Article

Experimental Dengue 4 Infection Increases the Production of NS1 Protein and Expression of MicroRNAs-15/16 Levels, Triggering an Apoptosis Caspase Induced Pathway

Samir Mansour Moraes Casseb[1,3*], Karla Fabiane Lopes de Melo[1], Carlos Alberto Marques de Carvalho[2], Carolina Ramos dos Santos[1], Edna Cristina Santos Franco[1] and Pedro Fernando da Costa Vasconcelos[2]

[1] Evandro Chagas Institute, Ananindeua, Pará, Brazil
[2] University of Pará State, Belém, Pará, Brazil
[3] Federal University of Pará, Belém, Pará, Brazil
* Corresponding author: samircasseb@ufpa.br

Abstract: The World Health Organization has estimated an annual occurrence of approximately 392 million dengue virus (DENV) infections in more than 100 countries where the virus is endemic, and this represents a serious threat to humanity. DENV is a serologic group with four distinct serotypes (DENV1, DENV2, DENV3, and DENV4) belonging to the genus Flavivirus, family Flaviviridae. Dengue is the most widespread mosquito-borne disease in the world. The ~10.7 kb DENV genome encodes three structural proteins (capsid [C], pre-membrane [prM], and envelope [E]) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The NS1 protein is found both as a membrane-associated dimer and as a secreted, lipid-associated hexamer. Dimeric NS1 is found on membranes both in cellular compartments and on the cell surface. Secreted NS1 (sNS1) is often present in patient serum at very high levels, which correlates with severe dengue symptoms. This study was carried out to find out how the NS1 protein, miRNAs 15 and 16, and apoptosis are related to each other during DENV4 infection in human liver cell line culture. The Huh 7.5 and HepG2 strains were infected with DENV4, and after different times of infection, miRNA-15 and miRNA-16, viral load, NS1 protein, and caspases 3 and 7 were quantified. This study demonstrated that miRNAs 15 and 16 are overexpressed during infection of HepG2 and HuH7.5 cells by DENV4 and have a relationship with NS1 protein expression, VDEN4 viral load, and caspase pathways 3 and 7, thus making these miRNAs interesting targets for markers of injuries during VDEN infection in human hepatocyte cells.

Keywords: dengue virus; microRNA; NS1; miRNA

1. Introduction

The World Health Organization (WHO) states that about 392 million dengue virus (DENV) infections happen every year in more than 100 countries where the virus is common, being a severe threat to public health. DENV causes the most common mosquito-borne disease in the world. It consists in a group of four different serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) that belong to the genus Flavivirus, Flaviviridae. Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS, now called severe dengue) are major causes of illness in tropical and subtropical areas (1–3).

The DENV is spread by the Aedes aegypti mosquito, which must feed on a person with the virus during the first 5 days of high viremia, when the person is just starting to feel sick. Asymptomatic individuals can still infect mosquitoes, and infected mosquitoes incubate the virus. The virus remains in the mosquitoes for 8–12 extra days (extrinsic incubation period) before it can be passed on to a susceptible human. The mosquito is infected for its whole life and, more importantly, can spread DENV to humans more than once. There is lack of evidence that the virus can be passed on in unusual ways, like

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 December 2022 doi:10.20944/preprints202212.0323.v1

through organ transplants, blood transfusions, or from an infected pregnant woman to her unborn child (4).

The DENV genome (~10.7 kb) encodes three structural proteins (capsid [C], pre-membrane [prM], and envelope [E]) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). They are 50-nm, icosahedral, lipid-wrapped particles that have the same structure and pathogenic properties, but different genetic and serological properties (5).

The NS1 protein can be found both as a membrane-associated dimer and a secreted, lipid-associated hexamer (sNS1). Dimeric NS1 is found on membranes both in cellular compartments and on the cell surfaces of virus-infected cells. It has been shown that the serum from patients contains secreted NS1, and the levels of this protein are often very high, which is linked to the start of DHF. NS1 also has effects on the innate immune response, and has been seen in multiple interactions with the complement system through factor H (6,7).

Identifying regulatory ~22 nucleotides (nt) non-coding RNAs has been one of the significant molecular biology tasks. MicroRNAs (miRNAs) are produced from transcripts that form a fold-back hairpin structure, which is cropped by the RNase III enzyme Drosha to a ~70 nt long pre-miRNA (7,8).

