

The utility of a three part differential hematology analyzer with CRP for assessing malaria

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Abstract

Background

Hematology analyzers display abnormal hematology parameters during malaria infection providing insightful information for suspecting and assessing malaria infection. The goal of this study is to demonstrate the potential of a three part differential hematology analyzer to assess malaria, provide information about the parasitemia, and discuss the importance of combining CRP with hematology parameters to obtain further information about the malaria infection.

Methods

The present study shows the results of a retrospective study involving comparison of raw instrument data from a three part differential hematology analyzer with CRP measurement and corresponding microscopic findings of samples obtained during the monsoon season of years 2018 and 2019 in Mumbai, India. Mann-Whitney U and Krustal-Wallis tests were applied for obtaining statistical significance of hematology parameters and CRP among *P. vivax*, *P. falciparum*, dengue and negative samples.

Results

24 The study considers 1008 non-malaria febrile cases, 209 *P. vivax*, 31 *P. falciparum*
25 positive malaria samples, five cases of mixed species malaria infection, and three co-
26 infection of malaria and dengue. Median values of WBCs, PLTs, PCT and %LYM were
27 lower in malaria and dengue samples compared to negative ones with statistical
28 difference ($p < 0.05$). Contrary, medians of MCHC, MPV, PDW, and %MON were
29 higher than negative samples.

30 The greatest difference in the analyzer results of malaria and dengue cases was
31 observed in between their medians of CRP levels, which is evidenced by the values of
32 the first quartiles of *P. vivax* and *P. falciparum* cases, and the third quartile of dengue
33 cases being 8.6 and 10.4 mg/L, respectively.

34 The parameters with a statistical difference for different levels of parasitemia were
35 WBC, PLT, %MON and CRP. An interfering abnormal peak was observed in the white
36 blood cell histogram, below 37 fl, in malaria infected samples, especially in *P. vivax*
37 cases, where the height of that peak showed a strong correlation with red blood cells
38 infected predominantly with larger parasitic forms.

39 **Conclusions**

40 A three differential part hematology analyzer has the potential to not only trigger
41 malaria diagnosis confirmation but also assess the severity of the infection when CRP is
42 considered.

43 **Keywords**

44 Malaria; hematology analyzer; WBC histogram; CRP

45 **Background**

46 According to the WHO an estimated 228 million cases of malaria occurred in 2018
47 worldwide and 85% of this burden was located in 19 sub-Saharan Africa countries and

48 India. For a rapid disease management and surveillance of malaria, the WHO
49 recommended two main strategies to eradicate malaria: closing the gaps of healthcare
50 access between urban and rural areas, and diagnosis before antimalarial treatment by
51 microscopy or malaria rapid diagnostic test (RDT) in all patients with malaria
52 suspicion. Fever is the main manifestation for suspicion of malaria infection. In malaria
53 endemic areas, it acts as a trigger for parasitology testing [1].

54 RDTs are currently replacing microscopy, the golden standard for diagnosing malaria,
55 especially in endemic countries. Thus, complementary information should be
56 considered to ensure correct interpretation and correct disease management
57 particularly in surveillance programs [2]. The two main limitations of RDTs are the
58 impossibility to obtain parasitemia quantification and, particularly in antibody based
59 RDT, the persistent positivity after treatment [2]. Microscopy can overcome both
60 limitations by quantifying the parasite density, however the activity is highly time-
61 consuming.

62 Complete blood count (CBC) is one of the most frequently requested laboratory tests in
63 patients with acute febrile illness symptoms to confirm infection status and/or severity
64 of disease to initiate proper treatment. For endemic and non-endemic countries,
65 abnormal hematology parameters might provide insightful information for managing
66 malaria infection.

67 **Malaria detection in standard automated cell counters (ACCs)**

68 The potential to diagnose clinically unsuspected cases of malaria for patients subjected
69 to CBC is one of the main benefits of flagging malaria in hematology analyzers [3]. A
70 comprehensive summary of studies for diagnosing malaria by ACCs without a specific
71 malaria-detection design is well described by Campuzano-Zuluaga [3], in which many
72 of the referred studies provide performances to detect malaria related to abnormalities

