Human antibody responses to dengue virus infection: involvement of antibodies against mosquito salivary proteins and alpha-gal glycan.

by

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Abstract

The family *Flaviviridae* member dengue virus (DENV), which is transmitted by *Aedes* mosquitoes, is the etiology of dengue fever. Dengue is one of the fastest-growing reemerging mosquito-borne diseases in the world and poses a threat to roughly half of the world's population with a 30-fold increase in the last five decades. During feeding, salivary proteins and viral pathogens are introduced into the host skin through mosquito salivation. These salivary proteins influence systemic and local (skin) immune responses. There is evidence that *Ae. aegypti* salivary gland extract, the Nterm peptide from *Ae. aegypti* 34-kDa salivary protein, and two new salivary proteins- *Ae. aegypti* bacteria-responsive protein 1 (AgBR1) and neutrophil stimulating factor 1 (NeSt1) have modulatory properties on flavivirus infection. In addition, an immunogenic glycan, galactose-alpha1,3-galactose (alpha-Gal or aGal), shown to be in DENV-2 envelope protein may function in dengue infection process. Therefore, in order to understand how human antibodies respond to *Ae. aegypti* salivary proteins as well as antibodies response to the aGal found on the DENV envelope associated with dengue infection, we used an ELISA technique to detect antibodies levels against salivary proteins in two-hundred and one serum samples and aGal in seventy-five serum samples from volunteers living in a dengue fever endemic region of Colombia in 2019 and 2020, respectively. We found that antibody levels against each salivary protein and aGal varied in the time course of dengue infection depending on the severity. An evolutionary analysis of DENV serotype 1, which circulates in the endemic region of Colombia with the highest frequency, showed clustering of the sequence with the typical South American DENV serotype 1 viruses. Our findings highlight the need for thorough studies on the roles and mechanisms of action of salivary proteins and aGal antibodies in dengue infection, as well as for ongoing epidemiological monitoring of dengue in the population.

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Dedication

This thesis is dedicated to everyone who didn’t give up on me. Especially to my mom who stood as both father and mother from birth till now.

Chapter 1 - Dengue virus

Dengue fever is a viral infection caused by any of the four serotypes of dengue virus (DENV): DENVs 1-4. Dengue virus belongs to the *Flaviviridae* family, and it is transmitted to humans through the bite of infected mosquitoes of the *Aedes spp*. - *Aedes aegypti* and *Aedes albopictus* mosquito vectors (1). For the past 50 years, dengue fever has emerged as one of the world's fastest-growing reemerging mosquito-borne diseases with a 30-fold increase (2). In recent decades, dengue has become a major public health problem worldwide with the significant increase of DENV global prevalence being linked to population increase, urbanization and climate change with serious social and economic consequences putting about half of the world's population at risk (3). Dengue fever is endemic in more than 100 nations in Africa, the Americas, the Eastern Mediterranean, South-East Asia, and the Western Pacific (4). It is primarily transmitted in tropical and subtropical climates with an estimated 390 million dengue infections occurring each year and an estimated 2.5 billion people at risk worldwide (4) (Fig 1.1).

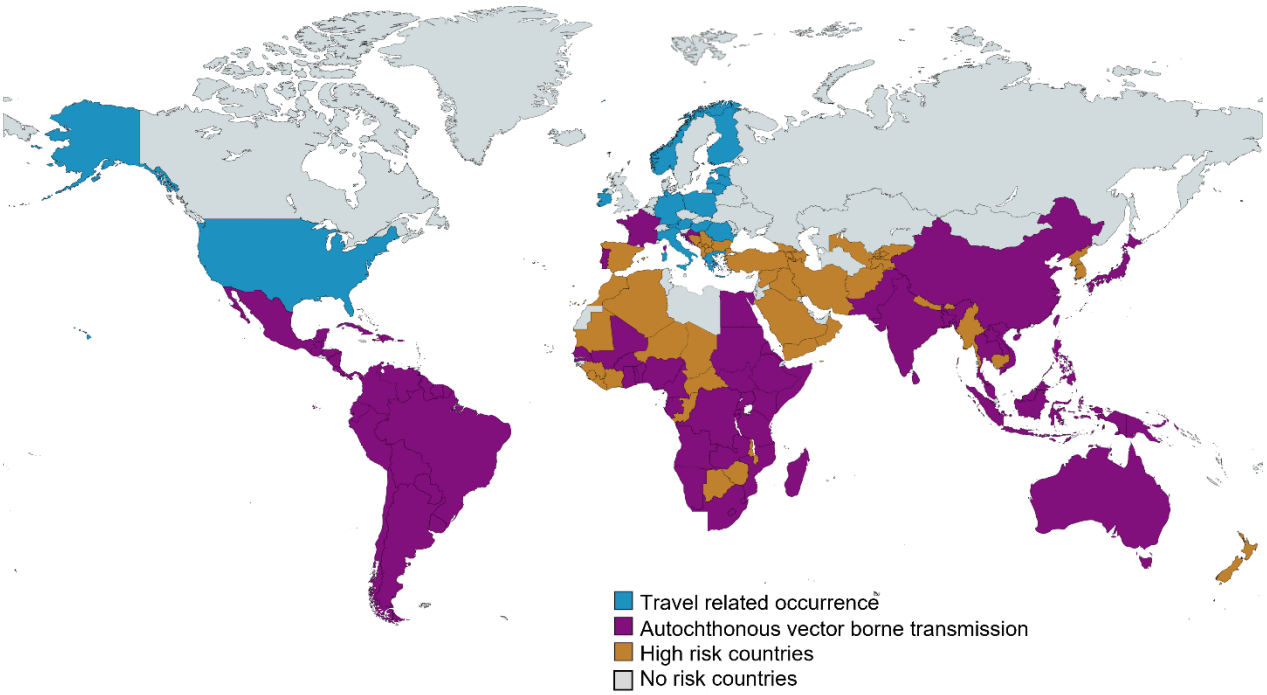

Figure 1.1. Global risk mapping for dengue fever occurrence (adapted from Leta 2018)

Dengue cases have dramatically increased in recent years all around the world, with the highest global record reported in 2019 (3). In the Americas, dengue has an endemo-epidemic pattern with outbreaks occurring every 3 to 5 years (6). During the 2019 outbreak, the Americas area reported about 3.1 million cases (3) with the top 10 dengue-endemic countries in the Americas including Colombia, Cuba, Dominican Republic, El Salvador, Guatemala, Honduras, Nicaragua, Panama, and Peru (7).

Dengue Symptoms

Dengue has a variety of clinical presentations, ranging from asymptomatic illness to a variety of symptoms with severe clinical manifestations. While 70% of DENV infections are asymptomatic, only 0.5–5% of dengue infections progress to the severe stage (3). Typical dengue symptoms include headache, fever, rash, and vomiting can last for up to a week (3,8). The

symptomatic infection may range from mild dengue fever (DF) to life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) according to the World Health Organization dengue guideline (9) (Fig 1.2).

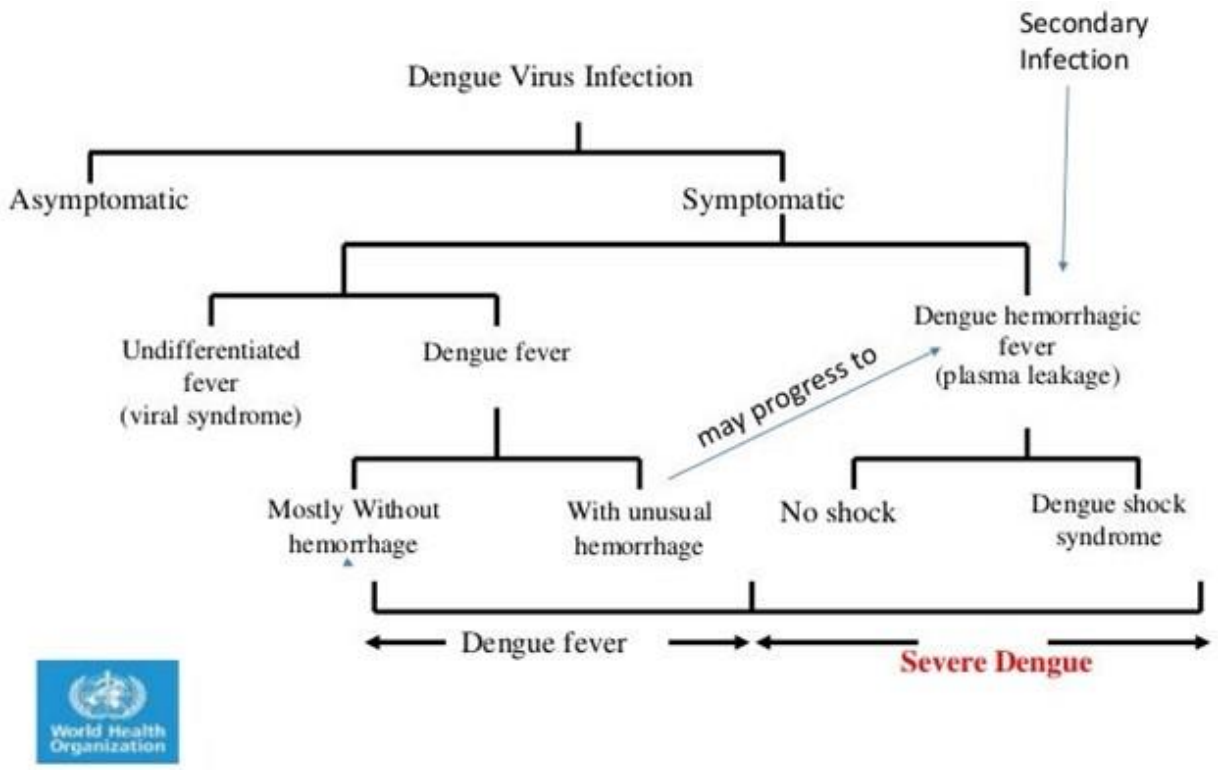

Figure 1.2. WHO flow chart of the classification of dengue infection and clinical manifestation (adapted from WHO)

In 2009, the WHO revised the guidelines for dengue symptoms classification into three levels of severity: dengue without warnings, dengue with warnings and severe dengue to improve sensitivity and specificity of the dengue case definition (10) (Table 1.1). It is believed that the classification into severity categories can accurately detect serious disease in 92% of cases (11,12).

Table 1.1. Clinical symptoms of dengue infection according to WHO severity classifications

Category	symptoms	Duration
Dengue without warnings	• Fever • Nausea, vomiting • Aches and pains • Leucopenia • Tourniquet test positive	2-7 days
Dengue with warnings	• Abdominal pain or tenderness • Persistent vomiting • Clinical fluid accumulation • Mucosal bleed • Lethargy, restlessness • Liver enlargement >2cm • Laboratory: increase in hematocrit • Rapid decrease in platelet count	After 3-5 days of fever
Severe dengue	• Severe plasma leakage • Severe bleeding • Severe organ involvement	After 3-5 days of fever

Although dengue do not currently have a particular treatment, early detection and appropriate medical care can reduce fatality rates to under 1% (3). In the case of dengue without warning signs, it can be treated at home with rest, painkillers, and fluids, with the likelihood that

patients will recover in approximately a week. On the other hand, dengue with warning signs can become severe within a few hours and should be managed by healthcare providers. Meanwhile, severe dengue, which has been a leading cause of serious illness and death in various Asian and Latin American nations, requires immediate management by medical professionals.

Dengue Vaccination and Prevention

After several attempts at dengue vaccine development, the first dengue vaccine, Dengvaxia (CYD-TDV), was licensed in 2015 in a few selected countries (13,14). However, controversy surrounding the phenomenon of potential vaccine-induction of more severe illness in children has prevented its use in naive populations and limited its administration to only those aged 9 to 45 who live in dengue endemic areas and who have previously experienced at least one episode of dengue infection (3). Alternatively, insecticides, physical devices (i.e., bed nets) and genetically modified control approaches have been used widely as the most effective vector control techniques for dengue prevention and management (15–18).

Dengue Transmission

Belonging to the order Diptera, the *Aedes* mosquito is one of about 3000 species of mosquitoes found in almost every part of the world with the exception of Antarctica and Iceland (19). Female *Ae. aegypti* mosquito is considered the primary vector of DENV. Unlike the male mosquitoes, which exclusively consume plant sugars, female mosquitoes consume blood in order to obtain the essential proteins needed for egg production. Dengue transmission starts with a female *Ae. aegypti* feeding on an DENV-infected person. During this blood-feeding process, viruses from infected humans are ingested by the female mosquito, replicate within the mosquito,

spread to tissues including the salivary glands, and the viral particles are mixed with saliva which then gets injected into a susceptible host during the following blood meal (Fig 1.3) (20).

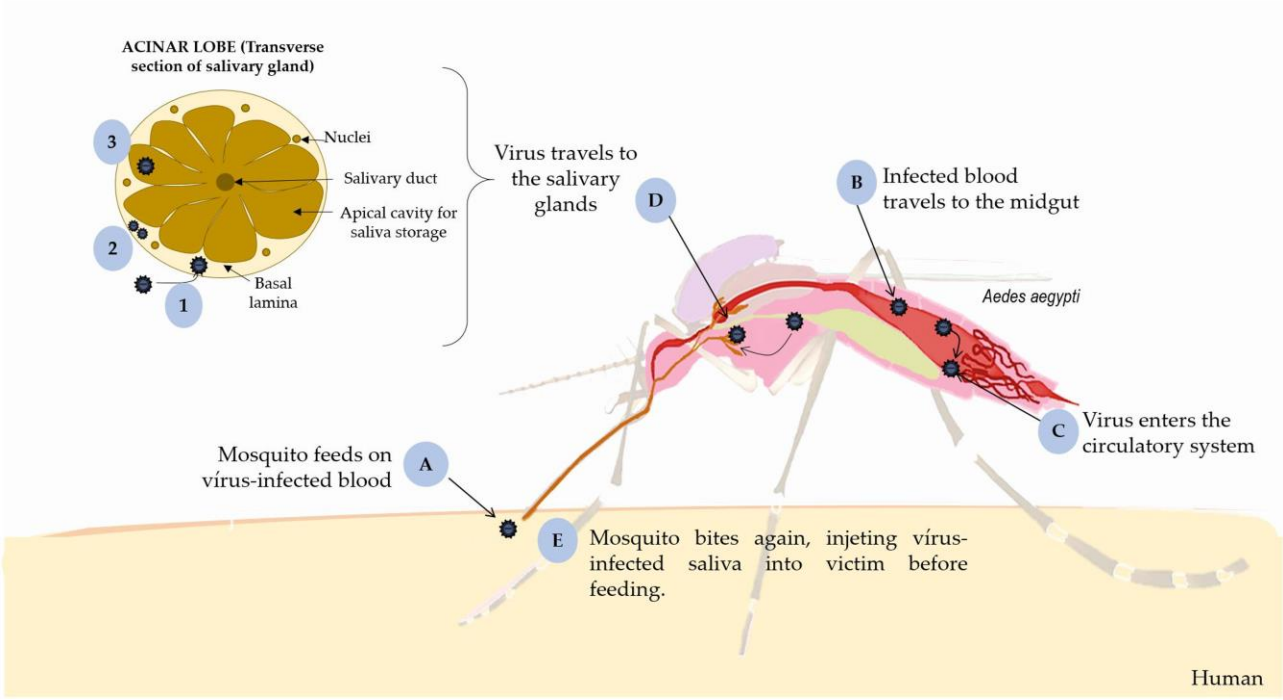

Figure 1.3. Viral dissemination in *Aedes* mosquito after blood feeding (adapted from Monteiro 2019)

The amount of time between consuming the virus and actually transmitting it to a new host referred to as extrinsic incubation period (EIP) is perhaps the most significant factor in determining a mosquito's capacity to transmit virus throughout its lifespan, as the earlier a pathogen enters the saliva, the greater the likelihood that it will be transmitted during a subsequent bite (21). The EIP takes about 8-12 days when the ambient temperature is between 25-28°C (3,22). After acquiring a virus, the mosquito can carry viruses for the rest of its life. Overall, DENV transmission can be influenced by interactions among humans, mosquito vectors, pathogens, and environmental factors (23,24).

Mosquito saliva and salivary proteins

During the feeding process, the mosquito secretes saliva to facilitate blood uptake. The saliva is composed of a wide range of molecules whose objective is to counteract the vertebrate hemostasis and facilitate blood uptake (25). In order to increase the likelihood of a successful meal, the salivary proteins can serve as anticoagulants, vasodilators, and or immunoregulators with some proteins displaying redundant functions (26). Several studies have shown different roles of specific *Ae. aegypti* salivary proteins during DENV infection such as the 34kDa protein promoting virus replication in human keratinocytes by decreasing type I interferon and anti-microbial peptides (27), serine protease enhancing dissemination of DENV into the mammalian host (28), aegyptin blocking collagen-induced platelet aggregation (29), D7 protein inhibiting DENV infection (30) and AaVa1 increasing viral replication in macrophages and dendritic cells (31).

Compelling evidence also indicates that the mosquito salivary proteins induce profound changes in immune responses in the vertebrate host both locally (at the feeding site) and systemically (32–34), with pathogens taking advantage of the immunomodulatory properties of the vector saliva to successfully establish infection (35,36). These salivary proteins induce significant humoral immune response specifically antibody induction and cellular immune responses in the host (37,38), and due to these salivary proteins immunomodulatory characteristics, there is evidence that protective immunity against vector-borne diseases may not only be directed against the pathogen, but also against the vector salivary components (39,40).

Given the limitations of the only licensed dengue vaccine and induction of antibodies against mosquito saliva antigens, mosquito-saliva-based vaccine approaches have actively been pursued to control mosquito-borne diseases by taking advantage of the immunomodulatory

characteristics of salivary proteins. A few studies have identified some salivary proteins from *Ae. agypti* and characterized them as potential vaccine candidates against Flaviviruses including DENV (Table 1.2) (41)**,** with the rationale that blocking the enhancing effect of such salivary proteins may block infection.

Table 1**.2. List of *Aedes aegypti* salivary proteins studied as potential DENV vaccine candidates (adapted from Olajiga 2021)**

Protein	Function	Phase	References
CLIPA3	Disrupts extracellular matrix allowing virus dissemination	Pre-clinic	(28)
D7	Inhibits DENV infection in vitro and in vivo	Pre-clinic	(30)
Aegyptin	Blocks collagen-induced platelet aggregation	Pre-clinic	(29)
AaVa-1	Increases viral replication in macrophages and dendritic cells	Pre-clinic	(31)

Chapter 2 - Phylogeny of dengue virus type 1 in Norte de Santander, Colombia

Abstract

Dengue fever is endemic in all regions of Colombia. There have been simultaneous co-infections and co-circulations of several dengue virus serotypes (DENV) in the Norte de Santander region on the Colombia-Venezuela border, with the most recent significant outbreak being linked to the predominance of DENV-1. The aim of this study was to perform phylogenetic analysis on the genetic diversity and origin of the DENV strains that are currently circulating in the Norte de Santander area. Forty-seven serum samples were collected from patients clinically diagnosed with febrile syndrome associated with dengue during the 2019 outbreak. These samples were tested for DENV, and dengue serotyping was performed using reverse transcriptase-polymerase chain reaction. Serotyping of 29% DENV-1, 17% DENV-2 and 10.6% of DENV-3 was confirmed. Surprisingly, the DENV-1 sequence obtained shared similarities with a strain from Peru, a neighboring country that is far from the Norte de Santander region. This phylogenetic analysis shows that transmission of dengue serotype and strain between neighboring countries is possible thereby highlighting the need for continuous monitoring of DENV transmission in the border regions of Colombia.

