VALIDATION OF THE RELATIONSHIP BETWEEN MRCI POLYMORPHISMS WITH DENGUE DISEASE IN A BRAZILIAN POPULATION

by

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ABSTRACT

Dengue is a viral disease characterized by mild febrile illness in the majority of cases. In a small percent of cases, it manifests more severe symptoms, and it can be fatal without proper treatment and support. It is a vector borne disease that is transmitted by the *Aedes* mosquitos. Currently up to 4 billion of the world's population lives in areas at risk for dengue transmission. This represents a public health risk.

The objective of this study was to evaluate the association of two single nucleotide polymorphisms (SNPs) located in the *MRCI* gene, rs2296414 and rs34039386, with dengue virus infection in a sample of patients from a cohort study of dengue patients and controls from Recife, Brazil. The *MRCI* gene codes for the mannose receptors of macrophages, which have been demonstrated to play an important role in viral infections. DNA was amplified using PCR. Genotyping of 179 individuals was done by three methods, fluorescence polarization, restriction digests, and sequencing. All samples were found to be in Hardy-Weinberg equilibrium across the sample population and when classified as cases of controls. No significant departures from Hardy-Weinberg were seen when samples were broken down by disease status. Odds ratios were calculated with novel data and combined with data from a previous study. The previous study examined the same two polymorphisms using a different sample from the same cohort study. A

statistically significant difference was found in the combined samples for the rs2296414 site with regards to case or control status, OR=1.46, p=.035. A statistically significant difference was also found for the rs34039386 site with regards to case/control status OR=2.76, p=.0029. This difference was only found in the novel data.

Further research with larger samples sized is needed to continue to understand the relationships between these sites and dengue infection, as the sample size for this study was small. Even when combined with data from the previous study, the data set was not particularly large. The public health significance of this research is produced by the increasing global importance of dengue and the need for more effective treatments and the development of a vaccine; advancement of the understanding of the relationships between the dengue virus and the *MRCI* gene may lead to improvements in the treatments available and in the production of a vaccine with high efficacy.

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1.0   INTRODUCTION

The purpose of this project is to further evaluate a previous observation found in a study by Erin Cathcart. This goal of this protect was to generate more results to see if similar trends present as they did in the earlier work. The previous study showed a possible connection between several single nucleotide polymorphisms (SNPs) and an increased risk of severe dengue infection in a sample of patients from Brazil. In addition to an association between these mutations and severity, other associations, including age and sex, were also evaluated. To maintain the similarities and replicability of these two studies, these factors will also be investigated using this new sample. Both the samples used in the previous study and the samples used in this study were selected at random from the same larger sample of DNA collected from patients in Recife, Brazil. Controls for this study were selected from patients presenting to hospitals with other complaints who were determined to be dengue-free. Controls for the previous study were selected from a control group of patients who presented to the Brazilian health service to receive a yellow fever vaccine, and were determined to be free of dengue.

Whenever possible, methodological approaches and data analysis techniques used in the previous work were used in this study as well. This was done to maintain as much similarity between the studies as possible to remove potential confounding factors when investigating the relationship of the results. Genotyping of patient samples was done using fluorescence polarization and restriction digest. Analysis of results was done in Excel and Stata, and several approaches

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were taken, including Hardy-Weinberg equilibrium testing using chi-squared tests for significance, and odds ratios to determine the impact of different alleles on the presence or absence of disease and disease severity.

1.1   BACKGROUND

Dengue is a dynamic and ever-changing disease that has the potential to affect up to a half of the global population[1]. It represents an important global infection whose impact is growing and will only continue to grow. The factors that make dengue an important global health concern are diverse and multifaceted. While it is not a new disease on the global stage, in recent years, multiple factors have converged to cause a significant resurgence and spread. Not only is the distribution of dengue changing, but it is also intensifying as an infectious disease. This means there will be more cases, more serious infections, and more deaths from the virus as its epidemiology continues to change and evolve[1].

1.1.1   Dengue Fever

Epidemiology

Current estimates disagree about the true global burden of dengue. However, despite the disagreements, no estimates place the burden as insignificant, and there is consensus that it is a growing and intensifying problem[2]. Morbidity and mortality estimates fall into a wide range depending on the source of the data. The World Health Organization (WHO) estimates that 2.5 billion people live in areas currently at risk for dengue infection. This represents about 40% of the

global population[3]. The Center for Disease Control (CDC) estimates fall closely in line with the WHO, but they differ slightly when determining the number of countries that are currently at risk for dengue[2]. The WHO lists 100 countries as at risk and the CDC has an additional four listed[2]. These numbers may be underestimating the true burden of the disease. A study in 2012 by researchers from the United Kingdom and the United States estimated that the number of at-risk countries is higher. They found that 128 countries have good evidence of dengue occurrence[2]. The new list they generated using multiple levels of data and analysis found 36 countries with a good likelihood of dengue occurrence that were determined to be dengue free by the WHO[2]. This new work increases the potential percentage of the global population at risk to up to 3.97 billion[2]. This is an extreme upper limit, but the evidence surrounding dengue infection makes it a plausible figure.

As with many infectious diseases, it is difficult to determine the true level of dengue in the population. In fact, it is particularly difficult with dengue because of the similarity to other diseases, the remote locations of cases, and the high percent of subclinical infections[1, 2]. Early stages of dengue look like many other common viral illnesses[2]. Because of this, misdiagnosis is common[2]. An additional issue with determining the global burden of dengue is the often remote locations of the healthcare facilities where cases are treated. In many situations, the diagnostic capabilities of the facility are not able to handle the complicated diagnosis of dengue infection[2]. Finally, a large portion of dengue cases go unnoticed because they manifest as subclinical infections. Estimates vary, but between 50% and 80% of dengue infections may be asymptomatic or subclinical[4-6]. These cases may be entirely non-symptomatic, or they may manifest as a nondescript and mild febrile illness that does not lead people to seek out medical care[5, 6].

The highest estimates place the total number of yearly dengue cases at 390 million with approximately 25% (96 million) of them being apparent, clinical disease[7]. These numbers were arrived at in a study by Bhatt et al and published in Nature that looked to more accurately predict the global burden of dengue using sophisticated modelling techniques[7] More conservative estimates from the WHO initially put that number considerably lower at 50 million cases[8], but recently they have updated information on the global burden of disease to include the numbers derived from Bhatt et al.'s study[3]. Of these 50-96 million clinical important cases, an approximate 500,000 are considered "severe dengue," and are potentially life threatening[9]. Like many of the numbers surrounding dengue, the mortality rates are an estimate and may be significantly underreported. Deaths from dengue are likely between 20,000 and 25,000[10]. While this number is much lower than the deaths from other common illness like malaria[11], the WHO classified dengue as the most important mosquito-borne viral disease in the world in 2012[10]. As previously discussed, the reasons for this classification by the CDC are varied, but the most important to consider is the significant global spread in recent years and increased potential for continued spread and changing and evolving disease patterns[10].

Dengue is firmly established as being present in over 100 countries[1]. Asia, Africa, and the Americas bear the highest burdens of the disease, with Asia holding the highest percentage at around 70% of the global burden[1]. Africa accounts for 14% of the burden and the Americas 13%[1]. Some of most affected countries include India, Mexico, and Brazil[1].

The global distribution of dengue has changed significantly over the past four decades[10]. All evidence suggests that dengue has been an important human pathogen for several centuries -- perhaps longer, as records from 992 AD in China have cases with symptoms compatible with dengue[10]. Outbreaks consistent with dengue continued in the Americas through the 17th, 18th,

19th, and 20th centuries[10]. However, the virus responsible for dengue was not determined and isolated until 1943[12]. Between the end of the 1940's and the 1970's, dengue was present in only a small number of countries and in isolated outbreaks[10]. After the cessation of control measures in the 1970's, dengue came back strongly over the next decades and is now firmly established as global disease[10]. Evidence strongly suggests that every WHO region has dengue transmission either in endemic or epidemic cycles[10].

Dengue is now hyperendemic, with locally acquired transmission in all but two countries in the Americas. Chile and Uruguay are the only countries in this region that have still not seen cases of locally-acquired dengue. Data suggests that there are close to two million cases annually in the American region countries[10].

Asia and the Pacific bear the largest brunt of the global dengue infections, as 75% of the exposed population lives in this region[10]. High morbidity and mortality is common in this region, and dengue represents the leading cause of deaths among children in the smaller region of southeast Asia[10]. In recent years, dengue has emerged in previously clear areas in China and Japan[1].

Dengue in Africa has been the subject of limited research, and is almost certainly underreported. At least 20 countries in Africa most likely have cases of locally-acquired dengue transmission and outbreaks have been recorded since as early as the 1960's[10]. Despite no official cases reported to the WHO, laboratory cases have been confirmed. The high presence of other illness similar in symptomology and the poor resource settings account for the lack of reporting of dengue in this region[1].

Dengue's presence in Europe and Middle East has only been established more recently, but in the past several decades local transmission has occurred in at least three European countries, and many more in the Middle East[10].

Understanding the global spread of dengue is impossible without understanding its vector. The dengue virus is carried and spread by two distinct but very similar species of the *Aedes* mosquito, *Aedes aegypti* and *Aedes albopictus* [13]. *Aedes aegypti* is the primary vector for dengue and is more competent at transmitting the disease[10], but the presence of the alternative vector *Aedes albopictus* should not be discounted, as it has been shown to be an effective transmitter as well[10]. The global spread of dengue, however, closely follows the spread of *Aedes aegypti*. Control measures of this vector are what allowed dengue to temporarily become nonexistent in the majority of the Americas. It was only when vector control measures were ceased that both *Aedes aegypti* and dengue returned to this region of the world[12]. Both *Aedes* species are now fully established on at least parts of all continents except Antarctica[13]. *Aedes aegypti* prefers and thrives in subtropical and tropical climates, but *Aedes albopictus* is able to survive in much cooler climates with more dramatic seasonal fluctuations[13, 14].

The fact that the *Aedes aegpyti* mosquito is the one able to transmit the dengue virus is part of the disease's remarkable ability to reach so many diverse regions. *Aedes aegypti* is a day-biting mosquito that prefers to feed and breed in urban and semi-urban areas[1]. The combination of these factors means that dengue is common in settings with high population density. Human migration and travel are key factors in the spread and transmission of dengue[10]. Because the primary vector for the virus is well established in a wide range of regions, the potential for transmission is always there.

This is important to the transmission cycle for the dengue virus. Dengue follows a mosquito-to-person-to-mosquito cycle of transmission with no important intermediate hosts, as are common in many other vector-borne disease[3]. Infected humans are the primary carriers and multipliers for the virus[3]. A mosquito may become infected after biting an infected human during a period of high virus load known as viremia. This is typically a period 4-5 days after initial infection, but it can last up to 12 days[3, 9]. An infected mosquito will then go on to bite a new, susceptible host, and the transmission cycle will continue (see Figure 1.).
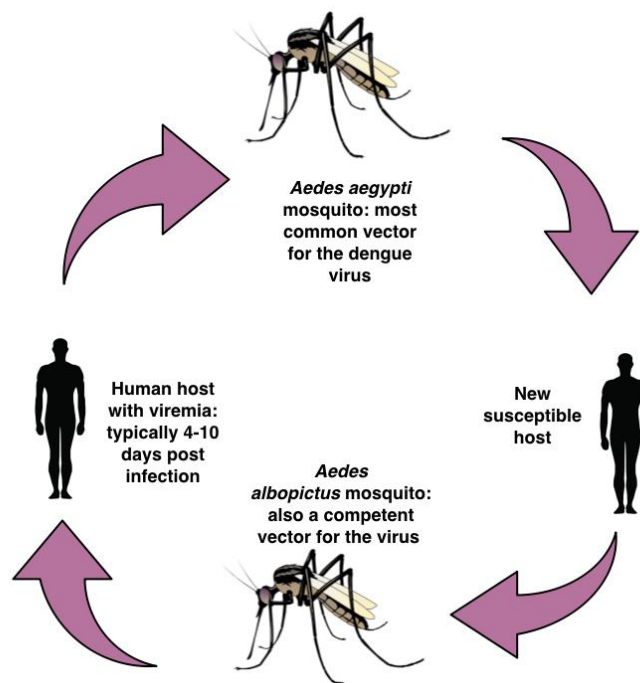
Figure 1. Dengue transmission cycle

Virus

Dengue belongs to a family of viruses known as the *Flaviviradae*, genus *Flavivirus,* which includes other important human pathogens such as Yellow Fever, West Nile, and Japanese Encephalitis[15]. Dengue itself has four phylogenetically distinct serotype labeled DENV1-4[16]. These four distinct types of dengue share about 65% of their genomes[9]. Despite sharing only a

little over half of their genomes, DENV1-4 are all highly related in their effects on their human targets and in their transmission and ecological niche[9].

