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Different aspects of platelet evaluation in dengue: Measurement of circulating mediators, ability to interact with the virus, the degree of activation and quantification of intraplatelet protein content

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ABSTRACT

Platelets play a role in hemostasis, coagulation, angiogenesis, inflammation and immune response is one of the most affected cells in dengue. Here we describe some aspects of platelets by observing their specific circulating mediators, the ability to interact with the virus and morphological consequences of this interaction, activation markers and intraplatelet protein contents in dengue. We conducted this study using dengue-patients as well as healthy donors. Immunoenzymatic assay, flow cytometry, transmission electron microscopy and intraplatelet proteins expression assays were carried out. Briefly, we found an increase in sCD62L, NO or TBX2 ratio in platelet count, mostly in patients with the worse clinical outcome. After in vitro DENV infection or during natural infection, platelets underwent morphological alteration with increased expression of platelet activation markers, particularly in natural infections. Analysis of intraplatelet protein contents revealed different angiogenic and inflammatory profiles, maintaining or not extracellular matrix integrity between DF and DFWS patients. Thus, platelets are frequently affected by dengue, either by altering their own functionality, as "carrier" of the virus, or as an antiviral and mediator-secreting effector cell. Thus, strategies aimed at recovering platelet amounts in dengue seem to be essential for a better clinical outcome of the patients.

1. Introduction

Dengue is an arbovirus spread by mosquitoes of genus *Aedes* in human-mosquito-human cycle. It is endemic in more than 120 countries, where 55% of the world population live at risk of infection. Therefore, dengue offers the greatest impact on public health worldwide with higher morbidity, albeit fortunately with low mortality rate (TDR/WHO, 2009). Dengue is usually an acute febrile disease of a broad spectrum of clinical manifestations ranging from a clinically unapparent infection in the form of an undifferentiated disease to severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DF and DHF are indistinguishable in early stages, though DHF is commonly associated with important hemorrhagic manifestations, plasma leakage and thrombocytopenia. However, thrombocytopenia is not necessarily restricted to severe forms of dengue fever, as small bleeds are observed in mild forms (Simmons et al., 2012; Bäck

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Table 1
Demographic and clinical-laboratory characteristics of the study population from Brazil in 2010 to 2013 outbreaks.

Characteristics	Health donors$^{n=10}$	DF$^{n=53}$	DFWS$^{n=31}$	Severe$^{n=15}$
Age, years	38 (25 - 30)	40 (26 - 54)	42 (24 - 54)	40 (20 - 51)
Gender, Female:Male	29:24	29:24	21:10	7:8
Post-infection days	nd	4 (2 - 6)	3 (2 - 4)	4 (3 - 6)
Bleeding[a]	nd	32%	65%	73%
Plasma leakage[b]	nd	8%	27%	60%
Leukocyte counts (mm^{-3})	5820 (5414-6123)	3450 (2608- 5250)*	3425 (2510- 4075)*	3900 (2680- 4855)*
Monocyte counts (mm^{-3})	386 (324-635)	313.2 (169- 607.7)	292.7 (112.6- 373)	572 (248- 759.9)$^{@}$
Lymphocyte counts (mm^{-3})	1780 (1511-2156)	1100 (648- 1787)	1337 (1112- 2231)$^{\$}$	2307 (1365- 3374)$^{!!!}$
AST/TGO (UI/L)	15 (13-20)	45 (27- 99)**	57 (36.5- 120)$^{\#\#}$	95 (70- 221)$^{\&\&!}$
ALT/TGP (UI/L)	14 (13-23)	43 (29- 82)**	51 (27.5- 129.5)$^{\#\#}$	88 (66- 195)$^{\&\&!}$
Platelet counts (x 10^3 mm^{-3})	276 (247.5-314.3)	143 (85.8- 189.8)**	96 (50- 152.5)$^{\#\#\$}$	21.5 (13.8- 50)$^{\&\&@@!!!!}$
NS1 positive	nd	38%	40%	57%
IgM positive	nd	77%	92%	100%
IgG positive	nd	79%	100%	80%
PCR positive	nd	52%	52%	31%
DENV-1		39%	60%	0%
DENV-2		48%	40%	100%
DENV-4		13%	0%	0%

Data are expressed as median (interquartile range, 25% to 75% with 95% C.I confidence interval) or number (%).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; TGO, glutamicoxalacetic transaminase; TGP, glutamic-pyruvic transaminase; nd, not determined. Mann-Whitney test in which *DF *vs* Healthy Control; $^{\#}$DFWS *vs* Healthy Control; $^{\&}$Sev *vs* Healthy Control; $^{\$}$DF *vs* DFWS; $^{!}$DF *vs* Sev; $^{@}$DFWS *vs* Sev. The significant P value is represented by a symbol when $p < 0.05$, two symbols $p < 0.01$, three symbols $p < 0.001$ and four symbols, $p < 0.0001$.
[a] Gingival bleed, vaginal bleed, gastrointestinal bleed, petechiae and/or exanthema.
[b] Ascites, pleural or pericardial effusion, edema.

and Lundkvist, 2013). A simplified categorization for classification of dengue severity was proposed by the World Health Organization (WHO) in 2009, reducing the percentage of patients unclassifiable previously, thus facilitating clinical management of patients (TDR/WHO, 2009). This classification was based on a multicenter study that deals with the illness as a dynamic and systemic event, including dengue fever without (DF) or with warning signs (DFWS) and severe dengue (Barniol et al., 2011). Many viral factors, including viral load, serotype, and virulence affect DENV pathogenesis (Vaughn et al., 2000; Guilarde et al., 2008). Moreover, immunopathogenesis of DENV infection is caused by host-specific immune responses, including antibody-dependent enhancement (Halstead et al, 1970, 1980), T-cell activation of cross-reactive memory described by the original antigenic sin theory (Mongkolsapaya et al., 2003). All hypotheses, to some extent, induce overproduction or a skewed profile of cytokine release, giving rise to cytokine storm/cytokine tsunami term (Rothman, 2011). Platelets are one of the major cell populations affected by dengue virus by direct and/or indirect infection mechanisms. However, its role in pathogenesis of dengue remains poorly understood.

Thrombocytopenia occurs when platelet formation (thrombopoiesis) is insufficient to balance physiological or pathological platelet consumption (de Azeredo et al., 2015). Two main events are associated with thrombocytopenia namely, reduced proliferative capacity of hematopoietic cells in bone marrow and/or increased destruction/clearance of platelets from peripheral blood (Nakao et al., 1989; Basu et al, 2008). Several studies have contributed to the understanding of mechanisms related to thrombocytopenia in dengue. Thus, activation of complement factor C3 followed by binding of C5b-9 complex to platelet surface is significantly linked with platelets destruction and thrombocytopenia in dengue patients (Avirutnan et al., 1998; Lin et al., 2001). Furthermore, *ex vivo* reports described direct correlation between micro particles derived from activated platelets and an important degree of thrombocytopenia in dengue patients (Punyadee et al., 2015). Recently, platelets increased expression of C3 factor and IgG after activation by DENV-2, making them more susceptible to phagocytes by monocytes (Ojha et al., 2017).

