) revealed a giant CAL in the LAD and left circumflex artery (LCX).

At the age of 6 (22 kg), 5 years from disease onset, stenosis was found in the proximal part of the LCA aneurysm; however, no ischemic changes were seen on the treadmill test due to the development of collateral arteries from the RCA. He weighed 22 kg, and his coronary artery diameter was predicted to be well-developed, but the surgeon did not want to perform CABG because there were no ischemic findings and the large aneurysm adhered tightly to the myocardial tissue, it would be difficult to excise. Due to those findings, CABG was postponed.

A treadmill test performed at 10 years of age, 8 years from disease onset, showed ST depression in the left chest lead during a regular check-up, and stress Tl myocardial scintigraphy showed extensive ischemia of the lateral wall; nevertheless, viability was maintained. CAG confirmed a huge aneurysm with calcification of the proximal left coronary artery and complete LAD occlusion at the outflow area of the aneurysm with collateral circulation from the RCA and stenosis of the left circumflex artery (LCX) (**Figure 1**). The RCA aneurysm disappeared completely. Left ventricular ejection fraction (EF) was 61%, indicating a decrease in sidewall contractions. Based on such findings, we believed that CABG was indicated. Surgery was performed using two arterial grafts: the left internal mammary artery (LITA)-LAD and the right internal mammary artery (RITA)–PL (posterolateral branch); the giant aneurysm (45 × 35 mm, severe calcification) was resected (**Figure 2**). The lumen of the giant calcified aneurysm was filled with a thrombus. The patient progressed well after surgery. Echocardiography at 1 year after the operation showed no changes in EF, which remained at 61%; however, coronary CT showed no stenosis or occlusion. Since the operation, the patient has been taking oral aspirin and carvedilol daily and living with exercise restrictions.

Case 2

The patient was a 7 year-old boy (107 cm, 16.4 kg). Disease onset occurred at the age of 2 months. Although IVIG was administered three times after IVIG and methylprednisolone pulse therapy, the inflammatory responses persisted, and bilateral coronary artery aneurysms were found 1 week after admission. The patient was transferred to our hospital on disease day 17. After admission to the intensive care unit, the patient was intubated to maintain sedation, and the heart rate and

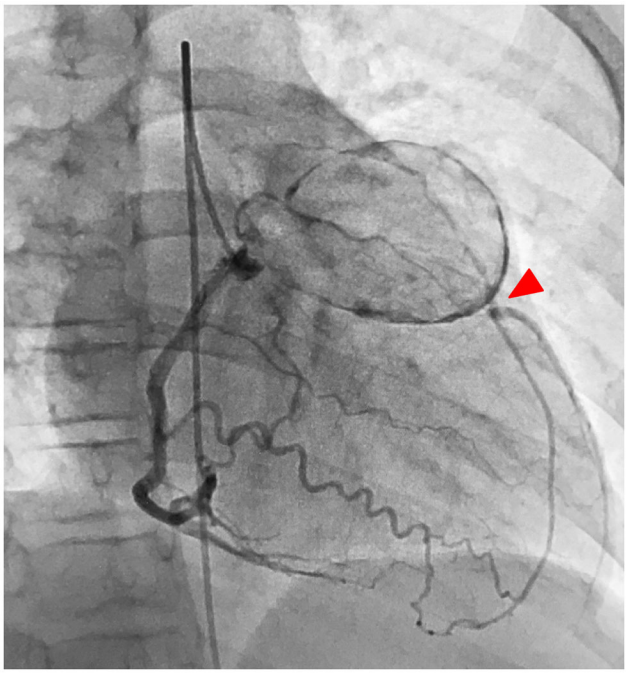

FIGURE 1 | Case 1: left CAG showed a huge aneurysm with calcification of the proximal left coronary artery, and the LAD is obstructed at the outflow of the aneurysm (red arrowhead), and the blood is supplied by the collateral circulation tract from the right coronary artery. LAD, left anterior descending artery; LCX, left circumflex artery.

