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Diana Fernández  
Universidad de Antioquia

Cesar Segura  
Universidad de Antioquia

Mònica Arman  
Hull York Medical School

Suzzane McGill  
University of Glasgow Institute of Infection Immunity and Inflammation

Richard Burchmore  
University of Glasgow Institute of Infection Immunity and Inflammation

Tatiana María Lopera Mesa  
Universidad de Antioquia  
mailto:tloperamesa@gmail.com  
https://orcid.org/0000-0002-9401-2779

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Uncomplicated *Plasmodium vivax* malaria: Mapping the proteome from circulating platelets

Diana Fernández¹, Cesar Segura¹, Mònica Arman², Suzanne McGill³, Richard Burchmore³, Tatiana Lopera-Mesa¹,

¹. Malaria Group, Facultad de Medicina, Universidad de Antioquia, Medellin, Colombia.
². Centre for Atherothrombosis and Metabolic Disease, Hull York Medical School, Faculty of Health Sciences, University of Hull, Hull, UK.
³. Institute of Infection, Immunity and Inflammation and Glasgow Polyomics, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

# Corresponding author:
Tatiana Lopera: tloperamesa@gmail.com
Phone: +57 4 2196490

Universidad de Antioquia, Medellin, Colombia
Sede de investigación Universitaria, Calle 62 # 52-59, Torre 1, Lab 610
ABSTRACT (350 words)

Background: Thrombocytopenia is frequent in uncomplicated Plasmodium vivax malaria. Contribution of platelets to pathogenesis is unknown and poorly understood. Our study explores the platelet proteome from uncomplicated P. vivax malaria patients to fingerprint molecular pathways in relation to platelet function. Also, plasma levels of platelet activation (Platelet factor 4 – PF4/CXCL4) and endothelial activation (Von Willebrand factor – VWf) markers, in conjunction with some in vitro interactions between platelets and P. vivax infected erythrocytes (Pv-IEs) were measured to explore platelet responses during infection and their effect on parasite development.

Methods: This study was performed in a cohort of 48 patients and 25 healthy controls. Platelets were purified from a subgroup of 5 patients and 5 healthy controls to be analyzed by LC-MS/MS. In all participants enrolled in this study, PF4/CXCL4 and VWf plasma levels were measured. Finally, a subsample of 10 P. vivax isolates were co-cultured with platelets to measure Pv-IE schizonts inhibition as well as platelet activation due to their interaction.

Results: In total 28 out of 215 proteins were significantly abundant in the proteomes from patients. The most significantly decreased protein was PF4/CXCL4 followed by other proteins related to platelet activation, cytoskeletal remodeling, and adhesion to endothelial cells. In contrast, acute phase proteins including SERPINs and Amyloid Serum A 1 (SAA1) were increased. High VWf plasma levels in patients suggested endothelial activation. Interestingly, PF4/CXCL4 plasma levels were similar between patients and controls, but high levels of this protein were found in co-cultures, and platelets inhibited Pv-IEs development to schizonts.

Conclusions: Platelet proteome from patients with uncomplicated P. vivax malaria suggests platelet degranulation, platelet activation, cytoskeletal remodeling, and adhesion to endothelial cells. According to the evidenced endothelial activation our study plus the suggested specific localization of PF4/CXCL4 during P. vivax infection due to the normal levels in plasma, and the inhibition of Pv-IE schizonts development; our study suggest that platelets are active players during the response to P. vivax infection. Future studies are needed to further investigate the molecular pathways of interaction between altered platelet proteins and host response; which could affect parasite control as well as disease progression.

Key words: Plasmodium vivax, thrombocytopenia, platelets proteome
INTRODUCTION

*Plasmodium vivax* infection is highly spread outside Africa (1). South America, Colombia, is a low endemic malaria region where *P. vivax* accounts ~50% of malaria cases (2). Malaria pathogenesis is complicated due to immune evasion mechanisms of *Plasmodium* parasites (3). Sequestration of *P. falciparum* infected erythrocytes (*Pf*-IEs) at the microvessels is a common strategy of immune evasion (4). However, the mechanisms of evasion developed by *P. vivax* infected erythrocytes (*Pv*-IE) and their potential link with severe malaria progression are unknown.

Thrombocytopenia (reduced blood PLTs (PLTs) count), is frequent in malaria, and it has been described in ~49% of *P. vivax* malaria patients in Colombia (5). The causes of thrombocytopenia in *P. vivax* infection are unclear, but they have been related to increased PLT destruction (including the production of temporary anti-PLTs antibodies and destruction by macrophages) or higher activation/consumption (6-13), rather than decreased production. Despite the high frequency of thrombocytopenia, PLTs – *Pv*-IEs interactions and the PLTs role in pathogenesis are poorly understood.

PLTs have several functions including regulation of hemostasis, vascular integrity, inflammation and immune response (14). In *P. falciparum* malaria, PLTs are involved in sequestration of IEs into the microvasculature (3, 15) by attaching to both IEs and endothelial cells (ECs) acting as bridges between them (7, 16). In suspension conditions, the direct binding between PLTs and *P. falciparum* IEs (*Pf*-IEs) can form mixed aggregates, a phenotype known as PLT-mediated clumping (11). Moreover, PLT activation is believed to contribute to cerebral vascular damage by inducing expression of EC adhesion molecules, leading to more PLT-endothelial interactions and cerebral vascular localization of IEs (16, 17). In contrast, in *P. vivax* malaria, IE sequestration is rare, but the imbalanced pro-inflammatory response is higher than in *P. falciparum* infection (18). The potential
binding of PLTs to \textit{Pv}-IEs causing clumping, and the involvement of PLTs in \textit{P. vivax}-related inflammatory processes are unknown. Importantly, a protective role for PLTs in malaria through PLT-mediated parasite killing via secretion of PF4/CXCL4 has been described in all significant \textit{Plasmodium} species infecting humans (13, 19, 20), and it requires further investigation.

PLT structure, functions, and molecular activation pathways are better understood in the context of cardiovascular diseases (21). An estimated ~5,000 PLT proteins, ~13,600 protein-protein interactions, and 229 PLT kinases have been described (http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de/plateletweb.php) based on several proteomic approaches (22, 23). These studies have shed some light on the function of PLTs in hemostasis. However, the role of PTLs in immune response to infections has been less studied. Recently, the PLT proteome from patients with Dengue Virus, where thrombocytopenia is a hallmark, evidenced alterations in PLT proteins expression (e.g., PF4/CXCL4 and HLA class I proteins associated with antigen processing and presentation) (24).

\textbf{MATERIALS AND METHODS}

\textbf{Ethical considerations}

This study was performed in accordance with relevant guidelines approved by the ethics committee of the School of Medicine at Universidad de Antioquia (Reference #002, February 2017). Patients and healthy volunteers accepted to participate in the study through the approval of a written informed consent. An appropriate guardian's consent also approved by the ethics committee was taken for patients under 18 years.

\textbf{Study population}
Patients with uncomplicated *P. vivax* malaria and healthy controls with similar age (∆ five years old difference) were enrolled from Quibdo (Choco, Colombia: latitude 5.6918802, longitude -76.6583481) and Apartado (Uraba, Colombia: latitude 7.8829899, longitude -76.6258698) to study their PLTs proteome as well as PLT – *Pv*-IEs interactions *in vitro*.

**Diagnosis of *P. vivax* mono-infection**

Detection of *P. vivax* in patients and healthy volunteers was performed by thick blood film as previously established (25). The diagnosis was confirmed by nested PCR as previously described for all experimental approaches described below (26). Whole blood with EDTA (4mL) was collected to prepare thick blood films and to visualize the differential stages of *P. vivax* by Giemsa staining (rings, mature trophozoites, schizonts, and gametocytes). Parasitemia was recorded using light microscopy by counting the number of parasites in 200 leukocytes (parasites/µL). Only isolates with >2,000 asexual parasites/µL were used for *in vitro* studies as previously suggested (27).