Besides quantitative reduction, dysfunction of platelets is implicated in prothrombotic complications associated with severe cases (George, Lam 1997; Michels et al., 2014). Platelets contain several preformed molecules, large amounts of mRNA, and packaged translational process required to synthesize new biologically active proteins, including growth factors, cytokines, and chemokines, acting as key effector immune cells (Weyrich et al., 2003; Weyrich and Zimmerman, 2004). Moreover, platelets act inducing permeation of leukocytes and progenitor cells to infection site or vascular injury inflow, interacting with neutrophils, monocytes and lymphocytes for activation forming thus platelet-leukocyte aggregates that immobilize pathogens and prevent of spreading. Also, presence of platelet surface receptors involved with immune response, such as Toll-like receptors show platelet impact on immunological response (Trzeciak-Ryczek et al., 2013).

Our study focused on some aspects of platelets by observing their specific circulating mediators, the ability to interact with the virus and morphological consequences of this interaction, activation markers and intraplatelet protein contents in dengue. Taken together, platelets play a role in hosting DENV, resulting in its activation. This would empower act on angiogenesis, inflammation, coagulation and extracellular matrix regulators. Thus, strategies aimed at recovering quantities of platelets in dengue seem essential for a better clinical outcome of patients.

2. Methods

2.1. Study population

To obtain blood samples, approval was obtained from the Ethics Committee for Human Research, registered in Platform Brazil (CAAE. no. 13318113.7.3001.0021). Patients were enrolled at Rio-Laranjeiras Hospital, Rio de Janeiro, and at Universidade Federal de Mato Grosso do Sul, Professora Esterina Corsini Day Hospital, Mato Grosso do Sul, Brazil. Dengue patients had diagnosis confirmation based on positivity in at least one of the laboratory tests used. Cases of dengue were classified according to criteria established in 2009 by the World Health Organization (WHO), as Dengue Fever (DF); DF with Warning Signs (DFWS) and Severe Dengue (Sev) (TDR/WHO, 2009). The remaining non-confirmed patients formed the other febrile illnesses (OFI) group. We included 10 healthy controls (HC) without fever episodes or other diseases in the last three months before sample collection. All patients and donors are characterized in Table 1.

2.2. Diagnosis

Positive for dengue case was considered when diagnostic assays met one or more of the following criteria: (1) IgM detection in acute samples by DENV-specific capture IgM enzyme-linked immunosorbent assay (ELISA) (PanBio Diagnostics), (2) DENV RNA detection in acute-phase serum by a serotype-specific RT-PCR assay as described elsewhere (Lanciotti et al., 1992), (3) DENV isolation in acute samples by inoculation onto *Aedes albopictus* C6/36 cells and subsequent immunofluorescent detection of viral antigens as described previously (Igarashi, 1978), and (4) detection of non-structural 1 (NS1) protein using NS1 antigen strip (Bio-Rad, Hercules, CA) in acute serum following manufacturer's instructions. Patients with primary infection were considered positive for any of the tests: IgM, NS1 and/or serotype by RT-PCR with IgG negative. With IgG positive, rate was of IgM/IgG > 2,0 indicating primary infection. In cases of secondary infection, rate was of IgM/IgG < 2,0 (TDR/WHO, 2009).

2.3. ELISA and Bio-Plex assays

In plasma samples from DENV-infected patients and healthy controls, ELISA was used to quantify soluble CD62 P (R&D Systems, catalog # BBE06), NO (R&D Systems, catalog # KGE001) and, TBX_2 (R&D Systems, catalog # KGE011) in accordance with manufacturer´s instructions. Moreover, Bio-Plex Pro Human Cytokine Standard (Bio-Rad, catalog# M50-0KCAF0Y) was used to quantify serum PDGF-BB and VEGF in accordance with manufacturer´s instructions.

2.4. Preparation of DENV-2 stock, titration, and infection

Preparation of ultracentrifuged DENV-2 (Thai strain 16,681), kindly provided by Dr, S. B. Halstead (Naval Medical Research Center, Silver Spring, MD, USA), and determination of viral titers were described previously (Torrentes-Carvalho et al., 2009). Ultracentrifuged DENV-2 was concentrated 20-fold (from the initial supernatant volume) in RPMI with 10% FBS, filtered through a 0.22-μm pore-size membrane and stored at −80 °C. Viral titers were quantified by determining 50% tissue culture infectious dose per ml by Reed-Muench method using C6/36 cell line (Schoepp and Beaty, 1984). Platelets (10^6/ml) were incubated with DENV-2 diluted to 10-fold, for 2 h at 37 °C. Cells were then washed with serum-free RPMI and incubated for 3 h in complete medium (RPMI with 10% FBS). Prior to incubation, cells were recovered for flow cytometry staining or electron microscopy.

2.5. Isolation of platelets

Ten mL of blood samples were collected into K_2EDTA containing tubes (BD Vacutainer®, catalog # 367862) from enrolled patients and healthy donors. Blood samples were placed over equal volume of OptiPrep™ density gradients (Axis-Shield, Dundee, Scotland). OptiPrepTM reagent was diluted in 0.85% NaCl, 20 mM Hepes-NaOH, 1 mM EDTA solution, pH 7.4. This dilution allows obtaining solution with barrier of density = 1.063 g/mL, recommended for isolation of a highly purified fraction of platelets from whole blood. Following centrifugation at 350 *g* for 15 min at room temperature (RT), recovered platelets ring was obtained, washed, and resuspended in phosphate buffered saline (PBS) containing 300 μM Prostaglandin E1 (Cayman, Michigan, USA). Another centrifugation was prepared at 1000 *g* for 10 min at RT. Platelet pellet was resuspended in PBS and counting performed by direct method in Neubauer chamber using liquid Rees & Ecker (composition: 3.8 *g* of sodium citrate, 0.2 ml of 0.38% formaldehyde solution and 0.1 *g* of brilliant cresyl blue to 120 ml of distilled water). Every process of platelet isolation from whole blood lasted about 4 h. Potential contamination from other cell types was excluded as leukocyte count was minimal (< 0.01 × 10^6/ml). Plasma was collected, aliquoted and stored at −70 °C.