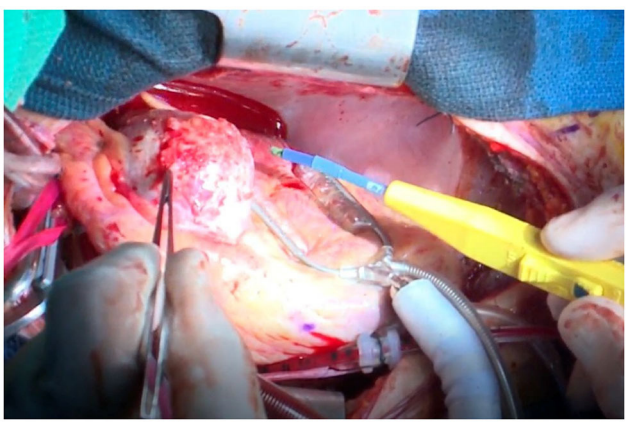

FIGURE 2 | Case 1: macrofindings of the LAD aneurysm during surgery. A giant coronary aneurysm prior to resection is observed. A hard and calcified mass >40 mm in diameter is present. The mass was filled with a red blood clot. LAD, left anterior descending artery.

blood pressure were suppressed using nicardipine and sedatives. Despite those therapies, large aneurysms were detected at all three coronary arteries. At 3 months of age, 1 month from disease onset, there were no symptoms, but deepQ wave in lead III on ECG suggested ischemic changes and the troponin and CK-MB levels were elevated on blood test. We diagnosed the patient with AMI, and CAG was performed. On CAG, a huge long segmental aneurysm on RCA was observed with a maximum diameter of 12 mm, the blood flow was almost arrested, and the presence of a thrombus was confirmed (**Figure 3**). On the left side, the aneurysmal size on LAD was 9.2 mm and that on LCX was 3.3 mm.

Emergency ICT using t-PA was performed for the RCA thrombus. The thrombus disappeared on the echocardiogram; however, the fever persisted, and infliximab was administered on day 18. The body temperature began to decline on the next day, and the heart rate stabilized. One week later, the patient was extubated, and sedation was discontinued. Therapy was continued with aspirin, ARB, and warfarin. Thereafter, the patient underwent regular follow-up evaluations with ECG, chest radiography, echocardiography, and coronary angiography.

At 1 year of age, 10 months after the acute phase, CAG revealed that the main RCA was completely occluded. However, peripheral blood flow was maintained from the LCX. The LAD vessel diameter still had aneurysmal change, but the diameter improved to 5 mm, no stenotic lesion was detected, and LCX also returned to its normal size. Subsequently, CAG exhibited similar findings as those observed at 1 year after the acute phase. At 2 years of age, 2 years from disease onset, CAG was performed and similar findings were observed. At that time, his body weight was <15 kg, and we speculated that performing CABG using RITA would be difficult at this age. Thus, CABG was postponed. After CAG, he went to the hospital for a regular check-up and underwent exercise ECG. He did not complain of any discomforts or chest pains, even while performing exercises at school. However, at 7 years of age (weight, 16.4 kg), 7 years from onset, he lost consciousness while exercising and underwent another CAG. The LAD was completely occluded and stenosis of the LCX of 0.9 mm was observed. Drug-loaded myocardial scintigraphy showed extensive ischemia of the sidewall; however, the left ventricular EF was maintained at 70%. He had symptoms and sufficient weight and ischemic findings on examination to warrant surgery. Then, CABG was performed. Anastomosis was performed at the following two places: LITA-LAD and RCA-GEA. However, the LITA was too small to be skeletonized; thus, anastomosis was performed using only milrinone injection. The RITA was also preserved for future stenotic episodes. Angiography performed at ∼1 year after surgery showed a good blood flow in both vessels (**Figure 4**).

Both patients provided written informed consent.

DISCUSSION

Examination and Indications of CABG

Guidelines have been developed regarding the indications of CABG for children with KD in Japan. The increasing number of KD cases involving surgery has enabled more detailed evaluations of the indicated pathological conditions, ages, and associated prognoses. In Japan, the indications for pediatric CABG are identical to those for adult cases and include ischemic findings and symptoms. Specifically, CABG is indicated if any of the four following conditions are present: (1) high-grade lesions in the left main trunk, (2) high-grade stenotic lesions in multiple arterial

