Immune thrombotic thrombocytopenic purpura plasmas induce calcium- and IgG-dependent endothelial activation: correlations with disease severity

by Edwige Tellier, Agnès Widemann, Raphaël Cauchois, Julien Faccini, Marie Lagarde, Marion Brun, Philippe Robert, Stéphane Robert, Richard Bachelier, Pascale Poullin, Elien Roose, Karen Vanhoorelbeke, Paul Coppo, Françoise Dignat-George, Gilles Kaplanski, and ENDO-13 study group: Dominique Bourdessoule, CHU de Limoges; Karine Clabault, CHU de Rouen; Cedric Daubin, CHU de C. Collaborative Groups: ENDO-13 study group (Dominique Bourdessoule, Karine Clabault, Cedric Daubin, Gabriel Choukroun).

Received: January 12, 2022.
Accepted: November 21, 2022.

Citation: Edwige Tellier, Agnès Widemann, Raphaël Cauchois, Julien Faccini, Marie Lagarde, Marion Brun, Philippe Robert, Stéphane Robert, Richard Bachelier, Pascale Poullin, Elien Roose, Karen Vanhoorelbeke, Paul Coppo, Françoise Dignat-George, Gilles Kaplanski, and ENDO-13 study group: Dominique Bourdessoule, CHU de Limoges; Karine Clabault, CHU de Rouen; Cedric Daubin, CHU de C. Collaborative Groups: ENDO-13 study group: (Dominique Bourdessoule, Karine Clabault, Cedric Daubin, Gabriel Choukroun).

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Immune thrombotic thrombocytopenic purpura plasmas induce calcium- and IgG-dependent endothelial activation: correlations with disease severity

Edwige Tellier¹², Agnès Widemann¹, Raphaël Cauchois²³, Julien Faccini¹², Marie Lagarde¹², Marion Brun³, Philippe Robert⁴, Stéphane Robert¹, Richard Bachelier¹, Pascale Poullin²⁵, Elien Roose⁶, Karen Vanhoorelbeke⁶, Paul Coppo²⁷, Françoise Dignat-George¹⁸, Gilles Kaplan²³ on behalf of the ENDO-13 study group⁹.

1. Aix-Marseille Univ, INSERM, INRAE, C2VN, Marseille, France
2. French Reference Center for Thrombotic Microangiopathies
3. Aix-Marseille Univ, APHM, INSERM, INRAE, C2VN, CHU Conception, Service de Médecine Interne et Immunologie Clinique, Marseille, France.
4. Aix-Marseille Univ, APHM, CNRS, INSERM, CHU Conception, Laboratoire Immunologie, Marseille, France
5. APHM, Service d’Hématrophèse, CHU Conception, Marseille, France
6. Laboratory for Thrombosis Research, IRF Life Sciences, KU Leuven Campus Kulak Kortrijk, Kortrijk, Belgium.
7. Service d’Hématologie, Hôpital Saint-Antoine, APHP.6-SU, INSERM UMRS 1138, Centre de Recherche des Cordeliers, Paris, France.
8. Aix-Marseille Univ, APHM, INSERM, INRAE, C2VN, CHU Conception, Laboratoire d’Hématologie, Marseille, France
9. ENDO-13 study group : D Bourdessoule, CHU de Limoges ; K Clabault, CHU de Rouen ; C Daubin, CHU de Caen ; G Choukroun, CHU Amiens, France.

Supported by a Grant from the Projet Hospitalier National de Recherche Clinique 2007 (#2007/23).

Correspondance to : Edwige Tellier, PhD, C2VN, Faculté de Pharmacie, 27 Bd Jean Moulin, 13005 Marseille, France ; E-mail address : edwige.tellier@univ-amu.fr

Running Heads : Ca²⁺-mediated Weibel-Palade exocytosis in iTTP

Abstract word number: 240
ACKNOWLEDGMENTS

This study was supported by research funding from the French ministry of Health (Projet National de Recherche Clinique 2007, #2007/23) to Gilles Kaplanski

AUTHOR’ CONTRIBUTION

ET, AW, MB, JF, RC and ML conducted the experiments and interpreted the data; MB, PP, PC, ENDO-13 study group, ER, KV and GK contributed to sample collection and analysis of clinical data; ET, SR and JF contributed to image capture and PR and ET to flow chamber experiments and fluorescence microscopy analysis; ER and KV provided TTP73 and ELH2-1 for the study and critically reviewed the manuscript; FDG handled funding and made a critical revision of the manuscript; RB critically reviewed the manuscript; GK took care of patients, handled funding and supervision of the project, helped with the interpretation of the experiments, and wrote the manuscript with ET.

DATA SHARING STATEMENT

The original data and protocols are available to others investigators without unreasonable restrictions.

CONFLICT-OF-INTEREST STATEMENTS

Paul Coppo is a consultant for Sanofi, Alexion, Takeda and Roche. Karen Vanhoorelbeke is a member of the scientific advisory boards of Ablynx-Sanofi and Shire-Takeda. Pascale Poullin is member of the scientific advisory boards of Ablynx-Sanofi. The other authors declare no competing financial interests.
ABSTRACT

Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is characterized by a severe ADAMTS13 deficiency due to the presence of anti-ADAMTS13 autoantibodies, with subsequent accumulation of circulating ultra-large von Willebrand Factor (VWF) multimers. The role of endothelial cell activation as a trigger of the disease has been suggested in animal models but remains to be demonstrated in humans.

We prospectively obtained plasma from the first plasma exchange of 25 patients during iTTP acute phase. iTTP but not control plasma, induced a rapid VWF release and P-selectin exposure on dermal human micro-vascular endothelial cell (HMVEC-d) surface, associated with angiopoietin-2 and endothelin-1 secretion, consistent with Weibel-Palade bodies exocytosis. Calcium (Ca\(^{2+}\)) blockade significantly decreased VWF release, whereas iTTP plasma induced a rapid and sustained Ca\(^{2+}\) flux in HMVEC-d which correlated in retrospect, with disease severity and survival in 62 iTTP patients. F(ab)\(^{2}\) fragments purified from the immunoglobulin G (IgG) fraction of iTTP plasma mainly induced endothelial cell (EC) activation with additional minor roles for circulating free heme and nucleosomes, but not for complement. Furthermore, two anti-ADAMTS13 monoclonal antibodies purified from iTTP patient B cells, but not serum from hereditary TTP, induced endothelial Ca\(^{2+}\) flux associated with Weibel-Palade bodies exocytosis in vitro, whereas inhibition of endothelial ADAMTS13 expression using small interference RNA, significantly decreased the stimulating effects of iTTP IgG.

In conclusion, Ca\(^{2+}\)-mediated endothelial cell activation constitutes a second “hit” of iTTP, is correlated with the severity of the disease and may constitute a possible therapeutic target.
INTRODUCTION

Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is a rare and life-threatening thrombotic microangiopathy (TMA) characterized by a severe thrombocytopenia (<30x10^9/L) and a mechanical hemolytic anemia. Consequently, ischemic events of variable severity occur, mainly affecting the brain, the heart, and the mesenteric tract. The diagnosis of iTTP relies on the demonstration of ADAMTS13 (A Disintegrin and Metalloprotease with Thrombospondin type I repeats, member 13) protease functional deficiency (<10%), due to the presence of anti-ADAMTS13 autoantibodies. ADAMTS13 is responsible for the cleavage of ultra-large von Willebrand Factor (UL-VWF) into smaller and less thrombotic multimers. Deficiency of ADAMTS13 activity leads to the accumulation of highly pro-thrombotic UL-VWF in the patient plasma inducing the formation of multiple platelet-rich thrombi into the microcirculation, consumptive thrombocytopenia, mechanical hemolysis and clinical symptoms. Despite treatments based on therapeutic plasma exchange (PEX) and immunosuppressive drugs, the mortality rate remains as high as 5 to 10%.

