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Madeira Dengue Outbreak

A tool to study primary symptomatic and asymptomatic infections

DOCTORAL THESIS

Paulo Marco Vasconcelos Henriques
DOCTORATE IN BIOLOGICAL SCIENCES

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Resumo

A febre da dengue é uma arbovirose que emergiu nas últimas décadas como uma séria ameaça à saúde mundial, colocando em risco quase metade da população mundial. A infeção por qualquer um dos quatro serotipos do vírus da dengue (DENV-1-4) resulta num amplo espectro de situações, desde infeções assintomáticas até doença severa que pode ser fatal. O desfecho assintomático parece ser o mais comum, podendo representar até 90% das infeções. Diferenças na virulência e capacidade de infeção de diferentes serotipos/genótipos de DENV, conjuntamente com a variabilidade genética dos hospedeiros, mecanismos de imunidade treinada e de tolerância à doença, são possíveis justificações para o desfecho assintomático e são extensivamente discutidos nesta tese.

Entre 2012 e 2013 ocorreu o primeiro e único surto de DENV-1 na ilha da Madeira, providenciando a oportunidade de investigar a resposta humoral em infeções primárias, bem como o seu impacto na saúde desta população exposta pela primeira vez ao DENV. Neste trabalho demonstra-se que os indivíduos que desenvolveram infeções assintomáticas durante o surto apresentam níveis muito baixos ou indetetáveis de anticorpos contra DENV-1 oito anos após a infeção. Por outro lado, as infeções sintomáticas parecem ter resultado numa resposta humoral forte, com níveis elevados de anticorpos contra DENV-1, e atividade neutralizante contra DENV-2. Sugerindo uma proteção não só homotípica, mas possivelmente também contra infeções heterotípicas. O impacto do surto de dengue no estado de saúde desta população, estudado através da caracterização retrospetiva dos sintomas experienciados durante o período de infeção, evidenciou que a maioria dos sintomas e as suas frequências são semelhantes às descritas noutros estudos e compatíveis com o desfecho menos severo característico de infeções primárias, exceto para os sintomas hemorrágicos, que foram mais frequentes do que se antecipava. Igualmente, verificou-se que as manifestações clínicas a longo termo ocorreram mais frequentemente e foram mais persistentes do que inicialmente pensado, influenciando o estado de saúde e bem-estar de uma porção considerável da população infetada. A análise adicional de parâmetros clínicos e laboratoriais, contribuiu para o conhecimento da dimensão clínica do surto de dengue na Madeira. Não obstante o desfecho clínico moderado, foram também encontradas

alterações laboratoriais sobretudo hematológicas e hepáticas, assim como sinais sugestivos de dengue severa em 5 pacientes, sugerindo um quadro de severidade relativamente amplo em infeções primárias.

Este trabalho conduziu a um melhor conhecimento da resposta humoral e do impacto clínico de uma infeção por DENV-1 numa população nunca anteriormente exposta a qualquer DENV.

Palavras-chave:

Febre da dengue; Infeção Assintomática; DENV-1; Fatores de Hospedeiro; Resposta Humoral; Desfecho clínico; Sintomas persistentes.

Abstract

Dengue fever (DF) is an arboviral disease that has emerged as a serious worldwide public health threat in the last decades, endangering almost half of the world's population. Infection with any of the four known serotypes of dengue virus (DENV-1-4) results in a broad spectrum of outcomes, ranging from asymptomatic to severe life-threatening illness. The asymptomatic outcome is thought to be the most common, which may reach up to 90% of all infections. Differences in virulence and infection capacity of the different DENV serotypes/genotypes, coupled with the host's genetic variability and mechanisms of trained immunity and disease tolerance, are likely determinants for the asymptomatic outcome of these infections and are thoroughly discussed in this thesis.

A primary and unique DENV-1 outbreak occurred in Madeira Island between 2012 and 2013, providing the opportunity to investigate the humoral immune response in primary infections, and the clinical impact of the outbreak on this naïve population. This first exposure of this population to DENV-1 is shown herein to have prompted a weak long-term humoral immune response in asymptomatic cases, with very low or undetectable DENV-specific antibody levels eight years post-infection. Conversely, symptomatic cases seem to have resulted in strong humoral responses, with high DENV-specific antibody levels, and cross-protective activity against DENV-2. These results suggest that symptomatic infections result in long-term immunity against homologous reinfection and maybe even against heterologous infection. Concerning the impact of the outbreak on the health status of this naïve population, a retrospective characterization of the symptoms experienced throughout the entire infection period revealed that most symptoms and their frequencies are in line with previous studies and are compatible with the milder outcome of primary infections, except for hemorrhagic symptoms, which were more common than anticipated. As for potential long-term manifestations, these occurred more frequently and persisted longer than previously thought, impacting the health status and well-being of a considerable proportion of the infected population. Further analysis of clinical, hematological, and biochemical parameters contributed to elucidate the clinical burden of the Madeira dengue outbreak. Despite the general mild clinical outcome, both hematological and liver abnormalities were also found, in both

adults and children, with 5 patients presenting signs compatible with severe dengue, suggesting a broader range of severity in primary infections.

Overall, this work allowed for a better understanding of the humoral immune response and the clinical impact of a DENV-1 primary infection, in a naïve population.

Keywords:

Dengue Fever; Asymptomatic Infection; DENV-1; Host Factors; Antibody Response; Clinical Outcome; Persistent Symptoms.

Abbreviation list:

ADE – Antibody dependent enhancement

ALT – Alanine aminotransferase

AR – Attack rate

AST – Aspartate aminotransferase

BCG – Bacillus Calmette-Guérin

C - Capsid

DCs – Dendritic cells

DC-SIGN - dendritic cell-specific ICAM-grabbing non-integrin

DENV – Dengue virus

DF – Dengue fever

DHF – Dengue hemorrhagic fever

DSS – Dengue shock syndrome

E – Envelope

ECDC – European Centre for Disease Control

ELISA – Enzyme-linked immunosorbent assay

ER – Endoplasmic reticulum

FCγRII – Fragment cristallizable region receptor II

FRNT – Focus reduction neutralization test

GAC-ELISA – IgG capture ELISA

HI – Hemagglutination inhibition

HLA – Human leukocyte antigen

IFN - Interferon

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IL - Interleukin

LCs – Langerhans cells

LDLR – Low-density lipid receptor

MAC-ELISA – IgM capture ELISA

MC – Mast cells

MMR – Measles-mumps-rubella

MOI – Multiplicity of infection

NAbs – Neutralizing antibodies

NAATs – Nucleic acid amplification tests

NK – Natural killer cells

NS – Non-structural proteins

PBMCs – Peripheral blood mononucleate cells

PCR – Polymerase chain reaction

PFU – Plaque forming units

PrM – Precursor membrane

PRR – Patter recognition receptors

RDT – Rapid diagnostic tests

RIG-I – Retinoic acid inducible gene 1

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT-PCR – reverse transcription polymerase chain reaction

Rus – Relative units

SR-BI – Scavenger Receptor B1

TAM – Tyro3, Axl and MerTK

TFH – T follicular helper cells

Th1 – T helper 1

TIM – T cell immunoglobulin and mucin

TLR – Toll like receptors

WHO – World Health Organization

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CHAPTER I

General Introduction

1. Introduction

Dengue fever (DF) is caused by 4 distinct, but closely related, serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4). Although considered a neglected tropical disease, dengue fever (DF) has represented a major health issue in the past few decades (Bhatt et al. 2013; Hotez and Kamath 2009), and it is believed that its incidence has increased 30-fold in the last 50 years, with dengue epidemics occurring annually in the Americas, Asia, Africa, and Oceania (WHO 2009). According to the World Health Organization (WHO), an estimated 50 million dengue virus (DENV) infections were thought to occur annually, of which 500,000 are cases of dengue hemorrhagic fever (DHF), resulting in 22,000 deaths, mainly among children (Gubler 2006; WHO 2009). However, more recent estimations point to approximately 400 million cases, of which around 300 million correspond to mild or inapparent infections (Bhatt et al. 2013), in over 100 countries worldwide, with roughly 3.6 billion people at risk of being infected (Diamond and Pierson 2015).

1.1. Dengue virus transmission and replicating cycle

DENV is an arthropod-borne virus of the *Flaviviridae* family, transmitted to humans by hematophagous mosquitoes of the genus *Aedes*, particularly *Aedes aegypti* (Rodhain and Rosen 1997) but also *Aedes albopictus* (Effler et al. 2005). Unlike most arboviruses that are spread through sylvatic transmission cycles, DENV establishes urban endemic/epidemic cycles in which humans are the primary amplifying host (Gubler 1998). Nonetheless, sylvatic transmission cycles between non-human primates and *Aedes* mosquitoes have been described in Asia and Africa (Diallo et al. 2003; Wolfe et al. 2001), although the contribution of this enzootic cycle to urban epidemic transmission seems to be limited (**Figure 1**).

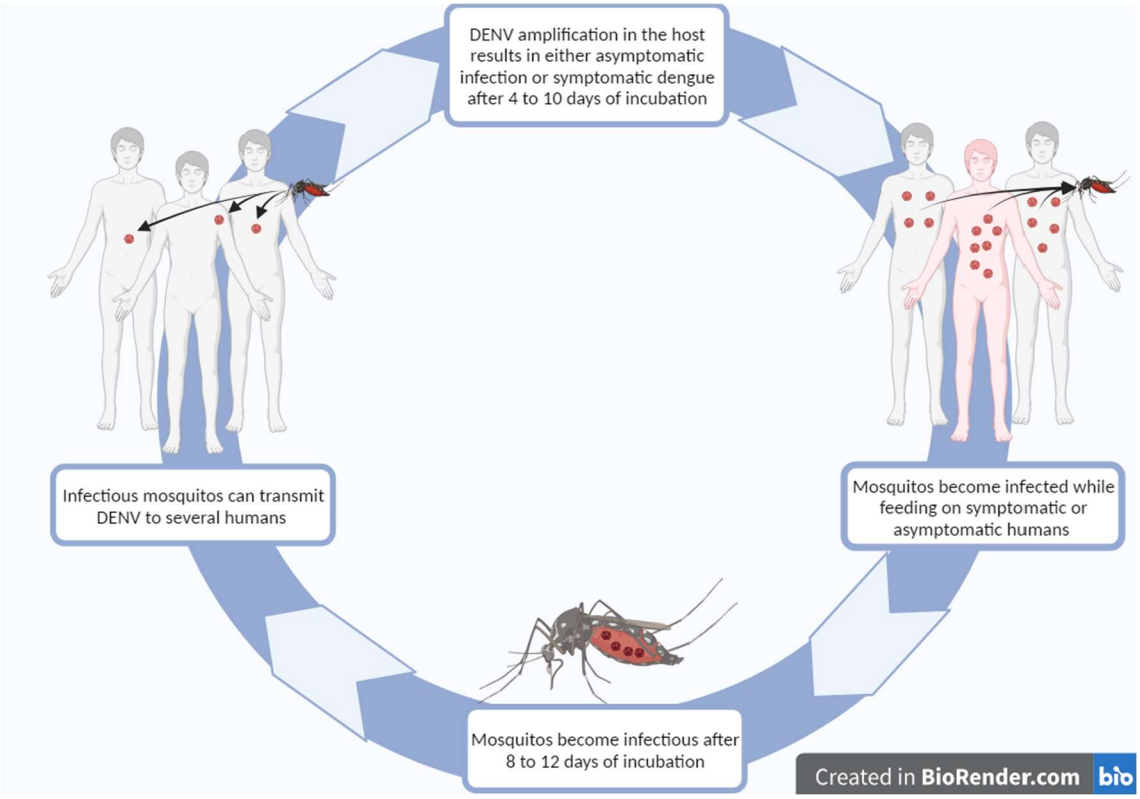

Figure 1 - Urban transmission cycle of dengue virus. Adapted from Guzmàn et al. (2016)

During the blood meal of an infected mosquito, *circa* 50,000 Plaque Forming Units (PFUs) of DENV are released into the epidermis of the host (Cox et al. 2012), where infection of skin cells, i.e. keratinocytes (Surasombatpattana et al. 2011), and immune cells such as Langerhans cells (LCs), macrophages (Halstead and O'Rourke 1977) and dermal dendritic cells (DCs) (Wu et al. 2000) occurs. Besides being permissive to infection, DCs capture virions and/or DENV antigens, transporting them to the draining lymph nodes (Marcial-Juárez et al. 2017; Schneider, Calvo, and Peterson 2021), where other cells of the immune system, such as blood-derived monocytes (Durbin et al. 2008), myeloid DCs (Wu et al. 2000) and T cells (Silveira et al. 2018) can be infected.

DENV is an enveloped virus, with a single-stranded positive-sense RNA of approximately 11kb in length encoding for a 3,411 amino acids precursor polyprotein, containing three structural proteins (capsid (C), precursor membrane (PrM) and envelope (E)), which are components of the mature virus particle, and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), which are only expressed in the infected cells (reviewed in Mukhopadhyay et al. 2005). Viral entry into

the cells (**Figure 2**) occurs via receptor-mediated endocytosis, starting with the binding of DENV's E protein to receptors in the membrane of target cells such as DC-SIGN, TIM (T cell immunoglobulin and mucin) and TAM (Tyro3, Axl, and MerTK), heparan sulfate, mannose receptors scavenger receptor B1 (SR-BI) (Chen et al. 1997; 2008; Li et al. 2013; Meertens et al. 2012; Miller et al. 2008; Tassaneetrithep et al. 2003). Upon internalization, the acidic pH of the endosome triggers a conformational change of the E protein, mediating the fusion of the viral envelope with the endosomal membrane. The viral nucleocapsid is then released into the cytoplasm, where the virus is uncoated, and the genome is released (Heinz and Allison 2003). Positive-stranded viral RNA is translated into a single polyprotein, further cleaved by viral and cellular proteases into structural and non-structural proteins. This process is followed by the synthesis of negative-stranded RNA, which serves as a template for the replication of the viral genome that, together with the viral proteins, is assembled into progeny virions (Clyde, Kyle, and Harris 2006). Nucleocapsid assembly occurs at the endoplasmic reticulum (ER) membrane, making use of the infected cell's machinery, where the newly synthesized RNA and the C protein are enveloped by the ER membrane. PrM and E structural proteins then encase the ER-enveloped nucleocapsid, forming immature virions. Immature viral particles are then transported to the Golgi apparatus, where furin-mediated cleavage of the PrM protein induces maturation of the virus (reviewed in (Aruna 2014).

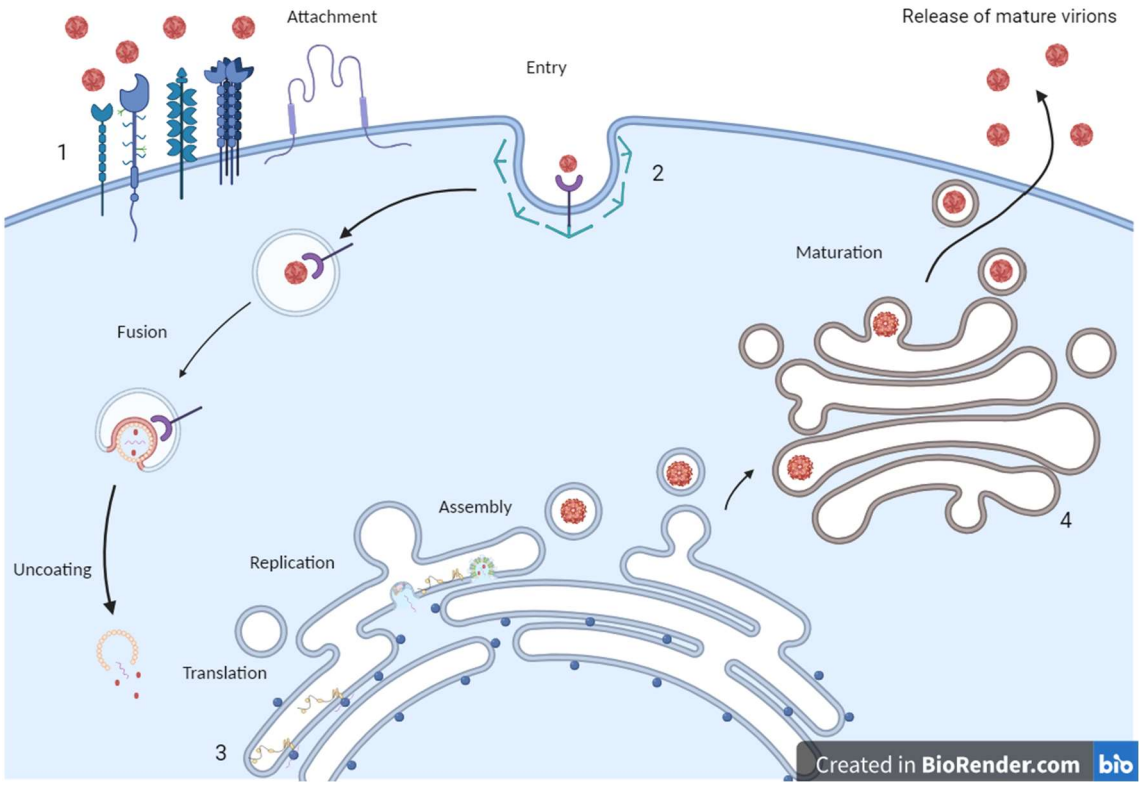

Figure 2 – Schematic representation of the replication cycle of DENV. 1- Binding Receptor (LECTIN, TIM, TAM, DC-SIGN, SR-BI); 2- Clathrin; 3- Endoplasmic Reticulum; 4- Golgi Apparatus. Adapted from Nanaware et al. (2021).

1.2. Clinical and Laboratory Diagnosis of DENV infection

DF is a systemic disease that presents a wide spectrum of clinical manifestations, including undifferentiated febrile illness, classical DF, and the more severe forms DHF and Dengue Shock Syndrome (DSS), which can lead to death (Gubler 2002). The diagnosis of DF relies on a combination of clinical diagnosis, epidemiological context, and laboratory confirmation (reviewed in Bäck and Lundkvist 2013). A probable case implies both, a clinical diagnosis, and compliance with epidemiological criteria, while a *confirmed case* is defined as a probable case with laboratory results confirming the presence of the virus (see below).

Concerning the clinical diagnosis of DF, the classification of WHO (WHO 2009) divides dengue cases into two categories: i) non-severe dengue, sub-divided into patients with and without warning signs, and ii) severe dengue. Non-severe dengue resembles undifferentiated viral infections, presenting fever and at least two of the following symptoms: nausea and vomiting; rash; aches and pains; and positive

tourniquet test and leukopenia, in patients that reside or have traveled to dengue endemic regions. In non-severe dengue, the emergence of warning signs foresees a possible progression to severe dengue. These warning signs include abdominal pain, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy and restlessness, hepatomegaly, and increased hematocrit, concurrent with a rapid decrease of the platelet count (WHO 2009). Severe dengue is characterized by serious plasma leakage, leading to hypovolemic shock and fluid accumulation with respiratory distress, severe bleeding, and critical organ impairment that can be detected by increased liver transaminases (Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT)), impaired consciousness, or other parameters indicative of the involvement of organs (WHO 2009).

Typically, the clinical course of dengue fever follows different phases. The first phase is called the febrile phase, which begins after a period of 3 to 14 days of incubation (Chan and Johansson 2012) and coincides with a sudden onset of symptoms such as fever, headache, arthralgia, myalgia, and anorexia, lasting up to 7 days (WHO 2009). From the second day of fever, patients may also exhibit leucopenia, thrombocytopenia, and rising hematocrit and, by the end of the febrile phase, cutaneous bleeding manifestations such as petechiae may arise. Patients with non-severe DF typically start to recover after defervescence. On the other hand, in a fraction of patients, the development of warning signs during the febrile phase may indicate an entry in the critical phase, which lasts up to 48 hours and is characterized by systemic vascular leakage, occurring with transient defervescence, and that may lead to severe shock and multi-organ dysfunction (WHO 2009). The critical phase is also associated with an increased risk of hemorrhagic manifestations and liver impairment (Kularatne 2015). Once the systemic vascular leak stops, the patient enters the recovery phase, in which a drop in the hematocrit and a swift increase in white cell and platelet counts signal the patient's improvement of health, and a full recovery occurs usually within one to two weeks (Kularatne 2015; Wilder-Smith et al. 2019).

The manifestations of DF can involve multiple systems. The detection of DENV RNA in post-mortem studies (Kularatne et al. 2014) suggests that DENV can infect multiple organs, causing inflammation and, consequently, possible organ failure. In fact,

hepatic manifestations such as liver injury and hemorrhagic necrosis of the liver have been reported by Fabre et al. (2001) and Kularatne et al. (2014), respectively. Cardiac involvement, such as myocardial impairment, arrhythmias, and fulminant myocarditis have also been reported (reviewed in Rahim et al. 2022; Weerakoon et al. 2011). Additionally, neurologic manifestations such as encephalitis, myelitis, meningitis, and Guillain-Barré Syndrome have also been described (Dalugama et al. 2018; Kularatne, Pathirage, and Gunasena 2008; Patey et al. 1993; Puccioni-Sohler, Rosadas, and Cabral-Castro 2013), as well as ophthalmologic conditions (Ng and Teoh 2015; reviewed *in* Trivedi and Chakravarty 2022). Chronic fatigue syndrome has been described as a long-term consequence of DF (Seet, Quek, and Lim 2007), similar to what has been reported for other viral infections (Garcia et al. 2014; Prins, van der Meer, and Bleijenberg 2006).

Probable dengue cases, assessed by clinical diagnosis and considering the epidemiological context, can then be laboratory-confirmed by the detection of viral antigen or nucleic acid, or specific anti-DENV antibodies (WHO 2009). However, the time window to successfully detect DENV in patient serum is short, since viraemia peaks before the onset of symptoms. By the time the patient seeks medical assistance, the viraemia might have already significantly decreased.

Commercial Rapid Diagnostic Tests (RDTs), which target the detection of the NS1 antigen (essential for viral replication) or IgG/IgM antibodies, are widely used to diagnose DENV infection due to their quickness, inexpensiveness, and simplicity of use. Several studies suggest that these tests are appropriate for the diagnosis of DENV infection (Kittigul and Suankeow 2002; Vajpayee et al. 2001; Vaughn et al. 1998). However, some studies report that the results of RDTs should be considered with caution, as they report highly variable sensitivity and, in some cases, variable specificity (Blacksell et al. 2006; Garg et al. 2019).

Several nucleic acid amplification tests (NAATs) have also been developed for the laboratory diagnosis of DENV infection (reviewed in Guzman et al. 2010), in particular reverse transcriptase PCR (RT-PCR) assays, targeting different genes and enabling serotype identification. The first assays to be designed were nested RT-PCR assays (Lanciotti et al. 1992), in which an initial reverse transcription and amplification step (using universal DENV primers targeting the C/PrM region of the genome) was followed

by a serotype-specific nested RT-PCR (Lanciotti et al. 1992). The products of these reactions were then visualized by electrophoresis in agarose gels, allowing for the identification of DENV serotypes based on the molecular weight of the amplicons. More recently, real-time RT- PCR one-step assays were developed, using primer pairs and DENV serotype-specific fluorescent probes, allowing for viral detection and quantification. These can detect a single serotype (singleplex) or several serotypes simultaneously (multiplex), without the need for electrophoresis (WHO 2009), being nevertheless useful only during viremia.

Due to the relatively high cost of NAATs, laboratory diagnosis of DENV infection is more commonly done by serological tests, aimed at detecting either the DENV NS1 antigen or anti-DENV specific IgM and/or IgG antibodies in serum and/or plasma, with several commercial test kits being currently available. NS1 is detectable in serum by enzyme-linked immunosorbent assay (ELISA), usually since fever onset up to 9 days post-infection (Alcon et al. 2002; Lapphra et al. 2008; Libraty et al. 2002; Schilling et al. 2004). Anti-DENV IgM antibodies become measurable 4 to 5 days after fever onset and remain detectable for up to three months. Anti-DENV IgG antibodies are detectable a few (~2) days later than IgM and may last for several years (**Figure 3**; reviewed in (Bäck and Lundkvist 2013). The IgM antibody-capture ELISA (MAC-ELISA) can detect anti-DENV IgMs in patients' sera by using capture antibodies specific for the μ-chain of human IgMs. DENV-specific antigens are bound to the captured anti-DENV IgM antibodies and detected by mono/polyclonal antibodies, directed mostly at the E protein, conjugated to an enzyme that produces a colorimetric reaction. Although this method has good sensitivity and specificity, it can only be used after the fifth day following fever onset and might be prone to cross-reactivity with other flaviviruses (WHO 2009). The IgG antibody-capture ELISA (GAC-ELISA) uses the same antigen as the MAC-ELISA and allows the detection of antibodies over a period of 10 months post-infection, being useful to detect primary or secondary DENV active or recent infections. A 4-fold or greater increase in IgG titers, in acute *versus* convalescent sera, or the IgM/IgG ratio, are currently widely used to identify secondary active infections (Nguyen et al. 2018). On the other hand, IgG Indirect ELISAs are usually used to diagnose past DENV infection, as these are more sensitive than GAC-ELISA, being able to detect lower levels of antibodies.

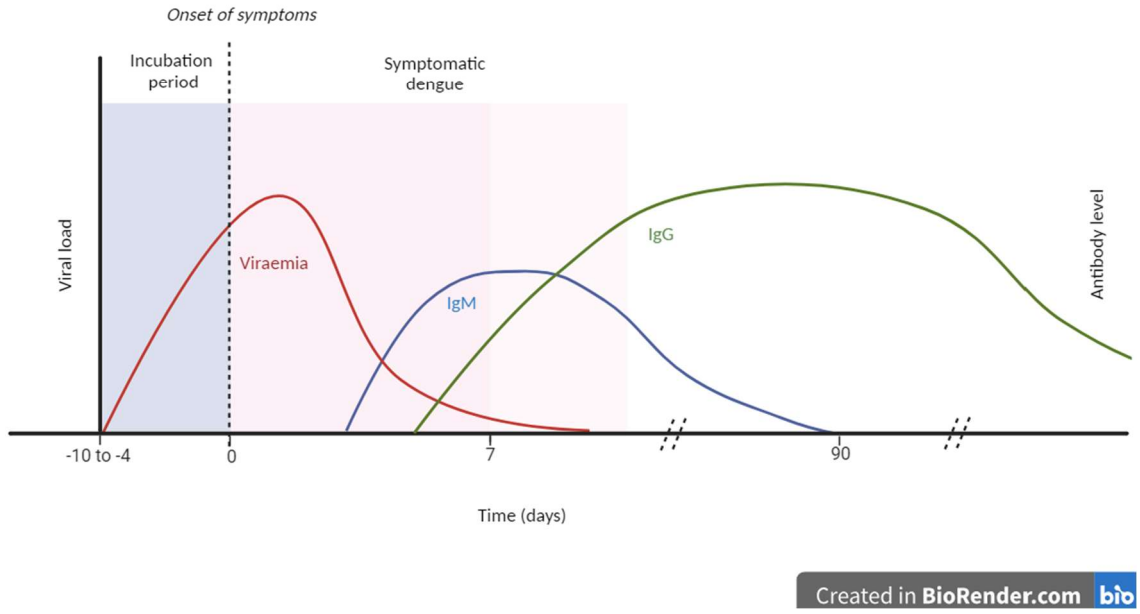

Figure 3 – Schematic representation of viral and antibody kinetics during primary DENV infection. (according to WHO, 2009).

For last, the hemagglutination inhibition (HI) assay is based on the ability of anti-DENV antibodies to inhibit agglutination of red blood cells and is considered the gold-standard for the detection of secondary infections. This assay also allows the detection of a 4-fold increase in antibody titers, from paired acute and convalescent sera, signaling recent DENV infections. A HI antibody titer of 1/2560 or higher is widely used as the hallmark of a secondary infection (Guzmán and Kourí 1996). However, it does not discriminate between infections by other closely related flaviviruses (WHO 2009).

Several commercial ELISA kits for the detection of the NS1 antigen have been also widely used in both endemic and non-endemic settings with variable sensitivity. Also, their capability to detect the NS1 antigen depends on the time at which the serum sample is collected, the infecting serotype, and whether it is a primary or post-primary infection (Duong et al. 2011).

1.3. The host immune response to DENV infection

As soon as DENV infects skin (Surasombatpattana et al. 2011) and Langerhans cells (Wu et al. 2000), viral replication begins, and the host's innate immune response is initiated. This response is mediated by innate immune cells expressing the so-called pattern recognition receptors (PRRs), such as monocytes, DCs, and macrophages, which recognize pathogen-associated molecular patterns (Navarro-Sanchez et al. 2003). PRRs include toll-like receptors (e.g. TLR-3 and TLR-7) and cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (e.g. RIG-I and MDA-5), which recognize single or double-stranded viral RNA, initiating a chain of signaling events critical to antiviral defense (Lester and Li 2014; Reikine, Nguyen, and Modis 2014). For instance, PRR signaling stimulates the production of type I α and β interferons (IFN-I α and IFN-I β) and other cytokines, through the activation of interferon regulatory transcriptional factors (Reikine, Nguyen, and Modis 2014; Schneider, Chevillotte, and Rice 2014). The induced IFN-I response results in cytokine and chemokine production, promoting an anti-viral state (reviewed in Uno and Ross 2018) and playing an important role in other innate and adaptive immune processes, such as promotion of T follicular helper cell activity (Cucak et al. 2009) and antibody class-switching responses (Le Bon et al. 2001).

Mast Cells (MCs) are likely among the first immune cells to contact with DENV after infection and to mount the initial immune response (Troupin et al. 2016). Brown et al. (2007) observed that infection of MCs is highly antibody-dependent and occurs through an FCγRII-dependent mechanism. Together with studies that correlate MC degranulation in DHF/DSS (Furuta et al. 2012; Syenina et al. 2015; Tissera et al. 2017), this could suggest that MCs response to DENV infection occurs mostly in secondary infections and could be associated to disease severity. Degranulation of MCs results in the release of proteases, cytokines (IFN-α and TNF), and chemokines (CCL5, CXCL10, and CXCL12) (St. John et al. 2011). These chemokines facilitate the recruitment of cytotoxic cells such as CD8+ T cells, natural killer cells (NK), and NK T cells (NKT) to the site of infection (St. John et al. 2011), which promote viral clearance, via killing of DENV-infected cells and production of cytokines such as INF-γ, which reduces the replication of DENV in infected cells (Costa et al. 2017; Gagnon et al. 2002). Meanwhile, DENV-activated DCs upregulate the expression of co-stimulatory molecules (CD80 and CD86;

(Ho et al. 2001) and simultaneously migrate to the draining lymph nodes, where they present the viral antigen to CD4+ and CD8 + T cells (Nightingale, Patkar, and Rothman 2008; Saron et al. 2018). The adaptive immune response is then initiated, taking place *circa* the 6th day of infection (Roy and Bhattacharjee 2021). Activated CD4+ and CD8+ T cells differentiate into effector T cells, responsible for cytokine production and for lysing DENV-infected cells (Rothman 2011). Additionally, CD4+ T cells when activated by DENV antigen presenting DCs can potentially become T Follicular helper (TFH) cells. These migrate to the periphery of the B cell follicle in the lymph nodes, where they promote the development of DENV-specific memory B cells and plasma cells (reviewed in (St. John e Rathore 2019), and lead to DENV-specific antibody production (Balakrishnan et al. 2011).

In a primary DENV infection, the humoral immune response is initiated soon after symptom onset, by the production of DENV-specific IgM antibodies, followed by IgG antibodies, mainly from the IgG1 subclass (Hofmeister et al. 2011; Koraka et al. 2001; Watanaveeradej et al. 2003). Serum levels of anti-DENV IgG antibodies peak several weeks after the disease onset and, subsequently, decline to levels that can nonetheless be detectable decades after (Sabin 1952). A large portion of this antibody response is cross-reactive with all DENV serotypes and even with other flaviviruses, while the functional neutralizing antibody (NAbs) response is more serotype-specific (reviewed in (Wahala and De Silva 2011). Humoral immunity against homologous secondary infections is thought to be long-lasting and mediated by NAbs that contribute to viral clearance (Sabin 1952). However, for heterotypic secondary infections, humoral immune protection seems to be limited in time, lasting for up to 2 years (Montoya et al. 2013). After this period, the risk of developing severe clinical manifestations increases, due to host-related mechanisms which are further detailed below. To note also that, the fact that homologous re-infections rarely occur has sustained the idea that NAbs epitopes are relatively conserved among strains belonging to the same DENV serotype (reviewed in (Wahala and De Silva 2011). Nevertheless, intra-serotype variation can result in significant differences in neutralization capacity. In fact, several studies have shown that serum neutralization capacity greatly varies between different strains/lineages of DENV (Endy et al. 2004; Guzman et al. 2007; Kochel et al. 2002). Also,

it has been shown that *in vitro* neutralization of DENV-1, DENV-2, and DENV-3 is dependent on the genotype used in the neutralization test (Brien et al. 2010; Martinez et al. 2020; Shrestha et al. 2010; Sukupolvi-Petty et al. 2010).

Several antigens and epitopes have been identified as potential targets of anti-DENV monoclonal antibodies, including both DENV's structural (E, PrM, and C) and non-structural proteins (NS1, NS3, and NS5) (Abubakar et al. 2002; Churdboonchart et al. 1991; Valdés et al. 2000). NAbs specifically target the E protein (AbuBakar et al. 1997; Lazaro-Olán et al. 2008; Santos et al. 2004), whose fusion loop region of the domain II and domain III have particularly been suggested as major targets of the anti-DENV antibody response (Crill et al. 2009; Lai et al. 2008). Additionally, some studies suggest that PrM is also a possible target for NAbs with variable neutralization capacity, ranging from poor to moderate (Alwis et al. 2011; Beltramello et al. 2010; Dejnirattisai et al. 2010). Nonetheless, the main epitope targeted by NAbs remains elusive (Wahala and De Silva 2011).

1.4. Factors influencing disease outcome in DENV infection

Factors influencing disease outcome in DENV infection are detailed in Chapter II, especially those involved in asymptomatic *versus* symptomatic infections. As such, this section will be focused on the factors influencing disease severity.

Several DENV factors have been identified as playing a role in determining the severity of this viral infection. For instance, the infecting DENV serotype has been found to influence the severity of dengue fever, however, there are conflicting reports on which DENV serotype results in more severe clinical manifestations. Some authors depict DENV-2 serotype as associated with more severe manifestations (Fried et al. 2010; Vicente et al. 2016). On the other hand, DENV-4 is usually associated with milder clinical manifestations and lower viraemia (Nisalak et al. 2003; Rocha et al. 2017; Thomas et al. 2008; 2014). These variations probably result from the different viral replication efficiency of different circulating serotypes/genotypes, resulting in different viraemia and consequently disease severity (Yung et al. 2015). The chronological sequence of infecting serotypes is also thought to influence clinical outcome, as a

correlate of pre-infection antibody titers (Aguas et al. 2019; Endy et al. 2004; Guzmán et al. 2000; Sangkawibha et al. 1984).

