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General Introduction, Scope and Summary of the Thesis

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1. General Characteristics of Dengue

Dengue virus (DENV) belongs to the *Flavivirus* genus of the family *Flaviviridae* together with yellow fever virus, West Nile virus and Japanese encephalitis virus[1]. It is transmitted by the bite of the female mosquito of the *Aedes* genus, of which *A. aegypti* is the main vector, followed by *A. albopictus*[2]. There are four different serotypes of DENV called DENV-1 to DENV-4.

Infection by DENV can be asymptomatic or result in a variety of clinical manifestations ranging from comparatively mild dengue fever (DF) to severe disease such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)[3,4].

Dengue viruses (DENVs) are present in the tropical and sub-tropical areas of the world and are endemic in more than 120 countries within Africa, America, Eastern Mediterranean, Asia, Australia, islands within the Indian Ocean, central and south Pacific and the Caribbean[4]. The average number of DF/DHF cases reported by the World Health Organization (WHO) has increased considerably in the past decades and dengue is expanding to new areas[5]. Recently, dengue outbreaks occurred in European countries including France, Croatia and Portugal (Madeira)[6]. Currently, it is estimated that 390 million dengue infections occur annually worldwide, including both symptomatic and asymptomatic cases[7]. Some of the reasons for the expansion of dengue are discussed below.

2. Factors Related to the Increase of Dengue Incidence

2.1. Uncontrolled Urbanization

In recent years, a considerable exodus from rural to urban areas of people looking for better economic conditions has taken place in tropical and sub-tropical countries[8]. As a consequence, an increase in population density in urban areas and the establishment of new, largely uncontrolled, urbanization has occurred[9]. Irregular water supply and the lack of trash collection are some of the principal characteristics of this unplanned urbanization[8,9].

The storage of water in containers and an increased use of non-biodegradable plastics that are left outdoors make appropriate mosquito breeding sites close to domestic areas[10,11]. *A. aegypti* is a domesticated mosquito with a fly range of approximately 100m in urban areas[12]. The mosquito feeds more than once in each gonotrophic cycle[13]. Because *A. aegypti* is very sensitive to movement, it interrupts its feeding several times and tries to take bloodmeals from the same or different individuals until its meal is completed[10]. The high population density is a risk factor for dengue infections due the short flying distance that the mosquito covers in order to take bloodmeals from one or

several individuals[14].

2.2. Spread of the Dengue Viruses

A boost in international tourism contributed to the globalization of the four dengue serotypes[15]. The number of international air passengers has increased 40 times between 1950 and 2011[16]. International movement of dengue-infected individuals is one of the main reasons of the global transmission of the disease[8]. Increased travel from Latin America to the USA and from Asia to Europe represents a major risk for dengue importation according to a geo-spatial model of the transmission of the disease by infected air passengers[17].

2.3. Spread of the Dengue Vector

The introduction of *A. aegypti* to Asia and the Americas probably occurred from Africa, where dengue outbreaks were reported among passengers traveling by ship[18]. Recently, the presence of *A. aegypti* and *A. albopictus* larvae/eggs has been reported in Europe. These early stages of the mosquitos have been found in used car tires and lucky bamboo plants transported by cargo ship from the USA and Asia to Europe[19,20]. Globalization has allowed the spread of the mosquito through international transport.

2.4. Inefficient Vector Control

In 1947, PAHO organized a plan to eradicate the vector of yellow fever virus (*A. aegypti*) from the Americas[21-23]. This plan, vertically established, mainly involved elimination of the mosquito larvae and the monitoring of larvae density[3,24]. In addition, adult mosquitoes were eliminated through outdoor fumigations using insecticide with residual activity such as DDT[3,21]. *A aegypti* was eliminated from 17 countries of South and Central America[25]. However, this campaign gradually deteriorated and was discontinued in the early 1970s without complete mosquito eradication from Cuba, the USA, Venezuela and other Caribbean countries[26].

One of the reasons for the weakening of the campaign was the declaration that the “war against yellow fever” had been won and the consequent re-direction of resources to the control of other diseases[22]. Another reason was the development of resistance to DDT and other organochlorine insecticides by the mosquito[26]. As a consequence, *A. aegypti* re-emerged in several countries producing new dengue epidemics[21,25].

