2. Review of Literature

2.1. History and Epidemiology of Dengue

The first cases of Dengue Fever (DF) were recorded in 1779 in Batavia, Indonesia, and Cairo (Gubler, 1994) For the past 200 years, pandemics have been recorded in tropical and subtropical climates at 10 to 30 year intervals In 1944, Albert Sabin successfully isolated the virus that causes DF and found that it belonged to the Flaviviridae virus family (Gubler, 1994) There are more than 70 known members of the Flaviviridae family Some examples include Yellow Fever and Japanese Encephalitis Virus

The first epidemic of DHF was in 1953 (Manila) and the disease remained localized in South-East Asia through the 1970s (Gubler, 1994) The South-East Asia Region of WHO comprises 10 countries, namely, Bangladesh, Bhutan, India, Indonesia, Democratic People's Republic of Korea, Maldives, Myanmar, Nepal, Sri Lanka and Thailand, with a total population of 1.45 billion Ever since the recognition of DF and DHF as a disease entity in Manila in 1953, the disease has spread to many countries in South-East Asia (Fig 1) Notably among them was Thailand which recorded a severe outbreak in 1958, followed by Indonesia in 1968, and Myanmar in 1970 (Prasittisuk and Andjapandze, 1996) The disease has now become endemic in all the countries of the Region, except Bhutan and Nepal, and the occurrence of outbreaks has become a regular feature Since 1994, there have been a growing proportion of cases with hemorrhagic manifestations, particularly in Sri Lanka and India, which has contributed to an increasing trend in the case fatality rates in the Region

DF has been recognized for many years in India, since the outbreak of dengue occurred in 1912 in Kolkata (Kennedy, 1912) The proportion of DHF or DF cases with hemorrhagic manifestations has increased in the last 5-6 years, all the states of the country have reported outbreaks The onset of the disease occurs immediately after the monsoon season which varies in duration from state to state, between July and November The attack rates in the outbreaks ranged from 20% to 80% of the population in affected localities All age-groups have been affected by DF, and all the four serotypes
of the virus have been isolated and are in circulation in the country; more than one serotypes are commonly present during many of the outbreaks in urban areas. The seropositivity of localities affected by dengue can be quite high ranging from 8% to 91% (Victor et al., 2002).

Fig. 1: Global distribution of Dengue (Halstead, 2007).

2.2. Disease burden

Over the decades DF and DHF have emerged as a global public health problem with countries in Asia and the Pacific sharing more than 70% of the disease burden. About 52% of the population at risk of DF/DHF lives in the WHO South-East Asia Region (Halstead, 2007). Dengue infections are a significant cause of morbidity and mortality and lead to adverse economic effects in many developing tropical countries. The average total economic burden was estimated to be US$27.4 million by India during the 2006 dengue epidemic (Garg et al., 2008).
2.3. Dengue virus

2.3.1. Dengue Serotypes and relationship with other flaviviruses:

There are three major complexes within flavivirus family—tick-borne encephalitis virus, Japanese encephalitis virus, and dengue virus. There are four dengue virus serotypes, called DEN-1, DEN-2, DEN-3, and DEN-4. All flaviviruses have common group epitopes on the envelope protein that result in extensive cross reactions in serologic tests. These make unequivocal serologic diagnosis of flaviviruses difficult. This is especially true among the four dengue viruses. Infection with one dengue serotype provides lifelong immunity to that virus, but there is no cross protective immunity to the other serotypes. Thus, persons living in an area of endemic dengue can be infected with three, and probably four, dengue serotypes during their lifetime (Gubler, 1997).

2.3.2. Viral Structure and genome:

Similar to other flaviviruses, mature dengue virions consist of a single-stranded RNA genome surrounded by an icosahedral nucleocapsid about 30 nm in diameter. This nucleocapsid is covered by a lipid envelope of about 10 nm deep. The complete virion is about 50 nm in diameter. The RNA genome of dengue virus is 10,862 nucleotides long and has an open reading frame of 10,233 nucleotides, which encodes a polypeptide of 3,411 amino acids (Noisakran and Perng, 2008). It is made up of a single polyprotein which is processed by the viral NS2B-NS3 protease and cell proteases to 3 structural proteins C, prM, and E, and 7 nonstructural proteins (NS) are 1, 2A, 2B, 3, 4A, 4B, and 5 (Fig. 2). The noncoding region at the 5' end is 118 nucleotides long followed by the first AUG codon at which translation is initiated. The first termination codon is encountered at nucleotide 10,352.