73 in the white blood cell (WBC) differentiation channel. ACCs with cytometry capable of
74 differentiating five WBC populations show a clear clustering for malaria population due
75 to specific scattering of infected red blood cells (iRBCs) that are resistant to lyse and/or
76 produce scatters due to residuals of the parasite infection. Pseudoeosinophilia resulting
77 from neutrophils containing haemozoin have been reported as an abnormality caused
78 by malaria infection [4 - 6]. Fourcade et al., demonstrated that large activated
79 monocytes are present on malaria infections by measuring the standard deviation of the
80 volume of LYM and MON calculating a malaria factor with sensitivity of 96.9% and
81 specificity of 82.5% [7]. Many studies have identified a particular cluster by inspecting
82 different angles of light after moderate lysing of RBC without using extra reagents.
83 However the majority of these studies have a lower performance to detect small or
84 immature forms of parasites due to their low lysing resistance and small size. For
85 example, a recent evaluation on a malaria parameter to evaluate the percentage of
86 iRBCs in the BC-6800 (Mindray) showed a lower sensitivity to detect *P. falciparum* in
87 comparison with detecting *P. vivax* (24.1% and 88.3%, respectively), and related to it
88 the difficulty to detect small size parasites and their low nucleic acid content [8], which
89 are prevalent in *P. falciparum* infection where trophozoites and schizonts are barely
90 seen in peripheral blood.

91 In spite of all promising results from ACCs to identify malaria, fewer studies have been
92 conducted for three differential ACCs. One study combined HGB, TLC, platelets and
93 RDW data in a KX-21 (Sysmex) achieving a 26.1% sensitivity and 96.3% of specificity in
94 a study mainly consisting of *P. vivax* infected samples [9]. The majority of small ACCs
95 utilize the Coulter-principle to classify WBCs in populations based on size and obtain an
96 RBC distribution using limited reagents. Identifying intracellular presence of malaria

97 parasite without inspecting the internal content of blood cells based only on abnormal
98 WBC histograms' distribution caused by the infection poses a difficult challenge.

99 **C-reactive protein (CRP) to distinguish malaria**

100 CRP is a member of the pentraxin family of proteins, an acute-phase reactant and a
101 biomarker for inflammation. Although CRP is not a specific biomarker for detecting
102 malaria infection, several studies have identified CRP to be a potential marker to assess
103 the severity of malaria infections [10-13]. A study in the south-western part of the
104 Brazilian Amazon has shown evidence in the association of *P. vivax* malaria infection
105 with inflammatory activation, including CRP, and cytokine imbalance [14].

106 Dengue and malaria infections, which frequently co-exist in endemic areas, present
107 with similar symptomatology and produce similar abnormalities in CBC parameters.
108 CRP has been observed to be a useful biomarker to discriminate malaria and dengue,
109 Epelboin et al., using CRP > 5 mg/L as cut off to differentiate between malaria from
110 dengue obtained sensitivity of 99% and specificity of 35% [15]. In addition, effective
111 management and surveillance of malaria require a screening method facilitating quick
112 transfer of patients to receive diagnostic confirmation and proper treatment. POCT
113 hematology analyzers providing information about malaria and other co-existing
114 infections such as dengue could play a major role to support this line of action.

115 The goal of this study is to demonstrate the potential of a three part differential
116 hematology analyzer to assess malaria, provide information about the parasitemia, and
117 discuss the importance of combining CRP with hematology parameters to obtain
118 further information about the malaria infection.

119 **Methods**

120 The present study shows the results of a retrospective study involving comparison of
121 raw instrument data from a three part differential hematology analyzer with CRP

122 measurement (LC-667G CRP, HORIBA) and corresponding microscopic findings of the
123 patients visiting at Dr. Dharap's Diagnostic Centre, Mumbai, India from samples
124 obtained in the monsoon season of years 2018 and 2019.

125 During monsoon season, the laboratory routinely caters to patients for diagnostic
126 testing in case of acute febrile illness which comprises a CBC, peripheral examination of
127 blood smear for malarial parasite and rapid screening tests for malaria and dengue NS1
128 being an endemic area with co-existent infections. Documented historical data of
129 known malaria, dengue positive and negative cases was used for the study. Persons for
130 routine health check-up without history of any specific ailments visiting the laboratory
131 during the corresponding periods were considered for normal or control group.

132 For the current study, the historical documented data was further anonymized in coded
133 fashion in order to protect patient confidentiality in accordance with local ethical or
134 IRB guidelines, according to national and international standards for the conduct of
135 clinical studies including 21 CFR Parts 50 and 56 and International Conference on
136 Harmonization (ICH) E6-Good Clinical Practice Consolidated Guideline. Ethical
137 clearance was not obtained as study was carried as a retrospective review of the
138 available raw instrument and historical patient data, without any collection of extra
139 specimen or monetary charges to patient and did not involve any communication of
140 results of the study to the clinician so as to affect the diagnostic and therapeutic
141 management of the patients.