In 1994, PAHO decided to abandon the concept of mosquito eradication. Instead, the limited resources were used to reduce the vector to levels of low importance to the health sector[12]. Within this new plan, strategies of prevention and control were developed in five key areas: 1) active disease surveillance, involving clinical and laboratory-based

dengue surveillance for early detection of epidemics; 2) rapid response in an emergency for efficient mosquito control; 3) contingency plan to hospitalize large numbers of patients with DHF; 4) medical education in clinical diagnosis and management of DHF patients and 5) involvement and integration of the community into mosquito control[27-29]. The impact of this plan on dengue incidence is still under evaluation.

3. Cell Biology and Immunology

3.1. Dengue Virus: Structure and Replication

Dengue virus is an enveloped virus with a positive-sense, single-stranded RNA genome. The RNA spans approximately 10.1 kb which is translated into three structural proteins (capsid C, membrane precursor prM and envelop E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5)[30] (Figure 1). The nucleocapsid is formed by a single copy of the RNA genome complexed to multiple copies of the C protein[31]. The nucleocapsid is surrounded by a lipid bilayer in which 180 M proteins and 180 E proteins are anchored[32,33]. The M protein is formed after the cleavage of the 'pr' portion from the prM protein by a cellular protease (furin) during the process of virus secretion[34]. The glycoprotein E is dimeric and is positioned parallel to the lipid bilayer. Each monomer consists of three domains. Domain I is the central domain that binds to both domains II and III. Domain II is dimeric and is related to virus-cell fusion activity. Domain III, has binding sites to cellular receptors and Ig antibodies (immunoglobulin)[30,35].

The non-structural NS1 glycoprotein can be found inside the cell, on the cell surface or can be secreted outside the infected cell[36]. NS1 has immunological importance and circulates in the blood from the first day of fever onset until day 9 when usually the disease is over[37,38]. Therefore, NS1 antigen has been used for the early detection of dengue infection[39].

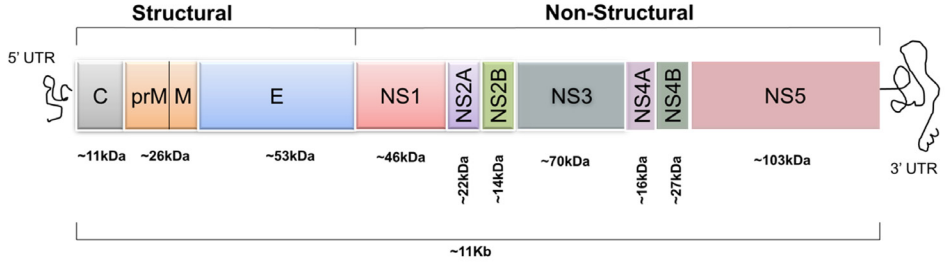

Figure 1. Representation of dengue virus genome. The single-strand of RNA encode three structural proteins, the capsid protein (C), the pre-membrane (prM) and membrane (M) protein and the envelop protein (E), and seven non-structural proteins mainly involved in viral replication. Adapted from Guzman *et al.*, 2010[5].

The main mechanism of DENV cell entry is clathrin-mediated endocytosis[40]. However, depending upon the serotype and target cell, the virus is able to use alternative internalization routes, consisting of caveolae and lipid rafts[41]. Principal target cells for DENV infection are monocytes, macrophages and dendritic cells[42]. Viral particles spread along the cellular surface and roll over different receptors for the E glycoprotein until they find pre-existing clathrin-coated pits[43,44]. Clathrin-coated pits evolve until they become clathrin-coated vesicles. These vesicles deliver their cargo to endosomes[45]. It is well established that the mildly acidic pH within the endosomal lumen produces a major conformational change in the E protein allowing the fusion loop of domain II to be exposed[46]. The highly hydrophobic fusion loop is subsequently inserted into the endosomal target membrane allowing the formation of an E trimer. This E trimer is necessary for the formation of a lipidic fusion pore and release of the genome into the cell cytosol[47].

As indicated above, the positive-sense RNA genome is translated into a single polyprotein which is processed by the host and viral proteases. Viral assembly is performed in the endoplasmic reticulum where particles with prM on their surface are formed. These immature particles are then transported to the trans-Golgi network where conformational changes on the viral surfaces occur within a mildly acidic environment during which the prM protein protects E from undergoing a premature conformational change[48,49]. Subsequently, the host protease furin cleaves the prM into M and “pr” peptides. When the viral particles are released from the cell, the change to neutral pH produces the dissociation of the “pr” peptide from the viral surface and infectious mature particles are formed[50,51].