This structure is exported to the cytoplasm and further processed by a second RNase III enzyme, Dicer, into the mature ~22 nt miRNA and its complementary star sequence. Mature miRNAs control gene expression by directing effector complexes made up of Argonauts (Ago) proteins to their cognate targets, which stops translation or speeds up mRNA degradation (9). Regulation depends on base pairing between the miRNA and target sites on the mRNA. The seed sequence, nucleotides 2–7 at the 5' end of the miRNA, is significant for this interaction (10).

Here, we show a correlation between the expression profile of the NS1 protein and the expression of miRNAs 15 and 16 during infection by DENV4 in cell culture, specifically HepG2 and Huh 7.5 cells of hepatic origin.

2. Materials and Methods

2.1. Cell Culture

HepG2 and Huh 7.5 hepatic human cell lines were cultured at 37 °C in a nutrient DMEM (Sigma-Aldrich) medium containing L-glutamine, HEPES buffer, and 10% fetal bovine serum (FBS, Gibco).

2.2. Viral Samples

At first, the DENV4 (Be H778494) isolate was grown in C6/36 cells with 5% FBS in L-15 medium at 28 °C. On the seventh day after infection, the culture supernatant was harvested and stored at -80 °C before infections of Huh 7.5 and HepG2 cell types.

2.3. Infection of Hepatic Human Cells

The HepG2 and Huh 7.5 cells were infected with DENV4 using the adsorption method for one hour at 37 °C (MOI 1). Subsequently, the cell cultures were washed with phosphate-buffered saline (PBS), put in a new medium, and grown again for the experiment. The extracted RNAs were kept at -80 °C until needed. The supernatants of the virus-infected cells were collected daily up to 120 hours post-infection (hpi).

2.4. RNA Extraction

The commercial Maxwell 16 LEV Simply RNA Cells kit (Promega, Madison, WI, USA) was used to extract the RNA from the experimental samples, as suggested by the manufacturer. The samples were then quantified using the commercial Qubit RNA High Sensitivity (HS) kit (Thermofisher, USA) on the Qubit 3.0 platform (Thermofisher, USA). The extracted RNAs were kept at -80 °C until needed.

 doi:10.20944/preprints202212.0323.v1

2.5. Quantification of Viral Load by Real-Time RT-qPCR

The primers and method described by Johnson et al. (11) were used to measure the viral load, along with the standard curve method and the plasmid pGEM Easy (Promega) to measure the exact amount.

2.6. Quantification of miRNA Levels by Real-Time RT-qPCR

The commercial kit TaqMan MicroRNA Cells-to-CT (Ambion, California, USA) was used to measure the levels of mir-15 and mir-16, according to the manufacturer's instructions. Endogenous controls were made from the targets of RNU48 (SNORD48) and RNU58a (the gene for ribosomal protein L17). The quantitative PCR was performed on the platform ViiA 7 (Life Technologies, California, USA).

The relative amounts of miRNAs in the samples were figured out and compared to the amounts in RNU48 and RNU58a, which were used as internal controls (12). We had previously written about how the comparative CT method was used to measure the expression.

2.7. NS1 Quantification

The amount of NS1 protein was measured with the commercial kit Platelia Dengue NS1 Ag (Bio-Rad, Hercules, California, USA) and a protein curve built with a synthetic protein made from an expressing plasmid, which uses NS1 protein standard curve. This quantification was performed using supernatants from cells infected with DENV4.

2.8. Analysis and Quantification of Apoptosis

Following the manufacturer's instructions, the commercial kit Caspase-Glo 3/7 Assay (Promega) was used on the Glomax-Multi+ (Promega) platform to analyze and measure the caspase 3 and 7 pathways. This test is based on the labeling of caspase pathways 3 and 7 with luciferin in order to measure how they function during a VDEN infection. For these tests, the MOCK caspase values were compared with the infection times.

2.9. NS1 fluorescent Imaging

Up to 120 hours after DENV4 infected Huh7.5 cells, they were moved to a microscope slide and fixed with 3.7% formaldehyde. After the cells were fixed, 3% bovine serum albumin (Sigma-Aldrich) was used to block nonspecific antibody binding sites for 1 hour. The cells were then put in a solution of 1:20 of FITC-conjugated DENV NS1 polyclonal antibody (Biorbyt) at 4 °C for 16 hours. After putting the coverslip on, samples were seen on an LSM 510 META laser-scanning confocal fluorescence microscope (Carl Zeiss, Colony, Germany) with excitation at 488 nm and emission collected from 500 to 550 nm. Images were processed using the software ImageJ v1.48 (National Institutes of Health, Bethesda, USA).

