OPEN ACCESS Freely available online

PLOS ONE

Angiopoietin-2 and Angiopoietin-2/Angiopoietin-1 Ratio as Indicators of Potential Severity of *Plasmodium vivax* Malaria in Patients with Thrombocytopenia

Luciano Teixeira Gomes[1,2,3], Eduardo Rodrigues Alves-Junior[2], Clebson Rodrigues-Jesus[1], Andreia Ferreira Nery[1,3], Thamires Oliveira Gasquez-Martin[1], Cor Jesus Fontes[1]*

1 Hospital Julio Müller, Federal University of Mato Grosso, Cuiabá, Brazil, **2** Univag University Centre, Varzea Grande, Brazil, **3** Facimed Cacoal Faculty of Medicine, Cacoal, Brazil

Abstract

Introduction: Angiogenic factors such as angiopoietin 1 (Ang-1) and angiopoietin 2 (Ang-2) are biomarkers produced during activation and dysfunction of the vascular endothelium in several infectious diseases. The aim of this study was to determine the serum levels of Ang-1 and Ang-2 and to establish their relationship with the main indicators of worst-case prognosis in patients with *P. vivax* malaria.

Methods: This is a retrospective case-control study nested within a cohort of symptomatic malaria patients. A potentially severe case was defined as a patient that presented at least one of the main indicators of the worst-case prognosis for falciparum malaria, as established by the World Health Organization. Ang-2 and Ang-1 and the Ang-2/Ang-1 ratio were used to analyze the role of angiopoietins as biomarkers in signaling potentially severe vivax malaria. ROC curves were generated to identify a cut-off point discriminating between the angiopoietin concentrations that were most strongly associated with potential infection severity.

Results: The serum levels of Ang-2 and the Ang-2/Ang-1 ratio were higher in the case group. In contrast, the serum levels of Ang-1 were lower in the cases than in the control patients. The blood count for platelets showed a positive correlation with Ang-1 and a negative correlation with Ang-2 and with the Ang-2/Ang-1 ratio. The area under the ROC curve (AUC) for serum angiopoietins, as an indicator of worst-case prognosis in a potentially severe *P. vivax* malarial infection, was larger in the subgroup of patients with platelet counts $<$75,000/μL.

Conclusion: This study showed that patients with predictors of worst-case prognoses for *P. vivax* malaria have lower Ang-1 and higher Ang-2 serum levels (and higher values for the Ang-2/Ang-1 ratio) than controls. Elevated serum levels of Ang-2 and high values for the Ang-2/Ang-1 ratio may potentially be used as predictors of worst-case prognoses for *P. vivax* malaria, especially in patients with thrombocytopenia.

Citation: Gomes LT, Alves-Junior ER, Rodrigues-Jesus C, Nery AF, Gasquez-Martin TO, et al. (2014) Angiopoietin-2 and Angiopoietin-2/Angiopoietin-1 Ratio as Indicators of Potential Severity of *Plasmodium vivax* Malaria in Patients with Thrombocytopenia. PLoS ONE 9(10): e109246. doi:10.1371/journal.pone.0109246

Editor: Claudio R. F. Marinho, Instituto de Ciências Biomédicas / Universidade de São Paulo - USP, Brazil

Received April 5, 2014; **Accepted** September 10, 2014; **Published** October 2, 2014

Data Availability: The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. Data are available from the Julio Muller School Hospital, Federal University of Mato Grosso, at the Ethics Committee for Researchers, who meet the criteria for access to confidential data. Data are from the Malaria Cohort Study whose authors may be contacted at Malaria Laboratory, Julio Muller School Hospital, Dr Luiz Phellipe Pereira Leite Street sn, Cuiaba (MT), CEP 78048-902, Phone/Fax: 55 65 3615-7254, E-mail: cephujm@ufmt.br.

Funding: The authors are thankful for financial support provided by the Brazilian National Council for Scientific and Technological Development and State of Mato Grosso Research Foundation–PRONEX/CNPq/FAPEMAT (Malaria Network). The authors declare that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: corfontes@gmail.com

Introduction

Over the past few years, the number of publications on severe *Plasmodium vivax* malaria has increased, moving away from the conventional idea that this infection is benign [1,2]. In several studies of patients diagnosed with severe *P. vivax* malaria, multiple syndromes have been described, similar to those caused by *P. falciparum* [3]. Although similar, the clinical symptoms are not the same nor of the same intensity. However, researchers have begun to use the same criteria to define the severity of malaria caused by *P. vivax* as those proposed by the World Health Organization [4] to define the severity of malaria caused by *P. falciparum* [5,6].

Compared to *P. falciparum*, *P. vivax* has a greater capacity to induce an inflammatory response, which results in a lower pyrogenic threshold [7]. However, the typical pathogenic mechanisms of cytoadherence and microvascular sequestration of parasites, present in severe infections caused by *P. falciparum*, are less frequent in severe vivax malaria [2,3]. There is evidence that links worst-case prognosis criteria for severe malaria caused by *P. falciparum* and severe infections caused by *P. vivax*, of which

severe anemia [8], acute pulmonary edema [6], coma [9,10], acute kidney injury [11], jaundice [11,12], and shock [13] are already known.