3.2. The Immune Response to DENV Infection

In a primary DENV infection, naïve DENV-specific B cells are selected and stimulated. These cells then differentiate into memory B cells and plasma cells (PCs). There are two types of PCs, short-lived PCs and long-lived PCs. Both of these PCs are able to secrete antigen-specific antibodies[52]. The short-lived PCs produce IgM antibodies in the acute phase of the disease (4-5 days after fever onset, Figure 2) which remain detectable for approximately 6 months[52,53]. The long-lived PCs migrate to the bone marrow where affinity maturation takes place. Here, only those clones with specific high-affinity antibodies survive for a long time[54]. These clones produce high concentrations of antigen-specific IgG antibodies (mainly IgG1 and IgG3)[52,55,56]. This antigen-specific IgG appears one week after the fever onset with a maximum production during the convalescent phase. Then, IgG concentrations decrease until levels that remain detectable for a long time[57].

In case of a secondary DENV infection by a heterologous dengue serotype, the

IgM response is variable and sometimes not detectable[57]. The memory B cells with homologous serotype-specific antibodies retained on their surface are rapidly activated. These memory B cells cross-react with the heterologous serotype[58]. The long-lived PCs produce IgG with high homologous serotype affinity, but cross-reacting and weakly neutralizing antibodies to the heterologous dengue serotype[56,58]. IgG antibodies that are specific for the heterologous dengue serotype are produced three months post-infection[56].

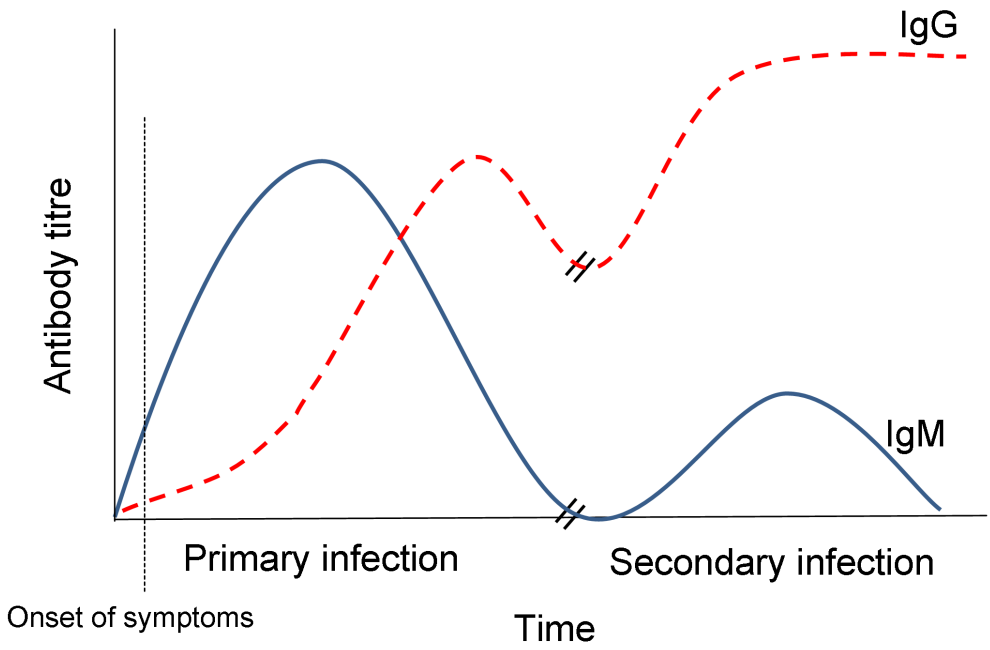

Figure 2. Antibody production during a primary and secondary dengue infection. In a primary dengue infection, IgM antibodies (—) can be detected since day 4-5 after fever onset and remained detectable for three months. IgG levels (----) can be detected a week after fever onset and remain detectable for long period of time. In a secondary infection with a heterologous dengue serotype, the IgM level is low and sometimes undetectable. IgG levels are higher than in a primary infection. Adapted from Peeling *et al.*, 2010[39].

