CHAPTER 4

Role of platelet activation in thrombocytopenia in Dengue infection

CHAPTER 4A: Assessment of platelet activation during Dengue infection in patients
4A.1 Introduction

Dengue infection poses huge economic burden on healthcare system of India [39] with an estimated 100,000 hospitalisations every year [40]. The clinical symptoms of DV ranges from mild febrile fever to severe forms such as Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS) [41]. Clinically Dengue pathophysiology is characterized by three different phases of illness i.e. febrile phase, critical phase and recovery phase which comprises of 10-12 days of infection [42]. The febrile phase is characterised by high fever, myalgia, arthralgia, headache, petechial rashes and chance bleeding during day 2-7 of infection, then it progresses into critical phase with symptoms like hypotension, hypovolemic shock, oedema and vascular leakage [43, 45]. The underlying mechanisms which lead to these pathophysiological changes in Dengue are still incompletely understood. It seems to be multifactorial interactions among the following factors; namely the virulence of the circulating strain, high density or efficiency of the vector, the wide circulation of the virus as well as host genetic and environmental factors which results in development of disease severity [46, 47].

Thrombocytopenia i.e. decrease in platelet count is a key clinical feature and is widespread in patients with febrile to critical forms of Dengue infection [48]. Several studies have implied that drop in platelet count is associated with bleeding complications in Dengue patients [49-51]. The platelet counts reduces below normal count (1.5-4.5x10⁵ platelets /μL) and may reduce further to lower level like <0.4x10⁵ platelets/μL during early days of fever in several patients [52]. A study performed with Dengue patients (n=225) suggested that bleeding occurred more commonly in patients with a platelet count below 20 x 10⁹/litre. In critical cases, patients need platelet transfusion in order to maintain their normal haemostatic function [53].
Another study has associated the disease outcome with the platelet count wherein they have shown that in severe cases DHF mortality was 6 fold higher in patients with platelet counts <0.5x10^5/μL in comparison to patients with platelet counts >0.5x10^5/μL[132].

Thrombocytopenia in Dengue patients mainly occurs because of two events: decreased thrombopoiesis i.e. production of platelets from progenitor cells in bone marrow itself is reduced and/or elevated destruction of platelets from peripheral blood [24]. DV by direct or indirect means can affect the bone marrow progenitor cells by impairing their function ultimately leading to reduced production of platelets. A study has shown that DV can induce bone marrow hypoplasia during critical phase of Dengue infection [56]. One more study has shown that increased TPO level in adult Dengue patients inversely associated with the platelet counts. In addition to this DV is reported to increase platelet utilization due to disseminated intravascular coagulation (DIC), enhanced decrease in count due to cross-reaction of platelets with anti-Dengue virus antibodies, increased peripheral sequestration, increased phagocytosis of platelets by macrophages in Dengue patients, increased platelet destruction by apoptosis or by increased complement mediated lysis and by involvement of antiplatelet antibodies [57-63].

Numerous studies depicted that dysfunction and activation of platelets is implicated in the thrombotic difficulties in acute cases of DHF and DSS [64, 65]. The suppression of platelet aggregation was depicted during the acute phase of DHF in both shock and nonshock patients with simultaneous increase in platelet factor 4 (PF4) and beta-thromboglobulin (BTG) release from platelets into plasma. Studies have investigated and shown that release of prostaglandin D2 (PGD2), Platelet activating factor (PAF)
and thromboxane B2 (TxB2) from leukocytes has increased regardless of primary or heterologous secondary exposure to DV2 which leads to platelet activation [247]. Few studies have reported the increased concentrations of nitrite or nitrate in DF patients when compared to patients with DHF and healthy individuals [248]. Release of nitric oxide is known to contribute in adhesion and aggregation of platelets to the vascular endothelium [249]. Platelet activation and apoptosis are implicated with early days (day 3-7) of Dengue infection [62, 63]. Platelets which normally exist at resting state in a healthy individual and is crucial to maintain the haemostatic balance becomes hyper-activated during several disease conditions.