Evidence strongly suggests that dengue was originally a monkey virus and was part of a sylvatic transmission cycle between non-human privates and mosquitoes. It is likely that the cross-species transmission to humans happened independently in all four of the distinct viral serotypes[16]. The emergence of the dengue virus into the human population most likely happened as recently as 500-1,000 years ago[9].

In the evolutionary sense, this makes dengue a relative newcomer in the world of viruses. Occurring even more recently was the cross-species transmission from monkeys to humans. DENV2 made the jump approximately 320 years ago and DENV1 even more recently, around 125 years[16]. The majority of the genetic diversity between dengue serotypes has occurred over the past 100 years[16]. Humans were likely incidental hosts in the sylvatic transmission cycle of the disease for the majority of its history[16]. The transition to the urban cycle of transmission observed today and the expansion of endemic areas was likely due to several factors, including a limiting number of nonhuman hosts, expansion of the vector, increased urbanization, and viral mutations[9, 10, 16].

The location of the emergence of the dengue virus is hard to determine with 100% confidence. Africa seems the obvious choice for several reasons. Part of the evidence supporting this assertion is that the primary vector for dengue, *Aedes aegypti*, is thought to have evolved in Africa. Additionally, a majority of the other mosquito-borne flaviviruses circulate primarily in Africa. This lends support to the claim of the flaviviridae family having evolved in this region, which would suggest that dengue may have evolved here as well[16].

Despite the strong evidence supporting Africa as the cradle for the dengue virus, Asia also has a strong set of evidence backing it up as the likely origin; the prevalence and burden of dengue is highest in this region, and all four serotypes of dengue exist in both humans and monkeys in this area. The resolution of this debate will require continued research and larger sample bases from Africa, as samples from this region are limited, so the complete evidence may not be available[16].

The dengue virus (DNV) is a single stranded RNA virus[9]. It is also a positive sense virus[17]. As with all viruses in the *Flavivirus* genus, dengue is an enveloped virus[17]. A lipopolysaccharide envelope surrounds a 40-50nm particle that is spherical in shape[9]. The RNA genome is around 11kb in length and encodes a total of only 10 proteins. Three of these proteins are structural, while the remaining seven proteins are non-structural[18]. The 11kb genome codes for a single polyprotein which is then broken down in the cytoplasm of the host cell into the separate structural and non-structural proteins[18]. The structural proteins include a capsid or C protein, a membrane or M protein, and an E or envelope protein[9, 17]. A host-derived lipid bilayer surrounds the nucleocapsid which is derived of multiple copies of the C protein. Within this lipid layer, 180 copies of the smaller M protein (8kDa) and the larger E protein (52kDa) are anchored[19]. The E protein is composed of three distinct domains, and its unique structure plays an important role in different stages of infection. The E protein is arranged in a herringbone pattern of 90 head-to-tail dimers organized in sets of three and positioned parallel to the viral surface. The non-structural proteins are NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (see Figure 2). These proteins are all responsible for parts of the viral life cycle including viral replication, virion assembly, and escape from the host's immune system[18]. NS2A is responsible for blocking host interferon (INF) signaling. NS2A, NS4A, NS4B, and NS5

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also work to block INF by reducing STAT activation[19]. NS1 is particularly important in dengue pathogenesis and diagnosis[18, 19].
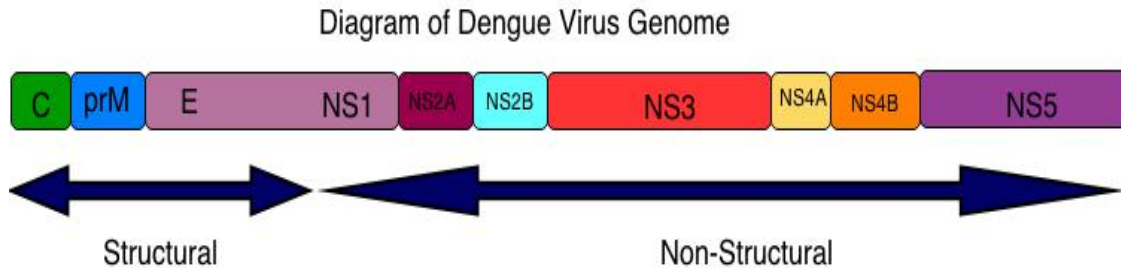
Figure 2. Dengue genome

The viral life cycle for dengue is complex, and elements of it are still not understood fully After viral entry into the host cell, a single polyprotein is produced from the 11kb genome, and is then processed by both host and virus proteases into the separate proteins[19]. The variety of NS proteins are responsible for replication of the viral genome after initial protein processing and folding is completed. After the production of new viral RNA, it is wrapped by the C protein capsid. The PreM and E proteins are arranged facing into the lumen of endoplasmic reticulum and formed into heterodimers. The processes of the association with the nucleocapsid are still unclear[19].

Pathogenesis

Primary cellular targets for DNV are of the mononuclear phagocyte lineage and include monocytes, macrophages, and dendritic cells[19]. Langerhans cells and dendritic cells residing in the skin are often the site of the first infection[19]. Viral entry into this primary cellular target is the most important factor in establishing infection, but it is also the least understood[18]. Viral entry involve many possible cellular targets[18, 19]. The process begins and is mediated by the viral E glycoprotein. These viral proteins bind to multiple cellular receptors, some known and

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some unknown. These will be discussed in more detail later, as they are an important component of this research.

There is strong evidence to suggest that the primary path for cellular entry by the dengue virus is receptor initiated: clathrin mediated endocytosis[18, 19]. Membrane fusion allows the viral RNA to enter the host cell[18]. More recent research has suggested that in addition to clathrin mediated uptake, certain strains of the dengue virus are able to enter the cell through clathrin independent pathways[19]. This is achieved by DENV-2 dynamin and is also completely independent of micropinocytosis or phagocytosis[18].

Cellular targets of the dengue virus are still not well-established and are an important area of research focus. On initial infection of immature dendritic cells in the skin, the virus enters the cell using cell surface molecules known as non-specific receptor dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN)[18]. DC-SIGN is a C-type lectin transmembrane protein[18]. DC-SIGN is expressed in abundance in these immature dendritic cells and is involved in their migration, as well as T-cell priming, antigen recognition, and presentation[18]. Other important possible cellular receptors include heparin sulfate, CD14, HSP90/HSP70, the glucose protein 78, the TIM and TAM proteins, the laminin receptor, and (most importantly for our discussion) the mannose receptor[18], which will be discussed in more detail later.

After the initial infection of the immature dendritic cells in the skin, the virus is taken up and disseminated to the lymph nodes. After dissemination, it continues to target cells of the mononuclear lineage. This stage is when the pathogenesis of the infection becomes known as macrophages begin to secrete inflammatory mediators[17].

The pathogenesis and host immune response of the dengue infection are closely tied together, and one must be discussed when discussing the other. Multiple theories have been

proposed to explain the process of immunopathogenesis and the progression of the infection, and each must be examined to fully understand this complex infectious process. Antibody-mediated or dependent enhancement theory, altered peptide ligand theory, and the cytokine storm theory will all be discussed. First, a brief overview of the immune response is necessary.

As with all infections, the body's initial defense against the virus is the components of the innate immune system. The initial defense after infection is the production of interferons: type I and type II interferons are produced within hours by all dengue infected cells[19]. During this time period of infection, natural killer cells are also activated. This whole process and the production of interferons is mediated by viral communication with pathogen recognition receptors on the cellular surfaces. Receptors involved in this include C-type lectins (DC-SIGN) and toll-like receptors (TLR), specifically TLR3 and TLR7. Pathogen recognition receptors begin a transcription cascade using various transcription factors which will ultimately lead to the production of interferons[19]. This stage also represents the first place the dengue virus is able to circumvent the immune system's response to its presence. The dengue virus is able to obstruct the interferon-alpha mediated innate antiviral response. This is done using several of its non-structural genes, particularly NS2A, NS4A,B and NS5[19].

Approximately six days following infection, the humoral immune response begins. Antigen presentation to cells of the humoral immune system initiates this process. The antibodies generated by B cells are mostly targeted at the viral envelope glycoproteins, the pre-membrane glycoproteins, and NS1. NS1 is both secreted by and expressed on the surface of infected cells[19]. The antibody production works to neutralize viral infection in several ways. Antibody production against NS1 starts the complement-mediated lysis of viral infected cells. Antibodies against the other protein types most likely work to directly influence the infectious ability of the virus, as well

as neutralization. Both strongly and weakly neutralizing antibodies are produced, and the number of them needed to effectively work to neutralize the virus is less or more respectively[19]. The most effective antibodies are produced to strain specific differences and are specific to the third domain of the envelope protein, whereas weaker antibodies are directed at the second domain. These neutralizing antibodies have been shown to work through blocking viral attachment to cellular receptors and by impeding other steps in the process of viral entry into the host cells[19].

T cell involvement in dengue is not well understood. Some studies have suggested that CD8+ T cells may play an important role in immune defense against the dengue virus, but limited models still leave questions surrounding this process. In contrast to the protective quality of T cell response, T cell responses may be responsible for the pathogenesis seen in the more severe cases of dengue infection[17, 19]. This topic will be discussed in more detail along with the various theories of pathogenesis.

One of the defining factors of dengue is the fact that secondary infection with a different strain is often responsible for a much more severe progression of the disease. Multiple epidemiological studies have corroborated this phenomenon[19]. The severe forms of dengue, dengue hemorrhagic fever and dengue shock syndrome, are much more likely to occur when a patient has had a previous dengue infection and is re-infected, this time with a different strain. Several theories have been proposed to explain this phenomenon and the process that leads to severe symptoms seen in dengue hemorrhagic fever and dengue shock syndrome.

The original and still widely accepted hypothesis to explain this is the theory of antibody-mediate enhancement or antibody-determined enhancement (ADE). This theory was first popularized by Halstead and O'Rourke in 1977[17] and seeks to explain why a secondary infection with a different serotype of the same virus would lead to more serious disease instead of protective

immunity, as is often the case with secondary infections, even when the serotypes are different. The primary tenet of this hypothesis is that something in the body's secondary immune response to the pathogen goes wrong, producing the symptoms associated with severe dengue. The most important component of this occurrence has been shown to be the presence of Fc receptors on cells of the mononuclear lineage, including dendritic cells, monocytes, and macrophages[20]. Antibodies produced in higher levels from memory B cells act to specifically direct the virus to cells that express Fc receptors, which happen to also be the primary target for the virus. This creates a higher viral load throughout the body and the impairment of several types of immune system cells[19, 20]. This enhancement of viral uptake seems to be facilitated primarily by enhancing immunoglobulin G (IgG), but IgM and complement factor 3 (C3) have also been demonstrated to be able to achieve the same ends[20]. These enhancing antibodies pull the virus into contact with the cellular surface of cells expressing Fc receptors. It seems like viral uptake into the cell is not achieved by the Fc receptors alone, but requires working with a normal viral receptor as a co-receptor.