4. Clinical Manifestations and Dengue Classification

4.1. Clinical Manifestations of DENV Infection

The majority of DENV infections are considered to be asymptomatic[24]. However, when the infections are symptomatic, after an incubation time of 3-8 days[59], the symptomatology of dengue begins with a sudden onset of fever that lasts 2-7 days and is frequently accompanied by myalgia, arthralgia, anorexia, headache, retro-ocular pain, rash and nausea[60,61]. During this febrile period, mild haemorrhagic manifestations

like petechiae and bruising at the venepuncture sites can also be present[4]. Laboratory tests show mild to moderate thrombocytopenia, leukopenia and moderate increase of hepatic aminotransferase levels[62]. The critical phase starts 24h-48h after the fever recedes. A small proportion of patients may develop increased vascular permeability, demonstrated by haemoconcentration, pleural leakage and ascites[63]. When the loss of plasma volume is critic, the pulse pressure narrows (≤ 20mmHg) and the patient can develop shock. To avoid shock, medical doctors must be alert to warning signs like persistent vomiting, severe abdominal pain, hepatomegaly, high levels of haematocrit or increased levels of haematocrit accompanied by a quick decrease in platelet levels, mucosal bleeding, lethargy or restlessness in order to give appropriate and timely treatment[62]. In the recovery phase, a gradual reabsorption of extravascular fluid occurs after [48-72]. A second rash may appear with itchy lesions and adults can present with fatigue for several weeks[4].

4.2. Dengue Classification

Initially, dengue was classified as DF, DHF (grade I,II) and DSS (DHF grade III and IV)[3]. This classification has been widely criticized, because it was too rigid. Patients with suspected DHF had to meet all four criteria (fever for 2-7 days, positive tourniquet test or spontaneous bleeding, platelets level ≤ 100 x 10^9 per litre, plasma leakage determined by changes of haematocrit levels and/or pleural effusion) frequently resulting in the inappropriate and delayed categorization of patients with severe dengue[62,64,65]. Several countries started to develop local adaptations of the 1997 dengue classification[66,67]. Therefore, epidemiological comparisons between counties became almost impossible[68]. Currently, WHO has proposed a new classification for dengue disease as follows: a) dengue with or without warning signs and b) severe dengue (defined as severe plasma leakage or severe bleeding or severe organ involvement)[4] (Figure 3). The main objectives of this new dengue classification are the timely identification of severe cases or potential severe cases for the proper management of the patient and, to use the limited resources for those that really need them[68]. Currently, this new classification is under evaluation in more than 12 countries of Asia and America[68].

CRITERIA FOR DENGUE ± WARNING SIGNS		CRITERIA FOR SEVERE DENGUE
Probable dengue Fever and 2 of the following criteria: ▪ Nausea, vomiting ▪ Rash ▪ Headache/ retro-ocular pain ▪ Myalgia and/or arthralgia ▪ Tourniquet test positive ▪ Leukopenia ▪ Any warning sign [live in /travel to dengue endemic area]	**Warning signs*** ▪ Abdominal pain or tenderness ▪ Persistent vomiting ▪ Clinical fluid accumulation ▪ Mucosal bleed ▪ Lethargy, restlessness ▪ Liver enlargement >2 cm ▪ Laboratory: increase in HCT concurrent with rapid decrease in platelet count *(requiring strict observation and medical intervention)	**Severe plasma leakage leading to:** • Shock (DSS) • Fluid accumulation with respiratory distress **Severe bleeding** as evaluated by clinician **Severe organ involvement** • Liver: AST or ALT >=1000 • CNS: Impaired consciousness • Heart and other organs
Laboratory-confirmed dengue (important when no sign of plasma leakage)		

Figure 3. Dengue classification according to the World Health Organization guideline (2009). Dengue is classified as: a) Dengue without warning signs, b) Dengue with warning signs and c) severe Dengue. Adapted from WHO, 2009[4].

4.3. Dengue Versus Other Febrile Illness

At the beginning of dengue illness, the patient presents with non-specific symptoms such as fever, headache, myalgia, arthralgia, retroocular pain, nausea, vomiting and rash. These symptoms can be easily confused with other febrile illnesses like influenza, leptospirosis, malaria, rickettsiosis, typhoid fever or chikungunya infection among others[69-72]. Up to now, there are no accepted guidelines for the early recognition of dengue infection[73]. Several clinical, haematological and biochemical parameters have been proposed to discriminate dengue from other febrile illnesses (OFI) at the early stage of the disease. The presence of rash, myalgia and mild haemorrhagic manifestations have been the most common clinical manifestations associated with dengue[73-77]. The mild haemorrhagic manifestations have been mainly represented by the presence of petechiae and positive tourniquet test. Among the haematological parameters, a majority of studies has reported a decrease of platelet counts, white blood cells and lymphocytes in dengue patients compared to OFI[77-79]. Indications of liver damage have been determined by biochemical parameters. Increased levels of transaminases, such as aspartate aminotransferase and alanine aminotransferase, have been associated with dengue patients[73,77,80,81].

