Monocyte Platelet Aggregates in Children with Kawasaki Disease- a Preliminary Study from a Tertiary Care Centre in North-West India

Pandiarajan Vignesh (vigimmc@gmail.com)
Post Graduate Institute of Medical Education and Research https://orcid.org/0000-0001-8490-5584
Amit Rawat
Post Graduate Institute of Medical Education and Research
Jitendra Kumar Shandilya
Post Graduate Institute of Medical Education and Research
Man Updesh Singh Sachdeva
Post Graduate Institute of Medical Education and Research
Jasmina Ahluwalia
Post Graduate Institute of Medical Education and Research
Surjit Singh (surjitsinghpgi@rediffmail.com)
Post Graduate Institute of Medical Education and Research

Short Report

Keywords: Kawasaki Disease, Platelet activation, Monocyte platelet aggregates, Flow cytometry, India

Posted Date: July 2nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-39407/v1

Version of Record: A version of this preprint was published on March 12th, 2021. See the published version at https://doi.org/10.1186/s12969-021-00515-3.

Abstract

Background:

Platelet activation is an integral part of pathogenesis of Kawasaki disease (KD). However, there is paucity of literature on flow-cytometry based assessment of platelet activation in KD. We aimed to analyse monocyte-platelet aggregates (MPAs), one of the sensitive markers for platelet activation, by flow cytometry in children with Kawasaki disease (KD).

Findings:

In this single-centre prospective study, we have enrolled 14 children with KD and results were compared with age-matched febrile (n = 15) and healthy (n = 13) controls. After gating monocytes in side-scatter plot, MPAs were identified based on CD14 and CD41 expression. Two (2) ml of blood samples for children with KD were collected at 3 phases of illness- acute stage before start of intravenous immunoglobulin or aspirin, 24 hours after completion of IVIg infusion, and 3 months after acute episode of KD.

Children with KD had a significantly higher MPA% values [Median (IQR)- 41.3% (26.6, 52.7)] when compared with febrile [Median (IQR)- 5.98% (2.98-9.72)] and normal [Median (IQR)- 4.48% (2.57-5.59)] controls, $p<0.01$. On follow-up, the MPA% showed a gradual decline in children with KD, but even at 3 months, the value [Median (IQR)- 7.55% (4.15-14.6)] was higher compared to healthy controls [Median (IQR)- 4.48% (2.57-5.59)].

Conclusions:

Our results suggest that MPA% was significantly elevated in acute stages in children with KD and activated platelets may continue to persist even after systemic inflammation has subsided. Future studies are warranted whether objective evidence of platelet activation may guide the use of immunomodulatory and anti-platelet therapy in KD.

Introduction

Kawasaki disease (KD) is a multi-systemic vasculitis that predominantly affects young children (1). It is considered to be the most frequent cause of acquired heart disease in children in developed countries. Hospital-based data from our centre have also shown a sustained increase in number of cases of KD since the mid-1990 s (2). Vasculitis in KD can result in endothelial cell damage and platelet activation. Activated platelets can in turn secrete several chemokines and enhance inflammation in vascular walls (3, 4).

Markers of platelet activation such as leukocyte-platelet aggregates and P-selectin expression are elevated in coronary artery disease (CAD) and are implicated in progression of cardiovascular events (5). Occurrence of coronary thrombosis and premature arteriosclerosis in KD suggests that excess activation

of platelets and coronary vasculitis may play an important role in progression of vascular damage in KD. Among all leukocytes, monocytes have the highest affinity towards platelets to form aggregates (6). Monocyte-platelet aggregates (MPAs) are considered a better marker for activated platelets than P-selectin expression (6). Though platelet activation has been studied in KD, there is paucity of literature on flow cytometry-based assessment of leukocyte-platelet aggregates in KD and there are no data from developing countries (7–13).