Animal models demonstrate that in addition to ADAMTS13 deficiency, endothelial UL-VWF exocytosis is necessary to reproduce the disease, suggesting that endothelial cells (EC) may participate to a “second hit” of the disease. UL-VWF is the main constituent of endothelial Weibel-Palade Bodies (WPB), from which it can rapidly be released upon EC activation. To obtain a TTP-like disease, ADAMTS13 KO mice need to be crossed with mice expressing high intracellular concentrations of VWF, then to be injected with shigatoxin to induce WPB degranulation leading to UL-VWF release, showing that inactivation of the adams13 gene is not sufficient to induce TTP-like manifestations. In agreement, injections of large concentrations of recombinant VWF can induce TTP in ADAMTS13 KO mice. Moreover, vWF gene deletion results in complete protection in the shigatoxin-induced TTP murine model, demonstrating the absolute requirement of VWF to develop TTP. Another TTP model consisting in injection of murine anti-ADAMTS13 inhibitory monoclonal antibodies (mAb) into wild type mice, led to plasma ADAMTS13 deficiency and UL-VWF accumulation without TTP-like symptoms. The additional injection of recombinant VWF in this model induces an
iTTP-like disease, further demonstrating the essential role of large concentrations of circulating VWF.

In a primate model of TTP, injection of a murine anti-human ADAMTS13 inhibitory antibody induced TTP, as demonstrated by the appearance of severe thrombocytopenia, hemolytic anemia, elevated LDH, schistocytes and the occurrence of microthrombi in kidney, heart, brain and spleen. However, the baboons do not develop end-stage disease, suggesting once again that inhibition of ADAMTS13 alone, may not be sufficient to reproduce a full-spectrum human TTP.

Thus, these experimental models suggest that induction of TTP in animals is a “two-hits” process requiring first, ADAMTS13 protease inactivation and second, increased VWF release by activated EC. Similar mechanisms however, remain to be demonstrated in humans. In this context, we asked whether iTTP-patient plasma was able to induce WPB exocytosis and tried to identify possible endothelial activators in iTTP-patient plasma. Using plasma prospectively collected from patients during the acute phase of iTTP, we observed induction of UL-VWF release from EC via WPB exocytosis in a calcium (Ca\(^{2+}\))-dependent pathway. We identified IgG from iTTP-patient plasma as the main inducer of endothelial activation and observed that Ca\(^{2+}\)-dependent endothelial activation intensity correlated with disease severity.
METHODS

Patient characteristics

We conducted a prospective study between 2008 and 2011 consisting in a National Clinical Research Project (#2007/23) approved by the Ethical Committee of the “Assistance Publique-Hôpitaux de Marseille”. Informed consent was provided according to the Declaration of Helsinki. Detailed information about the sixty-two patients can be found in supplementary methods and Table 1.

Immunofluorescence studies (IF)

Confluent human microvascular dermal EC (HMVEC-d) were grown in EC growth basal medium-2 (EBM2) containing 1% fetal bovine serum for 16h and activated for 1h with either control or iTTP plasma (1/100 in EBM2), washed twice in phosphate buffer saline (PBS), fixed in 1% paraformaldehyde for 10min and labelled with anti-VWF, anti-P-selectin antibodies or with rabbit non-immune serum like described in supplementary methods.

To visualize ADAMTS13, HMVEC-d were activated for 20min with control or iTTP IgG (30µg/mL), washed in PBS and labeled like described in supplementary methods. To exclude the influence of possible endotoxin contamination of biological samples, all experiments were performed in the presence of 10µg/mL polymyxin B (Sigma-Aldrich, Saint Louis, USA).

Soluble VWF, endothelin-1 and angiopoietin-2 measurements

Specific enzyme-linked-immunosorbent-assay (ELISA) were performed to determine the concentrations of soluble VWF (American Diagnostica, Stanford, USA), endothelin-1 or angiopoietin-2 (R&D system, Minneapolis, USA), according to the manufacturer’s instructions.

Intracellular Ca²⁺ flux

HMVEC-d were incubated for 1h with 5µM fluo-4 AM fluorescent dye (ThermoFisher Scientific, USA) in PBS containing 1% bovine serum albumin (BSA), washed and incubated in PBS 1% BSA for 30 min at 37°C. Cells were stimulated with A23187 (10µM in EBM2), thrombin (4IU/mL or 0.5µM), control or iTTP plasma samples (1/100 in EBM2) for 20 to 1800 seconds, or with IgG (30µg/mL in EBM2). Fluorescence analysis used a Cytoflour®, fluorescence multi-well plate reader (PerSeptive-
Biosystems, Framingham, USA). Total fluorescence intensity was expressed in arbitrary unit (AU). Intracellular Ca\textsuperscript{2+} flux was also measured by microscopy (supplementary methods).

**Preparation of IgG-depleted plasma and purification of the IgG fraction**

After washing with binding buffer (PBS containing 100mM Na\textsubscript{2}HPO\textsubscript{4} at pH 7.4), 100µL of protein A magnetic sepharose beads (GE Healthcare) were incubated for 30min at 4°C with 300µL of 1:5 diluted plasma from control or iTTP patients. Beads were retrieved using a magnetic bench and plasma was used for Ca\textsuperscript{2+} flux induction and IF studies. IgG were eluted using 100µL of elution buffer (100mM glycine at pH 2.8) and 5µL of 1M TRIS hydrochloride at pH9.

**Human anti-ADAMTS13 mAb**

The two anti-human ADAMTS13 mAb TTP73-1 and ELH2-1 were isolated and cloned from B-cells of 2 different iTTP-patients. As previously reported, these 2 antibodies recognize cryptic epitopes in ADAMTS13\textsuperscript{14} and were used at 30µg/mL in experiments.

**ADAMTS13 gene Silencing**

Downregulation of ADAMTS13 mRNA level was achieved using siRNA directed against ADAMTS13 (Ambion, ThermoFisher-Scientific, USA) and Jet Prime kit (Polyplus-transfection, USA) according to the manufacturer’s instructions. Because the decrease in ADAMTS13 mRNA level was achieved 24h after transfection by RT-qPCR and remained stable until 96h, Ca\textsuperscript{2+} flux experiments were performed 48h after transfection.

**Statistical analysis**

Graphpad Prism v9.2.0 software (GraphPad-Software Inc., San Diego, USA) was used for statistical analysis. Further details of the methods are available in the supplementary methods.
RESULTS:

Plasmas from iTPP patients induce VWF string formation on the surface of HMVEC-d via WPB exocytosis

First, we examined VWF string formation on HMVEC-d by confocal microscopy using anti-VWF labelling on permeabilized HMVEC-d. After incubation with control plasma samples, anti-VWF labelling showed punctuated fluorescent spots, whereas incubation with iTTP plasma samples induced the appearance of typical UL-VWF strings (Figure 1A). Quantification of the VWF string fluorescence was performed after 30 minutes incubation and demonstrated higher VWF fluorescence intensity (FI) when HMVEC-d were stimulated with iTTP than with control plasma samples (6.77[4.8-8.79] vs 0.53[0.36-0.87] AU/cellx10^4, respectively, P<.0001, Figure 1B). Kinetic studies showed that VWF strings on the endothelial surface in response to iTTP plasma was observed after 5 minutes incubation and increased for up to 60 minutes (Figure 1C) (6.75[4.81-7.38], 8.62[7.03-14.82], 14.5[10.1-21.68] vs 0.76[0.71-2.99] AU/cellx10^4, P=.0159, P=.0079, P=.0079 respectively at 5-, 15- and 60-min vs 0 min, Figure 1D)

Similar data were obtained in various conditions such as dynamic using a flow chamber device (supplementary Figure S1A), macrovascular HUVECs (supplementary Figure S1B), or when using either plasma obtained on citrated tubes or serum, instead of plasma from PEX in order to eliminate a possible Ca^{2+} overload due to the PEX process (supplementary Figure S1C). To exclude possible passive absorption of soluble VWF contained in iTTP plasma on HMVEC-d surface, we used a VWF-affinity column to completely eliminate soluble VWF contained in plasma. Using ELISA, we measured soluble VWF in the supernatant of HMVEC-d stimulated with either VWF-free iTTP or VWF-free control plasma samples for 1h and observed significantly higher concentrations of endothelial soluble VWF in the former condition (2.13[1.76-2.50] vs 1.05[0.98-1.61] ng/mL, P=.0159, supplementary Figure S1D).

