Original article

Serum biomarkers differentiating Kawasaki disease from febrile infections: A pilot case-control study

Asad Aziz Khan [a], Junu Vazhappully George [b], Sania Mazin Shareef Al Hamad [b], Richard L. Jayaraj [b,*], Hassib Narchi [b]

[a] Al Ain Hospital, Al Ain, United Arab Emirates
[b] Department of Pediatrics, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates

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ABSTRACT

Although some serum biomarkers are elevated in both Kawasaki disease (KD) and infections, these conditions have not been compared by individual or combined biomarkers. The aim of this study, undertaken between January 2016 and May 2018 in a large teaching hospital, was to compare the serum concentration of cytokines, metalloproteinases (MMP) and heat shock protein (HSP) between cases defined as children with Kawasaki disease (KD) and those with febrile infections (controls). Serum concentrations of tumour necrosis factor-alpha (TNF-alpha), interleukins (IL 1beta, 6, and 8), heat shock proteins (HSP 60 and 70) and matrix metalloproteinase (MMP 9) were measured on admission in 17 children under six years of age with a temperature >38.5 °C for ≥five days, and compared between the two groups. The median age was 25 months and the median duration of fever eight days. Seven children were diagnosed with KD and ten had a febrile infection. Only the serum concentrations of IL-6 and TNF-alpha were significantly higher in the former than in the latter group ($P = 0.01$ and 0.04 respectively). To differentiate between the two groups with the best sensitivity and specificity, the optimal cut-off value for IL-6 was 12.6 pg/mL, and for TNF-alpha 47.9 pg/mL. Their combined increase, however, outperformed their individual concentrations. The characteristic diagnostic "signature" of the combined elevation of IL-6 and TNF-alpha serum levels has the potential, in febrile children, to differentiate early KD from febrile infections, allowing the institution of appropriate therapy.

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1. Introduction

Kawasaki disease (KD) is an acute systemic vasculitis of unknown aetiology, affecting medium-sized arteries. Its diagnosis is exclusively made by clinical criteria: prolonged fever, bilateral non-purulent conjunctivitis, diffuse mucosal inflammation, polymorphous skin rashes, angioedema of the hands and feet, and non-suppurative cervical lymphadenopathy, in the absence of an infectious cause (McCrindle et al., 2017). Although it is usually self-limited and resolves within a few weeks, 15–25% of patients develop coronary artery lesions (CAL) (Shi et al., 2019). High-dose intravenous immunoglobulin (IVIG) associated with aspirin (ASA) reduces the systemic inflammation and the incidence of CAL (McCrindle et al., 2017). KD has been reported in more than 60 countries with wide differences in its incidence. For example in Japan, South Korea and China the prevalence of KD is between 10 and 30 times higher than in Europe and the United States (Kim, 2019). Unfortunately, reports of prevalence in the Middle East are still scarce (Lin and Wu, 2017). Interactions between immunological, environmental and genetic factors have been hypothesized to play a major role in KD etiology. The diagnosis of KD is challenging in its early stages when appropriate therapy would be most beneficial, because of difficulty differentiating it from more common febrile infections, hence the recommendation to always consider it even in infants and children with prolonged fever with no alternative diagnosis (McCrindle et al., 2017).

The underlying immunopathogenic mechanisms of KD remain unclear. Immune activation with cytokines, such as interleukins and tumour necrosis factor (TNF)-α, contribute to its pathogenesis

* Corresponding author at: Department of Pediatrics, College of Medicine and Health Sciences, United Arab Emirates University, PO Box 17666, United Arab Emirates.

E-mail address: richardlj@uaeu.ac.ae (R.L. Jayaraj).

([Wu et al., 2019; Hu et al., 2017](#)). Cytokines, produced by macrophages, B and T lymphocytes, mast cells, endothelial cells and fibroblasts contribute, through receptors, to cell signalling. In the immune system they modulate the balance between humoral and cellular responses. Elevated serum cytokines have also been studied in febrile illnesses associated, or not, with infectious diseases but without direct comparison with KD ([van der Galien et al., 2018; Chandrashekara et al., 2016; Zheng et al., 2017](#)).