This process alone does not explain the whole story behind the progression to severe disease, and other theories are needed to supplement it. It is also important to note that not every secondary infection leads to more severe disease; in fact, in a majority of cases it still produces an asymptomatic or mild infection, as is true for dengue rates as a whole[20].

The potential role of T cells in the production of symptoms during severe dengue infection is explained in the theory of "original antigenic sin" (OAS). The hypothesis of original antigenic sin is that on secondary infection by a new serotype of the dengue virus, the host's immune system activates memory T cells from previous infection. These T cells possess too low an affinity for the

new serotype present. This inappropriate response hinders the development of a strong enough response with high affinity, and instead leads to a state of immunopathology[17].

Closely tied into original antigenic sin is the cytokine storm. This is theorized to occur when the over-activation of T cells causes the production of vast quantities of antiviral cytokines. These cytokines, which under normal levels are vital for fighting infections, result in cellular damage. It is this process that is believed to be responsible for the loss of endothelial integrity, vascular leakage and hemorrhage that is a hallmark of severe dengue infections[19]. This abnormal immune response results in high levels of multiple cytokines including TNF-alpha, IFN-gamma, IFN-alpha, IL-1, IL-2, Il-4, IL-6, Il-7, Il-8, IL-10, Il-13, and Il-18[19]. This increased cytokine production cannot be fully explained by OAS only, and other factors including ADE are also likely causing it to a certain degree. [3, 17, 19, 20].

Clinical

More often than not, dengue manifests as an uncomplicated febrile disease with minor symptoms, similar to the flu and many viral illness. It is considered a self-limiting illness, meaning that in most cases it will resolve without medical intervention and the patient will recover. This self-limiting illness usually last only a week, with some symptoms lingering longer in some populations[6]. Dengue infection is classified into three stages by the World Health Organization, 1.) febrile, 2.) critical, and 3.) recovery[6].

The first stage in the natural history of dengue is the febrile stage. In addition to the high fever that gives this stage its name, this stage also comes with other symptoms. Frequently seen symptoms include fatigue, rash, joint and muscle pain, and headache. Gastrointestinal symptoms are less common but do occur including nausea and vomiting[6]. A critical component of this stage that is an important factor in diagnosis and transmission is the high viral load in the blood.

Viremia and a low platelet count are present during this stage of the disease[6]. The febrile stage typically last 2-7 days[6].

The disappearance of detectible viremia and a rapidly decreasing platelet count mark the beginning of the second stage. The critical stage is named such because it is this stage that determines the progression of the disease[6]. Capillary permeability present during the critical stage presents as a rising hematocrit and decreasing platelets. This stage resolves on its own in 24-48 hours in most cases but in a minority of cases the symptoms may progress to internal bleeding and shock which are the hallmarks of the more severe forms of dengue, dengue hemorrhagic fever and dengue shock syndrome[6].

Patients progress to the final stage of the disease if they do not develop either of the severe complications of the critical stage. During the recovery phase symptoms improve markedly and fluid lost during times of high capillary permeability is reabsorbed. Some symptoms may continue during the recovery phase and after, especially fatigue[6].

The clinical classifications of dengue have been revised and reworked several times during the history of the disease. The current classifications were set in 2009 by the World Health Organization and includes three new categories for dengue: dengue without warning signs, dengue with warning signs, and dengue hemorrhagic fever[6]. These new classifications were revised from the previously used ones implemented in 1997. The 1997 classifications include, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS)[6]. The presence of plasma leakage in dengue patients is what classifies DHF and the progression to bleeding and shock is classified as DSS[6]. These classifications were modified to the current 2009 recommendations because of concerns that the older categories did not do an adequate job of describing a classifying patients with more serious disease[6]. Several concerns

were raised about the 1997 classifications which were first adopted in 1975 and modified several times before reaching their final 1997 configuration[21]. Multiple case studies over the years after the 1997 adoption of classifications lead to a set of concerns about their applicability and usefulness. These concerns included the 1997 classifications being not well linked to disease severity, the potential for misdirecting clinicians in identifying severe disease, the fact that it is difficult to apply correctly because of the availability of tests and level of difficulty to conduct them, the fact that it is not useful for triaging in outbreak scenarios, and it leading to global differences in reporting because of the difficulty and availability of the test required to classify the cases[21]. The primary focus of the new system derived in 2009 is to reduce the difficulty in classifying cases and allow for better global reporting and reduction in mortality and morbidity[21]. The 2009 classifications are based on the presence or absence of several clinical signs and symptoms or laboratory tests. The full classification can be seen in Figure 3. Dengue without warning signs or "probable" dengue must meet several criteria, as patients must present with a fever and two or more other symptoms including aches and pain, leukopenia, nausea and vomiting, and rash[6]. In addition to the presence of these symptoms, travel or living in a dengue-endemic area is required[6]. It is also possible to use lab confirmation to confirm cases as well[6]. To be classified as dengue with warning signs, a patient must present with the above set of symptoms as well as one or more from a list considered warning symptoms for progression to severe disease[6]. These warning symptoms include abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, and liver enlargement[6]. Three criteria are required for a patient to be classified as having severe dengue: severe plasma leakage, severe bleeding, and severe organ impairment[6]. These criteria can be assessed by laboratory test of clinician evaluation[6]. Treatment of dengue is primarily supportive. Close fluid

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management is critical in cases of dengue with warning signs, in which cases the patients are

generally admitted to the hospital to manage care. Other supportive measures including pain

management are used to manage dengue cases[6]. For the purpose of our study, special

classifications common to Brazil will be used. These include Dengue Fever, Complicated

Dengue, and Dengue Hemorrhagic Fever.
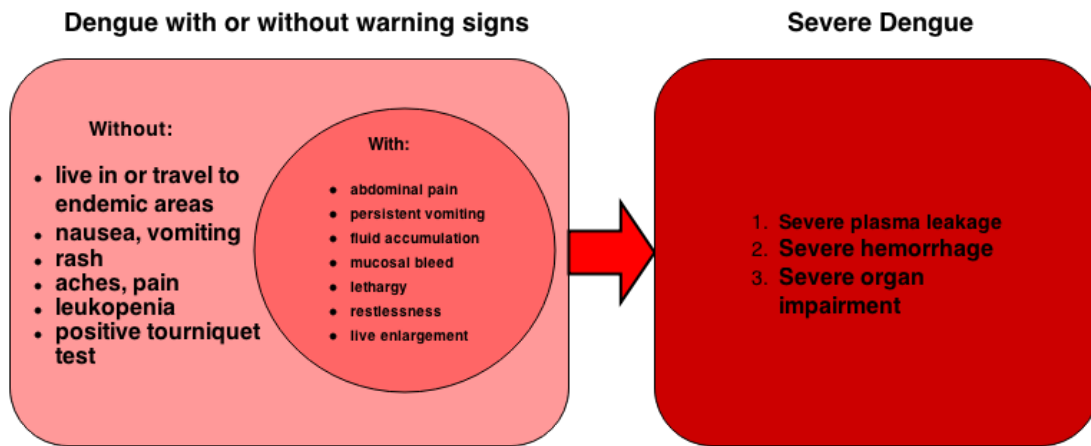
Figure 3. 2009 WHO classifications

1.1.2 MRC1 and the Mannose Receptor

The mannose receptor has been shown to have roles in immunity as well as specifically in viral

infections. The mannose receptor is made up of C-type lectin proteins. C-lectin type proteins are

transmembrane proteins expressed on multiple cell type surfaces[22]. These are especially

important on the surface of dendritic cells and macrophages. The mannose receptor is composed

of three extracellular domains, a cysteine rich domain (CR), a fibronectin type 2 repeat domain

(FNII), and a carbohydrate recognition domain (CRD). The receptor possesses one of each of the

CR and FNII, but has eight CRDs. The mannose receptor has the ability to bind both endogenous

and exogenous ligands, which supports its ability to perform multiple functions within the immune system[22, 23].

Studies have shown that the mannose receptor may play a role in cell-cell signaling, innate immunity, antigen presentation, and infection of macrophages. Evidence suggests that the mannose receptor acts as pattern recognition receptor and may play a role within the innate immune system's ability to fight microbial infections[22, 23]. An important piece of evidence that supports the mannose receptor being a good candidate for a method of entry of pathogens into macrophages is the fact that the mannose receptor is regularly internalized into the cell and recycled back out to the cell surface without the necessity of a substrate binding [22, 23].

The role of mannose receptors in the process of viral infections is still not well understood, but in recent years more and more research has been conduct to determine what role or roles it may play in different viral infections. Important to our discussing is recent findings linking the mannose receptor to dengue infection. Recent evidence has pointed to the mannose receptors of the macrophages and monocytes as being an important cellular target for the dengue virus. A study done in 2008 by Miller et al out of the University of Oxford found that all four serotypes of dengue are able to bind to macrophages and monocytes mannose receptors [22]. Not only is dengue able to find to the mannose receptor, but active infection of the cells was found shortly after binding[24]. This may indicate that the mannose receptor is necessary to infect macrophages[24]. This study has critical implications to this research, as it shows the mannose receptor may play a role in dengue's ability to infect host cells. These findings present the basis of the rationale for investigating the role of these specific polymorphisms of the MRCI gene and their relationship with dengue disease.

The mannose receptor of macrophages is coded for by the *MRCI* gene. It is located on chromosome 10 on the p arm at location 13[25]. This region of chromosome 10 has also been shown to have genes that code for a few other C-type lectin proteins. The *MRCI* gene is composed of 30 exons and 29 introns. All of the 30 exons are separated by an intron. Exons 3 and 7 are important for this discussion, as they contain the single nucleotide polymorphisms (SNPs) of interest to this study. Exon 3 codes for the one fibronectin type II repeat domain of the mannose receptor. Exon 7 codes for the carbohydrate recognition domain 2 of the mannose receptor. This domain is not fully coded by exon 7; other parts of it are coded by exons 8 and 9. The fibronectin type II repeat domain coded for by exon 3 is extracellular and believed to be necessary and sufficient for the binding of collagen. The carbohydrate recognition domain 2 is particularly important for infection[25, 26].

It is important to note that polymorphisms of the *MRCI* gene have been linked to several infectious diseases by previous studies[27-29]. Two studies in Chinese populations found significant associations between SNPs in exon 7 of *MRCI* and susceptibility to pulmonary tuberculosis. One study discovered that SNPs in this exon may confer protection against pulmonary TB[29]. Another study conducted on other SNPs found that they are significantly associated with susceptibility to tuberculosis[27]. Another study found association between leprosy susceptibility and polymorphism of exon 7 of *MRCI*[28].

1.1.3   Single Nucleotide Polymorphisms (SNPs)

Two SNPs of the *MRCI* gene were investigated for a relationship with dengue disease. One polymorphism is located on exon 3, and the other on exon 7. The first SNP of exon 3, rs2296414, is a change from C to T. It is a missense mutation that results in an amino acid change in the protein

from a threonine to asparagine[30]. This exon codes for fibronectin type II repeat domain[24]. The SNP of exon 7, rs34039386, is also a missense mutation. An A is changed to a G and results in an amino acid sequence change from a glycine to a serine[24, 31]. As previously mentioned, it codes for a portion of the second carbohydrate recognition domain of the mannose receptor[24]. For both of these specific mutations, the clinical significance is unknown[19, 30, 31].