Introduction

The dengue virus (DENV) genome is a single-stranded positive-sense ribonucleic acid with approximately 10,700 bases that codes for three structural proteins - capsid (C), membrane (M), and envelope (E) and seven non-structural proteins -NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (1) (Fig. 2.1). According to studies on the E gene, each serotype contains a significant amount of genetic variation as a result of RNA polymerase mutations and intra-serotype recombination (42,43). These genetic variations further form phylogenetic groups called genotypes and intra-genotype lineages that differ based on geographic distribution (44).

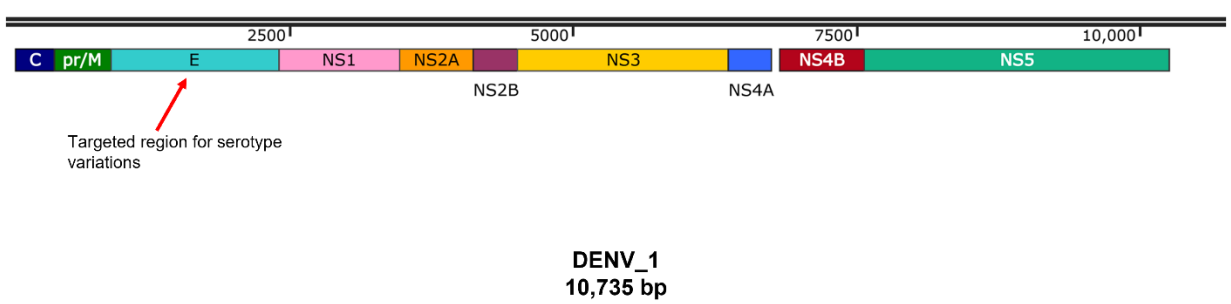

Figure 2.1. DENV genome coding for capsid, membrane, envelope and 7 non-structural proteins

Colombia is a dengue-endemic country and has experienced five major dengue fever outbreaks since 1971 when DENV-2 caused the first outbreak in the Atlantic coast region (45). Following that, DENV-3's occurrence was first reported in 1975, while DENV-1 and DENV-4 were later discovered in 1978 and 1982, respectively (45). Due to the co-occurrence of all four DENV serotypes, the country is considered hyperendemic for dengue (46). The most recent outbreak in 2019 had a total of 127,553 reported cases with predominance of DENV-1 followed by DENV-2 (47). Several departments of Colombia reported dengue cases with the highest annual

dengue incidence in Valle del Cauca, Santander, Antioquia, Huila, Tolima, and Norte de Santander (Fig. 2.2) (48–50).

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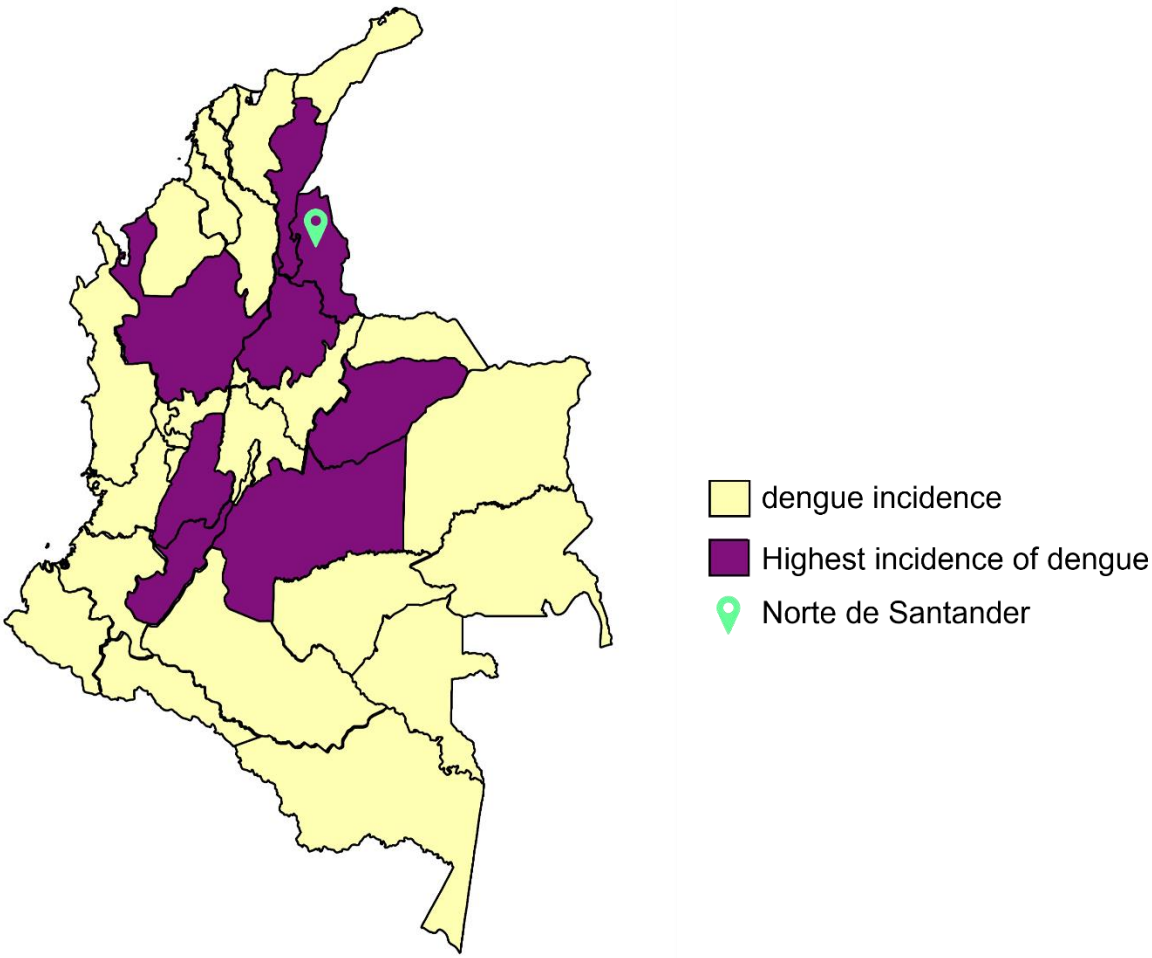

Figure 2.2. 2019 dengue incidence in Colombia

(Map created using MapChart.net and SIVIGILA 2019 dengue report).

The Norte de Santander department, which is at the border between Colombia and Venezuela has been confirmed to have all four DENV serotypes in the population (51). During the 2019 dengue outbreak, the two major municipalities of the department,- Cúcuta and Ocaña, both reported having a significant number of dengue cases (50). Therefore, the aim of this

research was to perform evolutionary analysis of DENV circulating in Cúcuta and Ocaña during a significant period in order to determine how the cross-border population serves as a DENV reservoir. The molecular characterization and phylogenetic reconstruction of viruses circulating in this population expands the understanding of epidemiological patterns and the effects of human migration on the transmission of viruses (52,53).

Materials and Method

Ethical Consideration

The protocols and methods for this study were reviewed and approved by the Kansas State University Ethics Review Board (IRB#8952, approval date- 10/11/2017). The Cúcuta and Ocaña Hospital Board also approved the methods and the performance of the study in their institutions. Before sample collection, each potential participant (adults, guardians, or parents of minors) was given a thorough explanation of the study's objectives, and written informed consent was obtained from individuals willing to participate. Blood samples were collected in compliance with the regulations on ethics of research in human participants for Colombia and the United States.

Samples

All volunteers who reported DENV-like symptoms and sought medical attention at the Hospital Universitario Erasmo Meoz in Cúcuta and the Hospital of Emiro Quintero Caizares in Ocaña between January and December 2019 had their blood samples (5 mL) collected in sterile tubes. Whole blood was used to extract the serum, which was then stored at -20 °C until testing. The study included 47 participants in total. Participants' ages, genders, places of residences, and the number of individuals in each household were all recorded on a questionnaire. An aliquot of each sample was utilized in a DENV (NS1)-based IgM ELISA or RDT Xerion DENGUE-NS1

antigen (Xerion—IMEX group, Bogota) according to manufacturer's recommendation to confirm DENV infection in serum.

RNA extraction

Serum samples were homogenized at a ratio of 1:5 in TRI Reagent (Zymo Research, Cat# R2050-1-200) for 15 seconds, followed by the addition of 200 ul of chloroform (Fisher Scientific CAS# 67-66-3) and a 5 minutes incubation period at 30 °C. The mixture was then centrifuged for 15 minutes at 4 °C at a speed of 12000g rpm. The supernatant was transferred into a new 1.5 ml tube, where RNA was precipitated with an equal volume of isopropanol (Fisher Scientific CAS# 67-63-0), and the tube was incubated at 30 °C for 20 minutes. The mixture was then centrifuged at 4 °C for 10 minutes at a speed of 12000g rpm, and the supernatant was discarded. RNA pellet was washed with 75 % ethanol twice (centrifuge at 4 °C at a speed of 7500g rpm for 5 minutes and supernatant discarded). The tube containing pellet was placed in a clean chamber to dry at room temperature for 30 minutes before the addition of 20 ul of DEPC treated water (Fisher Scientific Catalog# FERR0601) to dissolve the RNA pellet. A NanoDrop spectrophotometer was used to measure the quantity and quality of RNA.

DENV serotype detection

Reverse transcription polymerase chain reaction (RT-PCR) was used to determine DENV serotype using primers and probes from the multiplex CDC DENV 1-4 real-time RT-PCR kit (CDC, Catalog# KK0128) and the Luna Universal Probe One-Step RT-qPCR Kit (NEB, Catalog# E3006S). The following were the RT-PCR conditions used: RT Step 1: 55 °C for 30 minutes, then 95 °C for 2 minutes, 95 °C for 15sec, 60 °C for 1min for 45 cycles of amplification (cool down at 4 °C for 30sec). The CDC kit A positive control of DENV1-4 dengue virus from C6/36 cell

culture supernatant was used as positive control while molecular grade water was used as a negative control during RT-PCR runs. DENV RNA was detected using a reporter dye amplification curve; DENV1- FAM, DENV2- VIC, DENV3- TEXAS RED, and DENV4- CY5.

RT–qPCR and sequencing

The cDNA Synthesis Kit (Lamda Biotech Cat# G236) was used to reverse transcriptase extracted RNA under the following conditions: 18 ℃ for 5 minutes, 50 ℃ for 60 minutes, 85 ℃ for 10 minutes, and finally 4 ℃ for cooling. The synthesized cDNA serving as template was utilized along with primers designed to amplify the DENV-1 envelope (E) (Table 2.1) for RT-qPCR using the Pfu PCR PreMix (Bioneer Cat# V1/2016-02-15) under the following touchdown procedure- First 11 cycles of 94 ℃ for 3 minutes, 94 ℃ for 30 seconds, 60-55 ℃ for 30 seconds, and 72 ℃ for 1 minute, then another 20 cycles of 94 ℃ for 30 seconds, 94 ℃ for 30 secs, 55 ℃ for 30 secs, and 72 ℃ for 1 minute, followed by 5 cycles of 94 ℃ for 30 secs, 55 ℃ for 30 secs, 72 ℃ for 3 minutes and 72 ℃ for 5 minutes for final extension.

The yield and amplicon size of the PCR product were then verified using 1 % agarose gel electrophoresis. A gel photograph was taken with Bio-Rad Gel Doc EZ Imager and Image Lab Software (version 5.2.1). The PCR results were then subjected to PCR cleanup using the NucleoSpin Gel and PCR Clean-up kit (TaKaRa Cat# 740609.50), which was followed by confirmation using 1 % agarose gel electrophoresis and a photograph. Purified PCR products and DENV-1 envelope amplifying primers were sent GENEWIZ (USA) for sanger sequencing. Figure showing primer target region on DENV Envelope and raw sequencing data available in appendix (Table. A.1).

Table 2.1. List of DENV-1 primers used for the sequencing

Name		Sequence	Base pair
P1	F1	TCGTTGAAGGACTGTCAGGAGCAA	931 bp
	R1	TCCCAAGAGACCTGGAACAGACAA	
P2	F2	AGAACAGTTCCATGCTGGGTCTCA	899 bp
	R2	TGCTCTGTCCAAGTGTGGACTTCA	

Phylogenetic analysis

The sequences obtained (1546 nt length) were edited and assembled using SnapGene 5.3.1 software. Subsequently, they were confirmed using the basic local alignment search tool (Nucleotide BLAST). Next, datasets were constructed with sequences taken from the GenBank. Complete genome DENV-1 sequence was downloaded, belonging mainly to genotype V (NC_001477.1 10735 bp ss-RNA). In addition, these sequences included information on the country and sampling dates. Afterwards, the sequences were aligned using the CLUSTAL W program in MEGAX software 11.0 (54), that generated an E and partial NS1 gene sequence alignment for DENV-1 with a length of 2541 nt. Thereafter, phylogenetic trees were constructed using the maximum likelihood (ML) method with default settings in the MEGAX software 11.0, with a bootstrap value of 1000. The DENV-2 strain GC868592 served as an outgroup for DENV-1.

Results

RT-qPCR confirmed DENV serotypes 1-3 with absence of DENV-4 in sample

Our study included 13 (27.7%) patients from Cúcuta and 34 (72.3%) from Ocaña municipality. The mean age of patients was 11.87 years (SD ± 10.03 years). Patients' symptoms typically last 2-15 days, and 2 (4.3%) of the patients reported to have been previously

diagnosed of dengue fever. Twenty-two (46.8%) patients were female, and twenty-five (53.2%) patients were male. Twenty-one (44.7%) of the patients presented mild dengue symptoms (dengue without warnings), while twenty-three (48.9%) presented dengue with warning symptoms and 3 (6.4%) presented severe dengue symptoms. RT-PCR confirmed 29 samples were positive for DENV-1, 8 samples positive for DENV-2, and 5 samples positive for DENV-3 while no sample was positive for DENV-4. Additionally, 1 serum sample was positive for both DENV-1 and DENV-2, while 2 sera were positive for both DENV-1 and DENV-3, and 2 sera were positive for both DENV-1 and DENV-3 (Table 2.1).

Table 2.2. RT-qPCR confirmed DENV serotype positives

Serotype	Positive (%)
DENV-1	29 (29%)
DENV-2	8 (17%)
DENV-3	5 (10.6%)
DENV-4	-
DENV-1/2	1
DENV-1/3	2
DENV-2/3	2
Total	47

Phylogeny of DENV-1

We selected sample #97 which showed high titer DENV-1 by a low threshold cycle value (Ct = 25.9) in the CDC diagnostic test for PCR amplification and sequencing. This serum was from a 9-years old female with dengue without warnings symptoms and confirmed positive for DENV-1. Envelope protein sequenced generated 1546 nucleotide length was analyzed by NCBI

nucleotide blast to obtain highly similar sequences which were utilized to build the Maximum Likelihood tree of DENV-1 strain. The tree composed of DENV genotypes I and V with America, Asia and Europe origins (Fig 2.3). Most American sequences on the tree belonged to genotype I with the exception of Panama (MH879236; MH879247), Argentina's Buenos Aires (KC692514), Mexico: Sinaloa (KY346989) and French Guiana (MH279620) belonging to genotype V. Using location to group into clades, the clade I was comprised of American origins: Venezuela, United States, Peru, Colombia, Ecuador, Esmeraldas, Panama, Puerto Rico, Argentina, Mexico, Brazil and French Guiana while clade II was comprised of Asian origins: Myanmar, Thailand, Comoros, India, China, Mauritania, and clade III was of European origin (French Polynesia). This study sequence grouped with its America clades; however, it was interesting to observe that the sequence was grouped together with sequence from Peru (ON123599; isolate FPY01536/LOR-PER/2021) and they both descended from a common ancestor (Bootstrap value of > 75%).

Figure 2.3. Maximum Likelihood phylogenetic tree of DENV-1 Norte de Santander Isolate

The evolutionary tree for the DENV-1 sequences obtained from GenBank shows three clades of origin based on geographic location: green colored strains originating from America, magenta colored strains originating from Asia and black colored strain from European origin. DENV-2

from Colombia was included as outgroup on the tree. Nodes with bootstrapping values are indicated.

Discussion

Since the 1970s, when DENV was first discovered, dengue incidence rates have expanded around the world, predominantly in southeast Asia, the western Pacific, and America (55). Colombia is the third-highest reporting country for dengue cases in North and Central America and the Caribbean attributed to multiple epidemics of all four dengue serotypes (53). The increase in human population density, inadequate vector control measures enhanced by poor household infrastructure, weather fluctuations from seasons of drought to high rainfall, and several impediments to health services accessibility have all been implicated in dengue's behavior in Colombia (47).

One of the Colombian departments with a high dengue case report during the 2019 outbreak was Norte de Santander, which is in northeastern Colombia. The department shares an east-north boundary with Venezuela (51). Currently, Venezuelan migrants use this area as a route into Colombia (53). The issue of massive immigration into Colombia has been discovered to have an impact on the health of its residents because many Venezuelans, the neighboring nation with the highest number of immigrants into Colombia, lack access to necessary medications and vaccines, which has led to the resurgence of infectious diseases that had been under control (56,57).

Although epidemiological surveillances have been conducted by the National Institutes of Health, and research studies have been performed to understand the origin of DENV serotypes circulation in Colombia (47,52,53,58), the relevance of the Norte de Santander region remains a concern and there is a prompt need to understand the origin and genetic diversity of DENV

circulating along the northeastern border. As a result, we performed a phylogenetic analysis on a DENV-1 sequence that was collected from a febrile patient in Norte de Santander in 2019.

Using location to group clades, the phylogenetic tree produced three DENV-1 clades that grouped with our sample sequence, with clade I originating from the Americas. clade II originating from Asia and clade III originating from Europe. Our strain isolate was placed in clade I, which includes other American nations and showed a strong degree of similarity to the genotype I isolate from Peru (FPY01536/LOR-PER/2021), with a bootstrap value > 75%. It is important to highlight that the two major departments sharing border with Peru are the Amazonas and Putumayo departments and are both in the southern Colombia while Norte de Santander department is in the northeastern part of Colombia. It is also surprising that despite reports of all four DENV serotypes circulating in Peru, DENV-1 infections have been relatively sparse in the population (48,59,60,61).

The main limitation of this study was the low detection sensitivity of the PCR, which could be related to our prolonged sample storage under less-than-ideal conditions from the time of sample collection to the time of testing. However, the fact that one high purity sequenced strain from the Norte de Santander region in our study had similarity with a neighboring country strain suggested the possibility of disease transmission between nearby countries and also supports the notion that DENV migration can occur between countries due to their close proximity (62,63). This further demonstrated that DENV-1 genotypes and/or clades/lineages might be introduced and reintroduced into Colombia via various border locations. Therefore, in order to determine the viral origins and routes of transmission, which would help to understand the dynamics of the epidemiological situation and prepare for prospective outbreaks, it is

important to maintain continual surveillance for DENV and other arboviruses along all Colombia's borders.

Chapter 3 - IgG levels against *Aedes aegypti* salivary proteins in a cohort of subjects with dengue-like symptoms from a dengue-endemic region in Colombia.

Submitted as: Olayinka, O.M., Marin-Lopez, A., Cardenas, J.C., Maldonado-Ruiz, L.P., Gutierrez-Silva, L.Y., Gonzales-Pabon, M.U., Fikrig, E., Park, Y., and Londono-Renteria, B. 2022. *Aedes aegypti* anti-salivary proteins IgG levels in a cohort of DENV-like symptoms subjects from a dengue-endemic region in Colombia. *Frontiers in Epidemiology: Infectious Disease Epidemiology (submitted).*

Abstract

Dengue fever, caused by the dengue virus (DENV), is currently a threat to about half of the world's population. DENV is mainly transmitted to the vertebrate host through the bite of a female Aedes mosquito while taking a blood meal. During this process, salivary proteins are introduced into the host skin and blood to facilitate blood acquisition. These salivary proteins modulate both local (skin) and systemic immune responses. Several salivary proteins have been identified as immunogenic inducing the production of antibodies with some of those proteins also displaying immunomodulatory properties enhancing arboviral infections. IgG antibody responses against salivary gland extracts of a diverse number of mosquitoes, as well as antibody responses against the *Ae. aegypti* peptide, Nterm-34kDa, have been suggested as biomarkers of human exposure to mosquito bites while antibodies against AgBR1 and NeSt1 proteins have been investigated for their potential protective effect against Zika virus (ZIKV) and West Nile virus infections. Thus, we were interested in evaluating whether IgG antibodies against AgBR1, NeSt1,

Nterm-34kDa peptide, and SGE were associated with DENV infections and clinical characteristics. For this, we tested samples from volunteers living in a dengue-endemic area in Colombia in 2019 for the presence of IgG antibodies against those salivary proteins and peptides using an ELISA test. This pilot study is important to determine the involvement of antibody responses against salivary proteins in dengue disease progression.