![Fig. 2: The dengue viral genome (Noisakran and Perng, 2008). Noncoding regions with their terminal structures are indicated by black lines.](image-url)
2.4. Transmission cycles

2.4.1. Vectors:

The primitive enzootic transmission cycle of dengue viruses involves canopy-dwelling *Aedes* mosquitoes and lower primates in the rain forests of Asia and Africa. Current evidence suggests that these viruses do not regularly move out of the forest to urban areas (Rico-Hesse, 1990). An epidemic transmission cycle may occur in rural villages or islands, where the human population is small. Introduced viruses quickly infect the majority of susceptible individuals in these areas, and increasing herd immunity causes the virus to disappear from the population. A number of *Aedes* (*Stegomyia*) spp. may act as a vector in these situations, depending on the geographic area, including *A. aegypti*, *A. albopictus*, *A. polynesiensis* and other members of the *A. scutellaris* group. The viruses are maintained in an *A. aegypti*-human-*A. aegypti* cycle with periodic epidemics. Often, multiple virus serotypes cocirculate in the same city (hyperendemicity) (Service, 1992). The vectors, *A. aegypti* and *A. albopictus*, are widespread in India and their local densities can be quite high; however, the role of *A. albopictus* has not yet been established (Victor et al., 2002). Most of the DF/DHF outbreaks have occurred in localities where the larval house index was more than 20%. There is no regular vector surveillance and control programme in India.

2.4.2. Disease Transmission:

The female mosquito, which feeds during daytime, has the ability to spread dengue virus to another host after feeding on a viremic host or can transmit the virus 8-10 days after it has amplified in the salivary gland (Halstead, 1984; Gubler and Rosen, 1976). Once mosquitoes are infected with dengue virus, they have the ability to transmit the virus throughout their entire life. *A. albopictus*, which has a higher biting frequency than *A. aegypti*, originated in Southeast Asia, and has been introduced into the United States, Europe, and Nigeria. The strain of *A. albopictus* found in Europe is a cold-resistant strain, and it has been suggested that it may result in future dengue outbreaks (Kautner et al., 1997).
2.4.3. *Vector control:*

Prevention and control of dengue and DHF currently depends on controlling the mosquito vector, *A. aegypti*, in and around the home, where most of the transmission occurs. Space sprays with insecticides to kill adult mosquitoes are not usually effective unless they are used indoors. The most effective way to control the mosquitoes that transmit dengue is larval source reduction, i.e., elimination or cleaning of water-holding containers that serve as the larval habitats for *A. aegypti* in the domestic environment (Malavige *et al.*, 2004).

There are two approaches to effective *A. aegypti* control involving larval source reduction. In the past, the most effective programs have had a vertical, paramilitary organizational structure with a large staff and budget. These successful programs were also facilitated by the availability of residual insecticides, such as DDT, that contributed greatly to ridding the mosquito from the domestic environment. Unfortunately, in all of these programs, without exception, there has been no sustainability, because once the mosquito and the disease were controlled, limited health resources were moved to other competing programs and the *A. aegypti* population rebounded to levels where epidemic transmission occurred (Malavige *et al.*, 2004).

In recent years, emphasis has been placed on community-based approaches to larval source reduction to provide program sustainability. The rationale is that sustainable *A. aegypti* control can be accomplished only by the people who live in the houses where the problems occur and by the people who help create the mosquito larval habitats by their lifestyles. Community participation in prevention programs requires extensive health education and community outreach. Unfortunately, this approach is a very slow process. Therefore, it has been proposed that a combination top-down and bottom-up approach be used, the former to achieve immediate success and the latter to provide program sustainability. The effectiveness of this approach remains unknown (Igarashi, 1997).
2.5. **Clinical manifestations**

Dengue virus infections may be asymptomatic or lead to a range of clinical presentations, even death. The incubation period is 4–7 days (range 3–14). Typically, DF is an acute febrile illness characterized by frontal headache, retroocular pain, muscle and joint pain, nausea, vomiting, and rash (Kalayanarooj *et al.*, 1997; Cobra *et al.*, 1995).

Leukopenia and mild thrombocytopenia are frequent (Kalayanarooj *et al.*, 1997; Trofa *et al.*, 1997). Less frequent, but not rare, are haemorrhagic manifestations such as petechiae, epistaxis, gingival bleeding, gastrointestinal bleeding, microscopic haematuria, and hypermenorrhoea (Nimmannitya, 1994). A positive tourniquet test (more than 20 petechiae in a square patch of skin 2.5 X 2.5 cm (>20/in^2) may be found in over one-third of patients with DF (Nimmannitya, 1994). Clinical findings alone are not very helpful to distinguish DF from other febrile illnesses such as the chikungunya, measles, leptospirosis, typhoid or malaria (Trofa *et al.*, 1997; Halstead and Yamarat, 1965).