2.6. Measurements of platelets surface markers by flow cytometry

Platelet pellets were washed in 0.4% EDTA solution with centrifugation at 1000 *g* for 10 min at RT. Following, platelets were then incubated in ice for 40 min in darkness with fluorochrome-conjugated monoclonal antibodies and, washed twice with PBS. Platelets subpopulations (CD31 + cells) were enumerated by a four-color flow cytometry method using the following monoclonal antibodies: fluorescein (FITC) conjugated anti-CD31 (Southern Biotech, catalog # 9381-02), allophycocyanin (APC) conjugated anti-CD62 P (BioLegend, catalog # 304910), phycoerythrin cyanine dye (PE Cy5) conjugated anti-CD41 (BioLegend, catalog # 303707), phycoerythrin (PE) conjugated anti-CD40L (Southern Biotech, catalog # 9821-09) or, Alexa Fluor 546 conjugated anti-Fibrinogen (Molecular Probes, catalog # F-13192). Appropriate matched-isotype control antibodies were used to discriminate between positive and negative populations/subsets. A minimum of 3,000 platelets' gated events were acquired using Accuri™ C6 flow cytometer (BD Biosciences), and analysis was performed using Flow Jo v.7.6.1 software (Tree Star, Ashland,OR).

2.7. Platelets processing for transmission electron microscopy analysis

10^6 platelets from dengue patients or donors were processed and submitted to analysis by transmission electron microscopy (TEM). Platelet placed in microfuge tubes were fixed in 1% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 (ElectronMicroscopy Science), post-fixed in 1% buffered osmium tetroxide (Electron Microscopy Science), dehydrated in acetone (Merck), embedded in epoxy resin (Electron Microscopy Science) and polymerized at 60 °C over the course of three days (Barreto-Vieira et al., 2015; Barth et al., 2016). Ultrathin sections (50 ± 70 nm thick) were obtained from resin blocks. Sections were picked up using copper grids, stained with uranyl acetate and lead citrate (all ElectronMicroscopy Science) and observed using Zeiss EM-900 transmission electron microscope.

2.8. Intraplatelet proteins analysis

Human Angiogenesis Array Kit (R&D Systems, catalog# ARY007) was used to evaluate intraplatelet protein expression profiles of platelets lysates obtained from dengue patients. Following manufacturer's instructions, angiogenesis array data on developed X-ray film were quantified using Quantity One image analysis software (Version 4.6.3, Bio-Rad). A template analyze pixel density in each spot of the array was created and subtraction of averaged background signal from each one was performed. The average signal (pixel density) of each pair of duplicate spots, representing each -related protein was calculated and corresponding signal comparison on different arrays were used to determine relative change in protein levels between controls and patients' samples. A normalization strategy of signal intensity based on positive and negative controls values was also performed using Prism statistics 5 software (GraphPad Software, San Diego, CA. USA).

2.9. Statistical analysis

For quantitative variables with non-normal distribution median values and interquartile were used (25–75%) or Box and Whiskers (2.5–97.5%). Statistical analysis between healthy control and dengue groups were carried out using non-parametric Mann-Whitney test. All tests were performed with Prism statistics 5 software (GraphPad Software, San Diego, CA. USA). P values < 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of cohort and donors

Among 99 DENV-infected adult patients, 53.5% (53) were classified as DF and 31.3% (31) as DFWS and, 15.2% (15) presented the severe form based on WHO guidelines (Basu et al., 2008). Characteristics of all patients and donors are depicted in Table 1. No differences in age, gender distribution and post-infection days comparing all groups were observed. DF clinical manifestations included: fever, headache, retro-orbital pain, muscle pain, arthralgia, and nausea, vomiting and rash. Apart from petechiae, hemorrhagic manifestations were less common. DFWS/Sev patients presented: severe abdominal pain, severe nausea and vomiting, bleeding episodes such as skin hemorrhages, epistaxis, bleeding gums, gastrointestinal bleeding, urinary tract hemorrhage or metrorrhagia. The most Sev patients presented signs of vascular leakage as pleural or pericardial effusion and ascites. Additionally, severe gingival bleeding and hypotension were documented.

Leukopenia was presented in all dengue patients compared to controls, associated with monocytopenia in DFWS compared to Sev patients and lymphopenia in DF compared to DFWS and Sev patients. In addition, increase in AST and ALT levels and decrease in platelet counts were observed in all dengue-patients, more pronounced according to severity, compared to controls.

Almost all cases regardless of clinical outcome, anti-dengue IgM confirmed 85% of the patients, whereas NS1 antigen detection was 42% and 49% had RT-PCR positivity. Most of them already experienced secondary infection (90%). DENV-2 serotype was more frequent, followed by DENV-1 and less frequently by DENV-4. Among 15 Sev dengue patients, we performed RT-PCR or viral isolation in 13 of them. Nine of the 13 (69%) did not have the infectious serotype identified, while the remaining 4 were identified as DENV-2 (31%).

3.2. Molecules derived from activated platelet granules are secreted in dengue

To investigate activation status of platelets in dengue, granule secreted molecules derived from platelets in serum from a cohort was quantified, comprising dengue-infected patients with clinical symptoms and ranging from mild to severe dengue syndromes. Soluble P-selectin protein (sCD62P) is secreted exclusively by platelets. Surprisingly, sCD62P did not show differences between dengue patients when related to severity, nor between dengue patients versus healthy donors (16.96 [13.43–21.29] from donors compared to 12.42 [10.03–15.19] from DF, 13.87 [11.72–22.48] from DFWS and, 16.49 [10.24–24.81] from Sev by the Mann-Whitney test) (Fig. 1A). We expanded this analysis considering that DFWS and Sev patients showed lower platelet counts than DF, which could directly influence secreted sCD62P content. In this way, ratio between sCD62 P measurements on platelet counts was calculated and, therefore, their exclusive relationship was considered. From there, we found a greater ratio sCD62 P / platelet count in patients with DFWS (0.29 [0.17–0.86]) and Sev (0.75 [0.23–1.38]) compared with healthy controls (0.07 [0.06–0.11]) (both < 0.001). In addition, a drop ratio sCD62P / platelet count in DF compared to DFWS ($p < 0.0001$) and Sev ($p < 0.0001$), but similar ratios between DFWS and Sev (Fig. 1B), confirming a greater profile of platelet activation with severity dengue.