2.10. Statistical Analysis

Expression Suite v1.0 (Applied Biosystems, California, USA) and the project's qPCR.Ct package were used to do the statistical analysis of the data. Analysis of variance (ANOVA) and Pearson correlation were carried out using the Jamovi Project 2.3 platform (jamovi.org). P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Viral Load Profile During Infection

At first, we looked at the amount of virus in VDEN4-infected cell lines 120 hours post infection (hpi), then it was possible to find out when the number of viruses was increasing the most (Figure 01).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 December 2022 doi:10.20944/preprints202212.0323.v1

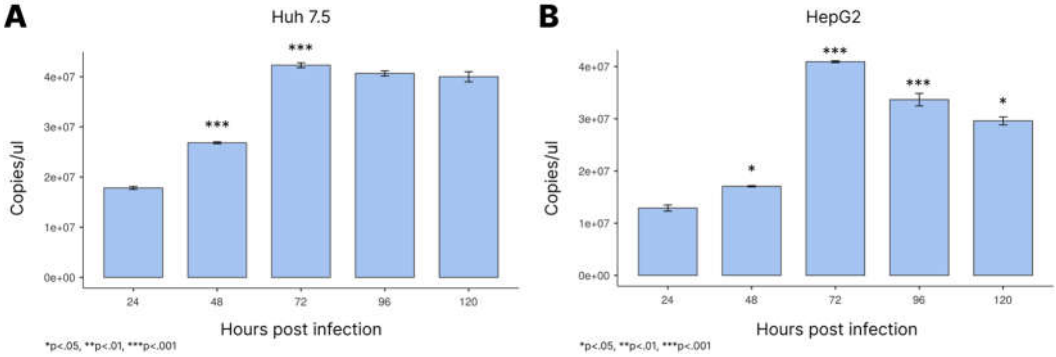

Figure 1. – Viral load as a function of time after DENV4 infection of Huh7.5 (A) and HepG2 (B) cells.

After analyzing these results, it is clear that 72 hpi is the time when both strains have the highest viral load, and that HepG2 strain has the lowest viral load at 72 hpi.

3.2. Expression levels of the miRNAs 15 and 16 During Infection

As stated above, DENV4 was used to infect Huh7.5 and HepG2 cells. At different times after infection, qRT-PCR was used to look at the levels of miRNA-15 and miRNA-16 in culture supernatants.

During the infection, the level of miRNA-15 slowly went up until it reached 120 hpi in both cell types. At 72 hpi, it reached a significant level in both cell lines (Figures 2A and 2B). After this time, the level of expression was stabilized and remained high for the rest of our experiment.

By analyzing the miRNA-16 expression values, we can see that the expression values in both cell types are significantly higher after 48 hpi (Figures 2C and 2D).