Interactions between matrix metalloproteinases (MMPs) and equivalent tissue inhibitors of MMPs (TIMPs) regulate the remodelling of the extracellular matrix (ECM), which constitutes the basement membrane structurally supporting the vasculature involved in the vascular lesions of KD. The MMPs, endopeptidases degrading extracellular matrix proteins, are produced by fibroblasts and macrophages in response to various cytokines, and play a significant role in cell proliferation, migration, differentiation, angiogenesis and apoptosis. Their distinctive role in the vascular pathology of KD may, therefore, prove useful for its diagnosis.

An unusual phenomenon in KD is the reactivation of the Bacillus Calmette Guerin (BCG) scar during its acute phase and its regression with the other inflammatory signs after specific therapy with intravenous immunoglobulins ([Loh et al., 2019; Novais et al., 2016](#)). It is attributed to an immunologic cross-reactivity between mycobacterial-derived heat shock protein 65 (HSP-65) and its human homologue HSP-63 (self P1 antigen) ([Nagata et al., 2009; Yin Ji et al., 2007](#)). HSPs are produced by cells in response to exposure to stressful conditions, including wound healing or tissue remodelling. Thus, considering their unique role in KD, they might also prove useful for its diagnosis.

The concentration of these biomarkers varies among different febrile conditions. No previous studies have directly compared such a panel of biomarkers between children with KD and those with other febrile infections. We, therefore, developed this pilot comparative study to test if the serum concentration of selected biomarkers, alone or in combination, could reveal a characteristic "signature" or "profile" in KD allowing its distinction from febrile infections ([Saary, 2008](#)). We included in the panel proinflammatory cytokines (IL-1β, IL-6, and TNF-alpha) as they modulate immune responses and augment the action of other cytokines. We also incorporated the proinflammatory chemokine IL-8, which induces directed chemotaxis, as well as MMPs and HSPs because of the mechanisms discussed earlier. Our null hypothesis is that there will be no difference in the serum concentration of each biomarker analysed, between children with KD and those with febrile infections.

2. Methods

2.1. Ethics approval

The study was approved by the Institution's Human Ethics Committee (No. 11/55). The parents of all participating children signed an informed consent form for this study.

2.2. Patients

We enrolled, between January 2016 and May 2018, children under six years of age admitted to a 400-bed secondary care hospital in the United Arab Emirates, for a temperature >38.5 °C for five days or more and without a clear diagnosis on admission. We excluded children whose parents refused consent, those with fever of less than five days or below 38.5 °C, or who had already received intravenous immunoglobulins, or known to have underlying diseases making them susceptible to infections, such as immunodeficiencies (congenital or acquired), malignancies, corti-

costeroid therapy or with intravascular catheters. The management of all children was at the discretion of the admitting physicians who were unaware of the results of the biomarkers.

2.3. Main outcome

The cases were children subsequently diagnosed with typical or incomplete/atypical KD diagnosed by the established clinical diagnostic criteria of the American Heart Association ([McCrindle et al., 2017](#)). Children with febrile infections (controls) were those in whom KD was subsequently excluded and who were then diagnosed with a confirmed alternative diagnosis with clear laboratory or radiological diagnosis of an infectious cause for the fever.

2.4. Data collection

On admission, demographic, clinical, and laboratory data were collected: gender, age, temperature peak, physical signs, serum concentrations of sodium, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), haemoglobin (Hb), C-reactive protein (CRP), as well as white blood cell (WBC), neutrophil and platelet counts and erythrocyte sedimentation rate (ESR).