Host factors have also been identified as having a determinant role in the clinical outcome of DENV infection. Age is one of such factors, with a raised risk of increased severity in infants, especially those 6 to 8 months old, in dengue-endemic/hyperendemic regions (Halstead et al. 2002; Hause et al. 2016; Kliks et al. 1989). This is usually explained by antibody-dependent enhancement (ADE) occurring in infants due to pre-existing cross-reactive sub- or non-neutralizing antibodies of maternal origin. These antibodies bind to different DENV serotypes but are not able to neutralize them, and thus contribute to an enhanced viral uptake and infection of Fc receptor-bearing cells (Halstead 2007). This is believed to result in a higher viral load, due to increased viral replication, which triggers an exacerbated host inflammatory response leading to severe disease (Halstead and O'Rourke 1977; Halstead et al. 2002). Children with one to two years old show a decrease in risk of developing severe dengue, as a result of the loss of these antibodies (Halstead et al. 2002). In endemic areas, the risk of increased severity increases once again in older children, most likely linked to secondary infections (Hammond et al. 2005). DHF and DSS in children could also be explained by their intrinsically more permeable vascular endothelium (Guzman et al. 2010). In fact, severe dengue cases are more commonly found in patients with secondary heterologous infections, being widely considered a risk factor for severe dengue (reviewed in Guzman, Alvarez, and Halstead 2013) and explained by ADE, as described above (Halstead and O'Rourke 1977; Halstead et al. 2002). It has also been suggested that memory T-cells, acquired during primary infection, might play a role in the pathogenesis of severe dengue in a secondary infection, due to shifts in cytokines production (Bashyam, Green, and Rothman 2006; Mangada and Rothman 2005; Sanchez-Vargas et al. 2021).

The hosts' health status also plays a role in the severity of DENV infection. A higher risk of developing severe dengue has been linked to pre-existing co-morbidities, such as diabetes (Bravo, Guzmán, and Kouri 1987; Lee et al. 2020; Pang et al. 2017; Singh et al. 2022) and chronic renal disease (Chen et al. 2020). In diabetic patients with sub-optimal glycemic control, the increased risk of severe dengue is likely due to immune

and endothelial dysfunction, caused by diabetes mellitus (Geerlings and Hoepelman 1999; Hsueh, Lyon, and Quiñones 2004). Obesity has been identified as a prognostic factor for severe dengue in children (Baiduri et al. 2020; Kurnia and Suryawan 2019), likely explained by increased production of inflammatory mediators, which are known to increase capillary permeability and cause plasma leakage (Bosch et al. 2002; Calabro et al. 2005; Juffrie et al. 2001). Bronchial asthma has also been implicated as a risk factor for increased dengue severity (Bravo, Guzmán, and Kouri 1987; Cunha et al. 1999; González et al. 2005) possibly due to the activation of the allergic inflammatory cascade (Pang et al. 2017). Other co-morbidities also known to influence dengue severity are cardiac disorders (Thein et al. 2013), peptic ulcers, and sickle cell anemia (Bravo, Guzmán, and Kouri 1987).

More recently, it has been proposed that exposure to certain immunostimulants or to infections with other pathogens might trigger a more robust and unspecific response against unrelated agents. This phenomenon, defined as innate immune memory or trained immunity, is thought to confer mid-term protection against unrelated pathogens, lasting from several months to years (Drummer et al. 2021; Netea et al. 2020; Tercan et al. 2021). Unlike adaptive immunity, which results from clonal expansion of cells with a specific rearranged receptor, trained immunity is the result of metabolic and epigenetic reprogramming at the transcriptional level (Kleinnijenhuis et al., 2012), resulting in a fast and unspecific innate response, mediated by the secretion of high levels of inflammatory cytokines and reactive oxygen species (ROS), and by phagocytosis (Kaur et al., 2022). Several triggers of trained immunity have already been identified. Vaccines such as BCG (Bacillus Calmette-Guérin), influenza, polio, and measles-mumps-rubella (MMR), as well as fungal and viral infections, have been shown to provide heterogeneous protection in murine models (Barton et al. 2007; Björkström et al. 2010; Chumakov et al. 2021; Covián et al. 2020; Debisarun et al. 2021; Eastman et al. 2019; Foley et al. 2012; Grassmann et al. 2019; Hole et al. 2019; O'Neill e Netea 2020; Quintin et al. 2012; Yager et al. 2009;). In a clinical trial with the yellow fever attenuated vaccine, the BCG vaccine has been shown to later induce heterogeneous protection, which results in low viraemia due to epigenetic reprogramming in monocytes (Arts et al. 2018). Additionally, several studies using murine models have reported that DCs, the

main target of DENV infection, can be primed by BCG vaccine, resulting in a robust production of IFN-γ and Th1 cytokines when exposed to a subsequent challenge (Eastman et al. 2019; Hole et al. 2019). Trained immunity has also been described in endothelial cells (reviewed in Drummer et al. 2021), a possible target of DENV infection, which actively participate in innate immune responses, by secretion of cytokines and recruitment of immune cells (Shao et al. 2020). On the other hand, it has also been suggested that the innate immune training of endothelial cells may also contribute to dengue pathogenesis, by increasing the viremia and capillary permeability (reviewed in Drummer et al. 2021; Dalrymple and Mackow 2012). Nevertheless, it is still unclear whether trained immunity plays a role, either protecting against clinical dengue or contributing to disease severity.

Finally, the clinical outcome of DENV infection might be determined by tolerance to disease, in which the host's health is promoted, independently of its neutral-to-positive effect on pathogen fitness (McCarville and Ayres 2018). This can occur due to mechanisms that prevent the onset or support the resolution of immunopathology in host's tissues (Ayres and Schneider 2012). Another mechanism of disease tolerance is the crosstalk between host's immune response and metabolism. For instance, metabolic adaptations triggered by the immune response can induce changes in the pathogen, affecting its virulence and promoting host survivability (McCarville and Ayres 2018). Additionally, cellular metabolism can act as a regulator of T cell exhaustion, which can also be viewed as a disease tolerance mechanism, for instance in autoimmune disorders. However, no current evidence is available to support that these mechanisms play a role in dengue infection outcome.

1.5. The dengue outbreak in Madeira Island 2012/2013

1.5.1. The vector *Aedes aegypti*

The presence of *Ae. aegypti* mosquito, the main dengue vector, was detected in Funchal for the first time in the parish of Santa Luzia, in 2005 (Margarita et al. 2006). According to Seixas et al. (2019), the introduction of *Ae. aegypti* in Madeira Island results

from two independent introduction events, possibly occurring at different time-points, with the most likely geographic origin being Venezuela and, perhaps, Brazil. Since the detection of *Ae. aegypti*, a vector control program was placed in action to limit the mosquito's breeding sites, with adult and larval elimination through the application of insecticides and raising awareness of the local community (Seixas et al. 2013). Nevertheless, this species continued to expand throughout the southern coast of the island (Gonçalves, Gonçalves Silva, and Biscoito 2008), reaching its maximum effective population size in 2012, which was coincident with the dengue outbreak that occurred in Madeira Island (Seixas et al. 2019).

1.5.2. Epidemiological characterization

In late September 2012, the first two cases of autochthonous dengue infection in Madeira Island were reported, thus ensuing the first dengue fever outbreak to occur in Europe since the outbreak in Greece, in 1927/28 (Halstead and Papaevangelou 1980). DENV-1 (genotype V) was identified as the viral serotype responsible for the outbreak, while its most likely source of introduction is thought to be a traveler from Venezuela (Franco et al. 2015; Wilder-Smith et al. 2014). Soon after, the number of cases rapidly increased towards the end of October 2012. A peak incidence occurred at week 43 of 2012, with 60 to 70 cases *per* day (Tomasello and Schlagenhauf 2013), and then decreased until March 2013, when the outbreak was declared extinct. During these 23 weeks, a total of 2168 probable cases were reported, of which 1080 were laboratory-confirmed (ECDC 2014). In the same period at least 78 cases of dengue were diagnosed in other European countries, in travelers returning from Madeira Island (ECDC 2014).

The male-to-female ratio of cases in the Madeiran population was 1:1.4 (ECDC 2014), with *circa* 900 dengue cases affecting men and 1267 women, representing an attack rate (AR) of 68.6 cases/10,000 men and 86.5 cases/10,000 women. Interestingly, younger men had higher ARs, which decreased with age, while for women ARs increased with age, peaking between the ages of 35 and 64. The authors explain this higher AR because of women within this age range being more exposed to the vector, due to more prolonged home stays (ECDC 2014).

Most of the reported dengue cases occurred in the municipality of Funchal (77.0%), followed by Santa Cruz (8.3%) and Câmara de Lobos (5.8%). The remaining 9.9% of cases were reported in different municipalities (Figure 4). Of note, however, that the reports only considered the place of residence of dengue cases and, as such, some cases outside Funchal may, in fact, reflect infections that have occurred at their workplaces/schools in Funchal in people residing in a different municipality. The higher ARs in the southern coast of the island are likely due to the higher population density, coupled with the distribution of the mosquito vector, at the time also restricted to the southern coast (ECDC 2014).

Source: IASAUDE; discretisation method: natural break (Jenks); map projection: EPSG:3061 – Porto Santo/UTM zone 28N; software: Quantum GIS 1.8; map reference: ECDC_CIR_n2168

Figure 4 - Cumulative incidence rate for probable and confirmed dengue cases per 10.000 inhabitants, by parish of residence. (Source: ECDC 2014).

Within the city of Funchal, the parish of Santa Luzia had the highest attack rate (AR) of dengue cases per 10,000 inhabitants, followed by the neighboring parishes of Sé, São Pedro, Imaculado Coração de Maria, and Santa Maria Maior (ECDC 2014). The distribution of cases is likely related to the existence, in these parishes, of abundant mosquito breeding sites (residential buildings with backyards, terraces full of ornamental plants, abandoned buildings, and the sewage system) (ECDC 2014). The existence of several schools and commercial buildings in the most affected parishes

could have also contributed to increased transmissibility to non-residents. The high attack rate in these areas, coupled with a ratio of symptomatic to asymptomatic infection estimated at 1:4 is likely to have contributed to the establishment of herd immunity. This immunity can contribute to protect the population against a re-emergence of DENV-1 but probably not against a possible secondary heterotypic infection, which may result in an increased severity of dengue cases (ECDC 2014).

Aiming at the control and limitation of the dengue outbreak, the local and national public health authorities strengthened the vector control measures, which included increased geographical vector surveillance, and campaigns of public awareness, to reduce possible breeding sites and to promote the use of insecticides and repellents, and strict surveillance on airports and harbors (Tomasello and Schlagenhauf 2013). The case definition was also established by the national and local health authorities, with probable dengue cases being defined according to clinical criteria, including the acute onset of fever and at least two of the following symptoms (headache, retro-orbital pain, myalgia, arthralgia, exanthema, hemorrhagic manifestations, or leukopenia), and epidemiological criteria, such as being resident or having visited a dengue-affected area during the 21 days prior to the onset of symptoms. A proportion of the probable cases were confirmed by the presence of dengue-specific IgM antibodies, a significant increase in dengue-specific IgG antibodies in 2 consecutive measurements, and/or dengue virus nucleic acid or antigen detection in blood or cerebrospinal fluid. Furthermore, the Madeira Dengue Surveillance System was established by the local public health authorities, in collaboration with the ECDC, allowing the local health authorities to follow the spatiotemporal evolution of the outbreak.

Noteworthy, screening procedures for early detection and prevention of dengue transmission through blood transfusions were implemented by the Blood and Transfusional Medicine Service of Funchal Central Hospital, as soon as the first dengue case was confirmed. A total of 1948 blood donations were screened by RT-PCR during the outbreak, of which 43 tested positive. Of these positive donors, only two developed symptoms, while the remaining 41 persisted asymptomatic. Most of these asymptomatic donors were called back for serological follow-up in the subsequent

months (IgM and/or IgG Capture ELISA), of which only two had detectable seroconversion (ECDC 2014).

1.5.3. Clinical outcomes of dengue infections in Madeira

Most symptomatic dengue infections during the outbreak resulted in mild/ undifferentiated febrile illness, with patients having a full recovery without obvious complications. Although no severe clinical forms were reported, 122 patients had to be hospitalized (Tomasello and Schlagenhauf 2013).

The first two autochthonous cases were described by Alves et al. (2013) as presenting fever, myalgia, asthenia, gastrointestinal symptoms such as nausea, vomiting, diarrhea, diffuse abdominal pain, anorexia, and petechiae in the upper and lower limbs. Later, Freitas et al. (2014) studied, retrospectively, the clinical manifestations of 67 adults hospitalized with dengue fever during the period of the outbreak and found that the most frequent symptoms was myalgia (42.3%), followed by nausea (26.9%), headache (25.4%), abdominal pain (10.4%), exanthema (7.5%), retro-orbital pain (5.9%), diarrhea (4.5%) and arthralgia (4.5%). Hemorrhagic manifestations were scarce, being the most common petechiae (26.9%), metrorrhagia (2.9%), hematemesis (1.5%), epistaxis (1.5%), and hematuria (1.5%). Hepatic dysfunction (88%), thrombocytopenia (86.5%) and leukopenia (80.6%) were the most frequent laboratory findings among these hospitalized patients. Overall, compared to other studies in hospitalized patients in different endemic regions (Martelli et al. 2011; Teixeira, Nogueira, and Nascentes 2017), the adult population hospitalized during the outbreak in Madeira Island showed a lower frequency for most signs and symptoms.

Additionally, the retrospective descriptive study of Castro et al. (2014) depicted the clinical and laboratory findings of a pediatric cohort (0-14 years) of 182 confirmed dengue cases, during the period between the 39th week of 2012 and the 9th week of 2013, with a total of 29 (15.9%) hospitalized patients. The authors found that the most common symptoms were fever (98.3%), headache (75.2%), myalgia (66.5%), and exanthema (51.6%). Retro-orbital pain (29.7%), asthenia and anorexia (15.0%), and arthralgia (11.0%) were less frequently reported. Gastrointestinal non-warning

symptoms such as nausea and vomiting (42.3%), and diarrhea (8.8%), as well as respiratory tract symptoms, such as odynophagia (11.5%), cough (5.5%), and nasal obstruction (2.7%), were also described in this cohort. Lastly, hemorrhagic symptoms were present in 9.9% of these pediatric patients, with epistaxis being the most frequent (6.0%) followed by petechiae (1.1%), thrombocytopenic purpura (1.1%), gingival bleeding (1.1%) and hematemesis (0.5%). Leukopenia and thrombocytopenia were also observed, with minimum levels of leukocytes and platelets being detected at the 4th and 7th day of disease onset, respectively. Despite the relatively small number of hospitalizations, the authors state that 48.9% of the pediatric cases of dengue presented warning signs, namely rising hematocrit (40.7%), abdominal pain (28.0%), mucosal bleeding and sudden defervescence (7.1%), persistent vomiting (4.4%), and low platelet count (<50.000 platelets/µL) (3.3%). The clinical outcome found in this pediatric cohort agrees, in general, with previously reported clinical outcomes of DENV infections in other studies in hospitalized children (Khan et al. 2021; Rathod, Ansari, and Singh 2018), although with fewer hemorrhagic manifestations. A putative explanation for this milder clinical outcome is that in primary infections, patients typically experience a self-limited febrile illness, accompanied by mild-to-moderate hematological and biochemical abnormalities (Castro et al. 2014; Wilder-Smith et al. 2019). Concerning age groups, de Souza et al. (2013) found differences in the clinical outcome of DENV infections between children and adults from Brazil. For instance, vomiting was more prevalent in children than in adults, while hemorrhagic symptoms were more common in adults. However, the frequency of specific symptoms varied between many of the above-mentioned studies. In Madeira, and contrary to what de Souza et al. (2013) reported, epistaxis was the most frequent hemorrhagic manifestation in children (Castro et al. 2014), while petechiae were the most common in adults (Freitas et al. 2014).

No severe cases were reported during the Madeira outbreak (ECDC 2014), suggesting mild clinical outcomes, typical of primary infections. However, the complete picture about the clinical manifestations during the dengue outbreak in Madeira Island is still not entirely described. Both, Freitas et al. (2014) and Castro et al. (2014) focused on specific study populations, either hospitalized adult patients, which likely overrepresents severity, or a pediatric cohort. Additionally, none of the mentioned

studies focused on laboratory parameters, which are known to be of interest in DENV infections, apart from liver enzymes, platelet count and hematocrit. Therefore, the knowledge about clinical and laboratory findings concerning most of the affected population, mainly adults that did not require hospitalization, is scarce. These aspects need to be studied more widely and comprehensively, to better characterize the clinical outcome of a primary DENV infection.

As with most arbovirus infections, it is to be expected that during the dengue outbreak in Madeira Island, most of the infected remained asymptomatic. The prevalence of asymptomatic infections is substantially variable among studies, ranging from 31% to 95% (Baaten et al. 2011; Balmaseda et al. 2006; 2010; Duong et al. 2015; Endy et al. 2002; Velandia-Romero et al. 2020; Wilder-Smith et al. 2009). Considering a ratio between symptomatic and asymptomatic cases of 1:4, as estimated by ECDC experts (ECDC 2014), almost 11,000 infections would have occurred during the outbreak, which would represent around 4% of the Madeira population. Auerswald et al. (2019), in a randomized retrospective serologic study performed 3 to 4 years after the outbreak, found a seroprevalence of anti-DENV antibodies of 7.8%. Based on that, the authors suggest that the number of infections might have been as high as 16,606, with a symptomatic to asymptomatic ratio of 1:7. However, the risk of being exposed to DENV was not the same for all the inhabitants of Madeira Island. The vector population was mostly restricted to the south coast, with different degrees of abundance (ECDC 2014; Gonçalves, Gonçalves Silva, and Biscoito 2008), and the outbreak was also limited to the southern coast, namely the municipalities of Funchal, Santa Cruz, and Câmara de Lobos, with different degrees of prevalence. As such, the number of total infections cannot be directly estimated based on the seropositive cases in the sample, and their extrapolation to the total population of Madeira Island, because there is a high risk of overestimation. Also, it is not clear if the sampled population in Auerswald et al. (2019) strictly represents asymptomatic infections or if it includes symptomatic infections. These last are more likely to participate in studies of this nature and thus, the rate of seropositivity described in this study will not reflect the real picture of infections during the outbreak and, if based on symptomatic/asymptomatic ratio, will also result in an overestimation of total cases. Additionally, Auerswald et al. (2019) refer that nearly a

third of their participants and nine of the seropositive samples had previous travel history to DENV endemic regions. Based on IgG capture ELISA results, they suggest these cases represent secondary infections. In fact, four of these cases had inconclusive IgG capture ELISA results, while the other five had high enough IgG titers to be detected by capture ELISA, and it is possible that these represent secondary infections. However, the use of IgG capture ELISA to identify secondary infections in samples collected three years after the outbreak is questionable. Given their travel history to DENV endemic regions, these cases might not represent recent secondary infections but, in fact, reflect a previous infection, with a different origin than the Madeira Island outbreak. On the other side, they may only represent the higher spectrum of a variable response within a population. Notwithstanding the existing body of evidence concerning the serological status of the population of Madeira, further studies focusing on the antibody response of asymptomatic and symptomatic DF cases are essential to elucidate the full scope of the humoral response to DENV infection in this population.

1.6. Objectives

The single DENV outbreak in Madeira Island offers a unique opportunity to study the epidemiology of dengue fever and the host factors providing protection against clinical dengue in primary DENV infections. One of the main confounding factors in the study of asymptomatic infections, which is particularly relevant in endemic or hyperendemic regions, resides in the difficulty of accurately discriminate between primary asymptomatic infection and the absence of symptoms in previously exposed individuals. As such, studies in these regions rely on identifying asymptomatic infections through several serological methods applied to differentiate primary and secondary infections. However, these methods have better performances in serum samples during the acute phase of the disease (Nguyen et al. 2018), are prone to cross-reactivity issues, and need to consider the kinetics of antibody responses. Also, since the individual humoral response to DENV infection is variable, high antibody titers might reflect this variability, instead of being indicative of a secondary infection. Considering that the population of Madeira Island largely represents a naïve population, where the risk of previous exposures to DENV was limited to travelers visiting dengue-endemic regions

during transmission periods, it allows for a greater degree of certainty in the identification of possible asymptomatic infections.

Additionally, secondary DENV infections are widely considered to result in more severe clinical outcomes. Thus, if studies in endemic/hyperendemic regions do not differentiate between primary and secondary infections, it is expected that the overall frequency of certain signs and symptoms in these populations represents both, those experienced in primary infections and more severe symptoms resulting from secondary infections. Studying the signs and symptoms of a DENV infection, in a naïve population can therefore provide useful insights into the clinical spectrum and outcome of a dengue primary infection.

The aim of the work herein reported is to retrospectively characterize the Madeira dengue outbreak and to investigate immune and biochemical host factors involved in the protection against DENV-1 clinical infection, in the Madeira Island's population. For this purpose, the following objectives were defined:

The first objective was to investigate the serological status of two sample sets of asymptomatic or symptomatic infections from the population of Madeira Island, eight years after their first and single exposure to DENV. This objective was accomplished by determining whether the humoral immune response differed between asymptomatic and symptomatic infections. To this end, anti-DENV IgG Indirect ELISA was used to measure serum antibody levels and titers in both studied groups. The possible association between antibody titers and the clinical outcome of DENV primary infection was also investigated. Lastly, the homologous and heterologous neutralizing capacity of anti-DENV antibodies were assessed, in order to provide further insights regarding the protection status of the studied population against future DENV infections. These analyses established the overall picture of the humoral immunity against DENV-1 in Madeira Island;

The second objective was to retrospectively characterize the clinical symptoms experienced during a primary DENV infection, to access the prior and posterior health status of DF patients, and to investigate the possible occurrence of long-term manifestations resulting from DENV infection, deepening the knowledge about the real

burden of the dengue outbreak in Madeira Island. For this purpose, an online questionnaire was applied to participants identified as having a clinical and/ or laboratory diagnosis at the time. This questionnaire inquired the participants about signs and symptoms during the acute and convalescent phases of DENV infection, pre-existing chronic diseases, recurrent previously non-existing symptoms, and chronic diseases detected in the few years after the infection. Additionally, a more thorough characterization of clinical, hematological, and biochemical parameters presented during the acute phase of DF was carried out. To that end, we analyzed clinical and laboratory data of DF patients provided by SESARAM E.P.E.R.A.M., stratified by age and gender. The analyses of these data allow an enhanced characterization of the clinical burden of a primary DENV-1 infection in the population of Madeira Island and is essential to estimate the real magnitude of the outbreak, in terms of health effects and disease severity, and to better evaluate its long-term consequences. Once analyzed, these data allow an enhanced characterization of the clinical burden of DENV-1 infection, during the outbreak and in the long-term, in the population of Madeira Island.

The present Ph.D. thesis is structured into seven chapters. The present chapter (Chapter I) provides a general introduction, establishing the state-of-art of dengue infections and setting the scope of the following chapters. Chapter II refers to the literature review about the asymptomatic clinical outcome of DENV infection, recognizing the current knowledge and identifying the existing gaps in the knowledge about asymptomatic infections. Chapter III focuses on the humoral immune response to DENV infections in the population of Madeira Island, resulting from a single exposure to the DENV-1 virus in 2012/2013. Chapter IV is centered on the clinical symptoms reported by the population of Madeira during both the acute phase of the disease and the following years, reflecting the occurrence of long-term symptoms and possible sequelae. Chapter V addresses the characterization of clinical, hematological and biochemical parameters determined for DF patients during the acute phase of the disease and establishes their relationship to the clinical outcome of DENV infection. Finally, the final chapter (Chapter VI) provides a general discussion of the body of work herein presented, the ongoing work, and future perspectives.

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CHAPTER II

Flying under the radar - asymptomatic flaviviral infections: The dengue example

Preamble

Infection with DENV results in a wide range of outcomes, varying from asymptomatic infection to severe life-threatening illness. Even though most DENV infections result in an asymptomatic outcome, most studies focus on the factors influencing disease severity. However, it is noteworthy that asymptomatic infections should play a role in the maintenance of the dengue transmission cycle. Another important aspect concerning asymptomatic infections is their potential role on the development of specific immunity by the host, including a protective role against secondary symptomatic infections or, on the other side, a contribution to the pathogenesis of post-primary DENV infections. Also, very few is known about the factors that determine the asymptomatic outcome in a DENV primary infection. As such, it was considered necessary to carry out and include in this Ph.D. thesis, a literature review manuscript encompassing the characterization of the asymptomatic outcome of DENV infection, with emphasis on the viral and host factors contributing to the asymptomatic outcome of DENV infection, here presented in this Chapter II.

The work depicted in the present chapter resulted in a review article submitted to *Frontiers in Cellular and Infection Microbiology – Research Topic: Reviews in Virus and Host.*

Flying under the radar - asymptomatic flaviviral infections

The dengue example

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Abstract

The clinical outcome of DENV and other Flaviviruses infections represents a spectrum of severity that ranges from mild manifestations to severe disease, which can ultimately lead to death. Nonetheless, most of these infections result in an asymptomatic outcome that may play an important role in the persistent circulation of these viruses. Also, although little is known about the mechanisms that lead to these asymptomatic infections, they are likely the result of a complex interplay between viral and host factors. Specific characteristics of the infecting viral strain such as, its replicating efficiency, coupled with host factors, like host gene expression of key molecules involved in the immune response or in the protection against disease, are among the potential candidates. This review revisits recent data on factors that may contribute to the asymptomatic outcome of flaviviruses infections, focusing on the world widespread DENV, highlighting the importance of silent infections in the transmission of these pathogens and on the host's immune status.

Introduction

In the last few decades, flaviviral diseases have become progressively more common in human populations, propelled by the spread into regions where these were previously absent, representing nearly 30% of all emerging infectious diseases in humans in the early 2000s (Jones et al., 2008). Examples include the introduction of dengue virus serotype 1 (DENV-1) in the Madeira (Portugal) in 2012, resulting in an

outbreak with 2,168 probable cases (ECDC, 2014), and Zika virus (ZIKV) introduction in South America in 2015 (Campos et al., 2015), leading to an outbreak of 1.3 million suspected cases, in at least 33 territories (Hennessey et al., 2016; WHO, 2016). The re-emergence of flaviviral diseases in regions where they had previously been controlled or eradicated, as it happened with DENV in Brazil in 1981 (Osanai et al., 1983), and with yellow fever virus (YFV) in Kenia in 1992 (Okello et al., 1993), has also contributed to an increase in the global number of cases.

Clinical manifestations of flaviviral infections range from mild to severe disease (Hollidge et al., 2010) but, nevertheless, possibly up to 95% of these infections in humans are asymptomatic (Balmaseda et al., 2010; Johansson et al., 2014;). Asymptomatic infections are thus of high epidemiological significance since their estimation is crucial to determine the real burden of infection and the risk of transmission in a population. Further, little is known about the underlying factors of asymptomatic outcome, which may also be common to other flaviviral infections. The purposes of this review are to revisit recent data on factors contributing to the asymptomatic outcome of flaviviral infections, focusing on the widespread DENV, and to stress the importance of silent infections in the transmission of this pathogen and on the immune status of the host.

Classification and transmission of Flaviviruses

Flavivirus is a genus of viruses of the Flaviviridae family, which share common features such as the presence of envelope, icosahedral nucleocapsids, and +ssRNA genomes of about 10-11kb. This genus includes the DENV, YFV, ZIKV, West Nile Virus (WNV), Japanese Encephalitis Virus (JEV), as well as several other viruses causing encephalitis (Shi, 2012) and insect-specific flavivirus (McLean et al., 2015). Flaviviruses are transmitted from infected hematophagous arthropods to different enzootic vertebrate reservoir hosts, such as domestic animals, and humans, and hence are classified as arboviruses. When viremia is sufficiently high in these amplifying hosts, permissive arthropods become infected during a blood meal, allowing for the maintenance of the cycle. For WNV (Bowen and Nemeth, 2007) and JEV (Weaver and Barrett, 2004), humans are often dead-end hosts, given that the typically found low

viremia is unlikely to allow reinfection of feeding arthropods and/or the chances of reencountering a vector in areas of low population density are scarce, therefore interrupting the viral lifecycle (Hollidge et al., 2010). By contrary, DENV, YFV, and ZIKV can establish active transmission cycles in areas with high population density, using humans as the primary amplifying hosts' (Nimmannitya et al., 1969; Padbidri and Gnaneswar, 1979; Weaver and Barrett, 2004; Barrett and Higgs, 2007; Saiz et al., 2016), and therefore having the greatest impact as human pathogens.

The DENV journey in the mammalian host

Following the bite of an infected arthropod, it is likely that the inoculation of the DENV into the dermis and epidermis of the mammalian host (Guzman et al., 2016) results in infection since, besides skin dendritic cells, other phagocytic cells such as monocytes and macrophages, as well keratinocytes are also targeted by the virus (Wu et al., 2000; Marovitch et al., 2001; Surasombatpattana et al., 2011). Infected cells can then migrate from the initial site of infection to lymph nodes, where the adaptive immune response is initiated. The resulting activation of effector T cells (Silberberg-Sinakin et al., 1976; Macatonia et al., 1987) prompts the recruitment of other monocytes and macrophages, that become further targets for the virus (Guzman et al., 2016) (see **Box 1** for details on immune response against DENV). The dissemination of the infection throughout the lymphatic system is then facilitated, with the subsequent infection of other cells of the mononuclear lineage, including monocytes, myeloid dendritic cells, and liver and splenic macrophages (Marovitch et al., 2001).

Once in the mammalian host, all flaviviruses follow similar steps in their replication cycle (reviewed in Fernandez-Garcia et al. (2009)). The DENV replication cycle initiates with the binding of the envelope (E) proteins of mature viral particles to the host target cell receptors, followed by internalization by endocytosis. *In vitro*, DENV showed to be able to use a wide variety of cellular receptors to attach and/or enter target cells, although their contribution to natural human infection is not fully established (reviewed in Cruz-Oliveira et al. (2015)). For example, *in vitro* DENV infection has been positively correlated to an increased expression of dendritic-cell-specific intercellular adhesion molecule 3'-grabbing non-integrin (DC-SIGN, CD209) in human

monocytes derived DCs and of its homologue L-SIGN (CD209L) in the endothelial cells of the liver and lymph nodes (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). The lipopolysaccharide-binding protein (LPS) receptor (CD14) and the macrophage mannose receptor (CD206) were shown to be involved in DENV binding to monocytes/macrophages (Chen et al., 1999; Miller et al., 2008). Other candidate receptors such as heparan sulphate receptor, expressed in hepatocytes, and heat shock proteins HSP70 and HSP90 have also been described as targeted by DENV, in *in vitro* systems (Germi et al., 2002; Reyes-Del Valle et al., 2005). Also, apolipoprotein-A-I (ApoA-I) scavenger receptor class b type I (SR-BI) seems to facilitate DENV entry into cells (Li et al., 2013), and endocytosis via LDL receptor has been reported in several flaviruses (Agnello et al., 1999). After the humoral response has been established, the virus may also form a complex with non-neutralizing antibodies, facilitating its entrance into cells expressing receptors for the Fc region of the antibodies, like monocytes. This phenomenon is called antibody-dependent enhancement (ADE) and it is likely implicated in the pathogenesis, especially during secondary heterologous DENV infections (reviewed in Halstead (2003)).

Once inside the host cell, pH-mediated rearrangement of the E proteins facilitates the fusion of the viral envelope and the endosomal membrane, releasing the nucleocapsid into the cytoplasm. With its further disassembly, viral genomic (+)ssRNA is translated into a long polyprotein, which is cleaved into individual E, membrane (M), and capsid (C) structural proteins, and several non-structural (NS) proteins, by both host and viral NS proteases. Viral RNA is replicated by RNA-dependent RNA polymerase and, together with C proteins, will assemble into new nucleocapsids. The precursor forms of the membrane (prM) and E proteins are embedded into the endoplasmic reticulum membrane and will surround the newly formed nucleocapsids as they bud into the lumen of this organelle, constituting immature viral particles. The pH acidification along the secretory pathway allows the maturation of the DENV particles, including the rearrangement of the E proteins and the cleavage of prM into the mature M protein. The infective mature DENV then exits the host cells via exocytosis, with the release of the pr peptides. New DENV virions can nevertheless retain uncleaved prM protein on its

surface, remaining in an immature non-infective form, or even present a mix of M and prM proteins, with unknown infectiveness status (reviewed in Guzman et al. (2016)).

Box 1 - The host immune response

Innate and adaptive immune responses are known to participate in the control of viral infections. In innate response, host pattern recognition receptors such as membrane toll-like receptor 3 (TLR3) and TLR7, and cytoplasmic retinoic-acid inducible gene I (RIG-I)-like receptors (RLRs) are expressed by the phagocytic cells and represent one of the first lines of antiviral defence, by sensing viral nucleic acids (reviewed in Guo et al. (2018)). These sensor molecules trigger the activation of two important families of transcription factors - interferon regulatory factors (IRFs) and NF-kB - that prompt the production of type I interferons (IFN)α/β and inflammatory cytokines, and therefore establish a cellular antiviral status in the infected and adjacent cells, and activate and recruit immune cells such as Natural Killer (NK) cells, which are important in the antiviral response (Shresta et al., 2004; Navarro-Sanchez et al., 2005; Bourne et al., 2007).

Regarding adaptative immunity, cellular immune response mediated by T-helper 1 (Th1) and cytotoxic T (CTL) lymphocytes is the most effective antiviral mechanism once the virus enters the host cell. Induced by IL-12 secreted by dendritic cells, Th1 lymphocytes produce inflammatory cytokines, namely IL-2, IFNγ, and TNFα, while CTL produce IFNγ, contributing to the clearance of the infection. IL-12 also induces CD8 T cells to differentiate into cytotoxic cells, triggering apoptosis of the infected cells. Humoral immunity, through neutralizing antibodies, is also thought to protect against DENV infections, by limiting infection dissemination and promoting viral clearance (Henchal et al., 1988; Clapham et al., 2015). Following a primary symptomatic DENV infection, DENV-specific IgM antibodies are detectable in serum 4 to 5 days after the onset of symptoms, remaining measurable for up to 3 months. DENV-IgG antibodies appear later, about a week after the onset of symptoms, peaking several weeks after the infection, and then declining to lower levels that, nevertheless remain detectable for decades (reviewed in Wahala and de Silva (2011); Salje et al., 2018). These antibodies are mainly from the IgG1 subclass (Koraka et al., 2001; Watanaveeradej et al., 2003; Hofmeister et al., 2011), indicating a Th1-based immune response. A large fraction of anti-DENV-IgG cross-react with all DENV serotypes and even with other flaviviruses. Effective protection is long-term against the homologous serotype from the primary DENV infection, but only transient (up to 2 years; Anderson et al., 2014 and Montoya et al., 2013) against heterologous serotypes (Sabin, 1952; Guzmán et al., 2000; Hubert and Halstead, 2009; Salje et al., 2018). After that period, there is also a higher risk of developing severe dengue in heterologous infections, likely facilitated by the ADE mechanism (reviewed in Rothman (2011)).

The outcomes of DENV infections

The clinical outcome of DENV infections, for which 4 circulating serotypes are known (DENV-1-4), results from the interplay between host and pathogen factors. As for other flaviviral infections, their manifestations may range from a mild influenza-like illness to severe disease, that may result in long-term physical impairment or even death (Hollidge et al., 2010).

According to the WHO guidelines, symptomatic dengue was classified up to 2009, as dengue fever (DF), dengue haemorrhagic fever (DHF), and dengue shock syndrome (DSS), this last being the most severe form. The more recent classification system established by the WHO in 2009 divides DENV infection in non-severe dengue (with or without warning signs) and severe dengue, depending on the severity of the clinical manifestations. The non-severe form is characterized by high fever, lasting for 2 to 5 days, likely accompanied by nausea, vomiting, rash, aches, and pains, and/or leukopenia. The following warning signs may be present: abdominal pain, persistent vomiting, fluid retention, mucosal hemorrhage, malaise and drowsiness, hepatomegaly, high hematocrit, and thrombocytopenia. Besides these symptoms and warning signs, severe dengue patients exhibit serious hemorrhage, organ impairment and plasma leakage, which leads to fluid accumulation in the lungs and abdomen, conducting to respiratory distress and hypovolemic shock (WHO, 2009).

Nevertheless, it is well known that the most common outcome of DENV infections, including primary infections, are asymptomatic infections, in which the virus and/or seroconversion can be detected in the complete absence of symptoms (Endy et al., 2011; Montoya et al., 2013; Grange et al., 2014; Duong et al., 2015). The DENV infection may also result in a subclinical/unapparent infection, with insufficient symptoms to be detected by existing surveillance systems and healthcare providers, although verifiable by viral and/or seroconversion detection methods (reviewed in Grange et al. (2014)). Using cartographic approaches, a total of 390 million worldwide DENV infections per year have been estimated, of which almost 300 million are clinically silent or mildly symptomatic (Bhatt et al., 2013).