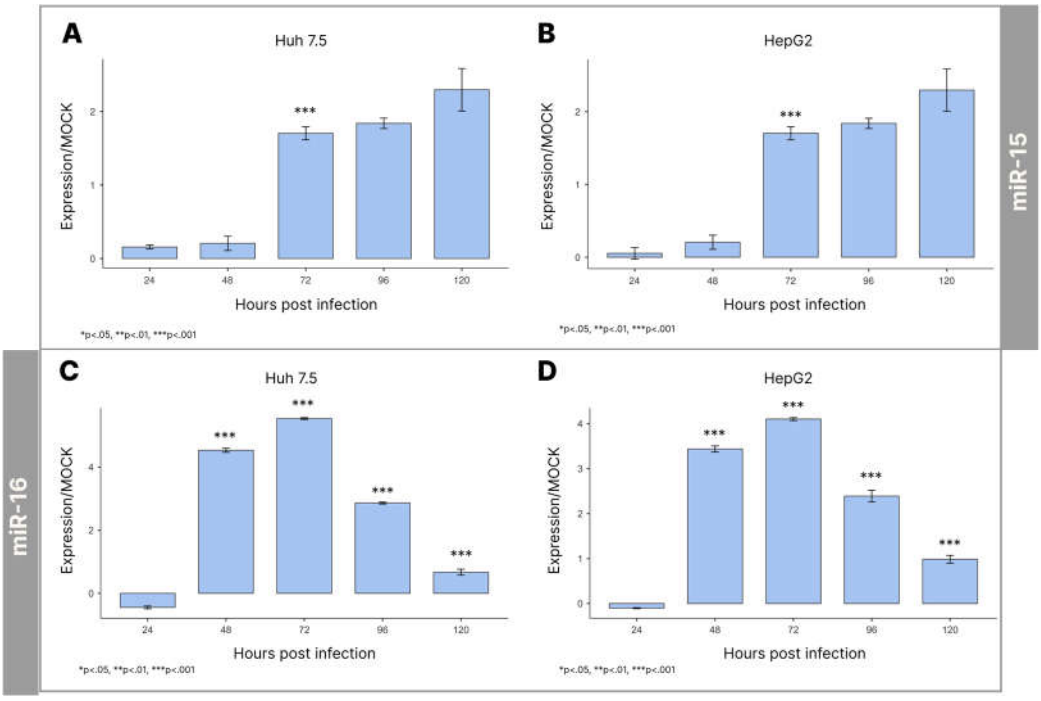

Figure 2. – Quantification of miRNA-15 levels as a function of time after infection by DENV4 into Huh7.5 (A) and HepG2 (B) cells. Quantification of miRNA-16 levels as a function of time after infection by DENV4 into Huh7.5 (C) and HepG2 (D) cells.

Between 96 and 120 hpi, the amount of miRNA-16 intensely drops, which is interesting. Even though the Huh7.5 strain showed negative expression values at 24 hpi, there was no statistically significant difference between these values and the ones at the time before infection, which we call time "0" hpi.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 December 2022 doi:10.20944/preprints202212.0323.v1

3.3. Activation of Caspases3 and 7 During Infection

Next, we looked at how the apoptosis pathway was turned on by DENV4 infection by measuring the activity of caspase-3 and 7 (Figure 3).

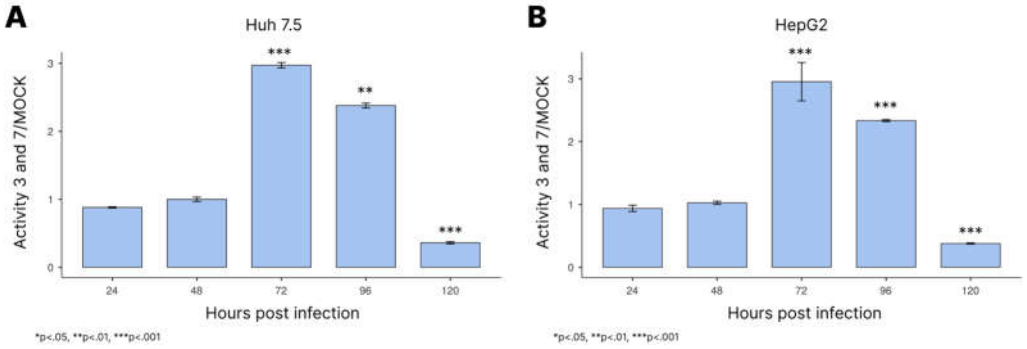

Figure 3. – Quantification of caspase-3/7 activity as a function of time after infection by DENV4 into Huh7.5 (A) and HepG2 (B) cells.

The results showed that caspase-3/7 activity was very high at 72 hpi in both cell types. This activity then went down at 96 and 120 hpi.

3.4. NS1 Expression During Infection

Then, we evaluated the expression of NS1 protein during VDEN4 infection (Figure 05).