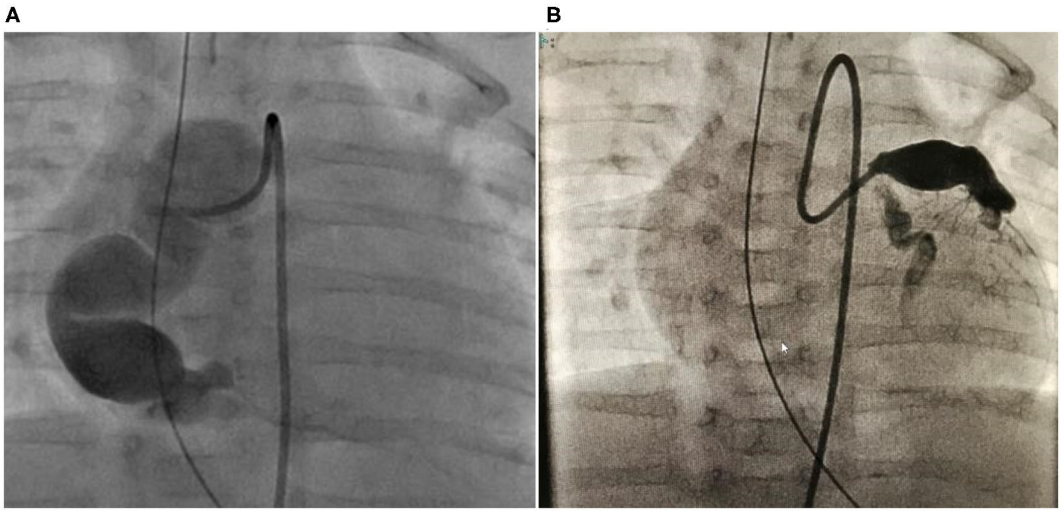

FIGURE 3 | Case 2: right and left CAG performed in the acute phase when the patient was 2 months old. **(A)** It exhibited a large RCA "sausage-like" aneurysm at segments 1–3 with a maximum diameter of 12 mm. **(B)** Let CAG exhibited aneurysms in the LAD at segment 6 with a maximum diameter is 6 mm and LCX with a maximum diameter of 3.3 mm at segment 11. CAG, coronary angiogram; RCA, right coronary artery; LAD, left anterior descending artery; LCX, left circumflex artery.

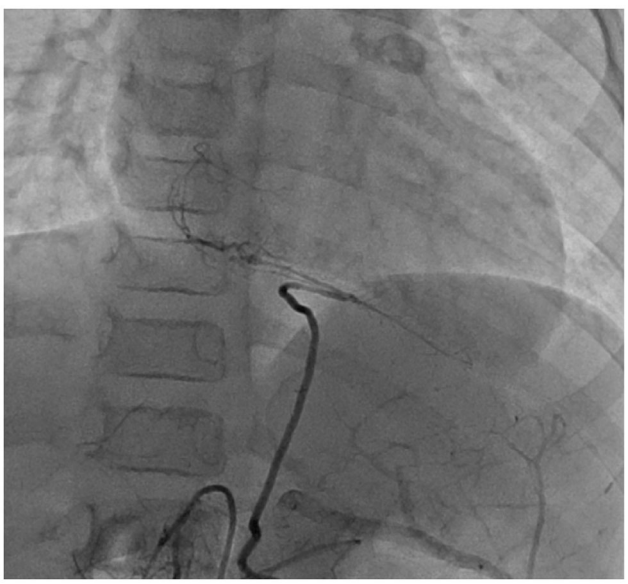

FIGURE 4 | Case 2: right CAG at 1 year after CABG. The blood flow supply from the GEA to the distal site of RCA was adequate. CAG, coronary artery angiogram; CABG, coronary artery bypass graft surgery; GEA, gastroepiploic artery; RCA, right coronary artery.

branches, (3) high-grade stenotic lesions in the proximal LAD, and (4) dangerous collateral pathways (3).

In contrast to adults, collateral pathways develop early in infants, and the blood flow is protected to some extent by collateral circulation, even if a stenotic lesion of the coronary artery main trunk progresses. Accordingly, the patient may be asymptomatic during infancy. However, upon reaching school age, the patient's level of activity increases drastically, and new ischemic findings may emerge even without a corresponding change in the degree of coronary artery stenosis. Therefore, a treadmill test or stress/rest myocardial perfusion imaging is useful for determining the indication for CABG. It is important to verify collateral circulation and left heart function by coronary angiography and left ventriculography and to determine the viability of the ischemic region accurately. The remaining coronary artery blood flow is another important determinant during the decision-making process for bypass surgery. In a patient with considerable remaining blood flow, CABG can result in the "string phenomenon," which involves two competing blood flows and depletion of blood flow from the bypass (4).