The asymptomatic outcome

The reported proportion of asymptomatic DENV infections is known to be highly variable, with studies pointing to values that ranges from 31% to 95% (Burke et al., 1988; Potasman et al., 1999; Endy et al., 2002; Porter et al., 2005; Balmaseda et al., 2006; Mammen et al., 2008; Balmaseda et al., 2010; Baaten et al., 2011; Salje et al., 2018; Tsang et al., 2019; Velandia-Romero et al., 2020) (summarized in Table 1). These differences can be due to several viral and host factors, and their combination. As discussed below in more detail, different DENV serotypes can produce distinct proportions of asymptomatic cases. The population genetic background may also affect the outcome of the infection, given its influence in the human host´s cells permissiveness and immune response to the infection. Additionally, the immune status of the population, built over previous infections and vaccination plans, may also account for differences in asymptomatic proportions. To illustrate this, in regions where dengue is endemic, secondary infections might result in a higher frequency of asymptomatic cases, since the protection window given by the presence of neutralizing antibodies lasts for approximately 2 years, in heterologous infections (Montoya et al., 2013), or can be lifelong, in homologous infections (Sabin, 1952). In that context, in dengue-endemic regions, the time elapsed between major dengue outbreaks can also influence the infection outcome.

Table 1 – Frequencies of asymptomatic DENV infections accessed by several studies.

Population	Study design	Year	Serotype	% asymptomatic	References	Observations
Thailand	Prospective cohort	1987	Nd	87%	Burke et al. 1988	Pediatric
		1998-2000	Nd	53%	Endy et al. 2002	Pediatric
		1998-2003	Nd	65%	Salje et al. 2018	Pediatric
		2004-2005	Nd	75%	Mammen et al. 2008	
Indonesia	Prospective cohort	2000-2002	Nd	75%	Porter et al. 2005	
Israel	Travellers survey	-	Nd	43%	Potasman et al. 1999	
Nicaragua	Prospective cohort	2001	DENV-2	93%	Balmaseda et al. 2006	Pediatric
		2002	DENV-1	85%		
Nicaragua	Prospective cohort	2004-2005	Nd	95%	Balmaseda et al. 2010	Pediatric
		2005-2006	Nd	83%		
		2006-2007	Nd	94%		
		2007-2008	Nd	75%		
Nicaragua	Estimated probability	2004-2010	DENV-1	90%	Tsang et al. 2019	Pediatric
			DENV-2	87%		
			DENV-3	76%		
			DENV-4	98%		
Colombia	Prospective cohort	2013-2015	Nd	31%	Velandia-Romero et al. 2020	
Netherlands	Travellers survey	2006-2007	Nd	64%	Baaten et al. 2011	

Study design may also affect the estimated proportion of asymptomatic DENV infections. In line with the above-mentioned, when studying non-naïve populations, this proportion is expected to be higher, given the difficulty to differentiate between true asymptomatics in primary infections and the lack of symptoms in individuals within the partial immunity window, following previous DENV infection. In fact, both situations are characterized by inapparent infections, but the underlying mechanisms are different. An overestimation of asymptomatic frequencies may also result from unidentified symptomatic mild cases occurring without febrile illness, as most studies track symptomatic cases through surveillance of body temperatures (Burke et al., 1988; Endy et al., 2002; Balmaseda et al., 2006). Further, studies focusing on determining asymptomatic frequencies at a given time point are also more likely to overestimate this proportion, since may not be able to distinguish between pre-symptomatic cases and truly asymptomatic cases, contrarily to studies encompassing a longer follow-up of their participants (Ly et al., 2019). To minimize these effects, some studies considered as asymptomatics only those individuals who had not experienced a documented febrile

episode linked to DENV infection but had a 4-fold or greater increase in total DENV specific antibody titer (Kuan et al., 2009; Balmaseda et al., 2010; Montoya et al., 2013). Similarly, some studies involving hyperendemic regions with co-circulation of different flaviviral infections are also thought to have a higher proportion of undetected DENV infections, likely due to induced cross-protective immunity (Ribeiro et al., 2018). Estimating the proportion of asymptomatic infections in naïve populations and studying the mechanisms underlining the absence of symptoms on a primary infection are therefore warranted to a better understanding of DENV infection, which likely has common features and may shed light to the asymptomatic outcome of other flaviviral infections.

The importance of asymptomatic DENV infections

Inapparent infections are recognized as an important component of the overall burden of flaviviral infections. However, their realistic epidemiological weight is still largely unknown (Chastel, 2011). Given their generally high frequency, ignoring asymptomatic infections can result in an underestimation of the rate of infection and transmission in a community, an inadequate evaluation of individual risk of severity in future infections, and an improper implementation of control measures. Asymptomatics are less likely to disrupt their daily routines and, therefore, have a greater potential to contribute to the epidemic spread of the virus, and to its persistent circulation during interepidemic periods.

Humans with DENV asymptomatic infection were, for long, considered dead-end hosts, as it was assumed that they did not reach sufficiently high viremia to infect feeding mosquitoes. Data on the viral titer of DENV asymptomatic infections is, however, not easily determined, since viremia depends on the day of the infection while, in symptomatics, the day of onset of symptoms is used as a reference. Nevertheless, Duong et al. (2015) were able to show that the average DENV viremia in asymptomatics is similar to that observed in symptomatics during the early and late viremic periods (2–3 days before or 5–8 days after symptoms onset, respectively). Moreover, these authors showed that pre-symptomatic or asymptomatic DENV infected individuals are, at any given level of viremia, more infectious to mosquitoes than symptomatic individuals

(Duong et al., 2015). It was suggested that the strong humoral immune response and high cytokine levels developed during symptomatic infections likely reduce human infectiousness to mosquitoes during this period (Lambrechts et al., 2012). In fact, Nguyet et al. (2013) have previously associated the increasing number of days of illness and the rise of IgM and IgG titers with a reduced risk of human-to-mosquito DENV transmission. Moreover, a slower decay of viremia observed in asymptomatic infection (Matangkasombut et al., 2020) may lead to longstanding infectious reservoirs. Subclinical DENV infections have, therefore, a significantly greater potential to contribute to viral transmission than previously recognized, including to the DENV persistent circulation during interepidemic periods. For example, Jamjoom et al. (2016) hinted at the possible influence of asymptomatic infections on the establishment of dengue in Saudi Arabia where, until recently, reported clinical cases of dengue were sparse, but a high seroprevalence was detected.

Asymptomatic infections can also play an important role in spreading the infection to new regions, where the arthropod vector is present in sufficient density to allow transmission. When returning from dengue endemic regions, asymptomatic travelers are less likely to be detected than symptomatics and represent a possible entryway for the virus. For instance, the phylogenetic study of the DENV virus by Franco et al. (2015) indicated Venezuela as the most probable origin of the DENV-1 strain responsible for the 2012 outbreak in Madeira, where the vector *Aedes aegypti* was present at considerable density. This origin was previously suggested by Wilder-Smith et al. (2014), considering the likelihood of dengue introduction from dengue-endemic countries based on their dengue incidence and travel volume to Madeira. The large emigrant community from Madeira living in these countries, particularly Venezuela, frequently travels back to the island, and introduction via an asymptomatic traveler is a very likely scenario.

The repercussion of asymptomatic infections on viral transmission is not limited to the likelihood of infecting mosquitoes, since there are also well-documented episodes of passive human-human transmission through blood donations and transplants (Wilder-Smith and Schwartz, 2005; Chuang et al., 2008; Tambyah et al., 2008; Stramer et al., 2012). These are probably underestimated routes of transmission, where

asymptomatic infections may have an increased responsibility, since symptomatic individuals are less likely to donate blood while sick. Furthermore, the asymptomatic or misdiagnosed clinical illness in a patient following a transfusion or a transplant, the pre-existent homotypic or recent heterotypic immunity in the recipient, or an infection incorrectly attributed to mosquito transmission, are factors contributing to the sub-evaluation of human-human transmission (Petersen et al., 2013).

By last, we would like to highlight that asymptomatic infections are also thought to play a protective role against a symptomatic secondary infection. In a prospective pediatric cohort study, Montoya et al. (2013) showed that the time interval between an inapparent DENV infection and a subsequent inapparent infection was significantly shorter (2.2 years) than that for a secondary symptomatic infection (2.7 years), suggesting effective immune protection during a window period of almost 3 years, induced by the primary inapparent infection. Moreover, the mean time interval between two consecutive symptomatic infections was estimated at 3 years, thus suggesting that the window of cross-protection induced by inapparent, or symptomatic infections is similar (Montoya et al., 2013). Similarly, as previously discussed for symptomatic infections, once the period of cross-protection is over, antibodies acquired during an asymptomatic infection might also contribute to a higher risk of severe forms of dengue during secondary heterologous infection. Since asymptomatic infections are largely unidentified, but are nevertheless coupled with an established antibody response, a greater number of people, other than those identified as symptomatic, are at risk of developing severe forms of dengue following the protection window.

Factors influencing asymptomatic outcome

The asymptomatic DENV infections and the kind and severity of clinical manifestations in symptomatics have been associated with viral factors, such as DENV serotype, and load (Gubler et al., 1978; Vaughn et al., 2000; Balmaseda et al., 2006), and host factors, such as age, lipid profiles, genetic background (Lan and Hirayama, 2011; Xavier-Carvalho et al., 2013; Yeo et al., 2014), immune factors, including their previous immunological experience (Gubler et al., 1978; Green et al., 1999; Vaughn et al., 2000; Navarro-Sánchez et al., 2005; Mathew and Rothman, 2008; Endy et al., 2011; Tisoncik

et al., 2012; Shresta, 2012; Salje et al., 2018) and antibody-dependent enhancement (Morens and Halstead, 1987), which will be further detailed below. Other factors, associated to more severe outcomes, such as the co-circulation of other microorganisms or the existence of chronic diseases (Bravo et al., 1987; Kouri et al., 1989; Teixeira et al., 2015; Pang et al., 2017), might also be implicated in protection against clinical manifestations.

Viral serotype and titer

Viral serotypes refer to closely related but genetically distinct viruses that, thanks to their different surface antigens, trigger different responses in the human host. In the case of DENV, the four known circulating serotypes share approximately 65% of their genomes and, even within the same serotype, genetic variation may exist (Anoop et al., 2012). Despite these variations, the different serotypes result in the same disease and range of symptoms.

An association between DENV-2 serotype and a higher proportion of severe dengue has been described (Balmaseda et al., 2006; Fried et al., 2010; Vicente et al., 2016). The efficient replication of this serotype, leading to high viral load is suggested as the main cause for more frequent severe manifestations (Vaughn et al., 2000; Thomas et al., 2008). In opposition, Yung et al. (2015) observed significantly higher risk of severe dengue and higher viral load in DENV-1 infections. On the other side of the spectrum lies DENV-4 serotype, which is typically associated with lower viral titters than other serotypes (Thomas et al., 2008) and milder forms of dengue fever (Nisalak et al., 2003; Thomas et al., 2014; Rocha et al., 2017).

Interestingly, Salje et al. (2018) observed a much higher proportion of asymptomatics in DENV-4 infections compared to the other 3 circulating serotypes, a result equally supported by Tsang et al. (2019). These results suggest that the distribution and proportion of serotypes found in most symptomatic surveillance-based studies may not be representative of circulating DENV serotypes in a given region, since the infections with some DENV serotypes could be more silent than others. It also suggests that the viral load, known to influence disease severity, might be partially responsible for asymptomatic infections, when at low levels. In fact, Duong et al. (2015)

were able to estimate that DENV viremia in asymptomatics (4.75 ± 0.39 log_{10} cDNA copies/mL) is similar to that observed in the early and late viremic periods of symptomatic viremia, but significantly lower than during the viremic peak, between days 1 to 4 of illness (6.12 ± 0.17 log_{10} cDNA copies/mL). Also, a slower decay of viremia was observed in asymptomatic infections when compared to symptomatic dengue, probably caused by a longer rate of clearance (Matangkasombut et al., 2020).

Another aspect, normally not addressed, is the size of the viral inoculum introduced during the mosquito feeding, that may trigger different initial immune response and/or lead to different viremia and thus influence the outcome of the infection outcome.

Concurrent infection with multiple serotypes is also believed to affect dengue severity (Bharaj et al., 2008; Vinodkumar et al., 2013; Lardo et al., 2016; Soo et al., 2016), although controversy on the subject exists (Gubler et al., 1985; Loroño-Pino et al., 1999). To our knowledge, no studies thus far report the effect of co-infections with multiple DENV serotypes in scenario of an asymptomatic outcome. Nevertheless, if such co-infection influences the viral load, we could expect that the outcome could also be affected.

Age

Host age seems to be associated with the likelihood of asymptomatic or symptomatic outcome following a DENV infection, particularly in children. Thai et al. (2011) estimated, in a Vietnamese pediatric cohort, that the risk of developing symptoms increases with age, during both primary and secondary infections, being lower for children under 10 years old. Similar results were obtained by Tsang et al. (2019) in a prospective pediatric cohort in Nicaragua, with children over 8 years old being more than twice as likely to develop symptoms upon infection. Also, Montoya et al. (2013) found that the mean age at which symptomatic dengue occurs is higher than for asymptomatic DENV infection (8.4 vs. 7.2 years old, respectively), in Nicaraguan children. Thai children aged between 10 to 15 years old were also more prone to develop symptoms during primary infections than those aged 4 to 9 years old (Burke et al., 1988). In opposition, Endy et al. (2011) found no relationship between age and the ratio of

asymptomatic:symptomatic infections in Thai children. This difference might be due to the limited number of studied children in the upper and lower age range and/or discrepancies in the study design.

Role of lipids

DENV infection and disease severity are believed to be directly linked to factors affecting lipid metabolism, serum lipoproteins, and their immunomodulatory effects, although their role is not perfectly clear (Cui et al., 2013; Biswas et al., 2015; Durán et al., 2015; Voge et al., 2016; Marin-Palma et al., 2019;). For instance, the presence of cholesterol and intact lipid rafts seem to be required for the activation of Jun NH(2)-terminal kinase and the p38 mitogen-activated protein kinases (MAPK) pathways during DENV infection of human macrophages (Ceballos-Olvera et al., 2010). Further, DENV entry into mammalian cells is associated with the expression of receptors associated with lipid rafts (Reyes-Del Valle et al., 2005). In fact, DENV infection promotes significant changes on cellular membranes to provide structures for the replication complex (Junjhon et al., 2014) and, possibly, to counteract the host cellular innate immune response (Uchida et al., 2014). DENV is also known to promote the activation of autophagy in infected cells (Lee et al., 2008), which is critical for viral replication in several viral infections, since autophagosomes have been proposed to be sites of active viral RNA replication (Wileman, 2006; Deretic and Levine, 2009), and because autophagy regulates lipid metabolism in infected cells. (Heaton and Randall, 2010) showed that DENV infection not only leads to the processing of cellular lipid droplets and triglycerides, but also that the depletion of lipid droplet correlates to an increase in autophagosomes and stimulation of β-oxidation in infected cells, to provide energy for DENV virus replication. Some authors have shown a reduction of lipid droplets in response to DENV infection, acting as an “energy sink” that is tapped during viral replication (Heaton and Randall, 2010; Tongluan et al., 2017), while others have reported an increase in lipid droplets (Samsa et al., 2009; Soto-Acosta et al., 2014), that act as binding sites for the DENV C protein during DENV assembly (Carvalho et al., 2012; Martins et al., 2012).

Evidence supporting a role of lipoproteins in the immunopathogenesis of dengue has been put forth by several studies that reported lower levels of high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol levels in more severe dengue cases (van Gorp et al., 2002; Suvarna and Rane, 2009; Biswas et al., 2015; Marin-Palma et al., 2019). In contrast, there is evidence of a direct association of serum apolipoprotein A-I (ApoA-I), the major protein component in HDL, with increased infectivity by Flaviviruses, as ApoA-I seems to facilitate their cell entry via scavenger receptor class B type (SR-BI) (Li et al. 2013). For other members of the Flaviviridae family, evidence also exists that low-density lipoproteins receptors (LDL-R) may be the main entrance in cells (Agnello et al., 1999). Moreover, it was hypothesized that DENV may form lipoviroparticles, which would constitute a novel step in DENV life cycle (Faustino et al., 2014). For last, it is worth mentioning that, besides the well-known cytokine-induced changes of lipoprotein profile during infection (Grunfeld and Feingold, 1996), HDL also has immunomodulatory properties, through the regulation of inflammasomes and SR-BI expression in macrophages (Song et al., 2015; Thacker et al., 2016).

Evidence highlighting the interaction between lipid metabolism and protection against clinical dengue was reported by (Sierra et al., 2017), whose genome-wide association study (GWAS) has identified two new genes – *OSBPL10* and *RXRA* - playing a role in infection resistance (more details in section *Host genetics*). Nevertheless, no comprehensive studies of lipids and metabolic profiles directed at asymptomatic individuals exist and are thus needed to better understand the role of lipids on the asymptomatic outcome.

Host Immune response

Studying the host immune factors underlying asymptomatic and subclinical arboviral infections is highly challenging, since many factors, such as differences in the host immune status and in the viral load between symptomatics and asymptomatics, can bias the results. In DENV infections, three main approaches have been used for this purpose, namely the comparison of gene expression profiles, cytokine serum levels, and *in vitro* stimulation of PBMCs between symptomatics and asymptomatics.

A few studies compared the gene expression profile of key molecules involved in the immune response of asymptomatic or symptomatic infections. Yeo et al. (2014) found a broad downregulation of host defense genes in asymptomatic infections when compared with symptomatics, and an up-regulation of a few specific genes. However, the patients analyzed in this study were already in the convalescent phase, with undetectable viremia. The results are, therefore, more likely to correspond to feedback mechanisms aiming to restore homeostasis, with little direct contribution to the knowledge about host immune factors influencing the clinical outcome. On the other hand, Simon-Lorière et al. (2017) compared the gene expression profiles and serum levels of inflammatory cytokines in viremic asymptomatic and symptomatic children, considering their viral load. Although the asymptomatic group was quite small (n=9), no major differences were found between the two studied groups for genes involved in innate immune pathways such as antiviral immunity, activation of pattern recognition receptors, or IL-8 signaling. Accordingly, in Raghupathy et al. (1998), serum concentrations of the inflammatory cytokines IL-6, IL-8, IL-15, CCL3, and CCL4 were not different between viremic asymptomatic and clinical dengue patients, with the main observed differences being associated with dengue severity. In contrast, the pathways involving antigen presentation and activation of T and B cells were differentially expressed between the two groups studied by Simon-Lorière et al., (2017). In particular, these authors observed an up-regulation of genes involved in the antigen-presentation pathway, and in dendritic cell maturation in viremic asymptomatic children. Serum concentrations of IL-12 and IL-23, both indicative of antigen-presenting cells (APCs) activation, were also increased, while the CD86 co-stimulatory molecule was significantly down-regulated in both CD14+ monocytes and Lin−CD11c+ dendritic cells. In addition, several T cell co-stimulatory pathways such as ICOS-ICOSL signaling in T helper cells, CD28/CTLA4 signaling in cytotoxic T lymphocytes, and expression of CD69 (an early activation marker of T cells), were up-regulated in asymptomatic children. In this same study, the serum concentration of IL-2, a cytokine associated with T cell activation and proliferation, was also increased, and the IL-2 signaling pathway was up-regulated. On the other hand, immune response feedback mechanisms were also increased in the asymptomatic group (Simon-Lorière et al., 2017). Higher activation of T cells has been previously associated with asymptomatic DENV infection, when

comparing *in vitro* stimulated PBMCs from healthy individuals, who subsequently developed either asymptomatic or symptomatic secondary DENV infections (Hatch et al., 2011; Friberg et al., 2018). Using this approach, the authors found a generally higher frequency of DENV-specific TNFα, IFNγ, and IL-2-producing T cells in the group that later developed secondary asymptomatic infections (Hatch et al., 2011), and a significantly lower secretion of IL-12, IL-2R, MIP-1α, RANTES, GM-CSF, and TNFα by PBMC from subjects who developed symptomatic infection (Friberg et al. 2018). In sum, asymptomatic DENV infections, at least secondary ones, seem to be associated with increased T cell activation and antiviral cytokine production coupled with proper immune response regulation.

Interestingly, a few studies addressing the symptomatic outcomes indicate that T cell activation may contribute to the pathogenesis of DENV infection through the production of inflammatory cytokines, leading to an exacerbated response (Chaturvedi et al., 2000; Mathew and Rothman, 2008; Rothman, 2011). However, other authors suggested that T cells, including CD8 and CD4 cytotoxic T cells, may play an important role in protection against severe dengue, allowing an efficient elimination of the virus without excessive immune activation and, hence, without causing severe disease (Weiskopf and Sette, 2014; Weiskopf et al., 2015). It is possible that asymptomatic primary infections lie at the extreme of this response, with a higher anti-viral T cells activation coupled with proper control mechanisms, allowing viral clearance without leading to clinical symptoms. A limitation of the above-mentioned studies is that none was carried out in primarily infected populations and thus, the pre-existence of memory T cells, cross-immunity to different serotypes, and window of antibody clinical protection may have influenced the results. More recently, based in a study involving primary and secondary infections, Rouers et al. (2021) suggest that outcome of a dengue symptomatic infection may result from an individual propensity, genetic and/or environmental determined, to produce particular adaptative cell phenotypes. It is possible that the baseline adaptative cellular profile of an individua may also contribute to the asymptomatic outcome. This may be true not only for the adaptative immune cells phenotypes but also for the innate immune cells, as consequence of the trained immunity.

Host genetics

In the last decades, there is increasing evidence that genetic polymorphisms play a role in the activation of different immuno-pathological mechanisms involved in DENV infection and, therefore, in its different outcomes. However, most studies focused on identifying host genetic factors correlated to disease severity (Coffey et al., 2009; Lan and Hirayama, 2011; Xavier-Carvalho et al., 2017; reviewed in Cahill et al. (2018)), while the genetic determinants of asymptomatic DENV infection have been largely disregarded.

The studies of García et al. (2010) and Mohsin et al. (2015) accessed the contribution of the *FcγRIIa* rs1801274 polymorphism to the clinical manifestations of dengue in Cuban and Pakistani populations, respectively. The H131 allele was found to increase the odds of developing clinical dengue, while R131 confers protection against the clinical forms of the infection. FcγRIIa is an Fc receptor, which binds to the Fc component of the IgG antibodies, and this SNP is known to change the affinity of this Fc receptor to different IgG subclasses. FcγRIIa-R131 receptors bind efficiently to IgG1 and IgG3 (van Sorge et al., 2003), which are the predominant immunoglobulins during DENV infection (Koraka et al., 2001), and their interaction with opsonized DENV activates phagocytes, leading to a more efficient control of the viral dissemination (Forthal and Moog, 2009). On the other hand, FcγRIIa-H131 receptors seem to preferentially interact with IgG2 (Clark et al., 1991), which may favor the viral dissemination by ADE (Moi et al. 2010). Surprisingly, Noecker et al. (2014) suggest a protective role for FcγRIIa-H131 in Mexicans, while the FcγRIIa-R131 associates with symptomatic dengue. According to the authors, the observed reverse association when compared to the findings of García et al. (2010) may be explained by differences between populations, either in other unstudied interacting genetic factors, age distribution, and history of infection (data absent in García et al. (2010)), and/or viral serotype (DENV-4 in Cuba and DENV-1 in Mexico).

Silva et al. (2010) analyzed the association of several candidate genes of the type I interferon (IFN) response pathway in Brazilian symptomatic and asymptomatic infections, being the top two most significantly associated polymorphisms located at the

5' end of *JAK1* gene. This work focused mainly on the effect of genetic variants on disease severity, by comparing SNP frequencies of DHF with DF, but the authors claim that the association remains true when comparing DHF against asymptomatics, though weaker (data not shown). JAK1 is one of the first components of the type I IFN receptor signaling pathway, known to control the response to flavivirus in mouse models (Perelygin et al., 2002). The associated SNPs may exert regulatory effects in *JAK1* expression and, thus, lead to the under-expression of type I IFN-induced genes, already described in severe dengue (Simmons et al., 2007).

More recently, SNP haplotypes in *MBL2* have been significantly associated with dengue severity in a sample set of Brazilian children with severe dengue, when compared to asymptomatic DENV IgG-positive controls (Ornelas et al., 2019). The mannose-binding lectin (MBL), a pattern recognition receptor encoded by *MBL2*, acts in the first-line response to DENV by triggering complement activation to promote viral neutralization (Avirutnan et al., 2011). *In silico* prediction has suggested that these SNPs act as expression regulators (Ornelas et al., 2019) and, thus, likely result in the deficiency of MBL already observed in severe dengue (Alagarasu et al., 2012; Figueiredo et al., 2016).

Polymorphisms in the *IL4R* and *IL6R* cytokine receptors were pinpointed as risk factors for clinical dengue in Colombian children (Useche et al., 2019), as these may introduce changes in the T cells signal transduction, and thus alter the Th subtypes activation in dengue. The upregulation of IL4R has been previously described in asymptomatic DENV infection, when compared to clinical dengue (Yeo et al., 2014), suggesting its protective role. The A-allele of the SNP in IL6R may result in lower levels of its soluble form sIL-6R, favoring classical IL-6 signaling (Ferreira et al., 2013), more associated with anti-inflammatory responses, which may impair the antiviral response against DENV. Note, however, that this study based its conclusions on the comparison between DENV IgG-positive symptomatics and a control group lacking symptoms, but not tested by serology. This being a dengue-endemic region, the control group may include both asymptomatics and non-infected individuals, considerably biasing the results.

The hypothesis-free GWAS conducted by Sierra et al. (2017) highlighted polymorphisms in *OSBPL10* and *RXRA* as inherited protective factors against DHF in Cubans with African ancestry, when compared with asymptomatics and population controls. Both genes are involved in the LXR/RXR activation pathway, which integrates lipid metabolism and immune functions, and are thus key players in viral entrance and replication, and in cytokine production. Their expression study in Cuban DHF cases and the analysis of a Thai dengue transcriptome dataset showed that both these genes are differentially expressed along disease progression. Furthermore, the *in vitro* knockdown of *OSBPL10* in THP-1 cells followed by DENV-2 infection, led to a significant reduction of viral replication, providing functional proof that the naturally occurring lower levels of OSBPL10 in Africans contribute to their natural resistance against clinical dengue.

The results of the above-mentioned studies are not without controversy, as some remain largely discrepant, while others lack functional evidence for their positive associations. Differences in the studies' design and data analyses exist, many times without clear biological reasoning. For instance, we found common practice in these studies to infer about the influence of genetic polymorphisms in dengue severity by comparing their frequencies in severe forms DHF/DSS against those in asymptomatics, instead of between groups with different symptomatic outcomes. Plusmore, we recognize one other important limitation: most of these studies were undertaken in dengue-endemic regions and, as retrospective studies, were unable to distinguish between primary and secondary infections. This will surely act as a confounding factor, making it difficult to study the natural, genetically conferred clinical protection in primary infections.

Concluding remarks

For many viral infections, including those caused by Flaviviruses, asymptomatics represent the unseen part of the iceberg. It is, therefore, crucial to understand their real impact on the spread of the pathogenic agent and its persistent circulation during interepidemic periods, as well as their contribution to herd immunity. It is equally imperative to determine the individual immune status induced by asymptomatic viral infections, the eventual emergence of long term sequalae, and, for certain viruses like

DENV, if the secondary infections pose an increased susceptibility of asymptomatics to more severe forms of the disease, as widely described for symptomatics. The very recent example of the SARS-CoV-2 illustrates how challenging it can be to understand these aspects. In fact, the perception of the impact of asymptomatic infections on viral transmission and on individual health has been changing since the beginning of the pandemic.

For a clearer picture, it is also essential to study the biological mechanisms conferring protection against clinical manifestations (resumed in **Figure 1**), observed in many flaviviral diseases. Why do some people become ill in a primary infection, while others are tolerant to the disease being able to clear the virus without changing their health status? Most studies concentrate their efforts on the determinants of the severe outcome *versus* mild infection, while asymptomatic infections remain disregarded. Furthermore, and especially in endemic areas, immunity acquired through a previous flaviviral infection can lead to clinical protection in a subsequent infection. This introduces a confounding factor for the identification of asymptomatics, which must be accounted for in the study design, since the mechanisms leading to the absence of symptoms within a window of immune protection are surely different from those experienced in an asymptomatic primary infection.

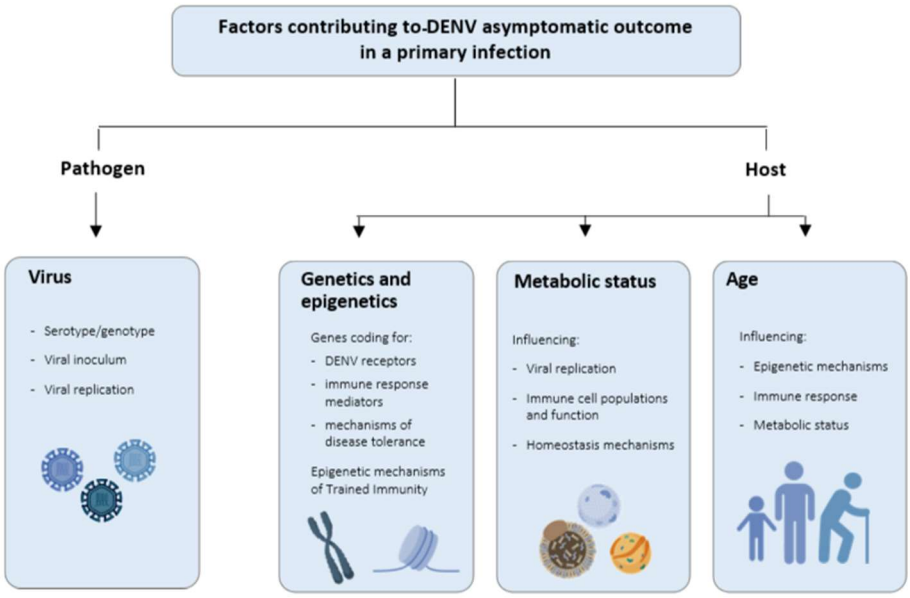

Figure 1 - Factors likely influencing the clinical protection in individuals experiencing a DENV primary infection. Different viral serotypes/genotypes may have different abilities to infect human cells and/or replication capacity, thus influencing the viral load and the outcome of the infection. in the same line, the viral inoculum during the mosquito blood meal may also affect it. on the other hand, host genetic polymorphisms, namely in genes coding for receptors used by DENV to infect human cells and mediators of the immune response or disease tolerance can protect against clinical manifestations, either by affecting viremia or by other mechanisms. additionally, the influence of trained immunity, as an epigenetic reprogramming of innate immune cells induced by previous exposure to unrelated pathogens, should be here considered. the metabolic status of the host can also influence clinical protection, either by affecting viremia or by modulating the immune response and/or the homeostasis mechanisms. nonetheless, metabolic status, immune response, and epigenetic mechanisms change according to age and, therefore, outcome of the infection may also be influenced by age. (Diagram was made using BIORENDER).

Additional evidence from adequately designed genetic and functional studies objectively addressed to asymptomatics is demanded, preferably in populations with unique epidemiological situations such as a primary infection by a single DENV serotype. These may strongly contribute to a better understanding of the mechanisms of the disease, by evidencing genes, cellular pathways and lipid profiles contributing to the natural protection against dengue. Hence, comprehensive knowledge of the physiological and immunological processes balancing the control of the flaviviral agent and the health status of the individuals, in asymptomatic infections, can be crucial for the rational design of effective therapies, and are likely common to other infectious diseases.

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CHAPTER III

Host immune response to DENV infection

Preamble

While in primary DENV infections the clinical outcome is likely influenced by a variety of viral and host factors, in secondary infections notwithstanding the influence of those same factors, it seems to be highly influenced by the serological status of the population. This is because pre-existing antibody titers that are protective against homotypic infections can lead to severe disease in heterotypic infections due to ADE. Following the Madeira Island dengue outbreak occurred between 2012 and 2013is important to evaluate the actual serological status of the population of Madeira Island. To that end we must consider both symptomatic and asymptomatic cases, which are likely the most frequent. Past infection can be identified by using Anti-DENV Indirect IgG ELISAs in conjunction with the epidemiological context. Information provided by this serosurvey, together with the information concerning their neutralization capacity, given by the FRNT assays, provides the opportunity to better characterize the serological status of this population and investigate potential differences in the humoral response between symptomatic and asymptomatic infections. Additionally, the identification of asymptomatic infection cases in a population with a single exposure to DENV, allows the development of studies aiming to identify the host factors that lead to the asymptomatic outcome.

The study of the serological status 8 years after a primary infection by DENV-1 in Madeira Island, and the ongoing work on the permissiveness of PBMCs to DENV infection and possible influence on the disease outcome is detailed herein in Chapter III.

The work depicted in the present chapter resulted in an original research article currently being prepared for submission.

Antibody response against Dengue in asymptomatic and symptomatic infections in Madeira Island

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Abstract

Dengue virus (DENV) is a Flavivirus with 4 known serotypes. Primary DENV infections are often asymptomatic but seem to generate immunity to the homologous strain. On the other hand, non-neutralizing antibodies generated during a primary infection have been suggested to enhance the risk of severe disease in a subsequent infection with a different serotype. Therefore, understanding the decay over time of IgG antibodies from dengue cases is not only crucial for the development of vaccination strategies, but also for the prediction of the risk of a population to develop severe disease in a subsequent outbreak. Most of the studies about dynamic changes of IgG antibodies have been conducted in dengue endemic areas and/or following symptomatic infections. Nevertheless, little is known about IgG antibodies produced in response to asymptomatic DENV primary infections, although 50-90% of primary infections have been reported to be asymptomatic.

Our study aims to investigate the humoral immune response to DENV infection in the population of Madeira Island, who experienced a primary and unique DENV-1 outbreak in 2012-2013. For this purpose, we have recruited 31 participants with asymptomatic infections, among blood donors which, at the time of the outbreak have tested positive by RT-PCR and did not develop any dengue symptoms. Current levels of anti-DENV IgG antibodies were determined by ELISA assays at 1:10 serum dilution and their neutralizing ability was assayed by foci reduction neutralization tests (FRNT). Sera from 119 participants who had experienced a symptomatic DENV infection during the outbreak were also tested for anti-DENV IgG at 1:100 dilution.

While current levels of anti-DENV IgG antibodies in symptomatic participants were high, these levels were very low or undetectable in blood donors with previous asymptomatic infections. Moreover, we found that symptomatic participants still have cross protective antibodies against DENV-2, 8 years after the outbreak, that correlate to total antibody titer.

These data suggest that asymptomatic DENV infections may induce a low humoral response, which is lost with time or does not lead to seroconversion at all, when compared to symptomatic infections.

1. Introduction

Dengue virus (DENV) is a *Flavivirus* with 4 known serotypes (DENV-1-4), which is responsible for the most important arthropod-borne viral infection worldwide. A recent report estimated that around 390 million infections caused by DENV occur yearly (Bhatt et al. 2013). Of those, only about 96 million are symptomatic infections, classified as dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) according to World Health Organization (WHO) criteria (Bhatt et al. 2013). The remaining 300 million annual infections represent mild or asymptomatic infections. Although asymptomatic infections can be a consequence of a memory immune response developed during an earlier exposure to the virus, primary DENV infections are also often asymptomatic, representing more than 50% of the primary infections (Endy et al. 2011; Bhatt et al. 2013).