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4.4. Risk Factors for Severe Dengue

Dengue severity is related to multiple risk factors such as secondary infection, viral virulence and ethnic background of the patient. Epidemiological evidence has shown that a secondary infection is a risk factor for severe dengue[2,60]. A widely accepted explanation for this phenomenon is the hypothesis of "antibody-dependent enhancement" (ADE) of disease proposed by Halstead[60]. People infected by one dengue serotype produce a lifelong protective antibody response to the homologous serotype and a short-lived (2-3 months) cross protection to heterologous serotypes[82]. After this period, the concentration of the non-specific antibodies starts to wane to sub-neutralising levels. These antibodies cross-react with heterologous serotypes facilitating the cellular uptake of the virus through Fc receptors[82,83].

Recently, prM-mediated ADE has also been proposed[84,85]. This hypothesis postulates that antibodies against the viral prM protein can potentially produce ADE. Cells infected by DENV release high levels of immature particles containing prM protein[86]. In a primary infection, antibodies directed against prM are being produced. These anti-prM antibodies are highly cross-reactive between the different dengue serotypes and are non-neutralizing even at high concentrations[84]. In an infection with a heterologous serotype, anti-prM antibodies facilitate the binding and cell entry of immature particles into Fc-receptor-expressing cells[85]. In either case, the consequence of ADE is a high viral load due to an increased number of infected cells[87]. A high viral load and the slow rate of viral clearance have been associated with severe dengue, resulting in immune activation for a prolonged period of time[88]. It has been postulated that a large mass of infected cells release high levels of cytokines and vasoactive mediators that increase vascular permeability[59].

Although, severe dengue is more likely to occur in secondary infections, only a small proportion of secondary infections results in severe dengue[89]. Moreover, the fact that severe dengue has also been observed in primary infections[89,90], indicates that, besides ADE, other unknown factors related to the host and/or virus are also involved in the evolution of severe disease. There is epidemiological evidence that relate some dengue genotypes with dengue epidemics and a higher proportion of cases of severe dengue (viral virulence)[91-93]. A higher proportion of individuals infected with DENV-2 Asian genotype and DENV-3 genotype III develop severe dengue compared to those infected with DENV-2 American genotype and DENV-3 genotype V, respectively[91,94].

In addition, it has been described that ethnicity is a risk factor for severe dengue. Sporadic DHF cases have been reported in Haiti despite the co-circulation of different dengue serotypes[95]. Caucasian and Asian individuals have a higher risk to develop severe dengue compared to those of African descent. This fact is evidenced by the low incidence of severe dengue in black populations of Cuba[96]. White Cubans generally

exhibit a stronger and remarkably cross-reactive dengue virus-specific memory $CD4^+$ T lymphocyte proliferation and interferon gamma (IFN-γ) release compared to the black Cuban population[97]. IFN-γ indirectly induces plasma leakage by enhancing tumour necrosis factor (TNF-α) production in activated monocytes[98].

5. Health-Seeking Behaviour

A significant association between the delay of healthcare seeking and DHF has been found[99]. Health or care seeking behaviour (HSB) has been defined by Kasl and Cobb's in 1966 as "any activity undertaken by individuals who perceive themselves to have a health problem or to be ill for the purpose of finding an appropriate remedy"[100]. According to Tipping & Segall (1995)[101], HSB could be addressed in two ways:

a) *Healthcare-seeking behaviour or utilization of the formal system:* mainly focused on the analysis of barriers to care that lie between patients and services, and the process of healthcare seeking. The latter involves the identification of pathways (from home-care to the formal system) to the formal healthcare system[102].

b) *Health-seeking behaviour or process to illness:* has a general point of view and varies according to the disease. The perception of the illness severity, the perception of susceptibility to the disease, perceived benefits of taking health action and perceived barriers to taking health action are the four main elements of individuals' health behaviour according to the health belief model[102].

Few studies on HSB have been performed in case of dengue. Most of them have been done in Asia. Symptoms recognition by caretakers and patients, the perception about the quality of care, social-economic factors, individual perception of the illness and previous experiences are factors that influence health-seeking behaviour[103-105].

Probably due to the high proportion of DF in endemic areas, dengue is considered a harmless disease that can be treated at home with traditional medical practices, self-medication and rest[103-105]. The perception of low susceptibility to infection among young adults and elderly in Malaysia is mainly due to the notion of a "natural ability to withstand infection" and the perception of low risk to be bitten by the mosquito, respectively[104]. Only when traditional medical practices or drugs fail to bring relief or when the health is perceived as declining, caretakers and patients decide to seek healthcare in health centres or by a formal practitioner; this normally occurs only after the third day of the onset of symptoms[105].