A number of laboratory markers have been used to assess inflammatory patterns in response to malaria and their relation to the severity of the infection [12]. Biomarkers for inflammation as a means of monitoring the clinical progress of patients infected with *P. vivax* have been described, including those for endothelial lesions, hemolysis, and oxidative stress. Numerous substances have been evaluated as biomarkers for the severity of malarial infections such as proteins associated with iron metabolism and anti-oxidant enzymes, as well as various cytokines and chemokines [14,15,16]. Although not considered to be a criterion for severe malarial infection, thrombocytopenia has been frequently reported in *P. vivax* infections, and its association with severe cases has been established [12,17].

An understanding of the relationship between the presentation of the disease and the production of a biomarker may help in clinical decision-making and could minimize the risk of disease complications and consequently, the suffering of patients [16]. Angiogenic factors such as angiopoietin 1 (Ang-1) and angiopoietin 2 (Ang-2) are biomarkers produced during activation and dysfunction of the vascular endothelium in non-infectious as well as infectious diseases, including malaria [18,19]. Ang-1 and Ang-2 are antagonistic ligands of the Tie-2 receptor that is expressed on the surface of the vascular endothelium. In healthy individuals, the levels of Ang-1 are higher than those of Ang-2, thus, promoting the stability of the endothelium and preventing the activation of a proinflammatory response [20]. Inflammation, however, promotes the liberation of Ang-2, and the increased binding of the Tie-2 receptors with Ang-2 induces the generation of proinflammatory and prothrombotic responses [21].

Recent studies have elucidated the relationship between disease severity and plasma levels of angiopoietins in *P. falciparum* infections, as well as that between the levels of these proteins and the occurrence of cerebral malaria, placental malaria, retinopathy, and death [22,23,24,25]. However, studies on angiopoietins in *P. vivax* infections [26,27], as well as on associations between this biomarker and the occurrence of clinical and laboratory evidence for severity, are limited. The aim of this study was to determine the serum levels of angiopoietins (Ang-1 and Ang-2) and to establish their relationship with the main indicators of a worst-case prognosis in patients with *P. vivax* malaria.

Methods

Patients and method

This retrospective case-control study was performed within a cohort of malaria patients treated at the Hospital Julio Müller, in the city of Cuiabá, state of Mato Grosso, Brazil, between December 2011 and November 2013. The hospital is a reference center for the diagnosis and treatment of malaria in people living and traveling in the south and southeast of the Brazilian Amazon. Participation in the study was voluntary, with a free and informed consent form signed by the patient, or in the case of children, by the parents. This study was approved by the Ethics Committee of the Julio Müller School Hospital (Document 130/CEP/HUJM/2011).

Patients with symptomatic malaria were eligible to participate in the study if they had a confirmed monoinfection with *P. vivax*, as confirmed by microscopy of a thin blood smear and polymerase chain reaction (PCR) analysis. Following the confirmation of the diagnosis, the patients underwent clinical examination, as well as hematology and blood biochemistry tests, including those for serum levels of Ang-1 and Ang-2. To analyze the role of angiopoietin as a biomarker for potentially severe vivax malarial infections, a subgroup of patients were classified as potentially severe cases, if they presented at least one of the main indicators (for children and adults) of the worst-case prognosis for falciparum malaria, as established by the World Health Organization [4]. The defining indicators were as follows: hemoglobin concentration $<$ 5 g/dL; erythrocyte volume fraction $<$15%; serum creatinine $>$ 3 mg/dL; blood urea $>$60 mg/dL; blood glucose $<$40 mg/dL; total bilirubin $>$2.5 mg/dL; greater than threefold elevation in serum transaminases; parasitemia $>$250,000/μL. Control group patients, who did not present any of these indicators, were randomly selected.

Sample Collection and Storage

Ang-1 is present in platelet granules and is released upon platelet activation [28]. Therefore, to measure circulating levels of Ang-1, platelet-free serum should be collected for measurement. Five milliliters of blood were collected into serum-separating tubes and allowed to clot at room temperature for 30 min before centrifugation for 15 min at 1000×*g*. The serum was removed and aliquots were stored at −80°C. The serum was defrosted only once prior to the assay for angiopoietin.

Laboratory analyses

The hematological evaluation of the patients was performed using a Cell Dyn Ruby multi-parameter automated hematology analyzer (Abbott Laboratories, Illinois, USA). Blood biochemistry was analyzed by photometry using a BT-3000 Plus automated chemistry analyzer (Diamond Diagnostics, Massachusetts, USA).

Angiopoietin levels in the serum samples were assessed by enzyme-linked immunosorbent assay (ELISA) using standard commercial kits (R&D Systems, Minneapolis, USA) for Ang-1, the Quantikine Angiopoietin-1 kit (Lot 306702), and the Quantikine ELISA Human Angiopoietin-2 kit (Lot 308323) for Ang-2, according to the manufacturer's instructions.

Statistical analysis

Because of the non-normality of the data, non-parametric statistics were used for the analysis. The Mann–Whitney *U* test was used to compare the distribution of the classic indicators for potentially severe malarial infection between the cases and the control patients. Spearman's correlation test was used to analyze the association between angiopoietins and platelet counts. Because of heteroscedasticity, we used the non-parametric Kruskal–Wallis test to compare the values of the levels of angiopoietin and classified the patients into three groups according to their platelet counts: severe thrombocytopenia ($<$50,000/μL), moderate thrombocytopenia (50,000 to 150,000/μL), and no thrombocytopenia ($>$150,000/μL). Cuzick's test for trend was used to analyze the tendency of quantitative variables between platelet counts of the different groups analyzed. We generated receiver operating characteristic (ROC) curves and calculated the area under the ROC curve (AUC) to evaluate the diagnostic accuracy of angiopoietins for discriminating between patients with and without the indicators of the worst-case prognosis for potentially severe vivax malaria. The Youden 'J' index was used to define the cut-off points corresponding to the sensitivity and specificity values with the lowest probability of having occurred randomly [29]. P-values$<$0.05 were considered significant for all tests.