To confirm *P. vivax* mono-infection, a total of 200µL EDTA-whole blood were dried at room temperature in filter paper (Whatman #3) for DNA extraction following the manufacturer instructions (Qiagen DNA minikit, Ref: 51306). *Plasmodium* spp. genus was detected by universal PCR (uPCR), using generic primers (0.250µM) rPLU1 (5′- TCA AAG ATT AAG CCA TGC AAG TGA -3′) and rPLU5 (5´- CCT GTT GTT GCC TTA AAC TTC-3´), MgCl₂ (2mM), dNTPs (0.2 mM per nucleotide), Taq DNA Polymerase (2 U/µL) and 2µL DNA. The uPCR amplification was performed as follow: 1 cycle of 94°C x 4 minutes; 35 cycles of 94°C x 30 seg, 55°C x 1 minute and 72°C x 1 minute and one cycle of final extension at 72°C x 4 minutes. After that, *P. falciparum* or *P. vivax* species were detected by nested PCR (nPCR) using specific primers (0.250 µM) rFAL1 – rFAL2 (*P. falciparum*) and rVIV1 – rVIV2 (*P. vivax*), MgCl₂ (2mM), dNTPs (0.2 mM per nucleotide), Taq DNA Polymerase (2 U/µL) and 1µL of each uPCR product and (26). The nPCR amplification was performed as follows: one
cycle of 94° C x 4 minutes; 35 cycles of 94°C x 30 seg, 58°C x 1 minute and 72°C x 1 minute
and one cycle of final extension at 72°C x 4 minutes. All the nPCR products were visualized
using electrophoresis at 1.5% agarose with Gel Red staining.

PLT proteomes from patients with uncomplicated vivax malaria

For the PLT proteome study, a total of five patients with uncomplicated \textit{P. vivax} malaria and
five healthy controls with similar age (± five years old difference) were enrolled. Whole
citrated blood (30mL) was collected from each participant and sent to the Malaria Reference
Laboratory within 4-6 hours post collection to immediately extract the proteins. One limitation
of this study was the small sample size due to the difficult selection of proper samples for
shipping to the Malaria Reference Laboratory between 4 – 6 hours post collection, since it
is well-known PLTs are very susceptible to undergo activation.

Prostacyclin (PGI\textsubscript{2}) at 4\,\mu M was added to the samples to centrifuge at 100g x 20 minutes at
room temperature (RT) and separate PLT rich plasma (PRP). PGI\textsubscript{2} was added at 4\,\mu M to
the remaining whole blood, and PLT poor plasma (PPP) was obtained by centrifugation at
1,200g x 20 minutes at RT. The PPP was frozen at -20°C for further analysis (described
below). To obtain PLTs, PRP was mixed 1:1 with HEPES buffer plus PGI\textsubscript{2} at 0.4\,\mu M and
centrifuged at 200g x 15 minutes (RT) to precipitate the remaining leukocytes and red blood
cells. The supernatant was collected, and PGI\textsubscript{2} added at 0.4\,\mu M to precipitate the pure PLTs
at 900g x 15 minutes (RT). Pelleted PLTs were recovered and washed three times without
suspension of the pellet using washing buffer (sodium citrate 10mM, 150 mM NaCl, 1mM
EDTA, 1\% (w/v) dextrose, pH 7.4). The isolated PLTs were suspended in 200\,\mu L water milliQ
with Roche's Complete™ Protease Inhibitor Cocktail (1X) to extract proteins by five cycles
of freezing-thawing and centrifugation at 7,000g x 20 min (4°C). The quality and integrity of
extracted proteins were confirmed by 1D-electrophoresis (SDS-page) and Silver
staining (Fig 1). The samples were stored at -80°C until proteomic analysis at the Glasgow Polyomics Institute, University of Glasgow (United Kingdom).

The extracted proteins were digested using trypsin, and peptides were quantified using the filter-aided sample preparation (FASP) protocol previously described (28). A pooled internal standard (IS) was prepared with equal amounts of peptides from patients and healthy controls to normalize the data. Then, the peptides from samples and IS were tagged using 6-plex TMT® Mass Tagging Kit (Thermo Scientific) for LC-MS/MS analysis using an Orbitrap Elite MS (Thermo Scientific). Peptides were desalted and concentrated for 4 minutes on the trap column before being transferred to the analytical column using starting solvent conditions (5% solvent B). A water acetonitrile gradient was used; 5-45% v/v solvent B from 4-154 min, 45-100% v/v solvent B 154-154.1 min, held at 100% v/v solvent B 154.1-160 min and then re-equilibrated at starting conditions 5% solvent B for a total time of 165 minutes. A fixed solvent flow rate of 0.3 µl/min was used for the analytical column. The trap column solvent flowed at a fixed 25 µl/min using 1% acetonitrile with 0.05% formic acid. The Orbitrap Elite acquires a high-resolution precursor scan at 60,000 RP (over a mass range of m/z 380 – 1,800) followed by CID fragmentation and detection of the top 3 precursor ions from the MS scan in the linear ion trap. The three precursor ions are also subjected to HCD in the HCD collision cell, followed by detection in the orbitrap. Singly charged ions were excluded from selection, while selected precursors were added to a dynamic exclusion list for 180s. Data analysis was done using the software Proteome Discoverer (version 1.4), excel 2010, and GraphPad Prism 5.0. Protein identifications were assigned using the Mascot and SwissProt Human databases as a search engine to interrogate protein sequences in the NCBI Genbank database, allowing a mass tolerance of 10 ppm for the precursor and 0.6 Da for MS/MS matching. The Perseus software was used for statistical paired t-test analysis and volcano plot construction. The significant differences between groups were defined by p-value <0.05 for highly confident proteins with false discovery rate >5% (FDR), Mascot
score >100, and coverage >5%. The protein-protein interactions were explored with confidence > 0.9 using online software STRING (https://string-db.org/). PLT protein functions were searched using UNIPROT (http://www.uniprot.org) and platelet web database (http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de/plateletweb.php).

**PF4/CXCL4 and VWf levels in PPP from P. vivax malaria patients**

PF4/CXCL4 levels were evaluated in PPP using the Enzyme-linked Immunosorbent Assay (R&D Systems DY795), following manufacturer’s instructions. Additionally, VWf levels were measured in citrated plasma at the Laboratorio Clinico Hematologico (Medellin, Colombia). Briefly, a total of 48 patients with uncomplicated *P. vivax* malaria and 25 healthy controls were enrolled between February – September 2018. Patients were diagnosed with *P. vivax* mono-infection as explained above, and healthy volunteers accomplished with the eligibility criteria (no cardiovascular or chronic diseases and not chronic consumption of anti-aggregate drugs, no fever-related symptoms during the last month, no malaria diagnosis during the last two months and malaria negative during enrollment confirmed by thick blood film, rapid diagnostic test, and nested PCR).

**In vitro PLT activation by *Pv*-IE parasites**

*Preparation of PLTs:* A total of 10 mL of whole citrated blood was collected from healthy volunteers to collect the PLT rich plasma (PRP) and purified PLTs as described above. Pelleted PLTs were suspended in RPMI without supplements and counted in Neubauer camera by light microscopy for further *in vitro* assays with *Pv*-IEs.

*Collection and preparation of *Pv*-IEs:* Whole blood (10 mL) in heparin was collected from patients with *P. vivax* mono-infection, > 2,000 parasites/µL, and predominantly mature trophozoite stages (27, 29). Samples were sent immediately to the reference lab for processing between 4-6 hours. The leukocytes were removed by centrifugation, and *Pv*-IE were washed using RPMI media without serum supplement for further assays.
To evaluate the PLT – *Pv*-IE interactions *in vitro*, the isolated *Pv*-IEs were concentrated at 50 – 100% parasitemia using percoll gradients at 45% as previously reported (29). The concentrated *Pv*-IEs were suspended in RPMI medium at parasitemia 5%, and hematocrit 5% without serum supplement. Co-culture of *Pv*-IEs and PLTs (2.5 x 10⁶ PLT/mL counted in Neubauer camera) were incubated 60 minutes at 37°C in N₂ (90%), CO₂ (5%), O₂ (5%).