Circulating contents of soluble nitric oxide (sNO) and Thromboxane A2 (TXA_2) in serum of dengue patients was analyzed. Both are produced and secreted by platelets. Regarding sNO, we found no differences between dengue patients and healthy donors (12.08 [11.30–12.85] from donors compared to 12.85 [11.30–14.80] from DF, 12.85 [10.91–14.80] from DFWS and, 11.69 [11.30–13.05] from Sev by the Mann-Whitney test) (Fig. 1C). Likewise, we calculated ratio between sNO measurements in platelet counts. We found a greater ratio sNO / platelet count in patients with DF (0.09 [0.06–0.19]), DFWS (0.17 [0.12–0.37]) and Sev (0.50 [0.22–0.81]) compared with healthy controls (0.04 [0.04–0.05]) (all $p < 0.0001$). Also, a drop ratio sNO / platelet count in DF compared to DFWS ($p < 0.03$) and Sev ($p < 0.001$), but similar ratios between DFWS and Sev (Fig. 1D). Since NO is an important vasodilator, we evaluated whether there was a difference between dengue patients who presented plasma extravasation compared to those without plasma extravasation or among dengue patients who presented bleeding in relation to those who did not. None of these comparisons showed statistical significance (data not shown). Following our analysis, since TXA_2 is rapidly hydrolyzed into

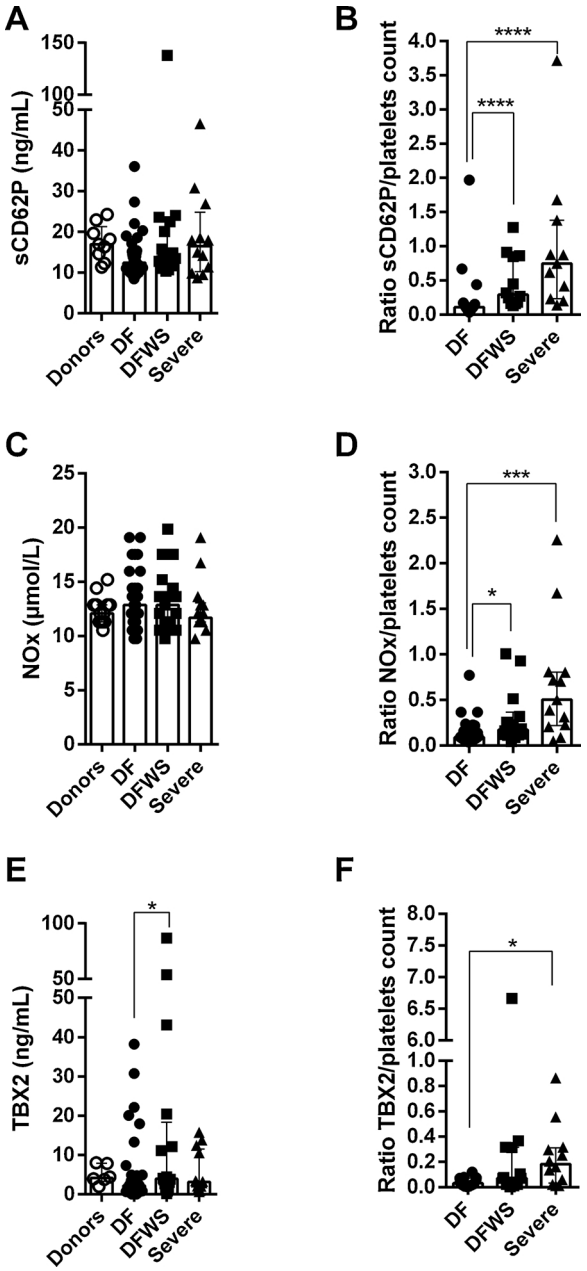

Fig. 1. Dengue triggers platelet activation and mediator's secretion. Concentration of (**A**) CD62 P, (**C**) NO and (**E**) Thromboxane B_2 in plasma from donors and dengue patients classified in mild (DF), dengue with warning signs (DFWS) and severe (Sev). Ratio was calculated by sCD62 P (**B**) NO (**D**) and TBX_2 (**F**) measurements on platelet count for each dengue patient. Each point represents an individual. Boxes indicate median and interquartile ranges and whiskers indicate minimum and maximum values in each group. * Represents $p < 0.05$ between pairs using Mann-Whitney U test for non-parametric distribution.

thromboxane B2 (TXB_2) which is more stable, TXB_2 is generally used to estimate TXA_2 levels. TXA_2 is vasoconstrictor and thrombus promoter. Importantly, TXB_2 content was statistically down-regulated in DF patients (2.21 [0.31–4.91]) compared to DFWS 3.92 [1.93–18.33], $p < 0.04$) (Fig. 1E). We calculated ratio of TXB_2 measurements in platelet count and found higher TXB_2 / platelet ratio in Sev (0.18 [0.03 – 0.31]) compared to DF (0.03 [0.01–0.07]) patients ($p < 0.05$). No other statistical difference was observed among the other groups in the TXB_2 / platelet ratio (0.02 [0.01 – 0.07] from donors and 0.07 [0.02–0.32] from DFWS) (Fig. 1F). Bleeding during dengue does not appear to be influenced by TXB_2 levels (data not shown). Surprisingly, increase in TXB_2 level was detected in dengue patients who showed signs of plasma leakage (n = 31, 3.55 [2.06–13.47]) compared to those who did not present it (n = 16, 0.66 [0.14–2.93]), p = 0.001 by the Mann-Whitney test).

3.3. Platelet susceptibility to dengue virus infection

Platelets were isolated with OptiPrep™ density gradient from 5 healthy volunteers and 18 dengue-positive patients with serological and molecular diagnostic confirmation to assess morphological changes and markers of platelet activation during dengue. Among 18 dengue patients, 10 were DF and 8 DFWS, but severe patients were available to go through this evaluation. Platelets from 5 healthy volunteers were stimulated with DENV, in vitro Mock culture medium, alone culture medium or Thrombin stimulation. After three hours, when compared to Mock-stimulated platelets, DENV infection appear to infect platelets using transmission electron microscopy (TEM). We can clearly observe several viral particles within platelet granules, confirming high susceptibility of platelets to DENV (Fig. 2A). When compared to medium-stimulated platelets (Fig. 2A and D), thrombin-activated platelets increased significantly percentage of CD62P or Fibrinogen-expressing CD31 cells in this same data point. Although significant DENV-2-infected platelets by TEM was observed, these platelets did not show an activated profile based on the expression of CD62P or Fibrinogen percentage when compared to Mock-stimulated platelets (Fig. 2C and E).

Interestingly, platelets from DF and DFWS patients showed a significant change in their morphological characteristics with the presence of filopodia, loss of cytoplasmic content and membrane dilatation (Fig. 2F). This morphological profile of activation was confirmed by increased CD62P expression in platelets derived from both dengue patients (74.40 [52.50–88.70] from DF and 44.50 [23.50–93.50]) from DFWS in relation to healthy volunteers (6.70 [2.33–24.80]) ($p < 0.001$ and $p < 0.02$, respectively) (Fig. 2G). However, among the three groups of individuals studied (Fig. 2H), expression of CD40L, used as another platelet activation marker, showed a tendency to increase, but no statistical significance was observed (0.87 [0.29–2.25] from donors, 2.34 [1.34–5.07] from DF and, 1.34 [0.76–2.69] from DFWS).