To determine whether VWF string formation on HMVEC-d was effectively due to WPB exocytosis, we measured the concentrations of endothelin-1 and of angiopoietin-2, two molecules stored in WPB.
Significantly higher concentrations of endothelin-1 (19.02[16.88-20.21] vs 11.55[5.79-17.9] pg/mL, respectively, \(P=.0102\), Figure 1E) and angiopoietin-2 (978[619.5-1200] vs 614.5[445.8-868.3] pg/mL, respectively, \(P=.0355\), Figure 1F), were present in the supernatants of HMVEC-d when cultured with iTTP compared to control plasma samples. In addition, membrane P-selectin expression on HMVEC-d was observed after stimulation for 10 minutes with iTTP, but not with control plasma samples (Figure 1G), associated with a 3-time-increased P-selectin Fl (290.3[188.2-596.6] vs 81.23[61.55-114.3] AU/cellx10^2; \(P<.0001\), Figure 1H). The same data concerning endothelin-1 secretion were observed when HUVEC were used instead of HMVEC-d (40.58[25.66-43.52] vs 19.28[16.45-21.56] pg/mL, \(P=.0070\), supplementary Figure S1E). The endothelin-1 secretion was equivalent when citrated plasma or serum of iTTP-patients were using instead of plasma from PEX (24.65[20.39-28.41] and 25.33[20.84-32.92] vs 24.10[19.9-24.9], supplementary Figure S1F). Altogether, these data are consistent with the fact that iTTP plasmas obtained after PEX were able to induce WPB exocytosis.

**WPB exocytosis induced by iTTP plasma is Ca^{2+}-mediated**

Two pathways of WPB exocytosis are known: a Ca^{2+}-dependent pathway and a cAMP-dependent pathway. To determine whether the Ca^{2+} signaling cascade was involved in WPB exocytosis induced by iTTP plasma, we used the pharmacological inhibitor MAPTAM. Compared with iTTP plasma samples alone, we observed a significant decrease of VWF Fl in the presence of MAPTAM (1.09[0.78-1.79] vs 7.59[7.52-8.85] AU/Cellx10^4; \(P=.0079\), Figure 2A and 2B). iTTP plasma-induced endothelin-1 secretion was also 92%-reduced in the presence of MAPTAM (82.1[73-134.8] vs 8.5[6.9-9.6] %, \(P<.0001\), Figure 2C).

We then measured the ability of iTTP plasma to induce Ca^{2+}-flux in EC, using a single-wavelength Ca^{2+} fluorescent dye. After a 20-second incubation, iTTP plasma samples induced a significantly higher Fl level than control plasma samples in HMVEC-d (94[78-125.5] vs 45[35.5-76] AU respectively, \(P<.0001\), Figure 2D) or in HUVEC (68[55.38-114.9] vs 41.75[37.75-54] AU, \(P=.0063\), supplementary Figure S1G). We also calculated the AUC for Ca^{2+} flux after 30 minutes of stimulation and observed that iTTP plasma induced significantly higher Ca^{2+} mobilization than control plasma samples.
The endothelial Ca$^{2+}$ mobilization was equivalent when using citrated plasma or serum instead of PEX plasma (74.5[59.5-94.75] and 66[41.75-85] vs 70[48.75-87.5] AU, supplementary Figure S1H).

**iTTP plasma-induced endothelial Ca$^{2+}$ flux correlates with iTTP severity**

To establish whether iTTP-induced Ca$^{2+}$ flux correlated with disease severity, we studied sera from 62 iTTP patients (25 from Marseille added with 37 from Paris) collected during the acute phase, and classified in 2 different groups depending on survival (Table 1). At 20 seconds, Ca$^{2+}$ flux appeared significantly higher in cells treated with plasma from non-survivor patients compared with those of survivors (157[119.7-262.3] vs 107[70-181] AU, respectively, $P=0.0031$, Figure 3A). Similarly, after 30 minutes, area under the curve (AUC) from non-survivors was much higher than those in survivors (6316[3066-8503] vs 1391[833.5-4235] cm$^2$, respectively, $P=0.0002$, Figure 3B). In addition, plasma from iTTP-patients in remission induced significantly less Ca$^{2+}$ flux than plasma from the same patients during the acute phase (52.5[45-69] vs 131.5[66-177] AU, $P=0.0013$, Figure 3C) and levels of Ca$^{2+}$ flux were not different from those induced by control plasma. In agreement, plasma obtained from patients in complete remission did not induce VWF release by HMVEC-d (Figure 3D) which was confirmed by fluorescence quantification (1.19[0.89-1.99] during remission vs 10.85[7.59-14.04] AUx10$^4$ during the acute phase, $P=0.0286$, Figure 3E).

**The purified IgG fraction from iTTP plasma induced Ca$^{2+}$-dependent WPB exocytosis**

In order to identify the soluble mediators contained in iTTP plasma involved in WPB exocytosis, we studied several candidates. As iTTP is an autoimmune disease, we first investigated the role of circulating IgG. After depletion of the IgG fraction from iTTP plasma, we observed a significant decrease of VWF FI release by HMVEC-d (Figure 4A). In agreement, the Ca$^{2+}$ flux was 47%-reduced in HMVEC-d cells treated with IgG-depleted plasma compared with complete plasma (127.5[83.5-188] vs 68[45.5-113] AU, respectively, $P=0.0003$, Figure 4B). Conversely, we tested the ability of the purified IgG fraction from iTTP or control plasma to activate HMVEC-d. VWF exocytosis was observed in HMVEC-d cultured with iTTP IgG, but not with control IgG (Figure 4C). Using ELISA, we measured
soluble VWF released in the supernatants of HMVEC-d and observed significantly increased VWF concentrations in HMVEC-d stimulated by iTTP IgG compared with control IgG (3.42[2.68-4.18] vs 2.72[2.08-3.03] ng/mL, respectively, \(P=0.0273\), Figure 4D). In agreement, we observed a significantly higher \(\text{Ca}^{2+}\) flux in HMVEC-d stimulated with iTTP IgG than with control IgG fractions (77.25 [54.88-109.8] vs 46.75 [41-54] AU, respectively, \(P=0.0011\), Figure 4E). These results were confirmed by directly measuring \(\text{Ca}^{2+}\) flux in living cells stimulated with either iTTP or control IgG, using microscopy (supplementary Figure S2A and S2B). In order to determine which fraction of the immunoglobulin was involved in EC activation, we purified the F(ab')2 fragments from the IgG fraction, and observed that F(ab')2 fragments induced both VWF string formation (Figure 4F) and a twice higher endothelial \(\text{Ca}^{2+}\) flux compared to the complete iTTP IgG fraction (130.8 [86.11-197.2] vs 265.3 [206.6-315.3], \(P=0.002\), Figure 4G).