Two negative controls were included: 1) *Pv*-IE cultured without PLTs, and 2) Uninfected erythrocytes (uEs) co-cultured with PLTs. Supernatants were collected from co-cultures and frozen at -80°C until analysis of PF4/CXCL4 levels by ELISA. All treatments were done in triplicate.

**Effect of PLTs on *Pv*-IE schizonts development**

To evaluate the effect of PLTs on *Pv*-IE schizonts development, *Pv*-IEs enriched with percoll as described above (section 2.6), were suspended in RPMI media at parasitemia 5%, hematocrit 2%, and AB+ serum 20% as previously reported (27). *Pv*-IEs were stimulated with 1) healthy PLTs previously purified (2.5 x 10⁶ PLT/mL); 2) releasates of healthy PLTs (2.5 x 10⁶ PLT/mL) previously activated by collagen [10µg/mL]; and 3) media as a control of parasite development to schizonts. All conditions were performed in triplicate on a plate of 48 wells.

The controls were monitored until schizonts (replicating parasites ≥ three chromatins) formation between 20 – 24 hours. Briefly, thick blood films from growth controls and treated wells were stained with Giemsa to determine the schizonts frequency (%) by light microscopy in a total of 100 asexual parasites (27). The frequency of gametocytes (sexual stage) was determined as a measure of parasite stress in culture. If ≥40% of schizonts were counted in growth controls, the isolate was considered successful for the analysis of PLT treatments. Schizont formation was compared between growth controls and PLTs treatments to determine the inhibition of schizonts' development.
**PLT-mediated clumping formation of Pv-IE**

PLT-mediated clumping of *Pv*-IE was measured as previously described by Arman et al, with some modifications (30). The *Pv*-IEs enriched with percoll as described above (section 2.6), were suspended in RPMI media at parasitemia 5%, and hematocrit 5% without serum supplement. Entire PLTs from healthy donors purified as described above (section 2.6), were co-cultured at different concentrations (2.5 x 10^6 – 1.0 x 10^7 PLT/mL) with *Pv*-IEs, to improve the probability of cell-cell interactions. The co-cultures were gently mixed for 60 minutes. Then, wet preparations containing acridine orange (5µg/mL) were done per duplicate, and slides were visualized by fluorescence microscopy (Leica DM500) at 200x magnification. A total of 500 *Pv*-IEs were counted per slide of wet preparation to determine the presence/absence of *Pv*-IE – PLT mediated clumping. When detected, the frequency of clumps was calculated as follows: One clump was defined as ≥3 *Pv*-IEs attached among themselves and PLTs. A *P. falciparum* reference strain from Colombia (FCB1) was used as a positive control for clumping.

**RESULTS**

**Clinical and epidemiological features from patients with *P. vivax* infection**

From a total of 48 patients and 25 healthy controls enrolled in this study, a subgroup of 5 uncomplicated *P. vivax* malaria patients (male: 5/5; 100%) and five healthy volunteers (male: 3/5; 60%) were enrolled for PLT proteome assays. All patients had *P. vivax* mono-infection and all healthy controls were negative, both confirmed by nested PCR. A total of 26 out 48 (54.2%) patients and 13 out of 25 (52.0%) of healthy controls were male. Table 1 shows the hematological parameters found in patients and controls, including normal hemoglobin and hematocrit levels in both groups. However, thrombocytopenia (PLT count <150,000/µL) was detected in 28 out of 48 (58%) patients, while all healthy controls showed normal PLT counts (150,000 – 450,000/µL).
The most common symptoms described in patients were: headache in 79% of cases, chills (79%), sweating (76%), adynamia (71%), myalgia (61%), arthralgia (58%), anorexia (58%), sickness (50%), low back pain (34%) and vomiting (32%).

Changes in PLT proteome from patients with P. vivax infection

Through TMT-labelling approaches, a total of 215 protein entries were identified at FDR=0.05% in both groups (P. vivax patients (Pv) versus healthy controls (HC)), in the SwissProt Human databank. The volcano plot depicts the protein abundances between Pv versus HC groups in LC-MS/MS and shows adjusted p-values (-log_{10}) versus the difference (Log₂) obtained by t-test analysis (Fig 2). A total of 38 PLT proteins were found to be decreased, and five proteins were increased in patients (Fig 2). However, only 23 decreased, and five increased proteins were selected for further analysis because of the high-quality identification, defined as Mascot score >100, FDR >5%, >2 peptides given match, and coverage >5% (Table 2). One limitation of this study was the low amount of PLT proteins (0.38 – 2.69 µg/µL) recovered in proteomes due to the low blood PLT count in our P. vivax malaria patients (Table 1 and Fig 1). Additionally, there was the logistic challenge of obtaining and transporting the samples from a place with remote health care access to the Malaria Reference Laboratory (Medellin, Colombia) for proteome extraction and further processing at Glasgow Polyomics Institute (UK).

Differentially expressed proteins in all-Pv patients

The decreased or increased proteins in PLT proteomes from uncomplicated P. vivax malaria patients compared to healthy controls are depicted in table 2. The decreased proteins included PF4/CXCL4 and its variant1 PF4V1/CXCL4L1, and myosins (e.g., Myosin light polypeptide 6 (MYL6), myosin regulatory light chain 12B (MYL12B), tropomyosin alpha-1 chain (TPM1) and myosin-9 (MYH9)), among others. On the other hand, the highly
increased proteins included the acute phase reactants Serum Amyloid A-1 protein (SAA1), and SERPINAs 1 and 3, followed by the immunoglobulin heavy constant gamma 2 (IGHG2).

**Altered proteins in PLTs from thrombocytopenic *P. vivax* patients**

Three out of five PLT proteomes came from patients with thrombocytopenia (PLTs <100,000/µl) and the remaining 2 PLT proteomes were from patients with normal PLT count (150,000 – 450,000/µl). In the thrombocytopenic patients (T-Pv), a total of 3 proteins were identified as more strongly decreased compared to healthy controls. Remarkably, GPV and the Immunoglobulin kappa variable 3D-11 (IGKV3D-11) had 2-fold lower expression when compared to healthy controls (Table 2). In contrast, the PF4/CXCL4 was found to be strongly decreased in T-Pv patients compared to healthy controls. The myosins MYL6, and MYL12B were similarly decreased in T-Pv patients and All-Pv, while SAA1 was increased in both groups of patients.

**Biological significance of altered proteins and network of interactions**

The function(s) of the PLT proteins identified in this study were recorded according to UNIPROT and the PLT web database (http://plateletweb.bioapps.biozentrum.university.wuerzburg.de/plateletweb.php) (Table 2). PLT expression and function of most of the identified proteins have been previously demonstrated at the experimental level, except for one protein (IGL1LC) that has been only inferred by homology to other species. Most of these proteins have been previously found in the secretome, membrane, α-granules, or extracellular vesicles released from PLTs. Moreover, the decreased proteins identified in our study are related to the dynamic process of PLT shape change, adhesion, and activation through calcium mobilization into the cell and maintenance of the endothelial barrier (Fig 3, Table 2).
The network of protein-protein interactions was built with all the differentially expressed proteins by the STRING web database. We also included the protein Von Willebrand Factor (VWF) in the network due to the substantial decrease of GPV in our proteomes, since it is well known that GPV is part of a protein complex (GPIb – GPIX – GPV) that interacts with VWF (41). As shown in fig 3, a total of 12 interacting proteins in 15 significant edges (p-value=0.0001) were found interacting between them at a high level of confidence (>90%), and seven interactions have been experimentally evidenced mainly between myosins. Further studies are needed to understand the role of these molecular interactions in the pathogenesis of \textit{P. vivax} infection and the progression of thrombocytopenia. Although a previous study reported that PLTs from malaria patients were highly activated (12), the relevance of PLT activation and blood coagulation in the pathophysiology of \textit{P. vivax} malaria needs more investigation.