142 **Laboratory techniques**

143 Testing for 'Acute Febrile Illness' in the laboratory comprises of a full blood count,
144 peripheral examination of blood smear for malarial parasite and rapid screening tests
145 for malaria and dengue NS1 being an endemic area with co-existent infections.

146 Full blood count is performed using a three part differential hematology analyzer (LC-
147 667G CRP, HORIBA) which additionally provides CRP level estimation. For peripheral
148 blood smear examination the following technique is used. Anticoagulated (K2-EDTA)
149 blood of patient is used to prepare thick & thin blood smear for peripheral blood smear
150 examination (PBSE). Both types of smear preparation are air dried. Thin preparation is
151 fixed with methanol whereas thick blood smear are subjected to 'process of de-
152 hemoglobinisation' with deionized water. Both smears are then stained by 'Field
153 staining technique' using automated slide stainer. Thin smears are examined for
154 presence of malarial parasite and species identification, presence of platelet aggregates,
155 macroplatelets and nucleated red blood cells, if any.

156 Malaria cases are further assessed for parasite density by counting parasite life cycle
157 forms against 100 white blood cells by an experienced pathologist. The life cycle forms
158 are grouped as small forms being ring and early ameboid, and large forms including
159 late ameboid with ample parasitic material, schizonts and gametocytes.

160 For additional screening and confirmation of malaria cases, Rapid Malaria Antigen
161 detection test kits manufactured by SD Biosensor Healthcare Pvt. Ltd., Gurugram,
162 India using monoclonal anti-*P. falciparum* HRP-II (0.75+/- 0.15 µg) with monoclonal
163 anti-*P. vivax* pLDH (0.75+/- 0.15 µg) respectively to detect presence of *P. falciparum* &
164 *P. vivax* related antigen is used.

165 For screening & diagnosis of dengue, RecombiLISA NS1 Antigen test utilizing pairs of
166 specific polyclonal & monoclonal anti-dengue antibodies of all four serotypes (DEN1, 2,
167 3, 4) and analytical sensitivity of 0.3 ng/ml for type 2 NS1 antigen, manufactured by
168 CTK Biotech, Inc., United States of America is used.

169 **Statistical analysis**

170 To determine the significance of the differences between the hematology parameters
171 extracted from LC-667G CRP of positive & negative samples of *P. vivax* & *P. falciparum*
172 samples respectively, as well as malaria & dengue positive samples, Mann-Whitney U
173 and Krustal-Wallis tests were applied due to their non-parametric distribution.

174 All the statistical calculations were done using Minitab 19.1.1. and histogram graphs
175 were created using R software 3.5.1.

176 **Results**

177 From the monsoon seasons of 2018 and 2019, based on selection criteria and
178 availability of corresponding data, we selected 209 (144 males, 65 females) *P. vivax*
179 positive cases, 31 (22 males, 9 females) *P. falciparum* samples, five cases of mixed
180 species malaria infection (4 males, 1 female), and three co-infection of malaria and
181 dengue (2 males, 1 female). In addition, 1008 non-malaria febrile cases were examined
182 including 197 (124 males, 73 females) dengue positive cases. The median and range of
183 age were 35 years (5-87) and 36 years (15-77) for only *P. vivax* and *P. falciparum* cases,
184 respectively.

185 Using Krustal-Wallis test to compare the four groups (see table 1), median values of
186 WBCs, PLTs, PCT and %LYM were lower in malaria and dengue samples compared to
187 negative ones with a statistical difference ($p < 0.05$). Contrary, the median values of
188 MCHC, MPV, PDW, and %MON were higher than negative samples. RBC, HGB, HCT,
189 MCV, MCH and %GRA showed no significant difference in median between the four
190 groups.

191 The medians of the parameters that had a significant difference between *P. vivax*
192 infected and negative samples were WBCs, MCH, MCHC, RDW, PLTs, MPV, PCT, PDW
193 and CRP. Similarly *P. falciparum* cases had the same parameters with significant
194 difference excepting MCH, MCHC and RDW.

195 Comparing both malaria infected cases with dengue, only PLT, PCT and CRP medians
196 had a significant difference, which makes difficult to differentiate between both
197 diseases when observing solely the hematology parameters affection. The greatest
198 difference of median between malaria infected patients and dengue was observed with
199 CRP. Figure 1 shows the first quartiles of *P. vivax* and *P. falciparum* to have a difference
200 with the third quartile of dengue patients of 8.6 and 10.4 mg/L, respectively.
201 In the current set of data only %LYM ($p = 0.029$) showed a significant difference
202 between *P. Vivax* and *P. Falciparum* samples.