Results

Eighty patients with symptomatic malaria caused by PCR-confirmed *P. vivax* monoinfection were included in the study. Of the 80 patients, 58 (72.5%) were men and 22 (27.5%) were women, with ages ranging from 5 to 78 years, with a median (1^{st}–3^{rd} quartile) of 39 (26–49) years. All had presented with fever within the 24 h prior to entering the study. Altered hematologic and biochemical parameters were infrequent except for platelet counts, for which the median (1^{st}–3^{rd} quartile) was 98,000 (62,500–168,000) platelets/μL for all patients. Severe and moderate thrombocytopenia were observed in 10 (12.5%) and 45 (56.2%) patients, respectively. Only 25 (31.3%) of the patients showed normal platelet counts. The clinical and laboratory characteristics of patients according to platelet count are shown in Table 1. Only total serum bilirubin and urea levels tended to decrease as the platelet count increased (P = 0.006 and P = 0.015, respectively).

Eighteen patients were classified as potentially severe cases in this study, showing one or more of the main indicators of the worst-case prognosis for malaria: severe anemia (n = 1), elevated blood urea nitrogen (BUN) (n = 3), hyperbilirubinemia (n = 10), and elevated serum transaminases (n = 4). The ages of the potentially severe cases did not differ from those of the controls (P = 0.870). As expected, however, the parameters signaling severe malarial infection were altered in the potentially severe cases, with significant differences in serum bilirubin levels (P = 0.008), aspartate aminotransferase (AST) (P<0.001), alanine aminotransferase (ALT) (P = 0.002), and parasitemia (0.010). No significant differences in hemoglobin (P = 0.760), hematocrit (0.471), serum urea (0.179), serum creatinine (P = 0.061), platelet count (P = 0.097), or axillary temperature (P = 0.126) were observed between cases and controls (Table 2).

Angiopoietin levels were not associated with other factors known to be related to the severity of malaria, such as age, level of parasitemia, or axillary temperature. When the analysis of the serum levels of angiopoietin was stratified according to the patients' platelet counts, we found that the median value of Ang-1 was lower (P = 0.0001) in the patients with thrombocytopenia than in those with normal platelet counts. The median values for Ang-2 and the Ang-2/Ang-1 ratio, on the other hand, were higher (P = 0.012 and P = 0.0001, respectively) in the patients with thrombocytopenia. As the platelet count increased, so did the levels of Ang-1 (P<0.001) but the levels of Ang-2 decreased (P = 0.003) as did the Ang-2/Ang-1 ratio (P<0.001). In summary, regardless of whether or not the patient met the worst-case prognosis criteria, the blood count for platelets showed a positive correlation with Ang-1 (r = 0.6; P<0.001) and a negative correlation with Ang-2 (r = −0.4; P = 0.001) and with the Ang-2/Ang-1 ratio (r = −0.6; P<0.0001) (Figure 1).

ROC curves were generated to identify a cut-off point to discriminate between angiopoietin concentrations that were most strongly associated with the potential severity of the case, including the subgroups of patients stratified according to the platelet blood count. From the ROC curve, a highly accurate cut-off level for serum angiopoietins was defined as an indicator for a worst-case prognosis of a potentially severe malarial infection. In the analysis of all patients, the AUC was 0.667 and the cut-off point for Ang-2 was 5.9 ng/mL (77.8% sensitivity and 50.0% specificity), whereas for the Ang-2/Ang-1 ratio, the AUC was 0.737 and the cut-off point was 0.75 (61.1% sensitivity and 83.9% specificity). Considering only the subgroup of patients with platelet counts <75,000/μL, the ROC curve showed an AUC of 0.833 and a cut-off point of 8.4 ng/mL for Ang-2 (87.5% sensitivity and 66.7% specificity), whereas for Ang-2/Ang-1, the AUC was 0.881, with a cut-off point of 1.2 (75.0% sensitivity and 90.5% specificity). In patients with platelet counts >75.000/μL, the AUC was 0.527 and the cut-off point was 3.85 ng/mL for Ang-2 (80.0% sensitivity and 29.3% specificity), whereas for Ang-2/Ang-1, the AUC was 0.645 and the cut-off point was 0.30 (60.0% sensitivity and 68.3% specificity) (Figure 2).

Discussion

In this study, low levels of Ang-1 and high values of Ang-2 and the Ang-2/Ang-1 ratio were associated with criteria that indicate the worst-case prognosis in patients with symptomatic *P. vivax* infections. In *P. falciparum* malaria, the production of angiogenic factors is principally associated with an increase in the cytoadherence of infected erythrocytes to the vascular endothelium [18]. Previous studies have demonstrated that *P. vivax* promotes cytoadhesion [30,31]. However, the mechanisms involved in endothelial activation and vascular alterations associated with *P. vivax* infections remain unclear. Only two studies have described the relationship between angiopoietins and malaria caused by *P. vivax*, and these presented conflicting results. One report showed

Table 1. Baseline clinical and laboratory characteristics of the patients in each platelet group.

