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1 **A distinct fingerprint of inflammatory mediators and miRNAs in**
2 ***Plasmodium vivax* severe thrombocytopenia**
3

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28

29 ## Abstract

30

31 ***Background.*** Severe thrombocytopenia can be a determinant factor in the morbidity of
32 *Plasmodium vivax* (*Pv*), the most widespread human malaria. Although immune
33 mechanisms may drive *Pv*-induced severe thrombocytopenia (PvST), the current data
34 on the cytokine landscape in PvST is scarce, and often conflicting. The analysis of the
35 bidirectional circuit of inflammatory mediators and miRNAs would lead to a better
36 understanding of the mechanisms underlying PvST.

37 ***Methods.*** We combined Luminex proteomics, NanoString miRNA quantification, and
38 machine learning, to evaluate an extensive array of plasma mediators in uncomplicated
39 *Pv* patients, whose blood platelet counts varied from reference values to PvST.

40 ***Results.*** Unsupervised clustering analysis identified PvST-linked signatures comprised
41 of both inflammatory (CXCL10, CCL4, and IL-18) and regulatory (IL-10, IL-1Ra,
42 HGF) mediators. As part of PvST signatures, IL-6 and IL-8 were critical to discriminate
43 *Pv* subgroups, while CCL2 and IFN-γ from healthy controls. Supervised machine
44 learning spotlighted IL-10 in *Pv*-mediated thrombocytopenia, and provided evidence for
45 a potential signaling route involving IL-8 and HGF. Finally, we identified a set of
46 miRNAs capable of modulating these signaling pathways.

47 ***Conclusions.*** The results place IL-10 and IL-8/HGF in the center of PvST and propose
48 investigating these signaling pathways across the spectrum of malaria infections.

49

50 **Key words**: *Plasmodium vivax*, malaria, thrombocytopenia, biomarkers, miRNA,
51 HGF, IL-10, IL-8, IL-6

52

53 **Running title:** *P. vivax*-driven severe thrombocytopenia

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56 **Introduction**

57

58 *Plasmodium vivax* is the most widespread of the human malaria parasites, placing 3.3
59 billion people at risk worldwide [1]. More recently, *P. vivax* (*Pv*) burden has been
60 aggravated by growing evidence of its presence across all regions of Africa [2].
61 Challenges to the control and elimination of *Pv* include its ability to relapse, its
62 remarkable transmission efficiency, and low-density blood-stage infections, often
63 undetected by routine surveillance [3].

64 It is currently a consensus that the virulence of *Pv* has been underestimated [4-
65 6], particularly in the presence of co-morbidities [7]. While there are critical gaps in the
66 current knowledge of *Pv* pathophysiology, it is well-established that vivax malaria is
67 associated with a systemic inflammatory response [8], perhaps more intense than its
68 counterpart *P. falciparum* [7, 9], which is more commonly associated with severe
69 malaria. Findings suggest that tissue accumulation of *Pv* may occur, with the hidden
70 biomass greatest in severe disease and capable of mediating systemic inflammatory
71 response [10][11].

72 *Plasmodium vivax*-induced severe thrombocytopenia (PvST), characterized by
73 blood platelet counts below 50,000 per mm^3, is a common clinical complication in *Pv*
74 malaria [12-14]. The mechanisms leading to PvST are unclear but may be related to
75 platelet activation, consumption and/or phagocytosis [15] [16] [17].

76 Growing evidence strengthen an essential role of platelets as mediators of
77 inflammation through their capacity to secrete numerous proteins upon activation or via
78 their interaction with the endothelium, or with leukocytes [18]. For example, we have
79 recently demonstrated that platelets enhance the inflammasome activity of innate
80 immune cells and amplify IL-1-driven inflammation [19]. Therefore, it is possible to

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81 speculate that platelets may be critical players in *Pv*-mediated systemic inflammatory
82 response.
83 The role of platelets in malaria is complex and multifaceted [20]. While platelets
84 can kill circulating parasites of all major human *Plasmodium* species through the release
85 of platelet factor 4 (PF4 or CXCL4) [21], most studies indicate a predominantly
86 deleterious role [22], which may involve the von Willebrand factor [23], the coagulation
87 cascade [24], and the protein C pathway [25]. Additionally, studies involving controlled
88 human *Pv* malaria infection (CHMI) suggested a link between platelets and endothelial
89 activation, an essential pathogenic process in severe malaria [26].
90 Given the multifactorial mechanisms through which platelets can impact *Pv*
91 malaria, we investigated here crucial factors and pathways that could underlie a
92 fingerprint of PvST. For that, we measured the plasma concentrations of cytokines,
93 chemokines, and growth factors in a cohort of *P. vivax* patients with varying degrees of
94 thrombocytopenia. To gain additional insights into possibly perturbed regulatory
95 pathways in PvST, we included a group of microRNAs (miRNAs), a class of small non-
96 coding RNAs that regulate gene expression and seem to be critical to regulate platelet
97 function [27]. By combining these highly sensitive methods with machine learning
98 algorithms, we provide here essential insights into the interplay between inflammatory
99 mediators, miRNAs, and *P. vivax*-induced severe thrombocytopenia.
100
101 ## Patients and Methods
102
103 ### Study participants and sample collection
104 Individuals who sought care at Brazilian malaria reference healthcare facilities in
105 endemic areas of the Amazon Region and presented *Pv*-positive thick blood smear were
106 invited to participate in the study. Exclusion criteria consisted of: (i) refusal to provide

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107 written informed consent; (ii) age below 17 years; (iii) self-reported pregnancy; (iv)
108 mixed malaria infections (PCR-based assays); and (v) any other traceable co-
109 morbidities. Upon enrolment, we used a standardized questionnaire to record
110 demographical, epidemiological, clinical and hematological data. Seventy-seven
111 symptomatic uncomplicated *Pv* patients, with a median age of 39 years and a proportion
112 male: female of 4.5: 1, were enrolled in the study (Supplementary Table 1). The
113 interquartile range of parasitaemia was 1,900 to 7,380 parasites per mm^3, with anaemia
114 present in 25 (32%) of *Pv* patients. Fifty-four (70%) patients present thrombocytopenia
115 (i.e., platelets below150,000/mm^3), with 9 (12%) of them classified as severe
116 thrombocytopenia (platelets below 50,000/mm^3), with no evidence of bleeding.
117 Peripheral blood sample (5 mL in EDTA) was collected for each individual; plasma
118 samples were immediately obtained after blood sampling (1,500 × g for 15 min at room
119 temperature) and stored at -80ºC until use. Additionally, plasma samples from nine
120 age-matched non-infected healthy adult from the same endemic areas were collected as
121 described above.

