Synopsis of the PhD work:

Dengue virus (DV) infects millions of people around the world and causes significant morbidity and mortality every year. Dengue pathogenesis causes several clinical complications including thrombocytopenia or platelet counts drop. Although many studies have investigated the mechanism of platelet clearance in Dengue infection, a clear insight into the pathogenesis of event is unclear. We have investigated the role of platelet activation in Dengue pathogenesis. We have explored in detail the mechanism and correlation between platelet activation and thrombocytopenia in Dengue infection. We have evaluated platelet profile in peripheral blood of patients during different days of infection (day 4, day 6, day 8 and day 10) along with platelet activation / apoptotic markers including P-selectin, PS expression and PAC1 binding on platelets. Elevated level of activation markers on platelet surface (P-selectin, PAC1 binding, PS-exposure) on early days of infection (day 4 and day 6) directly correlated with high genome copies of Dengue virus (DV) in platelets, and inversely correlated with low platelet count during early days of infection (day 4 and day 6) in Dengue infected patients. The number of platelets correlated with lower 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 of infection was restored to normal count by day 10 in Dengue patients. It indicated a clear association between platelet activation status and platelet count. These findings, mentioned in detail in chapter 4A of the present thesis, are a part of our published work in Scientific Reports, 2017. Since complement protein (C3) and Immunoglobulin (IgG) can bind to the surface of activated platelets and promote lysis and clearance of platelets from circulation, we have assessed the correlation between platelet activation and antibody and complement profiles and platelet lysis in dengue infection by estimating the level of bound C3 and IgG on platelets isolated from peripheral blood of patients on different
days of infection. We found increased level of C3 and IgG on platelet surface correlating with elevated platelet activation but low platelet count during day 3-4 of fever, and decreased level of C3 and IgG correlated with low platelet activation but normal platelet count on day 10 of infection in Dengue patients. The above finding is mentioned in chapter 4B of the present thesis and is a part of our published work in *Scientific Reports*, 2017. The findings from patient study clearly indicated a direct association between platelet activation and depletion of platelets from peripheral circulation.

Chapter 5 of the present thesis provides mechanistic insight into molecular events associated with Dengue virus induced platelet activation and lysis *in vitro*. We found that various MOIs of DV2 (ranging from 1.6 to 5), triggered platelet activation in a dose-dependent manner. The phosphorylation of signalling adaptor kinases including ERK and elevated expression of activation markers such as P-selectin, PAC1 binding in platelets were observed following treatment with different MOI of DV2. The study also demonstrated that the platelet activation inhibitor such as prostacyclin (PGI2) hindered significantly the DV2-induced activation of platelets. We also describe that the activation of signalling proteins involved in apoptotic pathway including caspase 9 and cyclophilin D as well as surface PS expression on platelets increased in MOI dependent manner following DV2 treatment. Further, we investigated the specific role of Dengue virus in platelet activation. We observed that the Japanese Encephalitis virus (JEV), another member of Flaviviridae family did not activate platelets in a MOI-dependent manner. We also elucidated the mechanism of DV-mediated platelet lysis, by checking the C3 and IgG binding on platelet surface *in vitro*. We found increased binding of both C3 and IgG on platelets incubated with DV2 in MOI dependent manner and the binding was abrogated by platelet inhibitor, PGI2 (50-100
ng/mL). We also showed that the DV2 treatment induced platelet lysis and as a result generated platelet-derived microparticles (MPs) in a MOI-dependent manner. DV2 mediated MP generation from platelets was abrogated by PGI₂. DV2 treatment to whole blood increased the adhesion of platelet and clot formation on endothelium monolayer in vitro. DV2 treatment to platelets increased the phagocytosis of platelets by monocytes (isolated from same healthy individuals) in vitro. These observations thus suggested the mechanism underlying depletion of platelets from circulation in patients with Dengue infection. This work is published in Scientific Reports, 2017. The above findings which are mentioned in details in chapters 4A, 4B and 5 of the present thesis are summarized in the schematic Figure-1, as shown below.

Figure.1.1. Schematic showing mechanism behind DV2-induced platelet activation and thrombocytopenia. DV2 induced platelet activation and apoptosis, and in turn induced mechanism of platelet destruction and clearance such as C3/IgG-mediated
lysis and clearance, platelet clot formation, etc. Further the inhibition of platelet activation by PGI2 reduced the DV2-mediated platelet destruction.

We further investigated the role of platelet activation in Dengue virus replication and propagation. Since it is reported that viral replication as well as maximum platelet activation occur simultaneously within 3-4 days of fever, we examined whether DV replication and propagation in monocytes differ upon engulfment of DV activated platelets or DV alone? We found that elevated replication of DV2 occurs in monocytes in the presence of either supernatant of activated platelets. We found one of the platelet chemokine i.e. Platelet factor 4 (PF4) at a concentration of 100 ng/mL to be increasing the DV2 replication in monocytes by 3-4 folds.

We validated the role of PF4 by using neutralizing antibody against PF4 or CXCR3 antagonists (PF4 receptor) and found that the use of neutralizing antibody against PF4 or CXCR3 antagonists (PF4 receptor) completely diminished PF4-mediated DV2 replication in monocytes in vitro. We further investigated the molecular mechanism behind this PF4 mediated elevated replication of DV2 in monocytes and found that PF4 is regulating the host cell anti-viral response pathway.

PF4 inhibits phosphorylation of p38MAPK and in turn decreases activity of STAT-2 and IRF-9 and finally decreases IFNα secretion in monocytes. We corroborated this finding in patients and found an elevated plasma PF4 correlated directly with high replication and propagation of DV in patients (n=20) during early days (day 3-5) of fever. The low viral replication was concomitant with low PF4 in plasma of patients on day 9 after infection. These observations together indicated a direct association between plasma PF4 and DV replication in Dengue patients. This work is published in *EBioMedicine*, 2019. The schematics below represent how platelet activation and thereby increased PF4 level promotes DV replication and propagation and the
associated mechanism.

Figure 1.2 A) Schematic showing the PF4 mediated elevated DV replication. Use of CXCR3 antagonist abrogated the PF4-mediated elevated DV replication. B) PF4 induces rapid replication of DV by targeting the anti-viral response pathway: by increasing the phosphorylation of p38MAPK and in turn decreasing activity of STAT2 and IRF-9 and less synthesis and secretion of IFNα in monocytes.

Therefore, our study provides detailed insight into the role of platelet activation in Dengue pathogenesis, specifically showing that platelet activation determines the outcome of the disease and platelet factors released upon activation, especially platelet factor 4 (PF4) plays crucial role in promoting DV replication and propagation. Inactivation of platelets limits the destruction and clearance of these cells, suggesting careful treatment with platelet-inactivating drugs may play important role in rescuing platelets from DV infection. Inhibition of PF4-CXCR3-IFNα axis abrogates DV propagation suggesting the potential target for developing therapeutics against this Flavivirus.
Major part of above work is published in following articles:

**Original articles**