Introduction

Interactions between people, mosquito vectors, pathogens, and environmental factors drive arbovirus transmission (69). Viruses from infected humans may be ingested by a female mosquito during a blood meal, replicate within the mosquito, and spread to tissues including the salivary glands, from where the virus is injected with saliva into a susceptible host during the next blood meal (70). Mosquito saliva not only facilitates blood uptake but also induces the production of antibodies (25). Our previous studies demonstrated that some of these proteins elicit a strong antibody response that is related to the intensity of mosquito bite exposure (71,72). Research also showed that antibodies against *Aedes* salivary proteins are associated with disease risk (72) and some play a role in DENV infection (28,30,72–74). One example is the 34kDa protein, observed to decrease type I interferon and anti-microbial peptides, promoting virus replication in human keratinocytes during DENV infection (27) and a significant peptide Nterm-34kDa identified as a highly immunogenic peptide in vertebrate hosts with the IgG antibody level correlating with the intensity of exposure to mosquito bites IgG antibodies (75). Antibodies against *Ae. aegypti* salivary gland extract (SGE) was also found as a biomarker of exposure to mosquito bites (71,72). Other proteins such as the Neutrophil-stimulating protein 1 (NeSt1), and the AgBR1 have been recently identified to modulate ZIKV infection and passive immunization with these proteins reduces ZIKV in mice preventing early viral replication and improving the survival rates (76–79) while the role of these salivary proteins in DENV infection is unclear.

DENV is a single-stranded positive RNA virus belonging to the *Flaviviridae* family with four serotypes (DENV 1-4) causing an acute febrile illness in humans (80). It is transmitted mainly by both *Ae. aegypti* and *Ae. albopictus* mosquitoes. Based on symptoms and disease management, the World Health Organization (WHO) has classified dengue fever severity into

three categories in 2019: dengue without warning signs (DWOWS), dengue with warning signs (DWWS), and severe dengue (SD) (10). Although 70% of DENV infections are asymptomatic, headache, fever, rash, and vomiting are common symptoms that can last up to a week (81) but between 0.5 and 5% of dengue infections progress to the severe stage (10).

The global prevalence of DENV has increased significantly in recent decades, with an estimated 100-400 million infections per year putting roughly half of the world's population at risk (3). DENV has emerged as a major public health issue in the tropics and subtropics, with serious social and economic consequences (82). In Latin America, Colombia is one of the countries with the highest dengue fever incidence rates, with the four dengue serotypes circulating concurrently at any given year (58,83). The department of Norte de Santander was among Colombia's departments with the highest dengue fever reported cases during the most recent dengue fever epidemic in 2019, which resulted in a total of 127,553 cases in Colombia (48).

In this study, we investigated the potential relationship between the levels of IgG antibodies against SGE, Nterm-34kDa peptide, AgBR1, and NeSt1 and the clinical presentations of dengue fever in volunteers living in a DENV endemic area in Colombia during the 2019 outbreak. This is the first study to evaluate a relationship between the two recently discovered salivary proteins AgBR1 and NeSt1 and dengue fever.

Materials and Methods

Ethical considerations

The protocols and methods for this study were reviewed and approved by the Kansas State University Ethics Review Board (IRB#8952, approval date- 10/11/2017). The Cúcuta and Ocaña Hospital Board also approved the methods and the performance of the study in their institutions.

Before sample collection, each potential participant (adults, guardians, or parents of minors) was given a thorough explanation of the study's objectives, and written informed consent was obtained from individuals willing to participate. Blood samples were collected in compliance with the regulations on ethics of research in human participants for Colombia and the United States.

Geographical location of sampled participants

This study was conducted in the department of Norte de Santander of Colombia. The department has 6 regions with 40 municipalities. Norte de Santander's capital, Cúcuta is located in the eastern part of the department with a level 3 hospital (advanced), where patients with severe diseases are transferred from level 1 and 2 facilities (basic and intermediate respectively), while the second-largest city, Ocaña, located in the western part with fewer densely populated areas, has a level 2 hospital (intermediate) where patients with mild diseases can seek health care. Norte de Santander shares a border with Venezuela and is the primary trade route between the two countries. Agriculture is the most important economic activity in the Norte de Santander Department. DENV infections peak in this region between mid-August and mid-October, as well as between December and February, with 1100 millimeters of annual rainfall between rainy seasons March–June, and September–December (51).

Sample collection and dengue fever diagnosis

Between January and December 2019, blood samples (5 mL) were collected in sterile tubes from all volunteers who reported DENV-like symptoms and within 3 to 15 days of fever seeking medical care at the Hospital Universitario Erasmo Meoz in Cúcuta and the Hospital of Emiro Quintero Cañizares in Ocaña. Serum was obtained from the whole blood and kept at -20° C until testing. A total of 201 dengue patients were enrolled in the study. To determine DENV infection in serum, an aliquot of each sample was tested DENV positive using a DENV (NS1)-

based IgM ELISA, RDT Xerion DENGUE-NS1 antigen (Xerion—IMEX group, Bogota) or qPCR testing following factory recommendations. Once dengue was confirmed, participants were categorized into DWOWS, DWWS, and severe dengue according to the WHO dengue fever classifications (10). We also included 22 non-febrile participants who accompanied patients to the healthcare facility and 39 febrile patients who tested negative for dengue on all three dengue tests conducted. A questionnaire was used to record participants demographics including age, gender, place of residence, and the number of people living in each household. No other demographic information was recorded from these patients. Also, no uninfected controls were included. It is important to state that these are "convenience samples" collected without prior sample calculations.

Immunoglobulin G against *Ae. aegypti* salivary gland and proteins

The levels of human IgG antibodies against mosquito salivary proteins were measured using an ELISA assay. We used whole salivary proteins from salivary glands dissected from *Ae. aegypti* mosquitoes as described in Londono-Renteria, et al. (4) and Nterm-34kDa peptide published by Elanga, et al. (75). Both AgBR1 and NeSt1 proteins were expressed in the Drosophila S2 cell line, as previously described (76,77). The ELISA procedures were optimized using checkerboard titration. ELISA microtiter plates (Santa Cruz Biotechnology, Dallas, TX) (96-well/per salivary protein antigen) were coated with 50 µl /well of 1 µg /ml SGE, 2 µg /ml Nterm-34kDa peptide and AgBR1, or 5 ug /ml NeSt1 prepared in the coating solution (Kierkegaard and Perry Laboratories, Gaithersburg, MD) and incubated overnight at 4°C. The plates were washed once with 1X PBS + 0.1 percent Tween 20 (Sigma–Aldrich, St. Louis, MO) wash solution before being blocked for 1 hour at 37° C with 2% milk powder in wash solution (blocking buffer). Plates were then incubated at 4° C overnight with a 50 µl/well 1:100 dilution of

patient sera in blocking buffer for SGE and Nterm-34kDa peptide, and a 1:50 dilution of patient sera in blocking buffer AgBR1 and NeSt1 proteins. The next day, plates were washed three times with wash solution before being incubated for two hours with 50 µl/well of a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Abcam, AB6858). Colorimetric development of plates was achieved using 50 µl/well tetra-methyl-benzidine (Biolegend; San Diego, CA) incubated at room temperature (37°C) for 2 minutes (SGE and AgBR1) and 3 minutes (Nterm-34kDa and NeSt1). The reaction was stopped with 50 µl/well of 1 M phosphoric acid, and the absorbance at 450 nm was measured.

Antibody levels were expressed as the ΔOD value: $\Delta OD = ODx - ODb$, where ODx represents the mean of individual OD in both antigen wells and ODb the mean of the blank wells. For each peptide tested, positive controls were used for the normalization of plate-to-plate variations. Assay variation of samples (inter and intra assay) tested in the study was below 20% and it was only included in the analysis of serum samples with a coefficient of variation ≤ 20% duplicates between duplicates.

DENV serotype detection

Serum samples were homogenized in TRI Reagent (Zymo Research, Cat# R2050-1-200) for viral RNA extraction. RT-qPCR was used to determine DENV serotype using primers and probes from the multiplex CDC DENV 1-4 real-time RT-PCR kit (CDC, Catalog# KK0128) and the Luna Universal Probe One-Step RT-qPCR Kit (NEB, Catalog# E3006S). The following were the RT-qPCR conditions used on the Roche Light Cycler 480: RT Step 1: 55 °C for 30 minutes, then 95°C for 2 minutes. 45 cycles of amplification (95°C for 15sec, 60°C for 1min), and 4 °C for 30 secs for cooling down. Positive control of DENV1-4 from C6/36 cell culture supernatant was used. Molecular grade water was used as a negative control instead of RNA during qRT-PCR

runs. DENV RNA was detected using a reporter dye amplification curve, DENV1- FAM, DENV2- VIC, DENV3- TEXAS RED, and DENV4- CY5.

Statistical analysis

The difference between two independent groups (i.e., antibody levels between dengue positive and dengue negative subjects) was determined using the Mann-Whitney test with a p-value <0.05 being considered significant. A comparison of more than three groups was tested with the Kruskal-Wallis test while the correlation between two independent parameters was done using the Spearman correlation method. All statistical analysis was performed using GraphPad Prism, version 9.2.0 (GraphPad Software Inc., La Jolla, CA).

Results

Cohort characteristics

A total of 201 DENV-infected patients who visited two different levels of Colombia healthcare facility in 2019 were included in the cohort: 43.2 % (87) patients from Cúcuta hospital and 56.7 % (114) from Ocaña hospital. DENV IgM testing revealed 100 % (87) of Cúcuta patients was IgM+, and 80.7 % (92) of Ocaña patients was IgM+. The Ocaña DENV IgM negative- (4) patients were further tested using DENV- NS1 and were confirmed positive. When it comes to dengue fever, 75.9 % (66) of DWOWS patients, 24.1 % (21) of DWWS patients visited Cúcuta health facility, while 32.5 % (37) of DWOWS patients, 67.5 % (77) of DWWS patients visited Ocaña health facility. The cohort's age ranged from 6 months to 67 years, with a median age of 13 years. The patients experienced dengue symptoms ranging from day1 to 15 days with median day of 6 days (Table 3.1).

Table 3.1. Cohort characteristics by hospital level.

Location	n	Gender		Dengue testing			Dengue fever classification		Age Median (range)	Symptom days Median (range)
		M	F	DENV IgM+	NSI IgM +	PCR +	DWOWS	DWWS		
Cúcuta	87	43	44	87	-	-	66	21	14.5 (0 - 67years)	6 (1-13 days)
Ocaña	114	48	66	92	4	47	37	77	10.1 (0 -53 years)	6 (3-15 days)
Total	201	91	110	179	4	47	103 (51.2%)	98 (48.8%)	13 (0-67 years)	6 (1-15 days)

Duration of symptoms was associated with anti-salivary proteins IgG level.

The duration of dengue-like symptoms in our study participants ranged from 1 to 15 days. We ran a correlation analysis between the level of anti-salivary protein IgG antibodies and the number of days from the onset of symptoms; both NeSt1 and Nterm-34kDa peptide IgG antibodies had a weak significant correlation with the number of days with dengue symptoms. NeSt1 IgG had a weak positive correlation with number of days with dengue symptoms (Spearman's correlation, r= 0.2079; p= 0.0037) (Fig. 3.1A) while Nterm-34kDa IgG had a weak negative correlation (Spearman's correlation, r= -0.3073; p< 0.0001) (Fig. 3.1C). However, AgBR1 and SGE IgG antibodies had no significant correlation with days with dengue symptoms (Fig. 3.1B and 3.1D).

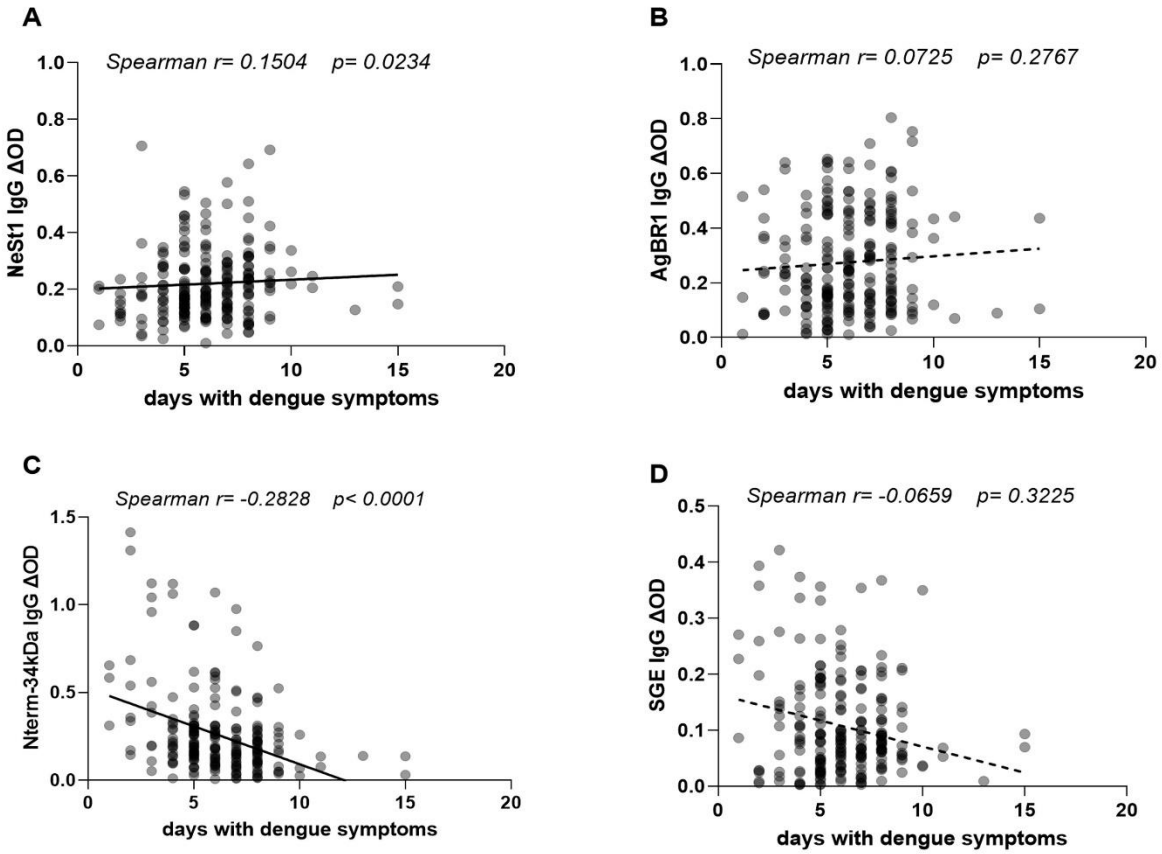

Figure 3.1. *Ae. aegypti* SGE, Nterm-34kDa peptide, NeSt1 protein, and AgBR1 protein IgG antibody levels in relation to patients' days with symptoms of dengue fever.

(A) NeSt1 protein, (B) AgBR1 protein, (C) Nterm-34kDa peptide, (D) SGE. Individual IgG levels are represented by black dots and a regression line with 95% confidence bands passing through the mean is shown. Antibody levels are measured in units of OD (optical density). The "r" and "p" values were measured using the pairwise non-parametric Spearman correlation test.

Anti-salivary proteins IgG levels differ between dengue fever classifications

Anti-salivary protein IgG antibody levels were compared between 22 non-febrile participants, 39 febrile patients with negative dengue results, 103 patients classified as DWOWS, and 98 patients classified as DWWS. Based on the dengue fever classification comparison of anti-salivary proteins IgG, we observed that non-febrile participants presented a higher level of anti-salivary IgG compared with febrile patients (either dengue positive or negative) in all salivary proteins tested (Fig. 3.2). Interestingly, both AgBR1 and Nterm-34kDa IgG levels were significantly higher in individuals presenting DWOWS than in those presenting DWWS (Mann-Whitney test, AgBR1 IgG p-value = 0.0053; Nterm-34kDa IgG p-value = 0.0171) (Fig. 3.2B and 3.2C) while both levels of NeSt1 IgG and SGE IgG were not significantly different between classifications (Mann-Whitney test, NeSt1 IgG p-value = 0.3533; SGE IgG p-value = 0.7821) (Fig. 3.2A and 3.2D).

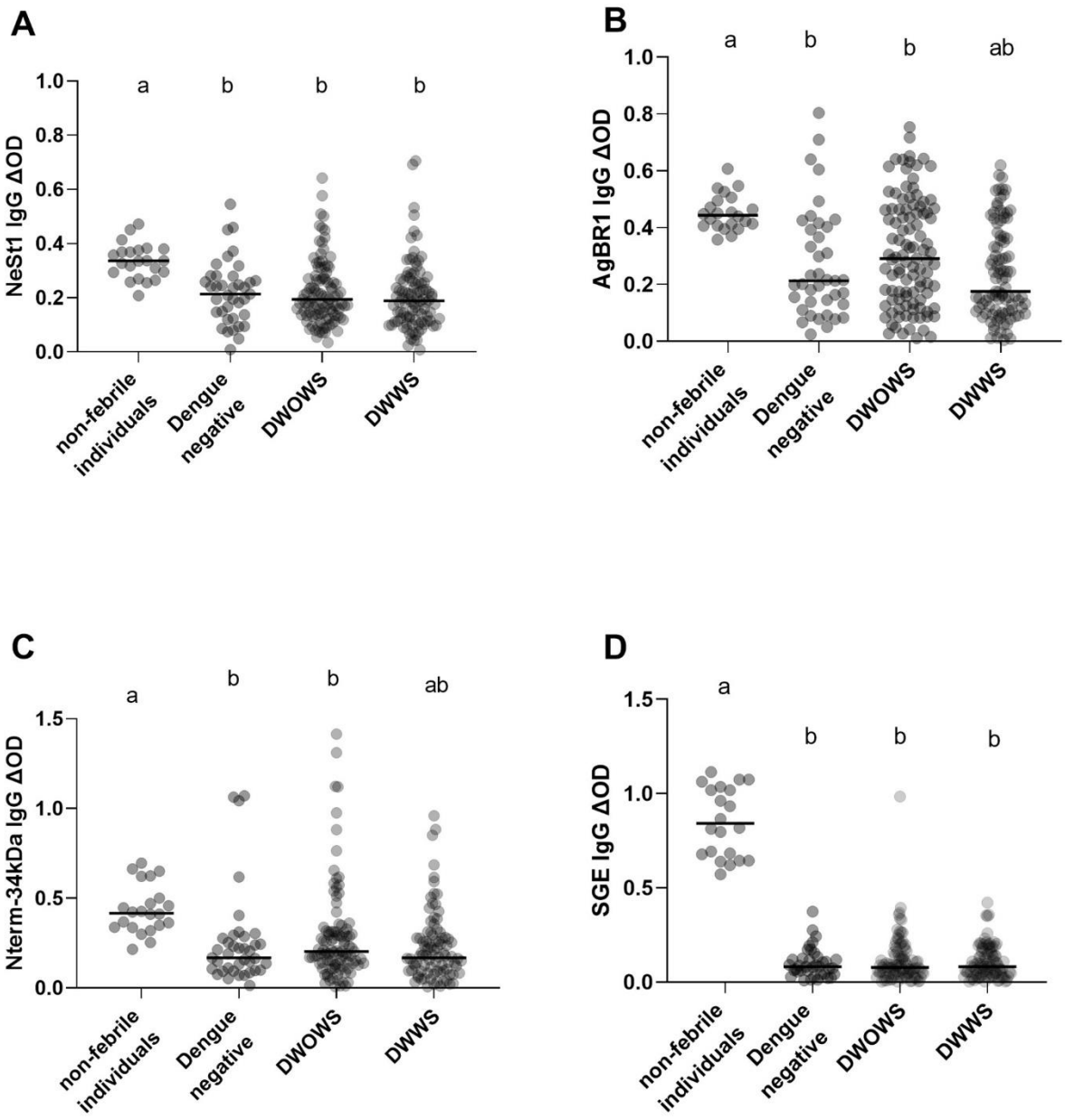

Figure 3.2. IgG antibody levels against *Ae. aegypti* SGE, Nterm-34kDa peptide, AgBR1 protein, and NeSt1 protein in comparison with dengue severity.