2.5. Enzyme-linked immunosorbent assay

A 2 mL blood sample, drawn on admission, was centrifuged and stored at −80 °C. The serum concentrations of IL-1β, IL-6, IL-8, TNF-alpha, MMP-9, HSP-60, and HSP-70 were measured by enzyme-linked immunosorbent assays (ELISA). The assays were performed according to the manufacturer's instruction using kits from R&D Systems (Minneapolis, MN, USA). Briefly, 100 mL of standard and prediluted serum (1:2) were added and incubated in anti-human IL-6, IL-1β, IL-8, HSP-60, HSP-70, TNF-alpha and MMP-9 well-coated plates at room temperature for two hours. After incubation and washing, 100 mL of prepared biotinylated antibody were added, and the samples were incubated at room temperature for two hours. After washing, streptavidin–HRP was added and the samples were incubated for 20 min at room temperature. Colour development was done with the provided substrate solution (tetramethylbenzidine). The plates were then developed for 20 min, in the dark and at room temperature. The reaction was then ended by adding 50 mL of stop solution. Absorbance was measured at 450 nm with the Emaxplus Reader (Molecular Devices, LLC, San Jose, CA). Biomarker's concentrations below the detection limit were assigned a value equal to half the lowest detection limit for that specific biomarker. The operator was blinded regarding the final diagnosis of the participants and the attending physicians were unaware of the results of the biomarkers, as these were available only after the patients were discharged.

2.6. Sample size

For a 1:1 design case-control study, based on a mean serum TNF-alpha level of 24 pg/mL in KD and 17 pg/mL with a standard deviation of 4 pg/mL in pneumonia, a sample size of seven children in each group (KD and acute febrile infections) is required to give the study a power of 80% with a 2-sided alpha error of 5%.

2.7. Statistical analysis

Proportions and percentages were compared with the Chi-squared or the Fisher exact test. Normally distributed variables (Shapiro-Wilk test for normality) were reported as means and standard deviations. They were compared between two groups with the unpaired *t*-test, and by analysis of variance (ANOVA) with Bonferroni correction for groups of three or more. The variables not

following a Normal distribution were reported as medians and interquartile ranges [IQR] (25th and 75th percentile) and were compared with the non-parametric Kruskal-Wallis test. For all tests, the STATA package version 15 (StataCorp, College Station, Texas) was used, and a two-tailed *P*-value < 0.05 defined statistical significance.

We estimated, for each biomarker, and for the combination of those with a significant difference between the groups, their diagnostic performance in differentiating between the two groups (sensitivity, specificity, positive and negative predictive value). We also calculated their optimal cut-off serum level using the Youden method in which its index is the sum of sensitivity and specificity minus one. It reflects the overall capacity of the best optimal cut-off serum level of a biomarker to make a diagnosis with the best possible combination of both sensitivity and specificity. For the biomarkers with significantly different values between the groups, we also determined their individual receiver operating characteristic (ROC) area under the curve (AUC).

We also constructed a spider (or radar) chart to compare the performance of the whole panel between both groups. This chart is useful for the comparison of the performance of the whole panel between both groups. It visually and simultaneously displays the multiple individual biomarkers concentration, and their individual contribution, to the overall performance of the panel between the groups. This chart is helpful when direct comparison of several biomarkers is not possible. This is because, in a single graph, it allows an overall judgment of the multiple results among the groups, which is otherwise difficult with large numbers of variables. Each axis displays the concentration of one biomarker in each individual group and, when the values on each axis are connected by lines, they form a polygon area representing the magnitude of the biomarker's profile. The values of all biomarkers are therefore easily compared, each along their individual axis, and overall differences are displayed by the size and shape of the polygons. For each biomarker, the magnitude of its value in each of the two polygon areas of the groups indicates the degree of its contribution to the panel performance.

3. Results

A total of 17 children (7 males, 41%) were enrolled (Flowchart). Their median age was 25 months [IQR 18, 53] and their demographic and clinical characteristics are described in Tables 1 and 2. There was no significant difference in the demographic or clinical presentation between the two groups.

3.1. Kawasaki disease

Seven patients were diagnosed with KD by established clinical diagnostic criteria, and received intravenous immunoglobulin therapy followed by oral aspirin, with a favourable response. One child had a coronary artery aneurysm on echocardiography (Table 2). There were no children with incomplete KD.