1.2 GOALS/HYPOTHESIS

1.2.1 What: Goals

The primary goals of this study are to twofold. The primary goal is to analyze a subset of samples from a patient cohort from Brazil. The aim of this analysis is to determine if there is a relationship present between the two SNPs in *MRCI* and dengue infection or dengue severity. The secondary goal is to produce further results to test the association found previously from a different sub-sample of the same cohort. Along with the novel analysis, data from the original study will be combined with data from this study to see if results remain consistent, and to increase the overall sample size. As with this current study, the previous study looked for an association between the SNPs and dengue infection and severity in this population.

1.2.2 How: Methodological Approaches.

This research was conducted by genotyping a subset of samples from the patient cohort to determine their genotypes at the two locations of interest. The genotyping was done using

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fluorescence polarization, restriction digest, and whole genome sequencing. After genotyping, the data was analyzed to test for Hardy-Weinberg equilibrium. This is a standard test performed on genotypic data, and departure from it could indicate either that genotyping errors had taken place, that demographic factors exist in our sample set that perturb equilibrium, or that one genotype may confer a selective advantage. Significance testing for Hardy-Weinberg was done using chi-squared analysis. In addition to this test, odds ratios were done to identify dominant/recessive relationship between the disease outcome and the different alleles present at the sites of interest. Odds ratios were done for the novel data alone, and then were retested after combining the novel data with data from the previous study.

1.2.3   Why: The supporting evidence

Previous studies have shown that there may be associations between SNPs in the *MRCI* gene and infectious disease susceptibility and severity [27-29]. Three studies have found evidence supporting this: one found an association between leprosy susceptibility and SNPs of *MRCI* [28], and two others found an association between *MRCI* polymorphisms and tuberculosis susceptibility[27, 29]. Especially important for this research is the fact that the macrophage mannose receptor which the *MRCI* gene codes for has been shown to mediate infection with the dengue virus[24], so it is reasonable to hypothesize that mutations on that gene may affect dengue infection.

2.0   METHODS

The 187 DNA samples for this study were pre-extracted on-site in Brazil. The samples arrived dehydrated, so rehydration was required before genotyping procedures could begin. The concentration of the samples was tested and determined by the Nanodrop 1000 spectrometer. The concentration of the samples was 100ng/ul. After determining the samples' concentration, primers were then selected for both of the SNPs locations. Fluorescence polarization and restriction digests were used to sequence the sample DNA. A database was created with the sample's genotype at both SNPs, which also contained important clinical and demographic information. Analysis was completed using Excel as a data organizer and test generator. Chi-Squared analysis and Odds Ratios were used to determine the significance between SNP and disease status.

2.1   SAMPLE SELECTION

The samples used in these studies were randomly selected from a large cohort study of dengue patients conducted in Recife, Brazil. The collection was done by the Department of Virology at the Aggeu Magalhães Research Center[32, 33], which is part of the National Oswaldo Cruz Foundation, also known as Fiocruz. The recruitment of the participants occurred between 2005 and 2009 with three hospitals involved in the study: The *Instituto Materno Infantil de Pernambuco*, *Hospital Esperanca*, and *Hospital Santa Joana*. Blood samples were collected on admission to any of these hospitals if dengue was suspected. Samples were collected at admission and several times after during the patients' stay[32, 33]. Controls were patients in the same hospitals that presented

with febrile illnesses that were determined to not be dengue. They were additionally tested to determine that they had not had previous infection with dengue.

The clinical classification of the patient was determined by following them over the course of the disease progression. New clinical classifications were developed for this study, as some cases did not meet the definition for WHO classifications of dengue fever, dengue hemorrhagic fever, or dengue shock syndrome. The classifications developed for the study were dengue, complicated dengue, and dengue hemorrhagic fever. These classifications were created before the revised WHO organization classifications were developed in 2009. The classifications used in this study closely follow those from 1997, with the added category of complicated dengue.

Case samples were also classified as either primary infection or secondary infection. Real Time-PCR, ELISA, and viral isolation were used to determine the infection as primary or secondary by analyzing the presence or absence of antibodies. Primary patients are classified as being without dengue specific IgG antibodies in acute serum during the first days after the beginning of a fever, but with dengue specific IgM followed by the development of an anti-dengue IgG response during recovery(8). Secondary cases were classified which had detectable anti-dengue IgG in the acute serum samples, but did not have anti-dengue IgM followed by the development of an anti-dengue IgM response in recovery[32].

2.2   SITE SELECTION

2.2.1   Single Nucleotide Polymorphism Selection

The SNPs selected for this project were chosen for various reasons. Because one of the aims of this study was to replicate results from previous work it was necessary to select the same sites that were used in that study[34]. The sites were originally selected for several additional reasons. RS2296414 has been studied previously, and several studies describe its allele frequencies in different population. The approximate frequency of the C allele is 0.854 and 0.146 for the T allele globally[30]. Rs34039386 was originally selected because of an association found in a previous study that showed it was significantly associated with a decreased risk of infection with *Mycobacterium tuberculosis*[29]. Because of the previously seen association with an infectious disease, it was speculated that it may affect the susceptibility to other infectious agents as well.

2.3   GENOTYPING

DNA from 179 samples was received pre-extracted. Before analysis could begin it was necessary to rehydrate the samples as they had been dehydrated for shipping. Each samples was rehydrated with 10micolitres of deionized water and the concentrations of DNA were tested on a random sample of the sample before proceeding. It was determined that the samples contained 100ng/micolitre. This was quantified using the Nanodrop 1000 spectrometer.

Genotyping was completed using two methods: fluorescence polarization (FP) analysis and restriction digests. FP analysis was the first method used. When the results from some of the

samples were not clear enough, restriction digest was used to confirm the genotypes of the remaining samples. Polymerase chain reaction was the first step performed for both of the analyses.

2.3.1 Polymerase Chain Reaction

Polymerase chain reaction was first used to amplify the DNA before genotyping. *Taq* polymerase was used for this PCR reaction. *Taq,* deoxynucleoside triphosphates, buffer solution, bivalent cations of magnesium, and sense and antisense primers were added to the DNA. Primers selected for this analysis were as follows: for rs2294614, sense-CAC TCA CAT TCC AAG TTC and antisense-GTC AGT AGT GGT TCC GCA C and for rs34039386, sense-GTG ATG TGC CTA CTC ACT G and antisense-GTA GAA AGG GGT GCT CCC. Rs2294614 samples were run with 2.5mg of magnesium chloride at 54 degrees Celsius. Rs34039386 samples were run with 2.5mg of magnesium chloride at 56 degrees Celsius.

2.3.2 Fluorescence Polarization

The primary method used to determine the genotypes of the samples was fluorescence polarization. This was done following the method of Chen et al[35]. After amplification by PCR, a cleanup step was performed with exonuclease I and shrimp alkaline phosphatase. This step is done to remove any nucleotide bases that were unused in the PCR amplification step. Next, the samples were cycled with thermosequenase, a heat-activated enzyme, FP probes and allele specific dyes. The FB probes used in this study were AGG CAA TGC CAA TGG AGC AA for RS2994614 and GTG GAT ACT TGT GAG GTC AC for rs34039386. A 1:8 CT dye was used. It was composed of r110

and TAMRA dye. After a final cycle, the samples were run through an FP machine. Data from this was collected and analyzed using the allele caller software. This software produces scatter plots of sample genotypes, from which genotypes can then be manually determined. A total of four plots were produced using this method, which corresponds to the four trays of samples that were run through. Two trays were done per RS number to cover the total 179 samples.

2.3.3 Restriction Digest

After FP analysis was completed, it was determined that some of the samples were not genotyped adequately with the FP analysis, and to fully determine their genotype at the two SNPs more analysis was needed. Restriction digest was used to complete this additional analysis. Restriction digest was only used to genotype samples for rs34039386. In total, 16 samples from tray 1 and a full tray 2 were done using this technique. A total of 98 samples were typed using restriction digest. *AciI* at a 1x concentration was used in this digest. They were run over night (12-18 hours) at 39 degrees Celsius. *AciI* is a an enzyme that digests the 589 base pair fragment amplified by PCR and produces cut fragments 350 and 239 base pairs long when a G allele is there. The occurrence of the A allele produces no cuts, and the resulting fragment stays 589 base pairs long. DNA fragments produced by the restriction digest were electrophoresed in a 2% agarose gel containing ethidium bromide for approximately 1 hour and visualized by UV trans-illumination using a Stratagene Eagle Eye II camera and read with Biorad Quantity One 4.4.0 software.

2.3.4   Sequencing

Finally, a few samples that were resistant to both previous forms of genotyping were genotyped with sequencing. Forty samples were genotyped using this method. The whole product of the initial PCR was sequenced. Sequencing was done on site at the University of Pittsburgh's sequencer.

2.4   STATISTICAL ANALYSIS

2.4.1   Population Demographics

All samples were analyzed for demographic characteristics using Microsoft Excel software. Samples were categorized by gender, age, and disease status to determine the characteristics of the particular sample of the population represented in this study.

2.4.2   Hardy-Weinberg Equilibrium

Observed genotype frequencies for each SNP of interest and for each disease status were used to calculate allele frequencies. The allele frequencies were then used to calculate the expected genotype frequencies in the sample under the Hardy-Weinberg equilibrium. Observed and expected genotype frequencies were then analyzed for significance using chi-squared tests.

2.4.3   Odds Ratios

In addition to chi-squared tests, odds ratios were calculated to determine the odds of disease based on observed genotypes. Odd ratios were conducted using STATA and Excel software.

3.0   RESULTS

3.1   POPULATION CHARACTERISTICS

3.1.1   Demographics

Population demographics were analyzed. The sample selected represented a closely even split between males and females, with just slightly more males represented. Females made up 41.9% of the population and males represented 58.1%. A wide spread of ages is seen in this population as well; the highest percent of the population is in the 40-49 group, followed by the 20-29 group. This distribution is similar to what is seen in the total population of Brazil. The lowest percentages are seen in the youngest and oldest groups. Figure 4 shows the distribution of the population by age and sex. In table 1, the total percent of each sex and age group are available.
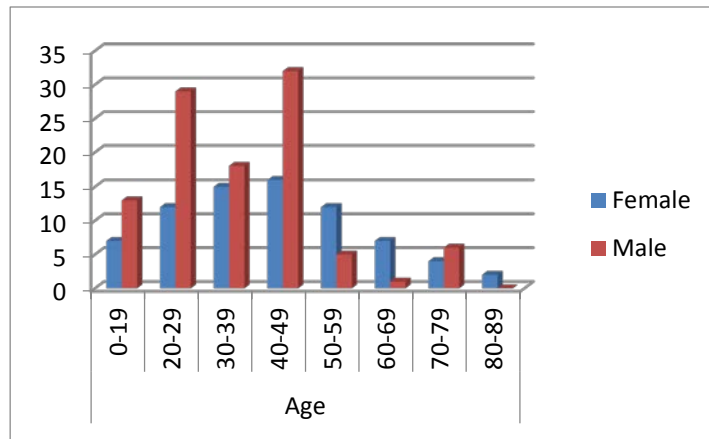
Figure 4. Age and sex distribution

Table 1. Age and sex distribution

Sex	Age								TOTAL	%
	0-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89		
Female	7	12	15	16	12	7	4	2	75	41.9%
Male	13	29	18	32	5	1	6	0	104	58.1%
TOTAL	20	41	33	48	17	8	10	2	179	
%	11.2%	22.9%	18.4%	26.8%	9.5%	4.5%	5.6%	1.1%	100.0%	