A first DENV symptomatic infection leads to the production of anti-DENV neutralizing antibodies, responsible for long-lasting protection against homologous infections, and for a window of protection of approximately 2 years against heterologous DENV serotypes (Sabin 1952; Montoya et al. 2013; Anderson et al. 2014; Katzelnick et al. 2016). Following this period of protection, an increased risk of developing severe dengue in a subsequent heterologous infection is observed (reviewed in Rothman (2011)). This ability of anti-DENV antibodies, of protecting or placing individuals at risk for a severer dengue outcome, has been associated with their decay over time (Katzelnick et al. 2017; Salje et al. 2018; Waggoner et al. 2020). When at sub-

neutralizing concentrations, anti-DENV antibodies bind to but do not neutralize the viruses, facilitating their entry into host cells by a process called antibody-dependent enhancement (ADE), and thus leading to an increased risk of a severer outcome (reviewed in Halstead (1988)).

Most studies regarding dynamic changes of anti-DENV IgG antibodies over time have been conducted in dengue endemic areas and/or following symptomatic infections. Two serological surveys carried out in different Chinese provinces found that, after a single symptomatic infection, the concentration of anti-DENV IgG antibodies decays over time (Luo et al. 2018; Wu et al. 2020), with around 4% of the individuals showing undetectable levels of antibodies in the following 3-year period. A significant decay in the concentration of anti-DENV IgG antibodies between the 2nd and 5th year after symptomatic infection was also observed by Wu et al. (2020), in a Chinese population. The level of pre-infection neutralization antibodies may determine clinical protection or risk of severe disease upon subsequent infection. In a pediatric study, Katzelnick et al. (2017) observed a half-life of 4.0 years for anti-DENV antibodies, and estimated that, by 3 years post-infection 22% of children had low titers (1:21 to 1:80) as measured by inhibition ELISA. Importantly, a correlation between anti-DENV antibody titers of 1:21 to 1:80 and the risk for DHF/DSS or severe dengue disease was found in this study. On the other hand, protective effects against symptomatic dengue were observed by these authors at titers above 1:320. Other studies, performed in adult (Ly et al. 2018) and pediatric populations (Katzelnick et al. 2016), also reported a correlation between high neutralizing antibody titers and protection against symptomatic dengue.

Despite their increasing incidence, antibody response and decay following DENV primary asymptomatic infections is much less known. In the asymptomatic cohort of Luo et al. (2018), established as IgM-positive individuals in the absence of disease symptoms, in an area where a single outbreak occurred, only 27% of asymptomatics remained IgG seropositive (at a 1:100 dilution) 3 years after the infection. These results suggest either i) a lowered antibody response and/or ii) a faster antibody decay following asymptomatic infections, or even iii) the absence of seroconversion in many of the asymptomatic cases. Thus, understanding the antibody decay following asymptomatic

dengue is also crucial, not only for the risk assessment of a population to develop severe disease in a subsequent outbreak but also for the development of vaccination strategies.

In 2012, the first and unique dengue outbreak took place in Madeira Island, Portugal, with a single serotype circulating (DENV-1). From September 26th, 2012, to March 3rd, 2013, of the 2168 probable cases reported, 1080 were confirmed but, fortunately, neither severe clinical forms nor fatalities were reported (ECDC 2014). Although a serological study was performed a few years ago in the population of Madeira Island by Auerswald et al. (2019), few is known about their current levels of anti-DENV antibodies, and no studies were conducted focusing on asymptomatic individuals. According to the European Center for Disease Control (ECDC 2014), among 1948 blood donations obtained at Hospital Central do Funchal during the outbreak, 43 donors tested positive for DENV-1 by RT-PCR but only one became symptomatic. Therefore, this population constitutes a unique opportunity to evaluate the antibody decay after symptomatic and asymptomatic infections and likewise, to have a more realistic picture of the present DENV serological situation in Madeira Island. In this study, we investigated the humoral immune response following primary asymptomatic and symptomatic DENV-1 infections, in the population of Madeira Island, 8 years after the single DENV-1 outbreak.

2. Material and Methods

2.1. Ethics Statement

The study protocol was approved by the Ethics Committee of SESARAM-E.P.E.R.A.M. (approval nº 23/2019). All procedures complied with the Declaration of Helsinki, and with regulations for the protection of human subjects, rights, and personal data of the enrolled participants. Written informed consent was obtained from all participants.

2.2. Recruitment of participants and sample collection

Confirmed asymptomatic participants were recruited among blood donors who tested RT-PCR positive for DENV infection during the outbreak that occurred in Madeira Island between September 2012 and March 2013, and did not develop any symptoms. Additionally, possible asymptomatic participants were recruited among co-inhabitants or co-workers of the symptomatic participants from the parishes of the municipality of Funchal that were most affected by dengue, namely Santa Luzia, São Pedro, Santa Maria Maior, São Roque and Sé, with no diagnosis of dengue fever and without travel history to dengue-endemic countries.

Symptomatic participants were recruited among probable or confirmed dengue cases, followed at the Central Hospital's emergency service or healthcare centers from the Regional Healthcare System Service (SESARAM-E.P.E.R.A.M.) during the outbreak, with clinical and/or laboratory (ELISA or RT-PCR) diagnosis, and aged between 13 and 65 years at the time of infection. Additionally, naïve controls, who did not reside in Madeira Island at the time of the outbreak and with no travel history to dengue endemic areas, were also recruited.

Before the collection of blood samples, each participant was informed of the purposes of the study and answered a questionnaire with relevant information, including demographic variables (age, gender), travel history to dengue-endemic countries, vaccination, and symptoms experienced during the infection, such as systemic (fever, myalgia, asthenia, muscle fatigue, headache, and arthralgia), gastrointestinal (nausea, vomiting/abdominal pain, and diarrhea), cutaneous (exanthema, palmar erythema, and rash), ophthalmological (retro-orbital pain, and maculopathy), hemorrhagic (epistaxis, petechiae, gingival bleeding, metrorrhagia, and hematuria), and respiratory (pharyngitis, rhinorrhea, otalgia, and cough) symptoms. Clinical data assessed during recruitment was complemented with clinical data provided by healthcare services, regarding symptoms at the acute phase of the disease. All blood samples were collected by venipuncture into PET BD Vacutainer Serum Separation Tubes II Advance (BD Biosciences, CA). They were immediately centrifuged, separated, and divided in several serum aliquots, which were stored at -80 °C until further analysis.

2.3. Detection of anti-DENV IgG by ELISA

31 confirmed asymptomatic, 49 possible asymptomatic, 119 probable and/or confirmed symptomatic, and 11 naïve participants (used as baseline controls) were screened for previous DENV infection using the Dengue IgG indirect Enzyme-Linked Immunosorbent Assay (ELISA) (ref. 266a-9601-1G, Euroimmun, Germany), according to the manufacturer's instructions for the quantitative method. Serum samples from symptomatic individuals were first screened at a 1:100 dilution, while asymptomatic and naïve serum samples were screened at a 1:10 dilution (Relative Units (RU) obtained from the calibration curve were then divided by a factor of 10). Symptomatic participants with borderline ELISA results were subsequently tested at 1:10 serum dilution. Following the manufacturer's guidelines, positive results were considered for samples with RU>22, borderline results for samples between 16 and 22 RU, and negative results for samples with RU<16. In order to ascertain the range of antibody titers, still detectable in symptomatic participants 8 years after DENV infection, positive serum samples were additionally tested at 1:160 and 1:1280 dilution, allowing to establish 3 level groups of participants: i) with high titers ≥1:1280), ii) with low titers <1:160 and iii) with medium titers (≥1:160 and <1:1280). The cut-off value was set at index≥1.1, following manufacturer´s instructions.

2.4. Focus reduction neutralization tests (FRNTs)

All confirmed asymptomatic participants (blood donor participants) were analysed by Focus reduction neutralization test (FRNTs), the most specific and sensitive test for detection of anti-DENV neutralization antibodies (Roehrig, Hombach, and Barrett 2008; Timiryasova et al. 2013). Possible asymptomatic participants with a positive or borderline ELISA result and symptomatic participants with borderline ELISA results were also further confirmed by FRNT. DENV-1 used on FRNT assays was originally isolated by the Department of Virology of the University of Helsinki, from a Finnish tourist infected in Madeira Island during the dengue outbreak (Huhtamo, Korhonen, and Vapalahti 2013). It was propagated in a *Aedes albopictus* C6/36 cell line (ATCC® CRL-1660, Manassas, VA, USA.) in completed Dulbecco's modified Eagle's medium (cDMEM;

DMEM, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.0 mM L-glutamine), supplemented with 2% heat-inactivated fetal bovine serum (FBS) (cDMEM-2) (GIBCO, Invitrogen, Carlsbad, CA) for 4 days at 33°C, 5% CO_2, and then stored at -80°C. Viral titer was determined by plaque assay on Vero cells (ATCC® CCL 81, Manassas, VA, USA), as previously described by Timiryasova et al. (2013). Briefly, FRNTs were performed in Vero cells, grown in cDMEM, supplemented with 10% heat-inactivated FBS (cDMEM-10), seeded in 48-well plates at a density of 40x 10^3 cells per well, and incubated at 37°C, 5% CO_2 for 48 hours. Patient serum was heat-inactivated at 56°C for 30 minutes, diluted at 1:5 in cDMEM without FBS (cDMEM-0), and mixed with equal volume of DENV-1, diluted in cDMEM-0 to yield 40-50 plaques/well, following incubation at 37°C for 30 minutes. Cell culture medium was removed from the 48-well plate, containing the Vero cell, and washed with Phosphate-buffered Saline (PBS) solution. The virus-serum mixture was transferred to a 48-well plate in triplicates and incubated at 37°C, 5% CO_2 for 90 minutes. The inoculum was then removed and replaced by 400 µL of 1,5% Sodium carboxymethyl cellulose (CMC, Sigma-Aldrich, St. Louis, Missouri, USA) in cDMEM supplemented with 5% heat-inactivated FBS (DMEM-5) and HEPES 1M (GIBCO, Invitrogen, Carlsbad, CA). Plates were then incubated at 37°C, 5% CO_2 for 4-5 days. Thereafter, the CMC overlay was aspirated from each well, which was washed twice with PBS. Cells were further fixed with 10% formaldehyde for 30 minutes, at room temperature (RT). Endogenous peroxidase was blocked with 0.3% oxygen peroxide for 20 minutes and cells were permeabilized with a PBS 0.1% BSA /0.1% Triton-X100 solution for 10 minutes at RT. Cells were then incubated overnight at 4°C with DENV anti-envelope protein biotinylated antibody (4G2) (Novus Biologicals, UK), at a dilution of 1:2000 followed by incubation for 60 minutes at RT with Avidin-HRP (Vector Laboratories, USA), at a dilution of 1:1000. Viral plaques were then visualized after incubation with True Blue (Biogen, Spain) for 25 minutes at RT. Washing steps with PBS 1x were implemented in between each step of the immunostaining procedure. Serum samples tested at a 1:10 dilution that manifested a reduction greater than 90% in virus infectivity, when compared to the average count of the virus-free serum controls, were considered to present specific neutralization antibodies.

FRNTs were also performed to further evaluate the neutralization capacity of serum antibodies from participants showing high, medium, and low antibody titers,

against other dengue virus isolates/serotypes. In these participants, using triplicates at 1:10 and 1:100 serum dilution, we have tested DENV-1 isolate from Madeira's outbreak described above, a different isolate from DENV-1 obtained from Brazil (DV1/BR/90, GenBank accession number S64849.1, Instituto Carlos Chagas/Fiocruz PR) (Silveira et al. 2018) and a DENV-2 isolate provided from Finland (Department of Virology of the University of Helsinki) isolated from a traveler coming from Ghana in 2005 (Cosmopolitan genotype, GenBank accession number F32.D2.05) (Huhtamo et al. 2008).

2.5. Statistical Analysis

All statistical analyses were performed with SPSS Statistics software (version 20.0, IBM Corp, USA) Datasets were first tested for normality using the Kolmogorov-Smirnov test, in order to determine which tests would be appropriate for further data analyses. Student T-test and Mann-Whitney U test were used to verify the existence of statistically significant differences in RU values of the different groups, according to sex, age, presence or absence of clinical manifestations, and groups of symptoms. Differences between classes of antibody titers (low, medium, and high) according to the sex and age were tested using Pearsons' Chi-square (χ^2) test. Statistical differences of the inhibition percentage for the different antibody titer groups were determined using the Wilcoxon signed-rank test. Results were interpreted at a significance level of 0.05.

3. Results

3.1. Characterization of participants

A total of 119 participants were enrolled as symptomatic. These participants had a clinical diagnosis of dengue by the regional healthcare system, 8 years before participation in this study. Only 10 participants with dengue symptoms at that time had no register in the healthcare system database but were included in the study, after confirmation of their seropositivity by ELISA and no travel history to dengue endemic regions. Of the total of 119 symptomatic participants, 72 (60.5%) were female. Their mean age at the time of enrolment in this study was 46.3 ± 12.6 years old (y.o.) (ranging from 21 to 69 y.o.), with no significant age differences between gender (48.3 ± 12.2 y.o.

for male *versus* 45.0 ± 12.8 y.o. for female, *p*= 0.166) (Table 1). Twelve (10.8%) symptomatics were aged ≥63 y.o., meaning ≥55 y.o. at the time of infection. Thirty-one blood donors with DENV-1 infection confirmed by RT-PCR but no symptoms during the outbreak, were enrolled as confirmed asymptomatic. The mean age of this group was 49.6 ± 10.4 y.o, ranging from 28 to 72 y.o., and most of these participants were males (90.3%) (Table 1). Forty-nine participants were also recruited as possible asymptomatic. The mean age of this group was 49.3 ± 9.0 y.o., ranging from 21 to 68 y.o., with 63.3% of these being female. The demographic characteristics of the participants are summarized in **Table 1**.

Table 1 *- Demographic characterization of participants*

		Gender		Mean age		
Participant	**Overall *(n)***	**Male *% (n)***	**Female *% (n)***	**Overall y.o. ± sd**	**Male y.o. ± sd**	**Female y.o. ± sd**
Confirmed Assymtomatic	31	90.3 (28)	9.7 (3)	49.6 ± 10.4	50.9 ± 9.8	36.7 ± 7.6
Possible Assymtomatic	49	36.7 (18)	63.3 (31)	49.3 ± 9.0	50.9 ± 9.9	48.2 ± 8.5
Symptomatic	119	39.5 (47)	60.5 (72)	46.3 ± 12.6	48.3 ± 12.2	45.0 ± 12.8

Abbreviations: y.o.-years old; sd- standard deviation

3.2. Anti-DENV IgG seroprevalence 8 years after DENV infection

Serum of all participants was screened for the presence of DENV IgG antibodies by ELISA. When ELISA results were positive but with a low RU value, or a negative result was obtained (including all asymptomatic participants), confirmation was made by FRNT assays with Madeira DENV-1 strain, as described in detail in Material and Methods. We observed that samples testing positive in ELISA, even at low RUs, showed more than 90% reduction in foci formation by FRNT at a 1:10 dilution, therefore validating the ELISA results. The exception was one confirmed asymptomatic participant who showed negative results in FRNT, suggesting a false positive result in ELISA, and thus being classified as seronegative. None of the samples testing negative by ELISA showed focus reduction by FRNT.

The seroprevalence of dengue IgG antibodies among the 31 confirmed asymptomatic group was very low: only 1 (3.2%) participant was positive for both ELISA and FRNT, at a 1:10 dilution (**Table 2**). Interestingly, a comparable seropositive rate (4.1%) was also found among the participants recruited as possible asymptomatic (**Table 2**). In contrast, for the participants who had experienced a symptomatic infection, the total serum IgG antibody positive rate was of 93.3% (111 out of 119) (**Table 2**). From these participants, 96 (80.7% of the total) were ELISA DENV IgG positive at 1:100 dilution, while 15 (12.6%) participants only had detectable IgG anti-DENV at a 1:10 serum dilution, by ELISA and FRNT (data not shown). Eight participants (6.7%) had no detectable antibodies, even at 1:10 dilution (**Table 2**). Of these last participants, one had clinical and laboratory diagnosis (NS1 and IgM positive) at the time of the outbreak, while the remaining 7 only had clinical diagnoses, since they were living in the most affected area. Although not statistically significant, the positive rate of serum IgG DENV antibodies in males (95.7%) was slightly higher than in females (91.7%). The positivity rate of serum IgG DENV antibodies in symptomatic participants aged ≥63 years y.o. (100%) tends to be higher than in participants <63 y.o. (92.5%) (**Table 2**). However, the mean age of those symptomatics without detectable antibodies was not significantly different from seropositive symptomatics (47,38± 12.71 y.o. *versus* 46.37± 12.66 y.o). Altogether, we found that the serum of 6.7% of the symptomatic and 96.8% of the asymptomatic participants had no neutralization activity against the DENV-1 strain responsible for the outbreak (**Table 2**). These results strongly suggest that, following a

primary DENV symptomatic infection, seroprevalence decreases but remains relatively high after 8 years, contrasting with the very low seroprevalence observed after asymptomatic infections.

Table 2 - *Seroprevalence of dengue IgG antibodies*

		Seroprevalence *			
Group	**participants *n***	**Positive % (n)**	**Negative % (n)**	Test	*p* value
Confirmed Asymptomatics					
Overall	*31*	3.2 (1)	96.8 (30)		
Possible Asymptomatics					
Overall	*49*	4.1 (2)	95.9 (47)		
Symptomatics					
Overall	*119*	93.3 (111)	6.7 (8)		
Male	*47*	95.7 (45)	4.3 (2)	χ^2=0.754	0.385
female	*72*	91.7 (66)	8.3 (6)		
≥ 63 y.o.	*12*	100 (12)	0 (0)	χ^2=0.962	0.327
< 63 y.o.	*107*	92.5 (99)	7.5 (8)		

* Determined by Elisa and FRNT>90%. Pearson's Chi-square (χ^2)

3.3. Concentration of anti-DENV IgG antibodies 8 years after infection

To evaluate the concentration of anti-DENV specific antibodies within seropositive participants, we analyzed the distribution of the Relative Units values (RU/mL) obtained by ELISA. Among the only 3 seropositive asymptomatic participants, the concentration of IgG antibodies 8 years after the infection was very low (11.3 ± 2.8 RU/mL, assessed at a 1:10 dilution and divided by a factor of 10). Conversely, 8 years after a symptomatic infection, the concentration of total IgG antibodies was 77.0 ± 41.7 RU/mL (at a 1:100 dilution), ranging from 6.1 to 154.5 RU/mL (**Figure 1a**). Interestingly, contrary to what was found for the seroprevalence, anti-DENV antibody concentration was significantly higher in females than in males (85.0 ± 38.4 RU/mL *versus* 65.3 ± 44.0 RU/mL, p=0.014, **Figure 1b**). Mean RU values between participants aged ≥63 y.o. and <63 y.o. was not statistically different (77.18 ± 45.47 RU/mL *versus* 71.49 ± 44.36 RU/mL,

respectively, data not shown). Also, no correlation between age and RU values was found (**Figure 1c**)

To figure out a possible association between the current concentration of DENV-specific IgG antibodies and signs/symptoms at the time of the active infection, we compared the differences in RU/mL distributions between participants reporting the presence or absence of each group of symptoms, namely systemic, gastrointestinal, cutaneous, ophthalmological, hemorrhagic, and respiratory. We found that patients with hemorrhagic symptoms had significantly higher mean RU values (mean RU/mL = 83.28 ± 41.13) than those without these symptoms (mean RU/mL =64.73 ± 45.05) (Mann-Whitney U=1260, p=0.019) (**Figure 1d**). No other significant differences were found for the comparison of participants with and without the other groups of reported symptoms.

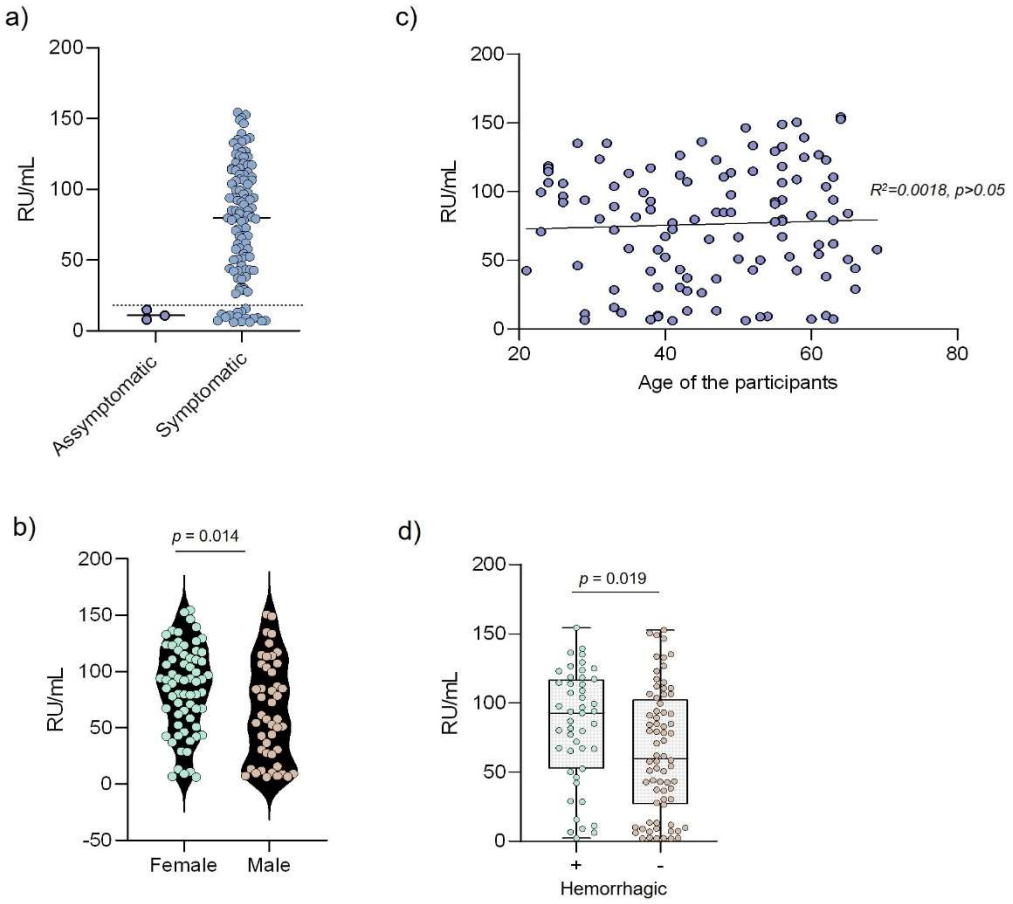

Figure 1 – DENV IgG antibodies concentration among seropositive participants, 8 years after DENV infection. a) Seric concentration of anti-DENV IgG antibodies (RU/mL) in symptomatic (*n*=111) and asymptomatic participants (*n*=3; includes seropositive participants from both confirmed and possible asymptomatic groups). The dotted line indicates the cut-off value for a positive test at 1:100 dilution (RU/ml = 22), as recommended by the manufacturer of the kit. The values below the cut-off were tested at 1:10 and the RU value was divided by a factor 10. b) Seric concentration of anti-DENV IgG antibodies (RU/mL) in symptomatic participants, according to gender (*n*=66 and *n*=45 for female and male, respectively. c) Correlation between age of the seropositive symptomatic participants (*n*=111) and concentration of their anti-DENV IgG (RU/ml) levels; d) Distribution of antibody concentrations according to manifestation or not of hemorrhagic signs. In figures a) to c) only participants with seropositivity confirmed by $FRNT_{90}$ are represented. *P*-values were determined by using Student *t*-test (b), Pearson's correlation (c) or Mann-Whitney U test (d).

In order to ascertain the range of antibody titers that were detectable in symptomatic participants and categorize them accordingly, serum samples obtained 8 years after dengue were tested by ELISA at 1:160 and 1:1280 dilutions, allowing to establish 3 subgroups of participants with: i) high titers (≥1:1280), ii) medium titers (≥1:160 and <1:1280), and low titers (<1:160). Our results indicated that 13 (10.9%) of

symptomatic participants had high antibodies titers (≥ 1:1280), while 24 (20.2%) had low titers or no detectable antibodies (<1:160), with the remaining participants being at intermediate titers of antibodies (**Figure 2a**). Among participants with low titers, males were 2-fold more frequent than females. Inversely, in the high titer group the opposite occurs with females being 2-fold more frequent than males. Even though differences in antibody titers weren´t statistically significant (χ^2=5.321, p=0.07). Interestingly, participants aged between 33 and 46 years old were not represented in the high antibody titer's group, although no correlation between age and RU values was found (**Figure 2b**).

Overall, our results revealed that, in a population exposed to a single dengue outbreak, the levels of anti-DENV IgG antibodies 8 years later are quite diverse. Still, these levels remain relatively high for most of the participants and, interestingly, higher in females than in males.

3.4. Neutralization capacity of the serum anti-DENV antibodies

The serological situation of a population accounts for the balance between risk and protection against a subsequent DENV infection, with the same or a different serotype. The proportion and the neutralizing capacity of antibodies are crucial for this balance. To study the serological status of our sample set, we performed FRNTs using two different serum dilutions (1:10 and 1:100), in order to evaluate the presence of antibodies able to neutralize different DENV serotypes, in participants with low, medium, and high titers of anti DENV-1 antibodies. The DENV-1 isolate responsible for the Madeira's outbreak, a different DENV-1 isolate from Brazil, and a DENV-2 isolate from Ghana were used (see Material and Methods for further details).

As expected, serum of all participants with detectable levels of antibodies strongly neutralized the DENV-1 isolate responsible for the Madeira´s outbreak (>90% of focus reduction), and even those belonging to the low antibody titer's group. Serum of participants with high antibody titers also strongly neutralized (>98% of focus reduction) the Brazilian DENV-1 and the Ghanaian DENV-2 isolates (**Figure 2c**). As for serum with medium titers of antibodies, these also exhibited high neutralizing activity against all viruses isolates (>96% of focus reduction), when tested at a 1:10 dilution. However, at a 1:100 serum dilution, only 46% reduction was observed against the DENV-2 isolate. The

neutralizing activity against DENV-1 was statistically different when compared to Brazil DENV-1 (Z= -2.666, p=0.008) or Ghanaian DENV-2 (Z= -2.549, p=0.011). Serum of participants with detectable but low antibody titers demonstrated ≥90% neutralization ability against the Brazil DENV-1 isolate, but only 87.3% and 1.8% against Ghanaian DENV-2, at a 1:10 and a 1:100 serum dilution, respectively. These differences in neutralizing activity against DENV-2 were statistically significant for both 1:10 serum dilution (z=-2.207, p=0.027) and 1:100 serum dilution (Z=-2.668, p=0.008).

Overall, these results demonstrate that, several years after a dengue infection, patients with detectable anti-DENV IgG antibodies are still able to neutralize different isolates of DENV-1 virus, even those presenting low antibody titers. In contrast, those with low antibody titers against the original isolate exhibit moderate neutralization against other strains of the same serotype (DENV-1) but low neutralization ability against a different serotype (DENV-2).

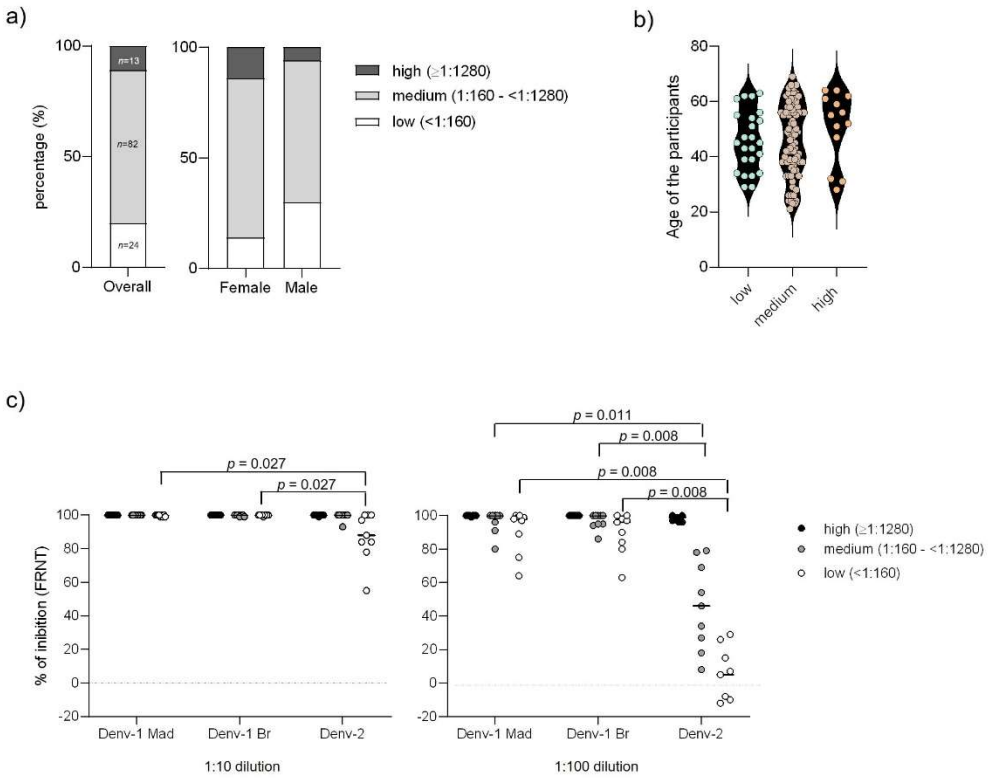

Figure 2 – Anti DENV antibodies titers and neutralizing activity against different DENV serotypes among symptomatic participants. a) Titer classification percentages overall or by gender (*n*=119). *p* values were determined by using Pearson's Chi-square. b) Age of the symptomatic participants (years old) according to antibody titer classification (*n*=24, *n*=82 and *n*=13 for low, medium, and high titers, respectively. c) Neutralization activity of serum from participants with high, medium, and low titers of anti-DENV antibodies. Samples were processed in triplicate. *p* values were determined by using Wilcoxon signed-rank test to compare the % of inhibition to each DENV isolate at a serum dilution of 1/10 and 1/100.

4. Discussion

In this study, we aimed at determining anti-DENV IgG seropositivity in confirmed asymptomatics, possible asymptomatics, and symptomatic participants (by ELISA, and FRNT when necessary), 8 years after primary infections, which occurred during the only dengue outbreak in Madeira Island. Among seropositive cases, the concentration of IgG antibodies was assessed, as well as its possible association to clinical signs and symptoms during the active infection. Moreover, the homologous and heterologous neutralizing capacity of serum anti-DEV antibodies were tested against isolates of the

DENV-1 strain responsible for the outbreak, one other DENV-1 strain, and one DENV-2 serotype.

Our study revealed that only 3.2% (1 out of 31) of the participants identified as asymptomatic during the Madeira outbreak had detectable anti-DENV antibodies and only detectable at 1:10 dilution. These asymptomatics were recruited among the 43 blood donors from Hospital Central do Funchal (HCF) with a PCR positive test during the outbreak. Of the PCR-positive donors, 33 were tested at that time for IgM and IgG in the subsequent weeks. Of these, only 3 showed detectable anti-DENV antibodies (one had become symptomatic and the other 2 remained asymptomatic) (ECDC 2014), meaning that only about 6% (2 out of 32) of those asymptomatic infections led to detectable seroconversion in the following weeks. The very low seroprevalence among confirmed asymptomatics suggests that, either the antibody response was very low and got rapidly lost over time, or there was no seroconversion for most asymptomatic infections. We cannot completely rule out the existence of seroconversion in asymptomatics, but rather that it leads to a low antibody response, below the detection limit of ELISA kits, which were developed to diagnose symptomatic cases. According to ECDC (2014), during the dengue outbreak, the healthcare services in Madeira used dengue IgM and IgG capture ELISA kits, for laboratory confirmation of the infections. These capture kits are known to detect only high antibody levels, and thus not being sensitive enough to detect low levels of antibodies. In an external quality assurance study of dengue serological diagnostic tests, the sensitivity of IgM capture ELISA tests was restricted to samples with titers above 1:3200 (Domingo et al. 2015). If seroconversion has occurred at the time but it was not detected by ELISA, this suggests that antibody levels would have been significantly lower in asymptomatics when compared to symptomatics, as these same ELISA kits were used to confirm the symptomatic dengue cases. Immediately after a primary dengue infection outbreak, Sun et al. (2012) established a cohort of asymptomatic individuals based on their IgM positivity, measured by indirect ELISA, and absence of medical visits during the outbreak. Three years later, Luo et al. (2018) observed that only 27% of these same asymptomatics were still seropositive (at a 1:100 serum dilution), suggesting a very fast decay of antibodies following asymptomatic infections. On the other hand, if our asymptomatic participants did not seroconvert at

all, this could mean that the prevalence of asymptomatic DENV infections may be much higher than that reported by several studies, since the establishment of many asymptomatic cohorts is based on their IgM seropositivity and absence of medical visits, instead of the detection of their viremia (Sun et al. 2012; Tsai et al. 2018). Further, these cohorts are also likely to include mildly symptomatic cases. It is also noteworthy that the seroprevalence among possible asymptomatics (around 4%; 2 out of 49), recruited between co-habitants or co-workers of the symptomatic participants from the most dengue affected parishes of Funchal, is very similar to the proportion of seropositivity among confirmed asymptomatics. These results hint at a high probability that these participants had an asymptomatic infection during the outbreak, suggesting quite high transmission rates and prevalence of asymptomatic infections in those parishes.

A previous study, performed 4 years after Madeira's dengue outbreak (Auerswald et al. 2019), found a seroprevalence of 8.7% among randomly selected individuals residing in Funchal. No information was presented about the Funchal parishes where these participants came from. In the same study, participants were not inquired about signs/symptoms of dengue fever during the outbreak and thus, it is not possible to know if these participants had dengue fever (with or without laboratory confirmation) or corresponded, in part, to asymptomatic cases. Taking into account the low antibody production and the fast decay following asymptomatic infections described in our study and also by others (e.g. 73% becoming seronegative in 3 years, (Luo et al. 2018), as well as, the fact that the blood samples were collected in the city main laboratories located at the mostly affected Funchal parishes (Auerswald et al. 2019), we question if the seropositivity found by Auerswald et al. (2019) might correspond mainly to previous symptomatic infections.

The absence of a long-term antibody response following asymptomatic infections does not mean that these individuals are not protected against subsequent dengue infections. Asymptomatic infections were, in fact, previously associated with a higher percentage of activated T cells and an increased adaptive response, compared to symptomatic infections (Simon-Lorière et al. 2017). Hence, DENV-specific memory T cells may develop and influence the outcome in a subsequent DENV infection (Jeewandara et al. 2015). Therefore, in future works, it would be important to assess

DENV-specific memory T cell developed after primary asymptomatic infection and their long-term protective role. It would also be interesting to investigate why individuals with asymptomatic infections do not develop a long-term humoral response, despite showing significant viremia (Matangkasombut et al. 2020; Morsy et al. 2020). This last aspect is particularly critical in understanding the best strategy for developing an effective vaccine, as most flavivirus infections are asymptomatic and vaccinal strategies may involve live-attenuated dengue vaccines.

In contrast to what we observed for the asymptomatic participants, 93.3% of our symptomatic participants had detectable anti-DENV IgG antibodies. Of these positive participants, most (80.7%) are positive at a 1:100 serum dilution, while 12.6% are only detectable at a 1:10 serum dilution and 6.7% are negative. Although specific anti-DENV IgG antibodies can be detected in individuals infected more than 60 years earlier (Ngwe Tun et al. 2016), continuous decay of the seropositive rate has also been observed after symptomatic infections. In a serological survey conducted in China´s Zhejiang Province, Luo et al. (2018) found DENV IgG-specific antibodies in 96.6% of participants, 3 years after a symptomatic DENV-3 infection. In a different Chinese province, similar results were found 3 years after the enrollment based on seropositivity detected 2 years after a symptomatic DENV-1 infection (Wu et al. 2020). In another serosurvey, conducted also in China following a DENV-3 outbreak (Ma et al. 2022), the anti-DENV IgG seropositive rate decreased significantly to 82.8% between the 4th and the 6th year after the infection.