\textbf{PF4/CXCL4 and VWF levels in \textit{P. vivax} infection}

To test whether the substantial decrease of PF4/CXCL4 in \textit{P. vivax} PLTs could be due to PLT secretion, the plasma levels of this protein were measured. While no significant differences were detected between PV and HC groups (Fig 4a), we found that PF4/CXCL4 plasma levels were significantly increased in patients with low PLT count compared to patients with normal PLT count (Fig 4b). PF4/CXCL4 plasma levels were not correlated with parasite density in any group (data not shown: \( r^2: -0.18; \ p=0.10 \)). In contrast to PF4/CXCL4, the plasma levels of VWF were increased in all-Pv patients compared to HC (Fig 4a and b).

\textbf{In vitro \textit{P. vivax} – PLTs interaction and PF4/CXCL4 secretion}

A total of 10 fresh \textit{P. vivax} isolates with mainly mature trophozoite stages (parasitemia median 6,800 parasites/µL; RIQ: 5,440 – 11,348) were used to test whether PLT activation, specifically the release of PF4/CXCL4, takes place in response to direct interaction with \textit{Pv}-IEs. PF4/CXCL4 levels were higher in supernatants of PLTs co-cultured with \textit{Pv}-IEs than
those of PLTs with uninfected erythrocytes (uEs). These findings suggest that PLTs can indeed get activated and release granular content upon *Pv*-IEs stimulation (Fig 5a).

**Effect of PLTs over intraerythrocytic *P. vivax* development**

In order to investigate whether entire PLTs (ePLTs) or PLT releasate compounds can impact *P. vivax* growth without the involvement of other vascular components, *Pv*-IEs were incubated *in vitro* with resting ePLTs or PLT releasates (e.g. releasates of collagen-activated PLTs). Four out of ten (40%) fresh *Pv*-IE isolates reached >40% of schizonts (Fig 5b). This percentage of successful maturation agrees with our previous study and is common in *P. vivax* cultures (42). Despite the small sample size of successful *Pv*-IEs developed to schizonts, there was found a significant reduction of schizonts in *Pv*-IE co-cultured with ePLT and PLT releasate (Fig 5c). Furthermore, after incubation with PLTs, characteristic features of dying *P. vivax* parasites were observed in Giemsa-stained samples, including the spread of parasite pigment (hemozoin) and picnotic or parasite crisis forms (Fig 5d, 5e). Also, an increase of gametocytes was observed that could be stress-related (data not shown).

Finally, none of the *Pv*-IE fresh isolates tested in the study showed the PLT-mediated clumping phenotype, compared to the quick formation of clumps by the *P. falciparum* reference strain FCB1, despite the increase on PLT concentrations (Fig 6).

**DISCUSSION**

Malaria illness is generally associated with periodic fever, chills, shivering, headache, nausea, vomiting, and many other clinical conditions (31). In *P. vivax* malaria, several clinical conditions are due to the imbalance in pro- and anti-inflammatory cytokine production (18, 32). In this study, our patients presented common malaria symptoms, and no one had criteria of severe disease. In patients with acute *Plasmodium vivax* infection, PLT counts
under 150,000/µL are frequent and vary between 24 – 94% of cases (33). The frequency of thrombocytopenia in our patients was high (58%), in agreement with previous reports in Colombia where ~50% of patients presented with low PLT counts (5, 34).

To date, the contribution of PLTs and thrombocytopenia to the progression of severe disease is poorly understood. Despite the high frequency of thrombocytopenia found in our patients (58%), we did not see differences in parasitemia between thrombocytopenic and non-thrombocytopenic Pv patients, and the median of parasitemia was <20,000 parasites/µL in all patients (e.g. values over 20,000 parasites/µL are a criterion of severity in *P. vivax* infection (5)). Although we did not select the cohort of patients according to specific ages, gender, previous malaria episodes, or clinical outcomes, those variables were not related to thrombocytopenia in this study. Further studies of larger numbers of patients with a range of Pv-related clinical conditions will be needed to address the relationship between PLT counts, parasite load, clinical outcomes, and disease severity.

In the present study, the PLT proteome from patients with acute *P. vivax* infection was analyzed to identify PLT alterations that could shed light on the PLT functional pathways that might be activated. Serum Amyloid A (SAA) was the highly increased protein detected in patients with a 2.7-fold change. SAA are acute-phase response proteins synthesized and secreted by the liver in response to inflammatory cytokines, and their plasma concentration can increase 1000-fold or more (35). Importantly, an increase in plasma levels of SAA in *P. vivax* infection has been previously reported (36) and SAA has been proposed as a potential predictive marker for malaria severity due to the activation of a proinflammatory state, as shown for previous increased in serum of *P. vivax* patients by proteomic approaches (36, 37). Since PLTs have scavenging properties (38), the increase of SAA could likely be due to PLT uptake from the serum. However, further studies are required to determine the ability of PLTs to uptake and recycle SAA proteins from the extracellular environment. Two important questions are whether SAA removal from the circulation can slow down the
transition to severe malaria, or whether PLT can secrete stored SAA with physiological or pathological consequences in *P. vivax* malaria.

Remarkably, most proteins identified in the PLT proteomes were decreased in *P. vivax* patients. Highlighting PF4/CXCL4, a small chemokine (7.8 KDa), and its variant 1 (CXCL4V1), both of them released from α-granules during PLT activation (39). PF4 has both cytokine and antimicrobial properties against bacteria, viruses, and parasites (20, 24, 40). In malaria, PF4/CXCL4 has been found as a killer of all intraerythrocytic *Plasmodium* parasites. Circulating PLTs act as hosts protectors binding directly and killing intraerythrocytic parasites of the four *Plasmodium* species infecting humans: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. knowlesi* (20).

As suggested by *P. falciparum* research in animal models, PLTs may have different roles at different phases of *P. vivax* infection, with a protective role by direct parasite killing, and an adverse proinflammatory role by interacting with ECs and leukocytes, among other vascular components (41). These multifaceted roles could also apply to PF4/CXCL4 since it has been shown to contribute to leukocyte cerebral vascular trafficking and damage in a murine model of cerebral malaria (41). Moreover, some clinical evidence has demonstrated that plasma PF4/CXCL4 is a predictive biomarker of cerebral malaria in humans (42), while other studies suggested this protein is not important in malaria pathogenesis (20, 43).

Despite the decrease in PLT PF4/CXCL4 cargo, we did not detect increased levels of PF4/CXCL4 in plasma from our uncomplicated *P. vivax* malaria patients. In combination with the proteomics results, our data supports the previously proposed model in which, upon PLT – *Pv*-IE interaction, locally released PF4/CXCL4 traffics into the digestive vacuole of *Plasmodium* parasites to induce death (19, 20). The intracellular localization of PF4/CXCL4 could explain the normal PF4/CXCL4 levels found in plasma from our patients. In agreement with this, a previous study has reported PLT – *Pv*-IE complexes and PF4/CXCL4 accumulation within IEs in the periphery of mild *P. vivax* malaria patients, without detectable
changes in plasma levels of PF4/CXCL4 or other markers of systemic PLT activation (20). However, we cannot exclude the possibility that megakaryocytes might be producing PLTs with lower intracellular PF4/CXCL4 amounts. Lastly, although PF4/CXCL4 is a very abundant protein in alpha granules (44), further investigations are required to test whether circulating PLTs with fewer PF4/CXCL4 (among other changes) have altered functions. In previous studies, functional changes in PLTs from *P. vivax* patients (e.g., impaired aggregation when *in vitro* stimulated with agonists) have been detected by light transmission aggregometry (10).

In *P. vivax* malaria, the link of *Pv*-IEs binding to PLTs, platelet activation, and thrombocytopenia is unclear. PLT – *Pv*-IE complexes as observed in other studies (20), and subsequent immune clearance, may be an additional contributor to malaria-induced thrombocytopenia. Furthermore, severe thrombocytopenia could be due to the high consumption of activated PLTs killing IEs. However, the high activity of PLTs could gradually induce, or contribute to intense inflammation, pathological changes, and severe disease. Due to the remote locations of sample collection, we were unable to measure PLTs - *Pv*-IEs binding in peripheral blood from our patients. It is important to note that standard hematological analyzers, like the ones used in this study, do not recognize PLTs bound to *Pv*-IEs or uninfected Es and, thus, our *Pv* samples might have had undetected PLT – *Pv*-IE complexes.