Materials And Methods

Children with KD diagnosed in the Allergy Immunology Unit, Advanced Pediatrics Centre, Postgraduate Institute of Medical Education and Research, Chandigarh, India, from July 2015 to December 2016 were enrolled. Written informed consent was obtained from parents. Diagnosis of KD was based on the American Heart Association (AHA) criteria (1). Age and sex-matched febrile children who had a diagnosis other than KD were taken as febrile controls. Siblings nearest in age to index patient were taken as healthy controls. In cases where a sibling control was not available or accessible, an age-matched control was enrolled from outpatient clinics.

The Institute Thesis Committee and the Institute Ethics Committee approved the study protocol. The Departmental Review Board has approved the manuscript. Standard treatment protocols were followed for management of KD (1).

Sample collection

Venepuncture was carried out under aseptic conditions and with minimal occlusion. Three (3) ml of peripheral venous blood was withdrawn into a vacutainer containing 0.106 mol/L of trisodium citrate (blood-citrate ratio 9:1) after discarding initial 1 ml of sample. Samples were collected at three different phases in children with KD- acute stage before administration of IVIg and aspirin; 24 hours after administration of IVIg; and approximately 3 months after onset of illness. Febrile and healthy controls had their blood drawn only once in a similar manner.

Measurement of monocyte-platelet aggregates

Blood samples were processed as quickly as possible to avoid time-dependent spontaneous aggregation and activation of platelets. Vortexing, centrifugation, and stirring were avoided before fixation of platelets. Fixation of cells and erythrocyte lysis was carried out at room temperature using *Becton Dickinson* FACS® lysing solution. After 10 minutes of incubation, samples were washed with phosphate-buffered saline and immuno-labelled with 8 µL of fluorescein isothiocyanate (FITC) conjugated GP IIb (CD41, BD Pharmingen™, *Catalogue no.*555466) and 8 µL of PerCP-conjugated CD14 (BD Pharmingen™, *Catalogue no.*555398) antibodies. After incubation for 20 minutes at room temperature, cells were acquired on a flow cytometer (Beckman Coulter, Navios). Using *Kaluza*® software, monocytes were gated from lymphocyte side scatter plot. MPAs were identified from monocyte population that expressed both CD14 and CD41 [Fig. 1].

Statistical Analysis

Means were compared using unpaired student 't' test and analysis of variance (ANOVA). Medians were compared using Mann-Whitney U test and Kruskal Wallis test. Categorical variables were compared by Fisher exact test. A 2-tailed p-value less than 0.05 was considered significant. Statistical analysis was performed with SPSS statistical software version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Fourteen (14) children with KD were enrolled during the study period. Fifteen (15) children were taken as febrile controls and 13 as healthy controls. Presumed aetiology of fever in febrile controls was viral upper respiratory tract infections (5), enteric fever (5), staphylococcal abscesses (3), and pneumonia (2). Median age (interquartile range (IQR)) in cases, febrile controls, and normal controls was 6 (3.0-7.25), 5 (3.6-9.0), and 5.5 (4.15–6.5) years, respectively. Male to female ratio among the 3 groups was 12:2, 13:2, and 11:2, respectively. Among cases with KD, median duration between onset of fever and administration of IVIg was 13.5 days [Table.1]. Two children had transient coronary artery abnormalities (CAAs) in form of brightness of coronary arteries and coronary ectasia. One child (7-month-old boy) developed giant coronary aneurysms in left main, left anterior descending, and right coronary arteries. All patients received IVIg (2 g/kg) infusion for treatment for KD. Two patients also received IV infliximab infusion (5 mg/kg) as they had severe disease and had developed CAAs at presentation. Antiplatelet dose of aspirin was continued for 3 months in one child as he persisted in having elevated platelet counts (640×10^9/L).

MPA% values were significantly high in cases [Median (IQR)- 41.3% (26.6–52.7)] when compared to febrile [Median (IQR)- 5.98% (2.98–9.72)] and normal controls [Median (IQR)- 4.48% (2.57–5.59)], $p < 0.01$ [Fig. 2A]. A significant drop in MPA% was noted after IVIg therapy. Serial MPA% in patients with KD showed a consistent drop in values from the time of diagnosis until the 3rd month of follow-up [Fig. 2B,2C].