	P. vivax (n = 209) Median (IQR)	P. falciparum (31) Median (IQR)	Dengue (n=197) Median (IQR)	Patients with fever but Non-malaria non-dengue (n=811) Median (IQR)	p_{KW}	p_{VF}	p_{VD}	p_{FD}	p_{VN}	p_{FN}
WBC ($10^3/\mu\text{L}$)	5.4 (4.1 - 6.6)	4.7 (3.8 - 5.5)	4.4 (3.4 - 6)	6.6 (5.1 - 8.5)	< 0.001	0.056	< 0.001	0.404	< 0.001	< 0.001
RBC ($10^6/\mu\text{L}$)	4.67 (4.3 - 5.15)	4.5 (4.09 - 4.89)	4.74 (4.4 - 5.18)	4.73 (4.36 - 5.09)	0.19	0.255	0.437	0.199	0.509	0.119
HGB (g/dL)	13.5 (12.32 - 14.67)	12.7 (11.8 - 14.4)	13.4 (12.5 - 14.6)	13.3 (11.95 - 14.8)	0.24	0.082	0.894	0.114	0.203	0.206
HCT (%)	40.05 (36.32 - 43.4)	37.7 (35.8 - 42.4)	40.3 (37.3 - 43.5)	39.7 (35.72 - 43.87)	0.318	0.136	0.436	0.053	0.601	0.187
MCV (μm^3)	86.05 (82 - 89.37)	84.2 (74.6 - 89.9)	84.8 (81.1 - 89)	85.4 (80.6 - 89.8)	0.437	0.148	0.667	0.309	0.358	0.335
MCH (pg)	29.15 (27.5 - 30.47)	28.7 (25.9 - 30.9)	28.6 (27.1 - 30.1)	28.6 (26.75 - 30.3)	0.123	0.185	0.139	0.669	0.04	0.593
MCHC (g/dL)	33.80 (33.2 - 34.75)	33.80 (33 - 34.4)	33.35 (33.1 - 33.9)	33.50 (32.9 - 33.9)	< 0.001	0.461	< 0.001	0.115	< 0.001	0.238
RDW (%)	14.45 (13.8 - 15.1)	15 (13.6 - 15.6)	14.5 (14.1 - 15.2)	14.7 (14.2 - 15.4)	< 0.001	0.333	0.152	0.09	< 0.001	0.698
PLT ($10^3/\mu\text{L}$)	102.5 (66.25 - 149)	109 (79 - 140)	174 (136 - 219)	223 (174 - 274)	< 0.001	0.991	< 0.001	< 0.001	< 0.001	< 0.001
MPV (μm^3)	8.4 (7.9 - 9)	8.6 (7.8 - 9.3)	8.2 (7.7 - 8.8)	8.1 (7.6 - 8.6)	< 0.001	0.168	0.732	0.14	< 0.001	0.001
PCT (%)	0.0865 (0.056 - 0.119)	0.088 (0.063 - 0.113)	0.144 (0.118 - 0.175)	0.179 (0.143 - 0.217)	< 0.001	0.769	< 0.001	< 0.001	< 0.001	< 0.001
PDW (%)	15.35 (13.9 - 17.3)	16.9 (13.8 - 19.4)	15 (14 - 16.4)	14.6 (13.7 - 15.9)	< 0.001	0.274	0.122	0.102	< 0.001	0.023
%LYM	25.9 (20.4 - 34.2)	22.8 (18.5 - 27)	23.4 (16.75 - 31.22)	26.1 (18 - 35.3)	0.016	0.029	0.015	0.919	0.406	0.117
%MON	5.6 (4 - 7.3)	5.8 (4.4 - 7.2)	5.5 (4.2 - 7.2)	5.2 (4 - 6.8)	0.04	0.658	0.217	0.636	0.055	0.156
%GRA	68 (59.1 - 73.8)	71.2 (66.3 - 77.2)	70.35 (61.55 - 79.25)	68.4 (58.32 - 77.6)	0.071	0.061	0.053	0.876	0.257	0.236
CRP (mg/L)	67.4 (35.7 - 111.2)	81.4 (37.5 - 122.3)	9.7 (3.3 - 27.1)	10.4 (2.9 - 27.9)	< 0.001	0.455	< 0.001	< 0.001	< 0.001	< 0.001

203 Table 1. Hematology parameters statistics of the LC-667G of data sets 2018 and 2019.

204 **p*-value of less than 0.05 was considered statistically significant

205 * p_{KW} , p_{VF} , p_{VD} , p_{FD} , p_{VN} , and p_{FN} corresponds to the *p*-value of Kruskal-Wallis test, *PV*
206 *vs PF*, *PV vs dengue*, *PF vs dengue*, *PV vs negative*, and *PF vs negative*, respectively