Acute form of Dengue infection display close association with molecular and cellular events such as platelet activation and elevated expression of receptors like αIIbβ3, CD63 and CD62-P. [250]. Further, platelet activation has been demonstrated in Dengue patients and increased platelet activation has been associated with disease severity [73, 79, 80-82]. It is generally marked by the presence of elevated surface P-selectin expression or with increased binding of conformation dependent PAC-1 antibody on the platelet surface receptor GPIIbIIIa. Increased caspases, increased phosphatidylserine (PS) expression over the platelet surface and inside-out activation of αIIbβ3 integrin all associated with activation and early apoptosis of platelets [251]. The activated platelets get deposited on the vascular endothelial wall which further results in more platelet loss. In agreement to this, platelet aggregates have been detected in micro vessels of post-mortem histology and skin biopsies from severe Dengue patients [103]. Aggregation of activated platelets with monocytes also contributes to thrombocytopenia in Dengue as observed by the presence of platelet-leukocyte aggregates in circulation of Dengue patients [43,164].
DV can infect circulating platelets and megakaryocytes in bone marrow [67, 55]. It binds with platelets and megakaryocytes through FcγRII receptor [68]. A recent finding reported direct binding of DV to surface receptors present on platelets (heparan sulfate proteoglycans and DC-SIGN) and its replication inside platelets [69, 70].

Although the underlying mechanisms behind immune mediated platelet lysis and clearance have been investigated, several queries remain unaddressed. This work is focused on exploring what role platelet activation plays in Dengue pathogenesis and whether it can act as determining factor for platelet lysis and clearance. In this chapter we investigated the close association between platelet activation and decrease in platelet count in Dengue patients on different days of infection which includes early days at which platelet count was very low in the patients and on later day points when platelet count was recovered to normal level. We measured the platelet count and platelet activation markers like P selectin, PAC-1 binding and PS expression as well as estimated the Dengue viral load in platelets of patients on different days of infection.

4A.2 Materials and Methods

4A.2.1 Materials

The pre-conjugated fluorescent antibodies like anti-human CD41a-PE, AnnexinV-FITC and PAC1-FITCalong with all isotype controls were purchased from BD Biosciences (San Jose, USA), Anti-human P-selectin (CD62P)-Fluorescein from R&D systems, Minneapolis, USA. Ambion TR Izol Reagent and SuperScript III First Strand cDNA Synthesis kit from Invitrogen and TaqMan universal PCR master mix from Applied Biosystems were purchased.
Majority of other laboratory chemicals including BSA and sepharose CL-2B beads for gel filtration chromatography were purchased from Sigma-Aldrich (St. Louis, USA).

4A.2.2 Human subjects

4A.2.2a Patients Samples

The approval for the Dengue patients sample collection was taken from the Institutional Ethics Committee for Human Research of Regional Centre for Biotechnology (RCB) and also from AIIMS (New Delhi) with Ref. No.RCB-IECH-3, 31.07.2014 and Ref. No.IEC/NP-39/13.03.2014 respectively.

Informed consent was provided and accordingly blood sample (5 mL) was collected from patients on different days of infections. The samples were collected on day 4, 6, 8 and 10 of fever from the admitted patients. Total number of Dengue patients enrolled at AIIMS for this study was 46 with average age of 38 years. Out of 46, only 19 patients were followed up for all day-points for sample collection. All the nineteen patients were found to be NS1 positive and the other three patients had high DV specific IgM level. Remaining patients had either withdrawn participation from study due to various associated medical conditions or citing other reasons. Surface markers for platelet activation such as CD62-P, PAC-1, AnnexinV and PMPs (microparticles released from platelets) were estimated from the collected samples. Total RNA was isolated from patients’ platelet pellet and DV viral genome copies were measured.

Inclusion Criteria: Dengue patients were enrolled at Medicine Department, AIIMS, New Delhi, India. All Dengue patients were recruited following given below inclusion and exclusion criteria:

- Age group between 18-65 years.
- Confirmation of presence of NS1 antigen or DV specific IgM in blood circulation.
- Presence of clinical symptoms like low platelet count, fever, viremia.
• Only Paracetamol was administered for fever management.

**Exclusion criteria:** In the present study patients were excluded who were having any of the following:

• Age group below 18 years
• Blood/plasma/platelets transfusion within 2 weeks
• Use of anti-thrombotic drugs on a regular basis like Aspirin and other NSAIDs.
• Multiple infections like AIDS and Hepatitis C
• Severe life-threatening problems like cardiovascular diseases and COPD.
• Expectant mothers.

### 4A.2.2b Healthy individuals (Control samples)

Blood samples were also collected from 10 healthy individuals after taking informed consent. All the above platelet activation parameters were estimated which served as normal reference.