The population was also examined with regards to disease status by sex and age. Females represent a slightly larger portion of the controls (ND) than males. They also represent higher proportions of dengue fever (DF) and dengue hemorrhagic fever (DHF). The only disease status where males have a higher proportion is complicated dengue (CD). Figure 5 shows the distribution. Examining the data by sex and age gives interesting information on the population characteristics as well.
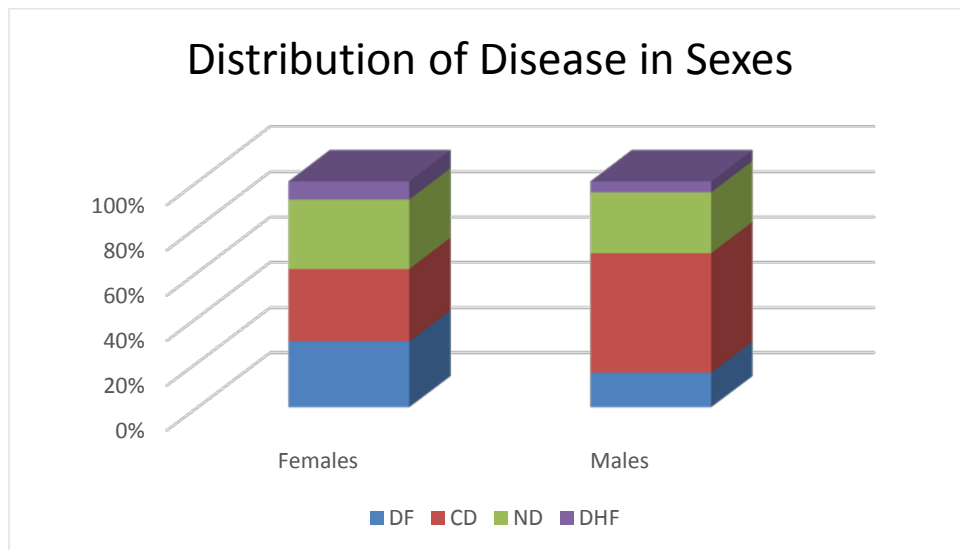
Figure 5. Distribution of diseases in the sexes

Among females (figure 6), the highest counts of DHF are seen in the older age groups. Both DF and CD dengue spike among the young to middle age adults. Cases are evenly distributed, with the notable exception of the youngest age group. The males in this sample are more skewed to the younger age groups (figure 7). The highest numbers of all forms of dengue can be seen in young to middle age adults. DF peaks in 40-49, CD peaks at 20-29 and 40-49, and DHF from 30-49. The controls in the males are not as evenly distributed as in the female samples.
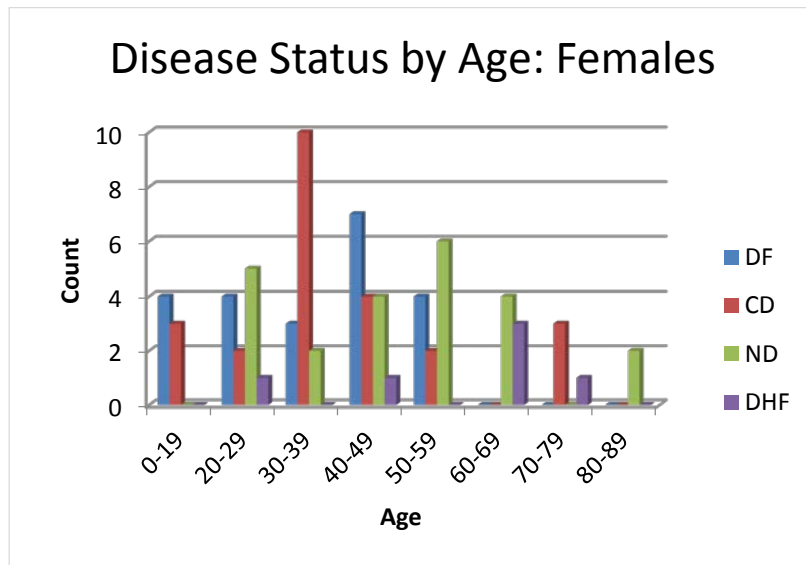
Figure 6. Disease status by age: female
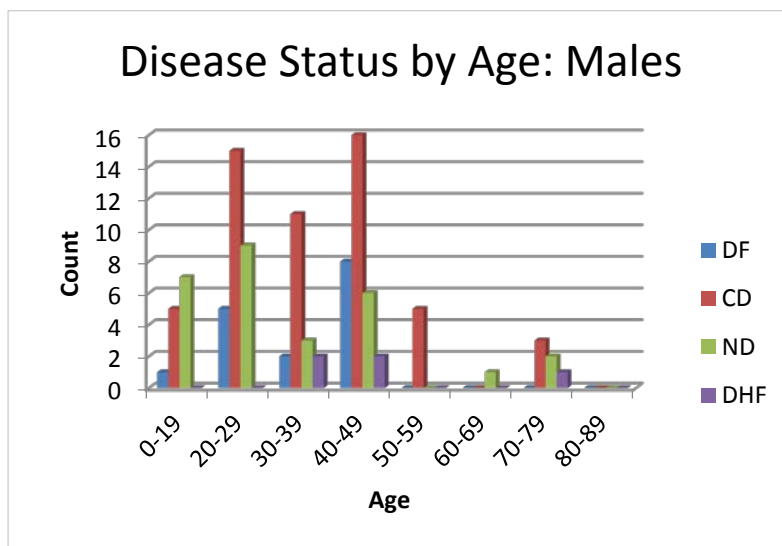
Figure 7. Disease status by age: males

Because sex can be a confounder when investigating the relationship between genotype and disease, it was first important to determine if there was any association present in the population between sex and case/control status. An odds ratio was calculated to test this. No significant result was found with regards to sex and disease status (table 2).

Table 2. Odds ratio for sex and case/control status

	Cases	Controls	Odds Ratio	95% CI	P
Female	52	23	0.83	0.43-1.60	0.58
Male	76	28			

3.1.2   Genotype Frequencies

The genotype frequencies for rs2996414 in the whole sample were .65 for CC, .33 for CT, and .017 for TT. These are summarized in figure 8. Genotype frequencies were also examined by disease status to see if there were visible differences in the frequencies between different levels of disease. The summary information for this can be found in table 3.
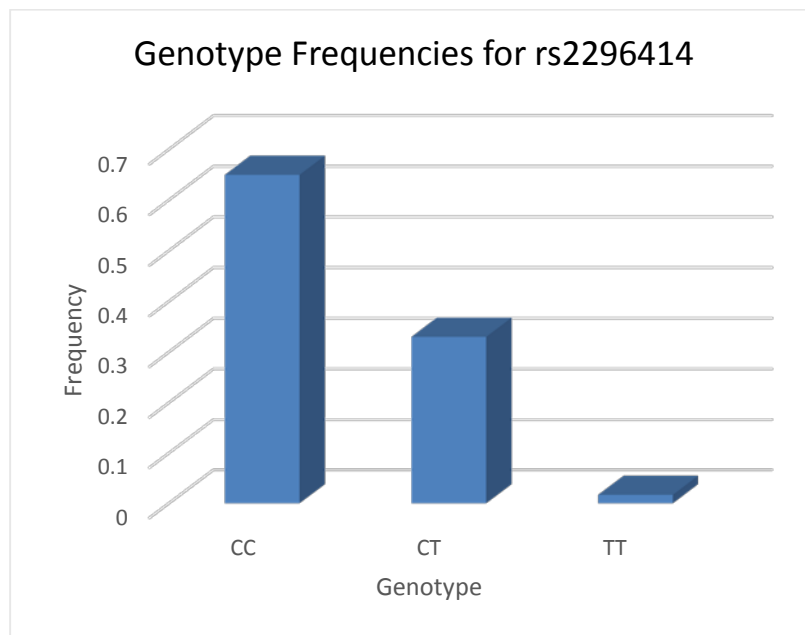
Figure 8. Genotype frequencies for rs2296414

Table 3. Genotypes by diagnosis: rs2296414

Genotype	Dengue Diagnosis				
	DF	CD	DHF	ND	TOTAL
CC	27	54	7	27	115
CT	10	24	2	23	59
TT	0	1	2	0	3
Total	37	79	11	50	177

The TT genotype is seen in the highest amount in DHF cases. The overall number of DHF cases is very small, however, because of the rare nature of the condition. CC and CT are relatively even throughout the diagnoses, with CT being slightly higher in ND and CC being slightly higher in DF (figure 9).
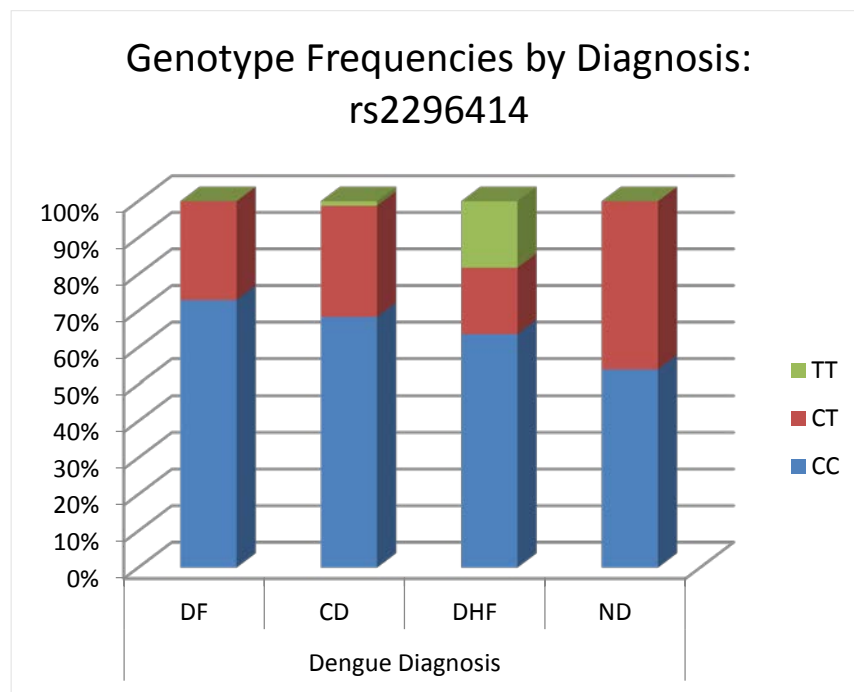
Figure 9. Genotype by dengue diagnosis: rs2296414

The genotype frequencies for rs34039386 were also characterized for the whole sample population as well as by disease (table 4). The frequency of GG was .43. The frequency for AG was also .43 and the frequency for AA was .13 (figure 10). The AA genotype is seen in the highest

amount in DF and CD. The AG genotype is evenly distributed among the cases, but is less frequent in the control samples. Likewise, GG is constant among the cases, but higher in the controls (figure 11).