No change in trend of fall of MPA% was noted even after exclusion of patients who were continued on aspirin [Fig. 2D]. MPA% values at 3 months post-diagnosis of KD [Median (IQR)- 7.55% (4.15–14.6)] were higher compared to normal controls [Median (IQR)- 4.48% (2.57–5.59)], p-0.072.

No significant differences in MPA% were noted between patients with and without CAAs [Table.2]. There was also no significant difference in MPA% values between patients with complete and incomplete presentation of KD [Table.3].

Discussion

Platelet function abnormalities in KD were first reported by Yamada et al in 1978 (14). Studies in platelet activation markers in KD have subsequently been undertaken in Japan, USA, and Italy [Table.4]. Present study has been undertaken to analyse one of the most sensitive platelet activation markers, i.e. MPAs, by

flow cytometry in a North Indian cohort of KD. To the best of our knowledge, MPAs has not been studied in acute and subacute phases of KD till date, and our study is perhaps the first attempt along these lines. Levels of MPA% were significantly elevated in children with KD compared to febrile controls and healthy controls (median levels of 41.3%, 5.98%, and 4.48% respectively, $p < 0.001$). MPA% levels significantly decreased after treatment with IVIg (median: 41.3% vs 18.5%, $p < 0.001$) [Fig. 2]. Elevations in MPA% suggest excess activation of platelets in KD compared to other common childhood febrile illnesses. Previous studies report that other platelet activation markers (e.g. neutrophil-platelet aggregates, platelet-derived microparticles, platelet VEGF levels, betathromboglobulin levels, PF4 levels, and platelet CD62P expression) are also elevated in acute phase of KD (7–13).

Median levels of MPA% on follow-up at 3 months were lower compared to levels measured 24 hours after IVIg therapy, but higher than values obtained in age and sex-matched controls. It suggests that patients with KD may have a prolonged endotheliitis even after control of systemic inflammation with IVIg therapy. Laurito et al analyzed MPAs in patients with KD several years after the acute phase (mean interval- 76 months). Authors reported that CD41 expression at baseline and after ADP stimulation was significantly higher in patients compared to controls. However, MPA% levels were not significantly different between two populations (9). In a recent study by Yahata et al., platelet-derived microparticles were found to have rebound elevations in 8 of 14 patients with KD after discontinuation of aspirin (10). In our study also, we found rebound elevation in MPA% levels in 2 patients at 3 months of follow-up. However, majority of patients had lower levels even after discontinuation of aspirin.

Ueno et al. reported that levels of neutrophil-platelet aggregates were significantly higher in patients with CAAs when compared with patients without CAAs (11). In our study, levels of MPA% were not different between patients with CAAs (n = 3) and those without CAAs (n = 11). Apparent discrepancy in results could be explained by differences in ethnicities of the study populations, small sample size in both studies, and differences in methodology of assessment of platelet activation. Ueno et al. also reported that levels of neutrophil-platelet aggregates were significantly lower in patients with KD treated with both IVIg and oral prednisolone than in patients who received IVIg alone (11). Corticosteroids were not used in any patients in our cohort.

A limitation of our study is the small sample size. This is understandable considering the fact that the protocol had to be completed in a limited span of time. Use of an imaging flow cytometry could have accurately captured the aggregates of monocytes and platelets (15), however, we could not use this modality in our study. To conclude, MPA% was significantly elevated in our cohort of children with KD when compared with age and sex-matched febrile and healthy controls. Future long-term studies are warranted to find out whether elevated MPAs in KD would have any clinical implications. This may provide a theoretical basis for additional immunosuppressive and/or antiplatelet therapy in KD.

Abbreviations

KD

Kawasaki disease
MPA
Monocyte-platelet aggregates
IVIg
Intravenous immunoglobulin
CAD
Coronary artery disease
AHA
American Heart Association

Declarations

Ethical approval and consent to participate: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional and National Research Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from parents of all individual participants included in the study.