122 The methodological aspects of this study were approved by the Ethical
123 Committee of Research on Human Beings from the René Rachou Institute – Fiocruz
124 Minas (protocols # 05/2008 and # 80235017.4.0000.5091), according to the Brazilian
125 National Council of Health. The study participants were informed about the aims and
126 procedures and agreed with voluntary participation through written informed consent.

127

128 **Multiplex determination of inflammatory mediators**

129 The plasma concentrations of 45 cytokines/chemokines/growth factors were measured
130 with the ProcartaPlex® 45-Plex Human array (eBioscience, USA) using Luminex®
131 xMAP technology (MAGPIX, Thermo Fisher Scientific, USA), as recommended.

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132

133 **Plasma RNA extraction and confirmation by real time PCR**

134 Circulating and exosomal RNA were purified from 400 µL of plasma using
135 Plasma/Serum Circulating and Exosomal RNA Purification kit (Norgen Biotek Corp.,
136 Canada), according to manufacturer instructions. In order to control extraction
137 efficiency, a panel contained 5 spiked in probes (ath-miR-159a, cel-miR-248, cel-miR-
138 254, osa-miR-414, osa-miR-442; IDT Technologies, USA) was added after second lysis
139 buffer incubation. After purification, RNA portion was concentrated using RNA Clean-
140 Up and Concentration kit (Norgen Biotek Corp., Canada), following manufacturer
141 instructions. As a control of the RNA extraction, samples were amplified for the U6
142 (RNU6-1) snRNA by using TaqMan® MicroRNA Reverse Transcription kit, followed
143 by qPCR amplification using QuantStudio 6 Flex (Applied Biosystems, USA),
144 according to fabricant's protocol. The U6 snRNA was successfully detected (data not
145 shown).

146

147 **Analysis of microRNA (miRNA) expression**

148 Detection of the miRNA was performed using NanoString nCounter technology (USA),
149 according to manufacturer's protocol. The Human v3 miRNA CodeSet allowed
150 multiplex assessment of 800 miRNAs by specific molecular barcodes. Raw data was
151 analysed with NanoString nSolver 4.0, using default quality control standards. For
152 normalization, spike in oligos (cel-miR-248 and cel-miR-254) were used. Following
153 manufacturer's instructions, only targets that presented average raw count above 100
154 were considered for further differential expression analysis in Partek Genomics Suite
155 7.0. Principal component analysis plot was also generated with the same software with

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156 batch effects removed. Targets presenting fold change equal or above 1.25 ($p<0.05$)
157 were considered differentially expressed.
158

159 Data analysis

160 Differences in medians were tested using Mann-Whitney test or Kruskal-Wallis test, as
161 appropriated. Correlation between variables was assessed by using either Pearson's or
162 Spearman's (rank) correlation (GraphPad Prism 7, San Diego, CA, USA) at significance
163 level of 5%. The ensemble of inflammatory mediators differentially expressed in the
164 study population was identified with the algorithm Comparative Marker Selection (fold
165 change ≥ 1.5, Bonferroni $p \leq 0.05$) followed by hierarchical clustering (Spearman's
166 correlation, average linkage) using the GenePattern platform (Broad Institute, MIT,
167 USA). The independent association between inflammatory mediator and the number of
168 platelets was evaluated by adjusting a negative binomial (NB) regression model with
169 stepwise backward deletion. NB regression analysis was performed using the statistical
170 package Stata version 12 (Stata Corp., Texas, USA). Covariates were selected for
171 inclusion in the regression models if they were associated with the outcome at the 15%
172 level of significance according to the exploratory unadjusted analysis. Associations with
173 p values < 0.05 were considered significant. Decision trees were used to select the
174 minimal set of phenotypic features that efficiently segregated groups. The J48 method,
175 present in Weka software (Waikato Environment for Knowledge Analysis, version
176 3.6.11, University of Waikato, New Zealand), was used for decision tree construction.
177 The Leave-one-out cross-validation (LOOCV) was calculated to estimate the accuracy
178 of the generated model. Functional enrichment analysis of the miRNAs targets with
179 experimental evidences was performed with Ingenuity Pathway Analysis (IPA)
180 (Qiagen) with default parameters.

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181

182 **Results**

183 **A cytokine/chemokine signature for *P. vivax*-induced severe thrombocytopenia**

184 To acquire a reliable assessment of the landscape of circulating cytokines/chemokines
185 in P. vivax malaria, we used high throughput Luminex cytokine arrays to profile the
186 concentrations of 45 human proteins (cytokines, chemokines, and growth factors) in the
187 plasma of P. vivax patients (Supplementary Table 1). As baseline comparison, we
188 included plasma from nine age-matched healthy volunteers from the same localities. We
189 found significantly altered plasma concentrations of 15 proteins in *Pv* patients compared
190 to healthy donors (Supplementary Figure 1A). Notably, several proteins in plasma from
191 *Pv* patients correlated with blood platelet counts (Supplementary Figure 1B, and Figure
192 1). Except for IL-1β and bNGF, the correlation for all other proteins was negative
193 (Supplementary Figure 1), suggesting an involvement of these proteins in the
194 pathogenesis of *P. vivax* associated with severe thrombocytopenia (PvST).