**iTTP IgG induced WPB degranulation partly via ADAMTS13 recognition on endothelial cells**

In order to identify the endothelial antigen target of iTTP-IgG, we concentrated on ADAMTS13, the main protein involved in iTTP pathogenesis. Since it was impossible to create an ADAMTS13 affinity column in order to eliminate anti-ADAMTS13 autoantibodies contained in iTTP-purified IgG, we used different procedures. First, we compared the ability of hereditary TTP (hTTP) serum samples which do not contain anti-ADAMTS13 autoantibodies, with those of iTTP. Compared to that induced by iTTP serum samples, we observed that VWF release induced by hTTP serum was 65% lower (6.61 [4.45-12.84] vs 17.27 [11.09-24.88] ng/mL, \(P=0.0317\), Figure 5A) and that the \(\text{Ca}^{2+}\) flux induced by hTTP serum samples appeared significantly reduced (113.5 [112.7-118.4] vs 126.7 [123.3-131.7] AU, \(P=0.0079\), Figure 5B). After we verified that extracellular expression of endothelial ADAMTS13 was not modified when EC were stimulated with IgG from iTTP-patients or control plasma samples (supplementary Figure S3A) (90.4[62.78-116.2] vs 116.3[73.37-144.7] %, \(P=0.3357\), supplementary Figure S3B), we inhibited endothelial ADAMTS13 expression using specific siRNA (70% of inhibition, 1[1-1] vs 0.29[0.16-0.41], \(P=0.0313\), Figure 5C,) and observed that inhibition of ADAMTS13 expression was associated with a significant reduction of the iTTP IgG-induced \(\text{Ca}^{2+}\) flux (33.44 [25.88-35.22] vs...
47.15 [31.42-73.9] AU, \( P = 0.0084 \), Figure 5D). We further tested the ability of two anti-ADAMTS13 antibodies, TTP73-1 or ELH2-1, previously isolated and cloned from the B cells of two iTTP-patients, to induce WPB exocytosis in HMVEC-d. Both TTP73-1 and ELH2-1 weakly induced VWF tethering on HMVEC-d membranes (Figure 5E). When cultured in the presence of TTP73-1 but not of or ELH2-1, higher soluble VWF concentrations were measured in HMVEC-d supernatants compared to stimulation with control IgG (3.7[3.3-4.3] for TTP73-1 and 1.7[1.6-2] for ELH2-1 vs 0.8[0.7-0.99] ng/mL, \( P = 0.0357 \) and \( P = 0.0571 \) respectively, Figure 5F), as well as higher endothelin-1 secretion (14.1[11.8-15.4] for TTP73-1 and 11.5[10.8-14.4] for ELH2-1, vs 6.4[4.2-11.5] pg/mL, \( P = 0.0317 \) and \( P = 0.0952 \) respectively, Figure 5G). In addition, an increased but non-significant \( \text{Ca}^{2+} \) flux was also induced by TTP73-1 and ELH2-1, compared to control IgG (15.01[11.55-19.31] and 14.91[6.26-18.39] respectively, vs 3.2[0.59-5.83] AU, \( P = 0.10 \), Figure 5H).

**Free heme and nucleosomes, but not complement, play minor roles in iTTP plasma-induced WPB exocytosis**

The concentrations of free heme were significantly increased in iTTP compared to control plasma samples (11.2[6.98-20.35] vs 9[8.2-9.6] \( \mu \text{M} \), \( P = 0.0369 \), Figure 6A), but returned to control levels after remission (0.63[0.44-1.03] vs 11.2[6.98-20.35] \( \mu \text{M} \), \( P < 0.0001 \), Figure 6A). Free heme concentrations however, were equivalent between survivor and non-survivor patients (10.96[8.2-14.07] vs 12.96[2.55-43.56] \( \mu \text{M} \), \( P = 0.6368 \), supplementary Figure S4A). Inhibition of heme by addition of large concentrations of hemopexin, weakly reduced iTTP plasma-induced VWF exocytosis (Figure 6B), endothelin-1 release (69.86[27.59-136.1] vs 79.86[54.79-162.6] %, \( P < 0.0494 \), Figure 6C) and iTTP-induced \( \text{Ca}^{2+} \) flux (30% inhibition, 133.3[89.25-242.8] vs 107.5[61.88-208.3] AU, \( P = 0.0063 \), Figure 6D). iTTP plasma also contained significant enrichment in nucleosomes concentrations compared to control plasma samples (6.34[3.37-12.14] vs 1.15[0.12-1.78] fold increase, \( P < 0.0001 \), Figure 6E) which was no longer observed after remission (0.81[0.53-1.52] vs 6.34[3.37-12.14] fold increase in acute phase, \( P < 0.0001 \), Figure 6E), in accordance with previous studies. Nucleosomes enrichment however, was not different between survivors and non-survivors (3.57[1.76-5.24] vs 3.51[1.24-7.49]
fold increase, $P=0.9295$, supplementary Figure S4B). Nucleosomes signalization is dependent of the
phosphatidylserine activation and can be inhibited by Annexin V. Addition of Annexin V to ITTP
plasma weakly but significantly reduced VWF exocytosis (Figure 6F) and endothelin-1 release by
HMVEC-d (10% inhibition, 72.62{[64.16-126.1]} vs 80.11{[67.52-149.6]} %, $P=0.0052$, Figure 6G), as well
as Ca$^{2+}$ flux induced by ITTP plasma (13% reduction, 216{[102.8-262.8]} vs 246.9{[128-304.5]} AU,
$P=0.0011$, Figure 6H).

To investigate the role of complement on ITTP plasma-induced endothelial activation, we performed
the same experiments with heated plasma or in the presence of eculizumab. None of these
treatments significantly influenced endothelial VWF (supplementary Figure S5A) or endothelin-1
secretion after 1 hour-stimulation (29.45{[23.61-41.76]} and 20.85{[16.01-27.10]} vs 23.27{[19.29-30.39]}
pg/mL, not significant, supplementary Figure S5B), as well as endothelial Ca$^{2+}$ flux intensity after 20
seconds (111.8{[74.85-148.8]} and 100.2{[79.43-287.4]} vs 99.41{[72.49-210.7]} AU, not significant,
supplementary Figure S5C). Since ITTP plasma is likely to contain thrombin, we reproduced the same
experiments in the presence of hirudin and observed no significant modification (187.5{[82.75-240]} vs
180{[89.75-262.5]} AU, not significant, supplementary Figure S5D). We also used the general serine
protease inhibitor PPACK on the ITTP plasma induced VWF release and Ca$^{2+}$ flux increase and
observed no significant difference (6.298{[5.859-6.924]} vs 6.345{[6.095-8.615]} ng/mL, not significant,
supplementary Figure S5E and 149.3{[134.2-154.8]} vs 144.4{[138.5-155.9]} AU, not significant,
supplementary Figure S5F respectively).
In the present study, we observed for the first time that plasma or serum from iTTP-patients in acute phase, but not in remission, were able to induce VWF secretion via WPB exocytosis by a mechanism involving a Ca\(^{2+}\) flux. We ruled out a possible passive adhesion of soluble VWF contained in iTTP plasma on the EC surface, since VWF tethering remained present on EC membrane even after complete depletion of VWF contained in iTTP plasma and concluded that VWF secretion was an active mechanism likely due to WPB exocytosis. WPB exocytosis was indeed confirmed by the fact that iTTP plasma not only induced VWF and P-selectin membrane exposure, but also endothelin-1 and angiopoietin-2 secretion by EC, all components of the WPB. WPB exocytosis is known to involve two different signaling pathways. One is rapid and Ca\(^{2+}\)-mediated, whereas the other is linked to adenylate cyclase activation. Both the WPB exocytosis and the rapid and sustained intracellular Ca\(^{2+}\) flux induced by iTTP plasma were inhibited by the Ca\(^{2+}\) chelator MAPTAM, demonstrating a Ca\(^{2+}\)-mediated signaling pathway. The possible Ca\(^{2+}\) overload of iTTP plasma due to the PEX process was excluded by the observation that the serum of the iTTP-patients had similar effects than plasma and that plasma of non-TTP patients but treated with PEX, was not able to activate EC.