Characteristics	Platelet groups (Median)			p*
	<50,000	50,000 to 150,000	>150,000	
Age (years)	47.5	38.0	38.0	0.435
Hemoglobin (g/dL)	13.0	12.9	12.1	0.488
Hematocrit (%)	38.0	37.9	35.6	0.367
Bilirubin (mg/dL)	1.7	1.3	0.9	0.006
Urea (mg/dL)	41.5	30.0	28.0	0.015
Creatinine (mg/dL)	1.1	0.9	0.9	0.159
Aspartate aminotransferase (U/L)	23.5	28.0	21.0	0.288
Alanine aminotransferase (U/L)	26.5	29.0	29.0	0.952
Parasitemia (/μL)	8,600	6,160	5,000	0.524
Axillary temperature (°C)	36.7	37.0	36.4	0.610

*Cuzick's test.

doi:10.1371/journal.pone.0109246.t001

Table 2. Baseline clinical and laboratory characteristics of patients that were used in defining a severe *Plasmodium vivax* infection.

Characteristics	Case n = 18 median (1^{st}–3^{rd} quartile)	Control n = 62 median (1^{st}–3^{rd} quartile)	p*
Age (years)	38.5 (25–55)	39.0 (28–49)	0.870
Hemoglobin (g/dL)	12.8 (12.6–14.0)	12.9 (11.1–14.3)	0.760
Hematocrit (%)	37.8 (37.0–41.2)	37.2 (32.8–41.8)	0.471
Bilirubin (mg/dL)	2.8 (1.9–3.4)	1.1 (0.8–1.4)	0.008
Urea (mg/dL)	34 (19–41)	29 (25–34)	0.179
Creatinine (mg/dL)	1.0 (0.9–1.2)	0.9 (0.8–1.0)	0.061
Aspartate aminotransferase (U/L)	54 (26–76)	22 (17–30)	<0.001
Alanine aminotransferase (U/L)	45 (31–104)	25.5 (16–37)	0.002
Parasitemia (/μL)	11,625 (7,200–17,500)	4,500 (1,900–9,500)	0.010
Axillary temperature (°C)	37.7 (36.6–39.0)	36.4 (36.0–38.0)	0.126
Platelet count (/μL)	78,500 (55,000–110,000)	113,000 (64,000–175,000)	0.097
Angiopoietin-1(ng/mL)	12 (9.8–17.6)	19.9 (13.1–29.2)	0.005
Angiopoietin-2 (ng/mL)	8.2 (5.9–14.8)	5.9 (3.9–7.9)	0.032
Angiopoietin-2/Angiopoietin-1 ratio	0.8 (0.3–1.5)	0.3 (0.2–0.6)	0.002

*Mann–Whitney *U* test.

doi:10.1371/journal.pone.0109246.t002

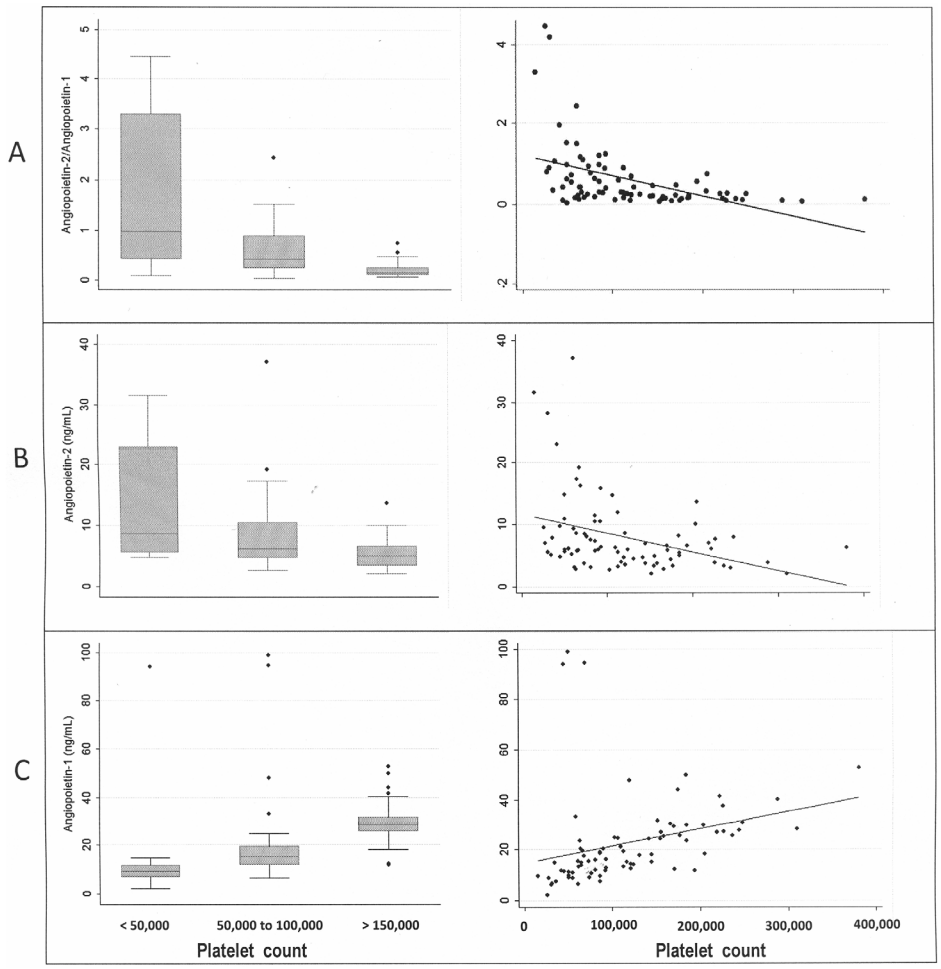

Figure 1. Boxplot and scatter graphs showing the association between the angiopoietin-2/angiopoietin-1 ratio (A), levels of angiopoietin-2 (B), and levels of angiopoietin-1 (C) with platelet counts in *P. vivax* malaria patients.

