1   **TITLE PAGE:**

2   **TITLE: Secondary Dengue Infection Elicits Earlier Elevations in IL-6 and IL-10 Levels.**

3   **Running title: High IL-6 and IL-10 in dengue infection**

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27

28   **ABSTRACT**:

29   **Introduction**: Dengue virus (DENV) represents a global health concern. Symptomatic infection

30   causes a wide range of clinical manifestations, from mild dengue fever to severe disease,

31   characterized by vascular permeability and bleeding. Previous reports indicated that the

32   exacerbated expression of some cytokines is implicated in the progression of the disease.

33   However, their time of expression within the infectious period remain uncertain. The aim of

34   this study was to assess IL-6 and IL-10 level kinetics distinguishing two phases within the febrile

35   stage in DENV infected patients.

36   **Methods**: We conducted a retrospective study on samples from 2016 and 2020 DENV

37   outbreaks in Argentina. Viremic patients were categorized in Phase I and II, based on anti-

38   DENV IgM presence. Cytokine levels, clinical parameters, and type of infection were analyzed.

39   **Results**: Our analysis included samples from 259 patients in the febrile stage. Of these, 184

40   patients (71%) were classified into Phase I, while 75 patients (29%) were in Phase II. Ninety-

41   nine patients showed secondary infection (38.2%). Notably, secondary infections exhibited

42   earlier IL-6 and IL-10 elevation than primary infections, suggesting pre-existing immune

43   memory priming the immune response. Thrombocytopenia and elevated AST were associated

44   with Phase II, secondary infection, and hospitalization. Elevation of IL-6 and IL-10 correlated

45   with low platelet counts, suggesting their association with clinical manifestations.

46   **Conclusion**: In cases of secondary dengue infections, cytokine levels increase at an earlier

47   stage of the disease compared to primary infections, where elevated cytokine levels are

48   typically observed during the later febrile phase. Further research with broader cytokine

49   panels is warranted to validate these findings.

50

51

INTRODUCTION

Dengue is a human disease caused by the infection with anyone of four genetically related dengue virus serotypes (DENV-1, -2, -3 and -4), which are transmitted to individuals through the bite of infected mosquitoes belonging to the *Aedes* species. Multiple factors have contributed to the reemergence of DENV infections as a significant global public health concern during the past 2 decades[1].

DENV infection can vary in presentation, ranging from asymptomatic cases or mild illness to severe disease. Severe dengue is characterized by vascular permeability, plasma leakage, massive bleeding, and, in some cases, liver compromise, organ impairment, and even death [12]. In patients who experience a sudden deterioration of symptoms during the critical phase, which typically occurs around 3-7 days after the onset of illness, close monitoring is essential to prevent complications and reduce the risk of mortality [2].

The known contributors behind severe and fatal cases of dengue are viral serotype [3], a second heterotypic infection which could cause an antibody-dependent enhancement (ADE) [4,5] and different aspects of the host innate and adaptive immune response [6]. Cytokines, mediators released because of complex interactions between DENV and host immune responses, have been implicated to play a role in the progression of severe dengue disease [7,8]. Excessive generation of pro-inflammatory cytokines, such as and IL-6 proved to contribute to the production of antiplatelet or anti endothelial cell antibodies, which results in a deficiency in coagulation, leading bleeding with dengue infection [9–11]. Conversely, the presence of anti-inflammatory cytokines such as IL-10 led to compromised immune clearance and persistent infectious effects during acute viral infection [12–14].

The current application of these potential markers remains uncertain by the limited understanding of how their expression evolves during the initial phases of the infection and whether their increase is protective or detrimental to the disease outcome.

77 Because previous reports indicated that the events triggering the release of inflammatory

78 mediators take place very early in illness (specifically, initial period of the febrile phase) [15] and

79 in line with the CDC's description of the febrile phase as having a biphasic nature, our study

80 introduces a classification of two sub-phases within the febrile phase. In this context, we

81 examined the levels of IL-6 and IL-10 to analyze their kinetics in patients with DENV across

82 these two distinct febrile phases.