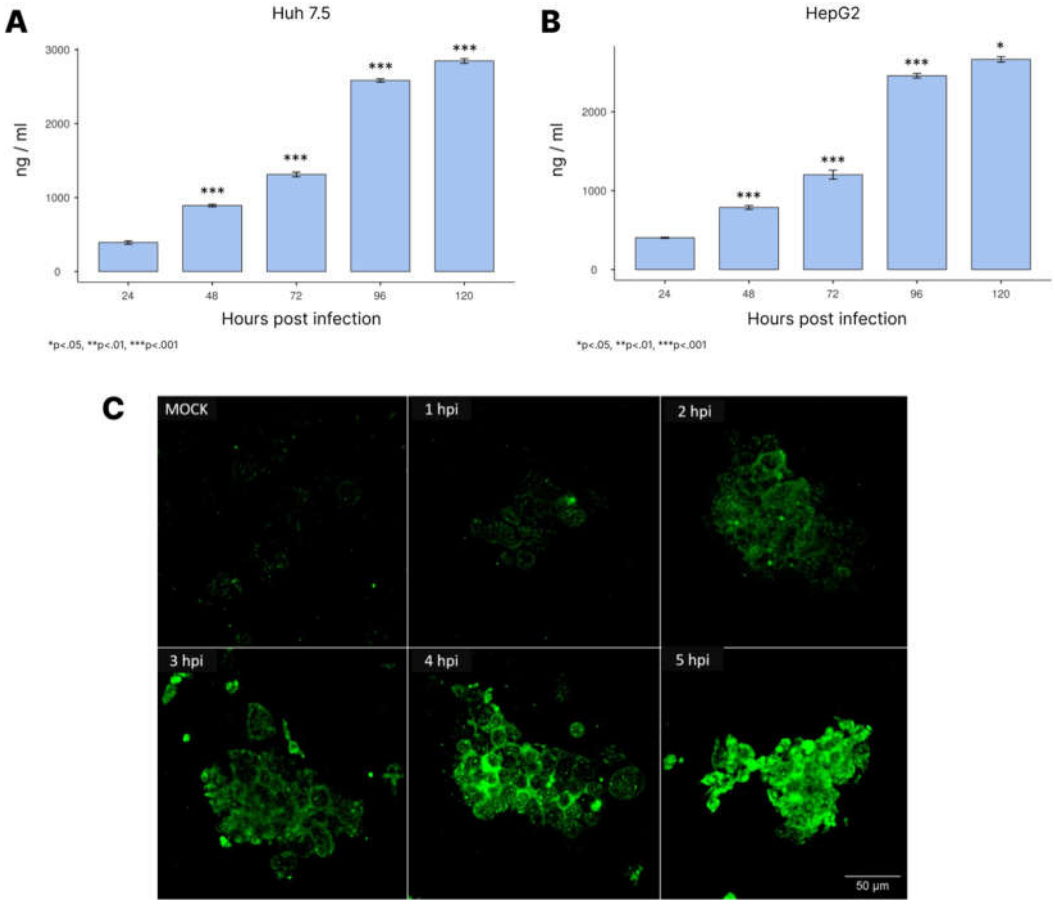

Figure 4. - Quantification of NS1 levels in Huh7.5 (A and C) and HepG2 (B) cells following DENV4 infection.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 December 2022 doi:10.20944/preprints202212.0323.v1

It is possible to notice that the quantification of NS1 was similar for both cell lines (HepG2 and Huh7.5) in this experiment. At 48 hpi, it becomes clear that NS1 is going up, and it continues to increase for the rest of the infection.

3.5. Correlation Viral Load, NS1 and Cellular Components

Then, a correlation analysis was performed between the data generated for this work. We correlated the time of infection with the viral load, the amount of NS1, and the expression of targets such as miRNA-15 and miRNA-16 (Figure 05).

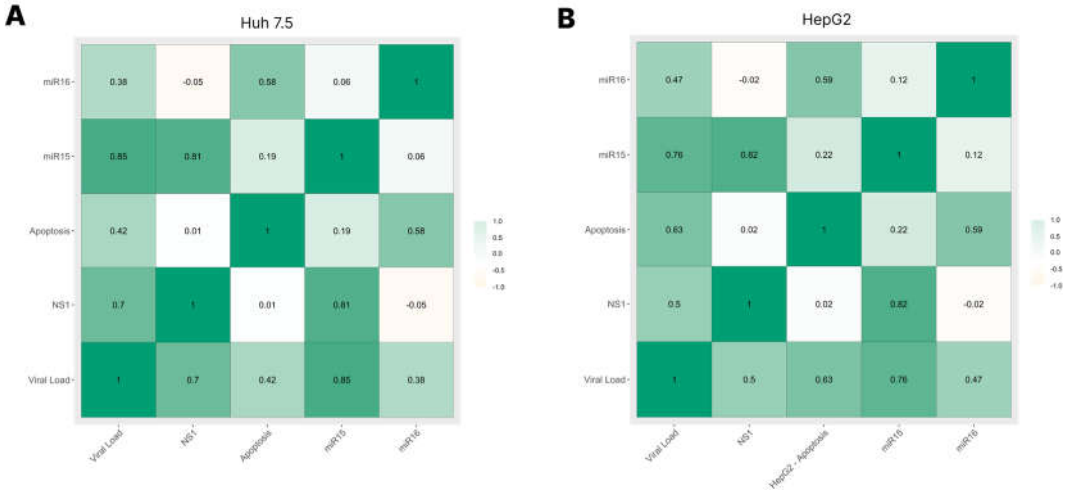

Figure 5. – Pearson correlation analysis between viral load, NS1 protein, cellular components, miRNA 15, and miRNA 16 during VDEN4 infection.

It was clear that the two cell lines behaved in the same way, and when we looked at the targets considering the amount of virus, this became even more evident. It is worth noticing the positive relationship between miRNA-15 and both NS1 and viral load, and also the lower relationship between caspases 3 and 7 and miRNA-16.