In line with the proteomic findings in patients, our *in vitro* assays showed that PLTs release PF4 in response to *Pv*-IE stimulation, and that both whole PLTs and PLT releasates directly inhibit *Pv* growth and cause phenotypic changes characteristic of dying parasites (namely spreading of parasite pigment suggestive of digestive vacuole dissolution). Importantly, these assays suggest that PLT functional responses to *Pv*-IEs can happen without the need of additional thrombotic or inflammatory signals. Our data agree with previous studies showing *in vitro* PLT killing of *P. falciparum* and *P. knowlesi* (13, 20, 45). Interestingly, the
variant PF4/CXCL4V1 was also found decreased and further studies are needed to elucidate its roles in hemostasis and immunity in malaria.

The proteomes also revealed decrease of GPV (which is a subunit of the PLT GPIb-V-IX receptor complex for VWf and thrombin) in thrombocytopenic patients, alongside the increase in plasma levels of VWf. GPV can be cleaved from the PLT surface during activation by thrombin (46). Ectodomain fragments of GPV are present in normal human plasma and may act as potential thrombotic markers or modulators (47). However, it is currently unclear whether the decrease in GPV detected in our study is due to shedding or whether megakaryocytes are producing PLTs with less GPV. It is also unclear how the decrease in GPV relates to plasma VWf levels, or if it is associated with malaria pathogenesis. Further studies are needed to elucidate the interactions between VWf and PLT receptors (namely the GPIb-V-IX complex) in *P. vivax* malaria, and the potential consequences in disease progression.

Additionally, a group of myosins, actins and tubulins (including MYH9, MYL6, MYL12B, MSN, TPM1, ACTC1, and TUBB1) were also decreased in PLT proteomes from patients suggesting alterations in the cytoskeleton of circulating PLTs during *P. vivax* infection. This might be related to PLT shape change and centralization of intracellular granules leading to secretion (perhaps in response to activation by *Pv*-IEs), which could be linked to the reduction of PF4 and GPV levels detected on these PLTs. Notably, ultrastructural changes on circulating PLTs in malaria, including granule centralization, have been detected previously (10). Further studies are needed to validate the data found here and to elucidate the morphological changes (and underlying molecular pathways) that take place in PLTs upon interaction with *P. vivax* parasite in the bloodstream. It is also important to evaluate potential changes in PLT functions that might be associated to all the proteomic changes that have been identified here.
An inflammatory response in our *P. vivax* patients was evidenced by the elevated levels of plasma VWf and increase of SAA1 amounts in PLTs. VWf is released from endothelial cells and α-granules in PLTs upon cell activation. Changes in plasma VWf levels are associated with endothelial dysfunction and the risk of bleeding/thrombosis (48). *P. vivax* infection is associated with elevated thrombomodulin, VWf, procoagulant activity, and thrombotic microangiopathy leading to other consequences of endothelial activation and altered hemostatic pathways (49, 50). These dysregulations could result in intravascular coagulation and endothelial inflammation through increased formation of large VWf multimers and PLT aggregates (51). However, more studies are needed to fully clarify the interactions between VWf and PLTs at different stages of malaria.

Interestingly, in human volunteers experimentally infected with *P. falciparum*, EC activation with release of active VWf (i.e., VWf in a PLT binding confirmation) was identified as an early malaria event, and VWf concentrations correlated negatively with PLT counts (52). Remarkably, although thrombocytopenia developed early, circulating PLTs had no increased expression of PLT activation surface markers (P-selectin/CD62P, CD63, and activated αIIbβ3), neither there was an increase of plasma PF4/CXCL4 concentration, among other PLT activation factors (53). The latter agrees with Kho et al. (20) and our observations in *P. vivax* patients. Interestingly, in the same experimental human model, concentrations of soluble glycoprotein-1b (sGP1b), the external domain of the PLT VWf-binding GP1b receptor, increased early too (53). Elevated sGP1b concentrations were also detected in patients with naturally acquired *P. falciparum* and *P. vivax* infections (53). The authors hypothesized that the increase in sGP1b concentrations might result from VWf-mediated GP1b shedding, which may prevent excessive adhesion of PLTs to activated ECs and IEs at early/mild stages of infection.

*P. vivax* has particular tropism to invade reticulocytes resulting in lower parasite biomass (e.g., only 1–2% of total erythrocytes in peripheral blood are reticulocytes) (54). This might
relate to the fact that events of parasite sequestration in *P. vivax* malaria are rare, and that patient mortality is rare compared to *P. falciparum* (15). *Pv*-IEs bind to endothelial cells via specific receptors, such as ICAM-1, with a 10-fold lower frequency than *Pf*-IEs (55).

Additional cytoadhesion phenotypes include rosetting, previously associated to anemia in patients with *P. vivax* malaria; and PLT-mediated clumping, associated with high parasitemia in *P. falciparum* infection (56, 57). PLT-mediated clumping of *P. falciparum*, only occurs during the mature intraerythrocytic stages, mediated by PLT receptors (e.g., gC1qR, CD36 and P-selectin), and specific variants of the parasite PfEMP1 family of adhesins, which are expressed on the IE surface at the mature stages of the intraerythrocytic cycle (30, 58, 59). However, the clumping phenotype has not been extensively studied in *P. vivax* malaria, and its significance is unknown (29, 60).

Despite reported limitations to culture fresh *P. vivax* isolates, in this study we were able to maintain ten fresh *P. vivax* isolates in short-term culture to evaluate *Pv*-IEs PLT-mediated clumping. Our study’s novelty was the enriched initial concentration of IEs (>90% of parasitemia) using percoll gradients, which allowed us to perform all the assays at 5% parasitemia. Additionally, PLTs were added at several concentrations (2.5 x 10^5 – 1.0 x 10^7 PLTs/mL) increasing the probability of PLT – *Pv*-IE interactions. However, the clumping phenotype was not observed in our isolates, consistent with previous reports (29, 60). It is worth noting that only patients with uncomplicated *P. vivax* malaria were enrolled in our study, which might have limited the possibility of detecting PLT – *Pv*-IE clumping if this phenotype is associated with malaria severity.

Although PLT-mediated clumping, which involves direct adhesion between PLTs and mature IEs, was not observed in our *P. vivax* samples, parasite death is believed to be PLT–IE contact-dependent both *in vivo* (13) and *in vitro* (20, 45). In this study, we found that whole PLTs can inhibit *P. vivax* growth, suggesting that PLT – *Pv*-IE proximity can induce PLT activation of PF4 secretory pathways, and it might also be essential to ensure that high
levels of PF4/CXCL4 reach the \( P_v \)-IEs. Moreover, PLTs have been found to bind IEs in all asexual stages in several \textit{Plasmodium} species (20). Altogether, these observations suggest that PLT – IE interactions are not restricted to PfEMP1 and PLT-mediated clumping. However, the molecular mechanisms that mediate PLT interaction with IEs of different \textit{Plasmodium} species (including the signals that trigger PLT PF4/CXCL4 release in response to IEs), are mainly unknown but likely to be diverse. \textit{P. vivax} and \textit{P. falciparum} could use different molecular mechanisms to interact with PLTs with different pathophysiological consequences.

CONCLUSIONS

In conclusion, we show the first PLT proteome from patients with uncomplicated \textit{P. vivax} malaria and thrombocytopenia as an effort to understand the role of PLTs in \textit{P. vivax} malaria. We have shown that not only PLT counts but also PLT protein content were altered in patients with uncomplicated \textit{P. vivax} malaria, including the increase of several acute-phase response proteins with hemostatic and immune functions, and the decrease of PF4/CXCL4 and GPV. In combination with plasma measurements and \textit{in vitro} assays, our study suggests that PLTs interact with both \( P_v \)-IEs and other vascular components during acute \textit{P. vivax} infection. The decrease of PF4 cargo in circulating platelets, and the direct interplay between \( P_v \)-IEs and PLTs observed \textit{in vitro} are compatible with a previously proposed model in which PLTs have a protective role in malaria by releasing PF4/CXCL4 to the local vicinity of IEs to cause parasite death. However, the changes in PLT SAA and GPV contents, and the increase of VWf plasma levels in \textit{P. vivax} malaria patients suggest that PLTs are also active players in the inflammatory processes that take place during acute disease.