83

84 **MATERIALS AND METHODS**

85 *Patients and study design*

86 This is a retrospective study carried out on archived plasma/serum samples collected from

87 patients in the city of Posadas, Misiones, Argentina, referred for diagnosis of dengue infection,

88 during 2 consecutive dengue epidemic periods, 2016 and 2020. A group of 259 samples within

89 the febrile stage, showing positive viremia (PCR-RNA+) or DENV NS1 antigen (+) were randomly

90 selected and included in our analyses. Samples were stored at −80°C until used for cytokine

91 and serotype assays.

92

93 *Dengue diagnosis and laboratory studies*

94 DENV diagnostic was carried out by immunochromatographic test, RDT, (SD BIOLINE Dengue

95 DUO kit, Abbott, USA) for DENV NS1 antigen detection or the RealStar® Dengue RT-PCR Kit 3.0

96 (Altona Diagnostics, Hamburg, Germany) for virus genome detection. Specific antibodies to

97 DENV, IgM and IgG, were determined by ELISA (Dia.Pro Diagnostic Bioprobes s.r.l., Milan,

98 Italy). DENV serotypes were determined using the CDC DENV 1–4 real time PCR assay modified

99 by Santiago et al. [16].

100 Routine lab determinations were performed, and parameters associated with bleeding were

101 assessed in our cohort. Furthermore, we analyzed the concentration of hepatic enzymes, ALT

102 (alanine transaminase) and AST (aspartate aminotransferase), as well as total protein, albumin,

103 and bilirubin. Certain parameters were categorized based on specific thresholds that were

104 previously associated with dengue severity [15,17]. For instance, white blood cell counts were

105 considered indicative of severity when they fell below 5,000/μL; similarly, platelet counts

106 below 100,000/μL. Liver enzyme concentration exceeding the upper limit of the normal

107 reference range (40 UI/mL) were considered elevated.

108

109 *Classification of phases and type of infection*

110 Patients within the febrile stage were categorized into two phases: the early phase (Ph I),

111 characterized by the presence of DENV-RNA (+) or NS1-Ag (+), and the late phase (Ph II),

112 characterized by the presence of viremia along with anti-DENV IgM. The type of infection was

113 defined based on the presence or absence of anti-DENV IgG antibodies. Secondary infections

114 were determined when NS1 antigen or viral genome were present concurrently with anti-

115 DENV IgG antibodies, and patients with viremia but without anti-DENV IgG antibodies were

116 categorized as primary infections, irrespective of the presence of anti-DENV IgM.

117

118 *IL-6 and IL-10 determination*

119 The concentrations of IL-6 (standard curve range: 0 - 300 pg/mL) and IL-10 (standard curve

120 range: 0 - 500 pg/mL) were determined using commercial reagents based on enzyme linked

121 immunosorbent assay (ELISA) (BD-Biosciences, San Diego, California, United States).

122 Procedures were carried out following the supplier's recommendations. Duplicates were

123 performed in selected samples to verify the accuracy of the results. A control group comprising

124 50 healthy blood donors, testing negative for dengue antibodies was included to establish

125 cytokine' reference ranges.

126

127 *Statistical Analysis*

128   Continuous variables were compared using either the student's t-test or the Mann-Whitney U

129   test. Categorical variables were assessed using the Chi-square test or Fisher's exact test.

130   Confidence intervals were set at 95% (CI95) and a p value <0.05 was considered statistically

131   significant. Statistical analysis was conducted using the SPSS statistical software package

132   release 23.0 (IBM SPSS Inc., Chicago, IL, USA) and graphical representations were generated

133   using GraphPad Prism 10.0.0 software.

134

135   *Ethical Aspects*

136   Patients included in the study were given and signed an informed consent. The experimental

137   protocols and procedures carried out in this work were approved by the Biosafety Review

138   Board, Ethical Committee of the Academia Nacional de Medicina, Buenos Aires (CEIANM) and

139   the Ethical Committee of the Investigación Provincial, Misiones (CEIP).