4. Discussion

Dengue virus is a Flavivirus with a vast worldwide dispersion. Even with the advances in this area, they remain. Along with the role of miRNA in dengue infections, one of them is the fact that viral proteins can act as signaling factors.

Studies such as the one by Casseb et al. (13) show that DENV infection changes how key miRNA-related proteins are controlled, and it is even possible to see the expression of these proteins go down. Other studies by Holanda et al. (14) and Castillo and Urcuqui-Inchim (15) support these results, both for DENV and for another flavivirus called Yellow Fever Virus (YFV).

It is important to notice that viral proteins may be related to miRNA. One of these proteins is NS1, which is released and can be taken up by hepatocytes and stored there. This makes it more likely that hepatocytes will get infected and makes it easier for them to take in NS1. As there is evidence that dengue affects liver function, especially in severe cases, and as NS1 interacts with hepatocytes, these cells are suitable for studying and test how NS1 causes immunopathology. In addition, there is evidence that NS1 can alter cell signaling pathways to help produce more viruses and bypass defenses (16–18).

This study analyzed how the expression of miRNAs 15 and 16 and the NS1 protein change over time when Huh7.5 and HepG2 cells are infected with DENV4. When DENV4 replicated in these human hepatocyte cell types, we observed viral load profiles similar to those described by Alhoot et al. (19). Furthermore, we demonstrated that the cellular effects are similar to those suffered by other flaviviruses, mainly related to the expression of key proteins.

 doi:10.20944/preprints202212.0323.v1

It has been said that miRNA-15 is a key regulator of apoptosis in cells, and low expression of this miRNA has been linked to cancer (20,21). Papillomavirus infections have also been shown to alter the levels of these miRNAs in cells, having important effects on the p53 signaling pathway (22–24). Even though studies have shown that DENV is harmful (25,26), the molecular basis of apoptosis is not well understood.

miRNA-15 is a lot like miRNA-16, which is also involved in apoptotic pathways and cell growth during oncogenic processes (25,27). The miRNA-16 levels have also been investigated in viral infections. In fact, a recent study by El-Abd et al. (28) showed that regulatory function is diminished when the hepatitis C virus causes a long-term infection, which can lead to hepatocellular carcinoma. In this study, however, we showed that the expression of both miRNAs goes up during an acute viral infection by DENV4, along with an increase in the expression of NS1 protein and the activity of the apoptosis markers caspases-3 and 7. This suggests that NS1 can initiate this apoptosis pathway.

In a recent study, the expression of miRNAs 15 and 16 was found to be opposite to the expression of Bcl-2, family of proteins that prevent cell death (Yu et al., 2014). According to (29), the downregulation of Bcl-2 protein levels by these miRNAs could be caused by a direct (miRNA-mRNA complementarity) or indirect interaction, which would lead to a change in the cell cycle and then apoptosis. In our study, DENV4 NS1 was made while the activity of caspases-3/7 was going up (Figure 3), suggesting that DENV4 infection of liver cells plays a role in apoptosis. This finding may be clinically important and may help explain why apoptosis was observed in the liver cells of people who died from dengue and yellow fever (21,30)..

Antibodies against NS1 are capable of a protective immune response and induce complement-fixing activity. However, the NS1 protein can prevent the immune system from functioning when a virus is present. When released into the extracellular medium, NS1 acts as a pathogen-associated molecular pattern (PAMP), which directly activates macrophages and peripheral blood mononuclear cells (PBMCs) via Toll-like receptor 4 (TLR4), causing them to produce and release cytokines, pro-inflammatory drugs and chemokines (17,31).

It was previously shown that during DENV1 infection of HepG2 cells, the NS1 protein can be found in lipid raft domains on the host cell surface, suggesting an involvement in signal transduction events (32). In fact, the authors of these studies found that the NF-kB p65 protein moves to the nucleus when NS1 is expressed in HepG2 cells. More DENV infections have recently been linked to NF-kB activation, which leads to endothelial cell death by apoptosis and bleeding symptoms (23,33).

5. Conclusions

This study showed that when DENV4 infects human hepatocyte cells, miRNAs 15 and 16 are overexpressed. NS1 protein production, viral load and apoptosis are linked to it, demonstrating an intense linkage between cellular expression components and NS1 expression during DENV4 replication. Thus, we can consider that miRNAs 15 and 16 have an important potential for use as markers of diseases during DENV infection in human hepatocytes.

