REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO
JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

BRIEF COMMUNICATION

http://doi.org/10.1590/S1678-9946202365043

# Interferon-gamma gene diplotype (AA-rs2069716 / AG-rs2069727) may play an important role during secondary outcomes of severe dengue in Brazilian patients

**Cristiane Campello Bresani-Salvi**[1], **Clarice Neuenschwander Lins de Morais**[1], **Heytor Victor Pereira da Costa Neco**[1], **Pablo Cantalice Santos Farias**[2], **André Filipe Pastor**[3], **Raul Emídio de Lima**[1], **Ulisses Ramos Montarroyos**[4], **Bartolomeu Acioli-Santos**[1]

## ABSTRACT

Dengue is a global and growing health threat, especially in Southeast Asia, West Pacific and South America. Infection by the dengue virus (DENV) results in dengue fever, which can evolve to severe forms. Cytokines, especially interferons, are involved in the immunopathogenesis of dengue fever, and so may influence the disease outcomes. The aim of this study was to investigate the association between severe forms of dengue and two single nucleotide polymorphisms (SNPs) in the interferon-gamma gene (IFNG): A256G (rs2069716) and A325G (rs2069727). We included 274 patients infected with DENV serotype 3: 119 cases of dengue without warning signs (DWoWS), and 155 with warning signs (DWWS) or severe dengue (SD). DNA was extracted, and genotyped with Illumina Genotyping Kit or real time PCR (TaqMan probes). We estimated the adjusted Odds Ratios (OR) by multivariate logistic regression models. When comparing with the ancestral AA/AA diplotype (A256G/A325G), we found a protective association of the AA/AG against DWWS/SD among patients with secondary dengue (OR 0.51; 95% IC 0.24-1.10, $p = 0.085$), adjusting for age and sex. The variant genotype at locus A325G of the IFNG, in combination with the ancestral genotype at locus A256G, can protect against severe clinical forms of secondary dengue in Brazilian DENV3-infected patients.

**KEYWORDS:** Cytokine. Interferon-gamma. Polymorphism. Single nucleotide. Dengue virus.

## INTRODUCTION

Dengue virus (DENV) is a flavivirus with four pathogenic serotypes (DENV1-4), which are transmitted by *Aedes* mosquitoes[1,2]. Half of the global population is under risk of DENV infection, with 400 million cases and 20 thousand deaths per year[1,2]. In Brazil, the incidence of DENV infection had an increase of 600% between 2016 and 2019, reaching two million people[3].

Dengue fever ranges from mild acute illness to severe. Severe dengue is a life-threatening form of vascular leakage syndrome, fluid loss, organ impairment, bleeding and shock[4]. Dengue fever is influenced by pathogen characteristics, environmental factors, and genetic, epigenetic and immunity of the human host. Severe forms are attributed to the adaptive immune response against a secondary infection by heterologous serotype, namely antibody-dependent enhancement (ADE)[5]. In turn, components of the innate immunity, such as T cell response and

[1]Fundação Oswaldo Cruz, Instituto Aggeu Magalhães, Departamento de Virologia, Recife, Pernambuco, Brazil

[2]Universidade Federal de Pernambuco, Departamento de Genética, Recife, Pernambuco, Brazil

[3]Instituto Federal de Educação, Ciência e Tecnologia do Sertão Pernambucano, Pernambuco, Floresta, Brazil

[4]Universidade de Pernambuco, Instituto de Ciências Biológicas, Recife, Pernambuco, Brazil

**Correspondence to:** Bartolomeu Acioli-Santos
Fundação Oswaldo Cruz, Instituto Aggeu Magalhães, Departamento de Virologia, Av. Prof. Moraes Rego, s/n, Cidade Universitária, CEP 50670-420, Recife, PE, Brazil
Tel: +55 81 21012564

**E-mail:** bartolomeu.santos@fiocruz.br

**Received:** 5 January 2023

**Accepted:** 4 May 2023

cytokines, can also play a role in severe dengue[6,7]. Amongst cytokines, the interferons (IFN) pathway stands out as a first line of host defense against DENV and can modulate the ADE[6].