Despite the observed decay in seropositivity over 8 years, our results strongly reinforce that primary symptomatic infection with DENV can also produce long-lasting immunity. Moreover, we found a significant variability in the concentration of anti-DENV antibodies in our symptomatic participants, ranging from not detectable to 155 RU/mL (at 1:100 dilution) and titers <1:160 to ≥1:1280. This reflects, either individual differences in their humoral response at the time of the infection, and/or in antibody decay over time. As expected, RU values observed in our symptomatic participants were lower than those determined in a Chinese study 5 years after a DENV-1 infection (77.0 ± 41.7 *versus* 120.0 ± 47.5; (Wu et al. 2020)) and using the same ELISA kit. Interestingly, we found a statistically higher concentration of anti-DENV antibody in females than in

males. Wu et al. (2020) had similar gender-bias results 5 years after a DENV-1 infection, but not earlier at 3 years, suggesting a faster antibody decay in men.

We should also point out that, although most studies stablish seropositivity based in ELISA methods performed at 1:100 serum dilution (according to the manufacture instructions), 12.6% of our symptomatic participants only had detectable antibodies at 1:10 serum dilution, further confirmed by the *in vitro* neutralizing test FRNT. Therefore, lower serum dilution should be considered when performing ELISA tests to evaluate the seroprevalence of a population, at least when FRNT can be used as confirmatory test.

No associations between the levels of antibodies and specific signs/symptoms reported by the participants were found, except for the hemorrhagic group of symptoms. Hemorrhagic signs may result from excessive production of inflammatory cytokines and chemokines, leading to disruption of the endothelium and increased vascular permeability (Raza et al. 2020) which may be associated with a stronger humoral response.

For a clearer scenario concerning the antibody status in our symptomatic participants, we categorized them according to their antibody titers in high, medium, and low titers. Unexpectedly, 8 years after a primary DENV infection, 10.9% of symptomatic participants still have high titers of antibodies (≥ 1:1280). This could reflect a boost in their humoral response subsequently to other unreported DENV exposure, besides the outbreak in Madeira. However, this possibility seems to us very unlikely since no other autochthonous dengue transmission was ever described in Madeira and only 1 of these participants reported traveling history to dengue-endemic countries. Nevertheless, we may consider that, within the period of the outbreak (September 2012 to March 2013), these participants may have had multiple exposures, given the high incidence of dengue and density of the mosquito vectors in the parishes of Funchal where they resided and/or worked, and this may have contributed to higher titers, at least in some cases. In fact, most (84.6%) of the participants with the highest antibody titers had dengue in the first 8 weeks of the outbreak and thus may have been exposed again during the outbreak.

While protection against homotypic re-infection is often indicated as long-lasting, cross-protecting antibodies against different serotypes are often suggested to be short-lived, with a half-life of several months to 3 years (Sabin 1952; Montoya et al. 2013; Anderson et al. 2014; Katzelnick et al. 2016). However, Guzman and Kouri (2008) found that heterotypic neutralization may persist for long-time, more than a decade, being dependent on the heterologous genotype (Kochel et al. 2002; Guzman and Kouri 2008). In our study, conducted several years after a primary dengue infection, antibodies from seropositive participants retain a strong ability to neutralize the original DENV-1 and a different DENV-1 isolate, even those with low total antibody titers (<1:160). In addition, significant heterologous neutralization of DENV-2 genotype by participants with high and medium antibody titers was also observed, suggesting that cross-protection can last longer than initially thought, at least in a proportion of symptomatic infections. On the contrary, the sera of participants with low antibody titers against the original isolate exhibit low neutralizing capacity against DENV-2. This means that a significant fraction of our participants, with low anti-DENV antibody titers, may be protected against a homotypic serotype but, probably not against a different serotype, and may even have an enhanced risk to develop severe disease. Nevertheless, we point out that the risk of developing a severe form of the disease seems to depend not only on the amount of pre-existing neutralizing antibodies, but also on the amount of enhancing antibodies amongst the total binding antibodies (Vo et al. 2022).

We performed the first study specifically to discriminate the seroprevalence status between symptomatic and asymptomatic infections following the unique DENV-1 outbreak at Madeira Island. Overall, most of the asymptomatics did not present detectable antibodies 8 years post infection. In contrast, a quite wide range of antibody concentrations were found in individuals who experienced symptomatic infections during the outbreak, some of them showing remarkably high titers. Moreover, our data suggest that the ones with low antibodies may be protected against a new infection with the same serotype (homologous protection) but, probably not against an infection with a different serotype (heterologous protection) and may have an enhanced risk to develop severe disease.

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Ongoing work

The work presented in this chapter allowed us to constitute two groups of participants with previous DENV-1 infection, according to whether they presented or not symptoms during the outbreak, and to investigate their actual serological status. Additionally, this work granted us the opportunity to investigate the host-factors involved in protective mechanisms against clinical manifestations of primary dengue infections. As extensively discussed in Chapter II different factors may determine if an infection will have an asymptomatic outcome, being the permissiveness of the DENV target cells one of those factors.

At the site of DENV inoculation during mosquito bite, the virus likely infects skin Langerhans or resident dendritic cells (Tassaneetrithep et al. 2003; Wu et al. 2000) and then spreads to different regions of the body where other cell types are likely to also become infected. There is still debate over which cells are permissive *in vivo* to DENV infection. For instance, Kou et al. (2008) found that monocytes, which bear recognized DENV receptors, but not primary T or B cells are permissive to DENV-2. Durbin et al. (2008) also showed that monocytes are the predominant target cell in the peripheral blood of dengue patients and, that once activated these may play a role in the immunopathogenesis of DENV. On the other hand, Silveira et al. (2018) reported that T cells are indeed permissive to all four DENV serotypes and are possible viral reservoirs, contributing to viraemia and T cell activation and thus having a potential role in dengue pathogenesis (Green et al. 1999) by inducing inflammatory cytokine secretion leading to a cytokine storm (Green et al. 1999; Kurane and Ennis 1992; Matos et al. 2015). Evidence has also been put forth that B cells are permissive to DENV infection (Upasani et al. 2020). While the infection of B cell seems to contribute poorly to viremia, it induces their proliferation and differentiation into plasmablasts and plasma cells (Upasani et al. 2020).

Given that cell permissiveness to DENV seems to play a role in dengue pathogenesis, it is reasonable to hypothesize that differences in permissiveness to DENV of these cells, in particular peripheral blood mononucleate cells (PBMCs), may contribute, at least in part, to the asymptomatic *versus* symptomatic outcome. As such, the *in vitro* permissiveness of different PBMC subsets to DENV-1 is currently being

evaluated and compared between asymptomatic and symptomatic participants. In this context, the expression of low-density lipid receptor (LDLR) and the scavenger receptor BI (SRBI), both potential DENV receptors, is also being examined by flow cytometry. The immune response to DENV infection is also being evaluated through the analysis of the cytokine profiles, in DENV infected PBMCs from both symptomatic and asymptomatic participants. An indirect measure of viraemia in the supernatants of DENV-1 infected PBMCs will also be determined, using plaque assays, to ascertain possible differences in viral replication capacity inside PBMCs between asymptomatic and symptomatic participants.

To this end, PBMCs from asymptomatic and symptomatic participants in the serologic study are being analyzed. PBMCs are infected with the DENV-1 isolate from the outbreak of Madeira Island (DENV-1 genotype V) at a multiplicity of infection (MOI) of 2, incubated for 48 hours at 37°C with 5% CO_2, after which the supernatant is collected and stored at -80°C for cytokine measurement, using a Cytokine Multiplex Assay. The PBMCs are then stained for flow cytometry, to identify infected cells and their phenotype/subset. Briefly, cells are stained with Zombie dye to identify live cells, followed by extracellular staining. Cells are then fixed and permeabilized for staining with the anti-DENV antibody. Three staining mixes are prepared, in order to identify different PBMCs subsets. Mix 1 containing CD3, CD4, CD8, and CD19 allows for the identification of B cells ($CD3^-$ $CD19^+$), $CD4^+$ ($CD19^-$ $CD3^+$ $CD4^+$ $CD8^-$) and $CD8^+$ T cells ($CD19^-$ $CD3^+$ $CD4^-$ $CD8^+$). Mix 2 containing CD3, CD19, CD14, HLA-DR, and CD56 allows for the detection of monocytes/macrophages ($CD3^-$ $CD19^-$ $CD14^+$ $CD56^-$ $HLADR^+$), DCs ($CD3^-$ $CD19^-$ $CD14^-$ $CD56^-$ $HLADR^+$) and NK cells ($CD3^-$ $CD19^-$ $CD14^-$ $CD56^+$ $HLADR^-$). Finally, mix 3 containing CD3, CD19, CD14 LDLR, and SRBI will allow to identify differences in the expression of LDL ($CD3^-$ $CD19^-$ $CD14^+$ $LDLR^+$), and SRBI ($CD3^-$ $CD19^-$ $CD14^+$ $SRBI^+$). The supernatant is used in the profiling of secreted cytokines, using a Human Inflammation panel that allows for the detection of IL-1β, IFN-α2, IFN-γ, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. To evaluate the virus' capability to multiply inside the PBMCs of different individuals, these are infected with DENV-1 at a MOI of 1 and incubated for 5 days at 37°C with 5% CO_2. Supernatants are collected and stored at -

80°C. Virus amount is then determined by supernatant titration, by focus assay, using Vero cells as described in Chapter III.

We expect that, once completed, these analyses will allow us to identify differences in terms of PBMC susceptibility to DENV, as well as cytokine secretion and receptors associated with lipid metabolism, between asymptomatic and symptomatic individuals.

CHAPTER IV

Primary Dengue and long-term health status in Madeira Island

Preamble

During the recruitment for the serological study (Chapter III), participants answered a questionnaire concerning demographic information and clinical symptoms experienced during DENV infection. Frequently, symptomatic participants mentioned that some signs and symptoms appeared in a later period of infection, after their visit to the regional healthcare services. Since patients, as a rule sought medical assistance at the initial phase of the disease, it is highly likely that symptoms typically manifesting at a later stage of the infection were underrepresented in the clinical information. Moreover, these participants also often referred to symptoms/signs that persisted for a longer time, even for years, after the infection. This prompted the need to better characterize the clinical manifestations of DENV-1 primary infection in Madeira Island, as to cover disease manifestations along the entire period of infection, and to assess possible long-term persistent manifestations. For that purpose, an online questionnaire was designed to retrospectively assess, the main symptoms presented during infection, the general health status before and after the infection, and the long-term persistence of previously non-existing symptoms.

The results of this retrospective questionnaire applied to participants who had a primary symptomatic infection by DENV-1 in Madeira Island are detailed herein in Chapter IV.

The work depicted in the present chapter resulted in an original research article submitted to *The American Journal of Tropical Medicine and Hygiene.*

Primary dengue and long-term health status in Madeira Island: a retrospective questionnaire-based study

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Keywords: dengue; questionnaire; retrospective cohort; symptoms; persistence of symptoms.

Abstract

Dengue is among the most important mosquito-borne viral diseases worldwide. Although its acute manifestations are well known, little is known about the long-term impact of dengue on the population's health status. Madeira Island experienced a single outbreak of autochthonous dengue in late 2012 – early 2013. To extend our knowledge about the clinical impact of the outbreak on this naïve population, we applied an online questionnaire to 168 adults diagnosed with dengue fever at the time, to retrospectively characterize their symptoms during the infection and to identify long-term manifestations possibly triggered by this disease. The most frequent symptoms during the clinical period and reported by more than three-quarters of our participants were fever, myalgia, extreme tiredness, and headaches, while vomiting, pruritus, nausea, retro-orbital pain, and arthralgia occurred in 35-50% of the participants. In the 8 years following dengue, 61.5% of participants reported at least one recurrent previously non-existing symptom, the most frequent being headaches, abundant hair loss, extreme tiredness, arthralgia, and myalgia, experienced by 25-35% of the respondents. Nearly 20% of the participants with persistent symptoms reported the onset of chronic illness in the 4 years after dengue, most frequently ophthalmological (9.7%) and autoimmune (7.8%) diseases, against only 2.2% of chronic disease onset in participants without persistent symptoms. Our results suggest that the

occurrence of persistent symptoms following a primary dengue virus infection might be more frequent than anticipated and may persist for several years, having an impact on the health status and well-being of a considerable proportion of the infected population.

1. Introduction

Dengue, a vector-borne disease caused by one of the 4 known serotypes of dengue virus (DENV), has become endemic in many tropical and subtropical regions,[1–3] where it represents a major health concern. During the past 5 decades, the incidence of dengue epidemics has increased 30-fold, reaching nowadays 390 million annual infections, with new countries being affected.[4,5]

Infected people may be asymptomatic (estimated to be 50%-98% of the infected)[6] or exhibit a wide spectrum of manifestations, from mild disease to severely debilitating or lethal disease, classified as "severe dengue" according to the World Health Organization (WHO).[7] Literature describing the manifestations of symptomatic dengue at the acute and convalescence phases of the infection is abundant.[8–11] The most common signs and symptoms are fever accompanied by myalgia/arthralgia, headaches, retro-orbital pain, maculopapular rash, leukopenia, abdominal pain and/or vomiting being, in most patients a self-limited infection, lasting up to 2 weeks.[7,11–13]

To better understand the real impact of a dengue outbreak on the health status of a population, it is crucial to evaluate the clinical manifestations during active infection but also those becoming more evident or appearing after the recovery phase, and chronically persisting. Despite the recognition of late complications and unusual manifestations by the WHO,[14] little is known about the long-term consequences of dengue. Some reports pointed out that muscle pain, arthralgia, or asthenia, among other symptoms, may persist for 1-2 months in 9-24% of patients.[15–17] A few studies have published evidence of an even longer duration of persistent symptoms, up to 2 years after the infection.[9,18,19] Additionally, long-term manifestations such as liver injury,[20] myocardial impairment, arrhythmias, and fulminant myocarditis (reviewed in Rahim et al.)[21], encephalitis, myelitis, and meningitis (reviewed in Puccioni-Sholer et

al.),[22] and ophthalmologic conditions (reviewed in Trivedi and Chakravarty)[23] have also been described. DENV infection can also favor Guillain-Barré[24] or chronic fatigue syndromes,[17] as observed in other viral infections.[25,26]

The only outbreak of autochthonous dengue in Madeira Island, Portugal, was caused by DENV-1 serotype, and happened between September 2012 and March 2013, leading to more than 2000 dengue fever cases assisted in public healthcare, without severe clinical forms or fatalities.[27] The clinical picture when seeking medical assistance, which happened essentially during the febrile period, may have been underestimated since some of the dengue manifestations only appear later on (e.g. petechiae) or may be temporary (e.g. rash)[28]. Additionally, many of the patients did not qualify for follow-up or further clinical surveillance, and thus no registry of expanded manifestations exists beyond the acute phase of the infection.

To deepen our knowledge about the clinical impact of the Madeira 2012' dengue outbreak, we applied an online questionnaire to dengue fever cases diagnosed at the time, aiming at i) a retrospective characterization of their symptoms during the entire infection period, and ii) an assessment of their health status before and after dengue virus infection, identifying long-term manifestations possibly triggered by dengue. We expect that the clinical manifestations reported here are a fair representation of DENV-1 infection in a naïve population, not biased by the protective or facilitating immune response established against previous infections.

2. Material and Methods

2.1. Ethics statement

The study protocol was approved by the Ethics Committee for Health of the SESARAM, E.P.E.R.A.M. (approval n. 49/2021). All participants gave their informed consent before starting the online questionnaire. All procedures complied with the Declaration of Helsinki of the World Medical Association and regulations for the protection of human subjects, rights, and personal data of the enrolled participants.

2.2. Selection of participants and questionnaire design

We hereby conducted, in 2021, a retrospective questionnaire-based study applied to patients with clinical and/or laboratory diagnoses of dengue fever in 2012-2013 by the Regional Healthcare Services of SESARAM, E.P.E.R.A.M., according to WHO diagnostic criteria[7] and following the guidance of ECDC experts, at the time assigned to accompany the Madeira outbreak. The invited participants were selected to have been aged between 16 and 62 years at the time of the outbreak.

For the purposes of this study, the questionnaire was structured into 3 main sections (**Figure 1**): Section 1 intended to characterize the signs/symptoms during the infection; Section 2 aimed at evaluating the general health status, before and after the infection, to infer about persistent manifestations after dengue; Section 3 assessed demographic data of the respondents. Questions with multiple answers had also an open-field option.

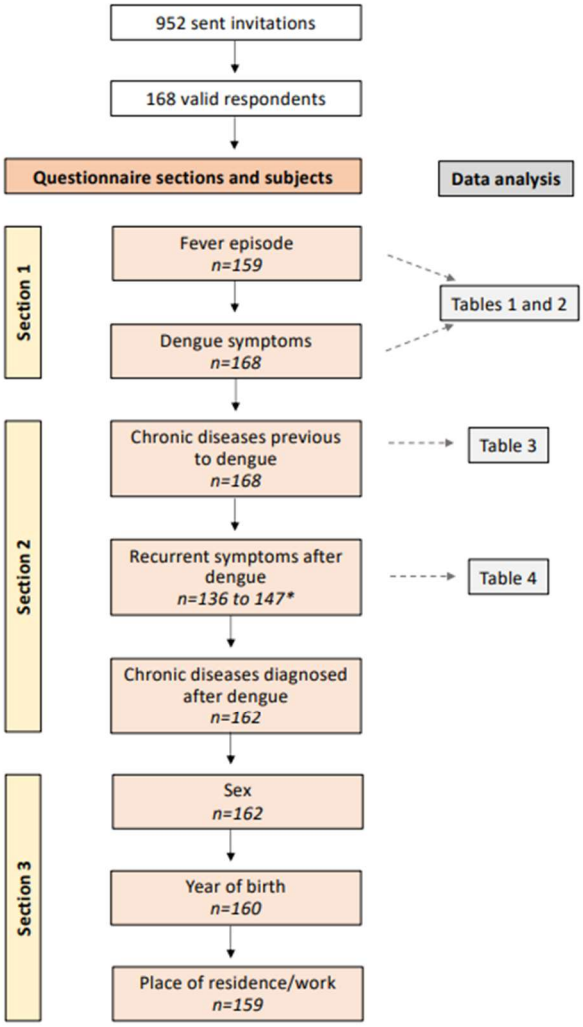

Figure 1 – Flow diagram of the sections of the questionnaire and the number of valid respondents per subject. * The number of valid respondents to each symptom in this question is variable, due to the different number of responders selecting "I don't know/I don´t remember" for each symptom

2.3. Data collection and analyses

The questionnaire was uploaded to the online *SurveyMonkey*® platform (Momentive Inc, San Meto, CAL), an open-source and cloud-based platform, which allows mobile data collection and complies with strict privacy and safety policies. An invitation with a link specifically generated for this questionnaire was sent to the mobile contacts of eligible participants, allowing them to respond anonymously and only once. Completed surveys were reviewed for data entry errors by the senior project members.

Data were extracted in *.xls* format and further edited for analyses in SPSS, version 28.0.0.0 (SPSS Inc, Chicago, IL). For each variable, the number of valid respondents was considered as those selecting "yes" or "no", while respondents indicating "I don't know/I don't answer" or leaving blank fields were excluded. The independence of categorical variables, overall and stratified by sex, was tested by univariate analyses using Pearson's Chi-square tests (or Fisher's exact tests, when at least one of the expected values was less than 5). Odds ratio (OR) and respective 95% confidence interval (95% CI) were calculated only for significant associations. Mean age differences, overall and per sex, were assessed by Mann-Whitney (or t-tests, according to the results of Shapiro-Wilk tests). Logistic regression was used to identify predictive variables for symptom persistence. All results were interpreted at a significance level of 0.05.

3. Results:

3.1. Characterization of participants

The invitation for participation in the survey was sent to 952 contacts (40.2% M: 59.8% F, aged 23-70 years old (y.o.), mean age 46.0 ± 13.4 y.o. (equivalent to a mean age of 38.0 y.o. at the time of the outbreak), ascertained from the SESARAM, E.P.E.R.A.M. database as having had clinical and/or laboratory diagnosis of dengue during the outbreak. Of the 178 respondents, not all were considered valid: few respondents did not answer the survey sections referring to signs/symptoms at the time of the infection (n = 6), to the general health status before the infection (n = 6), and/or to recurrent signs and symptoms after the infection (n = 10), which were considered relevant for the purpose of our study and were, therefore, excluded from the analyses. The universe of valid respondents refers to 168 participants, most of Portuguese nationality (98.1%). In terms of sex (32.7% M: 67.3% F) and age range (aged 24-70 y.o.; mean age 43.9 ± 11.9 y.o., equivalent to a mean age of 35.9 y.o. at the time of the outbreak), this is a representative subset of the invited participants, as no significant differences were found when testing for proportions and means (both p-values > 0.05).

3.2. Dengue clinical manifestations

During the active infection, 92.5% of the participants reported a fever episode, due to which 98.0% of them searched for medical assistance. Among other symptoms, the most frequently reported were systemic symptoms such as myalgia (85.7%), extreme tiredness (78.0%), headaches (76.8%), and arthralgia (55.4%), while hemorrhagic manifestations such as hematuria (5.4%), gum bleeding (4.2%) and epistaxis (3.6%) were the less frequently stated (Table 1). An unexpectedly high frequency of petechiae (32.7%) was also reported but as discussed later, should be interpreted with caution. Significantly different frequencies of signs/symptoms were observed according to sex, with pruritus, vomiting, nausea, extreme tiredness, palmar erythema, petechiae, and arm and leg swelling being 2.0 to 4.7-fold more frequent in females than in males (p-value < 0.046; Table 1).

Table 1 – Frequency of participants reporting different symptoms at the time of dengue infection (overall and per sex).

Symptoms	no. valid respondents	Frequency			Pearson's chi-square (or Fisher's exact test*) p-value	OR [95% CI]
		Overall % (n)	Males % (n)	Females % (n)		
Systemic						
Fever	159	92.5 (147)	92.0 (46)	92.2 (95)	1.000*	
Myalgia	168	85.7 (144)	84.9 (45)	86.2 (94)	0.820	
Extreme tiredness	168	78.0 (131)	64.2 (34)	84.4 (92)	**0.004**	**3.024 [1.409-6.489]**
Headaches	168	76.8 (129)	77.4 (41)	77.1 (84)	0.967	
Arthralgia	168	55.4 (93)	52.8 (28)	56.0 (61)	0.707	
Arm and leg swelling	168	16.1 (27)	5.7 (3)	22.0 (24)	**0.009**	**4.706 [1.348-16.426]**
Gastrointestinal						
Nausea	168	44.0 (74)	28.3 (15)	50.5 (55)	**0.008**	**2.580 [1.274-5.226]**
Vomits	168	35.7 (60)	22.6 (12)	42.2 (46)	**0.015**	**2.495 [1.182-5.267]**
Abdominal pain	168	24.4 (41)	22.6 (12)	25.7 (28)	0.673	
Diahrrea	168	23.2 (39)	22.6 (12)	24.8 (27)	0.766	
Cutaneous						
Pruritus	168	42.9 (72)	32.1 (17)	48.6 (53)	**0.046**	**2.004 [1.007-3.989]**
Palmar erythema	168	20.2 (34)	9.4 (5)	25.7 (28)	**0.016**	**3.319 [1.201-9.169]**
Opthalmological						
Retro-orbital pain	168	44.6 (75)	45.3 (24)	44.0 (48)	0.881	
Hemorrhagic						
Petechiae	168	32.7 (55)	15.1 (8)	41.3 (45)	**<0.001**	**3.955 [1.702-9.191]**
Hematuria	168	5.4 (9)	3.8 (2)	5.5 (6)	1.000*	
Gum bleeding	168	4.2 (7)	1.9 (1)	5.5 (6)	0.428*	
Epistaxis	168	3.6 (6)	1.9 (1)	4.6 (5)	0.665*	
Respiratory						
Breathing difficulty	168	10.1 (17)	7.5 (4)	11.9 (13)	0.393	
Productive cough	168	7.1 (12)	9.4 (5)	6.4 (7)	0.530*	
Others	168	16.1 (27)	18.9 (10)	14.7 (16)	n.d.	

Pearson chi-square or Fisher's exact (*) tests were used, with Fisher's p-values (*) shown when at least one of the expected values was less than 5; Odds ratio (OR) and 95% confidence intervals (95% CI) are indicated only for variables found to be significant; n.d. not determined for the "Others" category.

When comparing the age distribution of participants exhibiting or not each symptom, significant differences were found only for arm and leg swelling, more common in older participants (40.1 y.o. ± 12.0 vs. 35.1 y.o. ± 11.8, p-value = 0.037; Table 2). No significant differences were observed for the mean age of males and females presenting each symptom (Table 2).

Table 2 - Mean age of participants reporting or not each symptom at the time of dengue infection (overall and per sex).

Symptoms	Mean age at the time of the infection						
	Participants with symptom					Participants without symptom	
	no. valid respondentes	Overall y.o. ± s.d (n)	Males y.o. ± s.d (n)	Females y.o. ± s.d (n)	t-test (or Mann-Whitney*) p-value	Overall (y.o. ± s.d)	t-test (or Mann-Whitney*) p-value [a]
Systemic							
Fever	152	35.8 ± 12.2 (141)	37.2 ± 12.2 (46)	35.1 ± 12.1 (95)	0.330	40.1 ± 10.7 (11)	0.258
Myalgia	160	35.2 ± 11.9 (137)	36.3 ± 11.9 (45)	34.7 ± 12.0 (93)	0.448	40.0 ± 11.2 (23)	0.072
Extreme tiredness	160	35.1 ± 12.5 (124)	36.9 ± 12.8 (34)	34.4 ± 12.4 (91)	0.330	38.8 ± 9.3 (36)	0.094
Headaches	160	35.7 ± 11.9 (124)	37.6 ± 12.4 (41)	34.7 ± 11.6 (83)	0.201	36.8 ± 12.1 (36)	0.631
Arthralgia	160	36.8 ± 11.7 (89)	36.3 ± 10.9 (28)	37.1 ± 12.1 (61)	0.782	34.7 ± 12.2 (71)	0.273
Arm and leg swelling	160	40.1 ± 12.0 (27)	39.3 ± 5.9 (3)	40.2 ± 12.6 (24)	0.856*	35.1 ± 11.8 (133)	**0.037***
Gastrointestinal							
Nausea	160	36.1 ± 12.5 (69)	37.1 ± 11.6 (15)	35.9 ± 12.9 (54)	0.746	36.1 ± 12.5 (69)	0.837
Vomits	160	34.0 ± 12.9 (57)	36.8 ± 12.4 (12)	33.3 ± 13.0 (45)	0.409	37.0 ± 11.3 (103)	0.165*
Abdominal pain	160	35.3 ± 13.4 (39)	34.8 ± 12.0 (12)	35.5 ± 14.2 (27)	0.885	36.1 ± 11.4 (121)	0.719
Diahrrea	160	34.1 ± 12.8 (38)	37.4 ± 12.3 (12)	32.6 ± 13.0 (26)	0.285	36.5 ± 11.6 (122)	0.241*
Cutaneous							
Pruritus	160	37.4 ± 12.3 (68)	37.1 ± 13.5 (17)	37.5 ± 12.1 (52)	0.902	34.8 ± 11.6 (92)	0.174
Palmar erythema	160	36.9 ± 12.0 (32)	40.8 ± 5.8 (5)	36.1 ± 12.7 (27)	0.434	35.7 ± 11.9 (128)	0.609
Opthalmological							
Retro-orbital pain	160	36.5 ± 11.5 (71)	38.5 ± 9.7 (24)	35.5 ± 12.2 (47)	0.298	35.4 ± 12.3 (89)	0.570
Hemorragic							
Petechiae	160	33.7 ± 12.1 (52)	32.8 ± 11.7 (8)	33.9 ± 12.3 (44)	0.806	37.0 ± 11.7 (108)	0.121*
Hematuria	160	37.7 ± 16.0 (7)	49.0 ± 1.4 (2)	33.2 ± 17.1 (5)	0.381*	35.8 ± 11.8 (153)	0.683
Gum bleeding	160	43.0 ± 11.9 (6)	48.0 (1)	42.0 ± 13.0 (5)	1.000*	35.6 ± 11.9 (154)	0.138
Epistaxis	160	36.2 ± 16.9 (5)	48.0 (1)	33.3 ± 18.0 (4)	0.800*	35.9 ± 11.8 (155)	0.956
Respiratory							
Breathing difficulty	160	38.2 ± 13.8 (17)	37.8 ± 14.2 (4)	38.4 ± 14.3 (13)	1.000*	35.6 ± 11.7 (143)	0.396
Productive cough	160	35.7 ± 13.1 (12)	37.8 ± 17.2 (5)	34.1 ± 10.5 (7)	0.656*	35.9 ± 11.9 (148)	0.943
Others	160	39.2 ± 13.4 (26)	38.9 ± 12.2 (10)	39.4 ± 14.4 (16)	n.d.	35.3 ± 11.6 (134)	n.d.

T-test or Mann-Whitney (*) tests were used, according to the normality of distribution accessed by Shapiro-Wilk tests; [a] for comparison of the mean age of participants with and without symptoms; n.d. not determined for the “Others” category.

3.3. Health status before dengue virus infection

Participants were asked about the existence of chronic diseases before dengue virus infection in 2012/2013. Most (72.6%, n = 122) affirmed not having a diagnosis of chronic diseases at that time, while 27.4% (n = 46) had one or more chronic diseases (Table 3). The most frequently reported comorbidities by our participants were hypertension (n = 16), diabetes (n = 7), and respiratory disease (n = 5). It was also noted that, as expected, the mean age of participants with chronic diseases (41.2 ± 11.4 y.o.) was significantly higher than those without chronic diseases (34.1 ± 11.6 y.o) (p-value < 0.001). Also noteworthy, the mean age of participants with respiratory and autoimmune diseases was much lower (below 30 y.o.) than the mean age for other chronic diseases (over 44 y.o). Since hypertension was the most reported chronic disease, we tested the association of this condition to each symptom during dengue infection, by comparing their frequencies in hypertense and non-hypertense participants. For these comparisons, hypertension is suggested as a risk factor for arm and leg swelling (p-value = 0.025, OR = 3.743, 95%CI 1.231-11.380), but protective against retro-orbital pain (p-value = 0.029, OR = 0.256 (0.070-0.936) (data not shown).

Table 3 – Frequency and mean age of participants reporting chronic disease prior to dengue virus infection in 2012/2013.

Chronic disease before dengue	Frequency % (n)	Mean age y.o. ± s.d (n)
Without previous chronic disease	72.6 (122)	34.1 ± 11.6 (119)
With previous chronic disease	27.4 (46)	41.2 ± 11.3 (45)
Hypertension	9.5 (16)	44.1 ± 11.3 (16)
Diabetes	4.2 (7)	52.1 ± 8.6 (7)
Respiratory disease	3.0 (5)	29.0 ± 11.1 (5)
Hypercholesterolemia	1.8 (3)	48.3 ± 9.5 (3)
Autoimmune Disease	1.8 (3)	24.5 ± 12.0 (2)
Heart Disease	1.2 (2)	49.5 ± 10.6 (2)
*Others**	*8.9 (15)*	-

* Includes thrombin III deficiency, ulcerative colitis, kidney disease, venous thromboembolism, ophthalmological disease, and other non-specified chronic diseases.

3.4. Long-term persistence of clinical symptoms after dengue

In Section 2 of the questionnaire, participants were asked about persistent symptoms after dengue, among those symptoms referred to in Table 4. To distinguish between occasional symptoms of unspecific cause and recurrent clinical manifestations that could point to sequelae or chronic health problems, we have centered our attention on previously non-existing or rare symptoms, which became frequently reported (several times per year) after dengue. Considering that previous chronic diseases may influence the type and frequency of post-dengue recurrent symptoms, participants reporting comorbidities with onset before 2012-2013 were excluded from this analysis. Note the lower response rate in this and the following sections, with the dropout of 9 participants (4 with previous chronic disease and 5 without previous chronic disease) (Figure 1).

Of the 117 valid respondents, 61.5% (n = 72) reported at least one long-term persistent symptom after dengue, 68.1% (n = 49) of which sought medical assistance due to these symptoms. Systemic manifestations such as headaches (35.5%), abundant hair loss (30.8%), extreme tiredness (29.4%), arthralgia (25.7%), and myalgia (25.2%) were reported by more than one-quarter of these participants (Table 4). Also noteworthy, among participants with persistent symptoms, 18.1% (n = 13) described the onset of chronic illness within the 4 year-period following dengue, with the most frequent being ophthalmological (n = 4) and autoimmune (n = 4) disorders (data not shown). On the other hand, among the participants without persistent symptoms, only 2.2% (n = 1) developed a chronic auto-immune disorder, this proportion being significantly different ($p = 0.008$, OR = 9.565, 95% CI 1.584-212.400) when compared to the onset of chronic diseases in participants with persistent symptoms.

Table 4 – Frequency and mean age of participants reporting recurrent symptoms after dengue infection (overall and per sex).

	Frequency						Mean age at the time of the survey		
Recurrent symptoms since dengue infection	no. valid respondents	Overall % (n)	Males % (n)	Females % (n)	Pearson's chi-square (or Fisher's exact test*) p-value	OR [95% CI]	Participants with symptoms y.o. ± s.d (n)	Participants without symptoms y.o. ± s.d (n)	t-test (or Mann-Whitney*) p-value [a]
Headaches	107	35.5 (38)	20.6 (7)	42.5 (31)	**0.028**	**2.847 (1.099-7.377)**	37.1 ± 10.9 (38)	44.0 ± 12.2 (69)	**0.029***
Abundant hair loss	107	30.8 (33)	15.1 (5)	37.8 (28)	**0.019**	**3.409 (1.180-9.851)**	39.5 ± 12.8 (33)	43.0 ± 11.9 (74)	0.311*
Extreme tiredness	109	29.4 (32)	17.6 (6)	34.7(26)	0.071		46.2 ± 13.8 (32)	40.6 ± 11.4 (77)	0.092
Arthralgia	105	25.7 (27)	23.5 (8)	26.8 (19)	0.723		50.5 ± 14.7 (27)	39.6 ± 10.4 (78)	**< 0.001**
Myalgia	103	25.2 (26)	13.3 (4)	30.1 (22)	0.074		45.2 ± 15.3 (26)	41.0 ± 11.0 (77)	0.322
Bone pain	105	20.0 (21)	11.8 (4)	23.9 (17)	0.144		54.5 ± 11.2 (21)	39.0 ± 10.5 (84)	**< 0.001***
Palpitations	102	16.7 (17)	12.5 (4)	18.6 (13)	0.445		39.3 ± 13.1 (17)	42.5 ± 12.1 (85)	0.455
Blurred vision	106	16.0 (17)	14.3 (5)	16.9 (12)	0.730		43.3 ± 13.3 (17)	41.8 ± 12.1 (89)	0.953
Memory loss	99	14.1 (14)	12.5 (4)	14.9 (10)	1.000*		44.0 ± 11.8 (14)	41.7 ± 12.3 (85)	0.600*
Loss of apetite	105	10.5 (11)	2.9 (1)	14.1 (10)	0.099*		49.8 ± 14.5 (11)	41.1 ± 11.7 (94)	0.197*
Retroorbital pain	104	9.6 (10)	8.6 (3)	10.1 (7)	1.000*		39.2 ± 15.0 (10)	42.2 ± 12.1 (94)	0.563*

Participants with previous chronic disease (n = 46) were not considered in this analysis. Pearson chi-square or Fisher's exact tests were used for the frequency comparison among sexes, with Fisher's p-values (*) shown when at least one of the expected values was less than 5; Odds ratio (OR) and 95% confidence intervals (95% CI) are indicated only for variables found to be significant; For the comparison of the mean age of participants with and without symptoms, t-test or Mann-Whitney test were used, according to the normality of distribution accessed by Shapiro-Wilk tests.