207 **Co-infections of malaria and dengue or PV and PF were not contemplated*

208 **Insert here Figure 1**

209 **Parasitemia association with different hematology parameters**

210 To inspect the affection of the hematology parameters regarding the parasite density
211 observed in peripheral blood, only the data-set of *P. vivax* samples in the current study
212 has enough data variation to provide interesting results. The distribution of parasitemia
213 in *P. falciparum* samples of the present study is limited, more than 60% of the cases
214 have less than 0.2% of parasitemia, thus the analysis of associating parasitemia with the
215 impact on hematology parameters was performed solely for the *P. vivax* set of data.

216 The parasitemia percentage establishes the relation of the number of parasites found in
217 thin smear against the total count of RBCs and it was calculated with the following
218 formula:

$$\%Parasitemia = \frac{\frac{\#Parasites}{100WBC} \left(\frac{\#WBC}{\mu L} \right)}{\frac{\#RBC}{\mu L}} \times 100 \dots \text{Eq. 1}$$

219 **The total counts of WBCs and RBCs were retrieved from the hematology analyzer.*

220 The parasitemia ranges from 0.00005% to 2.15% and no cases of high parasitemia were
221 found (>5% in low intensity transmission areas). Table 2 summarizes the medians of
222 different hematology parameters provided by the LC-667G in relation with three
223 divisions of parasitemia (< 0.5%, 0.5% < parasitemia < 1%, > 1%). The parameters with
224 a statistical difference in the median were WBC ($p = 0.001$), PLT ($p = 0.022$), %MON (p
225 < 0.001) and CRP ($p < 0.001$). A negative slope in the significant different medians was
226 found in WBC, PLT, PCT and %MON, whereas CRP increases with its parasite load.

227 Figure 2 shows the association of CRP value with a more extended parasitemia
 228 classification where the median of each population has a positive correlation with the
 229 parasite load, however all groups have a wide variation (CV>20% in all groups).

Parameter	paras < 0.5 (n = 156)	0.5 <= paras < 1 (n=28)	paras >= 1 (n=14)	p-value
WBC (10 ³ /μL)	5.10	6.55	6.40	0.001
RBC (10 ⁶ /μL)	4.66	4.75	4.53	0.52
HGB (g/dL)	13.60	13.05	12.85	0.262
HCT (%)	40.30	37.95	38.40	0.341
MCV (μm ³)	86.10	85.80	84.55	0.723
MCH (pg)	29.20	28.90	28.60	0.663
MCHC (g/dL)	33.75	33.75	33.75	0.757
RDW (%)	14.55	14.55	14.65	0.366
PLT (10 ³ /μL)	104.0	81.0	72.5	0.022
MPV (μm ³)	8.40	8.55	8.35	0.49
PCT (%)	0.0875	0.0765	0.0685	0.05
PDW (%)	15.20	15.75	15.85	0.172
%LYM	25.75	31.05	31.10	0.065
%MON	6.1	4.2	4.0	<0.001
%GRA	68.65	64.50	63.50	0.49
CRP (mg/L)	61.50	98.40	116.65	<0.001

230 Table 2. Medians and statistical significance of hematology parameters in association to
 231 parasitemia of *P. vivax* samples. *Data set from years 2018 and 2019. Kruskal – Wallis,*
 232 *p-value of less than 0.05 was considered statistically significant.*

233 **10 samples from the original data set were incomplete on parasites counting,*
 234 *therefore they were not contemplated for the calculation of table 2 results*

235 **Insert figure 2**

236

237 For correctly associating the CRP with the affection of malaria infection, a
238 consideration on the age dependency has to be considered. Figure 3 shows the CRP
239 association separated in different age groups of *P. vivax* samples with parasitemia less
240 than 0.2%.

241 **Insert figure 3**

242 **WBC histogram interference in the RBC ghosting area**

243 In the LC-667G CRP HORIBA analyzer, an abnormal peak located approximately at the
244 37fL channel in the WBC histogram appears mainly in malaria *P. vivax* samples with
245 high density of large forms of parasites (see figure 4). Using the parasite density
246 information from datasets 2018 and 2019 of *P. vivax* samples, a statistically significant
247 multiple linear regression model ($p < 0.001$) was calculated obtaining the following
248 equation $y = 1229 + 7.771 \times 10^{-3} \alpha_1 + 48.1 \times 10^{-3} \alpha_2$ (see figure 5), where y represents the
249 events per μL from 19.3fL to 43.9fL in the WBC histogram (eq. 2), α_1 the coefficient
250 related to the small forms parasite density and α_2 to the parasite density of large forms.
251 The contribution of large forms is approximately 6.2 times bigger than the contribution
252 of small forms (α_2/α_1) and the percentage of variation of the model is 74.43%.