The interferon family includes type I (IFN-α and IFN-β), type II (IFN-γ) and type III (IFN-λ1:4). Generally, IFN-α and IFN-β can eliminate the DENV within the first hours of infection, by activating a cascade for infected cells to neutralize the virus[6]. In turn, IFN-γ seems to act by limiting viral replication and spread, and protecting against systemic vascular leakage[6,8]. Therefore, it is possible that variations in the IFN genes, such as single nucleotide polymorphisms (SNPs), can modulate outcomes in dengue. For instance, SNPs in the genes of IFN-λ1 and IFN-λ3 already were associated and included for dengue investigations in different populations[7,9].

In a previous DENV3 cohort in Northeastern Brazil, we had searched 322 SNP of innate immunity genes and found that a set of 13 SNP at 10 genes, including the interferon-gamma gene (IFNG), could accurately predict severe dengue[10]. Two SNPs in IFNG (A256G and A325G) presented high frequencies in our cohort, thus herein we investigated their interaction with severe forms of dengue.

## MATERIALS AND METHODS

We performed a case-control study nested within a previous cohort of DENV3 patients in Recife city, Northeastern Brazil. The protocol of the cohort study has been published[11]. We selected a subsample of 274 patients with a confirmed acute DENV3 infection. Subjects were classified as dengue without warning signs (DWoWS) (n = 119), dengue with warning signs (DWWS) (n = 130) or severe dengue (SD) (n = 25), according to guidelines of the World Health Organization (WHO)[4]. DWoWS is characterized by usual and mild manifestations of dengue fever; DWWS is defined by mucosal bleeding, ascites, abdominal pain or persistent vomiting; and SD presents major bleedings, shock or organ failures[4]. For this study, 274 dengue patients were selected and assigned between the case group (SD or DWWS) or controls (DWoWS).

Age, gender and sequence of infection (primary/secondary) were defined as covariables. The primary acute infection was characterized by the absence of anti-DENV IgG antibodies in the serum sample, whereas the secondary acute infection was characterized by detection of anti-DENV IgG in the acute serum sample and the absence of anti-DENV IgM.

Acute infection was confirmed in all cases and controls, based on the presence of two or more clinical symptoms of dengue and at least one of the following laboratorial criteria: anti-DENV IgM-positive; isolation of virus and/or viral RNA detection; anti-DENV IgG-negative in an acute sample followed by a positive one in a convalescent sample[11].

Peripheral blood samples were collected, and the DNA was extracted using the Wizard Genomic DNA Purification Extraction Kit (Promega, Madison, WI, USA). Our explicative variables were the IFNG SNPs A256G and A325G. Genotyping was performed by real time PCR (RT-PCR) using the TaqMan SNP Genotyping Assay Kit (Life Technologies©, Burlington, Canada), according to the recommended protocol, or Illumina GoldenGate Genotyping Kit® (Illumina, San Diego, CA, USA).

The SNPs frequencies were tested for Hardy-Weinberg's equilibrium and associations performed using the $x^2$ test. We calculated crude ORs and adjusted for age, gender and sequence of infection in a saturated multivariate logistic regression model, using Stata software (version 14, StataCorp©, College Station, USA). We also proceeded to an analysis stratified by sequence of infection, an interaction factor, and differences were considered significant at $p < 0.05$.

This study was approved by the committee on human experimentation of the Brazilian Ministry of Health (CEP Nº 68/02, CONEP Nº 4909, process Nº 25000.119007/2002-03) and by the institutional review board of the Johns Hopkins School of Medicine (JHM-IRB-3 Nº 03-08-27-01). All participants went through the informed consent process and signed the written consent form.

## RESULTS

The study population comprised 154 cases (DWWS/SD) and 119 controls (DWoWS), with a mean age of 28 years (5 to 64 years), 56% female (153/273), and 55.7% of patients with secondary dengue (151/271) (Table 1). Allelic frequencies were in Hardy-Weinberg's equilibrium for both the investigated SNPs ($x^2 = 0.0008$ and 0.0007). The frequency distribution of alleles (A and G) at locus A256G and A325G in patients with DWoWS was similar to patients with DWWS/SD, as well as the frequency distribution of genotypes: AA, AG and GG (Table 2). The ancestral allele A and genotype of AA of SNP A256G was present in more than 90% of the patients in case group (DWWS/SD) and controls (DWoWS), while around 4% and 8% of patients presented the allele G and genotype AG in each group respectively. Regarding the SNP A325G, allele A and genotype AA were present in around 60% and 38%, respectively, whilst allele G was around 37%, and genotypes AG and GG, around 48% and 13%, respectively (Table 2).