Consent for publication: All authors consent for final approval and inclusion of co-authors for this manuscript.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests: None

Funding: This work was partly supported by a Special research grant for MD/MS/DM/MCh from PGIMER, Chandigarh, India.

Authors' contributions: PV, SS, MS, JA- Inception of idea; PV, AR, JS- Carried out the flow cytometry experiments; PV- Literature review and framing of first draft; JA, SS, MS, AR- Critical review and editing of the manuscript; PV, SS- Editing of manuscript and final approval

Acknowledgments: Nil

Conflict of interest: All authors declare that he/she has no potential conflict of interest.

Authors' information: The study was presented in the 24th European Pediatric Rheumatology Congress held at Athens, Greece and the abstract was published in the conference proceedings.

Ref: Proceedings of the 24th Paediatric Rheumatology European Society Congress: Part one. *Pediatr Rheumatol Online J* 2017;15(Suppl 2):64.

References

1. Newburger JW, Takahashi M, Gerber MA, Gewitz MH, Tani LY, Burns JC, et al. Diagnosis, treatment, and long-term management of Kawasaki disease: a statement for health professionals from the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. Circulation. 2004;110:2747–71.
2. Singh S, Aulakh R, Bhalla AK, Suri D, Manojkumar R, Narula N, Burns JC. Is Kawasaki disease incidence rising in Chandigarh, North India? Arch Dis Child. 2011;96:137–40.
3. Orenstein JM, Shulman ST, Fox LM, Baker SC, Takahashi M, Bhatti TR, et al. Three linked vasculopathic processes characterize Kawasaki disease: a light and transmission electron microscopic study. PLoS One. 2012;7:e38998.
4. Chatterjee M, Geisler T. Inflammatory Contribution of Platelets Revisited: New Players in the Arena of Inflammation. Semin Thromb Hemost. 2016;42:205–14.
5. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J Am Coll Cardiol. 1998;31:352–8.
6. Gerrits AJ, Frelinger AL, Michelson AD. Whole Blood Analysis of Leukocyte-Platelet Aggregates. Curr Protoc Cytom. 2016;78:6.
7. Taki M, Kobayashi M, Ohi C, Shimizu H, Goto K, Aso K, et al. Spontaneous platelet aggregation in Kawasaki disease using the particle counting method. Pediatr Int. 2003;45:649–52.
8. Yokoyama T, Kato H, Ichinose E. Aspirin treatment and platelet function in Kawasaki disease. Kurume Med J. 1980;27:57–61.
9. Laurito M, Stazi A, Delogu AB, Milo M, Battipaglia I, Scalone G, et al. Endothelial and platelet function in children with previous Kawasaki disease. Angiology. 2014;65:716–22.
10. Yahata T, Suzuki C, Yoshioka A, Hamaoka A, Ikeda K. Platelet activation dynamics evaluated using platelet-derived microparticles in Kawasaki disease. Circ J. 2014;78:188–93.
11. Ueno K, Nomura Y, Morita Y, Eguchi T, Masuda K, Kawano Y. Circulating platelet-neutrophil aggregates play a significant role in Kawasaki disease. Circ J. 2015;79:1349–56.
12. Burns JC, Glode MP, Clarke SH, Wiggins J, Hathaway WE. Coagulopathy and platelet activation in Kawasaki syndrome: Identification of patients at high risk for development of coronary artery aneurysms. J Pediatr. 1984;105:206–11.
13. Ueno K, Nomura Y, Hashiguchi T, Masuda K, Morita Y, Hazeki D, et al. Platelet vascular endothelial growth factor is a useful predictor for prognosis in Kawasaki syndrome. Br J Haematol. 2010;148:285–92.
14. Yamada K, Fukumoto T, Shinkai A, Shirahata A, Meguro T. The platelet functions in acute febrile mucocutaneous lymph node syndrome and a trial of prevention for thrombosis by antiplatelet agent. Nihon Ketsueki Gakkai Zasshi. 1978;41:791–802.