195 To gain additional insights into the relationship between circulating
196 inflammatory mediators and PvST, we applied hierarchical clustering, an unsupervised
197 machine learning algorithm, to investigate patterns of plasma proteins that could
198 reliably report PvST. From all the cytokines that showed correlations with platelet
199 counts (Figure 1 and Supplementary Figure 1B), this analysis highlighted eight proteins
200 (CXCL10, CCL2, CCL4, IL-10, IL-1Ra, IFN-γ, IL-18, and HGF) as part of a specific
201 PvST signature, as compared to unexposed healthy controls (Figure 2A). When we
202 stratified *Pv* patients into subgroups with severe thrombocytopenia or non-
203 thrombocytopenia, this analysis additionally identified IL-8 and IL-6, but not IFN-γ and
204 CCL2 among the eight proteins found in the former comparison (Figure 2B). Using a
205 multivariate regression analysis, we excluded the effect of possible confounding factors,

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206 such as *Pv* parasitic density, hemoglobin concentrations and WBC counts. Notably, the
207 following mediators linked to PvST remained independently associated with platelet
208 counts in *Pv* patients: IL-8 (β= -0.0072; p=0.006), IL-10 (β= -0.0009; p=0.005), HGF
209 (β= -0.0026; p<0.001), and CCL2 (β= -0.0005; p= 0.006).
210 Next, we employed supervised machine learning to the complete dataset to
211 generate decision trees that could highlight key markers that predict severe
212 thrombocytopenia from possible noise of other mediators. From all inflammatory
213 mediators analyzed, the J48 algorithm generated best-fit decision trees that highlighted
214 (i) IL-10 to discriminate healthy controls from PvST (Figure 2C) and (ii) IL-8, HGF,
215 and IL-7 to discriminate PvST from non-thrombocytopenic patients (Figure 2D).
216 Notably, inclusion of additional biomarkers did not result in an appreciable increase in
217 classification accuracy. Altogether, these findings reveal a panel composed of IL-
218 8/HGF/IL-7 and IL-10 in the center of PvST.
219

220 **A signature of miRNAs for *P. vivax*-induced severe thrombocytopenia**

221 MicroRNAs (miRNAs) are well-known regulators of cytokine expression. To gain an
222 additional layer on the mechanisms driving PvST, we used a highly sensitive molecular
223 barcode NanoString approach to examine the miRNA profile of a representative subset
224 of the studied population. The samples were comprised of 26 age-matched *P. vivax*
225 patients, selected based on their blood platelet counts (as none, moderate, or severe
226 thrombocytopenia), as well as healthy individuals, as baseline controls. Malaria patients
227 and healthy individuals clustered into different expression ellipses in the principal
228 component analysis (Figure 3A), which explained 74% of the variation in the dataset.
229 While we detected 17 out of 800 miRNAs in *P. vivax* plasma, only six of them differed
230 between subgroups of *P.vivax* patients or healthy controls (Figure 3B). Within the

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231 differentially expressed miRNA between *P. vivax* patients and controls we found that
232 the miRNA pair hsa-miR-4454/hsa-miR-7975 was upregulated (1.957 fold, $p = 0.0434$)
233 while three other miRNAs were downregulated (< 1.5-fold) in *P. vivax* patients (Figure
234 3B).

235 The pair hsa-miR-4454/hsa-miR-7975 was additionally significantly increased
236 in patients with severe thrombocytopenia compared to other infections (none, or
237 moderate thrombocytopenia) (Figure 3B). The miRNA hsa-miR-122-5p showed the
238 highest expression levels in PvST patients compared to patients with moderate
239 thrombocytopenia, but this difference was not significant when compared to non-
240 thrombogenic patients. Together, these findings highlight these miRNAs as associated
241 with PvST. Supporting these findings, a correlation between the miRNA levels detected
242 by NanoString and the blood platelet counts in these 26 patients confirmed that platelet
243 counts are negatively associated with plasma levels of miRNAs (Figure 3C).
244 Importantly, plasma miRNA levels did not show significant correlations with other
245 blood parameters (data not shown).

246 In parallel, the expression of all but one miRNA linked to PvST increased
247 alongside the plasmatic concentration of cytokines, chemokines and growth factor
248 (Supplementary Figure 2), which were highlighted as part of a signature for PvST
249 (Figure 2). Lastly, data analysis of miRNA expression confirmed that the set of
250 miRNAs identified here are involved in key canonical pathways related to the signature
251 of PvST, including the signaling of IL-6, IL-7, IL-8/CXCL8, interferon and IL-10
252 (Supplementary Table 2). Additionally, signaling pathways related to several stages of
253 the inflammatory process and immune response were also identified.

254 Finally, the profile of the 17 miRNAs that were detected in plasma samples of
255 the studied subjects generated accurate decision trees based on a single miRNA for

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256 severe thrombocytopenia versus healthy controls (has-miR-4454/has-miR-7975) (Figure
257 4A), or non-thrombocytopenic *P. vivax* infections (has-miR-150-5p) (Figure 4B).
258 Together, our findings provide an intricate relationship between key
259 cytokines/chemokines with their regulatory miRNAs in *P. vivax* malaria that may be
260 useful to define PvST.

261

262 **Discussion**

263

264 Scarce, fragmented, and occasionally conflicting data are available on the
265 cytokine/chemokine network in PvST [14, 16, 28, 29]. Here, we hypothesize that the
266 combined analysis of circulating proteins and miRNAs could provide a more realistic
267 portrait of PvST. We, therefore, examined an extensive array of soluble plasma factors
268 in a cohort of *Pv* patients, whose most prominent hematological alteration was blood
269 platelet counts, which varied from normal ranges ($\geq$ 150 x10^3/mm^3) to severe
270 thrombocytopenia (< 50x10^3/mm^3). As expected, no bleeding was reported in our
271 patients, suggesting that megakaryocytes were able to release mega platelets in the
272 circulation to compensate for the low absolute number of platelets in the periphery [15].
273 In accordance, we found that the mean platelet volume (MPV), a measurement of the
274 platelet size that increases according to platelet destruction, correlated negatively with
275 the platelet counts ($r = -0.5426$, $p = 0.0002$).