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6. Dengue in Venezuela

6.1. History of Dengue Epidemics in Venezuela

Although the first description of dengue-like illness in the Americas dates from 1634[106], the first reports of such disease in Venezuela go back to 1828 and then to 1946[107]. However, these reports were only based on clinical manifestations. It was not until the year 1953, when dengue virus was isolated from a patient in Trinidad, that dengue cases were laboratory confirmed[108,109]. No dengue epidemics were reported in the Americas between 1946 and 1963, possibly related to the decrease of *A. aegypti* during the yellow fever eradication plan[110].

Since the year 1963 until 2010, Venezuela has reported eight dengue epidemics. These epidemics could be a consequence of:

a) Introduction of new serotypes: the epidemics in the years 1963, 1969, 1978 were attributed to the introduction of DENV-3, genotype V[21], DENV-2, American genotype (V)[109] and DENV-1, genotype III[109,110] respectively. The presence of DENV-4 in Venezuela was first reported in the year 1985 without producing an epidemic[111]. During these epidemics no cases of DHF were reported.

b) Introduction of new genotypes: the first DHF outbreak reported in Venezuela, and the second in the Americas, took place in 1989-1990. A total of 12,220 dengue cases were reported in Venezuela, of which 3,108 (25.4%) were DHF, and 73 individuals died[112]. During this epidemic DENV serotypes 1, 2 and 4 were circulating, but mainly serotype 2. Later, this serotype 2 was identified as the Asian genotype (III) which was gradually displacing the American genotype[91,113]. Due to unknown reasons, DENV-3 disappeared from the Americas[11] and was absent from Venezuela for 32 years[94]. In the year 2000 DENV serotype 3 was re-introduced producing the greatest dengue epidemic since 1989, with 104,282 reported dengue cases, including 8,727 cases (8.4%) of DHF[114]. The introduced DENV serotype 3 was of genotype III[94], which is generally considered a virulent virus[93,94]. Since the re-introduction of this serotype in the year 2000, the four dengue serotypes co-circulate in Venezuela[115].

c) DENV evolution "in situ": Phylogenetic analyses of samples taken during 1997-2000 in Aragua state, showed the circulation of a DENV-2 mixed genotype (American/Asian), indicating the evolution and the re-combination of this serotype *in situ*[116,117]. This serotype was positively associated with DHF/

DSS cases during the 1998 epidemic in Venezuela[116,117]. The shift of DENV-1 clades or lineages could be the reason of the dengue epidemic in 2007. The evolution of DENV-1 genotype V has been associated with high dengue morbidity in Aragua estate[118,119].

The most recent and biggest dengue epidemic in Venezuela occurred in 2010, with approximately 125,000 dengue cases, including 10,300 cases (8.6%) of DHF cases[120]. In this year, Venezuela was the third country with the highest number of dengue cases in the Americas and the second reporting a high number of DHF cases[114].

At the beginning, the reported dengue epidemics in Venezuela were relatively isolated in time and were caused by introduction of new serotypes. However, since 1980, dengue became hyperendemic in Venezuela with epidemic cycles of narrow intervals (3-5 years) and higher peak incidences each time[121]. The epidemic years are followed by inter-epidemic periods. The number of cases in each inter-epidemic period has increased[122]. The augmented dengue incidence in Venezuela has coincided with an increased number of cases in other American countries[123]. However, the augmented dengue incidence throughout time cannot be explained by the introduction of new serotypes or genotypes indicating that other factors are involved.

An important event in 2009 was the identification of *A. albopictus* for the first time in Venezuela[124]. *A. albopictus* is the second vector of dengue transmission and it has been involved in dengue epidemics[24,93].

6.2. Risk Factors for Dengue Infection in Venezuela

Although dengue has been present in Venezuela for more than 180 years, Venezuelan people underestimate the severity of the disease. A study performed during the 1990s on the knowledge of people in Venezuela about dengue, revealed that, although the majority of the individuals have heard about dengue, few of them could identify the mosquito vector, its habitat or the symptoms of the disease. Most of the people did consider dengue as a problem. However, they perceived this problem like an external situation in which they are not involved[125].