**Inclusion Criteria:** The study will include healthy volunteers both male and female, age ranging from 18-65 years.

**Exclusion criteria:** In the current study individuals with the following were not included -

• Prior history of a Dengue infection in the recent past.
• Individuals with regular use of anti-platelet agents like aspirin and clopidogrel
• History of intake of non-steroidal anti-inflammatory agents (NSAIDs)
• Known inflammatory/infective disease
• Coexisting conditions: diabetes, cardiovascular complications, cerebrovascular disease and associated complexities.
• Expectant mothers
4A.2.3 Platelet count of Dengue patients

Platelet count of blood samples collected on different days of Dengue infection were estimated using an automated cell counter (Sysmex XT-1800i from Diamond Diagnostics, USA).

4A.2.4 Platelet surface markers estimation using Flow cytometry

The expression of P-selectin (CD62-P) and phosphatidylserine (PS), and PAC1 binding to platelet surface was estimated by taking flow cytometry based approach. Briefly, within 2-3 hours of blood sample collection from healthy individuals and Dengue patients, the whole blood was centrifuged at 500rpm for 15 min without applying break at room temperature. The upper turbid fraction after centrifugation containing platelets and plasma referred to as platelet rich plasma (PRP) was collected. The PRP isolated from healthy individuals and Dengue patients was diluted with Tyrode’s buffer (pH-7.2) and stained by incubating either with FITC conjugated anti-P-selectin antibody, or PAC1 antibody or AnnexinV V antibody at a dilution of 1:50, 1:100 and 1:50 respectively for 30 min at 37ºC. Following incubation, platelet activation was measured using flow cytometer (BD FACSVerse). The percentage of FITC positive platelets was recorded.

4A.2.5 Estimation of Dengue virus genome copies in platelets of Dengue patients

The RNA was isolated from washed platelet pellet collected from patients on different days of fever (day 4, 6 and 8) with the help of TRIzol reagent. Total 2μg RNA was used for cDNA synthesis. For each qPCR reaction (10μl volume), 200nM of forward primer, 300nM reverse primer, 250nM probe and 1.6μL of cDNA of each sample was used along with the universal PCR master mix (TaqMan).
The qPCR conditions were amplification at 50°C for 2 min then denaturation at 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. DV genome copies detection primer pair used are forward primer 5’-GARAGACCAGAGATCCTGCTGTCT-3’ and reverse primer 5’-ACCATTCCATTTCTGGCGTT-3’.

4A.2.6 Statistical analysis

Mann Whitney U test was used for comparison between healthy individuals and Dengue patients and data was presented as median with interquartile range. When three or more groups were compared, two-way ANOVA was used followed by the Bonferroni post hoc test. Spearman correlation analysis was done wherever needed. The p-value less than 0.05 was considered as statistically significant. Graph Pad Prism 5.0 software was used for data analysis.

4A.3 Results

4A.3.1. Low platelet count on early days of infection

In order to determine the correlation between thrombocytopenia and activation status of platelets in Dengue patients, we first measured the platelet counts of the patients sample collected on various days of fever i.e. day 4, day 6, day 8 and day 1. On day 4 of fever, it was observed that the platelet count was very low with an average <5000/μL which recovered to the normal range (average >170000/μL) on day 10 of fever. (Fig 4A.1)
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Figure 4A.1: Platelet count at different day points in Dengue patients. Platelet count was estimated from samples collected on different day point (day 4, 6, 8 and 10) of infection from Dengue patients (n=19) and healthy individuals (n=10) using automated cell counter. Each dot represents each individual. Low platelet count was low on day 4 and recovered towards normal level on day 10. ***p<0.0007, ns=nonsignificant.

4A.3.2. Assessment of platelet activation and apoptosis marker in Dengue patients

We examined the platelet activation states of samples collected from Dengue patients and also from healthy individuals. We measured the surface expression of P-selectin and PAC-1 binding as platelet activation marker and annexinV binding on surface of platelets as an apoptotic marker. We then observed a higher percentage of P-selectin (Fig. 4A.2A&2B), PAC-1 binding (Fig. 4A.2C&2D) and PS (high annexinV binding) (Fig. 4A.2E&2F) positive platelets in Dengue patients as compared to healthy individuals. Platelets were gated on the basis of FSC and SSC parameters at log scale.
Figure 4A.2 Assessment of platelet activation. Level of platelet activation as assessed by the platelet surface P-selectin and activation of GPIIbIIIa in healthy individuals and Dengue patients. P-selectin FITC, PAC-1 FITC and AnnexinV FITC labelling was done respectively using flow cytometry. The representative dot plots and histograms were presented as the forward scatter or number of events versus platelet P-selectin (A) PAC-1 binding (B) respectively. [(a), (c) (e) Healthy individuals, (b), (d) (f) Dengue patient].