15. Hui H, Fuller KA, Erber WN, Linden MD. Imaging flow cytometry in the assessment of leukocyte-platelet aggregates. Methods. 2017;112:46–54.

Tables

Table 1
Baseline clinical and laboratory characteristics of children with Kawasaki disease at enrolment (n = 14)

S no	Clinical parameter	Result
1	Median age (IQR)	6 years (3.0, 7.25)
2	Male: Female	12: 2
3	Duration of fever [median (IQR)]	13.5 days (8.0, 15.0)
4	No. of cases with incomplete KD (%)	5 (35.7%)
5	Hemoglobin in g/dL [median (IQR)]	9.2 (7.6, 11.3)
6	White cell counts (x 10^9/L) [median (IQR)]	14.15 (10.6, 21.2)
7	Absolute neutrophil counts (x 10^9/L) [median (IQR)]	7.9 (6.2, 12.4)
8	Platelet counts (x 10^9/L) [median (IQR)]	385.5 (233.5, 598.5)
9	Erythrocyte sedimentation rate at 1st hour (mm) [median (IQR)]	45 (28.5, 69.5)
10	C-reactive protein in mg/L [median (IQR)]	48.5 (23, 81.8)
IQR: Inter-quartile range; KD: Kawasaki disease		

Table 2
Median percentage values of MPA (CD14 + CD41+) between children with KD patients with and without coronary artery abnormalities (CAA)

S no	Category	Children with CAA (n = 3)	Children without CAA (n = 11)	P value
1	At enrolment [median (Range)]	38.18 (17.42–56.67)	44.44 (9.58–54.2)	1.000
2	24 hours after IVIg [median (Range)]	18.26 (10.4-28.13)	18.84 (4.27–34.32)	0.885
3	Follow-up at 3rd month [median (Range)]	7.55 (2.84–14.6)	7.17 (1.59–27.32)	0.921

Table 3
Median percentage values of MPA (CD14 + CD41+) between children with complete (n = 8) and incomplete KD (n = 6)

S no	Category	Children with complete KD (n = 9)	Children with incomplete KD (n = 5)	P value
1	At enrolment [median (Range)]	45.3 (27.08–56.67)	25.17 (9.58–53.62)	0.083
2	24 hours after IVIg [median (Range)]	20.28 (5.56–34.32)	10.4 (4.27–22.4)	0.112
3	Follow-up at 3rd month [median (Range)]	8.34 (1.59–27.32)	5.21 (2.84–16.87)	0.792

Table 4

Comparison of studies on platelet functions in Kawasaki disease reported in English literature