More studies are required to further characterize the implications of all these PLT changes, and the interaction of PLTs with VWf and the vasculature, in uncomplicated \textit{P. vivax} infection and progression to severity. Importantly, this study gives evidence that to broaden our
understanding of the role of PLTs in different stages of \textit{P. vivax} malaria, a combination of experimental approaches will be required. Some challenges include the lack of good animal models, the difficulty in establishing \textit{in vitro} cultures from \textit{P. vivax} isolates, and the inability of culturing patient PLTs (e.g. enucleated cells) in the laboratory. Therefore, the use of proteomics to study patients from different clinical groups will be invaluable and should guide future \textit{in vitro} experimental designs.

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**COMPETING INTERESTS**

All authors declare that we have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript. We are also agreed to the publishing and editorial policies of the International Journal of Parasitology.
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AUTHOR’S CONTRIBUTIONS

Diana Fernández: Experimental design, laboratory research, data analysis, writing.

Cesar Segura: Experimental advisor, writing.

Mònica Arman: Experimental design of in vitro assays, writing.

Suzanne McGill: LC-MS/MS processing of samples

Richard Burchmore: Funding, experimental design of proteomics, writing

Tatiana Lopera: Principal investigator, Funding, administration of the project, experimental design, writing.
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FIGURE LEGENDS

Fig 1. Integrity of PLT proteomes in samples. The figure depicts the band patterns detected by 1D SDS-PAGE and Silver staining in PLT protein samples from 5 patients with *P. vivax* infection and 5 healthy controls. PLT counts in patients were P1, 57 x 10^3/ul; P2, 134 x 10^3/ul; P3, 70 x 10^3/ul; P4, 156 x 10^3/ul; P5, 66 x 10^3/ul. PLT counts in controls were C1, 228 x 10^3/ul; C2, 284 x 10^3/ul; C3, 328 x 10^3/ul; C4, 408 x 10^3/ul; C5, 263 x 10^3/ul. MW: Molecular weight; KDa: Kilo Daltons.

Fig 2. Differentially regulated PLT proteomes from patients with *P. vivax* infection. Volcano plots are shown from all shared protein entries and their abundance in the following conditions: A) All patients with *P. vivax* infection versus healthy controls by a paired *t*-test analysis; B) Patients with <100,000 PLTs/ul blood (thrombocytopenia) versus healthy controls by a paired *t*-test analysis. Each dot represents a protein mapped consistently to its –Log (*p*-value) on the ordinate axis and its difference (fold change) on the abscissa axis with FDR cutoff α (0.05). Red dots are increased proteins, blue dots are proteins decreased, and black dots are proteins equally expressed in all groups. The volcano plots were made using the Perseus Software platform.

Fig 3. Proposed model of protein interactions regulating PLTs roles in the pathogenesis of *P. vivax* infection. The figure depicts the protein-protein significant interactions found by STRING analysis (*p*-value<0.05) with a high level of confidence (Score > 0.9). Protein names: PF4/CXCL4 (platelet factor 4); SERPINA 1 (Alpha 1 Anti-trypsin); SSA1 (serum amyloid A 1 protein); FERMT3 (Fermitin 3); TPM1 (Tropomyosin 1); ACTC1 (Actin, alpha cardiac muscle 1); MYL12B (Myosin regulatory light chain 12B); MYL6 (Myosin light chain 6); MYH9 (Myosin heavy chain 9); GP5/V (Glycoprotein 5/V); RAP1B; VWf (Von Willebrand Factor). The lines depict the level of interaction evidence (Purple: experimental; blue light: databases; blue dark: gene co-occurrence; black: co-expression). Despite the fact
that VWf was not found differentially regulated in platelet proteomes, VWf plasma levels were higher in *P. vivax* patients (Fig 4), thus it was included in the interactome to analyze its relationship with the most important differentially regulated PLT proteins (Red arrow). The black arrow depicts the protein GPV exclusively decreased in the T-*Pv* group.

**Fig 4. PF4/CXCL4 and VWf expression in plasma from patients with *P. vivax* infection.**

A) Plasma concentration of PF4 and VWf from patients with *P. vivax* infection (PV) and Healthy controls (HC). B) Plasma concentration of PF4 and VWf in patients with normal PLTs counts (N-*Pv*), patients with thrombocytopenia (T-*Pv*), and healthy controls (HC). All values are median (interquartile range) unless otherwise indicated. Mann-Whitney U-test, significantly different to control patients (*p <0.05; **p<0.01; ***p<0.001).

**Fig 5. Platelet activation and parasite growth inhibition in vitro.** A) Platelet activation by *Pv*-IEs measured by PF4/CXCL4 release. Levels of PF4/CXCL4 from PLT releasates (rPLTs) stimulated for 1 hour at 37ºC with *Pv*-IE, uninfected erythrocytes (uEs), or RPMI media as a negative control. B) Measurement of PF4 in PLT releasates collected upon collagen stimulation. rPLTs were obtained after stimulating PLTs with collagen (10µg/mL) or PBS for 1 hour at 37ºC, aliquoted and stored at -80ºC for further *in vitro* assays with fresh *P. vivax* isolates (see section C). This figure shows PF4/CXCL4 levels in five collagen-stimulated sPLT aliquotes thawed at different times, to test PF4/CXCL4 stability in the frozen rPLTs. PF4/CXCL4 levels from sections A and B were measured by ELISA. C) Effect of PLTs and rPLTs on *P.vivax* IE schizonts development. Effect of entire PLTs (ePLTs), releasates of PLTs activated by collagen 10µg/mL (rPLTs), and RPMI media on *Pv*-IE schizonts formation measured after 20-24 hours of co-culturing with mature trophozoites. The frequency of schizonts (%) was counted in Giemsa stained thick blood films visualized by light microscopy. D) Frequency of *P.vivax* morphological alterations after culturing IEs with ePLTs or rPLTs. The frequency (%) of morphologically altered parasites was recorded in 100 *Pv*-IE. The morphological changes were defined as picnotic nucleus,
fragmented or condensed cytoplasm, nucleus degradation. **E) Representative images** of *P. vivax* morphological changes observed after culturing IEs with ePLTs or rPLTs. **E-1)** Control of *Pv*-IE schizonts; **E-2)** *Pv*-IE treated with rPLTs (collagen). **E-3)** Altered *Pv*-IE treated with ePLTs. Arrowheads represent parasites, and arrows are malarial pigment. Kruskal-Wallis test was shown statistical significance of mean and SD with *p*-value * <0.05; **p<0.01; ***p<0.001.