4A.3.3. High platelet activation and apoptosis in Dengue patients during early days of infection

To elucidate the close association between the reductions in platelet count with the platelet activation stage, we measured activation markers like P-selectin, binding of PAC1 and PS exposure on platelet surface at various days of infection. In contrast to the platelet count, all three platelet activation markers displayed increased values on
day 4 and 6, which reduced to lower level on day 10 (Fig. 4A.3A-C).

**Figure 4A.3: Platelet activation on different day points in Dengue patients.** Platelet activation markers like P selectin (Fig. 4A.3A), PAC-1 binding (Fig. 4A.3B) and PS expression (Fig. 4A.3C) were measured from samples collected on different day point (day 4, 6, 8 and 10) of infection from Dengue patients (n=19) and healthy individuals (n=10). All three parameters displayed increased level on day 4 and reduced further on day 10 (**p<0.0001, **p<0.001).

**4A.3.4. High genome copies of Dengue virus existed in platelet’s pellet of Dengue patients at early days of infection**

Next, estimation of DV genome copies in platelet pellets collected on different days of fever was done, and high DV genome copy number was found to be existing on day 4 which was reduced further on day 6 and day 8 which is directly correlated with platelet activation parameters (Fig. 4A.4).
Figure 4A.4: DV genome copies in platelets from Dengue patients at different day points. Dengue virus genome copies were measured platelets of Dengue patients (n=19) and healthy individuals (n=10). The DV copy number was higher in platelets on day 4 was further reduced on day 6 and day 8 (**p<0.0001, *p<0.0414).

4A.3.5. Inverse correlation existed between platelet activation parameters and total platelet count in Dengue patients.

We analyzed the correlation between platelet count and platelet activation markers like P-selectin and PAC-1 binding and found inverse relationship between the two parameters. The correlation coefficient (r) between Platelet count and P-selectin was r= -0.807, Platelet count and PAC-1 was r= -0.812, and Platelet count and PS was r= 0.741 indicating that higher the activation level of the platelet, lower is the platelet count in patients (Fig. 4A.5A-C).
Figure 4A.5: Correlation analysis between platelet count and platelet activation markers in Dengue patients. Correlation analysis between platelet counts (as in Fig. 4A.1) and P-selectin expression on platelets (mentioned in Fig. 4A.3A) (5A), PAC-1 binding to platelets (mentioned in Fig. 4A.2B) (5B), PS expression on platelets (mentioned in Fig. 4A.3C) (5C) [Ojha A. et al., 2017 Sci.Rep.]

4A.3.6. Direct correlation existed between high viral genome copies and platelet activation stage.

The DV genome copy numbers showed direct correlation with platelet P-selectin expression, r = 0.601 on all the above days, with PAC-1 binding on all days (r = 0.372); with PS exposure on all days (r = 0.238) (Fig. 4A.6). The analysis revealed
that DV viral load inside platelets was directly correlated with the platelet activation level showing that higher is the activation level of platelets, higher will be the viral load inside it and vice versa.

Figure 4A.6: Correlation analysis between DV genome copies and platelet activation in Dengue patients. Correlation analysis between the DV genome copies (as mentioned in Fig. 4A.4) and P-selectin expression on platelets (mentioned in Fig. 4A.3A) (6A), PAC-1 binding to platelets (mentioned in Fig. 4A.3B) (6B), PS expression on platelets (mentioned in Fig. 4A.3C) (6C) [Ojha A. et al., 2017 Sci.Rep.]

4A.5 Discussion

Platelet activation and thrombocytopenia are very common clinical manifestations in
Dengue [43, 65, 78, 93]. Decrease in platelet count can occur by various mechanisms which ultimately create a haemostatic imbalance in patients leading to haemorrhagic conditions accompanied by thrombosis, aberrant clotting, multiple organ failures and consequently death [11-13, 71-73]. To date, no studies have clearly described the direct correlation between platelet activation and thrombocytopenia. More specifically, how activated-platelets contribute to sudden drop in platelet counts in patients during Dengue infection.