Choice of Bypass Vessel

Kitamura et al. (5) reported the first use of an autologous great saphenous vein in a 4 year-old child with KD. However, this graft resulted in occlusion. In 1985, two adult cases of LITA-LAD anastomoses were performed using the left ITA. Subsequently, a study conducted in 1988 on 12 patients with LITA-LAD anastomoses reported a 100% patency rate, and the authors found that increases in the length and blood vessel diameter of the LITA paralleled the growth of the children (6). Recent literature includes reports of CABG involving the GEA, great saphenous vein, and radial artery bypass; most current cases involve the ITA, which is considered the optimal choice for use in bypass surgery in the majority of cases (7–10). Moreover, the use of the ITA requires anastomosis only with the coronary artery, and the minimal difference in the diameters of these vessels facilitates anastomosis. Moreover, the ITA is unlikely to be affected by KD-associated vasculitis and grows with age.

In the two cases reported in the present study, three of the four vessels were anastomosed to the ITA and only one was anastomosed to the GEA at the distal end of RCA, which did not

reach the ITA. The use of GEA is more complicated due to the requirement for laparotomy. Individual variations in the blood vessel diameter and length increase the likelihood of spasms and arteriosclerosis in adulthood.

The radial artery is also available as an alternative; however, the use of this artery has some disadvantages. In particular, it is not pedicled and growth cannot be expected. Some cases involving giant aneurysms of the RCA have been treated with reconstructive procedures rather than CABG, and these procedures have resulted in the establishment of stable blood flow (11).

Age at Surgery

Statistics regarding the age at surgery in Japan suggest that children as young as 1 year of age have undergone bypass surgeries. The achievement of good post-operative patency rates depends on the facility where the operation is performed. Some facilities would likely perform surgery even in a young child. Previous reports have described the post-operative development of occlusion in some cases. In the two cases reported in the present study, neither patient exhibited findings of ischemia due to the development of collateral pathways. Accordingly, surgery was delayed for several years after considering other factors. The first case also involved aneurysm removal, while the second case involved grafting to the GEA because the RITA could not reach the distal RCA. In the first case, the removal of the giant aneurysm during CABG in infancy may have caused damage to the thin myocardium. Furthermore, in the second case, the narrow GEA diameter and risk of post-operative stenosis were concerning. The surgeon also had had little experience in handling pediatric cases of coronary stenosis due to KD. Therefore, the operation in infancy was postponed until the patient is of school age.

Ultimately, decisions regarding the optimal timing of surgery depend on several factors, including the degree of coronary artery stenosis, condition of collateral circulation, degree of myocardial ischemia, experience of the surgeon, and comprehension on the part of the patient and family.

Acute Treatment

Both patients developed giant coronary arteries, despite the provision of treatment according to standard guidelines. Although the risk of rupture was high in both cases, this adverse outcome was avoided by imposing an adequate period of complete sedation, ventilator use, and antihypertensive drug therapy to control blood pressure levels. Currently, the acute-phase treatment is largely responsible for the ability of patients with even severe conditions to undergo successful CABG.

Explanation to the Patient

The prevention of sudden myocardial infarction, a serious complication, also critically depends on the patient's thorough understanding of their illness. The physician should explain the patient's condition adequately to both the parents and the child and should conduct thorough routine outpatient visits and examinations. These practices will enable the patient to remain aware of their illness and understand the need to avoid the types of play that elevate their heart rate.

Future Treatment and Follow-Up

Both our patients have lived without restrictions on their activities and have only used aspirin since surgery. In the future, it will be important to ensure the prevention of diseases associated with vascular disorders, including arteriosclerosis and diabetes mellitus, by incorporating practices such as daily exercise.

CONCLUSION

In this case report, we presented two cases of KD-associated coronary artery stenosis that were treated by CABG after a delay of several years. Both patients developed giant coronary arteries. Rupture was prevented by regulating the blood pressure and heart rate during the acute phase. Ultimately, both patients safely underwent delayed CABG. If the number of such cases increases in the future, the optimal surgical timing and technique for each patient should be examined further.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Written informed consent was obtained from the individual's legal guardian for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KA wrote the manuscript. MH, HO, and TF followed on these patients. KT, MK, and TS provided language and medical advice. AA, KN, and SK performed surgical treatment and provided advice about surgery. All authors contributed to the article and approved the submitted version.

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