2.5.1. **Dengue haemorrhagic fever**

DHF is defined as an acute febrile illness with minor or major bleeding, thrombocytopenia (≤10^5/μL), and evidence of plasma leakage documented by haemconcentration (haematocrit increased by at least one-fifth or decreased by the same amount after intravenous fluid therapy), pleural or other effusions, or hypoalbuminaemia or hypoproteinaemia (Pan American Health Organization, 1994). The major pathophysiological change that determines the severity of disease in DHF and differentiates it from DF is plasma leakage. Extravasation occurs through endothelial gaps, without necrosis or inflammation of the capillary endothelium (Chaturvedi *et al.*, 1997). Nevertheless, a recent clinical trial showed that a drug that counteracts capillary permeability induced by histamine or hyaluronidase, and is currently marketed in Asia (carbazochrome sodium sulphonate) did not prevent dengue vascular permeability or shock (Tassniyom *et al.*, 1997).

DHF commonly begins with a sudden rise in temperature and other symptoms resembling DF. The temperature is typically high (38–40°C) and continues for 2–7 days.
DHF and DSS usually develop around the third to seventh day of illness (World health organization, 1986). The most common haemorrhagic feature is a positive tourniquet test (50%) (Kalayanarooj et al., 1997). Petechiae and subcutaneous bleeding at venepuncture sites are present in most cases (Nimmannitya, 1994). The development of DHF provides warnings of an increased probability of shock. The first information to ascertain severe dengue disease is the time elapsed since onset of illness. DHF and DSS usually develop around day 3–7 of illness, at the time of defervescence (i.e., abatement of fever), which is an indication for intensified observation (World health organization, 1986; Pan American Health Organization, 1994). A progressively decreasing platelet count and a rising haematocrit (signaling abnormal capillary permeability) indicate increased probability of impending shock (Nimmannitya, 1994).

2.5.2. Dengue shock syndrome:

DSS is defined as DHF with signs of circulatory failure, including narrow pulse pressure (<20 mm Hg), hypotension or frank shock. The liver may be palpable and tender; and liver enzymes are usually mildly abnormal but jaundice is rare (Kalayanarooj et al., 1997; Rigau-Perez, 1997). The four warning signs for impending shock are intense, sustained abdominal pain; persistent vomiting; restlessness or lethargy; and a sudden change from fever to hypothermia with sweating and prostration. The developments of any of these signs or any suggestion of hypotension are indications for hospital admission and management to prevent shock.

2.5.3. Other severe dengue syndromes:

There are some unusual but well-described manifestations of dengue infection where the risk of death is high. These are DF with severe haemorrhage, hepatic damage, cardiomyopathy, and encephalopathy (Nimmannitya, 1994). Neurological manifestations such as altered consciousness, convulsions, and coma have been ascribed to an encephalopathy secondary to prolonged DHF and DSS, resulting from the leakage of plasma into serous spaces, haemorrhage, shock, and metabolic disturbances (Thisyakorn and Thisyakorn, 1994). However, invasion of the central nervous system (viral encephalitis) was documented as one of the cause (Lum et al., 1996). Vertical
transmission of dengue virus has been recorded in a small number of cases, leading to neonatal DF or even DSS (Chye et al., 1997). One case of nosocomial transmission from a needle stick injury has also been reported (De Wazieres et al., 1998).

2.6. Diagnosis

Diagnosis of dengue falls into two stages: stage I, fever and viraemia accompanied by NS1 antigens in blood; and stage II, the early post-febrile period lasting a few weeks when IgM and IgG antibodies are in higher levels (Halstead, 2007). During primary infection, viraemia more or less coincides with fever (Fig. 3). However, during a secondary infection; the duration of viraemia can be 2 or 3 days, whereas presence of NS1 antigens in blood lasts somewhat longer.

Serological diagnosis will not be positive until defervescence (Fig. 3). In individuals with DHF and DSS, vascular permeability is recognized usually at defervescence, at which time the IgM-capture serological test should be positive but tests to detect virions, dengue RNA, or dengue proteins could be negative. Commercial dengue serological tests in most countries are not subject to quality control (Blacksell et al., 2006).

Fig. 3: Course of dengue infection and timings of diagnosis (Halstead, 2007).
Attempts are being made to optimize and simplify PCR for diagnosis during the febrile period. With genus-specific and serotype-specific nested NS3 primers, Singh et al. (2006) were able to identify dengue virus in 80% of serum samples obtained within 5 days of onset of fever. Alternatively, dengue RNA can be detected rapidly with primers for all four dengue viruses in a one-step TaqMan real-time reverse transcriptase-PCR (Kong et al., 2006). An inexpensive, rapid, sensitive, and specific test is needed to diagnose dengue during the febrile stage. One such test is marketed by BioRad (Hemel Hempstead, UK) which uses a dengue group specific NS1 monoclonal antibody in an ELISA format to detect dengue NS1 antigen in blood.