To address the relationship among the platelet activation status with thrombocytopenia in Dengue infection, we measured platelet profiles from peripheral blood of patients on different days of infection i.e. day 4, day 6, day 8 and day 10. We investigated and measured the number of platelets as well as the markers for platelet activation / apoptosis including P-selectin, PAC-1 binding and PS expression on platelets. In our study, we observed higher level of surface P-selectin and high expression of phosphatidylserine (PS) on platelets of Dengue patients on early days of infection (Figure 4A.3A & C). P-selectin, the α-granules releasate from activated platelets is a well-studied platelet activation marker both in its membrane-bound form or soluble form and PS is exposed on highly procoagulant cells which undergo spontaneous apoptosis. In agreement with our finding, one study described the association between acute Dengue infection and platelet activation with elevated expression of platelet-activation markers like αIIbβ3, CD63 and P-selectin. In another study, P-selectin level in plasma was found to be considerably increased in patients with thrombotic thrombocytopenic purpura (TTP) [78]. In another study, the percentage of phosphatidylserine (PS)-bearing platelets was higher in Dengue patients compared to healthy individuals [79].
Our data also show that increased binding of PAC-1 is observed in Dengue patients indicating platelet activation. Activation of platelets has been associated with its clearance from the circulation. There are several studies which have reported increased PAC-1 binding to integrin GPIIbIIIa as an established parameter for assessing activation of GPIIbIIIa [77-79, 176]. Several lines of evidence indicate that PAC-1 is a specific monoclonal conformation dependent antibody that binds to only activated form of GPIIbIIIa complex [77, 210].

We investigated the correlation between number of platelets along with markers of platelet activation / apoptosis including P-selectin expression, PS exposure and PAC-1 binding on platelets. The low number of platelets with a mean value of <50000/μL on day 4 of Dengue fever, was rescued to normal count with a mean of >170000/μL on day 10 of infection; the platelet count from normal individuals was measured as reference with a mean of 1950000/μL. In contrast, all the platelet activation parameters including P-selectin expression, PAC-1 binding, PS-exposure and platelet derived microparticle (MPs) in plasma showed elevated level on days 4 and 6 of infection, which were reduced to lower level on day 10. The platelets counts clearly showed an inverse correlation with platelet activation as depicted by values between counts vs. P-selectin expression on various days of Dengue fever in patients. Altogether these findings suggest that there exists a fine link between the platelet activation level and drop in platelet count. The high activation and procoagulant state of platelets in Dengue patients thus suggest the crucial role of platelets in developing prothrombotic complications in these patients.

Recently it has been shown that DV can infect and replicate in platelets [211]. Therefore, in order to find the association between platelet activation and viral load inside them we estimated the viral genome copies inside the patient’s platelet.
It was observed that there were high copies of DV genome in platelets on day 3 of infection which reached below the detection limit on days 6-8. The DV genome load in platelets pellet showed a direct correlation with platelet activation markers such as P-selectin ($r = 0.601$). Cumulatively these observations indicate that platelet activation plays a crucial role in determining disease severity and pathogenesis. Higher the level of activation of platelets, the higher is the chance of an increased DV content inside them. Our results, in Dengue patients combined with published findings from other viral diseases where platelet activation occurs may provide a crucial link between thrombocytopenia and platelet activation and further raise the possibility of exploring the role of activated platelets in modulation of disease severity.

**4A.6 Conclusion**

In line with earlier findings our work describes in detail that platelets exist in active state. Our work clearly demonstrates that elevated level of activation markers exist on platelet surfaces (P-selectin and PS-exposure, and PAC-1 binding) during early days of infection (day 4 and day 6) which is directly correlated with high genome copies of DV in platelets pellet and inversely correlated with low platelet counts during early days of infection (such as day 4 and day 6) in Dengue infected patients. The number of platelets is correlated with less activation of platelets as well as non-detectable virus genome in platelets on day 10 of infection. Low platelet numbers on day 4 and day 8 and recovery on day 10 of infection clearly indicate the fine association between elevated platelet activation and decreased platelet count in Dengue patients.
CHAPTER 4

Role of platelet activation in thrombocytopenia in Dengue infection

CHAPTER 4B: Correlation between platelet activation and antibody-complement mediated platelet lysis during Dengue infection in patients.
4B.1 Introduction

The pathogenesis of Dengue infection is multifactorial and complement activation is one of the major dysfunctions. The complement system is suggested to be involved in Dengue infection particularly in the instigation of vascular leakage [159-165]. Studies have shown that platelet activation leads to complement activation [142, 146]. *In vitro* studies done using different platelet agonists demonstrated that binding of C3 on the surface of platelet has increased upon cell activation. Activation of the complement system is characterized by increased generation of the anaphylotoxin C3a and the C5b-9 complex. This study identified P-selectin as a C3b-binding protein. P-selectin acted as the activator of the complement system, which is designated by increased in C3b deposition, C3a production, and C5b-9 complex formation [12].