140

141   **RESULTS**

142   *Study Population*

143   Sera or plasma samples from 259 patients, referred for dengue infection diagnosis during two

144   dengue epidemic periods, were included in this study. Of these 128 (49.4%) samples were

145   from the 2016 outbreak, and 131 (50.6%) were from the 2020 outbreak. Among the patients,

146   137 (52.9%) were female with a median age (Q1-Q3) of 44 years (29-63). Our analysis of 205

147   serotypes revealed a prevalence of 86.3% (n=177) for DENV-1 and 13.7% (n=28) for DENV-4.

148   Additionally, 160 (61.8%) had primary infection, while the remaining 99 patients (38.2%)

149   exhibited evidence of secondary infection. Notably, our study revealed a significant association

150   between secondary infections and a higher incidence of individuals with platelet counts below

151   100,000/µl (p=0.024) compared to primary infections. Specifically, 12 out of 160 (7.7%)

152   patients with primary infections and 17 out of 99 (17.7%) with secondary infection presented

153   platelet counts lower than 100,000/µL.

154

Characteristics of Clinical and Immunological Parameters within the febrile stage

156   Within the febrile stage, we found 184 patients (71.0%) in Ph I, and 75 patients (29%) in Ph II.

157   Gender differences were not observed (p=0.891); however, patients in Ph I were, on average,

158   10 years younger than those in Ph II [41 years (29-60) vs. 51 (29-72), respectively, p=0.031].

159   Furthermore, as shown in Table 1, significant differences were also noted in DENV serotype

160   (p=0.007) and the type of infection (p<0.001). Analyzing clinical parameters and cytokines

161   between the early and late phases, a significant increase in the number of individuals with

162   platelet counts below 100,000/µL (p=0.007) in Ph II compared to Ph I was observed.

163   Additionally, a higher proportion of patients in Ph II had elevated AST values compared to

164   those in Ph I. Concerning the level of inflammatory and anti-inflammatory cytokines, only IL-6

165   exhibited higher concentrations during Ph I [7.67pg/mL for Ph I vs. 4.78pg/mL for Ph II,

166   p=0.045] while no significant differences were observed for IL-10 (p=0.611). Table 1 presents

167   all the epidemiological and clinical characteristics analyzed according to the phase within the

168   febrile stage.

169

170   The analysis of cytokine levels in relation to the febrile stage and the type of infection revealed

171   that IL-6 and IL-10 concentrations (p=0.005 and p<0.001, respectively) were elevated in Ph I

172   from secondary infections, compared to Ph I in primary infections. Conversely, primary

173   infections showed higher values in Ph II than that of secondary infections, for both cytokines

174   (IL-6, p=0.009; IL-10, p=0.003). Table 2 provides details on cytokine levels at different febrile

175   phases and types of infection. To establish a reference, levels of IL-6 and IL-10 were also

176   determined in healthy individuals (n=50) demonstrating higher levels in DENV-infected

177   individuals (n= 259). Specifically, IL-6 concentration was 3.48 pg/mL in healthy controls and

178   6.71 pg/mL in DENV-infected patients (p<0.001) while for IL-10, levels were 5.75 pg/mL in

179   healthy individuals and 16.73 pg/mL for those infected with DENV (p<0.001) (Figure 1).

180   As a final assessment, an analysis to evaluate the relationship between clinical parameters and

181   cytokine concentration during Ph II of secondary infections was performed. In summary, the

182   analysis revealed that elevated IL-6 levels were associated with low platelet counts and tended

183   to be associated with high ALT. On the other hand, elevated IL-10 levels were associated with

184   WBC >5000, low platelets, and increased AST (Table 3).

185

186   *Characteristics of hospitalized patients*

187   Hospitalization often indicates greater disease severity in dengue infection. Since 26 patients

188   (10%) required hospitalization due to DENV infection complications, we analyzed their clinical

189   and immunological parameters. Our study revealed that hospitalized patients were, on

190   average, 15 years older than outpatients (p=0.007). Among them, a higher proportion

191   exhibited platelet values below 100,000/µL compared to outpatients (p<0.001). Additionally,

192   hospitalized patients displayed elevated IL-6 and AST concentrations, highlighting the severity

193   of the condition (p<0.001 and p=0.047, respectively). Table 4 summarizes the epidemiological

194   and clinical characteristics of DENV infected patients based on hospitalization.