doi:10.1371/journal.pone.0109246.g001

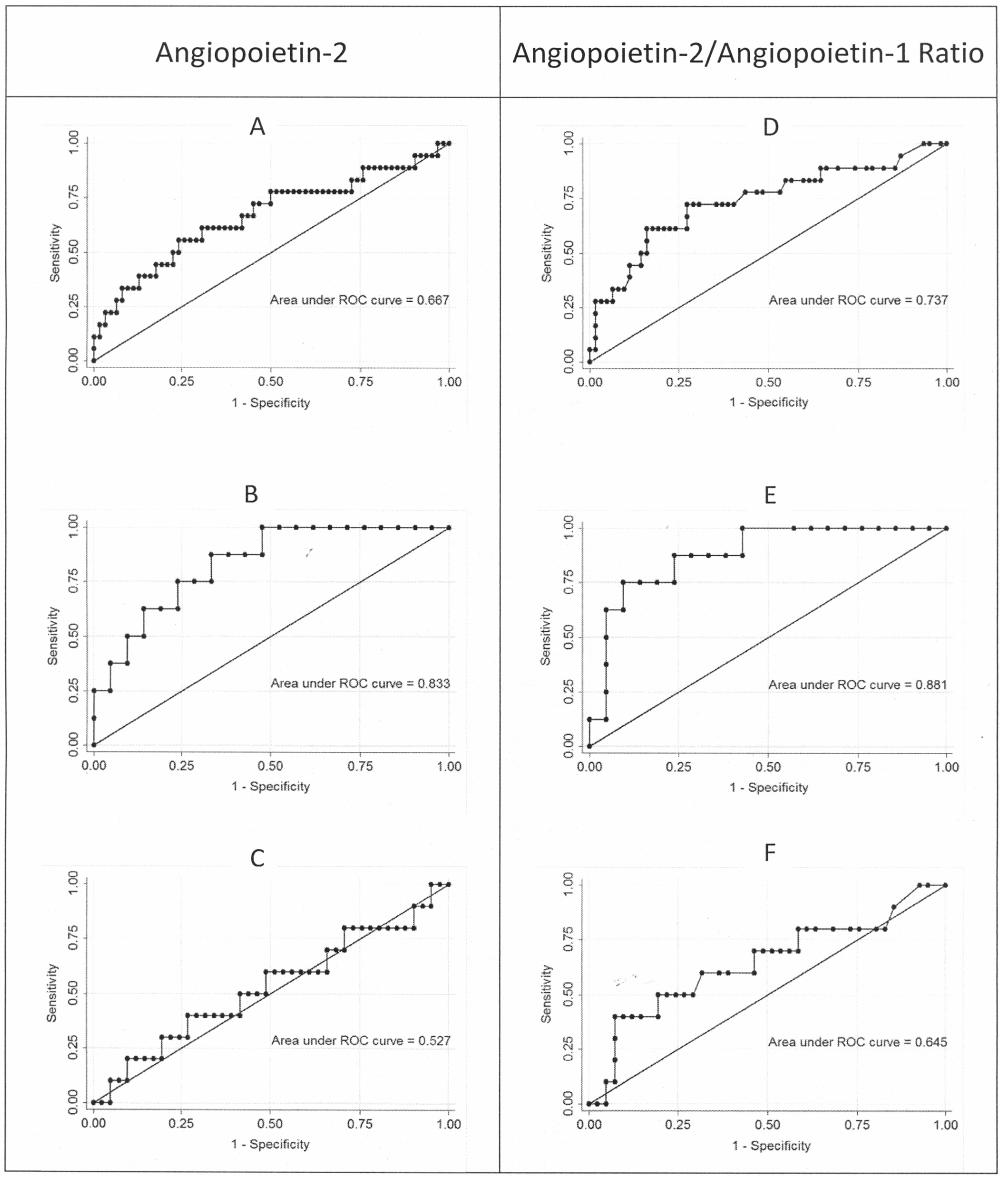

Figure 2. Assessment of the utility of Ang-2 levels and the Ang-2/Ang-1 ratio in discriminating between potentially severe malaria (cases) and controls using ROC analysis for all patients (A, B), patients with platelet counts <75,000/μL (C, D), and patients with platelet counts >75,000/μL (E, F). The reference line represents the ROC curve for a test with no discriminatory ability. The area under the ROC curve (AUC) is displayed on each graph.
doi:10.1371/journal.pone.0109246.g002

lower levels of Ang-2 in patients infected by *P. vivax* than in patients infected by *P. falciparum* [27], whereas the other report detected higher levels of Ang-2 in patients infected by *P. vivax* [26]. However, no information is currently available on levels of Ang-1 or the Ang-2/Ang-1 ratio.