The complement system once activated has been described to play a crucial role in DV infection either by protecting the host or by affecting disease pathogenesis [20]. Complement activation is mediated via any of the three known pathways namely, the classical, lectin and alternative pathways. Complement activation limits the viral infections by various mechanisms comprising of lysis of viral particles or destruction of the infected cells, by anaphylatoxins release, and inducing elevated T and B cell responses. The complement system works antagonistically in Dengue infection, either by controlling viral replication and protecting the host or by triggering an aggravated inflammatory response, increasing disease severity [220]. Most studies done on complement activation in Dengue have been focussed on patients with secondary DV infection and led to the conclusion that complement activation was mediated mainly via classical pathway. However, studies done on complement in children and infants experiencing primary DV infection implicated the involvement of alternative pathways in activating the complement system. It also revealed increased production of
complement split products correlated with increased fibrinogen levels and thrombocytopenia [218]. Moreover, numerous studies done using clinical and in vivo model systems, displayed that excessive utilization of complement proteins such as C3, factor B and C5 resulted in severe forms of the disease such as DHF and DSS, and increased levels of the products of complement activation (C3a, C5a) caused histamine release which ultimately leads to enhanced vascular permeability and vasodilatation in DV infections. Indeed, the level of anaphylatoxins in the blood of DHF and DSS patients correlated with symptoms of vascular leakage [22, 152, 154]. Furthermore, complement system also gets activated by anti-DV antibodies on the surface of endothelial cells resulting in formation of membrane attack complex (MAC) [25].

Ample experimental evidence suggests the complement activation as the leading cause of platelet activation and lysis. Reports also suggest that the activation of complement protein C3 followed by binding of MAC complex to platelet surface is significantly associated with platelet lysis and thrombocytopenia in these patients [26-28]. The C3a anaphylotoxin was described to induce activation and aggregation of platelets [14]. Also, incubation of platelets with sublytic concentrations of C5b-9 leads to transient membrane depolarization, granule secretion, release of platelet derived microparticles and apoptosis [132]. Further, the release of microparticles from activated platelets has been directly correlated with severity of thrombocytopenia by a patient based study [216]. In addition, platelet activation and apoptosis caused by complement mediated lysis leads to clearance of platelets from circulation leading to thrombocytopenia. Reports also described the pivotal role played by platelet-bound immunoglobulins having anti-DV activity in thrombocytopenia induction and disease severity in secondary Dengue infection [230, 231].
Although studies have explored the involvement of complement proteins in platelet lysis in DV infection, but we have investigated the association in detail between the activation statuses of platelets and their lysis and clearance by complement proteins leading to platelet destruction and thrombocytopenia in Dengue patients. We measured antibody-complement profiles from peripheral blood of patients on different days of infection i.e. day 4, day 6, day 8 and day 10. We have also examined the platelet lysis and release of microparticles from platelets into circulation of these patients.

4B.2 Materials and Methods

4B.2.1 Materials
Fluorescent conjugated antibodies such as anti-human C3-FITC and IgG-PE were purchased from BD Biosciences (San Jose, USA). Majority of other laboratory chemicals including BSA and sepharose CL-2B beads for gel filtration chromatography were purchased from Sigma-Aldrich (St. Louis, USA).

4B.2.2 Patients samples
The Dengue patient samples were collected on different days of infection as mentioned in detail previously in method section 4A.3.2a.

4B.2.3 Estimation of platelet count of Dengue patients
Dengue patients platelet counts on different days of infection were measured from whole blood as mentioned previously in section 4A.3.3.

4B.2.4 Estimation of C3 binding over the platelet surface during different days of infection
PRP isolated from patient samples was diluted in modified Tyrode’s Buffer (pH=7.2), followed by staining with fluorescent labelled antibodies against C3 complex for 30 minutes at 37°C for determining platelet activation. Each sample was subsequently
analyzed by flow cytometer and the percentage of FITC positive platelets was recorded.

4B.2.5 Estimation of IgG binding over the platelet surface on different days of infection

Blood from healthy individuals and Dengue patients were collected and centrifuged at 500rpm for 15 min without brake at room temperature. PRP was collected and processed as earlier described for estimation of P-selectin. The percentage of PE positive platelets was measured.

4B.2.6 Estimation of platelet derived MPs in Dengue patients

Citrate blood samples were taken from healthy controls and Dengue patients. Platelet Poor Plasma was separated by centrifuging the whole blood at 1500×g for 20 mins, followed by a second centrifugation at 15,000×g for 2 min which gives Platelet free plasma (PFP). The samples were incubated with platelet specific anti-CD41a-PE conjugated antibody at 37°C for 30 min followed by fixation with 1%PFA for 30 min and quantified using flow cytometry.