S no	Study, year, and Country	Platelet activation marker studied	Study population characteristics	Comments
1	Yokoyama et al (1980) Japan (8)	Platelet aggregation by optical density method	23 patients with KD (Age range: 3 months-6 years; M:F = 14:9);	Platelet aggregation in patients with untreated KD (82.4 ± 11.6%) significantly higher than normal controls (69 ± 5%) and patients with KD treated with low dose aspirin (66.8 ± 9.6%).
2	Burns et al (1984) USA (12)	Plasma beta-thromboglobulin	31 patients with KD	Levels significantly higher in patients with coronary aneurysms compared to patients without aneurysms measured 3 weeks after fever (72.3 ng/ml vs 29.4 ng/ml respectively, $p < 0.002$).
3	Taki et al (2003) Japan (7)	Platelet aggregation by particle counting method (mV count)	104 children with KD (mean age: 2.1 ± 1.9 years; M:F = 15:11); 9 normal controls	Spontaneous platelet aggregation was higher in patients with KD ($46.6 \times 10^3 \pm 13.2 \times 10^3$) compared to normal subjects ($9.4 \times 10^3 \pm 3 \times 10^3$). It reduced significantly after IVIg therapy ($22.8 \times 10^3 \pm 6.6 \times 10^3$).
4	Ueno et al (2009) Japan (13)	Platelet VEGF by ELISA	80 patients with KD (Mean age: 2.1 ± 1.8 years, M:F = 43:37); 26 controls	Levels significantly high in KD ($18.8 \pm 10.1 \times 10^{-8}$ pg) compared to controls ($8.1 \pm 3.0 \times 10^{-8}$ pg). The levels decreased in IVIg responders and remained elevated in IVIg non-responders.
5	Yahata et al (2014) Japan (10)	Platelet derived microparticles by ELISA	18 patients with acute KD (mean age: 2 years 7 months) in whom 14 received IVIg therapy; 33 children as febrile controls	Levels were significantly high in acute phase of KD (43.9 ± 13.5 U/ml) compared to febrile controls (15.4 ± 6.8 U/ml). The levels significantly came down with IVIg therapy and it rebounded after discontinuation of aspirin in 8 patients.
6	Laurito et al (2014) Italy (9)	Monocyte platelet aggregates by flow cytometry	14 patients with past history of KD (mean follow-up: 76 ± 58 months; M:F = 9:5); 14 controls	Mean %MPAs were similar in patients and controls even after ADP stimulation (18.3 ± 1.9% vs 17.2 ± 1.5%; $p = 0.09$). CD41 expression in MPA gate was higher in KD than controls after ADP stimulation (19.3 ± 1.3% vs 17 ± 1.7; $p < 0.001$).

KD: Kawasaki Disease; MPA: Monocyte-platelet aggregates; IVIg: Intravenous immunoglobulin; ADP: Adenosine diphosphate

S no	Study, year, and Country	Platelet activation marker studied	Study population characteristics	Comments
7	Ueno et al (2015) Japan (11)	Neutrophil platelet aggregates by flow cytometry	40 patients with KD (median age: 1.75 years, M:F ≈ 1:1); 7 febrile controls, and 9 normal controls	Rate of aggregates significantly high in KD compared to febrile and normal controls. Rate of decrease in aggregates was significantly high in patients who received prednisolone + IVIg compared to patients who had received IVIg alone.
8	Present study (2019) India	Monocyte platelet aggregates by flow cytometry	14 patients with acute KD (Median age: 6 years; M:F = 6:1); 15 febrile controls and 13 normal controls	Median %MPA significantly higher in KD compared to febrile and normal control (41.3 vs 5.9 vs 4.5%; $p < 0.001$). Levels significantly came down 24 hours after IVIg therapy (18.6%; $p < 0.001$).

KD: Kawasaki Disease; MPA: Monocyte-platelet aggregates; IVIg: Intravenous immunoglobulin; ADP: Adenosine diphosphate