Angiopoietins are proteins that are intimately involved in the stability of the endothelium [21] and their levels are altered in various inflammatory conditions, mainly due to endothelial activation [19] in malarial infections and other infectious diseases [32]. Under normal conditions, higher levels of Ang-1 promote quiescence in the vascular endothelium. In inflamed areas, however, Ang-2 levels are higher, principally in the endothelial cells and the smooth muscles, thus activating the vascular endothelium. *In vitro* and *in vivo* studies have demonstrated that higher concentrations of Ang-2 are associated with elevated production of TNF-α, nitric oxide, vascular endothelial growth factor (VEGF), and hypoxia [33,34].

In this study, a positive correlation was observed between platelet counts and Ang-1 levels in *P. vivax* malaria patients, whereas a negative correlation was detected between platelet counts and Ang-2 and Ang-2/Ang-1 levels. Although not a defining feature of severe malaria, vivax-associated thrombocytopenia occurs in more than 24% of patients with vivax malaria [35,36]. Its mechanism is multifactorial; it has been associated with oxidative stress, alterations in splenic function, invasion of the bone marrow by trophozoites, and destruction by immunoglobulins [36,37]. Vivax-related thrombocytopenia has been associated with severe manifestations [11,12] and the need for blood and platelet transfusions was reported in 5% of adults with severe vivax malaria [11].

The association between Ang-1, Ang-2, and the Ang-2/Ang-1 ratio was stronger in patients with thrombocytopenia. Some possible explanations for this association are given below. Firstly, Ang-1 is secreted by platelet granules, and thus its concentration in the plasma is dependent on the number of circulating platelets [38]. Our results support the findings of Browers et al. (2013), who detected elevated serum Ang-1 levels following the recovery of platelet numbers in patients treated for falciparum malaria [39]. Secondly, a prior study involving patients with vivax and falciparum malaria has shown increased thrombogenic activity and a reduction in platelet count associated with high production of cytokines and microparticles, and consequently, endothelial activation and an increase in angiogenic factors in the serum [40,41,42]. The observation of higher levels of Ang-2 and Ang-2/Ang-1 in patients with thrombocytopenia in this study supports these findings and validates the association between thrombocytopenia caused by *P. vivax* and the elevation of Ang-2 levels.

Although useful for clinical decision-making, biomarkers that predict the appearance of criteria indicating the worst-case prognosis for *P. vivax* malaria patients have not been identified to date. Disequilibrium in the expression and production of angiogenic factors is associated with the severity of malaria caused by *P. falciparum* [22,18,43,44]. It has also been found that Ang-2 is a better prognostic marker to signal imminent severity in *P. falciparum* malaria than lactate [18], which is the traditional laboratory marker for severity [4]. High plasma levels of Ang-2 have also been associated with worst-case prognoses for other diseases such as leukemia, metastatic melanoma, acute pulmonary damage, and acute hepatic and renal failure [19]. To summarize, Ang-2 and Ang-2/Ang-1 have been considered as predictive biomarkers for the severity of various pathologies, both non-infectious [45,46,47] and infectious [32,48], including falciparum malaria [15,23,49,50]. The Ang-2/Ang-1 ratio has already been considered as a more effective biomarker of severity than Ang-2 alone in falciparum malaria [50], as well as in other diseases [51,52].

To the best of our knowledge, this study provides the first evidence of the relationship between changes in angiopoietin levels and criteria indicating the worst-case prognosis for *P. vivax* malaria, especially in patients with low platelet counts. Once incorporated into routine hospital laboratory investigation, the systematic assay of angiopoietins could help isolate patients with the potential for developing severe malaria during clinical management of vivax malaria.

The main limitation of this study is the small number of patients examined, thus restricting the possibilities for extrapolating the results to other populations. It is also important to point out that the comorbidities of the patients studied may explain the findings suggesting a worst-case prognosis, as already shown in the literature [6]. However, in this study it was possible to exclude some acute morbidities, such as dengue, yellow fever, and types of viral hepatitis, leptospirosis, bacterial infections, pneumonia, heart failure, hepatic cirrhosis, and diabetes mellitus. However, further studies involving a larger number of patients and more research into comorbidities should be performed to validate our findings.

Conclusions

This study showed that patients with worst-case prognoses for *P. vivax* malarial infections have lower serum Ang-1 and higher serum Ang-2 levels (and higher values for the Ang-2/Ang-1 ratio) than controls. Furthermore, a positive correlation was observed between platelet count and Ang-1, and a negative correlation was observed between platelet count and Ang-2 (and the Ang-2/Ang-1 ratio). Elevated serum levels of Ang-2 and high values for the Ang-2/Ang-1 ratio may potentially be used as predictors of worst-case prognoses for *P. vivax* malaria, especially in patients with thrombocytopenia.

Author Contributions

Conceived and designed the experiments: LTG CJF. Performed the experiments: ERA CR. Analyzed the data: LTG CJF. Contributed reagents/materials/analysis tools: TOG. Contributed to the writing of the manuscript: LTG CJF. Performed the clinical evaluation of the included patient: AFN.