3.2. Febrile infections

Ten febrile children were diagnosed with an infection, one of whom had one KD sign while another had four. One child developed scarlet fever with evidence of Streptococcal A infection and the other had radiological lobar pneumonia, presumed bacterial. The eight other children had a viral infection diagnosed by polymerase chain reaction: three respiratory and five gastrointestinal.

3.3. Standard laboratory measurements

Only the serum CRP concentration, total white cell and neutrophil count were significantly more elevated and the haemoglobin concentration lower, in the children with KD than in those with febrile infections (Table 1).

Table 1

Characteristics of the 17 participants. Values are expressed as mean (standard deviation), and median [interquartile range], unless stated otherwise.

	KD n = 7	Febrile infections n = 10	*P*-value
Number of males (%)	3 (43)	4 (40)	0.900[†]
Age (months)	21 [12,25]	32 [19,55]	0.180[‡]
Highest temperature (^{0}C)	38.9 [38.8, 39.4]	38.9 [38.8, 39.4]	0.960[†]
Serum CRP (mg/L)	126 [68,163]	3.64 [1,134]	0.003[§]
Platelet count (x10^9/L)	390 (218)	295 (176)	0.360[‡]
WBC count (x10^9/L)	16.3 (2.2)	8.3 (3.2)	<0.001[‡]
Neutrophil count (x10^9/L)	9.8 (3.4)	4.8 (3.1)	0.010[‡]
Haemoglobin concentration (g/L)	108 (14)	124 (9)	0.030[‡]
Serum sodium concentration (mmol/L)	137 (3)	137 (1)	1.000[‡]
Serum albumin concentration (g/L)	29 (5)	37 (2)	0.030[‡]
Serum ALT concentration (U/L)	16 [15,25]	12 [12,47]	0.290[§]
Serum AST concentration (U/L)	42 (31)	53 (25)	0.600[‡]

KD: Kawasaki disease; CRP: C reactive protein; ESR: erythrocyte sedimentation rate; WBC: white blood cells; ALT: alanine aminotransferase; AST: aspartate aminotransferase; [†]Fisher exact test; [‡]Unpaired *t*-test; [§]Kruskal-Wallis test.

Table 2

Clinical characteristics and definitive diagnosis in 17 febrile children.

Participant	Age (months)	Gender	Peak temperature (^{0}C)	Final diagnosis
1	64	Female	39.0	KD
2	12	Female	39.9	KD
3	6	Male	38.9	KD
4	25	Female	38.8	KD
5	21	Female	38.9	KD
6	18	Male	38.8	KD, CAL
7	23	Male	39.4	KD
8	25	Male	39.0	Viral infection (respiratory)
9	19	Male	38.9	Viral infection (gastrointestinal)
10	72	Female	38.9	Viral infection (gastrointestinal)
11	53	Female	39.9	Viral infection (respiratory)
12	68	Male	39.4	Viral infection (respiratory)
13	37	Female	38.8	Viral infection (gastrointestinal)
14	7	Female	38.8	Viral infection (gastrointestinal)
15	19	Female	38.9	Viral infection (gastrointestinal)
16	55	Male	39.4	Scarlet fever
17	11	Female	39.0	Bacterial pneumonia

KD: Kawasaki disease; CAL: coronary artery lesion.

3.4. Biomarkers measurements

Among the tested serum biomarkers (Table 3), only IL-1β was significantly more elevated in bacterial than in viral infections (panel A, P = 0.036), but not vs KD (panel B, P = 0.104). Only serum IL-6 and TNF-alpha were significantly different between the three groups, viral, bacterial and KD (panel B, P = 0.023 and 0.38 respectively). To compare them between KD and any infection, we

Table 3
Concentration of serum biomarkers (pg/mL) in 7 children with KD and 10 febrile infections (8 viral and 2 bacterial). Values are expressed as mean (standard deviation), and median [interquartile range]