Table 4. Genotype by dengue diagnosis: rs3409386

	Dengue Diagnosis				
Genotype	DF	CD	DHF	ND	TOTAL
GG	15	27	4	31	77
AG	16	39	6	16	77
AA	7	13	1	4	25
	38	79	11	51	179
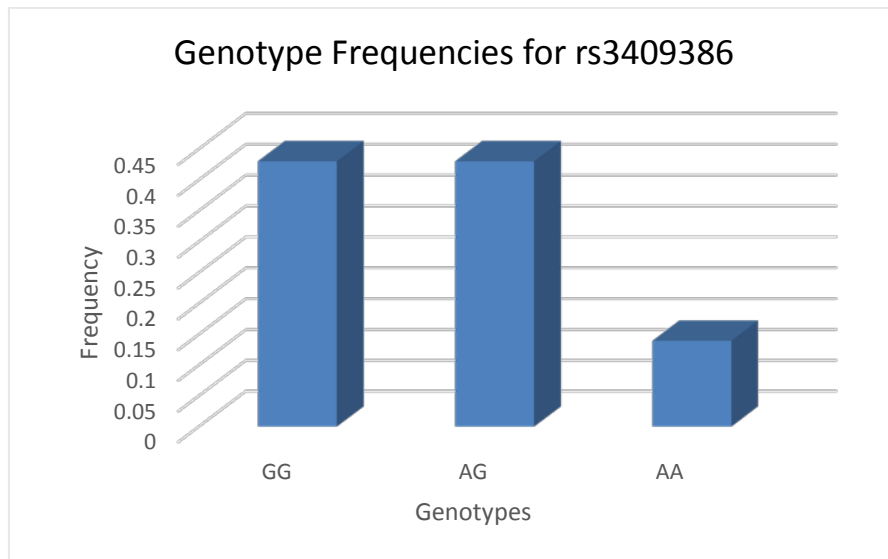
Figure 10. Genotype frequencies for rs3409386
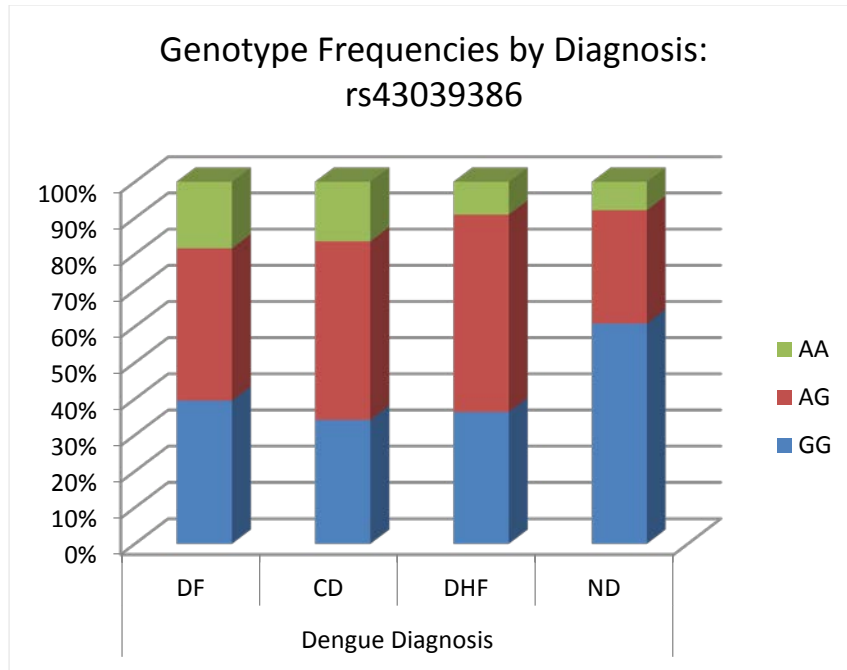
Figure 11. Genotypes by dengue diagnosis: rs34039386

3.1.3 Allele Frequencies

In addition to investigating genotype frequencies, it was also necessary to study the allele frequencies and how they might differ between diagnoses. The SNP rs2296414 was studied first. The frequency of the T allele in the whole sample population is .18 or 18%. The frequency of the C allele is .82 or 82% (figure 12).
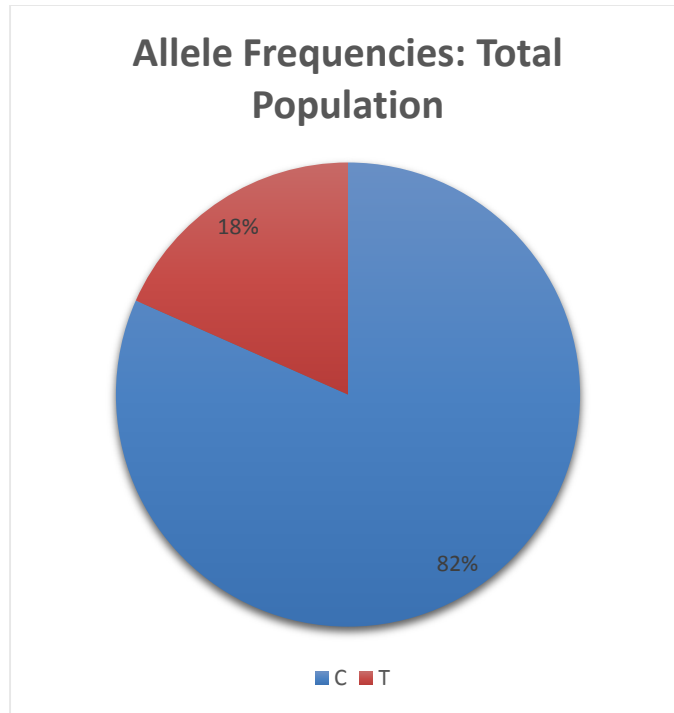
Figure 12. Allele Frequencies for rs2296414

When broken down by diagnosis, not much difference can be seen in the allele frequencies. There is a slightly higher proportion of T allele in the DHF cases than is seen in other diagnoses. The proportion of the T allele is lowest in DF cases. The proportion of the C allele is highest in DF cases and lowest in DHF cases (figure 13).

The proportion of the A allele of rs34039386 is .35 or 35% in the sample population and the frequency of the G allele is .65 or 65% (figure 14). When broken down by diagnosis, there is a somewhat clearer difference in the proportion of alleles than was seen at the other site. The highest proportion of A alleles is seen in CD cases and the lowest is seen in ND controls. Consequently, the highest proportion of G alleles is seen in control and the lowest is seen in CD dengue cases (figure 15).
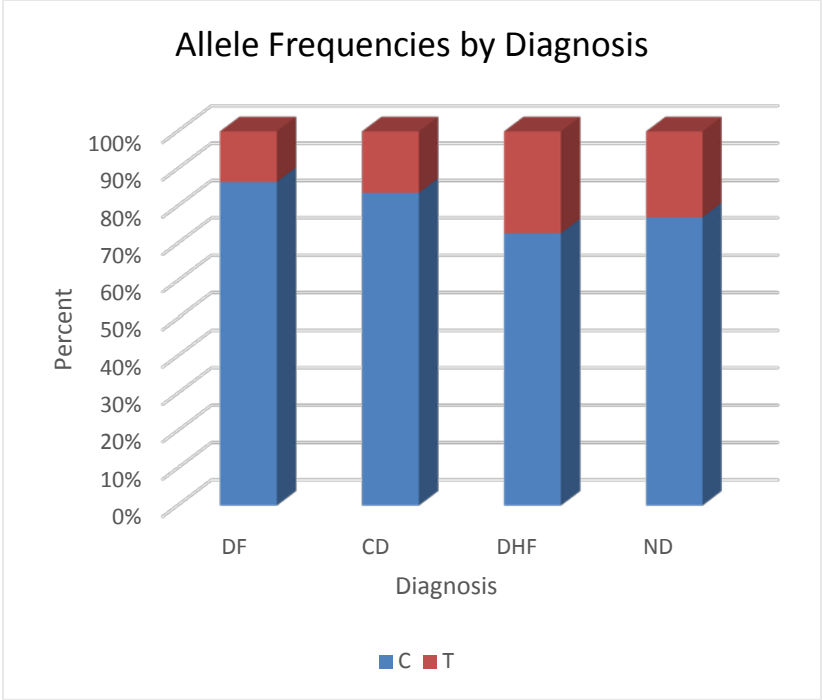
Figure 13. Allele Frequencies by diagnosis: rs2296414
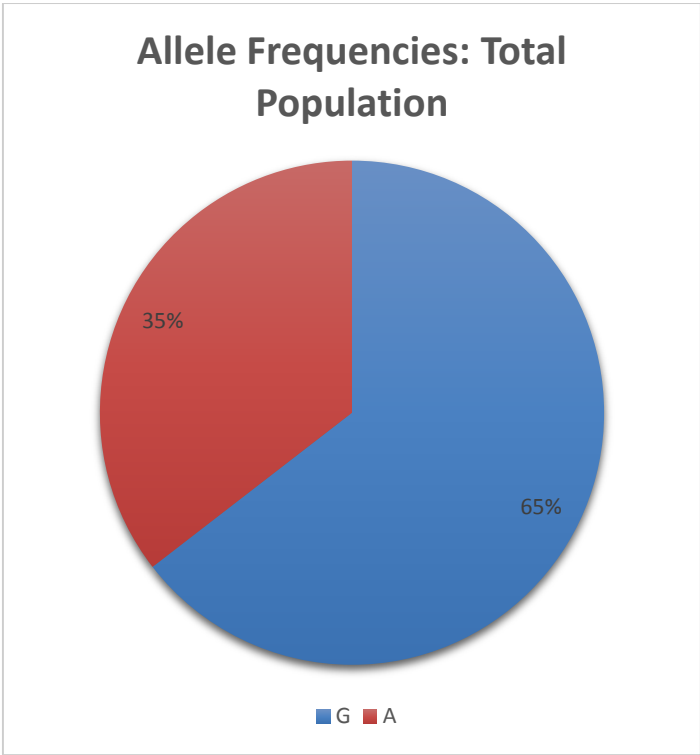
Figure 14. Allele frequencies for rs34039386
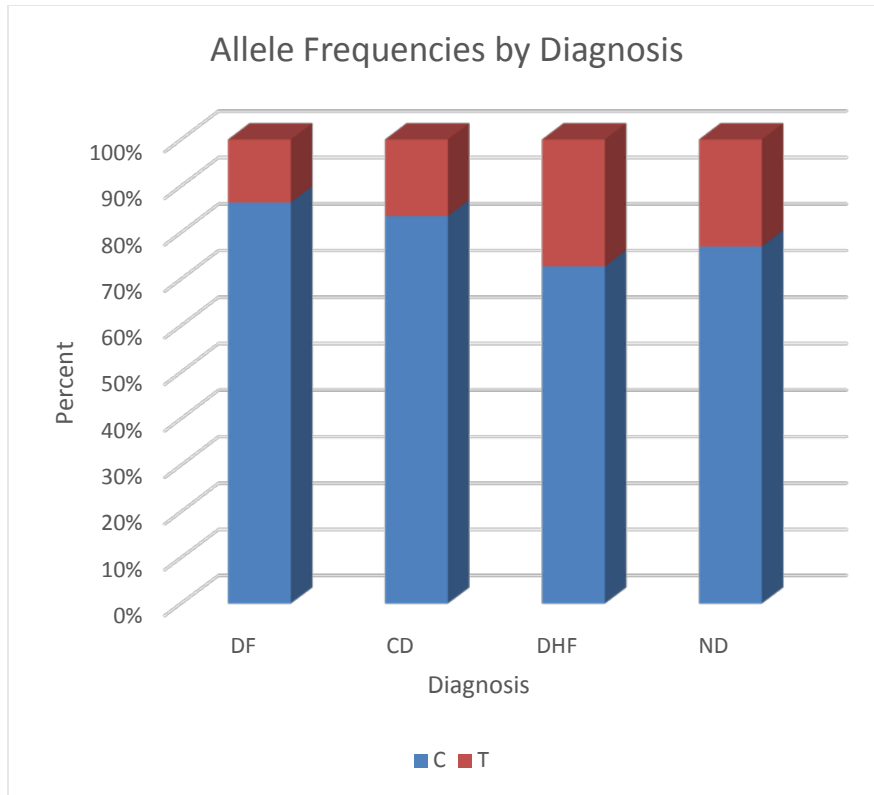
Figure 15. Allele Frequencies by Diagnosis: rs34039386

3.2   HARDY-WEINBERG EQUILIBRIUM

Observed genotype frequencies and observed allele frequencies were used to calculate expected genotype frequencies using the Hardy-Weinberg equilibrium equation,

$$p^2 + 2pq + q^2 = 1$$

Chi-squared analysis was used to determine if the sites were in Hardy- Weinberg and if there were any deviations within diagnosis groups. Departures from HWE could indicate errors in the genotyping itself, but could also suggest the presence of demographic features in the population (non-random mating, population substructure/admixture) as well as a selective advantage conferred by one genotype in the presence of dengue infection

3.2.1   rs2996414

The whole sample was found to be in Hardy-Weinberg Equilibrium with regards to rs2296414. A non-significant p-value from chi-squared analysis showed no departure from equilibrium (table 5). In addition to the whole sample, both the cases and controls are in Hardy-Weinberg equilibrium as well when considered separately (table 5).