253 Additionally, the small and large forms of parasitemia showed a small multicollinearity
254 (VIF = 1.05). For the case of positive *P. falciparum* multiple regression model, we could
255 not conclude a statistically significant association between the large forms parasite
256 density and the events in the above mentioned range of the WBC histogram ($p = 0.142$).

257 **Insert figure 4**

258 **Insert figure 5**

259
$$\text{Events per } \mu\text{L from 19.3fL to 43.9fL} = \frac{\text{Sum events 19.3fL to 43.9fL}}{\text{Total events of WBC hist}} \times (\#WBC \times 10^3) \left[\frac{\text{events}}{\mu\text{L}} \right] \dots \text{Eq. 2}$$

260 Large forms of *P. falciparum* samples are barely seen in peripheral blood due to
261 sequestration in the spleen, thus interference in the RBC ghosting area of the WBC

262 histogram will be smaller than in *P. vivax* samples were large forms of parasites
263 remains in peripheral blood, compare subplots of figure 4. In the analyzed set of data,
264 different interferences non-related to parasite inclusion located in the 37-fL area are
265 displayed in figure 4, these interferences include platelet aggregations, macro platelets,
266 and nRBCs.

267 Discussion

268 The most common reported affectations on hematology parameters observed in malaria
269 infection are thrombocytopenia, anemia and leucopenia [16, 17]. Thrombocytopenia in
270 malaria infection occurs due to complex platelet roles and abnormalities, including
271 enabling cytoadhesion of iRBCs to endothelial cells, clumping and killing iRBCs [18],
272 coagulation disturbances, bone marrow alterations, antibody-mediated platelet
273 destruction, systemic platelet activation, vascular pooling and oxidative stress [19].
274 Moreover, in hematological analyzers, PLTs complexed with RBCs are not identified,
275 therefore complexed particles would lead to an apparent platelet loss [20]. In the
276 present study, thrombocytopenia ($< 150 \cdot 10^3/\mu\text{L}$) was predominantly observed in
277 malaria *P. falciparum* cases (83.87%) than *P. vivax* (76.44%), which is similar to the
278 observations reported in [21, 22]. Authors in [20] have concluded that in malaria cases,
279 in addition to type of specie, the severity of thrombocytopenia is also affected by
280 parasite density. In present study, similar association with parasitemia ($p = 0.022$) is
281 seen in *P. vivax* infection with thrombocytopenia cases. While understanding
282 thrombocytopenia dependency on malaria specie, it must be analyzed considering the
283 parasite load and patient condition, as pointed out in a study where parasitemia was
284 significantly higher in individuals with thrombocytopenia only in *P. vivax* and not in *P.*
285 *falciparum* infections [23].

286 Anemia caused by malaria infection has a multi-factorial and complex pathogenesis
287 that is quite pronounced in *P. falciparum*, contrary to *P. vivax* which target mainly
288 young RBCs [24]. In the present analysis, although *P. falciparum* medians of RBC
289 counting, HGB and HCT were lesser than the rest of the inspected groups (see table 1),
290 no statistical significant difference was found in parameters concerning anemia
291 affection which might be related to the non-severe parasite invasion and small
292 parasitemia variability of *P. falciparum* current data.

293 In study performed using a five differential part ACC in Thailand, Tak Province the
294 neutrophil percentage, RBC count, MCV, MCH, MCHC and platelets count were found
295 to be significantly different between *P. falciparum* and *P. vivax* species [25]. In contrast,
296 in the current study, none of the above-mentioned parameters were significantly
297 different between malaria species excepting the leukocyte count ($p = 0.029$), which
298 most probably is caused by the interference of the WBC channel by the large forms of *P.*
299 *vivax* infection compared to *P. falciparum* samples.

300 Among the hematology parameters with significant statistical difference in medians
301 between malaria positive and negative samples, platelets related parameters, especially
302 MPV, should be further analyzed due to the strong affection with the elapsed time
303 between collection and measurement of sample. Unfortunately, due to the retrospective
304 nature of the study, the exact amount of elapsed time from collection to measurement is
305 not available.