	(A) Viral vs bacterial infections			(B) KD vs viral vs bacterial infections		(C) KD vs febrile infections	
	Viral n = 8	Bacterial n = 2	P-value	KD n = 7	P-value	Febrile infections (n = 10)	P-value
IL-1b	1.1 [0.9, 2.8]	11.8 [10.5, 13/2]	0.036*	1.8 [1.0, 5.9]	0.104*	1.2 [1.0, 7.3]	0.81*
IL-6	5.9 [3.1, 19.9]	49.1 [4.7, 93.5]	0.296*	47.9 [29.3, 53.3]	0.023‡	6 [4,28]	0.01*
IL-8	53.9 (114.7)	0 (0)	0.542†	47.7 (126.2)	0.839‡	43.1 (103.6)	0.93†
TNF- alpha	4.6 [0, 57.2]	323 [22.6, 623.4]	0.117*	201.4 [73.3, 437.0]	0.028*	15 [0,92]	0.04*
MMP-9	1846 (798)	1143 (951)	0.309†	2202 (662)	0.240‡	1705 (827)	0.20†
HSP-60	892 [874,1160]	1268 [865,1671]	1.000*	1031 [920,1622]	0.560*	892 [871, 1375]	0.30*
HSP-70	785 (1703)	0 (0)	0.550†	592 (406)	0.727‡	628 (1538)	0.95†

KD: Kawasaki disease; IL: interleukin; TNF: tumour necrosis factor; MMP: matrix metalloproteinase; HSP: heat shock protein; *Kruskal-Wallis test; †Unpaired *t*-test ‡Analysis of variance (ANOVA) with Bonferroni correction. Only serum IL-6 and TNF-alpha were significantly different between the three groups (viral, bacterial and KD) (B) as well as between all infections combined in one group vs KD in the other (C).

Table 4
Performance of serum biomarkers' concentrations (pg/mL) in differentiating children with KD (n = 7) from those with febrile infections (n = 10).