Additionally, female sex was suggested as a risk factor for the persistence of at least one symptom in our dataset, since these were reported by 72.6% of females compared to 51.0% of males (p-value = 0.012, OR = 2.553, 95% CI 1.273-5.118). On the other hand, the mean age of participants did not seem to influence the long-term persistence of at least one symptom. When assessing the frequencies of each recurrent symptom according to sex, we found significant differences in headaches (p-value = 0.028, OR = 2.847, 95% CI 1.099-7.377) and abundant hair loss (p-value = 0.019, OR = 3.409, 95% CI 1.180-9.851), with a higher frequency in females (Table 4). Additionally, the mean age of participants was found to correlate with persistent headaches (p = 0.029), more common in younger patients, while arthralgia (p < 0.001) and bone pain (p < 0.001) prevailed in older patients (Table 4).

When testing the association of symptoms at the active infection as prognostic factors for the long-term persistence of at least one symptom, by univariate analyses, we found breathing difficulties to be associated with a higher risk of persistence, as 93.8% of participants with breathing difficulties against 62.4% of participants without breathing difficulties state at least one persistent symptom (p-value = 0.012, OR = 9.034, 95% CI 1.160-70.368, data not shown). The logistic regression model suggests that breathing difficulties during the infection and female sex are predictors of symptoms' persistence (p-value = 0.033, OR = 9.489, 95% CI 1.195-75.297 and p-value = 0.009, OR = 2.670, 95% CI 1.275-5.589, respectively), corroborating the results of the univariate analyses.

4. Discussion

We hereby report the results of an online questionnaire applied to symptomatic dengue fever patients in Madeira Island, with clinical diagnosis in 2012-2013, at the time of the single dengue outbreak in this population. The questionnaire aimed at retrospectively characterizing symptoms during the active infection and at assessing health status before and after dengue diagnosis, identifying long-term manifestations possibly triggered by dengue. As mentioned before, we expect self-reported

manifestations to be a good representation of those verified at the first exposure of a population to the DENV-1 serotype, not biased by immune responses mounted against previous DENV infections.

The most frequent symptoms during the clinical period were fever, myalgia, extreme tiredness, and headaches, reported by more than three-quarters of our participants, while vomiting, pruritus, nausea, retro-orbital pain, and arthralgia occurred in 50% to 35% of the participants. In a subset (n = 67) of this same infected population, Freitas et al.[29] described similar clinical manifestations as the most common. However, their frequencies cannot be directly compared to our study, as they referred only to hospitalized patients and the most relevant symptoms motivating their search for medical care and/or whose onset occurred during hospitalization. Conversely, our study included non-hospitalized participants and retrospectively analyzed the entire period of symptoms, from the early-acute to the convalescence phase. It is here important to stress that symptoms are known to vary during the infection, and some even worsen after the acute phase.[9,15–17,30] Regarding the systemic and musculoskeletal symptoms, our results are in line with previous studies in other populations.[9–11,15–18,30–35] It is, however, noteworthy that gastrointestinal symptoms were 1.5 to 2.7-fold less frequent in our study when compared to other studies.[10,11,15,17,18,30,31,34] Hemorrhagic manifestations, when considered altogether, were mentioned by our participants at similar or slightly lower proportions than in other studies.[9,11,35] However, marked differences between studies are found when hemorrhagic signs are analyzed individually: for instance, gum bleeding and epistaxis were 1.6 to 4.2-fold less frequent in our study, while petechiae and hematuria were 1.4 to 5-fold more frequent in our study than in previous studies.[9,10,34,35] In 2015 and 2020, primary DENV-1 local transmission in non-endemic European regions occurred in France[36] and Italy,[37] respectively, and both describe similar clinical outcomes to our study. Nevertheless, direct comparisons to our study are made impossible by their small number of cases ($n \leq 12$). In Effler et al.,[35] in a likewise Hawaiian naïve population facing a DENV-1 outbreak, most symptoms were 1.4 to 2.2-fold more frequent than in our study, including hemorrhagic symptoms (except for petechiae, found at slightly lower proportions). As mentioned before, we recognized that there might be an

overestimation of petechiae in our study since, from our previous experience with a presential questionnaire, participants tend to misinterpret these by other non-hemorrhagic cutaneous manifestations.

Gastrointestinal and hemorrhagic manifestations are considered warning signs and predictors of severity,[38] more commonly present in secondary infections, and known to be influenced by DENV serotype/genotype (reviewed in Yuan et al.).[39] Nevertheless, mild hemorrhagic manifestations have been observed in non-complicated dengue, including in primary infections.[41,40] The results of different studies, including those presented here, suggest therefore that hemorrhagic manifestations in primary infections may be more common than anticipated. As for differences between studies addressing symptoms during the infection, we must consider that most studies were held in endemic areas and do not discriminate between primary and secondary dengue virus infections, while our data is compatible with an expected milder clinical outcome in primary infections. Furthermore, their different designs, including age and sex distribution, forms of assessment and duration of follow-up, and differences in DENV serotypes and host population backgrounds, with distinct genetics and immunological experiences (introduced by natural exposures and vaccination plans), hinder their direct comparison.

Most of our participants (72.6%) reported not having any chronic diseases before dengue, while more than one quarter had at least one chronic disease, including hypertension, diabetes, respiratory disease, hypercholesterolemia, autoimmune, or heart disease. This frequency of comorbidities is concurrent with the findings of Martelli et al.,[11] where 85.4% of the participants perceived their general health status before the infection as being very good or good, but discordant with Teixeira et al.,[18] in which 55.8% of the participants had previous comorbidities. The most frequently reported chronic diseases in our cohort are also not surprising, as these are among the most prevalent in our population background.[41] When investigating possible correlations between previous chronic disease and each symptom during dengue, hypertension was found to significantly correlate with risk for arm and leg swelling and protection against retro-orbital pain. Besides the influence of the pathophysiology of hypertension in the viral

infection, this can also be explained by the condition itself and/or therapeutic side effects.

The persistence of clinical manifestations after dengue has been previously reported,[9,15–19,30] and may be caused by systemic damage during the acute phase and/or host-related factors affecting their recovery. However, their prevalence and duration vary widely in the literature, ranging from 9% to 57% of the participants reporting at least one recurrent symptom, lasting from 1 month up to 2 years. In our study, 61.5% of the respondents reported at least one persistent symptom in the 8 years after dengue, of which 68.1% sought for medical assistance, thus representing a measure of intensity/persistence of their symptoms. The most frequent persistent symptoms were headaches (35.5%), followed by abundant hair loss, extreme tiredness, arthralgia, and myalgia, the latter experienced by approximately 25% of the respondents. Most of the existing literature describes similar persistent symptoms, although generally at much lower frequencies. Previous studies also indicate a resolution of symptoms in most patients within a few months after infection (persistence rates of 8.5 - 33.3% at 1-2 months[15–17,30] and 7.6 - 11.5% at 6 months[9,18]), ending their follow-up after these time windows. As mentioned before, discrepancies between studies can be due to a myriad of factors, namely differences in study design (age and sex distribution, forms of assessment, and time of follow-up), but also due to biological effects, such as differences in virulence, viral load, and tissue tropism between DENV serotypes, and host comorbidities, immunological status, and genetic susceptibility. We should also bear in mind that the perception of health status may be variable in different populational and cultural backgrounds.

To our knowledge, the only study reporting long-term persistent symptoms is that of Garcia et al.,[19] where 56.7% of Cuban participants had at least one chronic symptom 2 years after dengue. Their findings are similar to ours, except for more expressive differences noticed for persistent headaches, abundant hair loss (respectively, 2.4 and 3.3-fold less frequent than in our study), and retroorbital pain (1.5-fold more frequent than in our study). We should here stress that Garcia et al.[19] was undertaken in a dengue-endemic area, while ours refers to a primary dengue virus infection. In this context, our results suggest that, even in naïve populations, the

occurrence of persistent symptoms may be more frequent than previously thought and may prevail for several years. Nearly 20% of our respondents have described persistent ophthalmological signs such as blurred vision and/or retroorbital pains in the years following dengue, 4 of which have developed ophthalmologic chronic disease. There is, in fact, an increasing body of evidence about dengue-associated eye disease, whose onset has been described to occur immediately after[42] or a few months after the infection.[43,44]

Also noteworthy, our data are, to our knowledge, the first to investigate and suggest sex and age as good predictors of specific symptoms, either during the infection period or persistent symptoms. During the infection, pruritus, vomiting, nausea, extreme tiredness, palmar erythema, petechiae, and arm and leg swelling, were significantly more frequent in females, while arm and leg swelling were more common in older participants. The effect of these variables in an active infection is likely due to hormonal and psychosomatic differences and/or the co-occurrence of age-related conditions. As for persistent symptoms, headaches, and abundant hair loss were more common in females, while headaches were more frequent in younger participants, and arthralgia and bone pain in older participants. Although there is no consensus about the physiopathological mechanisms underlying the persistence of symptoms, it has been suggested that the excessive cytokine production during the acute phase[45] may cause neuroendocrine, musculoskeletal, and immunological damage,[46] which may affect males and females in different ways, and lead to more durable consequences in older patients, given their lower regenerative capacity of affected tissues.

Nevertheless, the results presented here should be approached with caution, as we recognize a few limitations of our study. All participants had clinical diagnosis of dengue according to WHO,[7] following the recommendations of ECDC to the regional healthcare authorities but given the anonymous nature of the questionnaire, it is not possible to estimate the proportion of participants with laboratory-confirmed dengue. Therefore, we must consider that our results may be biased by misdiagnosed cases, caused by other infectious or non-infectious health conditions. Nevertheless, an ongoing study of our team in a subset (n = 119) of invited participants showed that, 8 years after the outbreak, more than 90% are positive for ELISA anti-DENV IgG and Foci Reduction

Neutralization Test (FRNT) (Henriques *et al. in submission).* This is in accordance with publications in non-endemic areas, reporting a loss of seropositivity, in adults, of about 4% per 3-year period,[47,48] and makes us believe that most of our respondents were, indeed, true dengue cases. Also, we must consider the subjectivity of self-reported data, the misinterpretation of the questions/multiple answers, and a higher representativity of respondents with more severe complaints. Moreover, data reports to a reference period from 2012 to 2021, which introduces a recall bias to the participants. For last, we recognize that a sex and age-matched non-infected, asymptomatic, or population-based control sample would have been relevant for distinguishing persistent symptoms resulting from dengue from those naturally occurring in the population. However, as our study was undertaken 8 years after the outbreak, we found it very unlikely and very prone to recall bias that a sample set not affected by symptomatic dengue could identify recurrent previously non-existing symptoms.

In sum, and despite its limitations, the findings of this study contribute to the existing body of knowledge with a description of the symptoms during a primary dengue virus infection, in a naïve population where a single DENV-1 outbreak has occurred. Our study also illustrates that, even in a primary infection, dengue fever may have significant implications on the health status and well-being of a considerable proportion of the infected population, as recurrent symptoms may persist for several years. These data can, therefore, be considered in terms of healthcare policies, guiding new procedures and strategies for disease control and prevention, and strengthening the importance of the clinical follow-up of dengue patients.

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CHAPTER V

Clinical and laboratorial characterization of DF in Madeira Island

Preamble

Several clinical and laboratory parameters of DF are known to be useful predictors of severity and might play an important role in the implementation of future active surveillance measures. As such, it is paramount to extensively characterize the clinical manifestations and the laboratory parameters presented by dengue patients, during a primary infection. Although some attempts at characterizing these parameters, in the population affected by the DENV-1 outbreak in Madeira Island, have already been carried out, they have focused on very specific populations, namely a pediatric cohort and a subset of the hospitalized adults. In the previous chapter, a retrospective characterization of the entire clinical phase of dengue infection and its possible long-term manifestations was only achievable in a small sample of adults. This has, therefore, prompted us to perform the characterization of clinical and laboratory parameters in a more representative sample of the affected population, including children and adults, among hospitalized and non-hospitalized dengue patients, who sought medical assistance at Madeira healthcare services during the outbreak, as well as to evaluate parameters of dengue severity predictors. The present chapter describes the clinical and laboratory characterization of the DENV-1 symptomatic patients during the outbreak in Madeira Island.

The work depicted in the present chapter resulted in an original research article currently being prepared for submission.

Clinical and Laboratorial evaluation of the Dengue outbreak occurred in Madeira Island

Introduction

Dengue is a re-emerging arboviral disease caused by any one of four dengue virus serotypes, with 390 million estimated infections occurring annually, of which 96 million are clinically recognized (Bhatt et al. 2013). The rapid increase in dengue incidence has become a serious public health threat with a significant burden on health care (Alkhaldy and Barnett 2021; Bhatt et al. 2013) and in recent years, several European countries have reported an increasing number of autochthonous transmissions (Barzon et al. 2021; ECDC 2014; Succo et al. 2016).

Infection with DENV in humans is often unapparent (Endy et al. 2002) but there is a wide spectrum of clinical presentations that can range from dengue fever (DF) to a more severe outcome with a broader clinical spectrum as the potentially lethal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Castro et al. 2007; WHO 2009). Part of DF infections (0.5 to 5%) can progress to the more severe forms of the disease, mostly in children (Morens 2009). Therefore, early accurate diagnosis and proper treatment are determinants to reduce mortality.

After an incubation period of 4-6 days, the disease's initial phase is characterized by fever, typically with a sudden onset of high temperature (Simmons et al. 2012; WHO 2009). This febrile phase usually lasts 2 to 7 days and is accompanied by headache, myalgia, arthralgia, vomiting, and rash (Simmons et al. 2012; WHO 2009, 2011). Mild hemorrhagic manifestations can be seen, like petechiae and mucosal bleeding, and liver enlargement is commonly noted (da Silva et al. 2018; Simmons et al. 2012). As to the laboratory presentation, several hematological findings have been associated with DF, in particular, mild to moderate leukopenia (white blood cells (WBC) < 5000 cells/mm^3) and mild to moderate thrombocytopenia (< 150,000 cells/mm^3), and slightly increased hematocrit (5–10%) (WHO 2011). In addition, altered hepatic biochemical parameters such as high serum levels of alanine aminotransferase (ALT) and aspartate

aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP) and albumin concentrations have been found to associate with disease severity in DF patients (De Paula and Fonseca 2004; Khan et al. 2013). As such, these parameters can be useful in establishing the diagnosis and predicting the severity of dengue disease (reviewed in Kalluru et al. (2023); Phuong et al. 2004).

The WHO classifies dengue cases as either non-severe dengue, which can present with or without warning signs, or as severe dengue, following clinical, laboratory, and epidemiological criteria (WHO 2009). Non-severe dengue without warning signs is characterized by fever, accompanied by at least two other symptoms among nausea/vomiting, rash, aches and pains, leukopenia, and positive tourniquet test in patients who live or have recently traveled to a dengue-endemic area. When patients present warning signs such as abdominal pain or tenderness, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, restlessness, liver enlargement (>2 cm), increasing hematocrit with a concurrent rapid decrease in platelet count, they are classified as DF with warning signs and are at higher risk of progressing to severe dengue. This last outcome is characterized by severe plasma leakage, which can lead to shock (Dengue Shock Syndrome) and/or fluid accumulation, with respiratory distress, severe hemorrhage, and/or severe organ involvement (WHO, 2009).

Co-morbidities are also believed to represent a risk factor for developing severe dengue. Bravo, Guzmán, and Kouri (1987) showed that chronic diseases such as bronchial asthma, diabetes mellitus, and sickle cell anemia contributed to the development of severe dengue. More recent studies reinforce that diabetes (Htun et al. 2015; Lee et al. 2020; Pang et al. 2012), hypertension (Pang et al. 2012), renal disorders (Kuo et al. 2008; Lizarraga and Nayer 2014) and obesity (Tan et al. 2018) are also potential risk factors for the development of more severe forms of the disease.

The first and unique dengue epidemic on Madeira Island occurred in 2012 facilitated by the vector *Aedes aegypti* which had been introduced on the island in 2005. The outbreak resulted, until 3rd March 2013, in 2168 reported cases (probable cases) of which 1080 were laboratory-confirmed (ECDC 2014). The serotype responsible for the outbreak was identified to be DENV-1, probably imported from South America, more specifically Venezuela (Huhtamo, Korhonen, and Vapalahti 2013; Wilder-Smith et al.

2014). Clinical and laboratory characterization of two patients was provided by the case study of Alves et al. (2013) showing that, in both cases, thrombocytopenia and leukopenia were present. One patient also had elevated AST and ALT, while the other had slightly elevated AST, high lactate dehydrogenase (LDH), and very high creatine kinase (CK) serum levels.

Later, the study of Castro et al. (2014) characterized the pediatric dengue cases in Madeira Island, having observed mild clinical outcomes, typical of primary infections. For instance, the authors reported hematological parameters such as leukocyte count with a mean value of 2700 cells/µL, which evidenced mild leukopenia in the studied population. Thrombocytopenia was also observed, with a decrease in platelet count reaching a mean value of 106000 platelets/µL and a minimum platelet count of 3000 platelets/µL. Nonetheless, only one patient presented severe thrombocytopenia. Conversely, increased hematocrit was observed, with a mean value of 40.5% and a maximum of 48.2%. The authors also reported a progressive increase of transaminases during the first week of the disease, with an AST mean value of 141 U/L and an ALT mean value of 136 U/L. Absolute maximum values were 802 and 493 U/L for AST and ALT, respectively (Castro et al. 2014). The same parameters were registered for a cohort of adult hospitalized DF patients by Freitas et al. (2014). The authors observed that 80.6% of cases presented leukopenia, with a minimum of 1000 cells/µL. Similarly, thrombocytopenia was also frequent, with a prevalence of 86.5% (n=58) and a minimum of 1400 platelets/µL (Freitas et al. 2014). Concerning the increase of transaminases, 88.0% (n=59) of patients had either AST, ALT, or both elevated, with 31% (n 21) of patients reaching seric levels five-fold above the reference interval.

However, the above-referred studies were directed to specific subpopulations (adults hospitalized or children) and thus, to better characterize the clinical impact of the 2012 dengue outbreak in Madeira and to implement future active surveillance measures, a more detailed and broad evaluation of the clinical manifestations, hematological and biochemical parameters, in a broader number of DF patients, is required.

In this work, we propose to retrospectively characterize a primary dengue infection, by investigating the clinical manifestations, the laboratory features, and their age and gender influence, on probable and confirmed cases. Moreover, we intend to

determine which parameters are better predictors of DF severity and if the existence of co-morbidities had an impact on the clinical outcome of a primary infection. We expect the results of this study to contribute to the improvement of diagnosis and treatment of DF, and to the continuous active surveillance by healthcare providers, in order to implement prompt public health measures, in case of new foci of transmission.

Material and Methods

Sample characterization

This study analyzed data from probable and confirmed DF cases who sought medical assistance between October 2012 and March 2013 at SESARAM, E.P.E. Madeira healthcare services, which included Funchal Central Hospital, Hospital dos Marmeleiros, the four Healthcare Centers in the municipality of Funchal and some healthcare centers of other municipalities of Madeira Island. On admission, the first suspected cases were evaluated for clinical and laboratory findings, based on the WHO 2009 criteria for DF diagnosis (WHO 2009, 2011) and on the case definition from normative documents for the surveillance of dengue issued by the Portuguese Health Directorate (DGS 2012) and later from the Regional Health Authorities (IASaude 2012). Probable cases met both clinical and epidemiological criteria. Clinical criteria included acute fever onset and at least two of the following symptoms and/or signs: headache, retro-orbital pain, myalgia, arthralgia, exanthema, hemorrhagic manifestations, or leukopenia. Residents or visitors from the Madeira Autonomous Region during the three-week period prior to the onset of symptoms, met epidemiological criteria (DGS 2012; ECDC 2013). As to confirmed cases, these were probable cases with at least one of the following laboratory confirmation results: i) the presence of dengue virus-specific IgM antibodies in blood or cerebrospinal fluid (CSF); ii) a significant increase in the concentration of dengue virus-specific IgG antibodies (seroconversion); iii) detection of dengue virus nucleic acid or antigen in blood or CSF) (DGS 2012; ECDC 2013). With the progression of the outbreak, only a subset of these probable and confirmed dengue cases was evaluated for

laboratory findings, namely detailed hematological and biochemical parameters, including lipid profiles and serologic confirmation of dengue.

Specifically, two types of data were analyzed in this study:

i) Clinical data of probable or confirmed dengue patients (1369 cases) who sought medical assistance at Funchal Central Hospital, of which 1106 only had clinical records, 263 had clinical and laboratory data records (blood counts and biochemistry) and 60 of these also had laboratory confirmation of dengue.

ii) Laboratory data of probable and confirmed dengue patients (814 cases), for which no clinical records were available. Of these, 389 were serologically confirmed for dengue.

The clinical and analytic findings of these participants were extracted from SESARAM, E.P.E. databases by the clinicians participating in this study, and were anonymized prior to the statistical analyses.

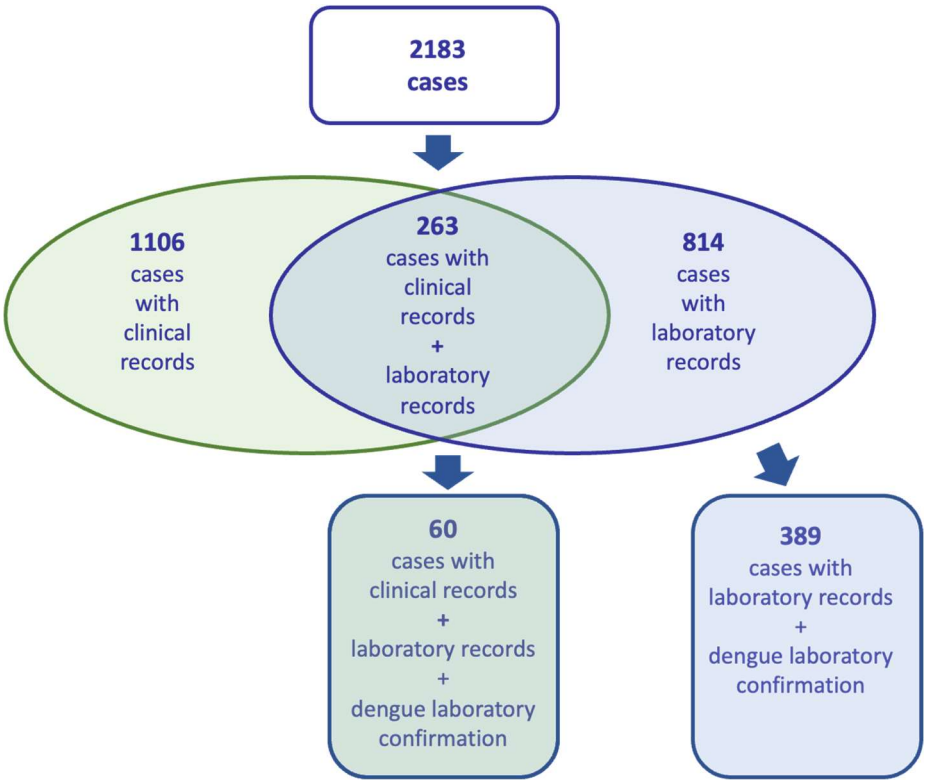

Figure 1. Clustering of dengue patients according to the analyzed data.

Statistical analyses

Descriptive statistics of the overall data, and per sex and age subgroups, included for categorical variables, the absolute and relative frequencies, while for continuous variables, means (and standard deviations), and confidence intervals, were calculated. Comparisons between categorical variables by gender and age were made using Pearson's chi-square test or Fisher's exact test (when applicable), with respective odds ratio (OR) and 95% confidence interval (CI) when statistics were found to be significant. When comparing differences in continuous variables by gender or age subgroups, Student T-tests or Mann-Whitney tests were used according to the previous assessment of the normality of data using Kolmogorov-Smirnov and Shapiro-Wilk tests.

Laboratory data were also compared with sex and age-matched reference values for the Portuguese population. All statistical analyses were performed using SPSS software (version 28.0.0.0, IBM Corp, USA), and interpreted at a level of significance of 5%.

Ethics approval and informed consent

Approval of the study was obtained from the Ethics Committee for Health of SESARAM, E.P.E. (approval n. 23/2019). No informed consent was requested from the participants since this was a retrospective study and data were analyzed anonymously. All procedures of the study complied with the Declaration of Helsinki.

Results

1. Clinical data

1.1 - Characterization of the sample population:

A total of 1369 patients were included in the clinical data analyses, of which 59,5% were females and 40.5% were males (Table 1). The overall mean age of patients was 38,8 ± 19.9 years old (y.o.; data not shown), of which 78.9% were adults (age > 18 y.o., mean age 45.9 ± 16.0 years), and 21.1% were children (age ≤18 y.o., mean age 12.2 ± 4.4 years), (Table 1).

Table 1. Characterization of the sample set with available clinical data.

	Overall	Adults					Children				
	n (%)	n (%)	Mean age (y)	SD	CI (95%)	Range	n (%)	Mean age (y)	SD	CI (95%)	Range
Total	1369	1080	45.9	16.0			289	12.2	4.4		
Male	555 (40.5)	409 (37.9)	43.8	15.3	42.3-45.3	19-85	146 (50.3)	11.9	4.2	11.2-12.6	1-18
Female	814 (59.5)	671 (62.1)	47.3	16.2	46.0-48.5	19-91	143 (49.3)	12.5	4.6	11.7-13.2	0-18
SD = Standard deviation; CI = Confidence interval											

These participants had their first visit to the healthcare system between days 0 and 142 of the onset of symptoms (on average at the 6th day), but more than half of the adult patients (56.9%) were observed on days 1-4 of clinical manifestations. Nearly 4% needed in-hospital management, on average for 5 days (varying from 2 to 12 days). Likewise, most children (61.6%) were assisted by healthcare services between days 1-4 of the disease (on average at day 5) and 3.4% were hospitalized, with a length of stay that varied from 3 to 8 days (on average 4 days).

1.2. Signs and symptoms from the clinical evaluation:

The clinical signs and symptoms of all patients enrolled in this part of the study are summarized in Figure 2 and Table 2. Given that the dengue cases included in this study were evaluated at different time points of the outbreak and by different clinicians, not all clinical parameters were systematically registered. Thus, for the analysis of each parameter/variable, missing entries were excluded, and only available data were considered.

Symptoms like fever, myalgia, and headache were the most frequently reported, respectively 89.7%, 87.5%, and 78.0% of the patients, while arthralgia was referred by 41.0%, retro-orbital pain by 38.8% and rash was noted in 35.2%. Hemorrhagic signs were observed in 4.9% of total patients, with petechiae in 2.1%, epistaxis in 1.8%, gastrointestinal bleeding, and hematuria in 0.4%, and metrorrhagia, which was observed in 16.3% of post-pubertal females (data not shown).

Several patients with ophthalmological complaints were referred to specialized ophthalmology services. Of 1301 total patients with available clinical data for this assessment, 1.0% presented maculopathy.

Also, palmar erythema was present in 4.9% of patients (data available for n=817), hypotension in 1.9%, hepatomegaly and pleural effusion in 0.7% (for these last, data was available for n=823). Ascites was observed in only 1 of these patients (0.1%).

When comparing the frequencies of signs and symptoms between the adult and pediatric sample sets (Table 2), fever was the predominant symptom in both, but significantly more frequent in children than in the adult population (96.9% vs 87.8%; $p<0.001$) along with headache (84.4% vs 76.3%; $p=0.002$). The febrile stage lasted from 1 to 10 days in children and from 1 to 12 days in adults, with 3 days being the most frequent duration in both groups. Also, overall hemorrhagic signs and rash (6.9% vs 4.3%, $p=0.082$; 40.1% vs 33.9%, $p=0.052$) tend to be higher in children, while arthralgia and myalgia were significantly more common in adults (24.2% vs 45.4%, $p<0.001$; and 78.5% vs 89.8%, $p<0.001$) (Table 2).

When comparing the frequency of signs and symptoms among gender within the adults' subgroup (Table 3), females had statistically significantly more frequent presentations of headache (78.9% in females vs 72.5% in males, p=0.018), rash (36.5% vs 29.0%, p=0.012), and hypotension (3.4% vs 0.4%, p=0.013). In contrast, males had a significantly higher incidence of pleural effusion than females (1.6% vs 0.0%, p=0.022). No statistically significant differences were found for other signs and symptoms between males and females.

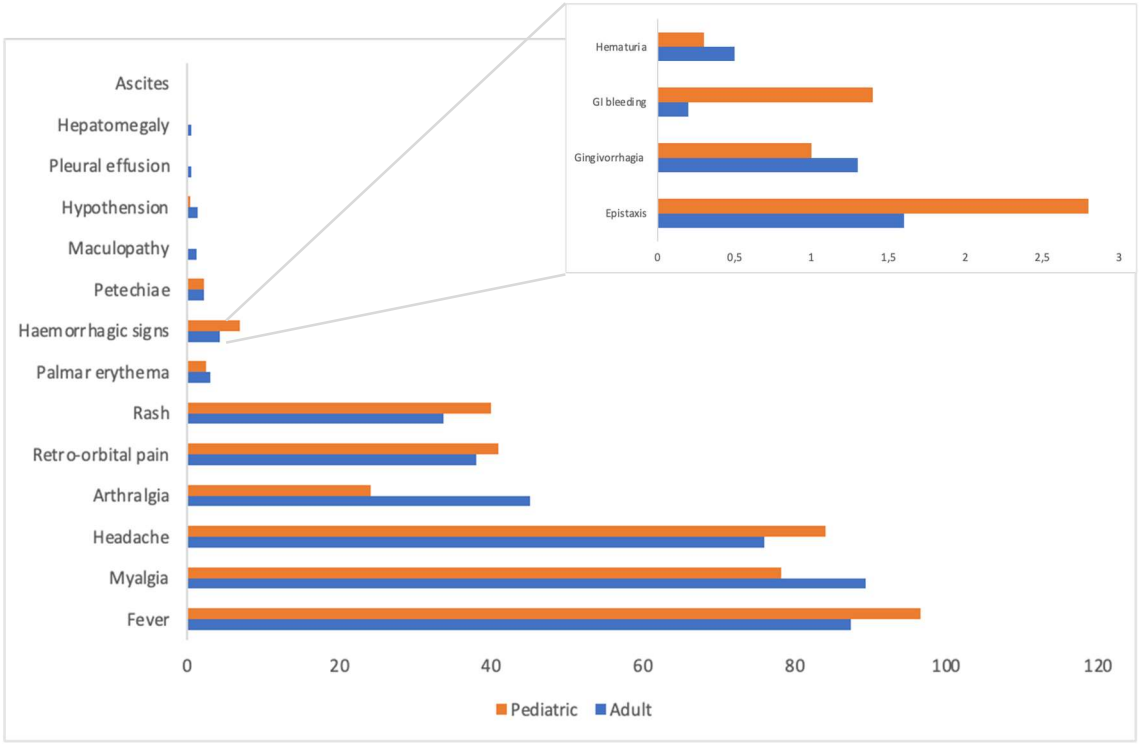

Figure 2: Relative frequencies (%) of clinical signs and symptoms from patients with dengue fever (adults and children).

Table 2. Relative (%) and absolute frequencies (n=1369) of clinical signs and symptoms of patients with dengue fever (overall and for adult and paediatric sample sets).

Signs / Symptoms	Frequency (%)			Fisher's Exact Test *p* -value	OR (95% CI)
	Overall (n)	Adult (n)	Pediatric (n)		
Fever	89.7 (1237)	87.8 (957)	96.9 (280)	**<0.001**	0.231 (0.116-0.460)
Myalgia	87.5 (1206)	89.8 (979)	78.5 (227)	**<0.001**	2.409 (1.710-3.393)
Headache	78.0 (1076)	76.3 (832)	84.4 (244)	**0.003**	0.595 (0.420-0.842)
Arthralgia	41.0 (565)	45.4 (495)	24.2 (70)	**<0.001**	2.603 (1.939-3.494)
Retro-orbital pain	38.8 (535)	38.2 (416)	41.2 (119)	0.377	
Rash	35.2 (485)	33.9 (369)	40.1 (116)	0.052	
Palmar erythema	4.9 (40)	5.1 (33)	4.0 (7)	0.693	
Hemorrhagic signs	4.9 (67)	4.3 (47)	6.9 (20)	0.089	
Petechiae	2.1 (29)	2.1 (23)	2.1 (6)	1.000	
Hypothension	1.9 (16)	2.3 (15)	0.6 (1)	0.216	
Epistaxis	1.8 (25)	1.6 (17)	2.8 (8)	0.211	
Gingivorrhagia	1.2 (17)	1.3 (14)	1.0 (3)	1.000	
Maculopathy	1.0 (14)	1.2 (13)	0.3 (1)	n.d.	
Pleural effusion	0.7 (6)	0.9 (6)	0.0 (0)	0.351	
Hepatomegaly	0.7 (6)	0.9 (6)	0.0 (0)	0.351	
Gastrointestinal bleeding	0.4 (6)	0.2 (2)	1.4 (4)	**0.020**	0.131 (0.024-0.719)
Hematuria	0.4 (5)	0.5 (5)	0.0 (0)	0.590	
Ascites	0.1 (1)	0.2 (1)	0.0 (0)	1.000	

OR = Odds ratio OR; 95% CI = 95% confidence intervals;. n.d. = not determined due to low n

Table 3. Relative (%) and absolute frequencies (n=1080) of clinical signs and symptoms of adult patients with dengue fever (overall and per sex).

Signs / Symptoms	Frequency (%)			Fisher's Exact Test *p* -	OR (95% CI)
	Overall (n)	Males (n)	Females (n)		
Fever	88.3 (949)	89.9 (366)	87.3 (583)	0.205	
Myalgia	90.0 (968)	90.2 (367)	90.0 (601)	1.000	
Headache	76.5 (822)	72.5 (295)	78.9 (527)	**0.018**	1.419 (1.066-1.889)
Arthralgia	45.6 (490)	42.3 (172)	47.6 (318)	0.089	
Retro-orbital pain	38.6 (415)	35.4 (144)	40.6 (271)	0.093	
Rash	33.7 (362)	29.0 (118)	36.5 (244)	**0.012**	1.409 (1.080-1.839)
Palmar erythema	4.9 (31)	4.1 (10)	5.5 (21)	0.571	
Hemorrhagic signs	4.3 (46)	3.2 (13)	4.9 (33)	0.214	
Hypothension	2.2 (14)	0.4 (1)	3.4 (13)	**0.013**	8.424 (1.095-64.808)
Petechiae	1.7 (18)	0.7 (3)	2.2 (15)	0.084	
Epistaxis	1.6 (17)	1.5 (6)	1.6 (11)	1.000	
Gingivorrhagia	1.3 (14)	1.7 (7)	1.0 (7)	0.409	
Maculopathy	1.3 (13)	1.6 (6)	1.1 (7)	0.569	
Hepatomegaly	0.8 (5)	0.4 (1)	1.0 (4)	0.654	
Pleural effusion	0.6 (4)	1.6 (4)	0.0 (0)	**0.022**	n.a.
Hematuria	0.5 (5)	0.7 (3)	0.3 (2)	0.373	
Gastrointestinal bleeding	0.2 (2)	0.0 (0)	0.3 (2)	0.529	
Ascites	0.2 (1)	0.0 (0)	0.3 (1)	1.000	

OR = Odds ratio OR; 95% CI = 95% confidence interval, n.a.= not applicable..

When analyzing the risk of hospitalization considering the presence of hemorrhagic signs, hepatomegaly, and duration of the feverish condition, by logistic regression, only the last one was found to be significant ($p<0.001$; OR=1.550; 95%CI 1.316-1.826; data not shown).