276 For the detection of soluble plasma mediators, we used a Luminex-based
277 technology that is highly reproducible compared to conventional cytokine bead arrays
278 [30]. The hierarchical clustering of samples allowed us to define two panels comprised
279 of inflammatory and regulatory mediators that discriminated PvST from health controls
280 or non-thrombocytopenic patients (Supplementary Table 3). Although the same

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281 regulatory mediators (IL-10/IL-1Ra/HGF) were included in both panels, decision trees
282 confirmed the levels of IL-10 as critical to classify PvST as compared with controls.
283 Previous studies supported a similar association between IL-10 and decreased blood
284 platelet counts [14, 16, 28, 29].

285 High levels of IL-10 are commonly observed in *Pv* infections and are primarily
286 associated with the immune system's effort to counteract excessive inflammation [31].
287 This oversimplified association of IL-10 with less severe vivax disease has been
288 challenged by studies that reported a lack of correlation between regulatory cytokines
289 and milder symptoms. On the contrary, high levels of IL-10 are linked to intense
290 paroxysms [32], increased disease-severity [33], parasite-related inflammation [10],
291 and the occurrence of recrudescence of blood-stage infections [34]. Although previous
292 studies highlight that IL-10 has complex and not well-characterized functions in *P.*
293 *vivax* pathogenesis [10], our findings strengthen this cytokine's critical contribution in
294 *P. vivax*-induced thrombocytopenia.

295 In addition to IL-10, our analysis identified IL-1Ra as associated with *Pv*-severe
296 thrombocytopenia. IL-1Ra is a naturally occurring member of the IL-1 family that binds
297 to IL-1 receptors and antagonizes IL-1α/IL-1β [35]. Notably, IL-10 is a potent inducer
298 of IL-1Ra, which may represent a mechanism whereby IL-10 exerts its anti-
299 inflammatory effects [36]. Interestingly, the decision tree further indicated HGF as
300 strongly associated with PvST, particularly to differentiate subgroups of *P. vivax*
301 patients. HGF exerts potent anti-inflammatory effects, which seem to involve a
302 signaling cascade leading to increased expression of IL-1Ra [37]. In agreement with the
303 upregulation of IL-1Ra in thrombocytopenic patients, we recently demonstrated that
304 low platelet counts in *Pv* malaria are associated with a progressive decrease in plasma
305 concentrations of IL-1β [19]. While IL-1Ra in *Pv* infections has been little explored,

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306 elevated IL-1Ra levels have been associated with increased disease severity in *P.*
307 *falciparum* malaria-infected children [38]. These findings suggest that an excessive anti-
308 inflammatory response may dampen the necessary inflammatory response able to
309 control the infection [39].

310 Combined with the abovementioned regulatory mediators, the panels capable of
311 differentiating PvST from other subgroups included well-known inflammatory
312 mediators (Supplementary Table 3). A range of different cell types produces these
313 cytokines/chemokines, which account for the cascade of events that lead to leukocytes
314 recruitment, trafficking, and amplification of inflammation and *Pv* pathogenesis [8, 10,
315 40]. Interestingly, the up-regulation of IL-6 and IL-8 was critical to discriminate
316 subgroups of vivax patients, but not from healthy subjects. IL-6 has an established role
317 in *Pv* infection, particularly as a marker of systemic inflammation leading to organ
318 dysfunction and disease severity [10]. The same authors demonstrated associations
319 between a decreased activity of plasma ADAMTS13 (a von Willebrand factor cleaving
320 protease), lower platelet counts, and increased concentrations of IL-6, a well-known
321 specific inhibitor of ADAMTS13.

322 An unexpected finding was the potential involvement of IL-8 in PvST, further
323 validated by the decision trees. IL-8, whose expression is induced by Toll-like receptor
324 (TLR) and IL-1R-stimulated-NF-κB signaling, is a key mediator of neutrophil
325 recruitment [41]. Though IL-8 has been largely underestimated in *P. vivax* infection, a
326 previous study noticed impaired chemotaxis of neutrophils towards an IL-8 gradient,
327 suggesting a possible mechanism for secondary bacterial infection during *Pv* malaria
328 [42]. On the other hand, earlier studies on *P. falciparum* have reported elevated IL-8
329 levels in patients suffering from severe disease [43, 44]. Although IL-8-mediated
330 thrombocytopenia has not been investigated in malaria, a significant body of evidence

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331 suggests its involvement in platelet production, destruction, and/or activation [45-48].

332 While the mechanism of IL-8 induced PvST is not known, our study identified a
333 potential route involving HGF. Notably, HGF binds to cMet (a receptor tyrosine kinase)
334 to regulate IL-8 expression [49]. HGF itself has been reported to affect the proliferation
335 and differentiation of hemopoietic stem and progenitor cells [50]. Intriguingly, a variant
336 beta-chain of HGF forms a molecular complex with IL-7, and this naturally occurring
337 hybrid cytokine IL-7/HGFβ exerts a potent influence on primitive hematopoietic cells
338 [51]. In the current study, the plasma levels of circulating IL-7 (a growth factor for
339 lymphocytes) were close to the detection limits of the assay (average 4 pg/mL), which
340 precluded definitive conclusions about its involvement in PvST; even though the
341 decision tree algorithm identified IL-7 as useful to classify a small part of *Pv* patients.
342 Despite that, reduced peripheral levels of IL-7 have been associated with inefficient
343 erythropoietic responses in *P. falciparum*-induced severe anemia [52]. Unfortunately,
344 no data related to blood platelet counts were available in the abovementioned pediatric
345 study. In our study, anemia was not a confounding factor as (i) multivariate regression
346 analysis confirmed that hemoglobin levels were not a confounding factor for the
347 association between plasma concentrations of IL-8 and HGF and peripheral platelet
348 counts (ii) the majority of enrolled patients does not present anemia. Collectively, our
349 results suggest that a mechanism involving the upregulation of IL-8 and HGF is
350 involved in PvST. . The potential contribution of the downregulation of IL-7 should be
351 further confirmed.