**Table 1 -** Distribution by age, gender, primary and secondary dengue in case (DWWS/SD) and control (DWoWS).

| Variables | All (N = 273) | DWoWS (N = 119) | DWWS/SD (N = 154) |
|---|---|---|---|
| Age (years) | 27.9 | 29.6 | 26.6 |
| Female – Nº (%) | 153 (56) | 68 (57.6) | 85 (55.2) |
| Primary dengue – Nº (%) | 120 (44.3) | 50 (42.4) | 70 (45.8) |
| Secondary dengue – Nº (%) | 151 (55.7) | 68 (57.6) | 83 (54.2) |
| Nº observations | 271 | 118 | 153 |

DWoWS = dengue without warning signs; DWWS = dengue with warning signs; SD = severe dengue; Nº observations = N.

**Table 2 -** Allele, genotypic and diplotypic frequencies of single nucleotide polymorphisms A256G" (rs2069716) and A325G (rs2069727) of the interferon-gamma gene in case (DWWS/SD) and control (DWoWS) groups.

| SNPs/ diplotypes | Study group | | | Logistic regression model | | | | Nº observations | |
|---|---|---|---|---|---|---|---|---|---|
| | All (N = 273) | DWoWS (N = 119) | DWWS/SD (N = 154) | Crude OR (CI) | *p* | Adjusted OR (CI)[a] | *p* | Crude OR | Adjusted OR |
| A256G rs2069716 | | | | | | | | | |
| AA | 250 (91.6) | 109 (91.6) | 141 (91.6) | Ref. | | Ref. | | 272[b] | 270[c] |
| AG | 22 (8.0) | 10 (8.4) | 12 (7.8) | 0.92 (0.39;2.23) | 0.867 | 0.91 (0.38;2.20) | 0.836 | | |
| GG | 1 (0.4) | 0 (0) | 1 (0.6) | ... | ... | ... | ... | | |
| A | 522 (95.6) | 228 (95.8) | 294 (95.4) | | | | | | |
| G | 24 (4.4) | 10 (4.2) | 14 (4.6) | | | | | | |
| A325G rs2069727 | | | | | | | | | |
| AA | 109 (38.8) | 39 (32.8) | 67 (43.5) | Ref. | | Ref. | | 273 | 271[e] |
| AG | 137 (48.0) | 64 (53.8) | 67 (43.5) | 0.61 (0.36;1.03) | **0.063** | 0.64 (0.38;1.09) | 0.103 | | |
| GG | 36 (13.2) | 16 (13.4) | 20 (13.0) | 0.72 (0.34;1.57) | 0.416 | 0.72 (0.33;1.58) | 0.418 | | |
| A | 343 (62.8) | 142 (59.7) | 201 (65.2) | | | | | | |
| G | 203 (37.2) | 96 (40.3) | 107 (34.8) | | | | | | |
| A256G/A325G | | | | | | | | | |
| AA/AA | 106 (38.8) | 39 (32.8) | 67 (43.2) | Ref. | | Ref. | | 272[d] | 270[f] |
| AA/AG | 114 (41.8) | 57 (47.9) | 57 (36.8) | **0.58** (0.34;1.00) | **0.049** | **0.61** (0.36;1.06) | **0.082** | | |
| AA/GG | 30 (11) | 13 (10.9) | 17 (11) | 0.76 (0.33;1.73) | 0.516 | 0.76 (0.33;1.75) | 0.521 | | |
| AG/AG | 16 (5.9) | 7 (5.9) | 9 (5.8) | 0.75 (0.26;2.2) | 0.593 | 0.77 (0.26;2.25) | 0.633 | | |
| AG/GG | 6 (2.2) | 3 (1.1) | 3 (1.9) | 0.58 (0.11;3.03) | 0.520 | 0.56 (0.10;2.97) | 0.495 | | |
| GG/AG | 1 (0.4) | 0 (0) | 1 (0.6) | ... | ... | ... | ... | | |