Comparison of IgG antibody levels between twenty-two non-febrile volunteers, thirty-nine febrile patients with dengue negative result (DENV (NS1)-based IgM and RDT Xerion DENGUE-NS1 antigen), one hundred and three dengue without warnings, and ninety-eight dengue with

warnings. (A) NeSt1 protein, (B) AgBR1 protein, (C) Nterm-34kDa peptide, (D) SGE. The individual IgG levels are represented by the black dots and horizontal red lines represent medians of individual antibody responses. Antibody levels were measured in units of OD (optical density). Different letters indicate statistically significant differences between groups p values <0.05 as measured using the pairwise non-parametric Mann-Whitney test.

Levels of anti-salivary proteins IgG between geographical locations

We compared patients from two hospital locations with different levels. This is because Cúcuta being the department's capital, has a level 3 hospital while Ocaña has a level 2 hospital. Eighty-seven Cúcuta hospital patients and 114 Ocaña hospital patients were analyzed, and we observed no significant difference in SGE IgG (Mann-Whitney test, p= 0.1753) (Fig. 3.3D) or AgBR1 IgG (Mann-Whitney test, p= 0.7877) (Fig. 3.3B). In the case of NeSt1 IgG, Cúcuta hospital patients had lower values than Ocaña hospital patients (Mann-Whitney test, p< 0.0001) (Fig. 3.3A), Contrastingly, Nterm-34kDa IgG levels was significantly higher in Cúcuta hospital patients than in Ocaña hospital patients (Mann-Whitney test, p< 0.0001(Fig. 3.3C). When comparing the level of anti-salivary IgG among dengue fever classification between the two different hospital location, it was surprising to observe that only Ocaña patients had significant differences in the level of AgBR1(Mann-Whitney test, p=0.0360) and NeSt1 (Mann-Whitney test, p= 0.0103) between DWOWS and DWWS while Nterm-34kDa and SGE IgG were not different in the two locations between DWOWS and DWWS (Fig.3 4).

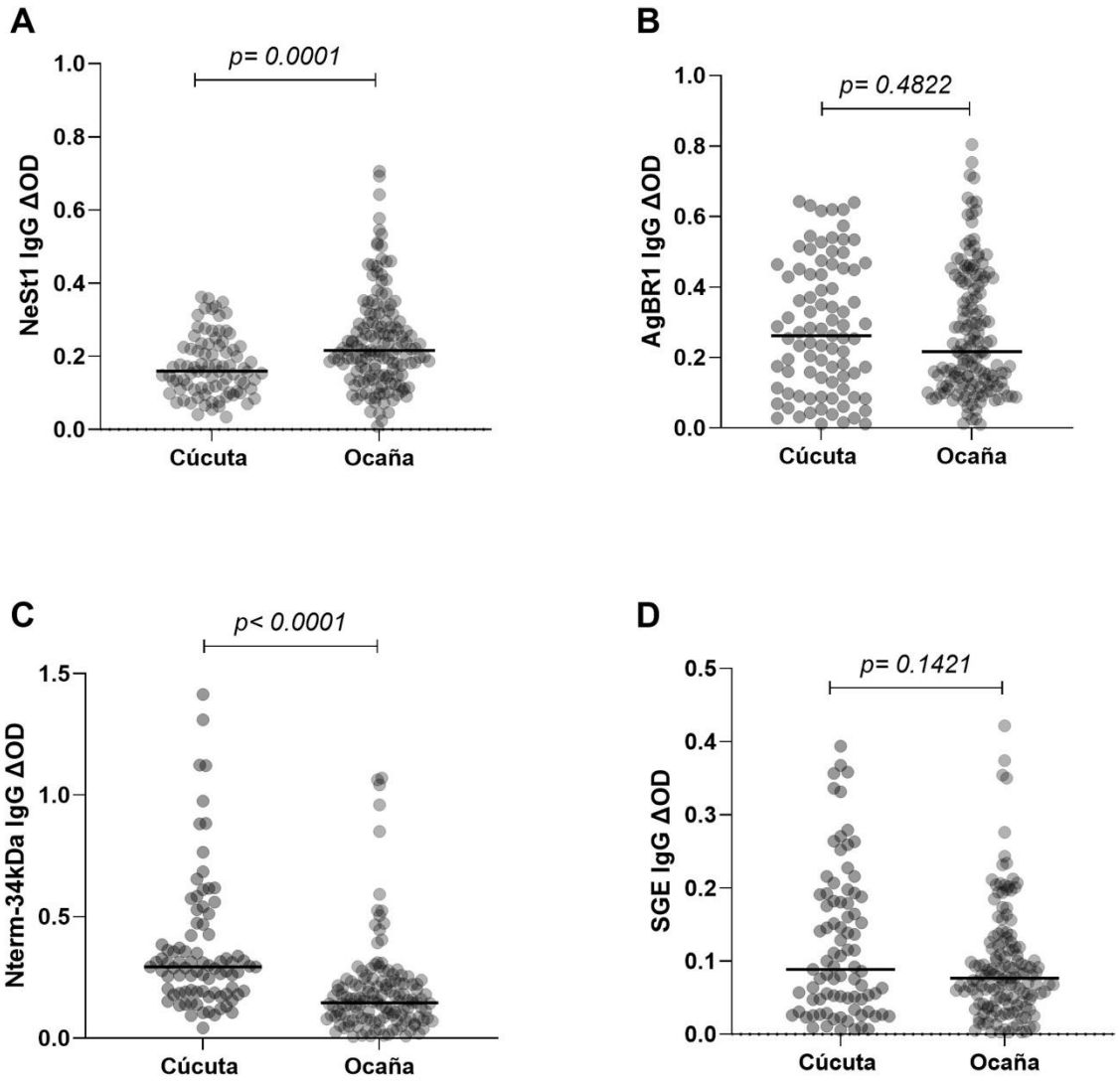

Figure 3.3. IgG antibody levels against *Ae. aegypti* salivary proteins between two major municipalities of Norte de Santander.

Comparison of IgG levels between participants from Cúcuta (n=87) and Ocaña (n=114). (A) NeSt1 protein, (B) AgBR1 protein, (C) Nterm-34kDa peptide, (D) SGE IgG antibody levels. Individual IgG levels are represented by black dots and horizontal red lines represent medians of individual antibody responses. Antibody levels were measured in units of OD (optical density). p-values were measured using the pairwise non-parametric Mann-Whitney test.

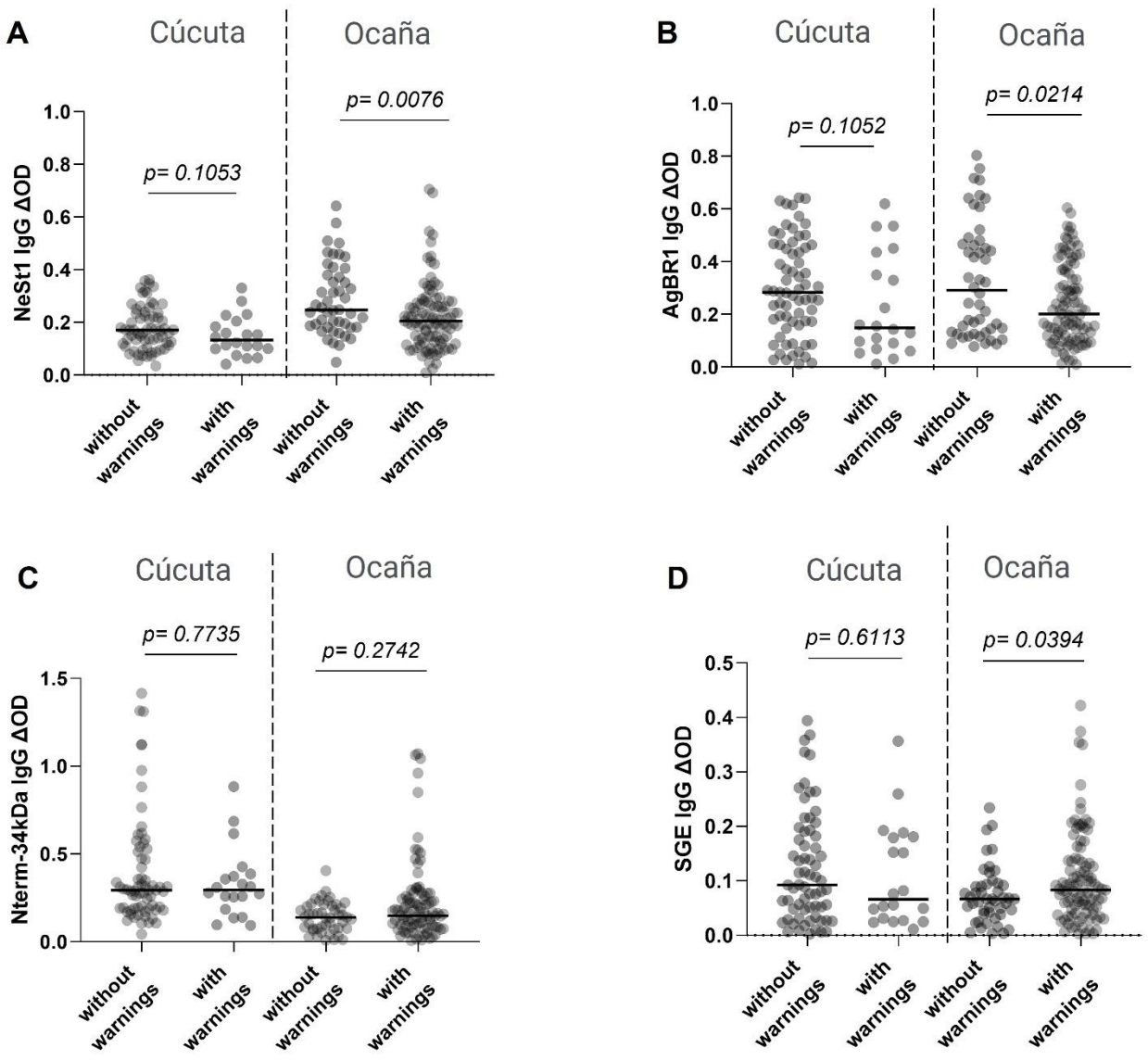

Figure 3.4. IgG antibody levels of *Ae. aegypti* SGE, Nterm-34kDa peptide, AgBR1 protein, and NeSt1 protein comparison between dengue severity and hospital location.

(A) NeSt1 protein; left -Cúcuta patients, right- Ocaña patients (B) AgBR1 protein; left -Cúcuta patients, right- Ocaña patients (C) Nterm-34kDa peptide; left -Cúcuta patients, right- Ocaña patients (D) SGE; left -Cúcuta patients, right- Ocaña patients. The individual IgG levels are represented by the black dots and horizontal red lines represent medians of group individual

antibody responses. Antibody levels were measured in units of OD (optical density). p-values were measured using the pairwise non-parametric Mann-Whitney test.

No difference in *Ae. aegypti* salivary proteins IgG levels between DENV serotypes

The levels of anti-salivary proteins IgG in confirmed dengue serotypes were examined. Of the 42 total identified multiplex qPCR dengue serotypes, DENV-1 (n= 29), DENV-2 (n= 8), and DENV-3 (n= 5) were confirmed, and these were used for the DENV serotype and anti-salivary protein IgG analysis. Pair-wise comparison using the non-parametric Mann-Whitney test revealed no significant differences between each anti-salivary protein IgG level and DENV serotypes (Figure 3.5).

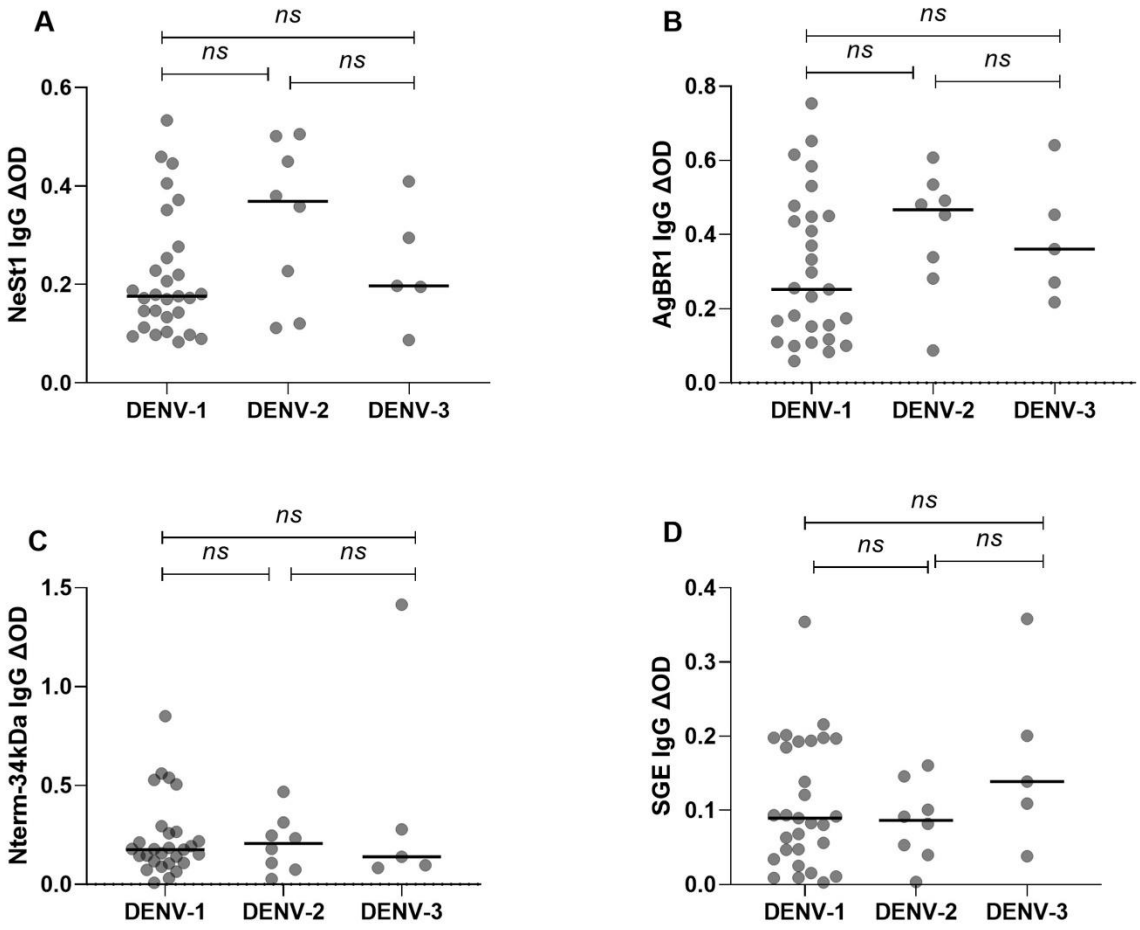

Figure 3.5. IgG antibody levels against *Ae. aegypti* salivary proteins in qPCR confirmed positive DENV serotypes.

Comparison of IgG levels between twenty-nine individuals positive DENV-1, eight individuals positive for DENV-2, and five individuals positive for DENV-3. Individual IgG levels are represented by colored dots and horizontal black lines represent medians of group individual antibody responses. (A) NeSt1 protein, (B) AgBR1 protein, (C) Nterm-34kDa peptide, (D) SGE. Antibody levels were measured in units of OD (optical density). p-values were measured using the non-parametric Mann-Whitney test with ns indicating non-significant p-value higher than 0.05

Age and gender had no influence on the levels of *Ae. aegypti* salivary proteins IgG in dengue patients.

To determine if age influences the levels of anti-salivary IgG in dengue patients, we assessed the level of anti-salivary proteins IgG in accordance with the age categories of 0–5 years, 6–10 years, 11–15 years, 16–20 years, and above 20 years. Fifty-nine patients are within the age range of 0–5 years, forty-nine patients within the age range of 6–10 years, forty-one patients within the age range of 11–15 years, eighteen patients were within the age range of 16–20 years and thirty-three patients were above 20 years of age. Among the various age groups tested using the Kruskal–Wallis test, NeSt1 IgG had a p-value= 0.2529, AgBR1 had a p-value= 0.7860, Nterm-34kDa had a p-value= 0.0566, and SGE had a p-value= 0.4480 (Fig. 3.6). We also proceeded to check if there were significant differences associated with gender on the levels of anti-salivary IgG in dengue patients. However, there were no significant differences in the levels of anti-salivary IgG between the ninety-one men and the one hundred and ten females. NeSt1 had a p-value= 0.4470, AgBR1 had a p-value= 0.5389, Nterm-34kDa had a p-value=0.2566 and SGE had a p-value= 0.0532 (Mann-Whitney test) (Fig. 3.7).

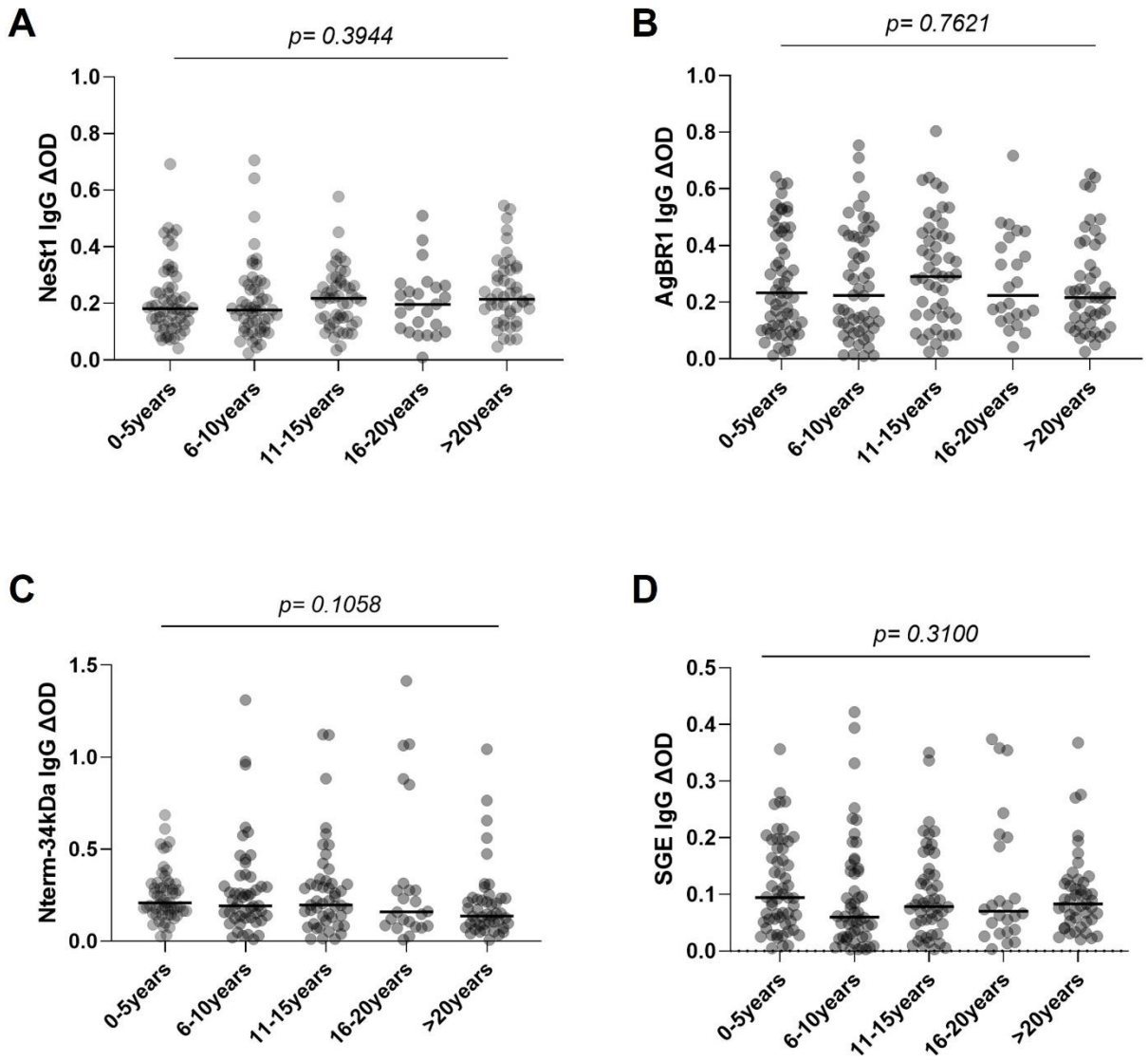

Figure 3.6. Anti-salivary protein IgG levels comparison between age group classification: Comparison of IgG levels between age 0-5years, 6-10years, 11-15years, 16-20years and above 20 years (A) NeSt1 IgG, (B)AgBR1 IgG, (C) Nterm-34kDa IgG, (D) SGE IgG. The individual IgG levels are represented by the black dots and horizontal red lines represent medians of group individual antibody responses. Antibody levels were measured in units of OD (optical density). p-values were measured using the pairwise non-parametric Kruskal–Wallis test for multiple comparisons and the Mann-Whitney testing for comparison between two groups.