352 Considering that cytokines/chemokines are among the most relevant proteins
353 whose expression is regulated by miRNAs [53], we further identify key regulatory
354 miRNAs that could be involved with the inflammatory profile of PvST. Remarkably, a
355 set of six miRNAs were differentiated expressed between PvST patients and other

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356 subgroups (non-thrombocytopenic or healthy controls), with the expression levels of all
357 but one miRNA increasing with the circulating levels of strategic mediators such as IL-
358 6, IL-8, IL-10, and HGF.

359 It is noteworthy that platelet-derived microparticles are transport vehicles for
360 large numbers of miRNAs [54]. Among the miRNAs linked to PvST, we detected
361 platelet-related miRNAs (e.g., miR-126-3p and miR-150-5p), which are known to
362 mediate platelet function and reactivity [54, 55]. This is a relevant observation as we
363 have previously demonstrated that platelets are major sources of circulation
364 microvesicles in *Pv* malaria [56] and that thrombocytopenia strongly correlates with
365 levels of circulating nucleic acids [57]. Lastly, the set of miRNAs identified here were
366 involved in key canonical pathways related to the signature of PvST, including the
367 signaling of IL-6, interferon, IL-8 and IL-10. Interestingly, the same set of miRNA
368 identified the signaling of IL-17A (IL-17, a key component of innate and adaptive
369 immunity) as associated with PvST. Notwithstanding, the plasma levels of IL-17A in
370 our *Pv* patients did not correlate with platelet counts. Perhaps IL-17A plays a more
371 complex role in the cascade of events that lead to PvST. IL-17A signaling mediates the
372 production of chemokines/cytokines such as IL-8, CCL2 and IL-6 [58], which were
373 associated with PvST in our analysis. Altogether, these findings are relevant, as the
374 miRNA profile in *P. vivax* remains poorly explored.

375 This study has limitations that should be considered when interpreting the
376 results. First, our study included a relatively small number of participants, which may
377 have underpowered some of the statistical analyses. Second, the multivariate model
378 used to control confounding variables was restricted to a small number of participants,
379 which may have excluded cytokines that could be independently associated with
380 platelets. Despite this limitation, key mediators such as IL-10, IL-8, and HGF were

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381 clearly identified as independently associated with platelet counts. Third, one single
382 time-point sampling is unlikely to provide insights into the sequence of events from
383 different levels of thrombocytopenia to the progression of clinical disease.
384 Notwithstanding, the direction of the associations with severe thrombocytopenia
385 revealed structural patterns in such data sets that allowed us to identify novel candidate
386 cytokines/chemokine patterns that may be critical for PvST. Notably, our analysis
387 highlighted key core markers such as IL-8 and HGF as potential, and unexplored,
388 signaling routes involving platelets and *Pv* malaria infection. Finally, we provide
389 evidence for an unprecedented role of the HGF signaling in a regulatory pathway
390 involving IL-10/ IL-1Ra. Coherently, this study identified a set of miRNAs capable of
391 modulating these vital chemokine/cytokine pathways, which should be investigated in
392 the context of thrombocytopenia across the spectrum of malaria infections.
393

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394 ## Acknowledgments

395

396 We thank malaria patients and healthy controls for participation in the study; the local
397 malaria control team in Porto Velho (RO) and Cuiabá (MT) for their logistic support;
398 Lucas Secchim Ribeiro for fruitful discussions and Cornelia Rohland for sample
399 shipment; the Programme for Technological Development in Tools for Health-PDTIS-
400 FIOCRUZ for use of the Real-Time PCR (RPT09D) facilities; the Coordination for the
401 Improvement of Higher Education Personnel (CAPES) and the Program for Institutional
402 Internationalization of CAPES-PrInt / FIOCRUZ is also acknowledged .

403

404 ## Figure Legends

405 **Figure 1.** Spectrum of plasma mediators in *P. vivax* infection. Luminex Cytokine plex
406 of cytokines, chemokines and growth factors measured in the plasma of *P. vivax*
407 patients. Patients were stratified according to their blood platelet counts as severe ($<$
408 50,000 mm3) or moderate (50,000 – 150,000 mm3) thrombocytopenia, and non-
409 thrombopenic ($\geq$ 150,000 mm3). For each boxplot, median and interquartile ranges
410 were represented as transversal lines in the center and lower/upper bounds of the box,
411 respectively, with whiskers the minimum and maximum values. P values for Kruskal-
412 Wallis multiple comparisons are shown.

413

414 **Figure 2.** Identifying a cytokine/chemokine signature for *P. vivax* patients with severe
415 thrombocytopenia (PvST). *A*-*B*, Agglomerative hierarchical clustering showed key
416 cytokines that could distinguish PvST (blue circles) from (*A*) healthy donors (green
417 circles), or (*B*) non-thrombopenic patients (NT, yellow circles). Data were represented
418 as heatmap of clustered proteins (rows) and individual plasma samples (column), with

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419 minimum and maximum normalized levels showed in blue and red scales, respectively.
420 Hierarchical clustering was performed based on Spearman's correlation coefficient,
421 using the average linkage method (GenePattern, Broad Institute). *C-D,* Best-fit decision
422 trees generated with the J48 algorithm (Weka software) identified IL-10 (*C*) and IL-
423 8/HGF/IL7 (*D*) as minimum mediations that efficiently segregated PvST from healthy
424 controls (HD) and from non-trombocytopenic patients (NT), respectively. Weighted of
425 the attribute (plasma levels) were placed in the root of the tree according to the
426 cytokine/chemokine value (pg/mL) that best divided groups. The total of classified
427 registers (correct and incorrect) for each class are given in parentheses for each terminal
428 node with the Full training (FULL) and Leave-one-out cross-validation (LOOCV)
429 accuracies. If incorrectly classified registers exist, they will appear after slash "/".
430