4A.2.7 Statistical analysis

Mann Whitney U test was used for comparison between healthy individuals and Dengue patients and data was presented as median with interquartile range. When three or more groups were compared, two-way ANOVA was used followed by the Bonferroni post hoc-test. Spearman correlation analysis was used wherever needed. The p-value less than 0.05 were considered as statistically significant. Graph Pad Prism 6.0 software was used for data analysis.

4B.3 Results

4B.3.1. Low platelet count on early days of infection in patients

In order to determine the correlation between thrombocytopenia and activation status
of platelets in Dengue patients, we first measured the platelet counts of the patients sample collected on various days of fever i.e. day 4, day 6, day 8 and day 1. On day 4 of fever, we observed that the platelet count was very low with an average <5000/μL which recovered to the normal range (average >170000/μL) on day 10 of fever. (Fig 4B.1)

![Platelet count on different days of infection in patients.](image)

**Figure 4B.1: Platelet count on different days of infection in patients.** The platelet count were estimated from samples collected on different day point (day 4, 6, 8 and 10) of infection from Dengue patients \(n=19\) and healthy individuals \(n=10\) using automated cell counter. Each dot represents each individual (**p<0.0001, ns=nonsignificant**).

### 4B.3.2. Assessment of C3-binding and IgG binding on the platelets collected from Dengue patients.

We have estimated the binding of complement factor C3 and IgG on the platelet surface in patients on different days of fever by using fluorescence conjugated antiC3FITC and anti-IgG-PE antibodies respectively using flow cytometry. We found increased binding of both C3 and IgG to platelets surface isolated from Dengue patients (Fig 4B.2B, D) as compared with the healthy individuals (Fig 4B.2A, C).
Figure 4B.2: Flow Cytometry gating strategy for estimating binding of C3 and IgG on platelets: Platelets from patients (B, D) and healthy controls (A, C) labelled with fluorescent antibodies for C3 and IgG were analysed using flow cytometer. Platelets from were gated using FSC and SSC characteristics.

4B.3.3. Increased level of C3-binding and IgG binding existed on the patients platelets surface during early days of infection.

We estimated the binding of C3 and IgG on the platelet surface in patients on different days of fever. Data shows the increased binding of both C3 and IgG onto the platelets when platelets are present at active conditions on day 4 and 6, which was further reduced on day 10 (Fig. 4B.3A, B) in patients.
Figure 4B.3: Increased complement factor C3 and IgG binding on platelets in Dengue patients during different days of infection: C3 binding (Fig. 4B.3A) and IgG binding (Fig. 4B.3B) estimated by flow cytometry as mentioned in Fig.4B.2. Increased levels of both C3 and IgG at day4 observed while decreased levels at day10 were observed (**p<0.0004, *p<0.001).

4B.3.4. Assessment of platelet derived microparticles in Dengue patients.

Platelet activation is accompanied by the release of MPs, which are the heterogeneous population of vesicles (< 1µM) (21), generated from the plasma membrane upon platelet activation or apoptosis by various stimuli. We first evaluated the size of MPs in platelet suspension relative to 0.5 and 1.7-2.2 µM diameter fluorescent beads or liposomes of size varying between 0.3-0.9 µM. MPs were distinguished from platelets on the basis of size. Briefly, the SSC/FSC (side scatter/forward light scatter) representation of activated platelets showed 2 populations: (1) a major population, corresponding to platelets (gated as P12), and (2) a smaller second population, corresponding to the MP fraction (gated as P6). This second population was absent in platelets under resting conditions (Fig. 4B.4A). Based on fluorescence dot plot, the percentage of MPs generated (Fig 4B.4B) from patient platelets were higher (40%) than that released from control platelets (Fig 4B.4C).
Figure 4B.4: Assessment of platelet derived microparticle from Dengue patient and controls. Resting or activated human platelets from control and patients were incubated with anti-hCD41 antibody for 30 min and analyzed for MP release using flow cytometry. (a),(b)&(c) SSC vs FSC dot plots showing the light scatter characteristics of the gated events from platelets.

4B.3.5. High number of platelet derived microparticles existed at early days of infection in Dengue patients.