195

196   **DISCUSSION**

197   IL-6 and IL-10 are two cytokines known to play critical roles in the pathogenesis of DENV

198   infection [18,19]. Their levels can vary depending on the type of infection and the severity of the

199   disease, warranting further research to better understand their significance in disease

200   progression since a persistent controversy has been observed [14,20]. Recent studies have

201   suggested that the increase in cytokine levels occurs in very early stages, triggered by the

202   innate immune response following virus entry. To investigate this, we divided the febrile

203   viremic stage into two phases and examined the kinetic of IL-6 and IL-10 levels.

204   One of our most notable findings is that in secondary dengue infections, cytokine levels rise

205   earlier in the disease course compared to primary infections, in which elevated cytokine values

206   are detected during the later febrile phase. This suggests that in secondary infections, the

207   immune system, guided by pre-existing immune memory, is primed for a more rapid response

208   to the virus, leading to an anticipated immune response. However, the mechanisms governing

209   differential clinical outcomes remain poorly defined.

210   Thrombocytopenia (platelet count<100,000/μL) is a hallmark of dengue hemorrhagic fever,

211   and it remains one of the current criteria for diagnosing the condition [21]. We observed a

212   significant increase in the individuals with low platelet counts, and elevated AST values in Ph II.

213   Focusing on the very early stages of the disease, when laboratory parameters are still within

214   normal ranges, may have limited our ability to observe further significant clinical alterations.

215   Additionally, patients in Ph II were older than those in Ph I, suggesting age may impact disease

216   outcomes, although this factor cannot be conclusively rule out.

217   Our study also identified a higher incidence of individuals with platelet count below

218   100,000/μL in cases of secondary infections, consistent with previous reports in which the risk

219   of severe dengue increases in patients with heterotypic secondary DENV infection [22,23].

220   However, it is essential to note that when sequential DENV infections are closely spaced,

221   significant cross-protection may occur, potentially altering the association between type of

222   infection and severity [24]. Remarkably, it is important to mention that DENV-1 was the

223   dominant serotype in both consecutive outbreaks analyzed here, with the occurrence of

224   DENV-4 in a lower percentage (30%) of the cases of 2020, a pattern consistent with previous

225   publications [25,26]. However, a limitation of our study is the lack of comprehensive information

226   on all serotypes, and the inability to determine whether secondary infections within our

227   population were heterotypic or homotypic.

228   It is worth emphasizing that cytokine values for healthy controls were similar to that observed

229   for healthy populations in previous reports [27,28] but lower than the levels exhibited in the group

230   of infected patients, as shown in Figure 1.

231   Although reports of elevated IL-6 levels in early acute phase in bleeding patients were

232   published [11], others observed significantly higher IL-6 levels associated with hemorrhagic fever

233   only in patients infected with DENV-2 but not with DENV-1 [29]. On the other hand, high levels of

234   IL-10 in dengue during early illness were indicator of an altered antiviral response and this

235   association was particularly observed in patients who progressed to dengue hemorrhagic fever

236   [30,31].

237   While direct correlation between cytokine levels in the acute phase and the severity of the

238   disease was not individually evaluated in this study, the analysis of hospitalized patients (Table

239   4) revealed an alteration in AST concentrations and platelets, combined with elevated levels of

240   IL-6, highlighting the role of these three parameters as warning signs in hospitalized

241   individuals. Additionally, the older median age in the hospitalized group compared to

242   outpatients may have contributed to the increased risk in these patients. It is essential to note

243   that cytokine elevation does not necessarily correlate with a worsened prognosis since

244   cytokines play a vital role in pathogen eradication [32]. However, in conjunction with other risk

245   factors, an exacerbated cytokine response may contribute to disease progression [33].

246   In Ph II of secondary infections, our analysis revealed the association between clinical

247   parameters related to dengue severity and the elevation of IL6 and IL10. Based on these

248   findings, it seems plausible that elevated IL6 and IL10 levels in Ph I might be indicative of

249   deteriorating conditions in Ph II.