Water supply became erratic and garbage collection less frequent in Venezuela due to the economic recession at the end of the 1980s[125]. In this context most of the entomological studies were performed. The majority of the people stored water in 208L metal drums which at the same time presented a high *Aedes* index compared to other containers[126,127]. A high correlation was found between the frequency and length of water supply interruptions and the proportion of households with at least one vector breeding site and the proportion of containers where the vector is present[126,127]. In the 1990s, high dengue incidences occurred during the raining season. However, more recently,

it has been reported that the frequency of dengue cases is similar in the rainy and dry season indicating a perennial transmission of the virus[121,128]. Factors related to the change of annual dengue transmission should be studied. In the years 1993-1998, the DF and DHF incidences of different municipalities of Maracay city were estimated using a geographical information system[129]. This study showed that a higher DF and DHF incidence was present in those municipalities with high population density. A persistent high dengue incidence was found in the same neighbourhoods of each municipality[129]. The presence of a high number of female *A. aegypti* inside the households has been related to neighbourhoods with high dengue incidence/persistence and accentuated deficiencies in the frequency and length of water supply interruptions, but also to those neighbourhoods with low dengue incidence/persistence that show any degree of deficiency in public services, indicating that other variables can determine the level and persistence of dengue transmission[121]. Identification of clusters ("hot spots") at block and household level and its relation with risk factors to dengue infection could explain the level and persistence of dengue transmission within these neighbourhoods with high dengue incidence/persistence. Furthermore, identification of high-risk areas of dengue transmission can be used to target surveillance and control measures to those locations in a cost-effective manner.

6.3. Clinical Presentation of Dengue in Venezuela

Clinical-haematological studies on dengue performed in Venezuela have mainly been focussed on fatal cases and on the differentiation of DF from DHF. A clinical-pathological study has demonstrated the development of leukopenia, neutrophilia, thrombocytopenia, increase in plasma levels of aminotransferase enzymes, prolongation of the partial time of thromboplastin and metabolic acidosis in fatal cases. Plasma leakage (pleural effusion and ascites) and an uncontrolled inflammatory process with multi-organ involvement mainly affecting the liver and lungs have also been found[130]. A decrease of platelet count and lymphocytes and an increase of neutrophil and IL10 levels have been associated with severe dengue[131,132]. However, the majority of Venezuelan people attend the health centres at the fourth or fifth day after fever onset[132] when patients could be already critically ill[133]. Indeed, it has been reported that patients with DHF have been hospitalized later (4.3 days) than patients with DF (3.2 days)[134]. A genetic study has indicated a gradual evolution in the pattern of gene expression in patients infected by DENV independent of the disease severity. The main change occurs at the fourth day after fever onset resulting in the separation of the early acute (0-3 days) and late acute phases (4-6 days)[131]. An early treatment intervention can reduce the case fatality from 20% to 1%[4,5,135]. Therefore, an early dengue recognition (<4 days) is required.

There are no reported studies in Venezuela that differentiate dengue from other febrile

illnesses (OFI) at the early stage of the disease. Diseases like influenza, leptospirosis and malaria are common in this country and their initial clinical manifestations are similar to dengue[69,71,72]. As mentioned before, there are no accepted guidelines for the early recognition of dengue infection in the world[73]. Serological tests confirms dengue infection at the late stage of the disease[136] and, laboratory techniques for early dengue virus detection are not available at primary health care services[75]. On the other hand, suspected dengue cases are clinically detected at the early days of the disease present low concordance with laboratory-confirmed cases in the American countries[137,138] creating unnecessary hospitalizations due to closer clinical monitoring. A decision-tree algorithm that discriminates dengue from OFI at the early stage of the disease is needed in order to avoid fatalities due to misdiagnosis and overburdening of health centres.

The delay in presenting to the health centres in case of dengue in Venezuela could be related to health-seeking behaviour (HSB). In order to achieve an early diagnosis and a proper dengue treatment, the reasons for the observed delay in health seeking among the Venezuelan population have to be elucidated. Studies on health believes and practices, HSB and access to care related to dengue have to be performed with the aim to find ways to improve early attendance to health centres and medical care.

Scope and Summary of the Thesis

Despite control measures, dengue has become a major public-health problem in Venezuela. Epidemics of increasing magnitude regularly occur against a background of an established endemic situation. Concomitantly, the number of severe dengue cases has risen over time. An early diagnosis and proper treatment of dengue cases can reduce the risk of development of severe disease. Furthermore, in Venezuela, patients with suspected dengue infection tend to seek medical help at a relatively late stage after fever onset.

The studies described in this thesis aimed to:

- determine dengue seroprevalence and identify current risk factors for dengue transmission in high incidence areas of Maracay city, Venezuela.
- find clusters (“hot spots”) of dengue transmission that could help target surveillance and vector control measures in an effective way.
- identify clinical and laboratory parameters that differentiate dengue from other febrile illness at the early phase of the disease.