Table 5. Observed and expected genotype frequencies for sample population: rs2296414

All Samples		Cases	Controls
CC	115	88	27
CT	59	36	23
TT	3	3	0
No Data	2	1	1
Informative Total	177	127	50
Alleles			
C	289	212	77
T	65	42	23
T frequency	18.36%	16.54%	23.00%
C frequency	81.64%	83.46%	77.00%
Expected Genotypes			
CC	117.97	88.47	29.65
CT	53.06	35.06	17.71
TT	5.97	3.47	2.65
Total	177.00	127.00	50.00
Chi-squared			
	0.330528	0.954914	0.107468

The p-value for the chi-squared test for Hardy-Weinberg equilibrium in the whole population is .33. For the cases it is .95, and for the controls .11. When investigated by diagnosis, the samples are still within Hardy-Weinberg equilibrium. No significant p-values were found when values of <.05 are considered significant. The observed and expected genotype frequencies as well as the allele frequencies and chi-squared results can be found in table 6.

Table 6. Observed and expected genotype frequencies by diagnosis: rs2296414

Observed Genotypes	DF	CD	DHF	ND
CC	27	54	7	27
CT	10	24	2	23
TT	0	1	2	0
No Data	1	0	0	1
Informative Total:	37	79	11	50
Alleles				
Total C Alleles	64	132	16	77
Total T Alleles	10	26	6	23
T frequency:	13.51%	16.46%	27.27%	23.00%
C frequency:	86.49%	83.54%	72.73%	77.00%
Expected Genotypes				
CC	27.7	55.1	5.8	29.6
CT	8.6	21.7	4.4	17.7
TT	0.7	2.1	0.8	2.6
Total	37	79	11	50
Chi-Squares				
	0.63657	0.647512	0.199146	0.107468

3.2.2   rs34039386

As with the previous SNP, the whole sample is in Hardy-Weinberg equilibrium with regards to rs34039386. The cases and controls are also in Hardy-Weinberg for this location. The p-value of the chi-squared test for significance for the whole sample population is p=.72. The values for cases and controls are p=.99 and p=.66, respectively (table 7). It was also found that the populations remain in Hardy-Weinberg equilibrium when broken down by diagnosis (table 8). No significant results are seen when a p-value of <.05 is considered significant.

Table 7. Observed and expected genotype frequencies for sample population: rs34039386

Observed	Whole Sample	Cases	Controls
GG	77	46	31
AG	77	61	16
AA	25	21	4
Total	179	128	51
Allele			
G	231	153	78
A	127	103	24
G frequency	64.53%	59.77%	76.47%
A frequency	35.47%	40.23%	23.53%
Expected			
GG	74.53	45.72	29.82
AG	81.95	61.56	18.35
AA	22.53	20.72	2.82
Total	179	128	51
Chi-Squared			
	0.721691	0.994744	0.657617

Table 8. Observed and expected genotypes by diagnosis: rs34039386

Observed Genotypes	DF	CD	DHF	ND	TOTAL
GG	15	27	4	31	77
AG	16	39	6	16	77
AA	7	13	1	4	25
Total	38	79	11	51	179
Alleles					
G	46	93	14	78	231
A	30	65	8	24	127
G frequency	0.61	0.59	0.64	0.76	
A frequency	0.39	0.41	0.36	0.24	
Expected Genotypes					
GG	13.92	27.37	4.45	29.82	77
AG	18.16	38.26	5.09	18.35	77
AA	5.92	13.37	1.45	2.82	25
Total	38	79	11	51	179
Chi-Squared					
	0.764649	0.985312	0.839136	0.657617	

3.3   ODDS RATIOS

3.3.1   rs2994614

Odds ratios were calculated for each site as well. The odds ratios were first calculated from the results of this study alone. To boost sample size and to further test the proposed association, results obtained from this study were combined with results of genotyping from the previous study[34]. In the combined test, the controls from the previous sample were used in place of combining control groups. This is because the controls for this sample were from a different group than the controls used in the previous study. The controls for this sample came from patients admitted to hospitals with other febrile diseases that were determined to be free of dengue. The controls used in the previous study were from individuals receiving a yellow fever vaccine that were determined to not have dengue. The controls from the previous study were used for the combined odds ratios because they gave a larger sample size. Odds ratios were calculated between comparisons of (TT+CT) and CC, and comparisons of TT and (CT+CC). This was done between cases and non-dengue controls and between severe dengue (CD+DHF) and classic dengue (DF), as well as dengue hemorrhagic fever (DHF) versus all other classifications of dengue. This analysis would reveal whether any allele was preferentially associated with disease status, and whether this association showed a Mendelian dominant or recessive pattern. The only significant results (OR=25.5, P=.01) from this analysis were found when DHFx(CD+DF) was compared for the TT genotype versus the other two genotypes (table 9). No other significant results were seen when the novel samples were analyzed alone (table 9).

Table 9. Odds ratios: rs2296414

	Dengue Cases	Non-dengue controls	Odds Ratio	95% CI	P-value
TT+CT	39	23	.52	0.27-1.02	.056
CC	88	27			
	Dengue Cases	**Non-Dengue Controls**	**Odds Ratio**	**95% CI**	**P-value**
TT	3.5	.5	2.84	0.14-55.97	.49
CT+CC	124.5	50.5			
	Severe Dengue (CD+DHF)	**Classic Dengue (DF)**	**Odds Ratio**	**95% CI**	**P-value**
TT+CT	29	10	1.28	0.55-3.00	.56
CC	61	27			
	Severe Dengue (CD+DHF)	**Classic Dengue (DF)**	**Odds Ratio**	**95% CI**	**P-value**
TT	3.5	.5	3.00	0.15-59.53	.47
CT+CC	87.5	37.5			
	DHF	**DF+CD**	**Odds Ratio**	**95% CI**	**P-value**
TT+CT	4	35	1.32	.36-4.8	.67
CC	7	82			
	DHF	**DF+CD**	**Odds Ratio**	**95% CI**	**P-value**
TT	2	1	25.56	2.1-309.56	**.01**
CT+CC	9	115			

When combined with the data from the previous study, a significant result (OR=1.92, p=.0013) was found when comparing cases and non-dengue controls. This significance was only seen between cases and controls and not between severe dengue and classic dengue (table 10). A

significant result was also carried over from the DHF versus (DF+CD) comparison. A highly
significant p value was seen (p=.0001).

Table 10. Combined Odds ratios: rs2296414

	Dengue Cases	Controls	Odds Ratio	95% CI	P-value
TT+CT	135	42	1.92	1.29-2.87	**.0013**
CC	259	155			
	Dengue Cases	Controls	Odds Ratio	95% CI	P-value
TT	9.5	3	1.59	0.43-5.90	.49
CT+CC	385.5	194			
	Severe Dengue	Classic Dengue	Odds Ratio	95% CI	P-value
TT+CT	101	50	1.52	1.00-2.30	.052
CC	148	111			
	Severe Dengue	Classic Dengue	Odds Ratio	95% CI	P-value
TT	8.5	1.5	3.77	0.66-21.61	.14
CT+CC	241.5	160.5			
	DHF	DF+CD	Odds Ratio	95% CI	P-value
TT	5	4	15.29	3.91-60.28	**.0001**
CT+CC	29	356			

3.3.2   rs34039386

As with the previous SNP, the odds ratios for rs34039386 were calculated first on their own and
then combined with the data from the previous study. Also, as previously stated, the controls used

in the combined analysis were only the controls from the previous study. Dengue cases versus non-dengue controls, severe dengue vs. classic dengue, and DHF vs. (DF+CD) were all investigated. A significant association was found between dengue cases and non-dengue controls (OR=2.76, p=.003). No significant association was found between severe dengue and classic dengue, or between DHF and (DF+CD). The results of the different odds ratios can be seen in table 11.

Table 11. Odds ratios: rs34039386

	Dengue Cases	Non-Dengue Controls	Odds Ratio	95% CI	P-value
AA+AG	82	20	2.76	1.42-5.39	**.003**
GG	46	31			
	Dengue Cases	Non-Dengue Controls	Odds Ratio	95% CI	P-value
AA	21	4	2.31	0.75-7.09	.14
AG+GG	106	47			
	Severe Dengue	Classic Dengue	Odds Ratio	95% CI	P-value
AA+AG	59	23	1.24	0.57-2.71	.58
GG	31	15			
	Severe Dengue	Classic Dengue	Odds Ratio	95% CI	P-value
AA	14	7	.82	0.3-2.21	.68
AG+GG	76	31			
	DHF	DF+CD	Odds Ratio	95% CI	P-value
AA+AG	7	75	.98	.27-3.54	.98
GG	4	42			
	DHF	DF+CD	Odds Ratio	95% CI	P-value
AA	1	20	.48	.05-4.05	.5
AG+GG	10	97			

When combined with the data from the previous study, the significant result seen between cases and non-dengue controls is no longer apparent. No significant results were seen in the combined data in either the cases/controls comparison or in the severe/classic comparison. Results from these odds ratios can be seen in table 12.

Table 12. Combined Odds Ratios: r34039386

	Dengue Cases	Controls	Odds Ratio	95% CI	P-value
AA+AG	197	100	1.18	0.81-1.70	.39
GG	134	80			
	Dengue Cases	Controls	Odds Ratio	95% CI	P-value
AA	47	33	.74	0.45-1.20	.22
AG+GG	284	147			
	Severe Dengue	Classic Dengue	Odds Ratio	95% CI	P-value
AA+AG	132	65	**1.41**	0.9-2.23	.14
CC	79	55			
	Severe Dengue	Classic Dengue	Odds Ratio	95% CI	P-value
AA	31	16	**1.12**	0.58-2.14	.73
AG+GG	180	104			

4.0 DISCUSSION

4.1 DEMOGRAPHIC RESULTS

The lack of significance between sex and disease represented a departure from what was seen in the previous study[34] and in the cohort from which the samples were selected[32]. Both Cathcart and Cordeiro found that female sex was significantly associated with dengue infection in this cohort. This departure may just be due to random variations within the samples or may result from the smaller sample size of this study. It is not well-understood what the connection between sex and dengue infection may be, so it is difficult to speculate what may be different within this specific set of samples.

4.1.1 rs2994614 Results

The observed genotypes open up some interesting questions. For rs2296414, a slightly higher proportion of TT genotypes can be seen in the DHF diagnosis group, while no TTs were found in the control group (figure 9). Because of the low frequency of the T allele in the global population (.146)[30], this sample had a small enough number of the rare homozygotes to make it difficult to determine if this difference may represent an actual difference or if it is just caused by random variation in this sample. Studies with larger samples sizes should be conducted to give enough power for this analysis to be possible. If this pattern can be established by larger studies, it may suggest that the TT genotype is associated with progression to DHF or other severe forms of

dengue infection. As can be seen in a later section some results from this study do suggest that this may be the case. This supports the evidence gathered from the previous research[34].