3.4. Proteomic analysis of platelets from dengue infected patients

To investigate changes in intraplatelet protein during dengue infection, platelets were isolated with OptiPrep™ density gradient from 8 dengue-positive patients, 4 DF and 4 DFWS and 4 healthy donors. Platelets were lysed in protease inhibitor cocktail and lysis buffer supplied by the Human Angiogenesis Array Kit as described in the methods section. Afterwards, Human Angiogenesis Array Kit approach was applied. Thirty of the 55 proteins analyzed did not detect spots. Within this set, amphiregulin, artemin, CXCL8/IL-8 and PlGF (Placental Growth Factor) are not reports stored or produced in platelets. In contrast, Angiopoietin-2 and TF (Tissue Factor/ factor III) spots were much lower when compared to other proteins, confirming their irrelevant stock inside platelets. The remaining 25 intraplatelet proteins

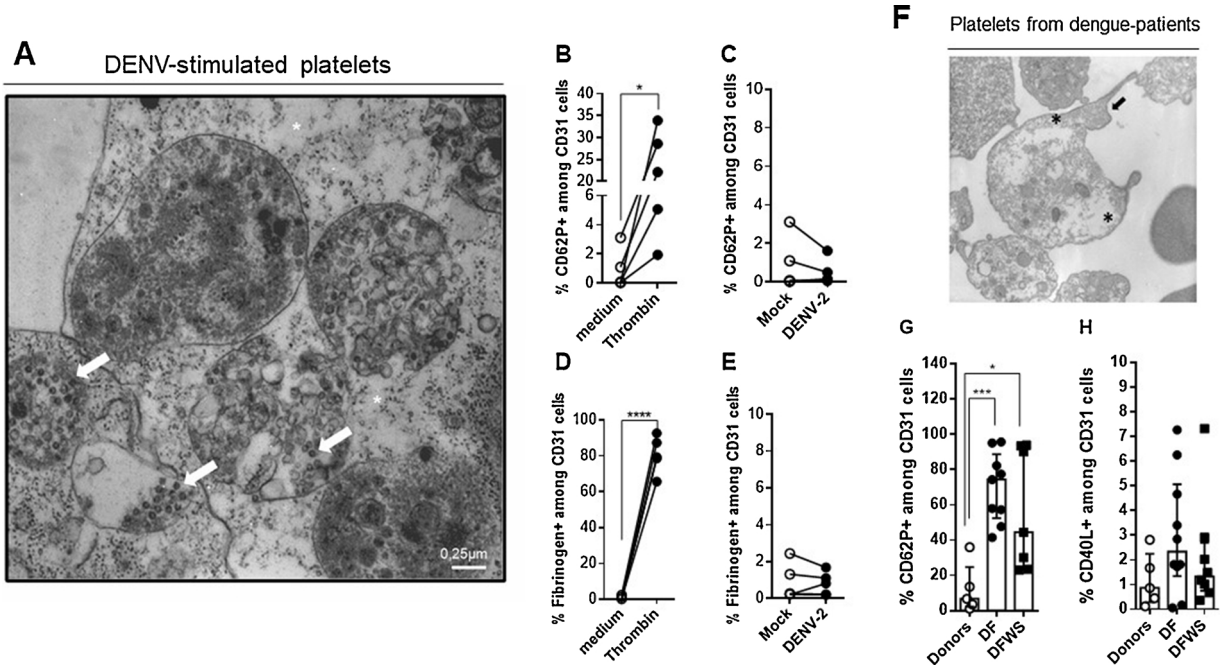

Fig. 2. Morphological changes and markers of platelet activation during dengue. Platelets were isolated with OptiPrepTM density gradient from healthy volunteers and dengue-positive patients. Platelets from healthy volunteers were stimulated with Mock or DENV culture medium in vitro for three hours. (**A**) DENV-2-infected platelets from donors show several viral particles (white arrow) within the platelet vesicles by transmission electron microscopy. (**B and D**) After Thrombin (100 U/mL, Sigma, catalog # T4393) or DENV-2 stimulation, P-selectin (CD62 P) and Fibrinogen expressions were observed on platelets from donors by flow cytometry analysis. In addition, (**F**) morphological alterations such as extensive filopodia (black arrow), loss of cytoplasmic content (*) and membrane dilatation (arrow head) of isolated platelets from one representative dengue patient was observed. *Ex vivo* analysis of (**G**) P-selectin (CD62 P) (**H**) and Fibrinogen expressions were observed on platelets from dengue-patients by flow cytometry. Boxes indicate median and interquartile ranges and whiskers indicate minimum and maximum values in each group. Each point represents an individual. * Indicates $p < 0.05$ between different stimuli using paired *t*-test and among dengue-patients versus donors using Mann-Whitney U test for non-parametric distribution.