DWoWS= dengue without warning signs; DWWS = dengue with warning signs; SD = severe dengue; CI = 95% confidence interval; Ref = Reference; OR = Odds Ratio; *p* = p-value; SNP = Single nucleotide polymorphism; N = Number of observations; [a]Logistic regression model adjusted for age, gender and sequence of infection; [b]Exclusion of a single patient with GG genotype; [c]Exclusion of two patients without information on the sequence of infection; [d]Exclusion of a single patient with GG/AG diplotype; [e]Exclusion of two patients without information on the sequence of infection; [f]Exclusion of two patients without information on the sequence of infection.

In our multivariate regression genotype models, adjusted for age, gender and sequence of infection, we found no association between severe forms of dengue (DWWS/SD) and genotypes of SNPs A256G and A325G (Table 2).

In Table 2, we display the frequency distribution of DWoWS and DWWS/SD among patients with each diplotype (AA/AA, AA/AG, AA/GG, AG/AG, AG/GG, GG/GG), showing that the highest proportion of DWWS/SD (43.5%) occurred among patients with the ancestral diplotype (AA/AA), followed by those who were AA/AG (37%) and AA/GG (11%). In the diplotype model, we found that the AA/AG patients had 40% less DWWS/SD, in comparison with the AA/AA patients. The OR and statistical significance remained similar after adjustment for age, gender and sequence of infection: OR 0.61; 95% CI = 0.36-1.06, $p = 0.082$ (Table 2).

Table 3 displays multivariate regression diplotype models stratified by sequence of infection, showing that the association between DWWS/SD and the genotype AA/AG remained only among patients with secondary dengue: OR = 0.51; 95% CI = 0.24-1.10; $p = 0.085$.

**Table 3 -** Association between DWWS/SD and the diplotypes of single nucleotide polymorphisms A256G/A325G (rs2069716/rs2069727), in each stratum of patients with primary and secondary dengue.

| Diplotypes A256G/A325G | All patients (N = 271) | Primary dengue | | | | | Secondary dengue | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | All primary dengue (N = 120) | DWoWS (N = 50) | DWWS/SD (N = 70) | Adjusted OR (CI)[a] | *p* | All secondary dengue (N = 151) | DWoWS (N = 68) | DWWS/SD (N = 83) | Adjusted OR (CI)[a] | *p* |
| AA/AA | 105 (38.7) | 50 (41.7) | 19 (38) | 31 (44.3) | Ref. | | 55 (36.4) | 20 (29.4) | 35 (42.2) | Ref. | |
| AA/AG | 113 (41.7) | 52 (43.3) | 24 (48) | 28 (40) | 0.70 (0.30;1.58) | 0.380 | 61 (40.4) | 32 (47) | 29 (34.9) | 0.51 (0.24;1.10) | **0.085** |
| AA/GG | 30 (11.1) | 11 (9.2) | 4 (8) | 7 (10) | 1.08 (0.27;4.39) | 0.911 | 19 (12.6) | 9 (13.2) | 10 (12.1) | 0.62 (0.21;1.78) | 0.374 |
| AG/AG | 16 (5.9) | 6 (5) | 2 (4) | 4 (5.7) | 1.06 (0.17;6.81) | 0.949 | 10 (6.6) | 5 (7.4) | 5 (6) | 0.61 (0.15;2.46) | 0.490 |
| AG/GG | 6 (2.2) | 1 (0.8) | 1 (2) | 0 (0) | ... | ... | 5 (3.3) | 2 (2.9) | 3 (3.6) | 0.84 (0.13;5.46) | 0.851 |
| GG/AG | 1 (0.4) | 0 (0) | 0 (0) | 0 (0) | ... | ... | 1 (0.7) | 0 (0) | 1 (1.2) | ... | ... |
| Nº Observations | | | | | 119[b] | | | | | 150[c] | |

DWoWS= dengue without warning signs; DWWS = dengue with warning signs; SD: severe dengue; CI = 95% confidence interval; Ref = Reference; OR = Odds ratio; p = *p* value; N = Number of observations; [a]Logistic regression model adjusted for age, gender and sequence of infection; [b]Exclusion of a single patient with GG/AG diplotype; [c]Exclusion of two patients without information on sequence of infection.