The presence or absence of Hardy-Weinberg equilibrium (HWE) is an important indicator of the accuracy of genotyping, as well as other, more complicated genetic influences. Deviations from HWE can indicate that a high proportion of samples were genotyped incorrectly. These samples were shown to be in Hardy-Weinberg for both cases and controls. Significance testing with chi-squared showed no deviations from HWE. When broken down by diagnosis, HWE is maintained throughout. It is clear that for this population, Hardy-Weinberg is maintained throughout. This is an important indicator that our samples were genotyped correctly in the majority of cases.

Odds ratios were conducted to test if each allele at this location is acting in a dominant or recessive nature with relationship to disease status. A significant association was found at this SNP when DHF was compared to all other dengue diagnoses. It was only seen when comparing the TT genotype with the other genotypes. This association suggests that there may be a relationship present between the TT genotype and the progression to DHF.

These results are limited, though, so it is not possible to determine what that relationship may be. It does open up research further into the question, however. The association between DHF and the TT genotype was the only significant result seen in analyzing the novel data. The test of the other associations may have been complicated by the very low number of TTs represented in this population. There were likely not enough TT genotypes present to get an accurate assessment of the true relationship. Because of the small sample size of the novel, this data was combined with data from the previous study. When analyzed, there was a significant association between cases and controls in relationship to the T allele (OR=1.90, p=.0013). This suggests that the T allele is

significantly associated in dominant manner with dengue disease. This corroborates the findings from the previous study which also found that the T allele was significantly associated with disease[34]. The significant result seen in the novel data when comparing DHF to (DF+CD) was also seen using the same comparisons in the combined data. A highly significant p-value was seen (p=.0001). This continues to support the evidence suggesting that in the case of DHF, the TT genotype and not just the T allele may play an important role in susceptibility.

This association between the TT genotype and disease is supported by what is understood of the process of dengue virus infection of macrophages. The mutation at rs2296414 results in a change from threonine to asparagine in the fibronectin domain of the mannose receptor. Because it has been shown that the dengue virus binds to the CRD, domain changes in the fibronectin domain most likely do not directly affect the entry of dengue into the cell, but it is possible that the amino acid switch may cause a conformational change in the receptor that makes binding or entry of the virus more difficult, if not impossible [22, 24, 25]. Research has shown that binding of the dengue virus to the mannose receptor is an important piece in the process of the virus infecting macrophages, so changes in this receptor may represent important factors in the infection process[24].

4.1.2   RS34039386

The SNP rs34039386 also showed some interesting results when genotypes were viewed. It can be seen in figure 11 that AG and AA are highest among the different dengue diagnoses, while GG is highest among the ND controls. This represents a possible association between the A allele and dengue infection. This is an interesting observation, as previous studies have found an association between this specific SNP and susceptibility to other infectious diseases[29]. Because a

relationship was seen between another infectious disease (tuberculosis) and this SNP, further research is definitely warranted. There may be important differences between these cases, however, because of the nature of the infectious diseases in question. Tuberculosis is a bacterium and dengue is a virus, so the mechanisms of infection and their relationship to the mannose receptor may be very different.

The distribution of genotypes seen for rs34039386 was also tested to determine if it was in Hardy-Weinberg equilibrium. It was found to be in equilibrium overall, as well as in cases and controls. When broken down by diagnosis, no departures from Hardy-Weinberg were found within these samples. The same results can be concluded from this as from the previous site.

Odds ratios for this SNP were also calculated. The relationship here was reversed from what was found at the other site with regards to the data analyzed separately and the data analyzed together. When the data from this study was analyzed on its own, a significant relationship was found between the A allele and disease (OR=2.76, p=.003). When the data was combined with the previous data, the association was no longer present. This is possibly because the previous study found no association at this site. It was speculated in that study that the fact that the population was out of Hardy-Weinberg for this SNP may have impacted the ability to tell if there was a true association present. This is supported by the fact that an association was found in a sample where the population was in Hardy-Weinberg equilibrium.

An association between disease and genotype at this location is very possible because of the nature of the site. The SNP rs34039386 is located in exon 7 of the *MCRI* gene which is an exon that has previously been shown to have associations with susceptibility to several infectious diseases [27-29]. This SNP codes for part of the CRD-2 domain of the mannose receptor[25] and

while dengue has not been shown to bind to this specific part of the CRD, it has been shown that this is the region of the receptor responsible for binding the dengue virus[24].

4.2   LIMITATIONS

This study has several limitations. Most importantly is the small sample size, which limits the power of the statistical testing and the likelihood of finding significant results. The biggest part of this limitation is the fact that the low frequency of the T allele of rs2296414 means that there are very few of these genotypes represented in these sample populations. This limits the ability to test for association between this genotype and disease status.

Another important limitation is the generalizability of the study. These samples only represent a small population of patients from a specific location in Brazil. Dengue epidemiology differs markedly between different global populations. In addition, it is common for allele frequencies to differ significantly between different populations and different ethnic and racial backgrounds. Because of these variations, it is possible that associations found may be specific to this Brazilian population and may not be generalizable to the larger global population. Additional studies with more diverse ethnic and racial backgrounds will be needed to determine how these associations may persist across different populations.

A third limitation is the lack of data on dengue type of dengue infection in the samples. This means if it was a primary dengue infection or a secondary dengue infection. Some samples had data on this, but it was not consistent enough across the whole data set to be looked at as a confounding factor or association. It is well documented that secondary infection is associated with

more severe disease[6]. This association was not seen in the population in this study, however[32]. Further studies will be necessary to test the association of genotype and infection type.

4.3   FURTHER DIRECTIONS

The results from this study and from the previous study suggest multiple directions that could be taken for further research. In addition to the obvious need for larger sample sizes and more diverse populations, there are many other directions to take. If more studies continue to find an association between these genotypes and dengue infection, there are many potential directions the research could take next. Haplotype and linkage analysis would more fully tell us what is happening at these particular SNPs and how their association with disease may be linked with other SNPs or alleles within the MRCI gene.

It would also be beneficial to investigate how the genotype in these cases affects the phenotype of the individual. Protein analysis could give answers on if and how these SNPs may be changing the way dengue infects macrophages through the mannose receptor.

As previously mentioned, it is not completely understood how dengue is internalized into host cells. Continuing to build understanding of that process would allow future research to better understand the potential mechanisms at play and how SNPs or other mutations within the mannose receptor or other candidates may change the viruses' ability to bind to or be internalized into host cells.

Finally, because the four serotypes are similar but distinct, there may be subtle but important differences between them. The host genetics that affect the susceptibility of a host to

one serotype may not have the same effect on the others. Studies should be done to compare the different serotypes' association with these SNPs and other host genetic factors.

5.0   PUBLIC HEALTH SIGNIFICANCE

Dengue is one of the fastest-spreading global infectious diseases. There is no doubt of the critical public health significance of this disease. While there are many important things about this disease that make it an important focus of research and development, perhaps the most important is its unique relationship to global climate, shifting climate patterns, and global travel. Because dengue is a mosquito-borne disease, its spread and relevance as a global infection is directly tied into the habitat of the *Aedes* mosquito, its primary vector. Another area of public health significance is the difficulty and challenges of vaccine development for dengue. Because of this, a deeper understanding of all factors that play a part in infection (including host genetics) is critical for further developing an effective and safe vaccine.

5.1   DENGUE AND CLIMATE CHANGE

Over the past several decades, shifting global climate has allowed for the expansion of the range of the primary vectors for dengue, *Aedes aegypti* and *Aedes albopictus*, to almost all areas at lower and middle latitudes[13]. These increased ecological niches for the vectors of dengue and other disease carried by these mosquitos poses a significant public health risk, as a large portion of the world population lives in these areas. Some estimates suggest that close to 4 billion people live in areas that put them at risk for dengue[2]. This number may continue to grow as climate shifts and global warming continues. In the past decade, dengue has been found in locations where it had previously been absent. Beginning in 2009, sporadic outbreaks dengue began in Monroe County

in southern FL. It was determined that these cases were not all travel-associated and that local transmission of the disease was occurring[36]. Local *Aedes aegypti* mosquitos were found to be infected with strains of dengue that were linked to strains from Nicaragua, Mexico, and Puerto Rico. Dengue is endemic in these areas, and the potential for continued introduction to the local mosquito population through travel is very high[36].

Another worrying trend is the spreading habitat of the other *Aedes* species able to transmit dengue, *Aedes albopictus*, or the Asian tiger mosquito. This mosquito is not as competent a vector for dengue as its cousin, but studies suggest that it is able to outcompete the *aegypti* species as larva. It is an incredibly aggressive mosquito that bites throughout the day and prefers urban areas and humans as its blood meals[14]. The range of the Asian tiger mosquito is also continuing to expand. It can be found as far north as New Jersey and New York, and as far west as western Pennsylvania. Because of the increasing range of this vector, the potential for introduction of the virus into the local mosquito populations is high. Global travel has made it easier for diseases to spread quickly and easily from distant locations. The fact that the closest locally-acquired dengue cases are in southern Florida may not stop the disease from becoming established among more mosquito populations within the United States.

5.2   VACCINE DEVELOPMENT CHALLENGES

Vaccine development for dengue is very challenging. The main goal of the development of an effective vaccine for dengue is the necessity for it to protect against all strains. This is critical because of the lack of a cross-reactive response from the immune system between the different stains and because of the theory of antibody-dependent enhancement. A vaccine that does not

provide effective protection against all strains may protect well against one strain, but cause a much more serious reaction on a secondary infection with a different strain. Because of these challenges, no effective vaccine is yet available despite several trials[37], and vaccine targets are a growing area of research. The mannose receptor is considered an important vaccine target that may be able to increase the immunogenicity of vaccine antigens because of its ability to internalize targets and load them onto MHC complexes[22]. A better understanding of the effects of polymorphisms within the *MRCI* gene may be an important factor in designing an effective vaccine.

5.3   GLOBAL BURDEN

Dengue's increasing global burden and the lack of a vaccine make research into this disease an important focus of continuing public health research. Genetic research into host factors that affect the disease provide an interesting and increasingly important area of research. A better understanding of the complex factors that determine a person's susceptibility to diseases may allow researchers to create more specific and targeted treatments, and may lead to the development of a vaccine that is effective and safe across all serotypes, as well as the diverse populations at risk.

6.0   CONCLUSION

Dengue is an important and emerging infectious disease that has critical public health implications on a global scale. Detailed and ongoing research on the dynamic nature of this disease is important in continuing to understand how it spreads globally and infects its hosts. This study found a significant association between both of the SNPs investigated and dengue for certain tests. The rs2296414 SNP was found to be in Hardy-Weinberg equilibrium. The population was found to be in Hardy-Weinberg equilibrium with regards to cases and controls. When broken down by disease status, no departure from equilibrium was seen. A significant association was found between DHF and the TT genotype. A previous study (Cathcart) found an association between rs2296414 and dengue infection and an association between rs2296414 and severe disease outcomes. When data from this study was combined with data from the previous study, a significant association was seen between cases and controls (P=.0013). No significant association was seen when comparing severe dengue and classic dengue in either the novel samples or the combined samples.

As with rs2296414 Hardy-Weinberg equilibrium was tested using chi-squared analysis to determine significance for rs34039386. The population was found to be in Hardy-Weinberg equilibrium, as were the populations when broken down by disease status. Odds ratios were also calculated for rs34039386, and some significant results were found. There was a significant association found between the A allele and disease that indicates that it works in a dominant manner. This finding was not duplicated when the data was combined with the previous study's results. This may be because the previous study population was out of Hardy-Weinberg equilibrium for this SNP.

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This study, along with previous work, opens up an important area of research that has received limited attention. Further in-depth analysis with larger sample sizes and from more diverse global populations may find that these SNPs do have an effect on dengue infection or dengue severity, or it may be found that there are other critical SNPs within *MRCI* and other similar genes. Developing this research more may lead to more avenues for developing vaccines to dengue and designing more effective treatments.

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