As to the presence of comorbidities in adult patients (data available for n=550), hypertension and dyslipidemia were the most frequent (26.5 and 17.4%), followed by heart disease (9.7%), diabetes (7.2%), respiratory disease (5.2%) and obesity (3.8%). Hematological, autoimmune diseases and renal, diseases were less frequent (1.4, 1.3 and 0.7%, respectively). However, none of these was significant in predicting the need for hospitalization.

2. Laboratory data

Laboratory data from a total of 1077 DF patients were assessed, 39.6% of these were males, 60.4% were females and their mean age was 39.1 ± 20.1 y.o. (data not shown). When analyzing by age group, adults had a mean age of 46.6 ± 15.9 y.o. and children of 12.1 ± 4.4 y.o. (Table 4).

Table 4. Characterization of the sample set with available laboratory data.

	Overall	Adults					Children				
	n (%)	n (%)	Mean age (y)	SD	CI (95%)	Range	n (%)	Mean age (y)	SD	CI (95%)	Range
Total	1077	845	46.6	15.9	45.5-47.6	19-91	232	12.1	4.4	11.5 -12.6	0-18
Males	427 (39.6)	310 (36.7)	44.4	15.2	42.7-46.1	19-85	117 (50.4)	11.6	4.1	10.8-12.4	1-18
Females	650 (60.4)	535 (63.3)	47.8	16.1	46.4-49.2	19-91	115 (49.6)	12.5	4.5	11.6-13.3	0-18
SD = Standard deviation; CI = Confidence interval											

Descriptive statistics of hematologic parameters of adult and pediatric patients, determined at the time of the active infection, are given in Tables 5 and 6, respectively.

The mean complete blood counts are illustrated, showing that leukopenia (essentially due to lymphopenia and neutropenia), thrombocytopenia, and elevated prothrombin times, presented the main deviations from the reference values in both adult and pediatric DF populations. In the DF adults, leucocytes' mean values were below the reference ranges in both sexes (4.22 ± 2.35 x 10^3/µL, M; 3.57 ± 2.10 x 10^3/µL,

F) being significantly lower in females ($p<0.001$). Lymphocytes' blood counts in DF males ranged from 0.10 and 343.00 x 10^3/µL (mean ± SD: 1.55 ± 7.21 x 10^3/µL) and were found significantly lower than in DF females (mean± SD: 2.63 ± 19.06 x 10^3/µL; $p=0.008$). In fact, in DF adults, leucocytes and lymphocytes were below the reference intervals in 66.7 and 74.5% of males and 77.2 and 77.4% of females, respectively. As for DF children, leukopenia and lymphopenia were present in 69.1% and 57.8% of the patients, with the lymphocyte blood count found to be significantly lower in DF children (mean ± SD: 1.31 ± 1.18 x 10^3/µL) than in DF adults (mean ± SD: 2.24 ± 15.82 x 10^3/µL; $p < 0.001$). Neutropenia was also observed in both DF populations more specifically in 26.0% of adult males, 58.5% of adult females, and 62.5% of children.

The mean blood counts for monocytes, eosinophils, and basophils were within the reference intervals but showed to be significantly lower in DF females than males (monocytes mean ± SD: 0.39 ± 0.24 x 10^3/µl, F; 0.53 ± 0.30 x 10^3/µl, M; $p < 0.001$; eosinophils mean ± SD: 0.036 ± 0.092 x 10^3/µl, F; 0.057 ± 0.103 x 10^3/µl, M; $p = 0.001$). For these parameters, no significant differences were found between DF adults and children.

As to hematocrit, an important indicator of plasma leakage, although ranging from 25.5 and 55.0% its mean values in DF adults were within the reference intervals. Nevertheless, significant differences were obtained for mean hematocrit in DF males and females, and its increase was more significant in the female population (2.7% increase in females against 0.5% in males). As to the hematocrit of the pediatric DF population, it denoted more expressive changes, ranging from 27.8% to 50.0%, but 17.2% of children presented a high hematocrit. Furthermore, other hematological parameters in children showed hemoconcentration, as the increase in erythrocytes observed in 20.7%, and of hemoglobin in 16.4%.

In the overall patients, mean platelets were below the reference limits (mean ± SD: 139.74 ± 67.78 x 10^3/µL), ranging from 4.00 to 568.00 x 10^3/µL. In the adult population, this condition was observed in 72.1% of males and 64.8% of females, with the mean platelet count being significantly lower in males (mean ± SD: 133.42 ± 68.01 x 10^3/µL) than in females (mean ± SD: 143.35 ± 67.45 x 10^3/µL; $p = 0.011$). Thrombocytopenia, with platelet count under the reference limit, was also found in

53.0% of children, with mean ± SD platelet blood count 150.68 ± 69.12 x 10^3/µL. On the other hand, prothrombin time was found to be considerably increased in both populations, particularly 88.2% of adult males, 71.4% of adult females, and 92.9% of children, while the sedimentation rate was above the reference range in 75.0% of adult males.

As to the other hematologic parameters, these were within the normal ranges, when comparing their mean values with the reference intervals, although differences were found between sex and age given their physiological and metabolic differences.

Table 5. Haematologic parameters of adult dengue patients, overall and by gender.

Blood counts	Overall			Males			Females			*p* -value*	Above	Below M/F
	n	Mean (SD)	Ref Values	n	Mean (SD)	Ref Values	n	Mean (SD)	Ref Values		M/F (%)$^{\Phi}$	(%)$^{\Phi}$
Erythrocytes (x10^6/µL)	839	4.57 (0.46)		305	4.83 (0.46)	4.50-6.50	534	4.43 (0.40)	3.80-5.80	**<0.001**	0.0 / 0.6	23.9 / 5.6
Hemoglobin (g/dL)	839	13.97 (1.34)		305	14.88 (1.24)	13.0-18.0	534	13.45 (1.10)	11.5-16.0	**<0.001**	1.5 / 0.9	7.5 / 3.6
Hematocrit (%)	839	41.39 (3.88)		305	43.91 (3.73)	40.0-54.0	534	39.96 (3.17)	37.0-47.0	**<0.001**	0.5 / 2.7	15.4 / 15.7
Mean corpuscular volume (MCV) (fL)	839	90.73 (4.64)	80.0-100.0	305	91.18 (4.56)		534	90.47 (4.67)		**0.036**	1.5 / 1.8	1.0 / 1.2
Mean Corpuscular Hemoglobin (MCH) (pg)	839	30.62 (1.85)	>27.0	305	30.90 (1.80)		534	30.46 (1.86)		**0.003**	n.a.	1.0 / 2.7
MCH Concentration (MCHC) (g/dL)	839	33.76 (1.20)	30.0-35.0	305	33.89 (1.16)		534	33.68 (1.22)		**0.010**	21.4 /13.6	0.0 / 0.0
Leucocytes (x10^3/µL)	836	3.81 (2.22)	4.5-11.0	305	4.22 (2.35)		531	3.57 (2.10)		**<0.001**	1.5 / 0.6	66.7 / 77.2
Neutrophils (x10^3/µL)	837	2.21 (1.52)		304	2.44 (1.63)	1.5-7.1	533	2.09 (1.44)	2.0-7.5	**<0.001**	3.5 / 0.6	26.0 / 58.5
lymphocytes (x10^3/µL)	837	2.24 (15.82)	1.5-4.0	304	1.55 (7.21)		533	2.63 (19.06)		**0.008**	1.5 /1.8	74.5 /77.4
Monocytes (x10^3/µL)	831	0.44 (0.27)	0.0-1.0	303	0.53 (0.30)		528	0.39 (0.24)		**<0.001**	6.5 / 2.4	0.0 / 0.0
Eosinophils (x10^3/µL)	835	0.044 (0.097)	0.0-0.5	303	0.057 (0.103)		532	0.036 (0.092)		**0.001**	0.5 / 0.3	0.0 / 0.0
Basophils (x10^3/µL)	835	0.0046 (0.021)	0.0-0.2	303	0.0050 (0.0217)		532	0.0043 (0.0204)		0.676	0.0 / 0.0	0.0 / 0.0
Platelets (x10^3/µL)	839	139.74 (67.78)	150-450	305	133.42 (68.01)		534	143.35 (67.45)		**0.011**	0.5 / 0.0	72.1 / 64.8
Sedimentation rate (mm)	24	20.00 (16.47)	<30	11	24.64 (20.67)		13	16.08 (3.13)		0.459	75.0 / 16.7	n.a.
Protombin Time (PT) (s)	73	16.80 (8.20)	11.0-13.5	30	18.28 (10.06)		43	15.77 (6.54)		**0.037**	88.2 / 71.4	0.0 / 0.0
Parcial Tromboplastin Time (PTT)	5	0.96 (0.15)		4	1.01 (0.11)		1	n.a.		n.d.	n.d.	n.d.

*Independent samples T-test or Mann-Whitney test used to compare gender according to the assumption or not of normally distibuted data. M = males; F = females; n.a. = not applicable; n.d. = not determined; $^{\Phi}$Percentage of individuals with values above or below the reference range.

Table 6. Hematologic parameters of dengue pediatric patients.

Blood counts	n	Mean (SD)	Ref Values	*p*-value[Ψ]	% Above[Φ]	% Below[Φ]
Erythrocytes (x10^6/µL)	232	4.75 (0.39)	3.91-5.07	**<0.001**	20.7	0.9
Hemoglobin (g/dL)	232	13.62 (1.41)	11.9-14.9	**0.005**	16.4	8.2
Hematocrit (%)	232	40.51 (3.93)	34.0-44.0	**0.018**	17.2	4.3
Mean corpuscular volume (MCV) (fL)	232	85.34 (5.28)	80.0-99.0	**0.000**	0.0	11.2
Mean Corpuscular Hemoglobin (MCH) (pg)	232	28.71 (2.28)	>27.2	**0.000**	83.2	15.5
HMC Concentration (MCHC) (g/dL)	232	33.62 (1.33)	32.0-36.0	0.227	2.2	3.9
Leucocytes (x10^3/µL)	228	3.87 (2.73)	4.2-10.8	0.544	1.7	69.1
Neutrophils (x10^3/µL)	228	2.03 (1.97)	1.9-7.2	**<0.001**	1.7	62.5
lymphocytes (x10^3/µL)	228	1.31 (1.18)	1.2-3.4	**<0.001**	4.3	57.8
Monocytes (x10^3/µL)	228	0.44 (0.33)	0.3-0.9	0.395	4.3	31.2
Eosinophils (x10^3/µL)	228	0.057 (0.134)	0.0-0.6	0.699	2.2	0.0
Basophils (x10^3/µL)	228	0.0057 (0.0251)	0.0-0.1	0.697	0.4	0.0
Platelets (x10^3/µL)	232	150.68 (69.12)	144.0-440.0	**0.006**	0.9	53.0
Protombin Time (PT)	14	16.05 (2.78)	10.0-12.5	0.210	92.9	0.0

[Ψ] Mann-Whitney U Test for comparison between adult and pediatric populations.
[Φ] Percentage of individuals with values above or below the reference range.

Table 7 summarizes routine biochemistry parameters, in the complete DF adult dataset and by gender. Mean serum glucose was increased in around one-third of the DF patients, both males and females, and in 26.2% of children (Table 8). Creatinine was also above the reference limits in 28.5% of DF males, while uric acid was below the reference levels in 37.5% of DF males. On the other hand, in children, creatinine and uric acid levels were below the reference limits in 21.3% and 9.1% of individuals, respectively.

Interestingly, mean serum HDL and LDL cholesterol were within normal levels, but HDL was found to be decreased in 50% of females, while LDL was increased in 30% of females. Although within the reference ranges, mean total proteins were decreased in almost a quarter of females (23.5%). Albumin was also decreased in half of the DF males but only in 8.3% of DF females. However, these results should be looked at with caution because these two parameters were determined in a limited number of patients ($n < 36$).

A moderate increase of several liver enzymes could be observed, indicating liver involvement. ALT and AST were moderately or even highly increased in a large proportion of patients, of both sexes. Their increase was nevertheless more common in females (increased ALT and AST in 62.0% and 76.8% of females, while 56.5 and 59.0 in males, respectively), whose mean values (ALT mean ± SD: 85.29 ± 139.72 U/L, F; AST

mean ± SD: 94.93 ± 155.59 U/L, F) were more than two-fold the upper reference limits. The severe increase of these enzymes (>= 2000 U/L) in one patient showed critical liver involvement. In children, AST was the enzyme that showed a larger increase, with 50.2% showing values above the reference limits (ALT: 58.8 ± 95.8 U/L; AST: 82.3 ± 97.7 U/L). GGT was also moderately increased in DF adults, ranging from 11.60 to 449.50 U/L. Mean values in both sexes were above the reference range (mean ± SD: 69.66 ± 60.19 U/L, M; 63.24 ± 71.80 U/L, F), with males displaying the highest proportion of DF patients with increased levels of γ-GT (47.2% M; 31.9 % F).

As to pancreatic amylase levels, they were above the reference limits in one-third of females (33.3%) against a small proportion of males (5.9%). Mean total bilirubin, indirect and direct bilirubin were within normal levels in adult patients, but total and direct bilirubin were above reference values mostly in males (12.5 and 28.1%, respectively).

Plasma concentrations of CK (mean ± SD: 318.12 ± 472.81, M; 222.67 ± 449.44, F) and LDH (mean ± SD: 316.43 ± 183.86, M; 324.33 ± 174.39, F) were clearly above the reference intervals, being CK levels significantly higher in DF males than in DF females ($p<0.016$). In children, CK mean values were almost four-fold the reference limit (mean ± SD: 525.80 ± 1406.50), and higher than in DF adults, although not significant ($p = 0.154$; not shown). CK was increased in around 50% of males and children and 37.6% of females, while LDH was increased in more than 60% of adults and children, but its mean values did not differ significantly between both groups.

C-Reactive Protein values in DF adults exceeded by more than twice the reference limits both in males (mean ± SD: 16.10 ± 36.88 mg/dL) and females (mean ± SD: 13.00 ± 20.78 mg/dL), with this increase being more frequent in the male (57.1%) than in the female sex (48.0%). In children, mean C-Reactive Protein values were slightly above the reference interval and above normal levels in 28.1% of the patients. To note also that mean values were significantly higher in adults than in children (mean ± SD: 14.10 ± 27.6 mg/dL and 6.5 ± 11.9 mg/dL, respectively; $p < 0.001$; not shown).

Electrolytes' mean values were found in the lower ranges of their reference intervals in both sexes, being below the lower limit in a considerable proportion of DF adults (sodium 42.0%, M; 35.5%, F; chloride 19.3%, M; 12.6%, F; and calcium 40.0%, M; 33.3%, F). Note, however, that this last parameter has only been determined in a limited number of patients (n=16). In children, the mean values of these electrolytes were similar to those in adults, with sodium below normal levels in 38.2% of patients.

Table 7. Biochemical parameters (mean ±SD) of dengue adult patients overall and by gender.

Biochemistry	Overall			Males			Females			*p*-value*	Above (%)ϕ M/F	Below (%)ϕ M/F
	n	Mean (SD)	Ref. Values	n	Mean (SD)	Ref. Values	n	Mean (SD)	Ref. Values			
Glucose (mg/dL)	670	109.2 (34.3)	74-110	246	112.08 (41.58)		424	107.46 (29.13)		0.125	31.6 / 33.3	1.3 / 0.8
Urea (mg/dL)	685	27.6 (10.5)	8-50	253	31.93 (9.91)		432	25.03 (9.96)		**<0.001**	4.2 / 1.9	0.0 / 0.0
Creatinine (mg/dL)	732	0.97 (0.23)		264	1.13 (0.24)	0.70-1.20	468	0.88 (0.16)	0.66-1.09	**<0.001**	28.5 / 8.2	0.0 / 2.4
Uric acid (mg/dL)	57	4.30 (1.37)		17	5.18 (1.59)	4.8-8.7	40	3.93 (1.08)	2.6-8.0	**0.001**	0.0 / 0.0	37.5 / 7.1
Total Cholesterol (mg/dL)	16	150.1 (29.1)	<200	5	138.40 (31.73)		11	155.36 (27.68)		0.295	n.d / 0.0	n.a.
HDL-Cholesterol (mg/dL)	15	42.1 (8.3)	40-60	5	40.40 (11.80)		10	42.90 (6.59)		0.602	n.d./0.0	n.d. / 50.0
LDL-Cholesterol (mg/dL)	15	79.2 (21.9)	<115	5	70.36 (31.29)		10	83.56 (15.78)		0.415	n.d. / 33.3	n.a
Triglicerides (mg/dL)	16	123.0 (49.8)	<150	6	127.67 (57.00)		10	120.20 (48.00)		0.783	n.a.	n.a
Total proteins (g/L)	36	70.3 (5.2)	66-83	9	72.62 (5.68)		27	69.48 (4.85)		0.116	0.0 / 0.0	0.0 / 23.5
Albumin (g/L)	20	38.3 (4.8)	35-48	5	38.40 (6.87)		15	38.28 (4.29)		0.963	0.0 / 0.0	50.0 / 8.3
Alcaline phosphatasis (U/L)	122	66.4 (22.2)	30-120	33	65.91 (21.73)		89	66.61 (22.53)		0.878	5.0 / 1.8	0.0 / 0.0
Gama glutamyltranspetidase (U/L)	221	65.5 (67.9)	7.0-50.0	77	69.66 (60.19)		144	63.24 (71.80)		0.504	47.2 / 31.9	0.0 / 0.0
Alanine aminotransferase (U/L)	826	85.6 (123.2)		300	86.20 (87.24)	<50.0	526	85.29 (139.72)	<35.0	0.919	56.5 / 62.0	n.a.
Aspartate aminotransferase (U/L)	793	91.4 (135.1)		292	85.35 (89.58)	<50.0	501	94.93 (155.59)	<35.0	0.336	59.0 / 76.8	n.a
Lactate dehydrogenase (U/L)	542	321.3 (177.9)	<246.0	205	316.43 (183.86)		337	324.33 (174.39)		0.617	64.4 / 68.6	n.a
Creatine Kinase (U/L)	575	258.0 (460.1)		213	318.12 (472.81)	<171	362	222.67 (449.44)	<145	**0.016**	50.0 / 37.6	n.a
Total bilirrubin (mg/dL)	116	0.60 (0.34)	0.3-1.2	47	0.720 (0.442)		69	0.527 (0.232)		**0.008**	12.5 / 0.0	0.0 / 10.9
Indirect bilirrubin (mg/dL)	116	0.46 (0.25)	<1.10	47	0.540 (0.323)		69	0.407 (0.174)		**0.005**	6.3 / 0.0	n.a
Direct bilirrubin (mg/dL)	116	0.14 (0.12)	<0.20	47	0.180 (0.159)		69	0.120 (0.078)		**0.019**	28.1 / 2.2	n.a
Pancreatic amylase (U/L)	48	39.5 (24.3)	<46.0	25	34.12 (16.31)		23	45.30 (30.11)		0.113	5.9 / 33.3	n.a.
C- Reactive Protein (mg/dL)	619	14.1 (27.6)	<6.10	220	16.10 (36.88)		399	13.00 (20.78)		0.181	57.1 / 48.0	n.a.
Sodium (mEq/L)	731	136.2 (3.0)	136.0-145	266	136.12 (3.00)		465	136.29 (2.94)		0.440	0.0 / 0.7	42.0 / 35.5
Potassium (mEq/L)	686	4.07 (0.45)	3.5-5.1	251	4.20 (0.43)		435	3.99 (0.45)		**<0.001**	3.6 / 1.1	2.4 / 10.5
Chloride (mEq/L)	731	101.1 (3.4)	98.0-111.0	266	100.43 (3.25)		465	101.46 (3.46)		**<0.001**	0.0 / 0.3	19.3 / 12.6
Calcium (mEq/L)	16	9.04 (0.57)	8.90-10.30	8	9.02 (0.74)		8	9.05 (0.46)		0.933	0.0 / 0.0	40.0 / 33.3

*Independent samples T-test or Mann-Whitney test used to compare gender according to the assumption or not of normally distibuted data; n.a. = not applicable; n.d. = not determined;

ϕPercentage of individuals with values above or below the reference range. M = males; F = females.

Table 8. Biochemical parameters (mean ±SD) of dengue pediatric patients.

Biochemistry	n	Mean (SD)	Reference values	Above (%)[Φ]	Below (%)[Φ]
Glucose (mg/dL)	130	96.9 (18.0)	74-110	26.2	4.6
Urea (mg/dL)	166	25.0 (6.6)	8.0-50.0	0.0	0.0
Creatinine (mg/dL)	164	0.86 (0.19)	0.70-1.20	3.7	21.3
Uric acid (mg/dL)	11	3.8 (0.80)	2.6--8.7	0.0	9,1
Total proteins (g/L)	7	74.1 (3.9)	66-83	0.0	0.0
Albumin (g/L)	14	43.0 (2.9)	35-48	0.0	0.0
Alcaline phosphatasis (U/L)	23	165.9 (98.0)	30-120	56.5	0.0
Gama glutamyltranspetidase (U/L)	41	21.9 (24.0)	7.0-50.0	4.9	0.0
Alanine aminotransferase (U/L)	221	58.8 (95.8)	<50.0	24.4	n.a.
Aspartate aminotransferase (U/L)	221	82.3 (97.7)	<50.0	50.2	n.a.
Lactate dehydrogenase (U/L)	72	329.5(206.8)	<246.0	63.9	n.a.
Creatine Kinase (U/L)	124	525.8 (1406.5)	<145	46.0	n.a.
Total bilirrubin (mg/dL)	23	0.49 (0.16)	0.3-1.2	0.0	4.3
Indirect bilirrubin (mg/dL)	23	0.38 (0.12)	<1.10	0.0	n.a.
Direct bilirrubin (mg/dL)	23	0.11 (0.04)	<0.20	4.3	n.a.
Pancreatic amylase (U/L)	3	35.3 (31.1)	<46.0	33.3	n.a.
C- Reactive Protein (mg/dL)	160	6.5 (11.9)	<6.10	28.1	n.a.
Sodium (mEq/L)	178	136.1 (2.4)	136.0-145.0	0.0	38.2
Potassium (mEq/L)	172	4.1 (0.5)	3.5-5.1	2.9	2.9
Chloride (mEq/L)	178	101.6 (2.3)	98.0-111.0	0.0	2.8
Calcium (mEq/L)	4	9.4 (0.2)	8.90-10.30	0.0	0.0
[Φ]Percentage of individuals with values above or below the reference range.					
Pvalue- Paedaitric vs adult Kruskal-Wallis test					

Dengue with warning signs and severe dengue:

When analyzing the hematological, biochemical, and clinical data considering warning signs and severe dengue criteria, according to the WHO dengue classification by severity (WHO 2011, 2012) and the proposed clinical endpoints by Tomashek et al. (2018) and Huits et al. (2023), the frequencies of patients presenting suggestive dengue with warning signs and severe dengue frequencies were determined for the overall adult population, by sex, and for children. The results are shown in Table 9.

As to the laboratory parameters, high hematocrit, (considered an increase of more than 10% of the upper reference limit (WHO, 2011), was found in 0.4% of adults (only females) and in 3.4% of children. Thrombocytopenia with platelet count below 100 x 10^3/µL (considered by the WHO as a warning sign for plasma leakage (WHO 2009)) was recognized in 29.3% of adults (33.5% of males and 24.6% of females) and 21.1% of children. Of these, 2.8% (n=24) adults and 2.5% (n=6) children presented platelet concentrations below 50 x 10^3/µL (not shown). Hematocrit increase combined with

thrombocytopenia was found in 2 adult females and 4 children. Additionally, levels of aminotransferases greater than 10 times the upper reference limits were identified in more than 2% of adults, slightly less frequent in males (ALT 0.0%, AST 0.6%) than in females (ALT 3.5%, AST 2.9%) and children (ALT 2.6%, AST 48.7%). Among the whole studied patients, only 5 had clinical findings of severe dengue, among which 1 female was diagnosed with ascites, and 4 males with pleural effusion.

Table 9. Frequencies of DF patients with laboratory and/or clinical findings suggestive of dengue with warning signs and severe dengue, in adults and children.

Warning Signs of dengue fever*				
	Overall Adults (n=845)	**Males (n=310)**	**Females (n=585)**	**Children (n=232)**
	% (n)	**% (n)**	**% (n)**	**% (n)**
High haematocrit (HHCT)	0.4 (3)	0.0 (0)	0.5 (3)	3.4 (8)
Thrombocytopenia (TCP)	29.3 (248)	33.5 (104)	24.6 (144)	21.1 (49)
HHCT*TCP	0.2 (2)	0.0 (0)	0.3 (2)	1.7 (4)
High ALT	2.1 (18)	0.0 (0)	3.5 (18)	3.0 (7)
High AST	2.2 (19)	0.6 (2)	2.9 (17)	2.6 (6)
Signs compatible with severe dengue*				
	Overall Adults (n=845)	**Males (n=310)**	**Females (n=585)**	**Children (n=232)**
	% (n)	**% (n)**	**% (n)**	**% (n)**
Ascites	0.2 (1)	0.0 (0)	0.3 (1)	0.0 (0)
Pleural Effusion	0.6 (4)	1.6 (4)	0 (0.0)	0.0 (0)

High haematocrit (>10% increase in hematocrit); Trombocytopenia (platelets below $100x10^3/\mu L$).
High ALT and/or AST (greater than 10 times the upper limit of normal); *WHO classification.

Discussion

We hereby characterized the clinical and laboratory findings of probable and confirmed DF patients with a primary DENV-1 infection, determined by the healthcare services in Madeira Island, during its only dengue outbreak, in 2012-2013. The literature focuses essentially on secondary infections in dengue-endemic regions, where the co-circulation of various DENV serotypes is common, and thus these data offer the opportunity to deepen our understanding of the clinical and laboratory parameters in a primary infection, the influence of gender and age in these parameters, and which may be better predictors of dengue severity.

The most frequent clinical signs and symptoms in more than three-quarters of the complete DF dataset (n=1369) enrolled in this part of the study were fever, myalgia, and headache. Arthralgia, retro-orbital pain, and rash were frequent in 35% to 41 of the patients and palmar erythema in around 5% of the patients. These results show the systemic involvement of the disease, probably due to endothelial cell activation and activation of inflammatory cytokines' cascades (Imad et al. 2020; Leong et al. 2007; Simmons et al. 2012). The most common symptoms in our study were similar to those presented in other studies (Barzon et al. 2021; Effler et al. 2005; Lin et al. 2019; da Silva et al. 2018; Succo et al. 2016), although not always with similar frequencies (Mallhi et al. 2015). Nevertheless, studies varied considerably in their sample sizes, and viral serotypes, and most cases were secondary infections, with multiple viral serotypes. The age-related variance between adult and pediatric populations was of notice, with arthralgia, and myalgia being significantly higher in the adult population, while fever and headache were significantly more frequent in children. Hemorrhagic signs and rash tended to be also more frequent in children, but not significantly different than in adults. In-hospital management was needed for 3.9% of the adult population and 3.4% of the pediatric population. This last proportion was lower than that of the studies of Capeding et al. (2010) and Endy et al. (2002) in infants, although this last concerned primary and secondary dengue infections. Sex-linked differences in clinical manifestations were also observed in adults, with significantly higher presentations of headache, rash, and hypotension in the female gender, and a higher incidence of pleural effusion in males. Differences between adults and children in both clinical and laboratory findings have also been described in other studies (Kittigul et al. 2007; Namvongsa et al. 2013; Wang et al. 2009), and epidemiologic studies have previously identified young age, female sex, virus strain, and secondary infection as risk factors for severe dengue (Anantapreecha et al. 2005; Endy et al. 2004; Halstead 2003; 2007; Sangkaew et al. 2021; Simmons et al. 2012; Wilder-Smith et al. 2019).

Concerning the hematological parameters in our study, leukopenia was observed in more than two-thirds of adults of both sexes and children. This is usually the earliest abnormality in blood count in dengue infection (WHO 2009), but these values were

much higher than those observed in other studies of primary infections (Giri et al. 2010; Lin et al. 2016), and even above the findings of (Avila-Aguero et al. 2004). Only 29.3% of our dengue patients had thrombocytopenia with platelet below 100 x 10^3/µL, this frequency being much lower than that of Giri et al. (2010) (100%) and (Avila-Aguero et al. (2004) (42.5%). Despite that, 72.1% of adult males and 64.8% females, and 53.0% of children, had platelets below the reference limits for our population, these frequencies being in accordance with those observed by Bhattarai et al. 2023, Ferede et al. (2018), Ho et al. (2013) and Lin et al. (2016). Platelet decrease seems to be one of the major causes of bleeding in dengue patients, likely due to their increased destruction via activation of the C3 complement factor or other unclear mechanisms, or to their lower production in the bone marrow (Michels et al. 2014; Ojha et al. 2017).

In our complete DF dataset, hematocrit ranged from 25.5 to 55.0%. This parameter has been used as an important parameter for monitoring patients with DF, constituting a warning sign of plasma leakage and probable progression to severe disease. Rising hematocrit together with rapid and progressive decreases in platelet counts may be the earliest signs of plasma leakage, which are usually preceded by leukopenia (WHO 2009). Avila-Aguero et al. (2004) found 51% DF patients with increased hematocrit in a primo-infection in Costa Rica. Oppositely, other studies of primary dengue infections did not show significant hematocrit changes (Bhattarai et al. 2023). In our study, no significant increase in hematocrit was observed, since only 0.5% and 2.7% of males and females respectively, presented with a hematocrit above the reference values for the population. Nevertheless, we cannot exclude a possible significant individual increase for some of the patients. Contrarily to adults, high hematocrit was observed in 17.2 % of pediatric patients (age ≤18), a slightly higher frequency when compared to that observed by Cardenas et al. (2021) in patients aged 7-15 years (12.5%). Also, Bodinayake et al. (2018), saw that children with dengue were more likely to have increased hematocrit when compared to other acute fever infections. Furthermore, an increase in erythrocytes' counting and hemoglobin was found in 20.7% and 16.4%, respectively, of our pediatric patients, which showed some hemoconcentration and possible plasma leakage. Lastly, in what concerns hemostasis markers, and likewise what was found by Avila-Aguero et al. (2004), prothrombin time

was elevated in DF adults from our study, especially in men, but also in DF children, although in this last group, it was only determined in a low number of patients (n=14).

As to the analyzed biochemical parameters, mean plasma glucose was increased in around 30% of the adult DF patients, both males and females, and creatinine plasma concentrations were above the reference limits, mostly in men (28.5%). On the other side, total proteins were decreased in almost a quarter of DF females. Additionally, low albumin levels were found in half of the males and only in 8.3% of females, concordant with previous studies (Cardenas-Perea et al. 2020; Bhagyanath and Jacob 2020; Kularatnam et al. 2019), and it was significantly lower in adults than in children of our dataset. Proteinuria and hypoalbuminemia are typically observed in dengue infection, due to protein loss, which is related to the changes in filtration characteristics of the glycocalyx (Simmons et al. 2012). However, these parameters were determined in a low number of patients and should thus be interpreted with caution.

Liver involvement is relatively common in dengue patients, with higher levels of hepatic enzymes being observed in severe disease (Huits et al. 2023; Leowattana and Leowattana 2021; Tomashek et al. 2018). AST is expressed in the liver but also in the heart, skeletal muscle, brain, and kidneys, while ALT is a better indicator of liver involvement because of its greater specificity (Tomashek et al. 2018). In our study, aminotransferases, ALT and AST, were moderately to severely increased in a large proportion (>57%) of DF patients, mostly females, whose mean values were more than two-fold the reference limits. Lin et al. (2019) also found elevated ALT and AST, but these occurred in 29.9% and 50.6% of DF patients, respectively. Additionally, elevated aminotransferases were also found in other primary DENV infection studies, like in Cardenas-Perea et al. (2020) denoting liver inflammation, which, in turn, has been positively associated with dengue severity. In our study, at least eighteen adult females and seven children had ALT greater than 10 times the upper limit of the reference range, compatible with acute hepatitis. These results are in accordance with other studies (Trung et al. 2010) and seem to correspond to an adverse effect of the host immune

response, likely proportional to the initial viral burden (Effler et al. 2005). The severe increase of both aminotransferases (>2000 U/L) in one female patient of 41 years, showed a more critical liver involvement. Besides, she had a highly increased LDH (>2000 U/L), leukopenia (1.9 x 10 / L), and lymphocytopenia (0.6 x 10 / L). In the pediatric population, ALT was above the reference limit in about a quarter of the patients, while AST showed an even larger increase, with 50.2% of these patients showing values above the upper limit. These results agree with the findings of Poovorawan et al. (2006), which suggest DF as an important cause of acute liver failure in children. In adult patients, the liver involvement was confirmed by moderately increased levels of GGT (47.2% M; 31.9 % F), and total and direct bilirubin (12.5 and 28.1%, respectively), with males displaying the highest proportion of patients within all the cases. Oppositely, only a minority of DF children (4.9%) displayed GGT values above the normal limit.

LDH and CK have been also positively associated with severe dengue (Yuan et al. 2022) and were clearly above the reference intervals in our studied population. LDH was increased in more than 60% of adults and children. This enzyme is widely distributed in several host tissues and probably reflects liver and muscle involvement since myalgia is one of the major symptoms of dengue. Around 50% of males and children and 37.6% of females had increased CK levels. These results may also be related to muscle affection and to cardiac involvement (Miranda et al. 2013), including myocarditis (Tomashek et al. 2018).

Concentrations of C-Reactive Protein, a marker of inflammation, were exceeded by more than twice the reference limits in adults, in males (57.1%) than in females (48.0%), and in 28.1% of children.

Additionally, children and adults with DF of both sexes were found to have hyponatremia (low sodium levels) in roughly 40% of cases. In fact, individuals with DF, particularly children, have been observed to have kidney dysfunction along with electrolyte and acid-base problems (Lumpaopong et al. 2010; Vachvanichsanong and McNeil 2015)

The presence of comorbidities like diabetes, dyslipidemia, obesity, respiratory, renal, cardiac, autoimmune, and hematological disease, immunodeficiency, and

hypertension, were not significant in predicting the severity of dengue symptoms, contrarily to what was described by Badawi et al. (2018). However, Al Awaidy et al. (2022), found that hypertension, and ischemic heart disease, did not increase the risk of severity although this might be related to the close clinical monitoring and early hospitalization of patients with comorbidities.

Warning signs of dengue were manifested in some of our patients, although in a quite low number. High hematocrit, the main indicator of plasma leakage, was found in a reduced percentage of adult females (0.4%), but in a higher proportion of children (3.4%). Thrombocytopenia, with platelets below 100 x 10^3/µL, was recognized in about one-third of adults and 21.1% of children. Remarkably, 2.8% of adults and 2.5% of children presented severe thrombocytopenia with platelet below 50 x 10^3/µL, with a high risk of plasma leakage and progression to severe disease. DF patients presenting a simultaneous increase of hematocrit and thrombocytopenia with platelets below 100 x 10^3/µL, a warning sign of plasma leakage according to WHO, were found in our sample set (2 adult females and 4 children). Moreover, moderate liver disease suggested by levels of aminotransferases greater than 10 times the upper reference limits, was identified in more than 2% of adults and children, revealing acute hepatitis in these patients. The analyses of our complete dataset are suggestive of severe dengue in 5 patients who, besides the altered laboratory findings, had ascites and pleural effusion. Several studies have been published reporting similar findings concerning the clinical spectrum of DENV infections (Barzon et al. 2021; Effler et al. 2005; Lin et al. 2016; Nunes-Araújo, Ferreira, and Nishioka 2003; da Silva et al. 2018; Succo et al. 2016). Although most studies varied considerably in what concerns the sample sizes and infecting viral serotypes, these usually focus on endemic regions, where most cases refer to secondary infections with different circulating viral serotypes. While secondary infection is considered to be the main risk for DHF, viral and host factors are also important (Guzmán and Kouri 2002; Ren et al. 2022; Srikiatkhachorn 2009) and may be determinants of a more severe outcome, even in primary infections (Soo et al. 2016), although the underlying mechanisms are less understood. Indeed, a greater percentage of severe dengue associated with DENV-1 primary infections was found in some studies. In fact,

Anantapreecha et al. (2005) and Yung et al. (2015) found that nearly 20% of DHF/DSS occurred in primary infections by DENV-1. Nevertheless, as referred by Soo et al. (2016), we should bear in mind that different WHO dengue classifications may affect the prediction of severity.