## DISCUSSION

Our analysis on the IFNG gene from patients with secondary dengue by DENV3 found that the diplotype AA/AG, was associated with a 40% lower chance of DWWS/SD, compared to patients with ancestral diplotype (AA/AA). The overall adjusted OR had a middle statistical significance ($0.05 < p < 0.10$), and remained similar in the sub-sample with only secondary dengue cases, indicating that our estimates were robust to suggest the IFN-γ as an important cytokine in the immune response against a second DENV infection.

Comparison of our results with previous publications is limited. According to the databases of Ensembl[12,13] and NCBI[14,15], there are no other studies investigating the SNPs A256G and A325G of IFNG gene in dengue fever, nor in other arboviruses. However, it is known that these SNPs may be related to IFNG upregulation, and together they may play an important role in the immune response against DENV. Perez *et al.*[16] had examined the SNP A+874T at IFNG and found no association between its genotypes and dengue hemorrhagic fever in secondary dengue. Santos *et al.*[17] also found no association of the SNP A+874T with dengue hemorrhagic fever, without adjustment for sequence of infection. Different from our study, these previous studies did not account for important confounders that may be implicated in a poor prognosis of dengue, such as DENV serotypes and ADE response to a secondary infection[6]. In fact, in studies on genetic factors of dengue, the comparison groups must be equally infected, having similar distributions of serotypes and primary/secondary dengue; otherwise results cannot be attributed to intrinsic genetic factors.

To address these confounders, we evaluated only patients with a confirmed DENV3 infection and included the sequence of infection (primary/secondary) in the multivariate regression models. We observed that a multivariate model, adjusted for age and gender, but not for sequence of infection, would yield a more significant association between the AA/AG diplotype and DWWS/SD ($p = 0.056$) than our final model adjusted for age, gender and sequence of infection ($p = 0.082$). Then, our stratified analysis showed that only patients with secondary dengue had the estimated association. These analyses indicate previous dengue infections as a critical confounder.

IFN-γ is produced indirectly by activated natural killer cells and lymphocytes in response to pro-inflammatory cytokines, such as IL-12 and IL-18, and seems to trigger a synergistic response against DENV[18]. Once produced, IFN-γ binds to specific receptors in neighbor cells, to stimulate transcription of genes involved in inflammatory regulation and production of chemokines and cytokines[19]. Increased levels of IFN-γ have been associated with dengue fever and SD[20]. Collectively, these data support the idea that IFN-γ may be a central key for both antibody and T cell responses to DENV infection, but how the IFNG might drive these responses remains an elusive question.

IFN-γ dosages and T cell experiments would be needed to fully interpret our results, but we had not performed such functional tests. Therefore, we can only speculate that the IFNG A325G variant works as a protection element when combined with the A256G ancestral genotype, in patients with DENV3 who were previously infected with another serotype. Our results agree with the hypothesis that the IFNG gene might modulate the immune response against severe forms of DENV infection, and indicate that examining single key genes can help to understand the

immunopathogenesis of flaviviruses. We suggest that IFN-γ should be better investigated in the pathogenic balance between a harmful *versus* protective response to secondary DENV infections.

## ACKNOWLEDGMENTS

The original text of this article has been revised by Sidney Pratt (The Johns Hopkins University).

## CONFLICT OF INTERESTS

All the authors declare to have no commercial or other conflict of interests.

## FUNDING

BAS was supported by the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), PAPES VII, grant Nº 41910/2015-6, and Fundacao de Amparo a Ciencia e Tecnologia do Estado de Pernambuco (FACEPE), PROEP, grant Nº APQ-1597-2.02/15.