250

251   *Study Limitations*

252   While the study boasts several strengths, such as its large sample size, inclusion of patients

253   from different outbreaks, and the separation of two febrile phases, it is essential to

254   acknowledge its limitations. This is not a sequential longitudinal study; rather samples from

255   different phases correspond to distinct individuals, each at a specific point in their infection.

256   Nevertheless, they all belong to the same population in Posadas, Misiones, Argentina.

257   Moreover, the study focused on a limited set of cytokines, and further studies should explore

258   the roles of other immune factors in dengue infection. Lastly, the small number of hospitalized

259   patients in our study precluded the possibility of conducting a logistic regression analysis.

260   *Conclusions*

261   In summary, our study provides valuable insights into the kinetics of IL-6 and IL-10 in different

262   febrile phases and types of infections. The association between particular cytokines and

263   specific disease phases highlights the importance of categorizing patients upon DENV diagnosis

264   based on the type of infection they have. Moreover, considering similar days since symptoms

265   onset will facilitate more precise comparisons. The measurement of cytokine levels that might

266   act as indicators for disease progression at this moment, could be an invaluable tool in making

267   earlier decisions regarding patient interventions, especially in the context of secondary

268   infections. Future studies should be conducted to validate our conclusions and explore the

269   relationship between a broader panel of cytokine and dengue pathogenesis.

272 **Conflict of interest statement**: On behalf of all authors, the corresponding author states that

273 there is no conflict of interest.

274 **Author contributions:**

275 SLE, FAD and PB: Conceptualized and designed the study, collected, and validated data,

276 performed statistical analysis and data interpretation, organized, and curated the dataset,

277 wrote the initial draft of the manuscript and its revision, created and approved the final

278 version to be submitted. MJP, AN, PS: conducted dengue diagnostic assays and cytokine ELISA

279 assays, collected and validated data, reviewed and approved the final version of the

280 manuscript to be submitted. CGM, AC, BMN, FJ, MM and CR: performed data analysis,

281 organized, and curated the dataset, reviewed the article critically for important intellectual

282 content, approved the final version of the manuscript to be submitted.

290 **Ethics statement:** The study was conducted in accordance with the Declaration of Helsinki and

291 approved by the Institutional Ethics Committee of the Academia Nacional de Medicina, Buenos

292 Aires (TI Nº13157/19/X) and the Ethical Committee of the Investigación Provincial, Misiones

293 (CEIP). Informed consent was obtained from all the individuals.

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411 **TABLES**

412 **Table 1. Epidemiological and Clinical characteristics by phase (n=259).**

Characteristics	Total (n=259)	Phase 1 (n=184)	Phase 2 (n=75)	p
Age* (years)	44 (29-63)	41 (29-60)	51 (29-72)	**0.031**
Female gender (%)	137 (52.9)	98 (53.3)	39 (52)	0.891
Dengue serotype (%) [#]				
1	177 (86.3)	131 (82.9)	46 (97.9)	
4	28 (13.7)	27 (17.1)	1 (2.1)	**0.007**
Infection (%)				
Primary	160 (61.8)	133 (72.3)	27 (36.0)	
Secondary	99 (38.2)	51 (27.7)	48 (64.0)	**<0.001**
WBC <5000/ μL (%)	170 (65.6)	119 (65.0)	51 (68.9)	0.663
Platelets <100,000/μL (%)	29 (11)	14 (7.8)	15 (20.8)	**0.007**
IL6 pg/mL	6.71 (3.4-15.2)	7.67 (3.5-15.8)	4.78 (3.2-10.7)	**0.045**
IL10 pg/mL	16.7 (9.8-41.1)	16.91 (10.1-40.2)	14.56 (8.4-63.2)	0.611
AST ≥ULN (U/L) [#](%)	98 (37.8)	65 (41.7)	33 (61.1)	**0.017**
ALT ≥ULN (U/L) [#] (%)	63 (24.3)	45 (28.8)	18 (33.3)	0.606
Albumin g/dL [#]	4.1 (3.9-4.4)	4.21 (3.9-4.4)	4.04 (3.8-4.2)	**0.005**
Bilirubin mg/dL [#]	0.49 (0.34-0.73)	0.48 (0.34-0.73)	0.50 (0.34-0.74)	0.391
Total protein g/dL [#]	6.99 (6.7-7.3)	6.99 (6.7-7.4)	7.00 (6.7-7.2)	0.446