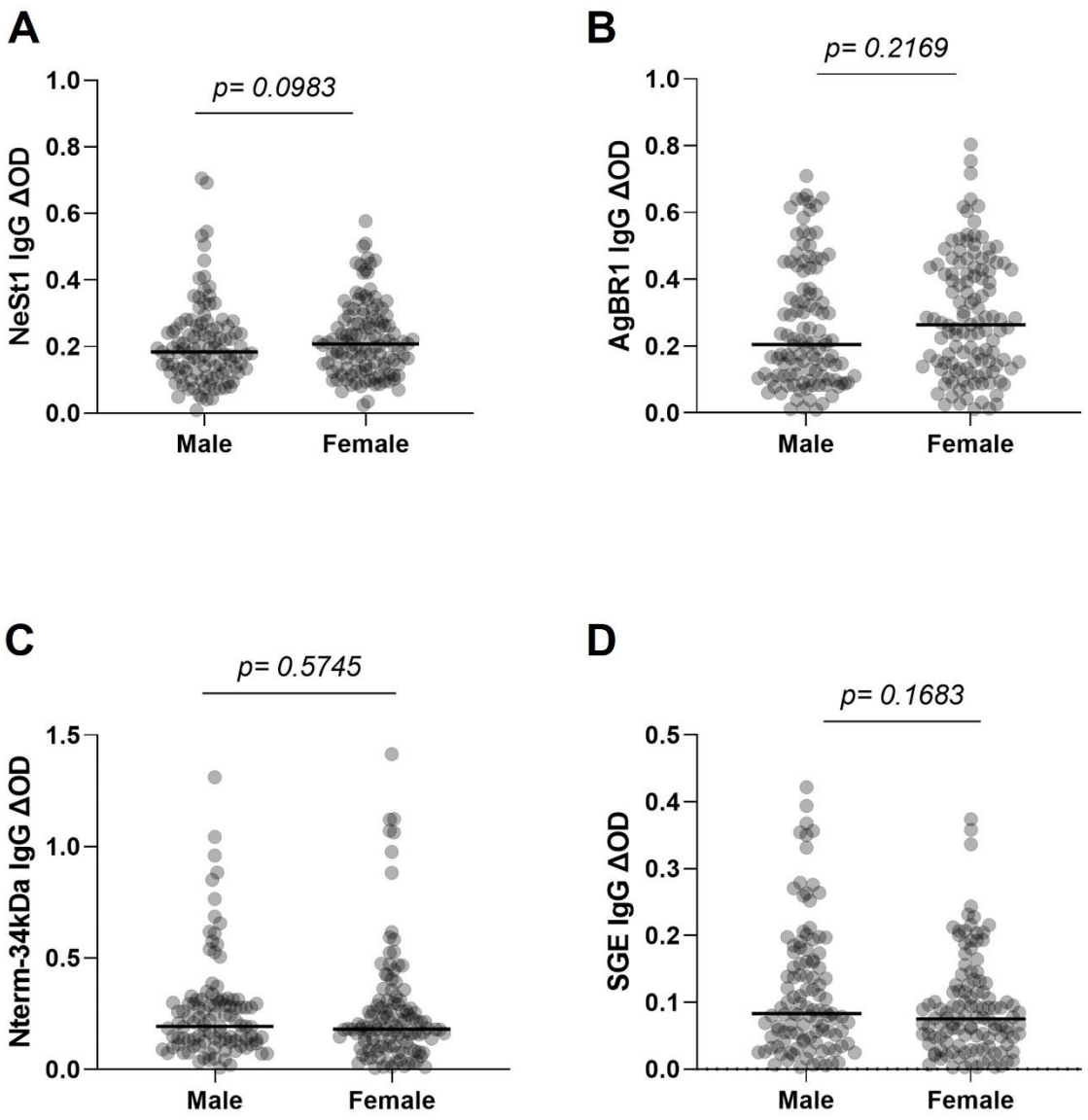

Figure 3.7. Anti-salivary protein IgG levels comparison between gender:

(A) NeSt1 IgG, (B) AgBR1 IgG, (C) Nterm-34kDa IgG, (D) SGE IgG. The individual IgG levels are represented by the black dots and horizontal red lines represent medians of group individual antibody responses. Antibody levels were measured in units of OD (optical density). p-values were measured using the pairwise non-parametric Mann-Whitney test.

Discussion

In addition to targeting arboviruses, host immunity also targets mosquito salivary proteins that may be modulating dengue pathogenesis. While the identification of specific mosquito salivary proteins opened the way for the development of serological toolboxes that could allow for the evaluation of human-vector interaction and disease transmission by the same vector (33,84), it could also be useful for epidemiological investigations, as well as for estimating the risk of transmission and the effectiveness of vector control measures. *Ae. aegypti* salivary gland extract has been proven to be immunogenic with seasonal exposure to mosquito bites inducing an increase in anti-mosquito SGE antibodies (85). These anti-mosquito SGE antibody concentrations are also short-lived and indicative of recent mosquito contact and wane when exposure to specific mosquito species is not sustained (32,71,86,87). We previously demonstrated that geographical location influences the level of SGE IgG with residents of houses harboring aquatic stages of *Ae. aegypti* having higher *Ae. aegypti* SGE IgG than people living in houses with no mosquitoes breeding (88). We also reported that febrile viremic individuals presented higher antibody levels against whole SGE than febrile non-viremic individuals (71).

Another helpful biomarker for exposure to *Aedes* mosquito bites has been the IgG response to Nterm-34kDa salivary peptide (89), although no conclusive connection to dengue risk has been shown. The NeSt1 protein from *Ae. aegypti* saliva enhances ZIKV pathogenesis and passive immunization with the NeSt1 antiserum showed some protection against ZIKV (76) while AgBR1 protein antibodies have been shown to delay deadly West Nile virus infection and modulate early Zika virus infection in mice (77,90). Interestingly, improved survival and decreased viral burden in blood were demonstrated with a combination of AgBR1 and NeSt1-specific antibodies through passive vaccination (79).

In this study, we evaluated the level of antibody responses against SGE, NeSt1, AgBr1, and Nterm-34kDa and their associations with DENV pathogenesis and clinical manifestation. Our result indicated correlation of anti-salivary protein IgG with the number of days patient has been experiencing dengue symptoms, variation of anti-salivary protein IgG between febrile patients, variation anti-salivary protein IgG between dengue fever severity, and variation anti-salivary protein IgG between patients location. Although the findings of this study contributed to the investigations of anti-salivary protein antibodies as biomarkers of dengue infection and prospective therapeutic tools that could be explored, future studies are necessary to determine the involvement of the anti-salivary protein IgG in dengue disease progression.

The symptoms of dengue can range from asymptomatic or mild to severe symptoms that can last up to 15 days (91). We found that the number of days with symptoms did not correspond to the levels of SGE, and AgBR1 IgG antibodies. However, both NeSt1 and Nterm-34kDa IgG showed clearly positive and negative correlations, respectively, which suggested that days experiencing dengue symptoms might be influencing NeSt1 and Nterm-34kDa IgG levels. It is also possible that the antibody levels against Nterm-34kDa decay at a different rate than AgBR1 or the concentration of such antibodies induced by a single exposure are driving these differences. However, more experiments are needed to determine the reasons for these observations and to establish if there is a causation relationship between levels of IgG antibodies against NeSt1 and Nterm-34kDa.

The diverse clinical manifestations of dengue prompted the WHO to classify dengue symptoms into three categories of severity: DWOWS, DWWS and severe dengue (10). Assessing the levels of anti-salivary proteins IgG among dengue fever classification revealed that dengue DWOWS had higher AgBR1 and Nterm-34kDa peptide IgG levels than those DWWS which

implies that the higher the antibody levels, the less severe the symptoms/infection. However, when location was used to group patients, Nterm-34kDa peptide IgG level was not significantly different between the dengue fever classes which suggested that Nterm-34kDa peptide IgG might be a reliable biomarker to detect variation in human exposure to *Ae. aegypti* bite (75) but cannot differentiate between dengue severity. Nevertheless, AgBR1 IgG was significantly different between dengue severity classification and hospital location which suggests that AgBR1 IgG may be a diagnostic tool to evaluate the risk of dengue fever severity in endemic regions. This could also imply that location abundance of mosquito or exposure to mosquito bites influences immune response to anti-salivary protein as earlier suggested (25,32). Furthermore, since antibodies against AgBR1 have been shown to modulate early Zika virus infection (77), higher level of AgBR1 IgG in DWOWS patients might be implying its modulating effect on dengue severity at the early stage of disease. However, since we used convenience samples, one limitation of this study is the absence of non-infected DENV individuals exposed to mosquito bites to determine how exposure to non-infected bites may have strengthened their immune response to salivary proteins and the potential protective mechanism against DENV infection or developing dengue symptoms. IgG against AgBR1 and Nterm-34kDa involvement in dengue severity is suggested in this pilot study, therefore, more research is needed to assess the risk of disease progression to severity through antibodies against these salivary proteins.

During outbreaks, the increased number of dengue cases in the Norte de Santander region is thought to be due to highly favorable climatic conditions for *Aedes* mosquito breeding in these years, as well as migration from neighboring countries (71). The disease hospital management in this region depends on the severity of the disease, with three tiers of hospitals: level 1 hospital (basic) is where patients with mild disease seek medical attention. Level 2 hospital (intermediate)

is where patients with mild and moderate disease seek medical attention. Level 3 hospital (advanced) is where severe patients are referred from level 1 or level 2 health care facilities. The Cúcuta hospital is a level 3 health care facility while Ocaña hospital is a level 2 health care facility. In comparing the levels of anti-salivary proteins IgG among patients visiting the two levels of hospital, we observed Cúcuta hospital residents having significantly higher Nterm-34kDa IgG than Ocaña hospital patients whereas NeSt1 IgG was significantly higher in Ocaña hospital patients than Cúcuta hospital patients. It was surprising that when we grouped the hospital location based on dengue severity, Ocaña patients presented distinct level of NeSt1 and SGE IgG between DWOWS and DWWS while Nterm-34kDa presented no significant differences in both Cúcuta and Ocaña patients between DWOWS and DWWSs (Fig. 3.4). The reasons for these variations are still unknown, but we are currently organizing a prospectus-controlled study to follow patients before and after mosquito exposure in each one of these localities.

Interestingly, levels of anti-salivary proteins IgG do not differ among serotypes suggesting that immune response to salivary protein during dengue infection may not be DENV serotype dependent. Unfortunately, the low sample size of multiplex confirmed dengue serotypes did not allow for more powerful analysis such as correlating monotypic vs multitypic status of the cohort (92). In addition, we did not find differences in the level of IgG anti-salivary protein among age groups neither was an association of age with the levels of the anti-salivary protein IgG. Previous studies have described a negative correlation between age and level of IgG antibodies mainly because of the nature of these antigens (93,94). Salivary proteins are known to elicit the formation of IgG4 antibodies associated with the induction of tolerance (95,96). However, another study found no correlation with age (97). It is possible that local and environmental factors other than those discussed in this study may influence the development of total IgG or specific IgG4.

In summary, the connection between anti-salivary proteins IgG and dengue severity, number of days with dengue symptoms and hospital disease management suggested that AgBR1 and NeSt1 IgG may be valuable tools to assess the risk of dengue severity progression and distinguish between exposure to DENV. It is noteworthy to highlight that AgBR1, regardless of location in Colombia, appears to be the best predictor of dengue severity distinguishing between DWOWS and DWWS. In the case ofNterm-34kDa IgG, conflicting observations were presented in this study, however, it has been established as a tool to measure human exposure to *Aedes agypti* bite (75). Although SGE IgG showed a no significant difference in all parameters evaluated, SGE is composed of all salivary proteins with competing roles in disease pathogenesis, indicating that each salivary protein needed to be independently examined for its role in DENV pathogenesis which would be of significance for disease diagnosis, pathogenesis, and management.

Chapter 4 - Association of dengue infection with anti-alpha-gal antibodies, IgM, IgG, IG1, and IgG2

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Abstract

Dengue virus (DENV) transmitted by the Aedes mosquitoes is the etiological agent of dengue fever, one of the fastest-growing reemerging mosquito-borne diseases on the planet with a 30-fold surge in the last five decades. Interestingly, many arthropod-borne pathogens, including DENV type 2, have been reported to contain an immunogenic glycan galactose-alpha1,3-galactose (alpha-Gal or aGal). The aGal molecule is a common oligosaccharide found in many microorganisms and in most mammals, except for humans and the Old-World primates. The loss of aGal in humans is considered to be an evolutionary innovation for enabling the production of specific antibodies against aGal that could be presented on the glycan of pathogens. The objective of this study was to evaluate different anti-aGal antibodies (IgM, IgG, IgG1, and IgG2) in people exposed to DENV. We observed a significant difference in anti-aGal IgG and IgG1 levels among dengue severity classifications. Furthermore, a significant positive correlation was observed between the anti-aGal IgG and the number of days with dengue symptoms in patients. Additionally, both anti-aGal IgM and IgG levels differ between the two geographical locations of patients. While the anti-aGal IgM and IgG2 levels were not significantly different according to the dengue severity levels, age was negatively correlated with anti-aGal IgM and positively

correlated with anti-aGal IgG2. Significant involvement of aGal antibodies in Dengue infection processes is suggested based on the results. Our results open the need for further studies on the exact roles and the mechanisms of the aGal antibodies in Dengue infection.

Introduction

Dengue virus (DENV) from the Flaviviridae family is transmitted by *Aedes (Ae.)* mosquitoes – mainly *Ae. aegypti* and *Ae. albopictus* (1). Dengue fever is an infectious disease caused by any of the four DENV serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) (3). Dengue fever has become one of the fastest-growing reemerging mosquito-borne diseases on the planet (98) with a 30-fold surge in the last five decades. The spread of this disease has been linked to population increase, urbanization, and climate change in more than 100 countries in the Asia-Pacific region, the Americas, the Middle East, and Africa (10,40,54,99). In South America, Colombia is one of the countries with the highest rates of dengue transmission, with all four serotypes circulating and DENV-1 and DENV-2 being the most common (47,58,83,100). As a result, Colombia is regarded as being a DENV hyperendemic area.

The most effective control measures for reducing vector-borne diseases to date have been the use of vector control tools such as pesticides, physical devices (i.e., bed nets) and Wolbachia-based mosquito control strategy to reduce both mosquito life span and pathogen transmission (17,101). However, the challenge of vector control sustainability, as well as the inadequacy of this intervention to reduce dengue infection burden, has prompted the development of additional disease control strategies such as vaccines (102). Due to the presence of several dengue serotypes, cross-reactivity with other flaviviruses, and antibody-dependent enhancement (ADE), the development of a dengue vaccine has been challenging (103), thereby making it difficult to control the spread of dengue fever with vaccination.

A study of N-glycomics of serotype 2 DENV, produced from mosquito cell, found various glycans including galactose-alpha-1,3-galactose (alpha-Gal, hereafter, aGal) (104) which is also found in many important pathogens including several arthropod-borne pathogens, *leishmania*

(105), *Trypanosoma* (106,107), *Borrelia* (108), and *Plasmodium spp.* (109). The aGal is a commonly found glycan in mammals except in old-world primates and human. Loss of alpha 1,3-galactosyltransferase enzyme in humans and old-world primates, lacking the endogenous terminal carbohydrate linkage of the aGal trisaccharide (110), allows production of antibodies against the exogenous aGal presented in the gut microbiota and often in the pathogens described above. The human immune system produces aGal specific natural antibodies, which account for up to 1-5 % of circulating IgM and IgG and 0.1 - 0.2 % of serum immunoglobulin (111,112). The roles of aGal antibodies in the processes of pathogen infections have been studied for infections of the *leishmania, Tripanosoma, and Plasmodium spp.,* (105–109,113–115) but is lacking in DENV infection.

We evaluated the association of DENV infection with levels of anti-aGal IgM, IgG, and two IgG subclasses; IgG1, which plays a key role in eliciting antibody responses against viral infections, and IgG2, responsible for anticarbohydrate IgG responses. Data from a cohort of 75 febrile patients, recruited in Norte de Santander, Colombia in 2020 was examined in this study, and suggests significant roles of anti-aGal in DENV infection.

Materials and Methods

Ethical Considerations

The protocols and methods for this study were reviewed and approved by the Kansas State University Ethics Review Board (IRB #8952, approval date- 10/11/2017). The Cúcuta and Ocaña Hospital Board also approved the methods and the performance of the study in their institutions. Before sample collection, each potential participant (adults, guardians, or parents 81 of minors) was given a thorough explanation of the study's objectives, and written informed consent was

obtained from individuals willing to participate. Blood samples were collected in compliance with the regulations on ethics of research in human participants for Colombia and the United States.

Experimental design

The focus of the study was to determine the association between dengue fever and IgM, IgG, IgG1, and IgG2 antibody responses to aGal. The variation in aGal antibody titers in participants from a dengue-endemic area in Norte de Santander were studied in terms of their infective status detected by dengue IgM testing, location, days of symptoms, and dengue disease severity classification.

Study participants and sample preparation

Samples were collected from a total of 75 febrile patients tested for dengue infection using the DENV [NS1]-based IgM ELISA (DENV-IgM) and 10 people from the same region who were asymptomatic (healthy) with unknown dengue status (5 males and 5 females, age range between 19-42 years old). Dengue participants were categorized into the patients showing typical dengue symptom without warning signs, with warning signs, and severe dengue symptom according to the WHO dengue fever classifications (5). A questionnaire was used to record participants demographics such as age, gender, and place of residence. All patients reported that their cases were the first time being diagnosed with dengue fever, indicating primary infections.