Author Contributions: Samir Casseb: Methodology, data analysis and manuscript writing. Karla Melo: Methodology and data analysis. Carlos Carvalho: Methodology. Carolina Santos: data analysis. Edna Franco: writing of the manuscript , Pedro Vasconcelos: revision of the manuscript.

Funding: This study was supported by grants from CNPq/CAPES/FAPESPA 573739/2008-0 and CNPq 301641/2010-2.

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable.

Acknowledgments: Thanks to Evandro Chagas Institute for providing the structure and viral strains used in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Horstick O, Tozan Y, Wilder-Smith A. Reviewing dengue: still a neglected tropical disease? PLoS Negl Trop Dis. 2015 Apr 30;9(4):e0003632.
2. Li G, Khandekar A, Yin T, Hicks SC, Guo Q, Takahashi K, et al. Differential Wnt-mediated programming and arrhythmogenesis in right versus left ventricles. J Mol Cell Cardiol. 2018 Sep 5;123:92–107.
3. Siqueira JB, Martelli CMT, Coelho GE, Simplicio AC da R, Hatch DL. Dengue and dengue hemorrhagic fever, Brazil, 1981-2002. Emerging Infect Dis. 2005 Jan;11(1):48–53.
4. Azeredo EL, Dos Santos FB, Barbosa LS, Souza TMA, Badolato-Corrêa J, Sánchez-Arcila JC, et al. Clinical and Laboratory Profile of Zika and Dengue Infected Patients: Lessons Learned From the Co-circulation of Dengue, Zika and Chikungunya in Brazil. PLoS Curr Influenza. 2018 Feb 15;10.
5. Álvarez-Díaz DA, Gutiérrez-Díaz AA, Orozco-García E, Puerta-González A, Bermúdez-Santana CI, Gallego-Gómez JC. Dengue virus potentially promotes migratory responses on endothelial cells by enhancing pro-migratory soluble factors and miRNAs. Virus Res. 2019 Jan 2;259:68–76.
6. Hafirassou ML, Meertens L, Umaña-Diaz C, Labeau A, Dejarnac O, Bonnet-Madin L, et al. A global interactome map of the dengue virus NS1 identifies virus restriction and dependency host factors. Cell Rep. 2017 Dec 26;21(13):3900–13.
7. Yen P-S, Chen C-H, Sreenu V, Kohl A, Failloux A-B. Assessing the potential interactions between cellular miRNA and arboviral genomic RNA in the yellow fever mosquito, *Aedes aegypti*. Viruses. 2019 Jun 10;11(6).
8. Ortiz-Dosal A, Rodil-García P, Salazar-Olivo LA. Circulating microRNAs in human obesity: a systematic review. Biomarkers. 2019 Sep;24(6):499–509.
9. Wang X, Wang Y, Dou Y, Chen L, Wang J, Jiang N, et al. Degradation of unmethylated miRNA/miRNA*s by a DEDDy-type 3′ to 5′ exoribonuclease Atrimmer 2 in Arabidopsis. Proc Natl Acad Sci USA. 2018 Jul 10;115(28):E6659–67.
10. Babion I, Snoek BC, Novianti PW, Jaspers A, van Trommel N, Heideman DAM, et al. Triage of high-risk HPV-positive women in population-based screening by miRNA expression analysis in cervical scrapes; a feasibility study. Clin Epigenetics. 2018 Jun 7;10:76.
11. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J Clin Microbiol. 2005 Oct;43(10):4977–83.
12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402–8.
13. Casseb SMM, Simith DB, Melo KFL, Mendonça MH, Santos ACM, Carvalho VL, et al. Drosha, DGCR8, and Dicer mRNAs are down-regulated in human cells infected with dengue virus 4, and play a role in viral pathogenesis. Genet Mol Res. 2016 May 9;15(2).

14. Holanda GM, Casseb SMM, Mello KFL, Vasconcelos PFC, Cruz ACR. Yellow Fever Virus Modulates the Expression of Key Proteins Related to the microRNA Pathway in the Human Hepatocarcinoma Cell Line HepG2. Viral Immunol. 2017 Apr 7;30(5):336–41.

15. Castillo JA, Urcuqui-Inchima S. Letter to the Editor "Drosha, DGCR8, and Dicer mRNAs are downregulated in human cells infected with dengue virus 4" - Genet. Mol. Res. 15 (2): gmr.15027891 - Drosha, Dicer, and TRBP mRNA are downregulated in Vero cells with the 3'UTR of Dengue virus. Genet Mol Res. 2016 Aug 30;15(3).