306 **Interference in the WBC channel and association with parasitemia**

307 In both three and five part differential ACCs a peak around the RBC ghosting area in the
308 WBC histogram has been identified as one of the main abnormalities to predict malaria
309 infection [26 - 29]. Briggs et al., observed this peak before the 35-fL threshold at WBC
310 histograms with malaria infection and used it for improving the specificity to detect

311 malaria in false-positives results coming from other infections [26]. In the present
312 study, the number of events within this abnormal peak were correlated with the
313 parasite density of *P. vivax* large forms, supporting the same observation of moderate
314 correlation ($r = 0.79$) in [28]. We concluded the abnormal peak under malaria infection
315 is caused mainly by the lysing-resistant large forms of parasites, regardless on the
316 specie, and probably affected by the presence of PLT aggregation and/or macro PLTs.
317 The abnormal peak is less often observed in *P. falciparum* infections as the larger forms
318 are sequestered by the spleen. Mature trophozoites and schizonts in *P. falciparum*
319 infection are barely present in peripheral blood samples, consequently according to
320 [30] the presence of schizontaemia, especially in *P. falciparum* infections, can be used
321 as a marker for disease severity. Therefore a semi-quantitative estimation of
322 schizontaemia can be inferred from the events before the 37fL channel of the WBC
323 histogram from the LC-667G and might help to assess the severity of malaria infections.
324 The abnormal peak in WBC histogram is not exclusively due to malaria iRBCs.
325 Common interferences include RBC lysed debris, PLT aggregations, macro-PLTs,
326 nRBCs, and others. Researchers used this abnormal peak along with two other
327 parameters of the LH780 (Beckman Coulter) to identify malaria infection having an
328 83.1% specificity but failed to discriminate from samples with nRBCs [28]. We feel that
329 the distribution pattern of the abnormal peak might provide a way to differentiate the
330 exact cause of it. We observed that PLT aggregations or macro PLTs show a
331 bigger/wider distribution due to the random nature of formation, whereas gametocytes,
332 schizonts and late stages of trophozoites show a narrower size distribution having a
333 smaller dispersion close to the abnormal peak area.

334 **A parameter to increase the specificity of detecting malaria and understand**
335 **better the parasite density: CRP**

336 In low cost ACCs where the Coulter principle is the only technology responsible of
337 differentiating the WBC population into three parts based on volume, the identification
338 of malaria or dengue infected samples is rather difficult due to the impossibility of
339 differentiating granulocytes populations and to distinguishing interferences of lysing
340 resistant particles in the WBC channel. Addition of CRP increases the specificity of
341 detecting malaria due to the inflammatory response secondary to parasite infection.
342 Some studies have positively correlated the parasite density of malaria infected samples
343 with CRP levels [31-34], even in afebrile malaria patients [34]. CRP has been proposed
344 for assessing the severity of malaria infection and for following-up response to
345 treatments [35]. However in the present study, a direct correlation between the CRP
346 value and the parasitemia was not found probably due to the non-specific CRP response
347 and the debated age dependency [36, 37]. Only when grouping the parasitemia in
348 different ranges, a positive association between parasitemia in *P. vivax* samples and the
349 CRP median was observed (see figure 2). Similar to results of [11], in this study no
350 significant difference of CRP value was observed between *P. vivax* and *P. falciparum*.
351 Dengue and malaria in many countries are co-endemic diseases that present similar
352 clinical presentation. The importance of distinguishing them has been reported by [38],
353 who developed a tree decision model for discriminating malaria and dengue infection.
354 Dengue samples collected in the present study had a lower counting of WBCs and
355 MCHC in comparison to both malaria species supporting observations of [38], however
356 only the difference in median with *P. vivax* samples was statistically significant. In
357 contrast, PLT medians of both species of malaria infections were lower than dengue
358 cases ($p < 0.001$). Discrepancy of results with [38] might be related to the severity of
359 each disease. For example, the hematology parameters affection in dengue infections is

360 greatly dependent on day of fever. Unfortunately current data has not any information
361 about the fever progression of dengue infected individuals.

362 By far, the CRP parameter showed the most significant difference between malaria and
363 dengue. Although CRP is not a specific biomarker for malaria, the response of CRP to
364 virus infections is rather small making a good parameter to understand the etiology of
365 the infection and help to differentiate malaria and dengue from similar hematology
366 parameters. Nevertheless, dengue and other non-viral infections can co-exist in a single
367 sample, thus relying only in CRP should not be an exclusive criteria to differentiate both
368 diseases.