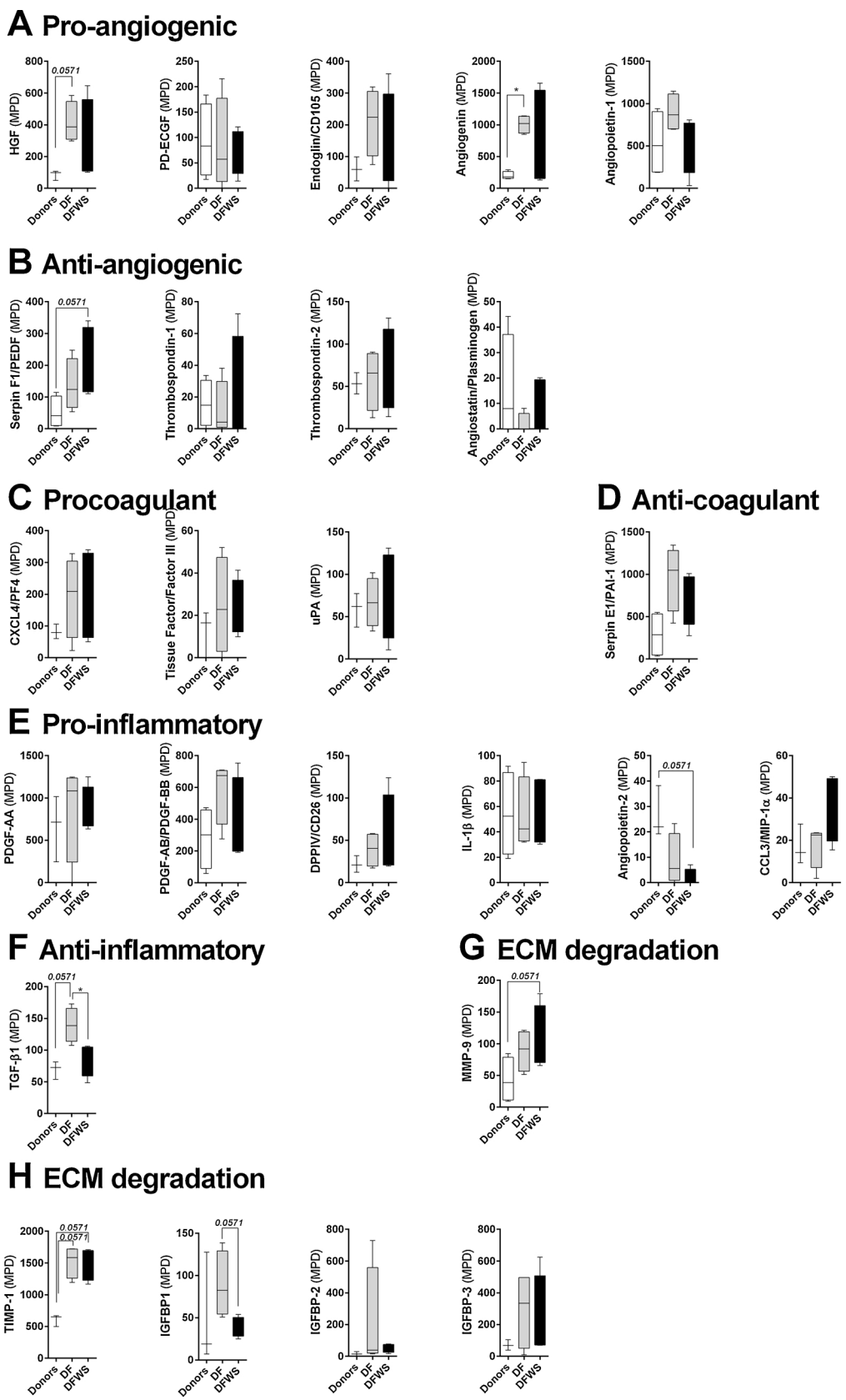

Fig. 3. Measurements of intra-platelet protein in dengue infection. Platelets were isolated with OptiPrep™ density gradient from eight dengue-positive patients (3 DF and 3DFWS) and three or four healthy individuals. Platelets were lysed in protease inhibitor cocktail and lysis buffer. Afterwards, Human Angiogenesis Array Kit approach was applied. Twenty-five of the 55 intra-platelet proteins were detected spots and classified according to their functions in: (**A**) pro-angiogenic; (**B**) anti-angiogenic; (**C**) procoagulant; (**D**) anti-coagulant; (*E*) pro-inflammatory; (**F**) anti-inflammatory; (**G**) proteins involved in endothelial cell matrix (ECM) degradation and finally, (**H**) those involved with ECM integrity. Boxes indicate median and whiskers indicate 2.5 to 97.5 percentiles values in each group. * Indicates $p < 0.05$ among patients with dengue syndromes compared to donors using Mann-Whitney U test for non-parametric distribution.

possible to detect some spot were regrouped according to their main function forming 8 groups: pro-angiogenic; anti-angiogenic; procoagulant; anti-coagulant; proteins involved in endothelial cell matrix (ECM) degradation and, ECM integrity; pro-inflammatory and finally, anti-inflammatory.

Among intraplatelet proteins with pro-angiogenic function, expression of HGF (Hepatocyte Growth Factor) and Angiogenin were highlighted, indicating increase in dengue patients, particularly in those

DFWS (for HGF 386.8 [308.8–547.1] and for Angiogenin 1020 [870.4–1134]) when compared to donor's individuals (for HGF 98.8 [48.89–106.9] and for Angiogenin 180.2 [156.9–264.5]) (both p = 0.0571) (Fig. 3A). Although not significant, anti-angiogenic Serpin F1 / PEDF (Pigment Epithelium-Derived Factor) was more expressed in DFWS (123.5 [66.55–221.0]) compared to donor's individuals (41.71 [9.72–102.9]) (Fig. 3B).

Regarding coagulant function, a balance between procoagulant proteins in the three groups of individuals was observed. However, increase of anti-coagulant Serpin E1 / PAI-1 (Plasminogen Activator Inhibitor-1) expression, although not significant, occurs in dengue patients, independent of clinical status, compared to donors (Fig. 3C–D).

Unexpectedly, most pro-inflammatory proteins in platelets showed similar levels among the three groups of subjects, except Angiopoietin-2, showing a tendency to decrease in DFWS in relation to donors. Interestingly, cytokine regulator TGF-β (Transforming Growth Factor beta) showed an increased expression in DF (95.98 [59.48–104.7]) compared to those with DFWS (138.6 [113.6–165.9]) ($p < 0.03$) and a tendency of DFWS compared to donors (72.71 [53.9–81.23]) (p = 0.0571) (Fig. 3E–F).

In relation to MMP-9 (Matrix Metallopeptidase 9) involved in degradation of ECM, DFWS platelets (94.84 [70.39–160.4]) showed greater expression of MMP-9 in relation to donors (38.94 [11.45–78.59]), although not significant (p = 0.0571) (Fig. 3G). However, TIMP-1 (tissue Inhibitor of Metalloproteinases 1), an important protein involved in the regulation of MMP-9 and maintenance of ECM integrity, also tended to increase in DF (1584 [1260–1721]) and DFWS (1529 [1228–1695]) patients than in controls (650.1 [496.3–668.7]) (p = 00,571), indicating a regulatory balance between these proteins with antagonistic functions. Next, IGFBP-1 (Insulin-like Growth Factor-Binding Protein), another protein involved with ECM integrity differentiated patients with dengue according to clinical classification. Increased IGFBP-1 expression in DF (82.46 [54.5–129.0]) compared to DFWS (38.70 [28.12–50.48]) (p = 0.0571) (Fig. 3H) was detected.

3.5. Circulating intraplatelet proteins related to thrombocytopenia in dengue

Circulating PDGF-BB (Platelet-Derived Growth Factor, beta, beta polypeptide) and VEGF (Vascular Endothelial Growth Factor) was measured in the above groups of dengue patients and donors. PDGF-BB shares two main functions, pro-inflammatory and pro-angiogenic. As clearly observed, an increase in PDGF-BB levels was detected in DF (960.9 [773.8–1332]) compared to DFWS (501.8 [296.1–648.6]) ($p < 0.03$) (Fig. 4A). Similarly, VEGF shares two other major functions, extracellular membrane matrix regulator and pro-angiogenic. Although not significant, a strong tendency for high levels of VEGF in DF (126.8 [100.0–255.6]) was detected when compared to DFWS (20.71 [0–98.05]) (p = 0.0571) (Fig. 4B).

4. Discussion

Platelets are mainly associated with coagulation and hemostasis, and more recently with other biological events such as inflammation, immune response, angiogenesis, and extracellular matrix synthesis. Focusing on other biological effects of platelets in dengue, we highlighted from this study: 1) ratio sCD62P/ platelet counts, NO/ platelet counts and TBX_2/ platelet counts can be good biomarkers to identify platelets activation status and even, predictors of dengue clinical outcome; 2) for in vitro assay DENV was able to enter in platelets without, however, activating expression of CD62P and Fibrinogen and; 3) Platelets from dengue-patients showed an activation status and; 4) Intraplatelet protein contents revealed a differential profile among DF and DFWS patients. These main points will be discussed below.