**Fig 6. In vitro assays of PLT-mediated clumping of *P. vivax* IEs.** A total of 10 fresh isolates of *Pv*-IEs were treated with percoll 45% to enrich >80% of *P. vivax* mature trophozoites. After enrichment, *Pv*-IE was adjusted to 5% parasitemia with the remaining uninfected erythrocytes (uEs) from the same sample and co-cultured at 5% hematocrit in RPMI media with freshly purified ePLTs from healthy donors at different concentrations (2.5 x 10⁶ – 1.0 x 10⁷ PLTs/mL) for 30 – 60 minutes at room temperature with gentle rotation at 10 – 12 rpm. **A)** The image is representative of a Colombian *P. falciparum* strain (FCB1) knob+ selected with gelatine and positive for PLT-mediated clumping in Giemsa and acridine orange staining (arrowheads). The reaction was done with 2.5 x 10⁶ PLTs/mL. Parasites *Pf*-IEs without PLTs were used as a negative control (not shown). This strain was used as positive control simultaneously with all fresh *P. vivax* isolates tested. The samples were stained by Giemsa and acridine orange. **B-C)** The top panel shows a Giemsa staining of a *P. vivax* isolate concentrated to 95% parasitemia by percoll gradient centrifugation. The bottom panel shows acridine orange-stained *Pv*-IEs (arrows) from the same parasite isolate adjusted to 5% parasitemia and co-cultured with 5.0 x 10⁶ PLTs/mL. **D-E)** The top panel shows a Giemsa staining of a *P. vivax* isolate concentrated at 65% parasitemia before assays. The bottom panel shows the same isolate adjusted to 5% parasitemia and stained with acridine orange after co-culturing with 1.0 x 10⁷ PLTs/mL. Clumping was not evidenced in any of the ten *P. vivax* isolates tested in total (data not shown).
Table 1. Characteristics of PV patients and HC in proteome and validation cohorts.

|                      | Proteome cohort | All subjects enrolled* |
|----------------------|-----------------|------------------------|
|                      | PV (n=5)        | HC (n=5)               | PV (n=48)                  | HC (n=25)                  |
|                      | Median          | RIQ                    | Median                      | RIQ                      | Median                      | RIQ                      | Median                      | RIQ                      |
| PLTs $10^3/µL        | 70.0            | 61.5 – 145.0           | 284.0                       | 245.5 - 368               | **0.004**                  | 133.0                     | 95.5 - 166.3                | 246                      | 203.0 - 277.5              | **<0.0001**               |
| MPV (fL)             | 11.6            | 10.0 – 13.1            | 9.3                         | 8.3 – 9.6                 | **0.014**                  | 9.2                       | 8.4 - 9.7                   | 9.5                      | 8.55 - 9.6                 | **0.2753**                |
| PDW (Ratio)          | 15.0            | 14.8 – 15.2            | 14.7                        | 14.6 – 14.8               | 0.207                      | 18.1                      | 16.9 - 19.5                 | 16.8                      | 16.2 - 17                  | **<0.0001**               |
| PCT (%)              | 0.08            | 0.07 – 0.12            | 0.26                        | 0.25 – 0.31               | **0.050**                  | 0.1                       | 0.08 - 0.14                 | 0.218                     | 0.192 - 0.264              | **<0.0001**               |
| Hemoglobin (g/dL)    | 13.2            | 12.9 – 13.5            | 13.9                        | 12.9 – 14.5               | **0.0712**                 | 12.9                      | 11.1 - 13.9                 | 13.2                      | 12.05 - 14.5               | **0.0972**                |
| Hematocrit (%)       | 38.9            | 38.7 – 40.5            | 41.5                        | 37.4 – 43.0               | **0.1548**                 | 38.4                      | 33.5 - 42.3                 | 39.7                      | 35.9 - 43                  | 0.1177                    |
| Age (Years)          | 18              | 10 - 43                | 39                          | 9 - 53                    | **0.840**                  | 23                        | 15 - 41                     | 33                       | 25 - 44                    | **0.0057**                |

*All subjects enrolled in the study include those from the proteomic cohort.

*a Mann-Whitney U-test, significantly different to healthy controls (p <0.05).

IQR: Interquartile. MPV: Media PLT volume. PDW: PLT distribution width. PCT: Plateletcrit.

PV: *P. vivax* patients. HC: Healthy controls.
Table 2. PLT proteins differentially expressed in patients with *P. vivax* infection and thrombocytopenia.

| Accession N’ (Uniprot DB) | Gene       | Protein                                      | Coverage sequence | # Unique peptides | Mascot Score | All PV versus HC | ‘T-PV versus HC | Molecular function                                                                 |
|---------------------------|------------|----------------------------------------------|-------------------|-------------------|--------------|-----------------|-----------------|------------------------------------------------------------------------------------|
| P10720                    | CXCL4L1    | Platelet factor 4 variant                    | 34.62             | 3                 | 259.00       | -3.14           | 0.229           | 0.1645 Inhibitor of endothelial cell chemotaxis in vitro (E).                     |
| P02776                    | CXCL4      | Platelet factor 4 / CXCL4                    | 35.64             | 1                 | 375.00       | -2.28           | 0.003           | 0.0084 Inhibits endothelial cell proliferation (E).                               |
| P60660                    | MYL6       | Myosin light polypeptide 6                   | 68.21             | 8                 | 638.00       | -2.16           | 0.001           | 0.001 Regulatory light chain of myosin.                                          |
| O14950                    | MYL12B     | Myosin regulatory light chain 12B            | 21.51             | 3                 | 163.00       | -1.96           | 0.0002          | 0.0025 Regulate the cell contractile activity via its phosphorylation.           |
| O95810                    | CAVIN2     | Caveolae-associated protein 2                | 17.88             | 7                 | 597.00       | -1.73           | 0.004           | 0.0171 Plays a role in caveola formation.                                         |
| Q01518                    | CAP1       | Adenyl cyclase-assoc. protein 1              | 15.16             | 6                 | 275.00       | -1.54           | 0.0007          | 0.0044 High specific activity and sensitivity to prostaglandins.                |
| P09493                    | TPM1       | Tropomyosin alpha-1 chain                    | 28.17             | 2                 | 601.00       | -1.50           | 0.016           | 0.1335 Plays a role in calcium-dependent regulation of cell contraction.         |
| P35579                    | MYH9       | Myosin-9                                     | 44.85             | 74                | 4,772.00     | -1.50           | 0.007           | 0.0029 Play a role in cytokinesis, cell shape, among others.                     |
| P04406                    | GAPDH      | Glyceraldehyde-3-phosphate dehydrogenase     | 49.25             | 12                | 972.00       | -1.47           | 0.002           | 0.0069 Delivery of nitric oxide to PLTs and decreased in PLTs activated by thrombin. |
| O75083                    | WDR1       | WD repeat-containing protein 1               | 12.21             | 7                 | 202.00       | -1.41           | 0.0006          | <0.0001 Decreased in PLTs activated by thrombin.                                |
| P68032                    | ACTC1      | Actin, alpha cardiac muscle 1                | 36.60             | 3                 | 2,649.00     | -1.41           | 0.007           | 0.009 Found in muscle tissues.                                                   |
| P40197                    | GPV        | Platelet glycoprotein 5                     | 7.74              | 2.14              | 195.00       | -1.40           | 0.025           | 0.0094 Mediate PLTs adhesion to blood vessels through VWF.                      |
| Q86UX7                    | FERMT3     | Fermitin family homolog 3                    | 21.44             | 10                | 1,167.00     | -1.36           | 0.0009          | 0.006 Activate the integrin β (1-3) for adhesion to endothelial cells.          |
| P00338                    | LDHA       | L-lactate dehydrogenase A chain              | 6.63              | 1                 | 195.00       | -1.34           | 0.003           | 0.0023 Involved in cadherin and kinase binding.                                  |
| P61224                    | RAP1B      | Ras-related protein Rap-1b                  | 48.91             | 6                 | 627.00       | -1.31           | 0.015           | 0.0007 Polarizing activity of ankyrin-1 and cadherin-5 in the maintenance of endothelial polarity. |
| P08567  | PLEK   | Pleckstrin | 48.57 | 17 | 1,688.00 | -1.30 | **0.005** | -1.29 | **0.0123** |
|---------|--------|------------|-------|----|-----------|-------|-----------|-------|-----------|
| A0A0A0MRZ8 | IGKV3D-11 | Immunoglobulin kappa variable 3D-11 | 7.83 | 1 | 114.00 | -1.30 | 0.534 | -2.54 | **0.0097** |
| Q9H4B7 | TUBB1  | Tubulin beta-1 chain | 20.40 | 6 | 602.00 | -1.24 | **0.002** | -1.05 | **0.0025** |
| P11142 | HSPA8  | Heat shock cognate 71 kDa protein | 17.18 | 9 | 488.00 | -1.24 | **0.002** | -1.18 | 0.0867 |
| P26038 | MSN    | Moesin | 8.49 | 5 | 131.00 | -1.21 | **0.014** | -0.99 | 0.075 |
| P07737 |PFN1   | Profilin-1 | 79.29 | 14 | 2,112.00 | -1.15 | **0.003** | -1.19 | 0.0516 |
| P0DP23 | CALM1  | Calmodulin-1 | 24.83 | 2 | 185.00 | -1.12 | 0.053 | -1.49 | 0.2806 |
| Q92686 | NRGN   | Neurogranin | 19.23 | 1 | 352.00 | -1.02 | **0.019** | -0.75 | 0.0583 |
| P01009 | SERPINA 1 | alpha-1antitrypsin | 35.89 | 16 | 136.00 | 1.33 | **0.001** | 1.22 | **0.0136** |
| P0DOX8 | IGL1LC | Immunoglobulin lambda-1 light chain | 40.74 | 3 | 753.00 | 1.49 | **0.002** | -1.60 | **0.0051** |
| P01011 | SERPINA3 | Alpha-1-antichymotrypsin | 11.11 | 5 | 136.00 | 1.65 | **0.048** | 1.00 | 0.2071 |
| P01859 | IGHG2  | Immunoglobulin heavy constant gamma 2 | 52.45 | 8 | 1,310.00 | 1.92 | **0.001** | 1.22 | 0.3589 |
| P0DJ18 | SAA1   | Serum amyloid A-1 protein | 64.75 | 3 | 357.00 | 2.73 | 0.065 | 2.81 | **0.0314** |