References

1. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, et al. (2007) Vivax malaria: neglected and not benign. Am J Trop Med Hyg 77(6): 79–87.
2. Baird JK (2009) Severe and fatal vivax malaria challenges 'benign tertian malaria' dogma. Ann Trop Paediatr 29(4): 251–252.
3. Anstey NM, Douglas NM, Poespoprodjo JR, Price RN (2012) *Plasmodium vivax*: clinical spectrum, risk factors and pathogenesis. Adv Parasitol 80: 151–201.
4. The World Health Organization (2013) Management of severe malaria – a practical handbook. Geneva.
5. Lança EF, Magalhães BM, Vitor-Silva S, Siqueira AM, Benzecry SG, et al. (2012) Risk factors and characterization of Plasmodium vivax-associated admissions to pediatric intensive care units in the Brazilian Amazon. PLoS One 7(4): e35406.
6. Lacerda MV, Fragoso SC, Alecrim MG, Alexandre MA, Magalhães BM, et al. (2012) Postmortem characterization of patients with clinical diagnosis of Plasmodium vivax malaria: to what extent does this parasite kill? Clin Infect Dis 55(8): e67–74.
7. Luxemburger C, Thwai KL, White NJ, Webster HK, Kyle DE, et al. (1996) The epidemiology of malaria in a Karen population on the western border of Thailand. Trans R Soc Trop Med Hyg 90(2): 105–111.
8. Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, et al. (2008) Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS Med 5(6): e128.
9. Lampah DA, Yeo TW, Hardianto SO, Tjitra E, Kenangalem E, et al. (2011) Coma associated with microscopy-diagnosed Plasmodium vivax: a prospective study in Papua, Indonesia. PLoS Negl Trop Dis 5(6): e1032.
10. Tanwar GS, Khatri PC, Sengar GS, Kochar A, Kochar SK, et al. (2011) Clinical profiles of 13 children with Plasmodium vivax cerebral malaria. Ann Trop Paediatr 31(4): 351–356.
11. Kochar DK, Das A, Kochar SK, Saxena V, Sirohi P, et al. (2009) Severe Plasmodium vivax malaria: a report on serial cases from Bikaner in northwestern India. Am J Trop Med Hyg 80(2): 194–198.
12. Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LM, et al. (2010) Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance. Malar J 9: 13.
13. Barber BE, William T, Grigg MJ, Menon J, Auburn S, et al. (2013) A prospective comparative study of knowlesi, falciparum, and vivax malaria in Sabah, Malaysia: high proportion with severe disease from Plasmodium knowlesi and Plasmodium vivax but no mortality with early referral and artesunate therapy. Clin Infect Dis 56(3): 383–397.
14. de Mast Q, Syafruddin D, Keijmel S, Riekerink TO, Deky O, et al. (2010) Increased serum hepcidin and alterations in blood iron parameters associated with asymptomatic *P. falciparum* and *P. vivax* malaria. Haematol 95(7): 1068–1074.
15. Erdman LK, Dhabangi A, Musoke C, Conroy AL, Hawkes M, et al. (2011) Combinations of host biomarkers predict mortality among Ugandan children with severe malaria: a retrospective case-control study. PLoS One 6(2): e17440.
16. Andrade BB, Barral-Netto M (2011) Biomarkers for susceptibility to infection and disease severity in human malaria. Mem Inst Oswaldo Cruz Suppl 1: 70–78.
17. Kochar DK, Saxena V, Singh N, Kochar SK, Kumar SV, et al. (2005) *Plasmodium vivax* malaria. Emerg Infect Dis 11: 132–134.
18. Yeo TW, Lampah DA, Gitawati R, Tjitra E, Kenangalem E, et al. (2008) Angiopoietin-2 is associated with decreased endothelial nitric oxide and poor clinical outcome in severe falciparum malaria. Proc Natl Acad Sci USA 105(44): 17097–17102.
19. Eklund L, Saharinen P (2013) Angiopoietin signaling in the vasculature. Exp Cell Res 319(9): 1271–1280.
20. Fukuhara S, Sako K, Noda K, Zhang J, Minami M, et al. (2010) Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis. Histol Histopathol 25(3): 387–396.