Five milliliter blood samples were collected in sterile tubes from all volunteers who reported DENV-like symptoms and within 3 to 15 days of fever seeking medical care at the Hospital Universitario Erasmo Meoz in Cúcuta and the Hospital of Emiro Quintero Cañizares in Ocaña. Samples were tested for DENV using either DENV NS1-based IgM ELISA, (Xerion—IMEX group, Bogota) following manufacturers' guidelines. Serum was collected and stored at -20 °C until it was shipped to the United States for analysis.

Determination of antibody titers against aGal

Enzyme linked immunosorbent assay (ELISA) procedures were optimized using checkerboard titration for standardization of each antibody including a positive control. The levels of human anti-aGal antibodies were detected using ELISA high absorption capacity polystyrene microtiter 96 well plates coated with 5μg/ml of Gal-alpha 1-3 Gal β1-4 GlcNAC conjugated to human serum albumin (14 atom spacer, Product Code: NGP3334) (Dextra, Shinfield, UK) in 100 μL/well in carbonate-bicarbonate buffer (5mM Na2CO3 (Acros Organics AC123670010) and 45mM NaHCO3 (Sigma-Aldrich S5761). After overnight incubation at 4ºC, coated plates were washed 3 times with 100 μL/well PBS containing 0.05 % Tween 20 (PBST) (Sigma-Aldrich P3563), blocked with 100 μL/well of 1% bovine serum albumin (BSA) (ChemCruz Cat# sc-2323) in PBST (Sigma-Aldrich) overnight at RT and then washed 3 times with 100 μL/well of PBST. Human serum samples were diluted 1:50 in PBST with final 1% BSA and 100 μL were added into the wells of the antigen-coated plates and incubated overnight at 37 ºC. Plates were washed 3 times with PBST and treated with 100 μl/well of detection antibodies; goat anti-human IgM conjugated to horseradish peroxidase (mu chain specific, Abcam cat# ab97205), goat anti-human IgG (IgG (H+L) specific, JacksonImmunoResearch Code# 109-035-088), mouse anti human IgG1 (Fc-specific, SouthernBiotech Cat# 9054-05), or mouse anti-human IgG2 (Fd-specific, SouthernBiotech Cat# 9080-05). Secondary antibodies diluted 1:10,000 for IgM, 1:1000 for IgG and IgG2, 1:100 for IgG1 v/v in blocking solution were added and incubated overnight at room temperature (RT). Plates were washed 3 times with 100 μL/well of PBST and 100 μL/well of 3,3', 5,5'-Tetramethyl-Benzidine substrate solution (Thermo Scientific, 124 34022) was added and incubated for IgM (5 mins), IgG (15 mins), IgG1 (40 mins), IgG2 (25 mins) at RT. Finally, the reaction was stopped with 100 μl/well of 1M phosphoric acid (H3PO4) (prepared using Fisher

Scientific Cat# 031101), and the optical density (OD) was measured in a spectrophotometer at 450 nm.

Antibody levels were expressed as the ΔOD value: $\Delta OD = ODx - ODb$ where ODx represents the mean of individual OD in two technical replications and ODb the mean of the blank wells. One spike control on each plate was used for normalization of plate-to-plate variations. Assay variation among samples (inter and intra assay) tested in the study was below 20% and it was only included in the analysis of serum samples with a coefficient of variation ≤ 20% between duplicates.

Statistical analysis

The ELISA OD values at 450 nm were compared between dengue IgM results, dengue fever classification, participants location of residence, and age by pairwise comparisons using the nonparametric Mann-Whitney U test ($p = 0.05$). Multiple data points in aGal antibodies in each Dengue severity level were compared by two-way ANOVA test, the test known to be highly tolerant for data sets with violation of normality (116,117). Spearman's rank correlation test was used to assess the strength and significance of a relationship between two independent variables. GraphPad Prism, version 9.2.0, (GraphPad Software Inc., La Jolla, CA) was used for all statistical analyses.

Results

Cohort characteristics

A total of 75 DENV-infected patients who visited two different levels of Colombia healthcare facility in 2020 were included in the cohort: 53.3% (40) patients from Cúcuta and 46.7 (35) from Ocaña, two cities in Colombia 201.2 km apart each other (Appendix Fig. B.1). Testing for DENV IgM is a diagnostic tool for identifying dengue infection in its active stage: According to the test, 67.5 % (21) of Cucuta patients were IgM+, and 68.6 % (24) of Ocaña patients were IgM+. When it comes to dengue fever, 42.5 % (17) of patients without warnings, and 57.5 % (23) of patients with warnings visited the Cúcuta health facility, while 31.4 % (11) of patients without warnings, 54.3 % (19) of patients with warnings, and 14.3 % of patients exhibiting severe dengue, visited the Ocaña health facility. The cohort's age ranged from 0 to 86 years, with a median age of 14 years. The patients experienced dengue symptoms for periods ranging from 2 to 15 days with median day of 6 days (Table 4.1).

Table 4.1. Cohort characteristics by hospital level

Location (Hospital level)	Total n	DENV IgM		Dengue fever classification			Age Median (range)	Symptom days Median (range)
		IgM+	IgM-	w/o warnings	w/ warnings	Severe		
Cúcuta (3)	40	21	19	17	23	0	14.5 (0-86 years)	5 (2-15 days)
Ocaña (2)	35	24	11	11	19	5	13 (3-62 years)	7 (3-15 days)
Total	75	45 (60%)	30 (40%)	28 (37%)	42 (56%)	5 (6.7%)	14 (0-86 years)	6 (2-15 days)

High levels of anti- aGal immunoglobulins in patients with DENV-IgM positive results

Measuring the levels of anti-aGal specific IgM, IgG, IgG1, and IgG2 in forty-five DENV-IgM (IgM+) and thirty DENV-IgM negative (IgM-) febrile individuals, we observed that DENV-IgM+ patients had significant higher anti-aGal IgM ($p = 0.0386$) and IgG ($p = 0.0264$) antibodies than those in DENV IgM- patients. No significant differences in their anti-aGal IgG1 ($p = 0.0757$) and anti-aGal IgG2 ($p = 0.1271$) titers (Fig. 4.1) were observed between dengue IgM+ and IgM- patients, although a moderately higher level of IgG2 in IgM- group was observed in IgM- group without statistical significance.

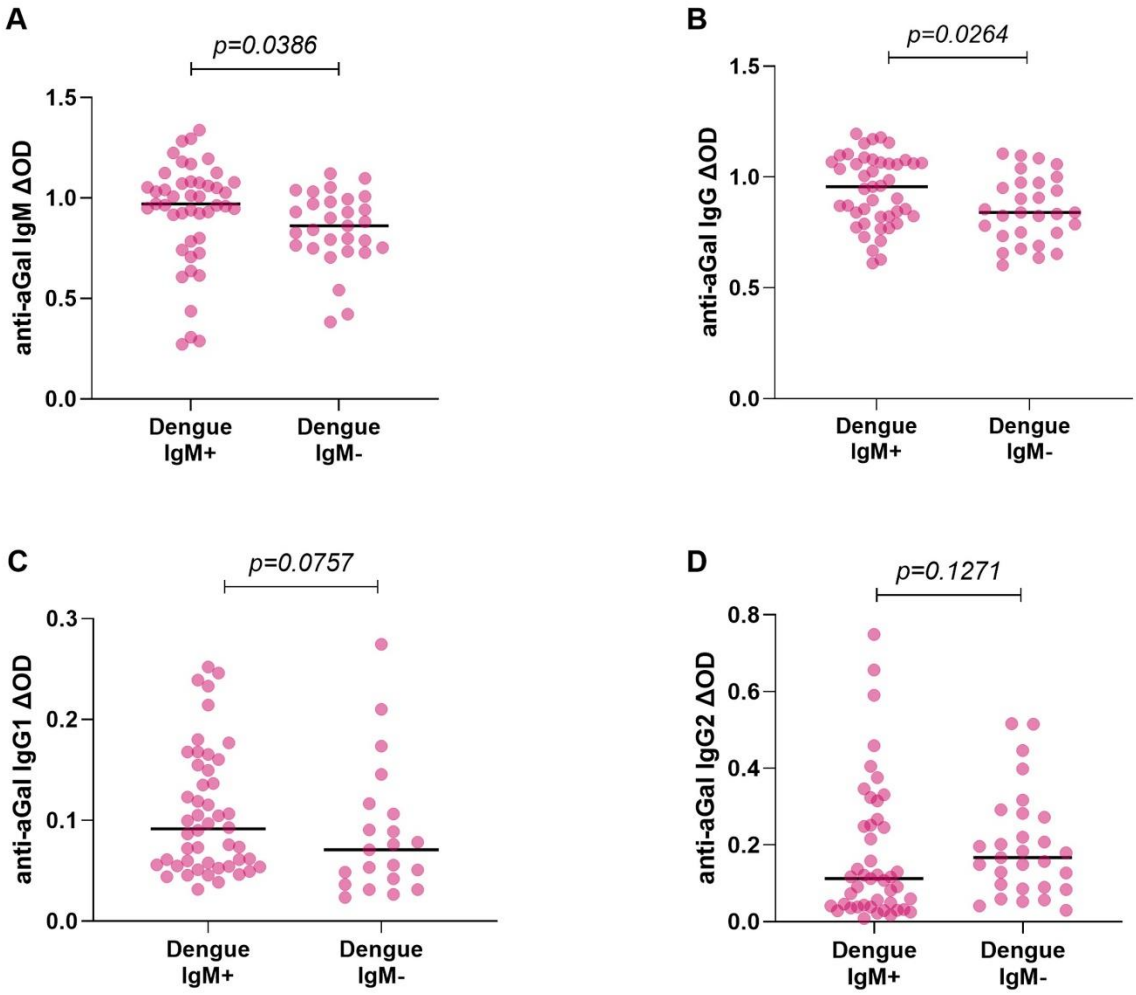

Figure 4.1. Specific anti- aGal IgM, IgG, IgG1 and IgG2 in dengue patients.

Dengue-IgM+ (n=45) and Dengue-IgM- (n=30). (A) Anti-aGal IgM antibody levels, (B) Anti-aGal IgG antibody levels, (C) Anti--aGal IgG1 antibody levels, (D) Anti-aGal IgG2 antibody levels. Individual anti-aGal antibody levels are represented by the colored dots and horizontal black lines represent medians of group individual antibody responses Antibody levels are measured in units of OD (optical density). "p" values were based on the pairwise non-parametric Mann-Whitney test.

Positive correlation between anti-aGal IgG and IgG1 levels and Dengue fever severity classification

We had different categories of dengue severity in our study sample: twenty-eight patients presented dengue without warning symptoms, forty-two patients presented dengue with warning symptoms while only five had severe dengue symptoms. We included ten healthy participants with unknown dengue status in this analysis. We tested whether the levels of anti-aGal antibodies was different between the four clinical classifications of dengue (Fig. 4.2). We observed that the levels of anti-aGal antibodies, anti-aGal IgG and IgG1, are significantly different depending on the dengue severity ($p < 0.0001$, $df = 3$, F value = 10.65). Further analysis with Mann-Whitney test revealed healthy participants with unknown dengue status had lower anti-aGal IgG than dengue patients without warnings ($p = 0.0337$), dengue patients with warnings ($p = 0.0001$), and severe dengue patients ($p = 0.0007$). The patients without warning symptoms had lower levels of anti-aGal IgG when compared to the with warnings patients ($p = 0.0231$) or compared with severe patients ($p = 0.0079$). Furthermore, anti-aGal IgG was also lower in with warnings patients when compared with severe patients ($p = 0.0409$).

Similar to IgG, anti-aGal IgG1 was also lower in without warnings patients when compared to the with warnings patients (p = 0.0196) and severe patients (p = 0.0004), although this trend between the patients with warnings and severe dengue patients was not significantly different (p = 0.0732). However, we find that anti-aGal IgM and IgG2 levels were not significantly different among the dengue fever classifications and participants with unknown dengue status (anti-aGal IgM p = 0.5740, df = 3, F value = 0.6473; anti-aGal IgG2 p = 0.7249, df = 3, F value = 0.2302) in the two-way ANOVA test. The data for anti-aGal IgG1 in healthy participants with unknown dengue status were not included due to shortage of these serum samples (Fig. 4.2).

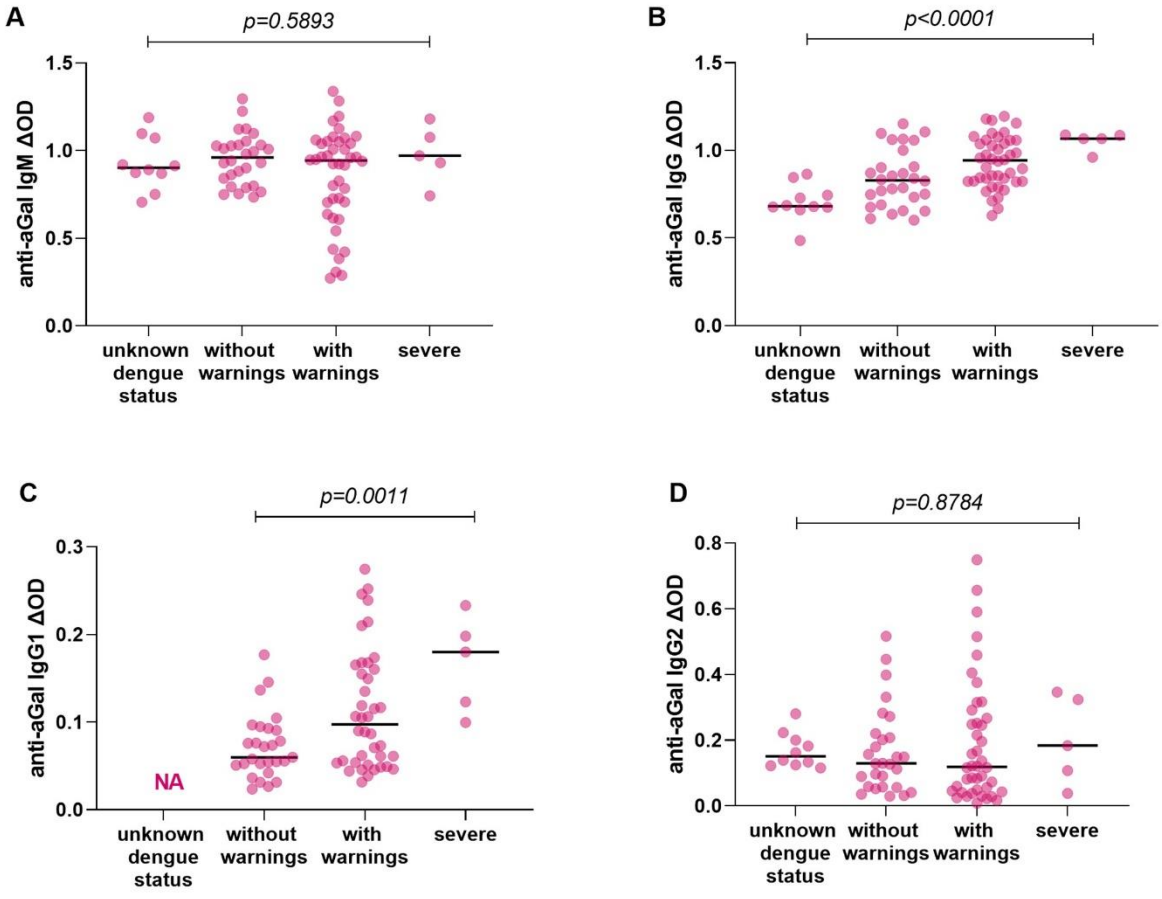

Figure 4.2. Specific anti-aGal IgM, IgG, IgG1, and IgG2 levels by dengue fever classification.

Those with unknown dengue status (n-=10), dengue without warnings (n=28), dengue with warnings (n=42), and severe dengue (n=5). (A) Anti-aGal IgM antibody levels, (B) Anti-aGal IgG antibody levels, (C) Anti-aGal IgG1 antibody levels, (D) Anti-aGal IgG2 antibody levels. Individual anti-aGal antibody levels are represented by the colored dots and horizontal black lines represent medians of group individual antibody responses. Antibody levels were measured in units of OD (optical density). "p" values were measured using the two-way ANOVA test.

Variation in levels of anti-aGal IgG and IgG1 antibody depending on the hospital level and locations

We compared the patients from two different locations- Cúcuta and Ocaña (Appendix Fig. B.2). The two hospitals in each region were vary in their levels of patient care. Norte de Santander's capital, Cúcuta, has a level 3 hospital where patients with severe diseases are transferred from level 1 (basic) or level 2 (intermediate) facilities, whereas Ocaña, the department's second-most populous city with fewer densely populated areas, has a level 2 hospital where patients with mild diseases can seek health care. Significant differences were observed in the anti-aGal antibodies. Among 40 patients from Cúcuta and 35 patients from Ocaña, the patients from Ocaña had higher anti-aGal IgG ($p < 0.0001$) and IgG1 ($p = 0.0343$) levels than those from Cúcuta (Fig. 4.3). In the case of anti-aGal IgM ($p = 0.7216$) and IgG2 ($p = 0.1050$), the levels of antibodies were not significantly different between the two locations.

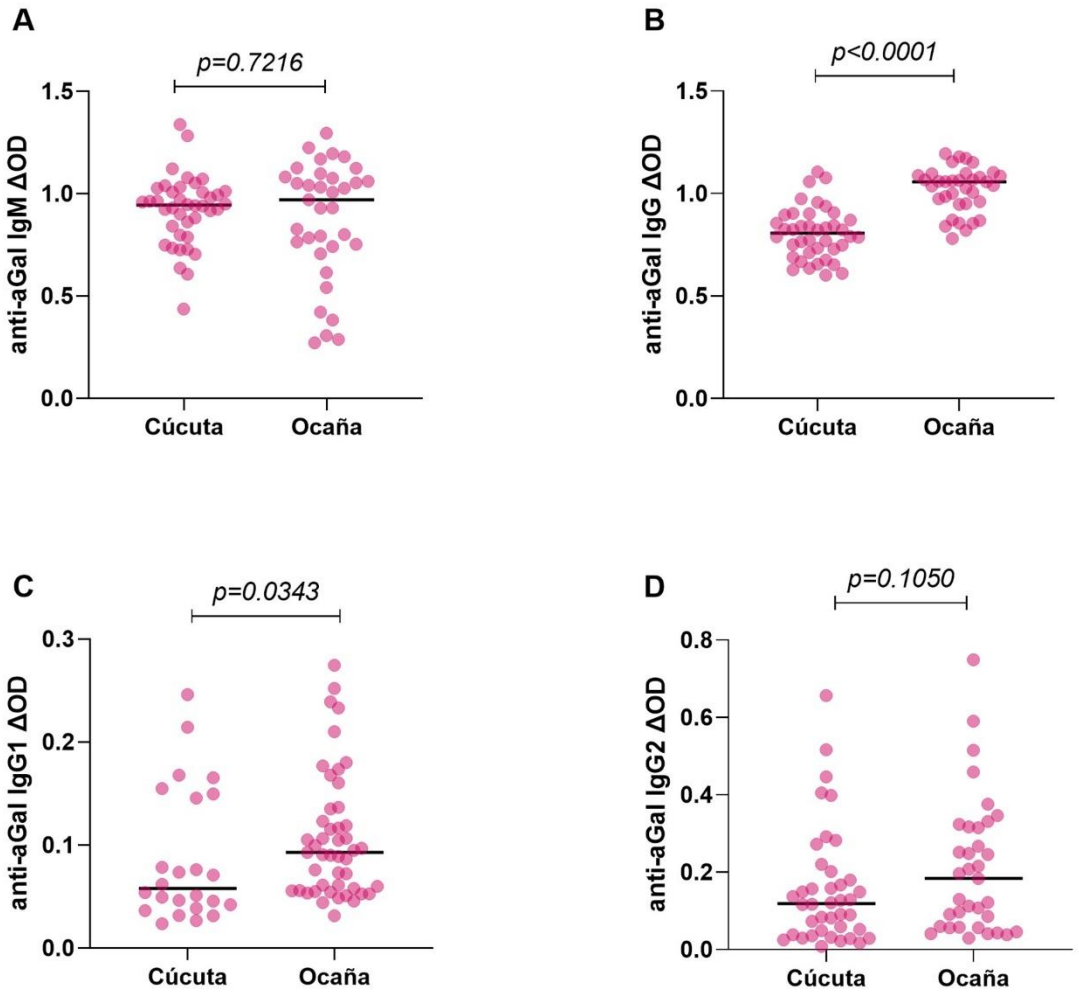

Figure 4.3. Specific anti-aGal IgM, IgG, IgG1 and IgG2 levels by patient locations with different hospital levels;

Cucuta (n=40) and Ocana- (n=35). A) Anti-aGal IgM antibody levels, (B) Anti-aGal IgG antibody levels, (C) Anti-aGal IgG1 antibody levels, (D) Anti-aGal IgG2 antibody levels. Individual anti-aGal antibody levels are represented by the colored dots and horizontal black lines represent medians of group individual antibody responses. Antibody levels are measured in units of OD (optical density). "p" values were measured using the pairwise non-parametric Mann-Whitney test.