|	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Correctly classified (%)	ROC AUC	*Optimal cut-off serum level (pg/mL)
IL-1b	0.00	100.00	NA	58.82	58.82	0.53	1.15
IL-6	57.14	80.00	66.67	72.73	70.59	0.85	12.60
IL-8	0.00	100.00	NA	58.82	58.82	0.48	327.60
TNF- alpha	42.86	90.00	75.00	69.23	70.59	0.79	47.90
MMP-9	42.86	80.00	60.00	66.67	64.71	0.68	2140.10
HSP-60	14.29	80.00	33.33	57.14	52.94	0.65	910.50
HSP-70	0.00	100.00	NA	58.82	58.82	0.75	199.10
Combined IL-6 and TNF- alpha rise	57.14	90.00	80.00	75.00	76.47	0.86	–
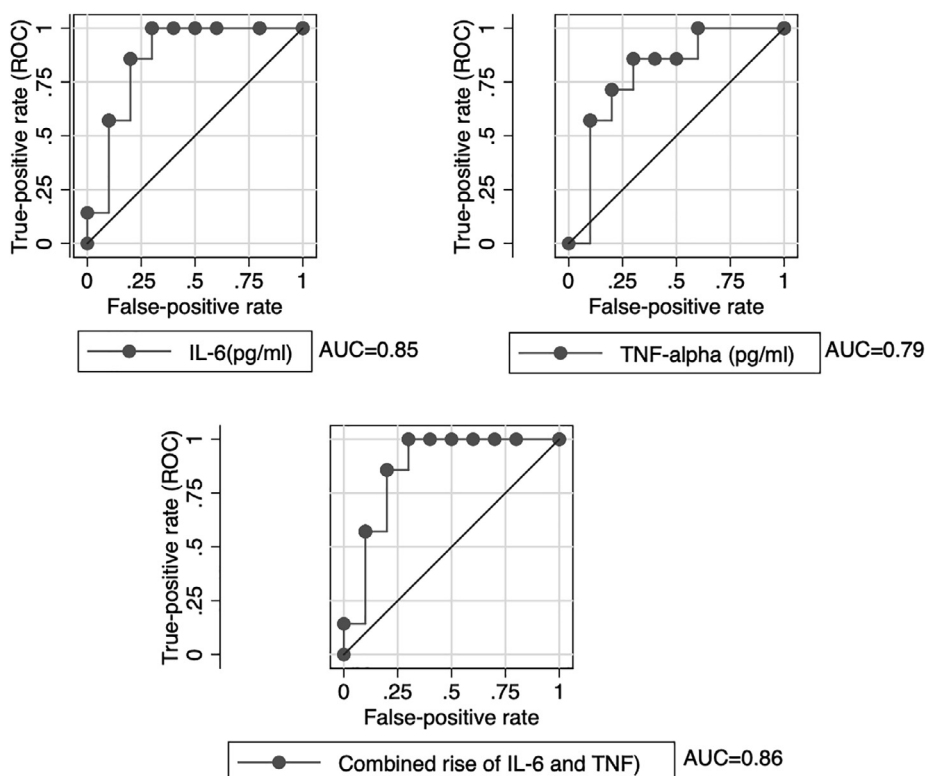
Fig. 1. Receiver operating characteristics (ROC) and area under the curve (AUC) of serum IL-6 and TNF levels to differentiate between children with Kawasaki disease (n = 7) and those with febrile infections (n = 10). The optimal cut-off value For IL-6 was 12.6 pg/mL and for TNF-alpha 47.9 pg/mL IL-6: interleukin 6; TNF: tumour necrosis factor.
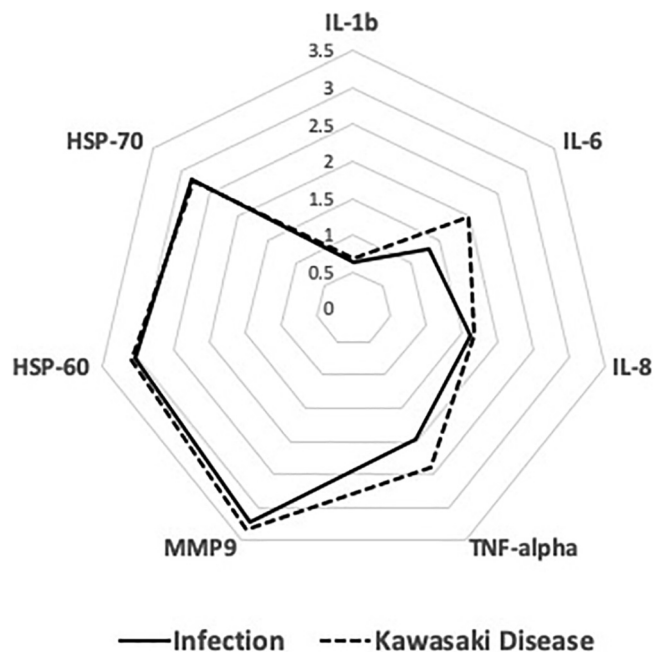
Fig. 2. Spider (or radar) chart of base-10 logarithmic serum biomarkers concentrations in children with Kawasaki disease (n = 7) compared to those with febrile infections (n = 10). It compares the performance of each biomarker between both groups by displaying its individual concentration on its own axis. The lines connecting the values on each axis form a polygon area which represent the magnitude of the biomarkers profile in each group and the overall differences are displayed by the size and shape of the polygons. For each biomarker, the magnitude of its value in each of the two polygon areas indicates the degree of its contribution to the panel performance. The concentrations are shown on a logarithmic scale because of the large number of biomarkers spanning a wide range of values, with many orders of magnitude, and to avoid skewness towards large values; IL: interleukin; TNF: tumour necrosis factor; MMP: matrix metalloproteinase; HSP: heat shock protein.

grouped in panel C bacterial and viral infections into a single group (febrile infections). When the latter was compared to KD, the same biomarkers, IL-6 and TNF-alpha remained significantly different between the two groups (panel C, P = 0.01 and 0.04 respectively), which we therefore used for all subsequent analyses.