21. Thurston G, Daly C (2012) The complex role of angiopoietin-2 in the angiopoietin-tie signaling pathway. Cold Spring Harb Perspect Med 2(9): a006550.
22. Einsenhut M (2012) Low angiopoietin-1 as a predisposing factor for cerebral vasospasm in cerebral malaria. Crit Care Med 40(12): 3333–3334.
23. Conroy AL, Glover SJ, Hawkes M, Erdman LK, Seydel KB, et al. (2012) Angiopoietin-2 levels are associated with retinopathy and predict mortality in Malawian children with cerebral malaria: a retrospective case-control study. Crit Care Med 40(3): 952–959.
24. Silver KL, Zhong K, Leke RG, Taylor DW, Kain KC (2010) Dysregulation of angiopoietins is associated with placental malaria and low birth weight. PLoS One 5(3): e9481.
25. Conroy AL, Liles WC, Molyneux ME, Rogerson SJ, Kain KC (2011) Performance characteristics of combinations of host biomarkers to identify women with occult placental malaria: a case-control study from Malawi. PLoS One. 6(12): e28540.
26. Yeo TW, Lampah DA, Tjitra E, Piera K, Gitawati R, et al. (2010) Greater endothelial activation, Weibel-Palade body release and host inflammatory response to *Plasmodium vivax*, compared with *Plasmodium falciparum*: a prospective study in Papua, Indonesia. J Infect Dis 202(1): 109–112.
27. MacMullin G, Mackenzie R, Lau R, Khang J, Zhang H, et al. (2012) Host immune response in returning travellers infected with malaria. Malar J 11: 148.
28. David S, Kümpers P (2011) Circulating angiopoietin-1 could be confounded by ex vivo platelet activation. Kidney Int 79(6): 687.
29. Bantis LE, Nakas CT, Reiser B. (2014) Construction of confidence regions in the ROC space after the estimation of the optimal Youden index-based cut-off point. Biometrics 70(1): 212–223.
30. Carvalho BO, Lopes SC, Nogueira PA, Orlandi PP, Bargieri DY, et al. (2010) On the cytoadhesion of *Plasmodium vivax*-infected erythrocytes. J Infect Dis 202(4): 638–647.
31. Lopes SC, Albrecht L, Carvalho BO, Siqueira AM, Thomson-Luque R, et al. (2014) Paucity of *Plasmodium vivax* mature schizonts in peripheral blood is associated with their increased cytoadhesive potential. J Infect Dis 209(9): 1403–1407.
32. Page AV, Liles WC (2013) Biomarkers of endothelial activation/dysfunction in infectious diseases. Virulence 4(6): 507–516.
33. Kim I, Kim JH, Ryu YS, Liu M, Koh GY (2000) Tumor necrosis factor-alpha upregulates angiopoietin-2 in human umbilical vein endothelial cells. Biochem Biophys Res Commun 269(2): 361–365.
34. Augustin HG, Koh GY, Thurston G, Alitalo K (2009) Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. Nat Rev Mol Cell Biol 10(3): 165–177.
35. Tan LK, Yacoub S, Scott S, Bhagani S, Jacobs M (2008) Acute lung injury and other serious complications of Plasmodium vivax malaria. Lancet Infect Dis 8(7): 449–454.
36. Lacerda MV, Mourão MP, Coelho HC, Santos JB (2011) Thrombocytopenia in malaria: who cares? Mem Inst Oswaldo Cruz Suppl 1: 52–63.
37. Kumar A, Shashirekha (2006) Thrombocytopenia-an indicator of acute vivax malaria. Indian J Pathol Microbiol 49(4): 505–508.
38. Li JJ, Huang YQ, Basch R, Karpatkin S (2001) Thrombin induces the release of angiopoietin-1 from platelets. Thromb Haemost 85(2): 204–206.
39. Brouwers J, Noviyanti R, Fijnheer R, de Groot PG, Trianty L (2013) Platelet activation determines angiopoietin-1 and VEGF levels in malaria: implications for their use as biomarkers. PLoS One 8(6): e64850.
40. De Mast Q, Groot E, Lenting PJ, de Groot PG, McCall M, et al. (2007) Thrombocytopenia and release of activated von Willebrand Factor during early *Plasmodium falciparum* malaria. J Infect Dis 196(4): 622–628.
41. Faille D, El-Assaad F, Alessi MC, Fusai T, Combes V, et al. (2009) Platelet-endothelial cell interactions in cerebral malaria: the end of a cordial understanding. Thromb Haemost 102(6): 1093–1102.
42. Campos FM, Franklin BS, Teixeira-Carvalho A, Filho AL, de Paula SC, et al. (2010) Augmented plasma microparticles during acute *Plasmodium vivax* infection. Malar J 9: 327.
43. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N, et al. (2009) Whole bloodangiopoietin-1 and -2 levels discriminate cerebral and severe (non-cerebral) malaria from uncomplicated malaria. Malar J 8: 295.
44. Conroy AL, Phiri H, Hawkes M, Glover S, Mallewa M, et al. (2010) Endothelium-based biomarkers are associated with cerebral malaria in Malawian children: a retrospective case-control study. PLoS One 5(12): e15291.
45. Ong T, McClintock DE, Kallet RH, Ware LB, Matthay MA, et al. (2010) Ratio of angiopoietin-2 to angiopoietin-1 as a predictor of mortality in acute lung injury patients. Crit Care Med 38: 1845–1851.
46. Goede V, Coutelle O, Neuneier J, Reinacher-Schick A, Schnell R, et al. (2010) Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumab-containing therapy. Br J Cancer 103(9): 1407–1414.
47. David S, John SG, Jefferies HJ, Sigrist MK, Kümpers P, et al. (2012) Angiopoietin-2 levels predict mortality in CKD patients. Nephrol Dial Transplant 27(5): 1867–1872.
48. Michels M, van der Ven AJ, Djamiatun K, Fijnheer R, de Groot PG, et al. (2012) Imbalance of angiopoietin-1 and angiopoetin-2 in severe dengue and relationship with thrombocytopenia, endothelial activation, and vascular stability. Am J Trop Med Hyg 87(5): 943–946.
49. Lovegrove FE, Tangpukdee N, Opoka RO, Lafferty EI, Rajwans N, et al. (2009) Serum angiopoietin-1 and -2 levels discriminate cerebral malaria from uncomplicated malaria and predict clinical outcome in African children. PLoS One 4(3): e4912.
50. Jain V, Lucchi NW, Wilson NO, Blackstock AJ, Nagpal AC, et al. (2011) Plasma levels of angiopoietin-1 and -2 predict cerebral malaria outcome in Central India. Malar J 10: 383.
51. Chen S, Guo L, Chen B, Sun L, Cui M (2013) Association of serum angiopoietin-1, angiopoietin-2 and angiopoietin-2 to angiopoietin-1 ratio with heart failure in patients with acute myocardial infarction. Exp Ther Med 5(3): 937–941.
52. Luz Fiusa MM, Costa-Lima C, de Souza GR, Vigorito AC, Penteado Aranha FJ, et al. (2013) A high angiopoietin-2/angiopoietin-1 ratio is associated with a high risk of septic shock in patients with febrile neutropenia. Crit Care 17(4): R169.