- recognize and understand the patterns of health-seeking behaviour (HSB) of Venezuelan people in order to improve early attendance to health centres.

Chapter 2: In this chapter a detailed description is given of the set-up of a prospective community-based cohort study in Maracay city, Venezuela. The characteristics of the study area, the recruitment process, the data collection in individual and household-structured questionnaires, the process of active surveillance as well as the training of the health personnel are fully described in this chapter. Approximately 2000 individuals belonging to three neighbourhoods of high dengue incidence were recruited into the study. Within the four years of follow-up, annual cross-sectional surveys were performed. During the recruitment process, a cross-sectional baseline study was accomplished in order to determine dengue seroprevalence and identify risk factor for dengue infection (the results which are presented in **Chapter 4**). Furthermore, the geoposition of each household was recorded with the aim to determine clusters ("hot spots") of dengue transmission (as further discussed in **Chapter 5**). Socio-economic data and intended pathways to care in case of fever or suspected dengue were collected during the third annual cross-sectional survey and presented in **Chapter 7** of this thesis.

Chapter 3: This chapter describes in detail the establishment of an observational health centre-based cohort study to identify clinical and laboratory parameters that differentiate dengue from other febrile illnesses (OFI). Inclusion criteria, data collection and documentation in a structured questionnaire are explained. Training of the health-centre medical personnel in the study procedure is also reported. Training of the study nurses on identification of febrile patients, dengue criteria, explanation of the study to the patients according to the writing informed consent/assent form are fully described. Data collected in this study are presented in **Chapter 6** of the thesis.

Chapter 4: The aim of this chapter was to determine the seroprevalence of dengue infection and the identification of risk factors for dengue transmission in the population recruited during the prospective community-based cohort study (as outlined in **Chapter 2**). For this purpose, a baseline cross-sectional study involving 2014 recruited individuals was carried out in the three communities of high dengue incidence. The population under study presented a high seroprevalence (77.4%) with 10% of people experiencing recent infections. Multivariate analysis demonstrated that socio-economic factors and environmental factors related to mosquito breeding sites determine the risk of acquiring a dengue infection in this population. Our data also suggest that people become infected mostly at home.

Chapter 5: In this chapter, the spatial distribution of dengue in the three selected neighbourhoods studied is presented. Risk maps identifying "hot spots" of dengue transmission at block and house level were generated. To these end, map technology and spatial analysis of epidemiological and seroprevalence data were used. We found that dengue prevalence is highly heterogeneous at the studied spatial scale. Also, our results suggest that transmission of dengue is very focal. These results can inform regional health authorities in order to reduce dengue transmission by direct the surveillance and the mosquitos control measures to those places of high dengue transmission.

Chapter 6: In this chapter, clinical and laboratory parameters that differentiate dengue from other febrile illness (OFI) are presented. Data were derived from a three year health centre-based cohort study that was established in three health centres serving the same communities, as presented in **Chapters 2** and **4**. In this study, 254 patients met the inclusion criteria of whom 112 (44%) were laboratory diagnosed as dengue and 142 (56%) were classified as presenting with OFI. The daily evolution of the most important clinical and haematological parameters in our study population is shown. Multivariate analysis of clinical and haematological parameters associated with dengue during the first 3 days of the illness and on days 4-7 are presented separately. A decision-tree algorithm that allows discriminate dengue from OFI during the first 3 days of the illness is proposed.

Chapter 7: In this chapter, intended pathways to seek care for suspected dengue are described with the aim to understand patterns of health-seeking behaviour (HSB). To this end, structured questionnaires were applied to 105 individuals belonging to the three communities studied in **Chapter 4** and recruited according to **Chapter 2**. Data were collected during the third annual survey. We showed that intended pathways to care differed for suspected dengue infection compared to fever, as well as for children and adults in case of suspected dengue. In case of fever, most individuals would firstly treat the disease at home before seeking medical care, while the contrary was reported in case of suspected dengue. Parents/guardians would take children earlier to the health facility than adults would seek care. Suspected dengue would prompt people to search medical help earlier than fever. For dengue, the appearance of new symptoms (77.1%) and high fever (74.3%) were the main reasons for seeking medical help. Primary health centres were the first intended choice to find medical care for nearly (93%) all participants. Dengue risk perception was high with a relative good general knowledge of the disease.

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Chapter 8: summarizes the results and discusses the most important conclusions of this thesis.

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