*Statistical significance was defined by p-value <0.05.*
Integrity of PLT proteomes in samples. The figure depicts the band patterns detected by 1D SDS-PAGE and Silver staining in PLT protein samples from 5 patients with P. vivax infection and 5 healthy controls. PLT counts in patients were P1, 57 x 10^3/ul; P2, 134 x 10^3/ul; P3, 70 x 10^3/ul; P4, 156 x 10^3/ul; P5, 66 x 10^3/ul. PLT counts in controls were C1, 228 x 10^3/ul; C2, 284 x 10^3/ul; C3, 328 x 10^3/ul; C4, 408 x 10^3/ul; C5, 263 x 10^3/ul. MW: Molecular weight; KDa: Kilo Dalton.
Figure 2

Differentially regulated PLT proteomes from patients with P. vivax infection. Volcano plots are shown from all shared protein entries and their abundance in the following conditions: A) All patients with P. vivax infection versus healthy controls by a paired t-test analysis; B) Patients with <100,000 PLTs/u1 blood (thrombocytopenia) versus healthy controls by a paired Pest analysis. Each dot represents a protein mapped consistently to its -Log (p-value) on the ordinate axis and its difference (fold change) on the abscissa axis with FDR cutoff a (0.05). Red dots are increased proteins, blue dots are proteins decreased, and black dots are proteins equally expressed in all group. The volcano plots were made using the Perseus Software platform.

Figure 3

Proposed model of protein interactions regulating PLTs roles in the pathogenesis of P. vivax infection. The figure depicts the protein-protein significant interactions found by STRING analysis (p-value<0.05) with a high level of confidence (Score 0.9). Protein names: PF4/CXCL4 (platelet factor 4); SERPINA 1 (Alpha 1 Anti-trypsin); SSA1 (serum amyloid A 1 protein); FERMT3 (Fennitin 3); TPM1 (Tropomyosin 1); ACTC1 (Actin, alpha cardiac muscle 1); MYL12B (Myosin regulatory light chain 12B); MYL6 (Myosin light chain 6); MYH9 (Myosin heavy chain 9); GP5N (Glycoprotein 5N); RAP1B; VWf (Von Willebrand Factory The lines depict the level of interaction evidence (Purple: experimental; blue light databases; blue dark gene co-occurrence; black co-expression). Despite the fact that VWf was not found differentially regulated in platelet proteomes, VWf plasma levels were higher in P. vivax patients (Fig 4), thus it was included in the interactome to analyze its relationship with the most important differentially
regulated PLT proteins (Red arrow). The black arrow depicts the protein GPV exclusively decreased in the T-Pv group.

Figure 4

PF4/CXCL4 and VIM expression in plasma from patients with P. vivax infection. A) Plasma concentration of PF4 and VWf from patients with P. vivax infection (PV) and Healthy controls (HC). B) Plasma concentration of PF4 and VWf in patients with normal PLTs counts (N-Pv), patients with thrombocytopenia (T-Pv), and healthy controls (HC). All values are median (interquartile range) unless otherwise indicated. Mann-Whitney U-test, significantly different to control patients ("p <0.05; "p<0.01; **"p<0.001).
Figure 5

Platelet activation and parasite growth inhibition in vitro. A) Platelet activation by Pv-IEs measured by PF4/CXCL4 release. Levels of PF4/CXCL4 from PLT releasates (rPLTs) stimulated for 1 hour at 37°C with Pv-IE, uninfected erythrocytes (uEs), or RPMI media as a negative control. B) Measurement of PF4 in PLT releasates collected upon collagen stimulation. rPLTs were obtained after stimulating PLTs with collagen (10pg/mL) or PBS for 1 hour at 37°C, aliquoted and stored at -80°C for further in vitro assays with fresh P. vivax isolates (see section C). This figure shows PF4/CXCL4 levels in five collagen-stimulated sPLT aliquotes thawed at different times, to test PF4/CXCL4 stability in the frozen rPLTs. PF4/CXCL4 levels from sections A and B were measured by ELISA. C) Effect of PLTs and rPLTs on P. vivax IE schizonts development. Effect of entire PLTs (ePLTs), releasates of PLTs activated by collagen 10pg/mL (rPLTs),
and RPMI media on Pv-IE schizonts formation measured after 20-24 hours of co-culturing with mature trophozoites. The frequency of schizonts (%) was counted in Giemsa stained thick blood films visualized by light microscopy. D) Frequency of P. vivax morphological alterations after culturing IEs with ePLTs or rPLTs. The frequency (%) of morphologically altered parasites was recorded in 100 Pv-IE. The morphological changes were defined as picnotic nucleus, fragmented or condensed cytoplasm, nucleus degradation. E) Representative images of P. vivax morphological changes observed after culturing Es with ePLTs or rPLTs. E-1) Control of Pv-IE schizonts; E-2) Pv-IE treated with rPLTs (collagen). E-3) Altered Pv-IE treated with ePLTs. Arrowheads represent parasites, and arrows are malarial pigment. Kruskal-Wallis test was shown statistical significance of mean and SD with p-value "<0.05; "p<0.01; "p<0.001.

Figure 6

In .ro assays of PLT-mediated dumping of P. v. .IFS. A total of 10 fresh isolates of 1.-IEs were tr.ted with percd1 45% to endch >80% of P ...mature Irophozoites. Aber enrichment IA9E was .trided to 5% parasiMmia with +c remaining uninfected erythrocytes (uEs) from the same samde and co-culture0 at 5% hernato. In 1,19811 media with freshly purbed ed_Ts from healthy donors at different concentrations (25 10° — 1.0 x 107 PL_Tdm14 tar 30-60 minutes al ram temper.re with gentle rotation at 10 —12 h. A) The triage s represent.. of a Colombian), ralciarpam sham (FCB1) Mod seMded with geld. arid positive for PLT-medid. doming in Giemsa and acridine c.nge staining (arrowdeads). The react. w+0 date with 25 x 10° PLT8.1_. Parasdes PLIES without PLTs were used as a negative control (not shown). This strain was us.
as poside con.l dmuneoudy with all fresh P. viv. i.a.t.ed. The .mdes were stain. by Giem. and acridine orange. B-C) The top panel shows a Giem® staining of a, wvax isolate concentrated to 95% par.dernia by percoll grad. centfllgldion. The bottom panel shows acridine orangAstained Pv-l. (arrows) horn the same paras+ isolate adjusted to 5% par.demia and co-cultured WM 50 x 10° PLTst.L. D-E) The top panel shows a Gmbsa staining of a P. wvar.ale conceda. al 65% parasdemia before assays. The lio.m panel shows the .me .1ate adjusted to 5% parasitemia and stained with acridine orange after co-cultudng WM 1.0 x 107 PL_Tdml_. Clumping was not .dencced in any of the ten P. viva,' isolates tested in total (data not shown).