Positive correlation between anti-aGal IgG and days with dengue symptoms

We investigated the levels of anti-aGal antibodies in relation to the number of days patients presented with dengue symptoms to provide insight into the possible influence of symptom duration on anti-aGal IgM, IgG, IgG1, and IgG2 levels (Fig. 4.4). We observed that IgG anti-aGal antibodies showed a significant positive correlation with days with dengue symptoms (Spearman correlation, r = 0.4877; p <0.0001). The levels of anti-aGal-IgM (p = 0.4571) were not significantly affected by the number of days with dengue symptoms. Positive correlations without statistical significance were observed in anti-aGal-IgG1 (p = 0.0849) and anti-aGal-IgG2 (p = 0.1470).

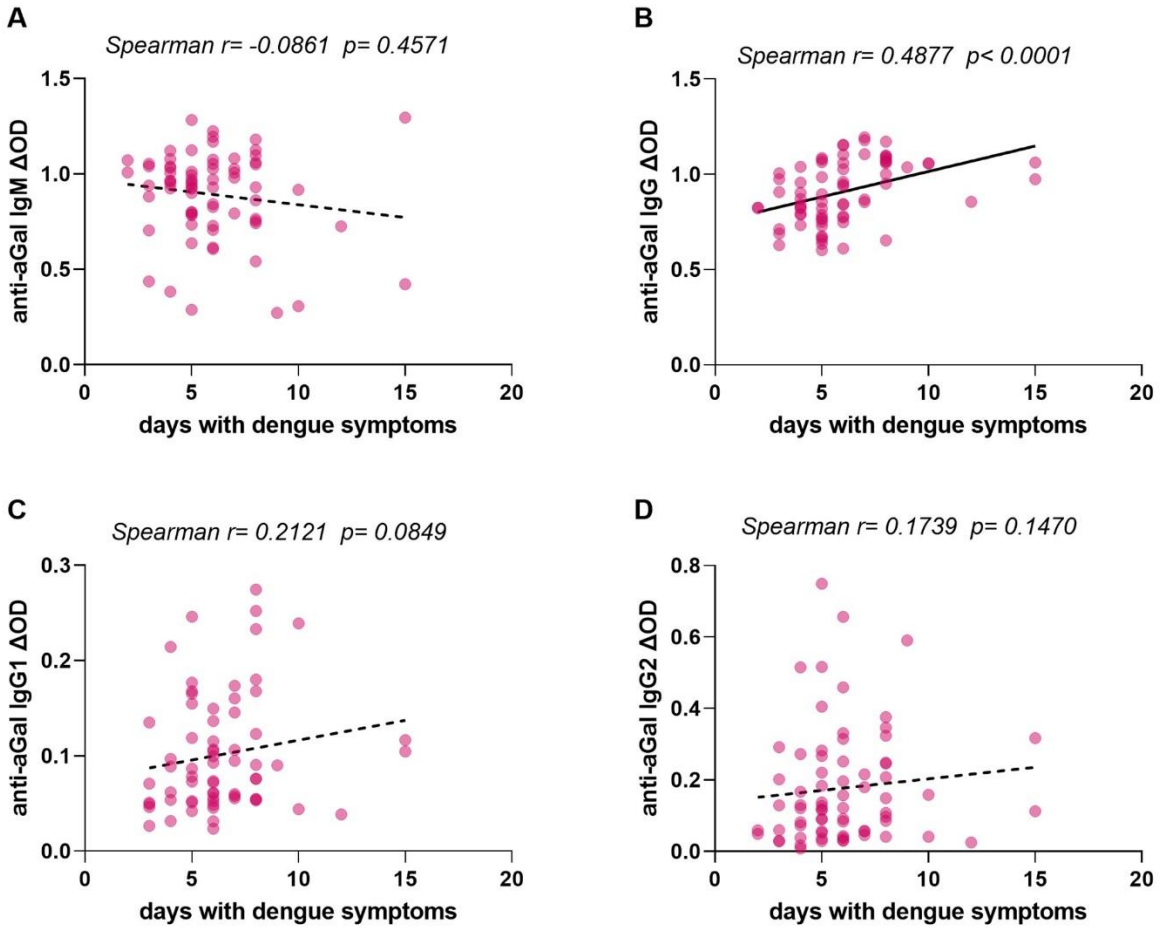

Figure 4.4. Correlation of specific anti- aGal IgM, IgG, IgG1, and IgG2 antibody levels and days with dengue symptoms in patients.

A) Anti-aGal IgM antibody levels, (B) Anti-aGal IgG antibody levels, (C) Anti-aGal IgG1 antibody levels, (D) Anti-aGal IgG2 antibody levels. Regression lines in each graph are shown by solid lines for $p<0.05$ and dotted lines for non-significant correlations. Antibody levels were measured in units of OD (optical density). "r" and "p" values were measured using the pairwise non-parametric Spearman correlation test.

Dengue fever patients' age affects anti-aGal IgM and IgG2 levels

Anti-aGal IgM was negatively associated with age (Spearman correlation, $r = -0.2491$; $p = 0.0312$), while anti-aGal IgG2 was positively associated with age ($r = 0.4678$; $p < 0.001$), but neither anti-aGal IgG ($r = 0.0718$; $p = 0.5403$) nor anti-aGal IgG1 ($r = -0.0711$; $p = 0.5556$) were 206 significantly correlated to patients age in the non-parametric Spearman correlation test (Fig. 5). Further analysis using the Mann-Whitney test (right panel) for the data categorized by age revealed that 0-10 year old patients' anti-aGal IgM levels were not significantly different when compared to those aged 11-20 years ($p = 0.9910$) Additionally, anti-aGal IgM levels in the 11-20 year old group were also not significantly different when compared with those patients above 20 years ($p = 0.1256$), but 0-10 years anti-aGal IgM were significantly higher when compared with those in above 20 years (p value = 0.0453). In the case of anti-aGal IgG2, the 0-10 years dengue patients had significantly lower levels when compared with 11-20 years ($p = 0.0062$) and above 20 years ($p = 0.0003$) but 11-20 years anti-aGal IgG2 was not significantly different when compared with above 20 years ($p = 0.5202$) (Fig. 4 5).

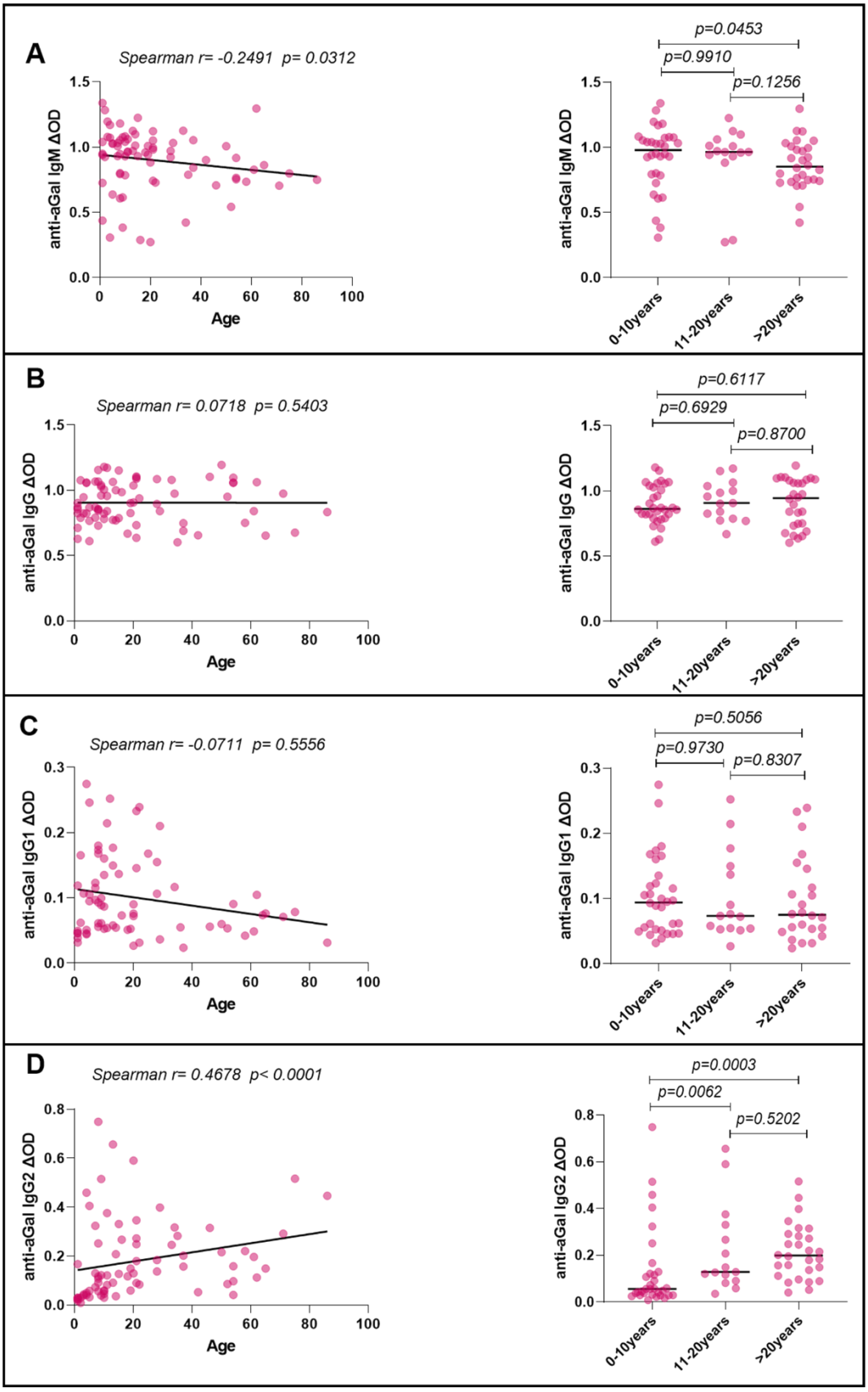
A
Spearman r= -0.2491 p= 0.0312
anti-aGal IgM ΔOD
Age
p=0.0453
p=0.9910
p=0.1256
0-10years
11-20years
>20years
B
Spearman r= 0.0718 p= 0.5403
anti-aGal IgG ΔOD
Age
p=0.6117
p=0.6929
p=0.8700
C
Spearman r= -0.0711 p= 0.5556
anti-aGal IgG1 ΔOD
Age
p=0.5056
p=0.9730
p=0.8307
D
Spearman r= 0.4678 p< 0.0001
anti-aGal IgG2 ΔOD
Age
p=0.0003
p=0.0062
p=0.5202

Figure 4.5. Correlation of specific anti- aGal IgM, IgG, IgG1, and IgG2 antibody levels with dengue patients' age.

A) Anti-aGal IgM antibody levels, (B) Anti-aGal IgG antibody levels, (C) Anti-aGal IgG1 antibody levels, (D) Anti-aGal IgG2 antibody levels. The left panel represents a correlation plot of anti-aGal antibodies with age. Regression lines in each graph are shown by solid lines for $p<0.05$ and dotted lines for non-significant p values. Both "r" and "p" values were obtained from the non-parametric Spearman correlation test. While right panel represent anti-aGal antibody with age distribution (0-10 years (n=32), 11-20 years (n=15), and >20 years (n=28)). Anti-aGal antibody levels are represented by the colored dots and horizontal black lines represent medians of group individual antibody responses. The "p" values were measured using the non-parametric Mann-Whitney test.

Discussion

Over the decades, dengue has become a major threat to human life in its endemic countries. Colombia has suffered five major dengue outbreaks in the years 1998, 2002, 2010, 2013, and 2019 with the 2010 outbreak being the worst dengue epidemic in the history of the country (6,47). The DENV envelope protein is an important component needed for the virus to fuse with the cell endosomal membrane and delivery of viral genome into the cytosol (118). Recently, Lei and colleagues identified five types of N-glycan including mannose, GalNAc, GlcNAc, fucose, and sialic acid having high mannose-type N-linked oligosaccharides and the galactosylation of N-glycans on DENV-2 produced in Ae. albopictus cell line C6/36 (104). The same study also identified aGal existing in the glycan profile of DENV-2 (104). The carbohydrates of DENV, including aGal, may be important in the process of virus infection and

also in the early phase of immune responses of the host although prevalence of aGal in different serotypes of DENV is yet unknown.

The aGal in DENV is likely a specific glycan of the virus produced from the mosquito salivary glands (104) but lacking in the DENV amplified in the human after the first wave of infection. The enzyme for production of aGal, alpha 1,3-galactosyltransferase, has not been identified in arthropod genomes in the homology-based searches (119–121), while multiple copies encoding alpha 1,4- galactosyltransferase and beta 1,4-galactosyltransferase were proposed to be the functional alpha 1,3- galactosyltransferase for production of aGal in arthropods (119,121). Considering that humans have a pseudogenized alpha 1,3-galactosyltransferase copy and are unable to produce aGal (110), the importance of aGal in DENV infection is likely determined by the aGal antibodies induced at the early DENV infection phase or pre-existing endogenously. Therefore, aGal antibody may be involved in the initial infection of DENV injected with mosquito saliva in dermal and epidermal (keratinocytes and fibroblasts) and skin resident immune cells, and spillover into the bloodstream (Langerhans cells, dendritic cells, and macrophages) (122).

We found that dengue IgM positive (+) patients with active infection presented significantly higher levels of both anti-aGal IgM and anti-aGal IgG (Fig. 4.2). The anti-aGal IgG and IgG1 levels were correlated with severe symptoms (Fig. 4.3) and anti-aGal IgG with days after the onset of the symptoms (Fig. 4.5). Although the data suggests that anti-aGal antibodies play significant roles in DENV infection, the consequences of anti-aGal antibody induction by DENV is yet difficult to predict. Two potential roles of aGal antibody resulting in two opposing effects could be; the role as the protective antibody against the DENV infections and the positive role in antibody-dependent enhancement (ADE) in DENV infection. These potential opposing

roles are not necessarily mutually exclusive because they could be antibody-titer dependent in the complex infection processes (123). Concentration-dependent roles of the anti-aGal antibodies in isolated systems, in addition to the current data from DENV patients, would be required to draw a conclusion.

A number of studies have shown that anti-aGal antibodies are neutralization antibodies against pathogens, such as *Plasmodium spp.* (115), *Leishmania spp.* (105), and *Trypanosoma cruzi* (124) with increased aGal antibody levels upon infection. Moreover, human anti-aGal antibodies were proposed to be the protective antibody against C-type retroviruses carrying aGal, derived from animal cells (125). Although the mechanism is not yet fully understood, aGal immunogen has also shown that it could act as an adjuvant (126–129). In field studies, patients with *Plasmodium vivax* infections had greater anti-aGal IgG and IgM levels than healthy people (130) like the case of DENV infection in this study. Trypanosomiasis and Leishmaniasis also have been associated with elevated anti-gal antibody levels (131). In contrast, patients with *Plasmodium falciparum* infections had lower anti-aGal IgG and IgM levels (109,131), and patients with *Mycobacterium tuberculosis* infections likewise had lower anti-aGal IgG and IgM levels (131). Although the neutralization activities of the anti-aGal antibody activities were shown in cell line studies for the above pathogens (105,115,120), interpretation of population studies for dynamics of anti-aGal antibodies appear to be more complicated.

In our study, higher levels of anti-aGal IgG levels in patient exhibiting severe symptoms (Fig. 4.3) and increased levels over the duration of the symptom (Fig. 4.4) supports that the anti-aGal IgG could be the antibody induced by the infection with DENV. This delayed anti aGal IgG productions is likely raised by the first wave infection of DENV injected through mosquito saliva, which could carry the immunogenic aGal. Therefore, the increased anti aGal IgG levels exhibited

by the DENV infection may function as a protective antibody like the cases in infections of other arthropod borne pathogens, although the host-dependent aGal production in virus is distinguished from autonomous aGal production in other pathogenic organisms. The delayed anti-aGal antibody induction, increased after the expected first wave of viremia, suggests that the increased anti-aGal IgG would function as a protective antibody only against the DENV transmission through additional mosquito bites, which is likely common in a Dengue hyperendemic area.

On the other hand, the anti-aGal IgG may function in the process of ADE in DENV infection. ADE occurs in hosts who already have antibodies reactive to DENV for enhanced infection of the virus into host cells through virus-antibody-Fcγ binding and results in severe dengue symptoms (123). The two opposing roles of the antibodies, neutralization or ADE, are likely determined by the antibody titer (123), specificity of the antibody toward certain protein/residues of the virus (132), and possibly divided roles in different types and subtypes of immunoglobulin. A study for evaluating titer-dependent roles of aGal antibodies in dengue infection would require the data for the anti-aGal immunoglobulin levels in the pre-DENV exposure of the same subjected patient. Furthermore, the roles in ADE for different types and subtypes of immunoglobulin need to be investigated because the significant associations between severe symptoms and high levels of IgG and IgG1 subtypes, combined with healthy individuals exhibiting the low levels (Fig 4.2), support the roles of anti-aGal IgG and IgG1 for ADE. Our study involves only patients who are categorized as first infection while ADE is described for the severe dengue cases in second infections. Therefore, the enhancement role of aGal antibodies in initial DENV entry to host cells, if it is the case, could be determined by pre-exposure to immunogenic aGal through various routes; tick bites, potentially mosquito bites with or without

pathogens such as malaria plasmodium, and the microflora and diet in the digestive system and milk.

Geographical differences in anti-aGal antibodies were observed. Both Cúcuta and Ocaña were among the most endemic cities for DENV in the country (96) (Appendix Fig. B.1). Cúcuta, the capital of Norte de Santander is the most urban settlement with a level 3 health facility where severe patients are referred to from level 1 (basic) or level 2 (intermediate) health care facilities 294 whilst Ocaña is an area with less urbanization than Cúcuta with a level 2 hospital where patients with mild disease seek health care (133,134). It was interesting that both healthcare facility patients had distinct levels of anti-aGal IgG and IgG1, albeit the 5 severe cases were all from the Ocaña healthcare facility. This further strengthens the assumption that anti-aGal IgG and IgG1 might be associated with the progression of dengue disease.

Our study indicated that anti-aGal IgM was negatively, and anti-aGal IgG2 was positively correlated with individuals' ages while age had no significant effects on the anti-aGal IgG and IgG1 levels (Fig 4.5). A previous study described positive associations of both anti aGal IgM and IgG levels with age in infants and children, but without age groups comparable to our study (135). Serum immunoglobulin levels up to 18 years old also showed generally increasing patterns of IgM and IgG over time (135,136). Interestingly, anti aGal IgE level, the immunoglobulin that is involved in the alpha gal syndrome (red-meat allergy) (137,138), was found to be negatively correlated to the anti aGal IgG2 level and positively to that of anti aGal IgG1 (139). Although we do not have the data for IgE levels in this study, comparisons of the levels of different immunoglobulin indicate positive correlations between anti aGal IgM and anti-aGal IgG1, and between anti-aGal IgG1 and anti-aGal IgG, implying that the levels of anti-aGal IgG2,

responsible for anticarbohydrate, may be independently regulated from the levels of anti-aGal IgG1, IgM, and possibly IgE.