## REFERENCES

1. World Health Organization. Dengue and severe dengue. [cited 2023 May 4]. Available from: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue
2. Messina JP, Brady OJ, Golding N, Kraemer MU, Wint GR, Ray SE, et al. The current and future global distribution and population at risk of dengue. Nat Microbiol. 2019;4:1508-15.
3. Pan-American Health Organization. Epidemiological update: dengue: 11 November 2019. [cited 2023 May 4]. Available from: https://www.paho.org/hq/index.php?option=com_docman&view=download&alias=50963-11-november-2019-dengue-epidemiological-update-1&category_slug=dengue-2217&Itemid=270&lang=en
4. World Health Organization. Dengue: guidelines for diagnosis, treatment, prevention and control. Geneva: WHO; 2009. [cited 2023 May 4]. Available from: https://www.who.int/publications/i/item/9789241547871
5. Guabiraba R, Ryffel B. Dengue virus infection: current concepts in immune mechanisms and lessons from murine models. Immunology. 2014;141:143-56.
6. Ngono AE, Shresta S. Immune response to dengue and Zika. Ann Rev Immunol. 2018;36:279-308.
7. Arayasongsak U, Naka I, Ohashi J, Patarapotikul J, Nuchnoi P, Kalambaheti T, et al. Interferon lambda 1 is associated with dengue severity in Thailand. Int J Infect Dis. 2020;93:121-5.
8. Prestwood TR, Morar MM, Zellweger RM, Miller R, May MM, Yauch LE, et al. Gamma interferon (IFN-γ) receptor restricts systemic dengue virus replication and prevents paralysis in IFN-α/β receptor-deficient mice. J Virol. 2012;86:12561-70.
9. Vargas-Castillo AB, Ruiz-Tovar K, Vivanco-Cid H, Quiroz-Cruz S, Escobar-Gutiérrez A, Cernas-Cortes JF, et al. Association of single-nucleotide polymorphisms in immune-related genes with development of dengue hemorrhagic fever in a Mexican population. Viral Immunol. 2018;31:249-55.
10. Davi C, Pastor A, Oliveira T, Lima Neto FB, Braga-Neto U, Bigham AW, et al. Severe dengue prognosis using human genome data and machine learning. IEEE Trans Biomed Eng. 2019;66:2861-8.
11. Cordeiro MT, Silva AM, Brito CA, Nascimento EJ, Magalhães MC, Guimarães GF, et al. Characterization of a dengue patient cohort in Recife, Brazil. Am J Trop Med Hyg. 2007;77:1128-34.
12. Ensembl Project. Variant: rs2069716 SNP. [cited 2023 May 4] Available from: https://www.ensembl.org/Homo_sapiens/Variation/Citations?db=core;r=12:68156535-68157535;v=rs2069716;vdb=variation;vf=96316609
13. Ensembl Project. Variant: rs2069727 SNP. [cited 2023 May 4] Available from: https://www.ensembl.org/Homo_sapiens/Variation/Citations?db=core;r=12:68153943-68154943;v=rs2069727;vdb=variation;vf=96316715
14. National Center for Biotechnology Information. Reference SNP (rs) report: rs2069716. [cited 2023 May 4]. Available from: https://www.ncbi.nlm.nih.gov/snp/rs2069716#publications
15. National Center for Biotechnology Information. Reference SNP (rs) report: rs2069727. [cited 2023 May 4]. Available from: https://www.ncbi.nlm.nih.gov/snp/rs2069727#publications
16. Perez AB, Sierra B, Garcia G, Aguirre E, Babel N, Alvarez M, et al. Tumor necrosis factor-alpha, transforming growth factor-β1, and interleukin-10 gene polymorphisms: implication in protection or susceptibility to dengue hemorrhagic fever. Hum Immunol. 2010;71:1135-40.
17. Santos AC, Moura EL, Ferreira JM, Moura AW, Ferreira AD, Bezerra RP, et al. Association of TNFA (-308 G/A), IFNG (+874 A/T) and IL-10 (-819 C/T) polymorphism with protection and susceptibility to dengue in Brazilian population. Asian Pac J Trop Med. 2017;10:1065-71.
18. Rathore AP, St. John AL. Cross-reactive immunity among flaviviruses. Front Immunol. 2020;11:334.
19. Carlin AF, Plummer EM, Vizcarra EA, Sheets N, Joo Y, Tang W, et al. Dengue viral resistance utilizes IRF-1 to stimulate type I and II interferon responses. Cell Rep. 2017;21:1600-12.
20. Sehrawat P, Biswas A, Kumar P, Singla P, Wig N, Dar L, et al. Role of cytokines as molecular marker of dengue severity. Mediterr J Hematol Infect Dis. 2018;10:e2018023.