369 **Conclusions**

370 The LC-667G CRP, a three differential part hematology analyzer has the potential to not
371 only trigger malaria diagnosis confirmation but also assess the severity of the infection
372 due to simultaneous CRP estimation. The current study suggests that CRP might be
373 helpful for distinguishing malaria from dengue, and for evaluating the severity of the
374 parasite load. In addition to CRP, the abnormal peak located around the 37fL channel
375 in the WBC histogram can provide a good estimation of the number of large parasites
376 present during the infection, however the calculation might be interfered by other
377 particles such as nRBCs and PLT aggregations. The combination of providing
378 hematology parameters plus CRP value in a hematology analyzer makes a preferred
379 method for screening malaria on routinely basis of any infection suspicion.

380 Current data-set is limited by the number of *P. falciparum* samples, the discussion and
381 observations provided were focused on *P. vivax* positive cases. Further research
382 following this study should be oriented to measure *P. falciparum* samples, evaluate the
383 potential of using hematology analyzers to provide additional information to the RDTs,
384 and monitor the anti-malaria drugs treatment.

385 **Abbreviations**

386 **WHO:** World Health Organization

387 **RDT:** rapid diagnostic test

388 **ACC:** automated cell counter

389 **CBC:** complete blood count

390 **WBC:** white blood cell

391 **LYM:** lymphocytes

392 **MON:** monocytes

393 **RBC:** red blood cell

394 **iRBC:** infected red blood cell

395 **HGB:** hemoglobin

396 **TLC:** total lymphocyte count

397 **RDW:** red cell distribution width

398 **CRP:** C-reactive protein

399 **POCT:** point of care testing

400 **PLT:** platelet

401 **PCT:** plateletcrit

402 **MCHC:** mean corpuscular hemoglobin concentration

403 **MPV:** mean platelet volume

404 **PDW:** platelet distribution width

405 **HCT:** hematocrit

406 **MCV:** mean corpuscular volume

407 **MCH:** mean corpuscular hemoglobin

408 **GRA:** granulocytes

409 **CV:** Coefficient of variation

410 **VIF:** variance inflation factor

411 **nRBC:** nucleic red blood cell

412 **Declarations**

413 **Ethics approval and consent to participate**

414 For the current study, the historical documented data was further anonymized in coded
415 fashion in order to protect patient confidentiality in accordance with local ethical or
416 IRB guidelines, according to national and international standards for the conduct of
417 clinical studies including 21 CFR Parts 50 and 56 and International Conference on
418 Harmonization (ICH) E6-Good Clinical Practice Consolidated Guideline. Ethical
419 clearance was not obtained as study was carried as a retrospective review of the
420 available raw instrument and historical patient data, without any collection of extra
421 specimen or monetary charges to patient and did not involve any communication of
422 results of the study to the clinician so as to affect the diagnostic and therapeutic
423 management of the patients.

424 **Consent for publication**

425 Not applicable

426 **Availability of data and materials**

427 The datasets used and/or analyzed during the current study are available from the
428 corresponding author on reasonable request.

429 **Competing interests**

430 JN and SR are employees of HORIBA Ltd. PD is an external consultant of HORIBA Ltd.

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433 consumables for the hematology analyzer and helped with the analysis and
434 interpretation of the hematology analyzer results.

435 **Authors' contributions**

436 PD, SR and JN designed the study. JN drafted the manuscript and performed the data
437 analysis. PD collected all samples, performed the smear inspections and clinical
438 observations. SR and PD reviewed and corrected the manuscript. All authors read and
439 approved the final manuscript.

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444 **Authors' information (optional)**

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551 **Figure Legends**

552 Figure 1. Box plot of CRP values on different groups. *Dengue (D)*, *malaria and dengue*
553 *(MD)*, *non-malaria-dengue samples (N)*, *P. falciparum (PF)*, *P. vivax (PV)* and the
554 *combination of both malaria species (PV + PF)* distributions are represented with
555 *their corresponding median value and outliers.*

556 Figure 2. Box plot of CRP in association with parasitemia of *P. vivax* samples. *All*
557 *individual points are displayed in the box plots*

558 Figure 3. Box plot of CRP in association with age of *P. vivax* samples with parasitemia
559 less than 0.2%. *All individual points are displayed in the box plots*

560 Figure 4. WBC histograms of 2019 data-set with interferences around 37-fL. *Bold lines*
561 *represent the median of the histograms.*

562 Figure 5. Multiple linear regression model between the events before 43.9fL and
563 parasite density. *The vertical axis corresponds to the number of events from 19.3fL to*
564 *43.9fL in the WBC histogram and the horizontal axis to the parasite density of small*
565 *and large forms of samples infected by malaria *P. vivax*. The regression model shows*
566 *that the contribution of the linearity is mainly provided by large forms of *P. vivax**
567 *malaria infection.*

568