431 **Figure 3.** The profile of circulating miRNAs in *P. vivax* patients. *A*, Principal
432 component analysis of the distribution of miRNAs detected in the plasma of a subgroup
433 of P. vivax patients (red, n = 26) and healthy donors (blue, n = 8); *B,* Six miRNAs
434 were differentially expressed in groups of *P. vivax* patients and healthy subjects, with
435 positive values of fold change (FC) highlighted in red and negative values in green,
436 considering p<0.05. *P. vivax* patients were stratified according to their blood platelet
437 counts as: severe (< 50,000 mm3) or moderate (50,000 to 150,000 mm3)
438 thrombocytopenia, and non-thrombocytopenic patients (≥ 150,000 mm3); *C,*
439 Correlations between plasma miRNA levels and blood platelet counts in *P. vivax*
440 patients from *A*. Correlation coefficients and P. values are shown.
441

442 **Figure 4 – Decision trees for the miRNAs linked to *P. vivax* severe**
443 **trombocytopenia**

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444 Accurate decision trees generated with the J48 algorithm (Weka software) identified
445 has-miR-4454/has-miR-7975 (*A*) and has-miR-150-5p (*D*) as minimum miRNA that
446 efficiently segregated PvST (ST) from health controls (HC) and from non-
447 trombocytopenic patients (NT), respectively. Weighted of the attribute (miRNA) were
448 placed in the roof of the tree according to the miRNA value (counts) that best divided
449 groups. The total of classified registers (correct and incorrect) for each class are given in
450 parentheses for each terminal node with the Full training (FULL) and Leave-one-out
451 cross-validation (LOOCV) accuracies. If incorrectly classified registers exist, they will
452 appear after slash "/"
453

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454

455 ## Notes

456

457 **Authorship**. Conception and study design: LHC and BSF. Fieldwork and recruitment
458 of participants: MLSS, CJFF, DBP. Performance of experiments: MLSS and IH. Data
459 analysis: MLSS, IH, TNS, CJFF, LHC, BSF. Bioinformatics analysis: RSC, MSG,
460 LRA. The drafting of the manuscript: LHC and BSF. All authors critically revised the
461 manuscript and approved the final version.

462

463 **Funding.** This work was supported by the European Research Council (PLAT-IL-1,
464 714175 to BSF); the National Research Council for Scientific and Technological
465 Development-CNPq (LHC); the Research Foundation of Minas Gerais- FAPEMIG
466 (LHC). BSF was further supported by the Germany's Excellence Strategy (EXC 2151 –
467 390873048) from the Deutsche Forschungsgemeinschaft (DFG, German Research
468 Foundation to BSF). The fellowships were sponsored by CNPq (MLSS, CJFF, TNS,
469 and LHC,), and by the Brazil-Germany Program of Cooperation -
470 CNPq/CAPES/DAAD (MLSS).

471

472 **Potential conflict of interest.** The authors do not have a commercial or other
473 association that might pose a conflict of interest.