Platelets, upon complement mediated activation, undergo apoptosis or get lysed and release microparticles. The release of platelet derived microparticles on different days of infection (day 4, 6, 8 and 10) from patient's plasma was estimated by using flow cytometry (Fig.4B.5A) and increased release of microparticle was found on the early
days of infection (day 4, 6 and 8) which reduced to normal level on later days of infection (day 10).

**Figure 4B.5: Platelet derived microparticle release at different day points in Dengue patients.** Patient’s plasma samples were labelled with anti-CD41a antibody to label the platelet-derived microparticles (MPs). The percent of CD41a positive population was estimated and segregated as MPs on FSC and SSC characteristics. MPs were released in highest amount on day 4 and lowest on day 10 in patients. (**p<0.0001).

**4B.3.6. Inverse correlation existed between complement mediated lysis and platelet counts in Dengue patients.**

The association between platelet counts vs. platelet derived microparticle release during different days of fever is estimated by performing Spearman correlation analysis. (r = -0.8075) An inverse correlation (r = -0.80) was found with platelet count data indicating that higher is the complement mediated lysis and release of microparticle from the platelets, lower is the platelet count in patients (Fig. 4B.6).
Figure 4B.6: Correlation analysis between platelet count and platelet lysis in Dengue patients. Correlation analysis between platelet counts (as in Fig.4B.1) and platelets derived microparticle release (mentioned in Fig. 4B.5).[Ojha A. et al., 2017 Sci.Rep]

4B.4 Discussion

Studies have shown that several proteins of the complement pathway interact with platelets, activate them and promote thrombosis. [13-15, 205, 214]. Studies have also shown that the reverse is also true: activated platelets can also mediate activation of the complement system [31]. Studies have also shown that mitochondrial dysfunction occurs in platelet which leads to its apoptosis and clearance from circulation [32]. An increased level of platelet associated IgG i.e. PAIgG is detected regularly in chronic idiopathic thrombocytopenic purpura (ITP) patients, is also present in various other diseases [33-35]. Although virus-mediated ITP like human herpes virus infection has been studied [36-38], but the association between elevated level of PAIgG and PAIgG-mediated thrombocytopenia has been poorly understood in case of Dengue infection. Therefore in order to elucidate the mechanism of association between the activation status of platelets, their lysis and clearance by antibody-complement mediated lysis, and thrombocytopenia in Dengue infection, we measured antibody-complement profiles from peripheral blood of patients on different days of infection i.e. day 4, day
6, day 8 and day 10. We also checked the release of platelet derived microparticles in circulation of the patients as platelets upon activation release microparticles in external milieu and then undergo apoptosis. Other studies have demonstrated the presence of active complement pathway proteins in circulation during Dengue infection which increases disease severity [39, 217]. On the other hand, one recent study [25] described the dual role of complement system in protection against as well as pathogenesis of DV infection. This study illustrated the mannose binding lectin (MBL) pathway can mediate neutralization of DV. Deficiency in MBL level or activity due to host polymorphisms in the MBL2 gene is associated with decreased level of DV neutralization. A study recently has demonstrated the inverse correlation between elevated platelet-associated IgG (PAIgG) and platelet counts during the acute phase of secondary Dengue infections (40). The findings depict that formation of PAIgG involving anti-DV IgG has indispensable role in inducing thrombocytopenia in secondary infections. As thrombocytopenia is more prominent in acute cases like DHF than classical fever [3], they hypothesized that the increased level of platelet bound IgG might be involved in determining the disease severity as well as thrombocytopenia.

In line with above findings we also observed higher binding of IgG and C3 on platelets during day 4 and 6 of fever. The levels of C3 and IgG were reduced on day 10 of infection. As mentioned above that the platelet counts were low on days 3-4 of fever in patients and recovered to normal counts on day 10. The levels of bound C3/IgG on platelet surface were higher at day 3-4 and low at day 10, clearly indicating the probable mechanism for complement mediated lysis and clearance of IgG-bound circulating platelets by phagocytes in patients.
4B.5 Conclusion

It is well reported that complement protein (C3) and Immunoglobulin (IgG) can bind to the activated platelets surface and promote lysis and clearance of platelets from circulation and upon lysis platelet release microparticles in extracellular milieu which is used as biomarker of platelet activation and lysis. We estimated the levels of bound C3 or IgG on platelets collected from peripheral blood of patients on different days of infection. Increased levels of C3 and IgG on platelet surface correlated with elevated platelet activation but low platelet count on days 3-4 of fever and the decreased levels of C3 and IgG correlated with low platelet activation but normal platelet count on day 10 of infection in Dengue patients.