413 *Median (interquartile range), WBC: White blood cells, ULN: upper limit of normal (>40 U/L),
414 [#]Available in: Serotype 205 patients, ALT and AST 210 patients, Albumin 160 patients, Total
415 protein, and bilirubin 150 patients.
416

417

418   **Table 2: Cytokine levels at different phases and type of infection (n=259).**

Cytokines	Infection / Phase	Primary Infection (n=160)	Secondary Infection (n=99)	p by type of infection
		(n=133)	(n=51)	
	Phase I (n=184)	6.59 (3.3-14.5)	10.26 (5.5-26.8)	**0.005**
IL-6 pg/mL		(n=27)	(n=48)	
	Phase II (n=75)	7.7 (4.3-30.9)	4.28 (2.7-8.4)	**0.009**
p by phase		0.200	**<0.001**	
		(n=133)	(n=51)	
	Phase I (n=184)	14.30 (9.0-30.4)	36.46 (14.1-84.6)	**<0.001**
IL-10 pg/mL		(n=27)	(n=48)	
	Phase II (n=75)	30.34 (12.1-110.9)	11.25 (6.5-32.1)	**0.003**
p by phase		**<0.001**	**<0.001**	

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Table 3. Cytokine levels by clinical parameters during phase 2 of secondary infections (N=48)

Characteristics	IL-6*	p	IL-10*	p
WBC 5000/ µL				
<	4.3 (2.9-7.6)		14.0 (9.8-37.6)	
>	3.8 (2.3-10.7)	0.891	6.6 (5.7-11.2)	**0.014**
Platelets 100,000/µL				
<	10.7 (3.5-15.7)		42.3 (11.6-100.6)	
>	4.2 (2.6-6.2)	**0.020**	10.8 (6.2-18.0)	**0.002**
AST ULN (U/L) [#]				
<	3.3 (2.0-8.5)		7.6 (5.7-13.5)	
≥	4.9 (3.5-10.7)	0.252	17.2 (11.3-42.3)	**0.006**
ALT ULN (U/L) [#]				
<	3.5 (2.8-6.3)		11.2 (6.3-20.5)	
≥	6.2 (3.5-15.2)	**0.082**	17.2 (8.3-30.5)	0.330

*Median (interquartile range),
WBC: White blood cells, ULN: upper limit of normal (>40 U/L),
[#]Available in 34 patients.

427 **Table 4. Epidemiological and Clinical characteristics of DENV infected patients by**

428 **hospitalization (n=259).**

Characteristics	Outpatients (n=233)	Hospitalized (n=26)	p
Age* (years)	42 (28-62)	57 (40-74)	**0.007**
Female gender (%)	121 (51.9)	16 (61.5)	0.235
Infection			
Primary	145 (62.2)	15 (57.7)	
Secondary	88 (37.8)	11 (42.3)	0.401
WBC <5000/ µL (%)	152 (65.8)	18 (69.2)	0.455
Platelets <100,000/µL	19 (8.4)	10 (40.0)	**<0.001**
IL6 pg/mL	6.21 (3.2-14.7)	11.07 (7.4-62.8)	**<0.001**
IL10 pg/mL	16.07 (9.4-40.8)	21.72 (11.5-62.6)	0.235
AST ≥ULN (U/L) [#]	83 (44.4)	15 (65.2)	**0.047**
ALT ≥ULN (U/L) [#]	56 (29.9)	7 (30.4)	0.566

429 *Median (interquartile range), WBC: White blood cells, ULN: upper limit of normal (>40 U/L),
430 [#]Available in: Serotype 205 patients, ALT and AST 210 patients.
431

432   **FIGURE LEGENDS**:

433   Figure 1: Cytokine values in DENV infected group (in black) and healthy population (in grey).

434   Median for IL-6, and IL-10 concentrations are expressed in log (pg/mL).

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