In sum, our study characterized the clinical and laboratory findings of a primary autochthonous DENV-1 infection in Madeira Island, Portugal, in a considerable number of patients, stratified by age and gender. The most common symptoms across all ages and genders included fever, headache, myalgia, arthralgia, retro-orbital pain, and rash. According to laboratory findings, the most prominent hematological abnormalities were moderate thrombocytopenia, leukopenia with lymphocytopenia and neutropenia, in almost two-thirds of DF adults and half of the children. Additionally, elevated hepatic enzymes in more than half of DF patients indicated liver involvement and significant elevations in CK and LDH suggested rhabdomyolysis. All these laboratory parameters are positively correlated with DF severity.

These results may be of utmost importance to better planning of future dengue-related sanitary emergencies in this and other regions at risk of dengue spread, which are predicted to further increase in Europe due to the population dynamics and climate changes.

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CHAPTER VI

General Discussion

General Discussion

Dengue displays a wide spectrum of clinical manifestations, ranging from a mild undifferentiated fever to severe dengue that can ultimately lead to death (Gubler 2002). Nonetheless, most DENV infections are asymptomatic (Endy et al. 2002; 2011; Grange et al. 2014; ten Bosch et al. 2018). Several studies have proposed different mechanisms to be responsible for the different clinical outcome of DF (Aguas et al. 2019; Bravo, Guzmán, and Kouri 1987; Endy et al. 2004; Fried et al. 2010; Guzmán et al. 2000; Halstead et al. 2002; Halstead and O'Rourke 1977; Hause et al. 2016; Kliks et al. 1989; Lee et al. 2020; Pang et al. 2017; Sangkawibha et al. 1984; Singh et al. 2022; Vicente et al. 2016), but few studies have focused on the factors influencing the asymptomatic outcome of DENV infections (Endy et al. 2002; Grange et al. 2014; Katzelnick et al. 2016; Montoya et al. 2013; OhAinle et al. 2011). Additionally, these studies were undertaken in endemic/hyperendemic regions, where the population has a higher risk of having been previously exposed to DENV, introducing confounding factors to the serological status of these populations. As such, one of the challenges of studying asymptomatic infections in these regions, consists in determining whether the absence of symptoms results from host intrinsic characteristics or is due to immunity acquired in a previous exposure to DENV, which require differentiation between primary and secondary infections in these regions. This can be achieved by several serological methods, which can nevertheless be prone to cross-reactivity, need to take in consideration antibody kinetics, and are best suited to be used during the acute phase of the disease (Nguyen et al. 2018). Consequently, the study of a population that has only experienced a single DENV outbreak allows to remove these confounding factors and provides a unique opportunity to study the epidemiology of DF and the protective host factors in primary DENV infections. In that sense, the population of Madeira Island is a prime candidate for studies of this nature, since it was exposed to a single DENV-1 outbreak, without further autochthonous cases being reported since 2012/2013.

Humoral immune response to DENV-1 infection in the population of Madeira Island

It is widely accepted that infection with any DENV serotype elicits long-lasting immunity against the same serotype but short-lived heterotypic immunity (Gubler 2002). In our study, the serological status of the population of Madeira Island was investigated eight years after the DENV-1 outbreak of 2012/13. The results herein reported show extremely low or absent antibody levels in confirmed asymptomatic participants (blood donors with PCR-confirmed DENV infection but without symptoms at the time of the outbreak), contrasting with high levels of antibodies in symptomatic patients. Only 3.2% of the confirmed asymptomatic participants had detectable IgG-specific antibodies, detected by indirect ELISA. These results suggest that asymptomatic patients either had no seroconversion or had a very low antibody response, and/or a rapid decay over time.

According to the ECDC (2014), a fraction (n=31) of the 42 PCR-confirmed asymptomatic blood donors were tested for seroconversion using IgM and IgG capture ELISA kits, at two distinct time points following viral detection. However, seroconversion was only detected in 6.3% (n=2) of these PCR-positive asymptomatic cases. It should be stressed that, Capture ELISA kits have lower sensibility and higher cutoff values than indirect IgG ELISAs (Domingo et al. 2015), usually correlating to a HAI titer of 1:1280 (Cordeiro et al. 2009; Mishra et al. 2018) and are thus unable to detect low levels of antibodies. Also, IgM capture ELISAs have been described as only being able to detect titers higher than 1:3200 (Domingo et al. 2015). These tests are therefore designed to detect high antibody titers and are used to identify secondary infections, which are characterized by an increase in IgG antibody titers. Since these same kits were used to detect seroconversion in symptomatic cases during the outbreak (ECDC 2014), we suspect that asymptomatic cases might have had seroconversion resulting in low antibody titers and therefore undetectable by capture ELISA, contrarily to symptomatic patients. Rapid antibody decay following asymptomatic infection might also explain the low seroprevalence found in our asymptomatic participants. A previous study by Luo et al. (2018) observed a rapid antibody decline three years after DENV infection, with only 27% of the asymptomatic participants remaining seropositive at a serum dilution of 1:100. Interestingly, eight years after the outbreak, we found a seroprevalence of 4.1%

in probable asymptomatic participants, recruited among co-inhabitants or co-workers of symptomatic cases from the parishes of Funchal most affected by the outbreak. Although we were not able to confirm their positivity during the outbreak, this rate is similar to that of our confirmed asymptomatic participants (3.2%), suggesting that many of these were, in fact, truly asymptomatic, and hinting at high transmission rates and a high prevalence of asymptomatic infections in these parishes.

Considering that asymptomatic infections might not have led to seroconversion, it is possible that the prevalence of asymptomatic DENV infections in Madeira Island was higher than that reported by a previous study. This stems from the fact that many asymptomatic cohorts are usually established by IgM seropositivity in individuals that do not seek for medical assistance, rather than by detection of viraemia (Sun et al. 2012; Tsai et al. 2018). A previous study focusing on the seroprevalence of anti-DENV IgG antibodies in the population of Madeira, performed four years after the onset of the DENV-1 outbreak found a seroprevalence of 8.7% (Auerswald et al. 2019). Nevertheless, the above-mentioned study did not provide information regarding whether the participants presented or not dengue symptoms during the outbreak, making it impossible to distinguish between participants with diagnosed DF (laboratory confirmed or not) from asymptomatic infections. Also, these authors found that several of the seropositive samples tested positive in IgG capture ELISA, suggesting that these cases may represent secondary infections eventually due to unreported DENV circulation in Madeira. However, in our opinion, considering that one third of the seropositive cases had travel history to DENV endemic regions, the origin of those secondary infections is difficult to determine. Another study by De Santis et al (2023), in the island of Reunion, reported a low prevalence of asymptomatic cases (2.62%) prompting them to suggest that asymptomatic cases are less frequent in studies of adult populations, since most studies reporting higher proportions of asymptomatic cases are mostly focused on pediatric cohorts (De Santis et al. 2023). Interestingly, their observation of low seroprevalence in possible asymptomatic cases is in concordance with our results. However, De Santis et al (2023) study focuses on a population with a distinct epidemiological setting, with more than one DENV serotypes in circulation, continuous DENV circulation between 2017 and 2021 (De Santis et al. 2023) and having had a

previous chikungunya outbreak between 2005 and 2006 (Borgherini et al 2007). Additionally, in their study, De Santis et al. (2023) used IgM and IgG capture ELISA to identify asymptomatic cases, which are unable to detect low levels of anti-DENV antibodies. Taking this in consideration, it is likely that the prevalence of asymptomatic infections in their study may be higher than what was estimated.

DENV infection is believed to elicit long-term serotype-specific protective immunity, yet it is unclear whether this protection results from sterile immunity or from other mechanisms protecting against clinical disease (Webster, Farrar, and Rowland-Jones 2009). Several studies have reported cases of homotypic reinfection 1 to 2 years after a previous symptomatic infection, suggesting that either, natural DENV infections might not always result in sterile immunity, the protection may be short-lived in some individuals (Waggoner et al. 2016), or the induced protection does not cover all genotypes of the same serotype. For instance, cases of possible reinfection by DENV-1 (Sirivichayakul et al. 2014) or DENV-2 (Endy et al. 2004; Gibbons et al. 2007; Sharp et al. 2014) have been reported. However, these studies relied on serologic evidence and information about the circulating serotypes, but were not able to unequivocally confirm, by molecular methods, the serotype of the first infection. Waggoner et al. (2016) reported that up to 13.8% of symptomatic reinfections in a Nicaraguan pediatric cohort were caused by homotypic DENV, with viremia confirmed by RT-PCR assays. Furthermore, anti-DENV antibody titers and neutralizing antibody titers evaluated annually in healthy individuals and suspected dengue cases, suggest that DENV infections for certain patients, may either result in a short-lived or in an indetectable humoral response, being the infection successfully controlled by cellular or innate immune responses (Waggoner et al. 2016). The latter may also apply to asymptomatic infections, shown to have low antibody response. If so, the absence of long-term antibody response found in the asymptomatic participants of our study does not necessarily mean that they are not protected against a subsequent homologous infection. Simon-Lorière et al. (2017) described an association between primary and secondary asymptomatic outcome and a higher percentage of activated T cells, compared to secondary symptomatic infections. As such, the outcome of a subsequent

DENV infection might be influenced by the development of DENV-specific memory T cells (Jeewandara et al. 2015).

DENV symptomatic infections are believed to result in higher IgG antibody titers than asymptomatic infections, which in part may be related to their higher viremia observed at the peak of the infection (Luo et al. 2018). In fact, symptomatic participants from our study, showed high seroprevalence eight years after their primary infection, with 93.3% having detectable anti-DENV IgG antibodies, with highly variable titers. Of these, 10.9% had high antibody titers (>1:1280), 68.9% had intermediate titers (>1:160 to <1:1280) and 20.2% had low antibody titers (<1:160)). The DENV outbreak lasted for 23 weeks and was mostly restricted to three main clusters in parishes of Funchal (Santa Luzia, Santa Maria Maior, and São Martinho), coinciding with the areas with higher mosquito densities. It is possible that higher antibody titers in some of the symptomatic participants resulted from a boost of the B cell response, due to a possible re-exposure to the virus during the outbreak, but without further clinical manifestations. In fact, 72.2% the participants with antibody levels higher than 120 RUs, reported living or working in the three cluster areas of Funchal with higher DF incidence, while only 46.4% of those with antibody levels lower than 120 RUs resided or worked in these same areas. Moreover, most (86%) of the participants with the highest antibody levels had dengue in the first 8 weeks of the outbreak and thus had longer periods until the end of the outbreak where a re-infection may had occurred. Nevertheless, we cannot exclude the possibility that higher antibody titers could also be due to secondary infections in participants with travel history to DENV endemic regions. When considering this aspect, in our cohort, only 7.7% of the participants with high antibody titers had travel history to DENV endemic countries, compared to 22.7% and 28.7% of those with low and medium antibody titers, respectively. Furthermore, no statistically significant differences were found for the distribution of antibody titers according to travel history ($p=0.615$), indicating that, even if there were secondary infections, these do not seem to affect overall antibody titers in the sampled population. Also, it should be noted that travel history by itself does not mean these individuals were exposed to DENV, since they might have traveled to endemic countries during time periods, or to regions, with no circulating virus. Lastly, we should consider that high antibody titers eight years after

the outbreak, could also reflect a boost of the immune response of these participants due to a later unreported DENV exposure in Madeira. However, this is highly unlikely, since no other autochthonous DENV transmission has been described in Madeira besides the 2012/2013 outbreak.

Genetic factors influencing host immune response can account for the broad range of antibody levels found in symptomatic participants. Scepanovic et al. (2018) investigated the role of host genetic factors on the humoral immune response to several viral infections and found that, in humans, variations on HLA class II molecules are critical in determining these responses. The authors suggest that small changes in the capacity of HLA II molecules to bind to specific viral peptides can have a measurable impact on downstream antibody production (Scepanovic et al. 2018). Also, genetic polymorphisms affecting IL-10 expression have been associated with an increased risk of severe DF (Perez et al. 2010). This anti-inflammatory cytokine is thought to promote humoral immune responses, by enhancing class II expression on B cells and inducing immunoglobulin production (reviewed in Saxena et al. 2015). Hence, changes in IL-10 expression might affect the humoral response against DENV infection, as seen in systemic lupus erythematosus, in which high IL-10 production enhances B cell proliferation, differentiation, and autoantibody production, contributing to disease pathogenesis (Llorente et al. 1995; Rousset et al. 1992).

Individual differences in antibody decay might also contribute to the wide range of antibody titers found in the symptomatic cohort. Although specific anti-DENV IgG antibodies can be detectable in serum from individuals over 60 years after infection (Ngwe Tun et al. 2016), several studies have pointed out an over-time seroprevalence decrease after symptomatic infections, likely due to the antibody decay (Luo et al. 2018; Wu et al. 2020). As anticipated, due to the longer period after the infection at which our study was performed, the RU values observed in our symptomatic participants were lower than those reported by Wu et al. (2020) in a Chinese population, five years after DENV-1 infection. Interestingly, a similar gender-bias in both studies favored females among participants with high antibody titer, suggesting a faster antibody decay in males. In fact, in Wu et al. (2020) the difference between males and females in the mean antibody concentrations was 5 RUs two years post-infection (males mean RU = 162 ± 34

vs. females mean RU = 167 ± 30), increasing to 10 RUs after five years (males mean RU = 114 ± 61 vs. females mean RU = 124 ± 35), while in our study it was of 20 RU, eight years after the infection (males mean RU = 65±44 vs. females mean RU = 85±38). These results suggest that antibody decay is more accentuated in males.

Considering the variable serological status within our sample, we hypothesized that symptomatic individuals with different anti-DENV IgG antibody titers might have experienced different symptoms. To test for this possible, association symptomatic participants were grouped according to the presence/absence of the reported signs/symptoms and mean RUs were compared among groups. No significant differences were found for most groups of signs/symptoms, except for hemorrhagic signs, whose participants reporting hemorrhagic signs had significantly higher antibody titers than participants without these signs (Mann-Whitney U=1260, p=0.019). Hemorrhagic signs are thought to result from the excessive production of inflammatory cytokines and chemokines which contribute to the disruption of the endothelium and to increased vascular permeability (Raza et al. 2020).

Protecting antibodies against heterotypic infections are often suggested to be short-lived, lasting from several months to up to three years (Anderson et al. 2014; Katzelnick et al. 2016; Montoya et al. 2013; Sabin 1952). Several authors, however, propose that heterotypic neutralization can persist long-term, possibly more than one decade, being dependent on the serotype of the post-primary infection (Guzman and Kouri 2008; Kochel et al. 2002). The results obtained in the present study indicate that, eight years after a primary DENV infection, seropositive individuals retain antibodies with strong neutralizing ability against the two tested DENV-1 isolates, from Madeira (genotype V) and from Ghana (genotype I), even for those sera with low antibody titers (<1/160). Also, sera from participants with medium and high antibody titers are still capable of heterologous neutralization of DENV-2 (Cosmopolitan genotype). This is suggestive of a possible longer-lasting heterotypic protection. Conversely, Auerswald et al. (2019) found that, after four years, sera of most participants were not able to neutralize heterologous serotypes, including the DENV-2 of the Asian genotype. This difference between studies probably results from the genotype used in the neutralization tests, since it is known that serum of DENV-1 infected individuals is less

likely to neutralize the Asian genotype of DENV-2 than the cosmopolitan genotype (Rodrigo et al. 2009). In our work, participants with low titers represented around 20% of the studies samples and demonstrated a markedly lower cross-neutralization activity against DENV-2. This suggests that even though protected against homotypic infections, these participants, are likely not protected against heterotypic infections, and may even be at risk of enhanced disease severity, due to ADE. However, it is noteworthy that disease severity might not be entirely dependent on the amount of pre-existing neutralizing antibodies, but also on the amount of enhancing antibodies (Vo et al. 2022).

Outcome of DENV-1 infection in the symptomatic population of Madeira Island.

Dengue results in a wide spectrum of clinical manifestations that can change throughout the course of the infection, with some symptoms occurring early in the febrile phase, when most of the patients search for medical assistance, while others occur more commonly after defervescence (WHO 1997). The study herein reported, aimed at furthering the knowledge about the clinical outcome of the primary DENV-1 infection in Madeira Island. An anonymous online questionnaire was applied to symptomatic DF patients with clinical diagnosis of dengue during the 2012/2013 outbreak, allowing to retrospectively characterize the clinical manifestations experienced during the entire infection period, determine the general health status of patients before and after dengue, and thus identify possible long-term effects.

The most frequent symptoms reported by the respondents to our questionnaire were fever, myalgia, extreme tiredness, and headache, followed by vomiting, pruritus, nausea, retro-orbital pain, and arthralgia. The overall picture of clinical manifestations is in accordance with that described by several studies in different regions of the world (Bonifay et al. 2019; da Silva et al. 2018; Dettogni et al. 2015; Effler et al. 2005; Hasan et al. 2021; Jain et al. 2017; Lim et al. 2020; Martelli et al. 2011; Seet, Quek, and Lim 2007; Teixeira, Nogueira, and Nascentes 2017; Teixeira et al. 2010; Tiga-Loza et al. 2020; Tristão-Sá et al. 2012). Interestingly, our participants reported a similar or slightly lower frequency of hemorrhagic manifestations than in other studies (Effler et al. 2005; Martelli et al. 2011; Tiga-Loza et al. 2020). However, the type of hemorrhagic manifestation differs between studies, with petechiae and hematuria being more

common in our study, and gum bleeding and epistaxis being less common, when compared to other studies (da Silva et al. 2018; Effler et al. 2005; Jain et al. 2017; Tiga-Loza et al. 2020). Also, gastrointestinal symptoms were less frequently reported by our participants than in previous studies (da Silva et al. 2018; Dettogni et al. 2015; Hasan et al. 2021; Jain et al. 2017; Martelli et al. 2011; Seet, Quek, and Lim 2007; Teixeira, Nogueira, and Nascentes 2017; Tristão-Sá et al. 2012). Overall, the reported clinical picture is consistent with the expected milder outcome of a primary exposure to DENV. Nonetheless, the results herein presented and those of Effler et al. (2005) suggest that hemorrhagic manifestations might be more common in primary infections than previously thought.

During the 8-year period after dengue, the persistence of at least one clinical manifestation was considerably high (61.5%) in our sample set. These participants reported that the most frequent persistent manifestations, defined as previously inexistent or rare manifestations whose recurrence several times per year happened after dengue, were headache, followed by abundant hair loss, extreme tiredness, arthralgia, and myalgia. The long-term recurrence of clinical manifestations after dengue has been formerly reported by several studies (Dettogni et al. 2015; García et al. 2011; Seet, Quek, and Lim 2007; Teixeira, Nogueira, and Nascentes 2017; Teixeira et al. 2010; Tiga-Loza et al. 2020; Tristão-Sá et al. 2012), with varying prevalence ranging from 9 to 57%, and lasting from 1 month to 2 years. Even though the persistent clinical manifestations reported in our study are similar to those previously reported, other studies usually report much lower frequencies and resolution of persistent symptoms within few months after dengue. Only García et al. (2011) described symptoms lasting for as long as 2 years, in a Cuban cohort. Differences between studies can be due to differences in study design, including age and sex distribution, forms of assessment, and time of follow-up, but also due to differences in virulence and tissue tropism between DENV serotypes and host genetic susceptibility and health and immunological status.

Interestingly, García et al. (2011) observed that patients presenting long-term symptoms after dengue had higher anti-DENV IgG titers. Following analysis of their antibody titers, combined with the detection of autoimmune markers such as C reactive-protein, immune complex, and antinuclear-antibodies, and considering the type of long-

term symptoms exhibited by these participants, the authors suggest that an autoimmune phenomenon might be responsible for the persistence of clinical manifestations after dengue. Given the anonymous on-line nature of our questionnaire, antibody titers were not determined in our respondents. In this sense, it would have been interesting to inquire the participants of our serologic study about persistent symptoms after dengue and compare with their antibody levels. Although no direct link can be established, it is interesting to note that many of the reported long-term symptoms are consistent with autoimmune disorders and 4 of the respondents were diagnosed with autoimmune diseases after dengue. The onset of autoimmune disorders associated to dengue have been previously reported (Jardim et al. 2012; Rajadhyaksha and Mehra 2012), suggesting that dengue may cause abnormal immune responses that lead to autoimmune disorders (Wan et al. 2013).

Even though no severe cases of dengue were reported in Madeira Island, we found that hemorrhagic manifestations during the infection, reported by the participants in the serological study, were more frequently associated to higher antibody levels detected 8 years after the infection. Interestingly, several studies have reported that DENV-induced autoantibodies might contribute to thrombocytopenia and plasma leakage (Wan et al. 2013). The cross-reactivity of anti-DENV antibodies with host proteins is likely due to molecular mimicry between the viral proteins and host platelets, endothelial cells, and coagulatory molecules, resulting in platelet dysfunction, endothelial cell apoptosis, coagulation defects, and macrophage activation (Lin et al. 2011; Liu et al. 2011). For instance, the action of both IgM autoantibodies against platelets, endothelial cells, and coagulatory molecules, and of IgG autoantibodies against fibrinogen, plasminogen, and human coagulation factors have been implicated in the pathogenesis of DF (Chungue et al. 1994; Falconar 1997, 2007; Lin et al. 2001; 2003; Oishi et al. 2003; Markoff et al. 1991; Saito et al. 2004). Despite autoantibody titers having been shown to decay during the convalescence phase, lasting only a few months (Lin et al. 2001; 2003), as mentioned above, autoimmunity has also been implicated in the long-term persistence of DENV-related clinical manifestations (García et al. 2011).

Clinical and laboratory characterization of dengue in Madeira Island

The analysis of clinical and laboratory data from probable and confirmed dengue patients, assisted in the SESARAM-EPERAM Healthcare Services during the 2012/13 outbreak, aimed at fully characterizing the clinical burden of a primary DENV infection and understanding if age and gender play a role in its clinical presentation.

Clinical signs and symptoms in the studied population evidenced the typical outcome of primary mild DENV infections with hemorrhagic symptoms being present in around 5% of the studied population. Nevertheless, it is noteworthy that ascites and pleural effusion, usually associated with plasma leakage (WHO 2009), were reported for one and four patients in our cohort, respectively. Several studies have reported similar findings (Barzon et al. 2021; Effler et al. 2005; Lin et al. 2019; Nunes-Araújo, Ferreira, and Nishioka 2003; da Silva et al. 2018; Succo et al. 2016) but most of them focus on endemic/hyperendemic regions, where most cases refer to post-primary infections. The study with closest similarities to ours, in terms of studied population, is that of Effler et al. (2005), who studied a Hawaiian population affected by the first DENV-1 outbreak in over 50 years. The clinical outcome of DF described by the authors was similar to that observed in our study, although with higher frequencies for most symptoms (Effler et al., 2005). Even though, no indicators of plasma leakage (ascites or pleural effusion) were reported by Effler et al. (2005), hemorrhagic manifestations were more frequent than in our study. These differences might be explained by differences related to the infecting genotype, as it is known that different genotypes have the potential to influence disease severity (Anantapreecha et al. 2005; Effler et al. 2005; Yung et al. 2015), or due to intrinsic characteristics of the infected population (Sierra et al. 2017). It is noteworthy, that severe manifestations are also known to occur in primary DENV infections (Soo et al. 2016). Moreover, DENV-1 infections have been associated with a more severe clinical outcome than the other DENV serotypes (Yung et al. 2015), even in primary infections (Anantapreecha et al, 2005), which is concordant with the five patients that presented signs of plasma leakage in our population.

In chapter IV, we reported the clinical outcome of DENV-1 infections in Madeira Island, through the analysis of retrospective self-reported symptoms. Overall, these results are in line with those described in chapter V for the adult population. Fever,

myalgia, and headache are the most frequent symptoms in both datasets, although frequencies do vary. Arthralgia and hemorrhagic signs are more frequently self-reported in our questionnaire-based study than those registered by the assistant clinicians during the outbreak, and we here note the particularly high reported frequency of petechiae. As stated before, we anticipate two possible explanations for this; if, in one hand, self-reported symptoms are more prone to misidentification due to the population's low medical literacy, it is also true that some DF symptoms, and especially hemorrhagic manifestations, appear later in the course of the disease, and can therefore be underrepresented in the dataset.

In chapter IV, we focused on the adult population, however age is implicated in the clinical outcome of DENV infections. As such, in chapter V we compared the signs and symptoms presented by adults and children. We found that arthralgia, and myalgia were significantly higher in the adult population. Conversely, fever and headache were significantly more frequent in children. Hemorrhagic signs and rash tended to be more frequent in children. Although with fewer hemorrhagic manifestations, our results are in accordance with the clinical outcome of non-severe dengue reported in other pediatric cohorts (Khan et al. 2021; Rathod, Ansari, and Singh 2018).

The assessment of hematological parameters such as platelet count, hematocrit, and leukocyte count, as well as biochemical parameters such as aminotransferase levels, are important not only for the diagnosis of DF, but also in determining its severity (Chaloemwong et al. 2018; Souza et al. 2007; Triana, Kurniati, and Wirastari 2020). In chapter V, we also provide a descriptive analysis of these parameters in the symptomatic DF patients during the DENV-1 outbreak of 2012/13. If on one hand, the clinical data here described point to a mild clinical outcome, it must be noted that the evaluation of laboratory parameters showed that a significant proportion of the studied population had these parameters altered and that some could even be at risk of progressing to a more severe outcome. In fact, leukopenia was observed in most of the adults and children (>50%) and more importantly, thrombocytopenia, with platelet count below $100x10^3/\mu L$, was present in 29.3% of the adult patients, and 21.1% of children. Also, considering an increase of the hematocrit of more than 10% as a risk of developing plasma leakage (WHO,2011), we found that 0.4% of adults and 3.4% of children were at

risk of developing plasma leakage. Regarding aminotransferase levels, suggestive of liver involvement (Tomashek et al. 2018; Trung et al. 2010), we found a moderate-to-severe increase, in the levels of AST and ALT, compared to the reference limits, in a large proportion of our primary DF patients (> 57% in adults of both sexes, and > 24% in children). Of interest, over 2% participants had ALT levels 10 times higher than the upper reference limit, and one of them with values suggesting a critical liver involvement. Lastly, we observed 5 patients with signs that are compatible with severe dengue, four males with pleural effusion and one female with ascites. Taking this data in consideration, it becomes clear that even in a primary infection, the clinical outcome of DENV infections is complex and represents a spectrum of severity that ranges from a mild outcome to a severe outcome with many levels of "severity" in between that have different health care needs.

Final Conclusions

The findings of the studies undertaken in the context of the present thesis contribute to increase the knowledge about the 2012/13 DENV-1 outbreak in the naïve population of Madeira Island, in terms of the current serological status of this population and a description of signs and symptoms presented during the entire clinical period of a primary dengue virus infection, and those persisting beyond the convalescence phase becoming recurrent in the years after infection. We would like to emphasize the following conclusions:

i. Primary asymptomatic DENV-1 infections in Madeira resulted in a weak humoral response characterized by very low to undetectable antibody titers eight years post-infection;
ii. Primary symptomatic DENV-1 infections, on the other hand, resulted in high antibody titers, that persisted for 8 years and likely conferring homotypic and heterotypic immunity, as sera from these participants retained high neutralization capacity against DENV-1 and DENV-2;

iii. Antibody levels were higher in females, likely due to differences in antibody decay rates between sexes;

iv. Anti-DENV antibody levels positively corelate to the occurrence of hemorrhagic symptoms, suggesting a possible involvement of the antibody-mediated response in the pathogenesis of dengue;

v. The clinical manifestations of dengue, as retrospectively reported by our participants, fall within the expected milder outcome of primary infections, with systemic and musculoskeletal symptoms being the most common, while gastrointestinal and hemorrhagic symptoms are less frequent. Nonetheless, hemorrhagic manifestations seem to be more frequent than previously thought;

vi. The occurrence of persistent symptoms, following a primary symptomatic dengue infection, might be more frequent than anticipated and may persist for several years, having an impact on the health status and well-being of a considerable proportion of the infected population;

vii. The clinical manifestations presented by the studied population, concerning a primary DENV-1 infection, show that the overall picture of DF outcome is complex. In brief, hematological parameters such as leukopenia, thrombocytopenia, and hematocrit were altered in a large proportion of the studied DF population. Also, the biochemical parameters associated with liver involvement in DF were moderately to severely augmented in both DF adults and children;

viii. The outcome of this primary infection can be considered mild, up to 2% of adult dengue cases with warning signs and a smaller proportion (0.6%) of cases whose hematological and biochemical parameters are suggestive of severe dengue. For last, in our studied population, no association between previous chronic disease and the clinical outcome of DF was found.

Future perspectives

Throughout the project leading to this thesis, the increase of knowledge promoted by the bibliographical research and the development of the work plan, made possible to identify research subject of interest and which could be developed in the future, and which are briefly described below.

It is known that heterotypic secondary infections are associated with a more severe clinical outcome, usually explained by ADE (Halstead & O'Rourke, 1977; Halstead et al., 2002). The population of Madeira, having been exposed to a single DENV serotype, while having developed homotypic immunity might be at higher risk of developing severe disease in a subsequent heterotypic infection. As such, investigating the risk of severe dengue during a secondary infection is of particular interest for the Regional Healthcare authorities. FRNT's are a useful tool to describe the serological status of a population, allowing us to infer about the titer of neutralizing antibodies. The same methodology can be adapted to indirectly determine the enhancement potential of antibodies present in the sera of seropositive individuals. To that end, FRNTs can be performed in cell lines that express FCγR such as, BHK-21 FCγRiiA. Briefly, a comparison of focus forming units between asymptomatic, symptomatic with low-, medium- and high-antibody titers, and seronegative controls, could be used to determine the relative risk of severe disease during a heterotypic secondary infection.

Memory T-cells are also thought to be involved in protection against symptomatic heterotypic DENV infection (Jeewandara et al., 2015), even though some studies suggest that these may contribute to dengue pathogenesis (Sanchez-Vargas et al., 2021). Studying these adaptive responses is of great interest, as they may be useful tools in determining the risk of severe disease during secondary heterotypic infection (Jeewandara et al., 2015). DENV-specific memory T cells have been detected in dengue patients 20 years after a primary infection (Sierra et al., 2002). However, T-cell responses to DENV have also been shown to be very low or undetectable 1 to 3 years post-infection (Sanchez-Vargas et al., 2021). Nonetheless, we foresee that ELISpots assays can be used to determine whether individuals infected by DENV-1 during the Madeira outbreak in 2012/13 still have detectable memory T-cells and for inferring possible differences between primary asymptomatic and symptomatic cases, thus contributing to the

understanding of how differences in the immune response to primary DENV infections can have a potential impact on secondary heterotypic infections.

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Annex I

Summary table of the questions included in the online questionnaire.

Objetivo	Código	Variável	Questão	Escala de medida e gama de valores
1 - Determinação do grau de exposição à dengue	Exp_Vector	Exposição ao vetor	A1. Foi picado por mosquitos entre 2012 e 2013?	Dicotómica
				1 - Sim
				2 - Não
	Freq_Picada	Frequência de picada	A2. Indique com que frequência era picado	Ordinal
				1 - Muito frequente
				2 - Frequentemente
				3 - Ocasionalmente
				4 - Raramente
				9 - Não sabe/Não responde
	Local_Picada	Local de ocorrência de picada	A3. Indique onde se encontrava quando era picado	Nominal
				1 - Em casa
				2 - No local de trabalho
				3 - Em deslocação
				4 - Outro
				9 - Não sabe/ Não se lembra
2 - Caracterização da sintomatologia associada à dengue	Febre	Ocorrência de episódio febril	B1. Indique se teve um episódio febril entre 2012 e 2013	Dicotómica
				1 - Sim
				2 - Não
	Assis_Med	Assistência médica	B2. Procurou assistência médica devido a esse episódio febril?	Dicotómica
				1 - Sim
				2 - Não
	Diagnóstico	Diagnóstico de dengue	B3. Foi diagnosticado(a) com febre da dengue?	Dicotómica
				1 - Sim
				2 - Não
	Sintomas	Sintomas observados	B4. Indique os sintomas que teve	Nominal
				1 - Dores de cabeça
				2 - Dores retro-orbitais
				3 - Dores musculares
				4 - Dores nas articulações
				5 - Cansaço extremo
				6 - Tosse
				7 - Dificuldade respiratória
				8 - Dores abdominais
				9 - Náuseas
				10 - Vómitos
				11 - Diarreia
				12 - Inchaço nos braços ou pernas
				13 - Comichão (Prurido)
				14 - Manchas arroxeadas na pele (Petéquias)
				15 - Sangramento nasal
				16 - Sangramento das gengivas
				17 - Urina avermelhada (Hematúria)
				18 - Palmas das mãos avermelhadas (eritema palmar)
				19 - Outro (indique qual)
	Comorb	Comorbilidades	B5. Indique os problemas de saúde que tinha em 2012/2013	Nominal
				1 - Diabetes
				2 – Colesterol elevado (Hipercolesterolémia)
				3 - Hipertensão
				4 - Doenças cardíacas
				5 - Doenças autoimunes
				6 - Nenhum
				9 - Não sabe/Não responde
3 - Impacto da dengue na qualidade de vida	Sequelas	Ocorrência de sequelas	C1. Indique os problemas de saúde que teve nos últimos 9 anos	Nominal
				1 - Cansaço extremo
				2 - Dores musculares
				3 - Dores nas articulações
				4 - Dores nos ossos
				5 - Dores de cabeça

				6 - Dores retro-orbitais
				7 - Visão desfocada
				8 - Perda de memória
				9 - Palpitações
				10 - Perda de apetite
				11 - Queda de cabelo abundante
				12 - Nenhum destes
	Freq_Sequelas	Frequência das sequelas	C2. Indique a frequência com que ocorrem os problemas assinalados anteriormente	Ordinal
				1 - Muito frequente (várias vezes ao ano)
				2 - Frequentemente (uma vez por ano)
				3 - Ocasionalmente (menos de 9 vezes nos últimos 9 anos)
				4 - Raramente (uma vez nos últimos 9 anos)
				9 - Não sabe/Não responde
	Assis_Med_Seq	Procura de assistência médica devido a sequelas	C3. Procurou assistência médica devido a estes problemas?	Dicotómica
				1 - Sim
				2 - Não
	Diag_Post_Dengue	Doenças diagnosticadas após dengue	C4. Nos últimos 9 anos foi-lhe diagnosticado	1 - Doença Respiratória (ex. Derrame pleural)
				2 - Doença Hepática (ex. Hepatite, Insuficiência hepática)
				3 - Doença Cardíaca (ex. Miocardite)
				4 - Doença Renal
				5 - Doença Autoimune (ex. Diabetes tipo I; Lupus; Artrite reumatoide)
				6 - Nenhuma
				9 - Não sabe/Não responde
4 - Caraterização demográfica da população	Sexo	Sexo	D1. Sexo	Dicotómica
				1 - Masculino
				2 - Feminino
	Data_nascimento	Idade	D2. Data de Nascimento	DD/MM/AAAA
	Nacionalidade	Nacionalidade	D3. Nacionalidade	Pergunta aberta
	Residência	Freguesia de residência	D4. Indique a freguesia onde residia em 2012/2013	Pergunta aberta
	Trabalho	Freguesia de local de trabalho	D5. Indique a freguesia do seu local de trabalho em 2012/2013	Pergunta aberta