Our objective was then to identify the main endothelial activators contained in iTTP plasma/serum and we showed that the IgG fraction purified from iTTP plasma was able to induce a Ca\(^{2+}\) flux and subsequent WPB exocytosis associated with VWF secretion, whereas IgG depletion almost 60%-reduced reduced EC activation by iTTP plasma. Noteworthy, was the fact that IgG stimulating effects were reproduced by the F(ab')2 fractions and thus linked to a putative antigen recognition on EC. Since iTTP is known to be due to acquired antibodies directed against ADAMTS13, we investigated the role of these antibodies in EC activation. We did not succeed in directly purifying anti-ADAMTS13 Ab from the iTTP IgG fraction, thus we used different ways to determine whether ADAMTS13 played a role in endothelial activation by purified IgG. First, we compared the ability of serum from iTTP with those of hTTP to induce endothelial activation. Hereditary TTP are due to ADAMTS13 genetic deficiency and do not contain anti-ADAMTS13 autoantibodies. We observed a
significant reduction (almost 60%) of endothelial activation using hTP serum samples. Second, we observed that efficient inhibition of ADAMTS13 expression in HMVEC-d using siRNA was associated with a significant reduction of EC degranulation induced by iTTP IgG fraction. Third, we used two previously described human anti-ADAMTS13 mAb, cloned from iTTP isolated B cells, and observed that both of them weakly induced Ca\(^{2+}\) flux in EC as well as VWF and endothelin-1 secretion, confirming that anti-ADAMTS13 IgG were at least in part, responsible for iTTP-induced Ca\(^{2+}\)-mediated EC activation. In most of the patients, anti-ADAMTS13 antibodies behave as inhibitors of the ADAMTS13 protease, but in 10-20% of iTTP-patients, these antibodies demonstrate other mechanisms of interaction with ADAMTS13, such as increased clearance, conformational changes, or increased metalloprotease activity without modifying ADAMTS13 quantification in ELISA. Our data suggest that in addition to these various interactions, anti-ADAMTS13 antibodies may also induce endothelial activation and VWF exocytosis, the well-known second hit of the disease. This observation may appear contradictory with animal models suggesting that anti-ADAMTS13 antibodies do not induce VWF release. Indeed, injection of an inhibiting anti-human ADAMTS13 mAb into baboons did not increase circulating VWF concentrations, but induced histological lesions of TTP, therefore suggesting that at least local VWF occured. In human also, the sole presence of anti-ADAMT13 autoantibodies seems not to be sufficient to induce the disease, which appears frequently related to precipitating events such as pregnancy, surgery or infections. These apparently contradictory results may have several explanations. First that our observations in vitro may not have an important significance in vivo. However, despite some heterogeneity, our results show a significant association of Ca\(^{2+}\)-induced endothelial activation in vitro with TTP prognosis and survival. Second, that not all anti-ADAMTS13 autoantibodies have the capacity to induce WPB degranulation or that iTTP IgG may recognize additional endothelial targets to induce this effect. This is consistent with the fact that in our experiments, inhibition of ADAMTS13 expression did not completely suppress the effects of iTTP IgG and that anti-ADAMTS13 mAb weakly activated EC in vitro. Other anti-EC antibodies, as suggested by previous reports in iTTP and other
autoimmune diseases, may participate in endothelial activation.\textsuperscript{23} Moreover, in support of the role of anti-ADAMTS13 in endothelial VWF release, it may be noticeable that during hTTP, even very low circulating concentrations of ADAMTS13 may not be associated with disease bouts, and that serum of hTTP which do not contain anti-ADAMTS13 auto-antibodies did not induce a strong endothelial activation in our experiments.

We also observed high free heme concentrations in iTTP plasma, in the order of magnitude of those observed in sickle cell disease and HUS patients.\textsuperscript{24,25} Heme induced VWF exocytosis and participated in iTTP-induced Ca\textsuperscript{2+}-mediated EC activation since VWF exocytosis was 30%-decreased by hemopexin. Free heme binds to TLR4 on EC and has been shown to induce WPB exocytosis.\textsuperscript{26,27} Interestingly, TLR4, also the receptor for LPS, may mediate Ca\textsuperscript{2+} flux and endothelial permeability, as shown in lung EC.\textsuperscript{28} Moreover, hemoglobin resulting from intravascular hemolysis has been shown to inactivate ADAMTS13.\textsuperscript{29} Furthermore, in accordance with previous reports showing that histones could induced WPB exocytosis\textsuperscript{30}, we observed elevated concentrations of nucleosomes in iTTP plasma samples which weakly participated in iTTP-induced Ca\textsuperscript{2+}-mediated EC. DNA/histones complexes known to contribute to thrombosis have been reported elevated in the plasma of TMA patients\textsuperscript{15} and shown to induce TTP in a zebrafish model, underscoring their potential importance in iTTP pathogenesis.\textsuperscript{31} Despite previous evidence showing the amplifying role of complement in endothelial activation\textsuperscript{32}, our experiments did not show a role for complement in this very initial endothelial activation during iTTP.

To date, except for markers of tissue ischemic injury, no real prognostic factor has been reported in iTTP, although anti-ADAMTS13 antibodies of various isotypes, including IgA, may be associated with more severe forms.\textsuperscript{33,34} In our study, the amount of in vitro endothelial Ca\textsuperscript{2+} flux induced by 62 iTTP plasma or serum samples appeared to be associated with disease severity and death, whereas neither free heme, nor nucleosome concentrations correlated with iTTP severity. This result may indicate that several plasmatic components including anti-ADAMTS13 IgG, free heme, nucleosomes and possibly others converge to induce Ca\textsuperscript{2+}-mediated EC activation. We have previously observed in
a prospective study that circulating EC counts correlated with disease severity.\textsuperscript{35} Thus, iTTP early prognosis and death may be related to the intensity of endothelial activation/injury. Moreover, the identification of Ca\textsuperscript{2+} as the main cellular messenger of iTTP-induced WPB degranulation may possibly help to design a new therapeutic strategy targeting endothelial activation.


TABLE 1: Demographics, clinical and biological data of the 62 iTTP patients included from Marseille and Paris.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>iTTP, n = 25, MARSEILLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median [IQR]</td>
<td>39.5 [26.5-52.8]</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
</tr>
<tr>
<td>Neurological involvement, n (%)</td>
<td>14 (56)</td>
</tr>
<tr>
<td>Mortality, n (%)</td>
<td>3 (12)</td>
</tr>
<tr>
<td>Laboratory features</td>
<td></td>
</tr>
<tr>
<td>Platelet (G/L)</td>
<td>13 [11-21]</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>81 [69-99]</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>1228 [1027-1577]</td>
</tr>
<tr>
<td>Presence of schistocytes, n (%)</td>
<td>25 [100]</td>
</tr>
<tr>
<td>ADAMTS13 activity (%)</td>
<td>5 [0.5-6.5]</td>
</tr>
<tr>
<td>Anti-ADAMTS13 IgG (IU/mL)</td>
<td>87 [49.5-113.5]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Demographics</th>
<th>iTTP, n = 62, MARSEILLE (25) + PARIS (37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median [IQR]</td>
<td>43 [28.8-54]</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>18 (30)</td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
</tr>
<tr>
<td>Neurological involvement, n (%)</td>
<td>29 (46.8)</td>
</tr>
<tr>
<td>Mortality, n (%)</td>
<td>21 (32.3)</td>
</tr>
<tr>
<td>Laboratory features</td>
<td></td>
</tr>
<tr>
<td>Platelet (G/L)</td>
<td>13 [10-21]</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>84 [68-101.8]</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>1535 [1115-2461]</td>
</tr>
<tr>
<td>Presence of schistocytes, n (%)</td>
<td>58 [93.5]</td>
</tr>
<tr>
<td>ADAMTS13 activity (%)</td>
<td>0 [0-5]</td>
</tr>
<tr>
<td>Anti-ADAMTS13 IgG (IU/mL)</td>
<td>94 [50-120]</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1: Induction of Weibel-Palade bodies exocytosis on endothelial cells after stimulation with iTTP plasma