Figures

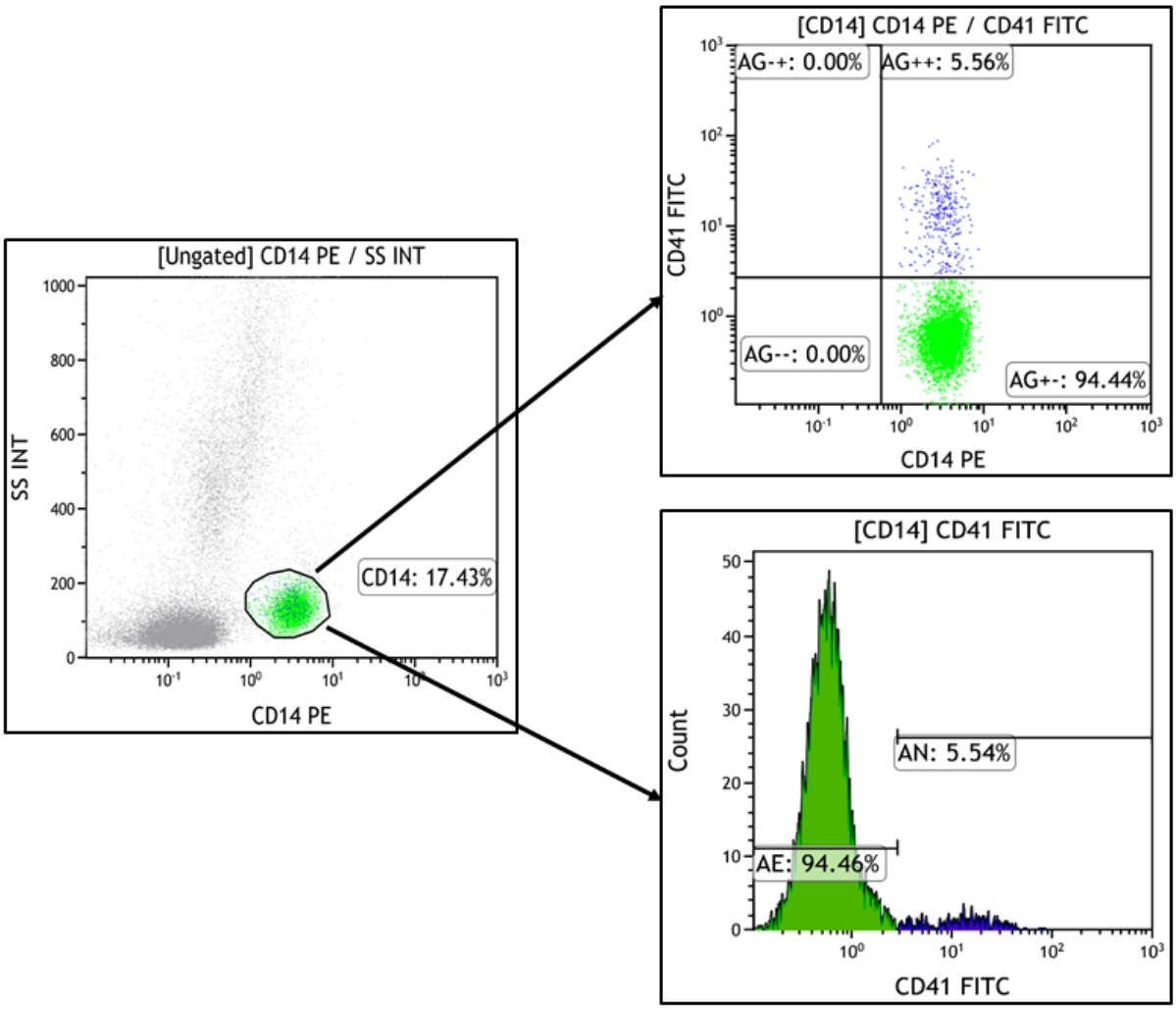

Figure 1

Representative flow cytometry plots that demonstrate gating of monocytes from the side scatter plot of lymphocytes, and analysis of MPA% by identifying monocyte populations that express both CD14 and CD41.