Using the recent dengue outcome classification (TDR/WHO, 2009), we observed similar values of sCD62P, NO or TXB_2 in plasma in all

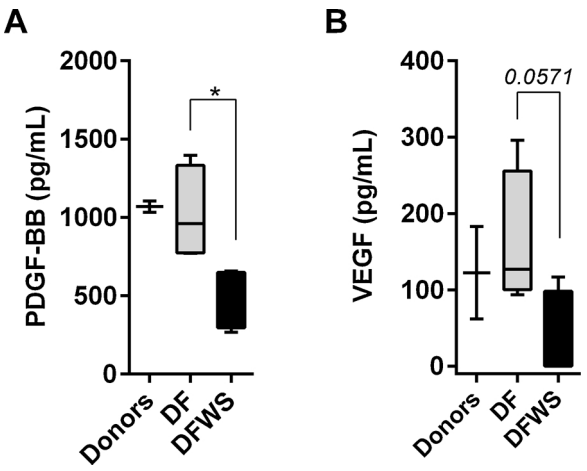

Fig. 4. Circulating PDGF-BB and VEGF in plasma from dengue-infected patients and not infected individuals. (**A-B**) Eight dengue-infected patients were grouped based on those DF and those DFWS. The white bars are donors individuals (n = 2–3). (**A**) PDGF-BB and (**B**) VEGF concentrations were measured in plasmas. Boxes indicate median and whiskers indicate 2.5 to 97.5 percentiles values in each group. * Indicates $p < 0.05$ among patients with dengue syndromes compared to donors using Mann-Whitney U test for non-parametric distribution.

patients with DENV when related to severity, and among patients with DENV versus healthy donors. Our data agree with Krishnamurti and colls. who found normal sCD62P levels in the acute samples of all DENV patients in the different disease, DF and DHF categories (Krishnamurti et al., 2001). The involvement of NO in dengue is still controversial. Increased NO levels were found in patients with the mild form of the disease; However, in the hemorrhagic form, levels like those of healthy controls were found (Valero et al., 2002). On the other hand, a study associated with increased vascular NO in dengue, with increased vascular permeability and compromised homeostasis, was considered useful as a predictor of DHF (Thein et al., 2015). Finally, there are few papers on TXB_2 and dengue. Plasma levels of TXB_2 in patients with DHF without shock did not present a statistically significant difference in relation to normal individuals; however, those DHF patients with shock had significantly lower levels of TXB_2 than normal patients and those DHF with shock (Preeyasombat et al., 1999). Subsequently, to estimate the contribution of the sCD62P shed from the platelets, intraplatelet NO production and TXB_2 synthesized by activated platelets, ratios of sCD62P, NO and TXB_2 to the actual platelet counts were reported by us. As previously shown, platelets are the main source of circulating sCD62P (Ferroni et al., 2009). In addition, NO is produced in human platelets and that changes in intra-platelet NO production has important physiological and pathophysiological implications (Carrizzo et al., 2014). Finally, Thromboxane A2 is a labile prostanoid synthesized by activated platelets through the sequential actions of the enzymes cyclooxygenase (COX) and TXA_2 synthase (Cheng et al., 2002). All proportions increased in the acute samples with increasing disease severity. Thus, we assume that most of the sCD62P, NO and TXB_2 originate from activated platelets and, once activated, the platelets are removed from the circulation. Thus, the ratios of sCD62P, NO and TXB_2 to actual platelet count, in addition to platelet counts *per si*, are better markers of disease severity.

Previous study described strong association between activation status of platelets and their destruction/depletion in febrile dengue patients (Ojha et al., 2017). Our data confirmed accentuated thrombocytopenia majorly in severe febrile cases of dengue (Michels et al., 2014; Krishnamurti et al., 2002; Hottz et al., 2013). However, in mild dengue when moderate thrombocytopenia occurs, circulating platelets are also in their active forms with maximum expression of CD62P, but less CD40L, presenting activated morphological alterations. We

speculated that an activation status of platelets could contribute to their sensibility to death as observed in severe dengue associated with other biological effects such as immunological role, majorly in mild dengue patients. Study found that activated and apoptotic platelets aggregate with monocytes during dengue infection and such interaction mediate IL-10 secretion that may contribute to pathogenesis of dengue interaction (Hottz et al., 2014).

Multiple pathways lead to platelet activation, including agonists such as collagen, adenosine diphosphate (ADP), thromboxane A_2 (TXA_2), epinephrine, serotonin, and thrombin (Brass, 2003; Davi and Patrono, 2007; Offermanns, 2006), through interaction with receptors on platelet surface, leading to release of its granular content, increasing intracellular Ca^{2+} levels and activation of fibrinogen receptor, αIIbβ3 integrin (Li et al., 2010). Previous data showed that plasmatic TXB_2 levels of DHF patients with shock decreased significantly than those of normal controls and DHF patients without shock patients, supposing that failure or inadequate TXB_2 production may eventually lead to shock (Preeyasombat et al., 1999). We concluded that TXB_2 levels in DF significantly decreased than those DFWS and severe patients. Release of TXA_2 from adherent platelets enhances recruitment and aggregation to the primary plug and activates platelets (Varga-Szabo et al., 2008). So, we suggested that high TXB_2 levels in donors could reflect protective homeostasis, while in DFWS, severe patients and those with plasma leakage could lead to pathologic thrombus formation. Conversely, platelet activity may be modulated negatively by substances like nitric oxide (NO) released by cells (Li et al., 2010). Our data showed similar levels of nitrite/nitrate content between control donors and dengue patients, independent of severity and of plasma leakage. However, when ratio sNO/platelet counts was performed, a higher ratio level was observed in DFWS and Sev compared to DF. Clinical studies in dengue that measured NO showed conflicting results. A South American study showed significant increased level of serum nitrite/nitrate content in DF when compared with DHF and donors and that, monocytes could be an important source of cytokine and NO production (Levy et al., 2010). Half-life of NO is short and most of its physiological effects derive from local sources, and studies did not assess vascular NO bioavailability as well as its effect associated with endothelial homeostasis and permeability in dengue. Recently a prospective longitudinal adult study showed vascular NO bioavailability measured as reactive hyperemia index (RHI) associated with a 4-fold greater odd of developing DHF (Thein et al., 2015). A study showed that intraplatelet l-arginine–NO pathway is activated in DHF patients by independent mechanism of NOS activity (Matsuura et al., 2012). We added an additional role of platelets as NO inducers during dengue.

Although dengue antigens have been observed in platelets (Noisakran et al., 2009 and Simon et al., 2015), dengue virus can enter platelets but replicate viral ribonucleic acid to a minimal extent and, therefore, cannot produce infectious virus (Kar et al., 2017). Intracellular virus was detected at 3 h by transmission electron microscopy, suggesting that the virus was entering platelets, although we were not able to demonstrate the ability of the virus to replicate or produce infectious viruses within the cell as reproduced by Kar and colls. (Kar et al., 2017). In addition, Núñez-Avellaneda and colls. (Núñez-Avellaneda et al., 2018) exposed platelets to DENV-2 for 4 h and by confocal microscopy analysis they observed some significant morphological alterations, including swollen and elongated cells, not observed in the mock control.