(A) Confocal microscopy analysis of permeabilized human microvascular endothelial cells from derm (HMVEC-d) incubated with either control plasma (a) or immune thrombotic thrombocytopenic purpura (iTTP) plasma samples (b) using rabbit anti-von Willebrand Factor (VWF) antibodies and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (original magnification X63, scale bar=10µm). (B) Quantification of VWF fluorescence intensity (FI) after HMVEC-d stimulation with either control plasma (Control: n=9) or iTTP plasma samples during the acute phase (iTTP: n=14. ***P <.001). (C) Kinetics of VWF HMVEC-d exocytosis visualized by fluorescence microscopy after incubation with iTTP plasma samples (original magnification X40, scale bar=10µm). (D) Quantification of VWF FI over time on HMVEC-d stimulated with iTTP plasma samples (iTTP: n=5, *P <.05, ** P <.01). (E) Endothelin-1 and (F) angiopoietin-2 concentrations in HMVEC-d supernatants stimulated with either control plasma (n=6) or iTTP plasma samples for 1h (n=25, * P <.05). (G) P-selectin expression detected by confocal microscopy analysis using anti rabbit P-selectin antibodies and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (green) in HMVEC-d incubated with control (a) or iTTP plasma samples (b) (original magnification X63, scale bar=10µm). (H) Percentage of P-selectin FI per cell on HMVEC-d stimulated with control plasma (n=10) (representing 100%) or iTTP plasma samples (n=23, *** P <.001).

Figure 2: Weibel-Palade body exocytosis induced by iTTP plasma is Ca²⁺-mediated

(A) VWF secretion detected by fluorescence microscopy, using rabbit anti-VWF antibodies and Alexa Fluor 594-conjugated donkey anti-rabbit IgG after incubation of HMVEC-d with iTTP plasma samples in the absence (a-b) or in the presence of the Ca²⁺ chelator MAPTAM (c-d) (original magnification X40, scale bar=30µm). (B) VWF FI quantification by cell comparing HMVEC-d stimulated with iTTP plasma samples in absence or presence of MAPTAM (iTTP: n=5, ** P <.01). (C) Endothelin-1 concentration measured in the supernatants of HMVEC-d incubated with iTTP plasma samples (n=10).
in the absence (representing 100%) or in the presence of MAPTAM (**P <.001). (D) Fluorescence intensity (AU) of the Ca\(^{2+}\) flux in HMVEC-d incubated for 20s with either control plasma samples (n=13), iTTP plasma samples (n=25, ***P <.001) or A23187 Ca\(^{2+}\) ionophore (n=3). (E) AUC (area under the curve) (cm\(^2\)) from Ca\(^{2+}\) flux curves after 30 min of HMVEC-d incubation with either control plasma (n=13), iTTP plasma samples (n=25, ***P <.001) or A23187 Ca\(^{2+}\) ionophore (n=3).

**Figure 3:** In vitro endothelial Ca\(^{2+}\) flux intensity induced by iTTP plasma is associated with clinical severity and prognosis

(A) Fluorescence intensity (AU) of the Ca\(^{2+}\) flux measured at 20 seconds in HMVEC-d stimulated with iTTP plasma samples of survivors (n=41) or non-survivors patients (n=21, **P <.01). (B) AUC from Ca\(^{2+}\) flux curves after 30 min of HMVEC-d stimulated with iTTP plasma samples of survivors (n=41) or non-survivors (n=21, ***P <.001). (C) Fluorescence intensity (AU) of the Ca\(^{2+}\) flux in HMVEC-d incubated for 20s with plasma samples from iTTP patients in acute phase (iTT-P-AP) or from same patients in remission (iTT-P-R) (n=11, **P <.01). Horizontal dotted line represents median Ca\(^{2+}\) mobilization induced in HMVEC-d by control plasma. (D) VWF secretion and tethering detected by fluorescence microscopy using rabbit anti-VWF antibodies and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (red) after 1h-incubation of HMVEC-d with iTTP plasma samples collected in acute phase (a) or during remission (b). Cell nuclei are labelled in blue using DAPI (original magnification X40, scale bar=10µm). (E) VWF fluorescence quantification after the HMVEC-d stimulation with iTTP plasma from patients in acute phase (iTT-P-AP: n=4), or in remission (iTT-P-R: n=4. * P <.05).

**Figure 4:** IgG contained in iTTP plasma are mainly involved in HMVEC-d activation

(A) VWF secretion detected by fluorescence microscopy after HMVEC-d incubation with complete iTTP (a) or IgG-depleted (b) plasma samples (original magnification X20, scale bar=30µm). (B) Fluorescence intensity (AU) of the Ca\(^{2+}\) flux induction in HMVEC-d after 20 seconds incubation with complete iTTP or IgG-depleted plasma samples (n=15, ***P <.001). (C) VWF secretion detected by fluorescence microscopy after HMVEC-d incubation with IgG purified from control (a) or iTTP plasma samples (b) (original magnification X40, scale bar=30µm). (D) VWF quantification by ELISA in the
supernatants of cells treated with IgG purified from control (n=7) or iTTP plasma samples (n=16, *P <.05). (E) Fluorescence intensity (AU) of Ca²⁺ flux induction in HMVEC-d after 20 seconds incubation with IgG from control (n=6) or iTTP plasma samples (n=16, **P <.01). (F) VWF secretion detected by fluorescence microscopy after HMVEC-d incubation with IgG (a) from iTTP plasma samples or F(ab)’2 fragments (b) purified from these IgG (original magnification X63, scale bar=20µm). (G) Fluorescence intensity (AU) of Ca²⁺ flux induction in HMVEC-d after 20 seconds incubation with medium alone (EBM2), A23187 calcium (Ca²⁺) ionophore, IgG (IgG) from iTTP plasma samples, or F(ab)’2 fragments purified from these IgG (n=10, **P <.01).

**Figure 5: ADAMTS13 is involved in iTTP IgG-induced Weibel-Palade bodies degranulation**

(A) VWF quantification by ELISA in the supernatants of cells treated with iTTP serum (n=5) or hereditary TTP serum samples (hTTP, n=5, *P <.05). (B) Fluorescence intensity (AU) of Ca²⁺ flux induction in HMVEC-d after 20 seconds incubation with serum from iTTP patients (n=5) or from hTTP patients (n=5, **P <.01). (C) ADAMTS-13 mRNA relative quantification in HMVEC-d cultured in the presence of control siRNA (Ct si) or siRNA targeting ADAMTS13 (ADAMTS13 Si) (n=6, *P <0.05). (D) Fluorescence intensity (AU) of Ca²⁺ flux induction in HMVEC-d transfected with control or anti-ADAMTS13 siRNA, then stimulated for 20 seconds with IgG from iTTP plasma samples (n=15, **P <.01). (E) VWF expression detected by fluorescence microscopy in HMVEC-d incubated with IgG from control patient samples (a) TTP73-1 mAb (b) or ELH2-1 mAb (c) (original magnification X40, scale bar=30µm). (F) VWF or (G) endothelin-1 concentrations in supernatants of HMVEC-d incubated with IgG from control patient samples (Control) (n=3), TTP73-1 or ELH2-1 mAb (n=5) (*P <.05). (H) Fluorescence intensity (AU) of Ca²⁺ flux measured at 20s in HMVEC-d stimulated in a similar fashion than in (G) (n=3, ns, Not Significant).