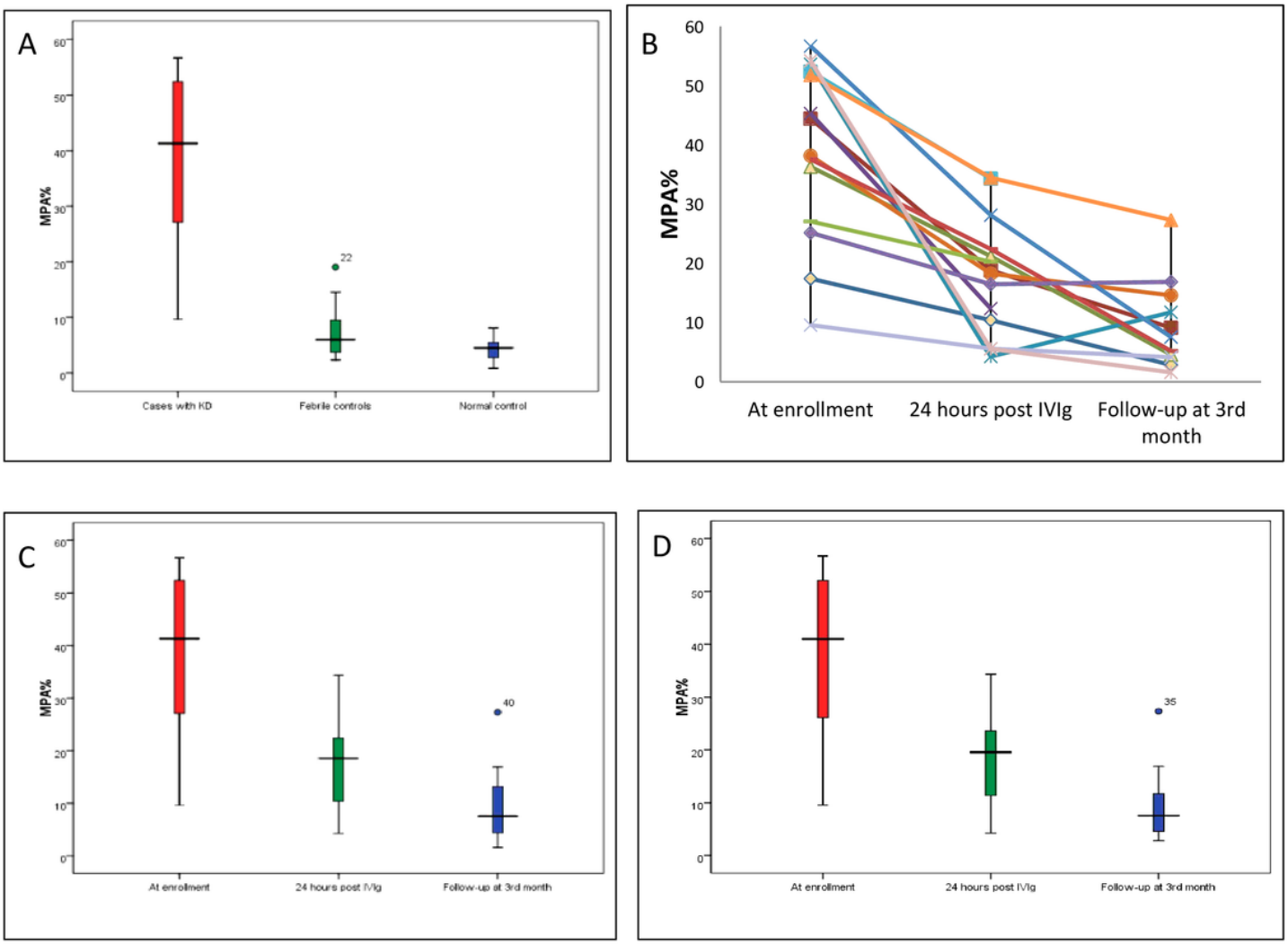

Figure 2

A. Boxplot showing MPA% between children with KD at enrolment (n=14) [Median (IQR)- 41.3 (26.6-52.7)], febrile controls (n=15) [Median (IQR)- 5.98 (2.98-9.72)], and normal controls (n=13) [Median (IQR)- 4.48 (2.57-5.59)], p <0.001; B. Trend of serial values MPA% among all 14 children with KD; C. Boxplot showing MPA% between cases at enrolment (n=14) [Median (IQR)- 41.31 (26.6-52.69)], 24 hours post-IVIg (n=14) [Median (IQR)- 18.55 (9.2-22.99)] , and at 3rd month follow-up (n=11) [Median (IQR)- 7.55 (4.15-14.6)], p<0.001; D. Boxplot showing MPA% (excluding 2 cases who were on aspirin at 3rd month follow-up) between cases at enrolment (n=12) [Median (IQR)- 41.01 (25.65-52.23)], 24 hours post-IVIg (n=12) [Median (IQR)- 19.56 (10.89-24.17)], and at 3rd month follow-up (n=9) [Median (IQR)- 7.55 (4.36-14.3)], p<0.001.