The performance of the biomarkers to differentiate between these two groups is shown in Table 4. IL-6 levels provided the highest sensitivity, while TNF- alpha the highest specificity. For IL-6, the optimal cut-off value was 12.6 pg/mL with a ROC AUC of 0.85, while for TNF-alpha it was 47.9 pg/mL with a ROC AUC of 0.79 (Fig. 1). Although both biomarkers outperformed all others in correctly classifying KD and febrile infections, the performance of their combination was superior. The ROC AUC for the combined elevation of IL-6 and TNF-alpha levels was 0.86 (Fig. 1) displaying the best diagnostic performance.

The overall distribution of the biomarkers' values between the two groups in the spider chart (Fig. 2) highlights a characteristic "signature" of simultaneously elevated IL-6 and TNF-alpha serum concentrations in children with KD compared to those with febrile infections.

4. Discussion

Only the serum concentrations of IL-6 and TNF-alpha were significantly higher in children with KD than in those with infections, with an optimal cut-off serum level of 12.6 pg/mL for IL-6, and 47.9 pg/mL for TNF-alpha. However, their combined increase outperformed their individual concentrations and correctly classified 76.47% of the participants.

Serum IL-6 levels were higher in children with KD than in those with infections, confirming previous reports (Tan et al., 2013; Wu et al., 2019). The higher concentrations of TNF-alpha in children with KD compared to febrile infections contrast with the results of a previous study (Tan et al., 2013). The reason might be our smaller sample size and that, unlike our study which included only acute febrile infections, the latter included children with recurrent episodes of fever, not always infectious and without providing details of those caused by infections.

The other studied biomarkers performed less well. The lack of increase in MMP-9 in KD is probably because only one out the seven affected children had coronary artery abnormalities, a complication known to be associated with elevated MMP-9 levels which leads to elastin breakdown in the inflamed coronary arteries, wall destruction and coronary artery aneurysm (Nagata et al., 2009; Yin Ji et al., 2007; Hoang et al., 2014; Korematsu et al., 2012; Kuo et al., 2017). The concentrations of HSPs, normally released by a proinflammatory response triggered by a release of lipopolysaccharides, were not different between KD and infections. This is not surprising as most infections in our study were viral compared to previous reports which included a predominance of bacterial infections known to release lipopolysaccharides (Triantafilou and Triantafilou, 2003). Another possible explanation is that in KD autologous HSP-60 functions as a regulator for control of inflammation, rather than a proinflammatory mediator as shown in infections (Yin Ji et al., 2007).

The study suffers from some limitations. The number of children with KD was small, and only one developed CAL. The febrile controls had mainly viral infections and no sepsis. The blood collected on admission occurred at different times throughout the illness. As the biomarkers were measured only on admission, their kinetics throughout the febrile illness were not studied. Although a few children might have received ibuprofen or antibiotics prior to admission, most parents were unable to recall the name or type of the medication, the number of doses or the duration of its administration, resulting in omission of this data from the analysis. Finally, our results cannot be generalized to febrile illnesses caused by conditions other than those included in this study. Future prospective studies with a larger sample size are therefore needed to address the limitations of this preliminary study and confirm our findings.

5. Conclusion

Although the serum concentrations of IL-6 and TNF-alpha were significantly higher in children with KD than in those with febrile infections, they were outperformed by their combined rise. If confirmed by future studies, these findings could stimulate the development of a rapid diagnostic assay kit for such a combined test, as the resulting characteristic "signature" would be very useful in diagnosing KD early and instituting appropriate therapy.

Ethics approval

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Institution's Human Ethics Committee (No. 11/55).

Consent to participate

The parents of all the participating children signed an informed consent form for this study.

Consent for publication

All authors confirm their consent for publication.

Availability of data and material

Available upon a reasonable request.

Authors' contributions

Conceptualization, Hassib Narchi; Data curation, Asad Aziz Khan, Sania Al-Hamad and Richard L Jayaraj; Investigation, Junu A George; Writing – original draft, Hassib Narchi; Writing – review & editing, Richard L Jayaraj and Hassib Narchi.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2020.09.034.

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