**Figure 6: Heme and nucleosomes participate of the iTTP patient’s plasma induced Ca²⁺-mediated endothelial activation**

(A) Free heme concentration (µM) measured in control (n=13), iTTP patients in acute phase (iTTP-AP, n=62) or iTTP patients in remission (iTTP-R, n=14) plasma samples (*P <.05, ***P <.001). (B) VWF
expression detected by fluorescence microscopy after HMVEC-d incubation with heme or iTTP plasma samples in the absence (a-b) or in the presence (c-d) of hemopexin (+Hx) (original magnification X40, scale bar=30µm). (C) Endothelin-1 concentrations in the supernatants of HMVEC-d incubated with iTTP plasma samples in the absence (representing 100%) or in the presence of hemopexin (n=14, *P <.05). (D) Fluorescence intensity (AU) of the Ca²⁺ flux induction in HMVEC-d after 20-seconds incubation with iTTP plasma samples alone or after preincubation with hemopexin (n=18, **P <.01). (E) Total nucleosomes in control (n=13), iTTP patients in acute phase (iTTP-AP, n=62) or iTTP patients in remission (iTTP-R, n=14) plasma samples (**P <.001). (F) VWF secretion detected by fluorescence microscopy after incubation of HMVEC-d with iTTP plasma samples in the absence (a) or in the presence of Annexin V (b) (original magnification X40, scale bar=30µm). (G) Endothelin-1 concentrations in the supernatants of HMVEC-d incubated with iTTP plasma samples in the absence (representing 100%) or in the presence of Annexin V (n=16, **P <.01). (H) Fluorescence intensity (AU) of the Ca²⁺ flux induction in HMVEC-d after 20-seconds incubation with either iTTP plasma samples alone, or in the presence of Annexin V (n=17, **P <.01).
FIGURE 5
SUPPLEMENTARY METHODS

Patient characteristics

Twenty-five patients were diagnosed in Marseille with immune thrombotic thrombocytopenic purpura (iTTP), using the following criteria: ischemic and/or hemorrhagic clinical events associated with mechanical hemolytic anemia (hemoglobin<100g/L and presence of schistocytes>2% on blood smears), peripheral thrombocytopenia (<150x10^9/L), low plasmatic ADAMTS13 activity (<10%) (TECHNOZYME® ADAMTS-13 (Technoclone, Vienne, Autriche)) and detection of anti-ADAMTS13 auto-antibodies in serum (>20 IU/mL) (Table 1). For each patient in the acute phase of the disease, platelet poor plasma was obtained from the first plasma exchange (PEX). For 10 patients, plasma on sodium citrate and serum were also obtained by peripheral vein puncture. Plasma from 14 of the 25 patients was also obtained during remission defined as normal hemoglobin and platelets, plasma ADAMTS13 activity >80%, and absence of anti-ADAMTS13 autoantibodies in serum 3-6 months after acute phase by vein puncture on sodium citrate. Plasma from patients with autoimmune neurologic diseases (myasthenia gravis or polyradiculoneuritis, n=13) treated by PEX constituted negative control. For 5 iTTP patients, von Willebrand Factor (VWF)-free plasma was prepared.

To study the correlation between endothelial activation and severity of the disease, in addition to the cohort from Marseille, we also included 37 iTTP patients from the French Reference center in Paris according to the same inclusion criteria for a total of 62 patients. (Table 1). Non-survivors patients were defined by the occurrence of an all-cause mortality in the acute phase of iTTP, i.e., occurring between the diagnosis and the remission phase.

To evaluate the role of anti-ADAMTS13 IgG, we also studied 5 serum samples from patients with acute episodes of hereditary TTP (hTTP) seen in the French Reference center, Paris. These patients were all tested negative for anti-ADAMTS13 autoantibodies.
**Cells**

Human microvascular dermal endothelial cells (HMVEC-d) and microvascular endothelial cell growth medium-2 bullet Kit (EGM2-MV) were from Lonza (Vivier, Switzerland) and were used between passages 3 and 7 in static conditions. Human umbilical vein endothelial cells (HUVEC) were grown in endothelial growth medium-2 bullet Kit (EGM2) from Lonza and used between passages 2 and 4.

**Flow chamber**

The formation of UL-VWF strings secreted by iTTP plasma-activated HMVEC-d was studied under flow conditions in a parallel-plate flow chamber device with HMVEC-d grown in endothelial cell growth basal medium-2 (EBM2) from Lonza (Vivier, Switzerland) containing 1% Fetal Bovine Serum (FBS). Briefly, a syringe pump connected to the outlet port injected thrombin, 1/100-diluted control or iTTP plasma samples through the chamber at 0.2ml/min in order to generate a shear rate of 4dyn/cm². After 1h of flow, the cells were washed, fixed in 1% paraformaldehyde for 10 minutes and labelled as described in immunofluorescence studies.

**Intracellular Ca^{2+} flux measurement by microscopy**

HMVEC-d were incubated for 1h with 5µM fluo-4 AM fluorescent dye in phosphate buffer saline (PBS) containing 1% of Bovine Serum Albumin (BSA), washed and incubated in PBS 1% BSA for 30min at 37°C. Cells were stimulated with 30µg of IgG from control or iTTP plasma samples, EBM2 or A23187 10µM. The fluorescence was captured every second during 120 seconds and total fluorescence quantified using a fluorescence microscope (Nikon ECLIPSE TE2000U) equipped with a CCD camera.

**Immunofluorescence experiments and quantification**

After 1h stimulation, confluent HMVEC-d were washed twice in PBS, fixed in 1% paraformaldehyde for 10 minutes and labelled with anti-VWF (Dako, Denmark), anti-P-selectin antibodies (1/400 dilution) (BD Biosciences, Europe) or rabbit non-immune serum (1/100 dilution) (ThermoFisher Scientific, USA) for 1h at RT. To visualize VWF and P-selectin, the secondary antibodies Alexa Fluor 594-conjugated or Alexa Fluor 488-conjugated donkey anti-rabbit IgG, respectively, were used (1/1000, ThermoFisher Scientific, USA) for 1h. Nuclei was then stained with DAPI (4’6-di-amidino-2-phenylindole)
To study Weibel-Palade Bodies degranulation, the same experiment was repeated in 1% Triton-X permeabilized HMVEC-d. Quantification of IF was performed using a previously reported method. Images of VWF secretion and membrane tethering were obtained with a fluorescence microscope (Nikon ECLIPSE TE2000U) equipped with a CCD camera. Images of VWF degranulation on 0.02% saponin permeabilized cells and of P-selectin were realized with a confocal microscope (Leica TCS SP5). For quantification of VWF immunofluorescence, a trained operator opened with a drawing program (Microsoft, USA) the TIFF-formatted 8-bit RGB fluorescence images, and surrounded in color each cell and its exocytosed VWF. A program written in Java (Oracle, USA) incorporated in ImageJ (National Institute for Health, USA) detected the border and counted the number of pixels \( n_i \) inside for each VWF-antibody fluorescence intensity \( i \) (from 0 to 255). Total signal for an image was \( S = (\sum_i i \times n_i) / N \) where \( N \) was the number of cells in the image. Images contained 6 or 7 cells and five images at least were analyzed per condition. For quantification of the P-selectin, the total fluorescence intensity (FI) was measured with ImageJ on each image and this FI was normalized to cell number. Images contained 3 to 5 cells and five images at least were analyzed per condition.

Some experiments were made in the presence of \( \text{Ca}^{2+} \) ionophore A23187 (10 \( \mu \)M), of calcium chelator MAPTAM (1,2-bis-5-methylaminophenoxylethane-NNN'-tetraacetoxymethyl acetate) (1\( \mu \)M) from Calbiochem (Merck Chemicals Ltd, Nottingham, UK), thrombin (\( \geq 2000 \) NIH units/mg, 4IU/mL or 0.5\( \mu \)M), hirudin (40U), hemopexin (170\( \mu \)M), Annexin V (10ng/mL), PPACK (10\( \mu \)M) from Sigma Aldrich (Saint Louis, USA), decomplemented-iTTP-plasma (1/100) (heating for 30 minutes at 56\(^\circ\)C) or anti-C5 monoclonal antibody 200\( \mu \)g/mL (Eculizumab, Alexion, France).