474

475

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634

Figure 1

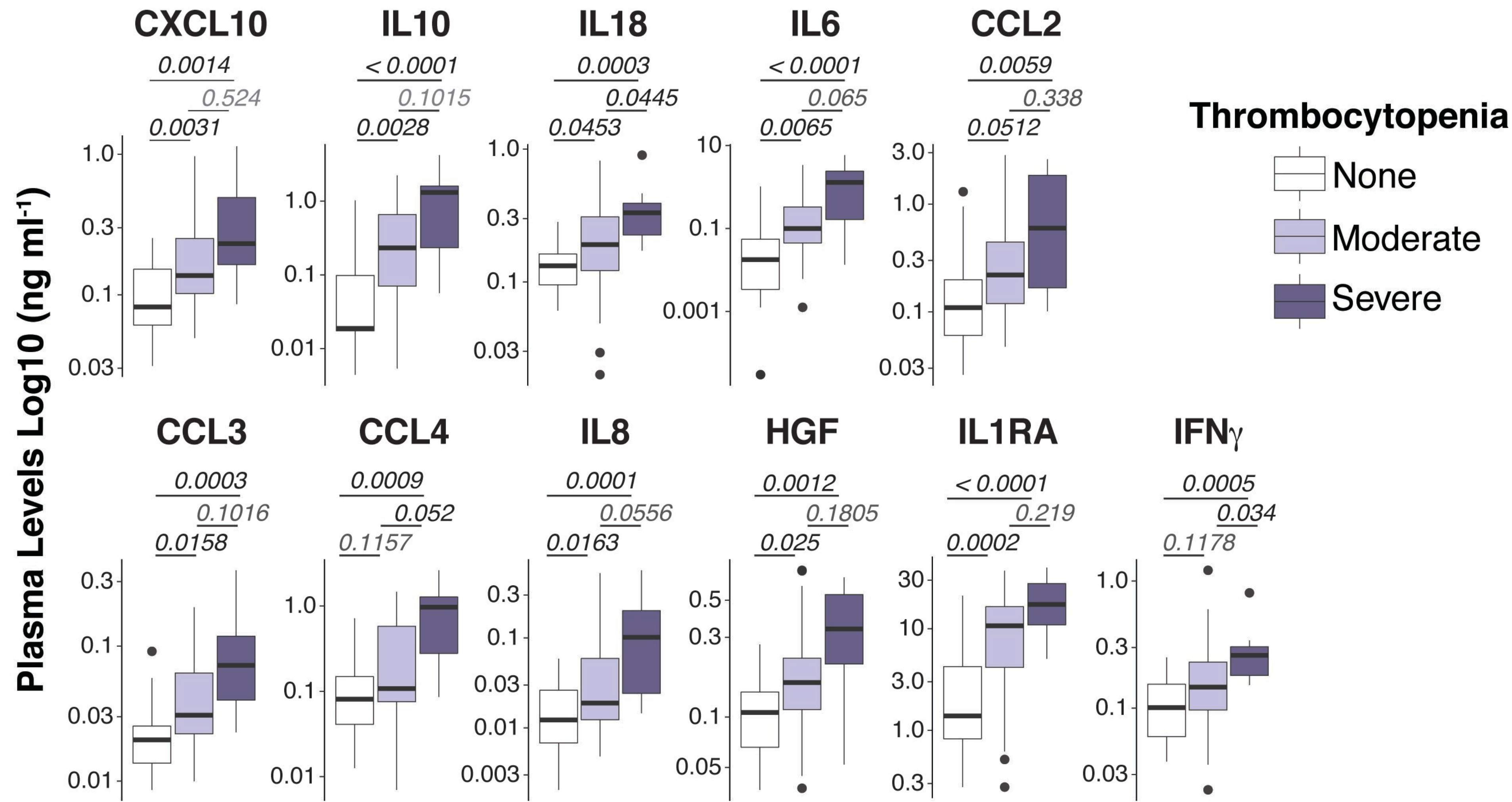
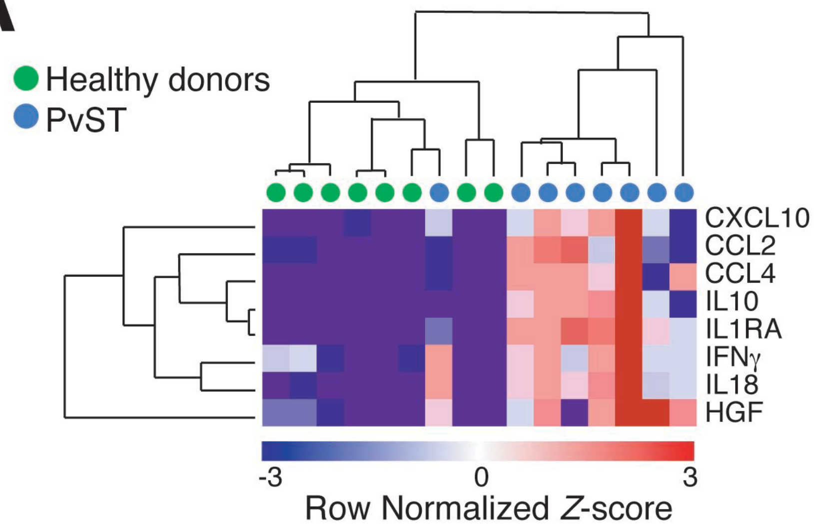
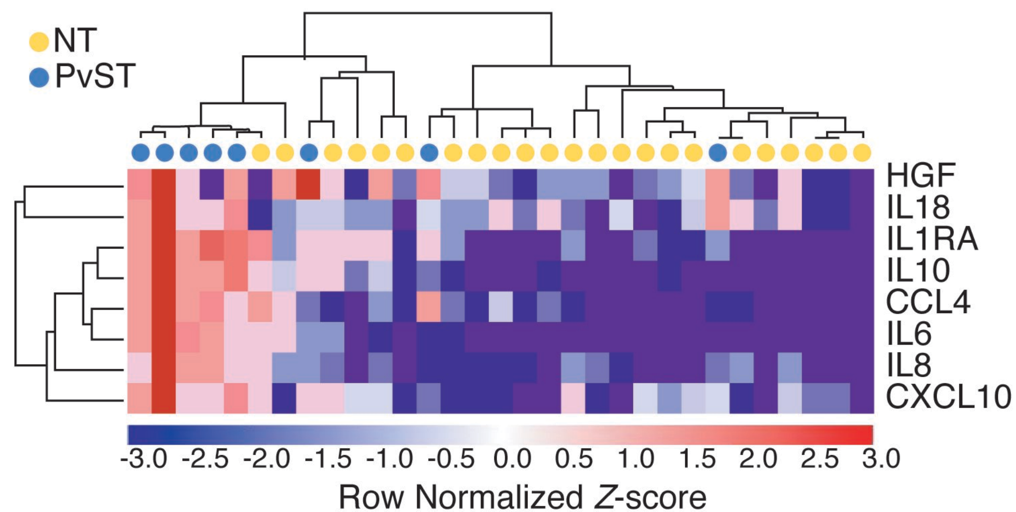
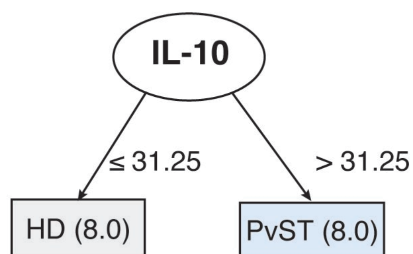