An intriguing question for us is that although dengue antigens were found on platelets, these platelets did not increase the expression of CD62P or Fibrinogen. A distinct kinetics of CD62P expression in platelets exposed to DENV-2 was detected by Hottz and colls (Hottz et al., 2013). Subsequently, Trugilho and Hottz and colls (Trugilho et al., 2017) confirmed that DENV infection significantly increased platelets expressing CD62 P after 6 h, but not after 3 h. Interestingly, a decrease in platelets expressing CD42b was observed after exposure to DENV in comparison to mock control. After that, although we observed a significant number of DENV-2 particles in the platelets, it is possible that these platelets could not show an activated profile due to several experimental reasons. Among them, the variation of the multiplicity of infection (MOI) of the DENV, kinetics of expression of the molecule activated in the platelets and the multiple possibility of activation molecules, such as CD40L, CD42b, CD62P, fibrinogen and class I of the MHC, regulated. Some of these factors will be tested in the future. The most interesting is that a typical activated morphological alteration by microscopy occurs in platelets from dengue patients indicating that besides infection *per si*, circulating mediators and interaction of other immune or non-immune cells contribute to activate these cells.

At the vascular injury site, platelets aggregate and release growth factors such as insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), endothelial cell growth factor (ECGF), and transforming growth factor-β (TGF-β) (Kaplan et al., 1979; Oka and Orth, 1983; Assoian and Sporn, 1986; Miyazono et al., 1987; Karey et al., 1989; Trugilho et al, 2017). We discussed initially the release of insulin-like growth (IGF-1), a peptide hormone whose concentration in plasma increases in response to growth hormone, largely due to increased production by the liver (Le Roith, 2003) and from α granules upon activation platelets (Karey and Sirbasku, 1989). The effect of IGF-1 is modulated by multiple IGF-binding proteins (IGFBPs), which binds IGF-1 and thereby serves as transporter proteins and storage pools (Karey and Sirbasku, 1989). Thus, our data showed high levels of IGFBP-1 in DF patients with more than 150,000 platelets compared to those DFWS with less than 150,000 platelets. We speculated that as high levels of IGFBP-1 decreases free IGF-1 concentrations, it may therefore play an important role in autocrine and paracrine regulation of platelet function in non-thrombocytopenic dengue patients (Hartmann et al., 1989).

Evidence of hepatocellular damage is common in dengue-infected individuals. In dengue, serum HGF level was significantly higher at the early febrile stage than at follow-up, indicating that it may be a useful predictor for clinical progression to DHF (Voraphani et al., 2010). Moreover, another study showed that HGF levels were increased significantly in severe and shock patients compared to patients with uncomplicated (van de Weg et al., 2013) and more recently, HGF levels also increased in patients with significant plasma leakage and secondary DENV infection (Her et al., 2017), suggesting that besides classical mediators, growth factors may play an important role in cytokine storm during severe DENV infection. Herein, intraplatelet levels of HGF were higher in DF patients with more than 150,000 platelets compared to controls. Our data are unprecedented since intraplatelet HGF levels were evaluated in dengue for the first time. We do not know if intraplatelet and plasma levels of HGF were comparable among our patients. We agree that there is up-regulation of HGF in dengue, but data found was not clear if intraplatelet HGF could be useful as a dengue prognostic biomarker.

Many findings showed increased levels of TGF-β in patients with severe dengue disease (Laur et al., 1998; Agarwal et al., 1999). We already demonstrated that TGF-β1 plasma levels increased in the acute phase and reached peak levels by day 11 onwards (Azeredo et al., 2006). For the first time, TGF-β intraplatelet levels were measured in dengue and our data showed significant higher level in DF patients, while those DFWS and controls presented similar levels. Similarly, to HGF, we do not know if intraplatelet and TGF-β plasma levels were correlated. The up-regulation of TGF-β1 in dengue patients non-thrombocytopenic might suggest that TGF-β activity may suppress inflammatory response, acting in an anti-inflammatory manner preventing possible harm to host.

In relation to other growth factors, PDGF and VEGF were measured both as intraplatelet proteins expression as serum levels, but no association was observed between these two approaches. Both PDGF and VEGF were generally lower in DFWS, relative to both DF patients and the control group (Rathakrishnan et al, 2012). No other approached the role of PDGF and dengue. An interesting work approached severe fever

with thrombocytopenia syndrome (SFTS) caused by a bunyavirus named SFTS virus (SFTSV) in which serum PDGF-BB levels were observed consistently to be decreased in SFTS patients than controls (Zhang et al., 2016). Our data showed that serum PDGF-BB levels were lower in DFWS thrombocytopenic dengue patients compared to DF and heathy donors' non-thrombocytopenic groups, which is quite consistent with Zhang's' data. Once BB isoform of PDGF is described as a key regulatory molecule in various physiological processes such as bone homeostasis, repair, and regeneration (DiGiovanni et al., 2012), it's highly possible that PDGF might play a role in pathogenesis of thrombocytopenia in dengue.

In recent study of Thakur and colls (Thakur et al., 2016), VEGF levels were higher in patients with severe dengue when compared to patients with non-severe dengue with and without warning signs. Moreover, they found significant correlation between raised VEGF levels and thrombocytopenia. In view of the above, low levels of VEGF in our DFWS group will be at odds. These conflicting findings emphasize the need for in-depth investigation of VEGF, observing days of illness and even different forms of clinical classification.

5. Conclusion

Taken together, our data showed that plasma circulation of CD62 P, NO and TBX_2, when assessed as ratio of platelet count, may be a good strategy to identify platelet activation status and as a predictor of the clinical outcome of dengue. Platelets undergo morphological alteration after in vitro DENV infection or during natural infection, although only during natural activation increased expression of activation markers as CD62P was observed. This activation status would empower regulation in angiogenesis, inflammation, coagulation and extracellular matrix regulators. Thus, strategies aimed at recovering quantities of platelets in dengue seem essential for a better clinical outcome of patients.

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Conflict of interest

No reported conflicts.

Author contributions

TAdCB, DBdO and LMdOP have designed experiments, reviewed data, and planned the experimental strategy. ATdC performed Human Angiogenesis Array Kit. DFBV, FCJ and OMB performed transmission electron microscopy analysis. NRdCF and RMRN performed all diagnostic tests. PCdCN and DCdSM assisting in Human Angiogenesis Array Kit assay. TAdCB, DBdO, CFK, ELdA, PVD and RVdC collected samples and provided clinical information. LMdOP conceived and directed the study and wrote the manuscript. All authors have critically read and edited the manuscript equally.

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