To visualize ADAMTS13, HMVEC-d were stimulated, washed in PBS and labeled with anti-ADAMTS13 (1/500 dilution) (ThermoFisher Scientific, USA) for 1h at 4\(^\circ\)C, then fixed in 1% paraformaldehyde for 10 minutes, before labeling with secondary Alexa Fluor 488-coupled anti-mouse IgG (ThermoFisher Scientific, USA) and total fluorescence quantification. DAPI labelling (1/5000 dilution) was performed for nucleus visualization.
Preparation of VWF-free plasma

VWF-free plasma was obtained using a purification column (Microlink protein coupling kit, Thermo Fisher Scientific,) coupled with rabbit anti-human VWF antibodies. VWF concentrations were measured by enzyme linked-immunoSorbent assay (ELISA) (Thermo Fisher Scientific, Rockford, USA) in control and iTTP plasma samples before and after passage on column and 99% of the VWF concentration was lost.

Quantification of IgG and purification of F(ab)’2 fragments

IgG quantification in native plasma, depleted plasma or eluted IgG were realized with Fast ELISA kit dosage (RD-Biotech, Besançon). F(ab)’2 fragments were isolated with PIERCE F(ab)’2 fragment preparation kit (Thermo Fisher Scientific, Rockford, USA).

Quantification of nucleosomes

Nucleosomes were quantified by ELISA (Cell Death Detection ELISA Plus, (Sigma Aldrich, Saint Louis, USA) according to the manufacturer’s instructions. One unit of nucleosomes refers to the average amount of nucleosomes quantified in plasma from negative controls.

Determination of plasma heme concentrations

Heme concentrations were measured in diluted plasma (1/10 in PBS) as described by Belcher et al. In diluted plasma (1/10 in PBS), the free heme concentration was determined using oxidation of the 3,3’,5,5’-tetramethylbenzidine (Turbo-TMB, Thermo Fisher scientific, Waltham, MA) by the pseudoperoxidase activity of heme. The reaction was stopped by adding sulfuric acid (2M) and the absorbance was measured at 450 nm.

Statistical analysis

Values are presented as median with interquartile range (25% and 75% percentile) for the indicated number of experiments. Data are compared using non-parametric Mann-Whitney two-tailed test with Prism software in all data, except to compare the effects of the plasma from the same patients obtained in acute phase or remission, with or without treatment with hemopexin, Annexin V, eculizumab, hirudin, plasma heating or depleted in IgG in which Wilcoxon matched-pairs signed rank
test was used. A p value less than 0.05 was considered statistically significant.
SUPPLEMENTARY REFERENCES


SUPPLEMENTARY FIGURE LEGENDS

Figure S1: iTTP plasmas induced-VWF release in endothelial cells in different conditions

(A) VWF string formation detected by fluorescence microscopy on HMVEC-d incubated with either control (a), or iTTP plasma samples (b) in dynamic conditions using a flow chamber (n=3) (original magnification X40, scale bar=10µm). (B) VWF string formation detected by fluorescence microscopy in HUVECs incubated with either control (a), or iTTP plasma samples (b-c) (original magnification X40, scale bar=10µm). (C) VWF string formation detected by immunofluorescence microscopy in HMVEC-d incubated with (a) iTTP plasma obtained by PEX, (b) iTTP serum or (c) iTTP citrated plasma from the same patient during the acute phase of the disease (original magnification X40, scale bar=10µm). (D) VWF concentrations in the supernatants of HMVEC-d stimulated with VWF-free plasma samples (n=5, * P<.05). (E) Endothelin-1 concentrations in the supernatants of HUVECs incubated with control or iTTP plasma samples (n=7, ** P<.01). (F) Endothelin-1 concentrations (pg/mL) in supernatants of HMVEC-d incubated with iTTP plasma obtained by PEX (n=8), iTTP serum (n=8, *P<.05) or iTTP citrated plasma (n=8, P: ns). (G) Fluorescence intensity (AU) of the Ca^{2+} flux in HUVECs after 20 second-incubation with either control (n=5) or iTTP plasma samples (n=17, ** P <.01). (H) Fluorescence intensity (AU) of the Ca^{2+} flux at 20s in HMVEC-d treated with iTTP plasma obtained by PEX (n=10), iTTP serum (n=10) or iTTP citrated plasma (n=10).

Figure S2: IgG-induced calcium flux on microscope observations

(A) Ca^{2+} flux filmed on living cells during 120s after stimulation with medium alone (EBM2), iTTP IgG or control IgG. (B) Quantification of the Ca^{2+} flux filmed on living cells treated as in (A). Time 0 correspond to cell stimulation with A23187 (orange line), iTTP IgG (red line) or control IgG (blue line). The quantification of each condition is normalized with the EBM2 stimulation (green line).

Figure S3: ADAMTS13 expression on endothelial cells is not modified by incubation with IgG from iTTP plasma patients

(A) ADAMTS13 detection by fluorescence microscopy on HMVEC-d incubated with medium alone (EBM2) (a), control IgG (b) or iTTP IgG (c) (original magnification X40, scale bar=50µm) (B) ADAMTS13
FI quantification in HMVEC-d incubated with either EBM2 (representing the 100%), control IgG or iTTP IgG (n=7, P = ns).

Figure S4: Heme and nucleosomes circulating concentrations are not correlated with iTTP severity
(A) Heme concentrations (µM) in survivors (n=41) and non-survivors (n=21) patient’s plasmas (P = ns).
(B) Nucleosomes quantification in survivors (n=41) and non-survivors (n=21) patient’s plasmas (P = ns).

Figure S5: Complement and thrombin are not involved in iTTP plasma induced endothelial activation
(A) VWF secretion detected by immunofluorescence on HMVEC-d incubated with either iTTP plasma (a), heated-iTTP plasma (b) or iTTP plasma samples in the presence of eculizumab (c) (original magnification X40, scale bar=30µm). (B) Endothelin-1 concentrations (pg/mL) in the supernatants of HMVEC-d incubated with either iTTP plasma (n=17), heated-iTTP plasma (n=9) or iTTP plasma samples in the presence of eculizumab (n=8). (C) Fluorescence intensity of the Ca²⁺ flux at 20s in HMVEC-d incubated with iTTP plasma (n=25), heated iTTP plasma (n=14) or iTTP plasma samples with eculizumab (n=12). (D) Fluorescence intensity of the Ca²⁺ flux at 20s in HMVEC-d incubated with iTTP from PEX in presence or absence of hirudin (n=18). Horizontal dotted line represents median Ca²⁺ mobilization induced in HMVEC-d by control plasma. (E) VWF concentrations (ng/mL) in the supernatants of HMVEC-d incubated with medium (EBM2), thrombin (THR), iTTP plasma (n=10) in presence or not of PPACK 10µM. (F) Fluorescence intensity of the Ca²⁺ flux at 20s in HMVEC-d treated with medium (EBM2), A23187 or iTTP plasma (n=10) in presence or not of PPACK 10µM.

SUPPLEMENTARY VIDEOS LEGENDS

Supplementary Figure S2A medium (EBM2): Ca²⁺ flux filmed on living cells during 120s after stimulation with medium alone (EBM2) (original magnification X10, scale bar=50µm).

Supplementary Figure S2A iTTP IgG: Ca²⁺ flux filmed on living cells during 120s after stimulation with 30 µg of IgG from iTTP patient’s plasma (original magnification X10, scale bar=50µm).

Supplementary Figure S2A Control IgG: Ca²⁺ flux filmed on living cells during 120s after stimulation with 30 µg of IgG from control patient’s plasma (original magnification X10, scale bar=50µm).
FIGURE S2

(A) Photographs of tissue samples stained with specific markers.

(See Online Supplementary Videos)

(B) Graph showing calcium flux over time for different conditions.

Calcium flux (AU) vs. time (sec)
FIGURE S4

A

B