16. Acosta-Ampudia Y, Monsalve DM, Castillo-Medina LF, Rodríguez Y, Pacheco Y, Halstead S, et al. Autoimmune neurological conditions associated with zika virus infection. Front Mol Neurosci. 2018 Apr 11;11:116.

17. Modhiran N, Watterson D, Muller DA, Panetta AK, Sester DP, Liu L, et al. Dengue virus NS1 protein activates cells via Toll-like receptor 4 and disrupts endothelial cell monolayer integrity. Sci Transl Med. 2015 Sep 9;7(304):304ra142.

18. Halstead SB, Russell PK, Brandt WE. NS1, Dengue's Dagger. J Infect Dis. 2020 Mar 2;221(6):857–60.

19. Alhoot MA, Wang SM, Sekaran SD. RNA interference mediated inhibition of dengue virus multiplication and entry in HepG2 cells. PLoS ONE. 2012 Mar 23;7(3):e34060.

20. Aqeilan RI, Calin GA, Croce CM. miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. Cell Death Differ. 2010 Feb;17(2):215–20.

21. de Oliveira LF, de Andrade AAS, Pagliari C, de Carvalho LV, Silveira TS, Cardoso JF, et al. Differential expression analysis and profiling of hepatic miRNA and isomiRNA in dengue hemorrhagic fever. Sci Rep. 2021 Mar 10;11(1):5554.

22. Chen C-L, Lin C-F, Wan S-W, Wei L-S, Chen M-C, Yeh T-M, et al. Anti-dengue virus nonstructural protein 1 antibodies cause NO-mediated endothelial cell apoptosis via ceramide-regulated glycogen synthase kinase-3β and NF-κB activation. J Immunol. 2013 Aug 15;191(4):1744–52.

23. Lin J-C, Lin S-C, Chen W-Y, Yen Y-T, Lai C-W, Tao M-H, et al. Dengue viral protease interaction with NF-κB inhibitor α/β results in endothelial cell apoptosis and hemorrhage development. J Immunol. 2014 Aug 1;193(3):1258–67.

24. Ouyang X, Jiang X, Gu D, Zhang Y, Kong SK, Jiang C, et al. Dysregulated Serum MiRNA Profile and Promising Biomarkers in Dengue-infected Patients. Int J Med Sci. 2016 Feb 18;13(3):195–205.

25. Kayesh MEH, Tsukiyama-Kohara K. Mammalian animal models for dengue virus infection: a recent overview. Arch Virol. 2021 Nov 10;

26. Nanaware N, Banerjee A, Mullick Bagchi S, Bagchi P, Mukherjee A. Dengue virus infection: A tale of viral exploitations and host responses. Viruses. 2021 Sep 30;13(10).

27. Wong RR, Abd-Aziz N, Affendi S, Poh CL. Role of microRNAs in antiviral responses to dengue infection. J Biomed Sci. 2020 Jan 3;27(1):4.

28. El-Abd NE, Fawzy NA, El-Sheikh SM, Soliman ME. Circulating miRNA-122, miRNA-199a, and miRNA-16 as Biomarkers for Early Detection of Hepatocellular Carcinoma in Egyptian Patients with Chronic Hepatitis C Virus Infection. Mol Diagn Ther. 2015 Aug;19(4):213–20.

29. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting

 doi:10.20944/preprints202212.0323.v1

BCL2. Proc Natl Acad Sci USA. 2005 Sep 27;102(39):13944–9.

30. Pagliari C, Quaresma JAS, Fernandes ER, Stegun FW, Brasil RA, de Andrade HF, et al. Immunopathogenesis of dengue hemorrhagic fever: contribution to the study of human liver lesions. J Med Virol. 2014 Jul;86(7):1193–7.

31. Arboleda JF, Fernandez GJ, Urcuqui-Inchima S. Vitamin D-mediated attenuation of miR-155 in human macrophages infected with dengue virus: Implications for the cytokine response. Infect Genet Evol. 2019 Apr;69:12–21.

32. Silva BM, Sousa LP, Gomes-Ruiz AC, Leite FGG, Teixeira MM, da Fonseca FG, et al. The dengue virus nonstructural protein 1 (NS1) increases NF-κB transcriptional activity in HepG2 cells. Arch Virol. 2011 Jul;156(7):1275–9.

33. Puerta-Guardo H, Glasner DR, Espinosa DA, Biering SB, Patana M, Ratnasiri K, et al. Flavivirus NS1 Triggers Tissue-Specific Vascular Endothelial Dysfunction Reflecting Disease Tropism. Cell Rep. 2019 Feb 5;26(6):1598-1613.e8.