Figure 2







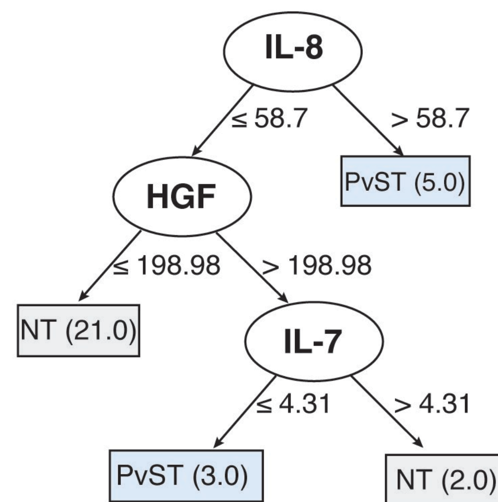
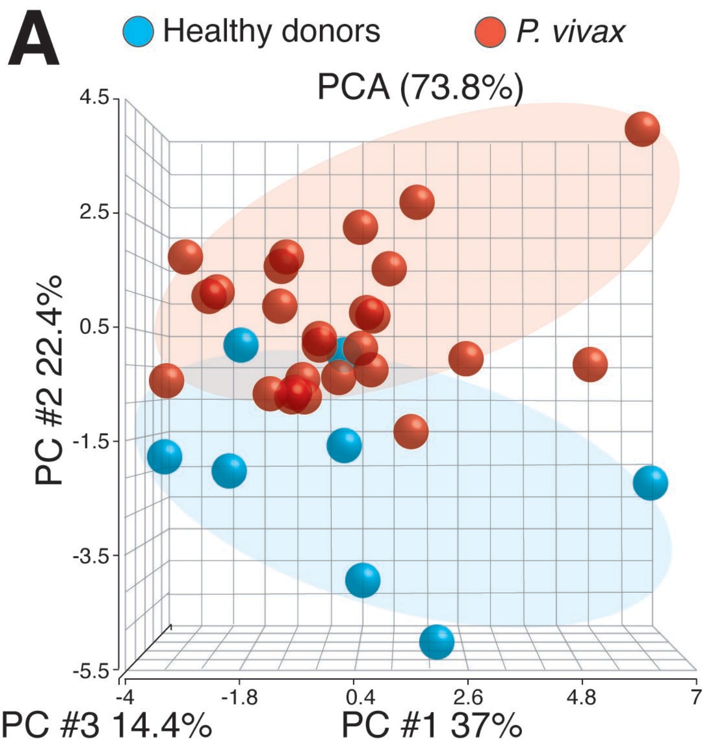
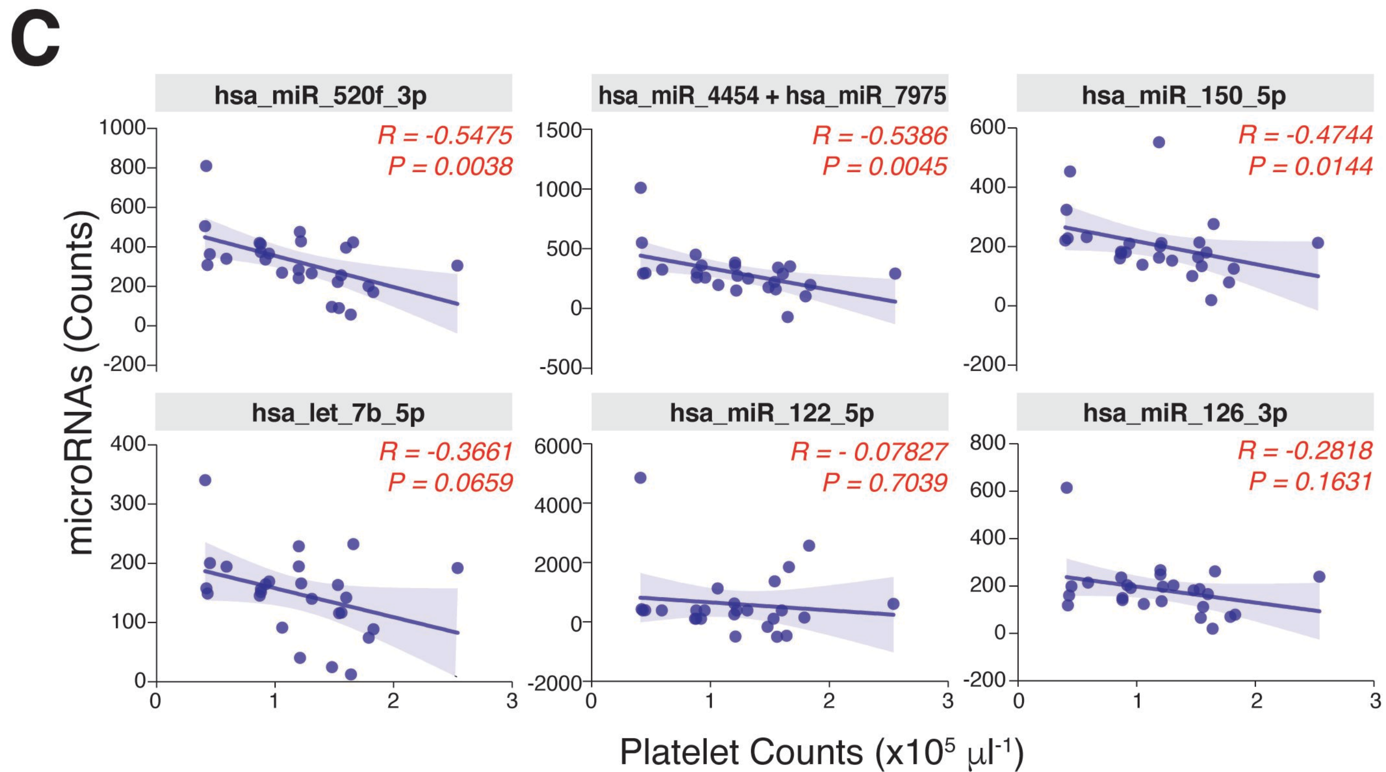


Figure 3





B

miRNAs	*P.vivax* v.s. Control		*P.vivax* Severe vs. Moderate		*P.vivax* Severe vs. None	
	P value	FC	P value	FC	P value	FC
hsa-miR-520f-3p	***0.00339***	-1,614	***0.0466***	1,497	***0.00403***	2,105
hsa-miR-4454 + hsa-miR-7975	***0.0434***	1,957	***0.00606***	1,862	***0.000881***	2,563
hsa-miR-150-5p	***0.029***	-1,536	0.126	1,496	***0.0348***	1,961
hsa-let-7b-5p	***0.000304***	-2,055	0.216	1,475	0.143	1,676
hsa-miR-122-5p	0.24	4,575	***0.0276***	5,449	0.144	2,245
hsa-miR-126-3p	0.701	-1,09	0.168	1,421	***0.0281***	2,041

Figure 4