An innovation in the evolution of the human immune system includes generation of expanded repertoires of anti-glycan antibodies, i.e., anti-aGal that is associated with the loss of endogenous aGal. Escalation of host and pathogen arms race to involvement of vector in production of aGal on the pathogenic DENV is the case that we examined in this study. Although we were unable to draw a solid conclusion for the roles of the aGal antibodies, whether anti-aGal antibodies are a positive or negative factor in DENV infection, a significant role in the infection process is proposed with the population data showing different levels of anti-aGal antibodies over dengue progressions.

Chapter 5 - Conclusions

Given that mosquito salivary proteins have been associated with infection establishment and modulation of host immune responses, this work aimed to contribute to the growing body of knowledge regarding the involvement of antibodies against salivary proteins in dengue fever. It was interesting that the study found a connection between AgBR1 and NeSt1 IgG and the severity of dengue, the duration of dengue symptoms, and hospital disease management while Nterm-IgG presented contradictory findings and SGE showed no significant associations with any of the measures tested. These findings demonstrated that AgBR1 and NeSt1 IgG are associated with dengue fever.

Having established the involvement of anti-salivary IgG with dengue fever, we hypothesized that immunogenic aGal molecules identified on DENV could be inducing antibody responses in dengue-infected individuals and that these antibodies in turn might be involved in dengue pathogenesis. Evaluating the level of antibodies IgM, IgG, IgG1, and IgG2 in people with dengue fever revealed anti-aGal IgG and IgG1 varying among dengue fever classifications and the number of days a patient experienced dengue symptoms correlated with anti-aGal IgG. Furthermore, anti-aGal IgM and IgG levels varied across the two geographical locations of patients, and anti-aGal IgG2 was related to patient age, thereby supporting our hypothesis that aGal antibodies are involved in dengue pathogenesis.

We also investigated DENV serotypes, and we were able to determine that three of the four serotypes were present in the study community. Further examination of the DENV-1 strain phylogeny revealed similarities between our study strain and that of a bordering country, indicating the possibility of travel-related DENV transmission among bordering countries.

Overall, the knowledge obtained in this study contributes to the knowledge of salivary protein and alpha-Gal antibody roles in DENV pathogenesis and epidemiological surveillance of dengue in the study population.

Limitations and future study

One of the study's limitations was not being able to determine the role of AgBR1 and NeSt1 proteins in dengue pathogenesis. Another limitation of the study was the lack of a follow-up study to understand the relationship between dengue infection and changes in anti-salivary IgG levels in relation to patient's survival or recovery. Therefore, future research should investigate the role of AgBR1 and NeSt1 in dengue pathogenesis to ascertain whether improving patients' immune responses to these specific salivary proteins can aid in the fight against dengue infection.

Although several investigations found aGal antibodies to be involved in other mosquito-borne human pathogens, this is the first investigation of aGal antibodies during dengue infection in humans, which presents challenges for understanding how the aGal antibodies affect dengue infection or the relief of dengue symptoms. Future studies should identify whether aGal is present also in the mosquito saliva or only on DENV if aGal antibodies are indeed generated during dengue infection. It would also be beneficial to investigate whether aGal antibodies can prevent DENV replication or viral entry into cells.

The limitation of our phylogenetic study was low detection of DENV in our samples due to suboptimal storage conditions and prolonged time of testing from sample collection. This hinders the sample integrity and sensitivity of the PCR testing and sequencing. Future studies into the evolutionary trends of DENV using sera samples should be done with better quality samples in order to improve sensitivity and specificity of detection.

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Appendix A - DENV-1 Notre de Santander strain sequence

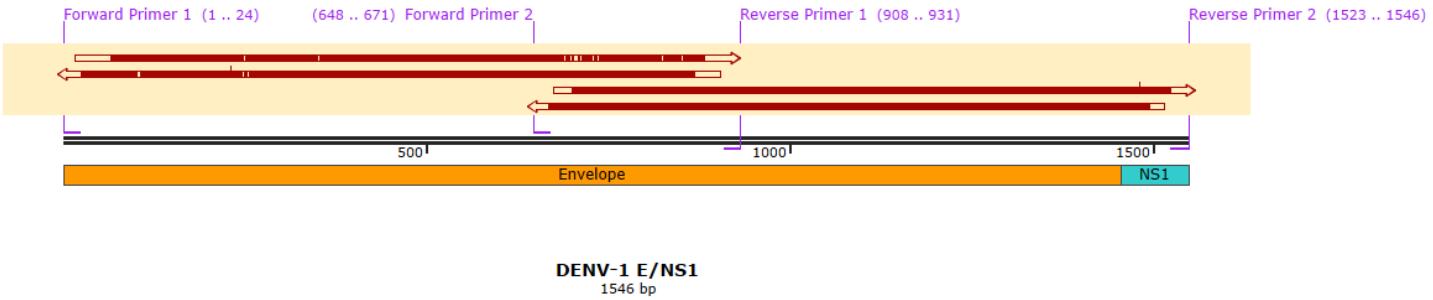

Figure A.1. Sequenced region

Table A.1. Sequence obtained

Primer	Sequence
F1	ACCATGGCAAAAAATAAACCAACATTGGACATTGAACTCCTGAAGACGG AGGTCACGAACCCTGCCGTCTTGCGCAAACTGTGCATTGAAGCTAAAATA TCAAACACCACCACTGATTCAAGATGTCCAACACAAGGAGAGGCTACAC TGGTGGAAGAACAAGACGCGAACTTCGTGTGTCGCCCAACGTTTGTGGAC AGAGGCTGGGGTAATGGCTGCGGACTATTCGGAAAAGGAAGTCTATTGA CGTGTGCCAAGTTCAAGTGTGTGACAAAACTAGAAGGAAAAATAGTTCA ATATGAAAACTTAAAATATTCAGTGATAGTCACTGTCCACACTGGGGACC AGCACCAGGTGGGAAACGAGACCACAGAACATGGAACAATTGCAACCAT AACACCTCAAGCTCCTACGTCGGAAATACAGCTGACCGACTACGGAGCCC TCACATTGGACTGCTCACCTAGAACAGGGCTGGACTTTAATGAGATGGTG CTGTTGACAATGAAAGAAAAATCATGGCTTGTCCACAAACAATGGTTTCT AGACTTACCACTGCCATGGACTTCGGGGGCTTCAACATCCCAAGAGACCT GGAACAGACAAGATCTGCTGGTCACATTCANGACAGCTNATGCNANAAA ACANGAAGTGGTCGTACTANGATCACANGAAGGAGCAATGCACACTGCG TTGACTGGGGCGACAGAAATCCAGACGTCAGGAACGACAACAATCTTCG

	CAGGACACCTGAAATGCNGACTAAAAATGGATAAACTGACTTTAANAGG GATGTCATATGTGATGTGCACAGGTTC
R1	CATGGGTGGACGTGGTATTGGAGCATGGAAGCTGCGTCACCACCATGGC AAAAAATAAACCAACATTGGACATTGAACTNNGAAGACGGAGGTCACGA ACCCTGCCGTCTTGCGCAAACTGTGCATTGAAGCTAAAATATCAAACACC ACCACTGATTCAAGATGTCCAACACAAGGAGAGGCTACACTGGTGGAAG AACAAGACGGCGAACTTCGTGTGTCGCNGAACGNNTGTGGACAGAGGCT GGGGTAATGGCTGCGGACTATTCGGAAAAGGAAGTCTATTGACGTGTGCC AAGTTCAAGTGTGTGACAAAACTAGAAGGAAAGATAGTTCAATATGAAA ACTTAAAATATTCAGTGATAGTCACTGTCCACACTGGGGACCAGCACCAG GTGGGAAACGAGACCACAGAACATGGAACAATTGCAACCATAACACCTC AAGCTCCTACGTCGGAAATACAGCTGACCGACTACGGAGCCCTCACATTG GACTGCTCACCTAGAACAGGGCTGGACTTTAATGAGATGGTGCTGTTGAC AATGAAAGAAAAATCATGGCTTGTCCACAAACAATGGTTTCTAGACTTAC CACTGCCATGGACTTCGGGGGCTTCAACATCCCAAGAGACCTGGAACAG ACAAGATCTGCTGGTCACATTCAAGACAGCTCATGCAAAGAAACAGGAA GTGGTCGTACTAGGATCACAGGAAGGAGCAATGCACACTGCGTTGACTG GGGCGACAGAAATCCAGACGTCAGGAACGACAACAATCTTCGCAGGACA CCTGAAATGCAGACTAAAAATGGATAAACTGACTTTAAAAGGGATGTCA TATGTGA
F2	TGCAAAGAAACAGGAAGTGGTCGTACTAGGATCACAGGAAGGAGCAATG CACACTGCGTTGACTGGGGCGACAGAAATCCAGACGTCAGGAACGACAA CAATCTTCGCAGGACACCTGAAATGCAGACTAAAAATGGATAAACTGAC TTTAAAAGGGATGTCATATGTGATGTGCACAGGTTCATTTAAGCTAGAGA AGGAAGTGGCTGAGACCCAGCATGGAACTGTTCTAGTGCAGGTCAAATA CGAAGGAACAGATGCGCCATGCAAGATCCCCTTCTCGACCCAAGACGAG AAAGGAGTGACCCAGAATGGGAGATTGATAACAGCCAATCCCATAGTTA CTGACAAAGAAAAACCAGTCAACATTGAGACAGAACCACCTTTTGGTGA GAGCTACATCGTGGTAGGGGCAGGCGAAAAAGCTTTGAAACTAAGCTGG TTCAAGAAAGGAAGCAGCATAGGGAAAATGTTCGAAGCAACCGCCCGAG GAGCACGAAGGATGGCTATTCTGGGAGACACCGCATGGGACTTCGGTTCT

	ATAGGAGGAGTGTTCACATCTGTGGGAAAATTGGTACACCAAGTTTTTGG AACCGCATATGGGGTTCTGTTCAGCGGTGTTTCTTGGACCATGAAAGTAG GAATAGGGATTCTGTTGACATGGTTGGGATTAAATTCAAGGAGCACGTCA CTTTCGATGACGTGCATTGCAGTTGGCATGGTCACACTGTACCTAGGAGT CATGGTTCAAGCGGATTCGGGATGTGTAATTAACTGGAAAGGGCAGAGA ACTTAAATGTGGAAGTGGCATCTTTGTTACTAATGA
R2	CAAGATCTGCTGGTCACATTCAAGACAGCTCATGCAAAGAAACAGGAAG TGGTCGTACTAGGATCACAGGAAGGAGCAATGCACACTGCGTTGACTGG GGCGACAGAAATCCAGACGTCAGGAACGACAACAATCTTCGCAGGACAC CTGAAATGCAGACTAAAAATGGATAAACTGACTTTAAAAGGGATGTCAT ATGTGATGTGCACAGGTTCATTTAAGCTAGAGAAGGAAGTGGCTGAGAC CCAGCATGGAACTGTTCTAGTGCAGGTCAAATACGAAGGAACAGATGCG CCATGCAAGATCCCCTTCTCGACCCAAGACGAGAAAGGAGTGACCCAGA ATGGGAGATTGATAACAGCCAATCCCATAGTTACTGACAAAGAAAAACC AGTCAACATTGAGACAGAACCACCTTTTGGTGAGAGCTACATCGTGGTAG GGGCAGGCGAAAAAGCTTTGAAACTAAGCTGGTTCAAGAAAGGAAGCAG CATAGGGAAAATGTTCGAAGCAACCGCCCGAGGAGCACGAAGGATGGCT ATTCTGGGAGACACCGCATGGGACTTCGGTTCTATAGGAGGAGTGTTCAC ATCTGTGGGAAAATTGGTACACCAAGTTTTTGGAACCGCATATGGGGTTC TGTTCAGCGGTGTTTCTTGGACCATGAAAGTAGGAATAGGGATTCTGTTG ACATGGTTGGGATTAAATTCAAGGAGCACGTCACTTTCGATGACGTGCAT TGCAGTTGGCATGGTCACACTGTACCTAGGAGTCATGGTTCAAGCGGATT CGGGATGTGTAATTAACTGGAAGGGCAGAGAACTTAAA

Appendix B - Association of dengue infection with anti-alpha-gal

Gal antibodies

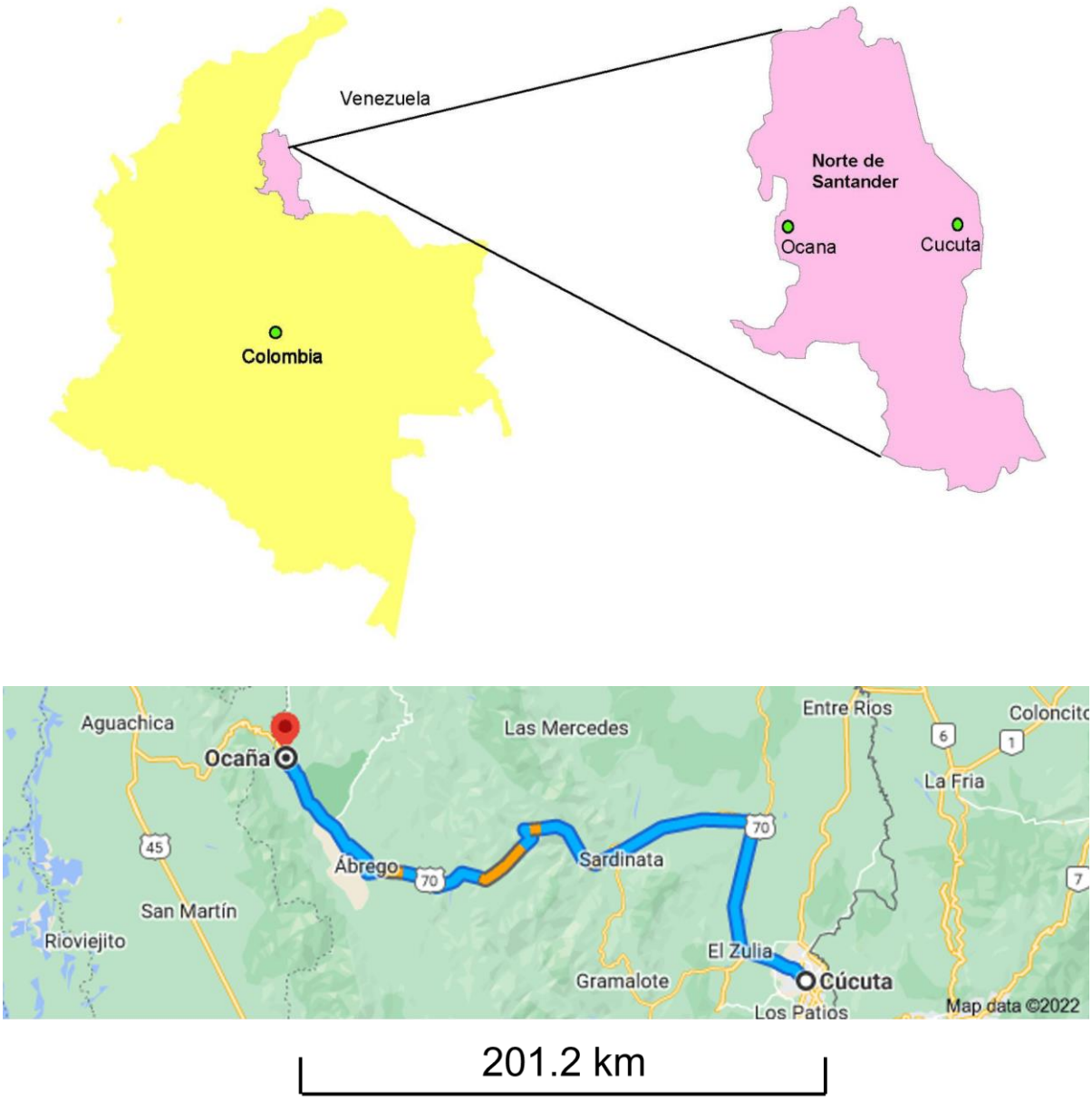

Figure B.1. Study location map

Map showing the geographical location of study samples. The yellow map shows Colombia while the pink map shows the two municipalities of Norte de Santander where sera were collected. The green map shows the distance of 201.2km between Cúcuta and Ocaña municipalities. Map created using MindtheGraph, Mapline, and Google.

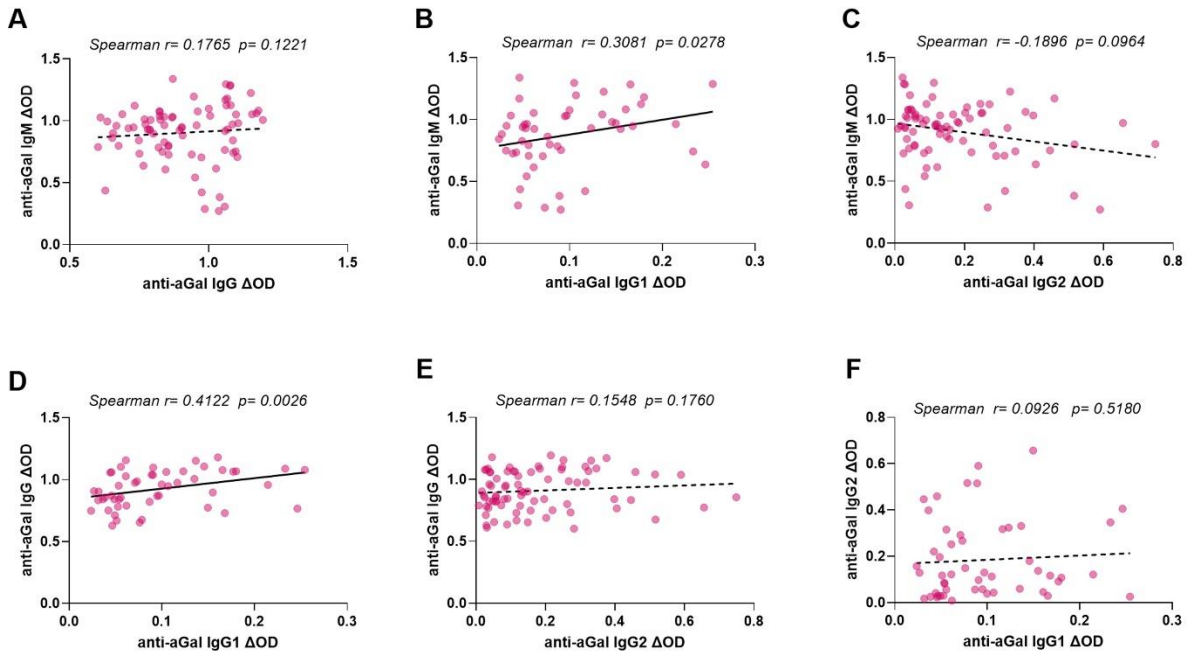

Figure B.2. Anti-aGal IgG1 correlates positively with increasing anti-aGal IgM and IgG

We used the non-parametric Spearman correlation test to assess the different levels of anti-aGal immunoglobulins, and we observed anti-aGal IgM had no significant correlation with anti-aGal IgG (r= 0.1765; p= 0.1221), anti-aGal IgG had no significant correlation with anti-aGal IgG2 (r= 0.1548; p= 0.1760), anti-aGal IgM had no correlation with anti-aGal IgG2 (r= -0.1896; p= 0.0964), anti-aGal IgG2 had no significant correlation with ant-aGal IgG1 (r= 0.0926; p= 0.5180) but anti-aGal IgM was having significant positive correlations with both anti-aGal IgM (r= 0.3081; p= 0.0278) and anti-aGal IgG1 (r= 0.4122; p=0.0026).