2.7. Case management

Patients with DF require rest, oral fluids to compensate for losses via diarrhoea or vomiting, analgesics, and antipyretics for high fever (acetaminophen) but not aspirin; so that platelets function will not be impaired. Steroids in DSS are not helpful. With the earliest suspicion of threatened severe illness, an intravenous line should be placed so that fluids can be provided. Monitoring of blood pressure, haematocrit, platelet count, hemorrhagic manifestations, urinary output, and level of consciousness is important. Plasma leakage in DHF is very rapid and the haematocrit may continue to rise even while intravenous fluids are being administered; however, the "leaky capillary" period is short and intravenous isotonic solutions are usually required for only 1–2 days (Rigau-Perez et al., 1998).

Monitoring should be continued for at least a day after defervescence. Once the patient begins to recover, extravasated fluid is rapidly reabsorbed, causing a drop in haematocrit. Before discharge, the patient should meet the following criteria: absence of fever for 24 h (without antipyretics) and a return of appetite; improvement in the clinical picture; hospital care for at least 3 days after recovery from shock; no respiratory distress from pleural effusion or ascites; stable haematocrit; and platelet count greater than 50,000/µL (Rigau-Perez et al., 1998).
2.8. Pathogenesis

2.8.1. Integral Hypothesis:

For years, DHF pathogenesis has been a controversial matter. Some workers argued that secondary infection was the main factor in the severity of this disease, whereas others pointed to viral virulence (Hammon, 1973; Sangkawibha et al., 1984; Rosen 1989; Halstead, 1989; Kouri et al., 1987). Currently, the major view is that secondary infection is one of the risk factor for DHF; however, other factors such as viral virulence and host characteristics are also of utmost importance. DHF occurs as a result of a very complex mechanism where virus, host, and host immune response interrelate to give this severe disease (Guzman and Kouri, 2008). An integral hypothesis for the development of DHF epidemics was published taking into account the international experiences on DHF (Fig. 4). The intersection of three groups of factors (host, viral, and epidemiological factors) determine the occurrence of a DHF epidemic.

Fig. 4: Integral hypothesis for occurrence of DHF epidemic (Guzman and Kouri, 2008).
2.8.2. **Immunology and Immunogenetics:**

A. Host Immune factors

Large body of evidence, mostly obtained *in vitro*, suggested that Antibody dependent enhancement (ADE) leads to development of DHF (Halstead, 1970; Gubler, 1998). Briefly, infection with a single dengue serotype induces both serotype-specific CD8+T lymphocytes and serotype-cross-reactive CD4+memory T cells. These cross reactive heterotypic, nonneutralizing antibody binds with dengue virus, facilitating the entry of the virus into cells of the monocytic line leads to development of DHF. These data, along with epidemiologic observations that the majority of patients with reported DHF cases were experiencing a secondary infection, form the basis for this hypothesis. The lack of a good animal model for human disease and limitations of human clinical studies have made it difficult to confirm this hypothesis.

Kurane and Ennis (1992) have proposed a model of immunopathogenesis based on these observations. Briefly, it is hypothesized that dengue virus infections of monocytes/macrophages is enhanced by ADE. This enhancement is facilitated by the fact that the dengue virus-specific CD4+T lymphocytes produce interferon (IFN)-γ, which in turn up-regulates the expression of Fc receptors. The increased number of dengue virus-infected monocytes/macrophages results in increased T-cell activation, which results in the release of increased levels of cytokines and chemical mediators. These workers hypothesized that the rapid increase in the levels and the synergistic effects of mediators such as tumour necrosis factor (TNF) -α, interleukin (IL)-2, IL-6, IFN-γ, platelet activating factor, C3a, C5a, and histamine induce increased vascular permeability, plasma leakage, shock and malfunction of the coagulation system, which may lead to haemorrhage.

In summary, available evidence suggests that both viral and host immune factors are involved in the pathogenesis of severe dengue disease. Unfortunately, the role of each factor is not fully understood as stated earlier and the lack of an animal model makes this a difficult area to study.
B. Human Leucocyte Antigen and Dengue viral infection

Little is known of the role of human leucocyte antigen (HLA) alleles or non-HLA alleles in determining resistance, susceptibility or the severity of acute viral infections. Dengue fever and DHF are suitable models for immunogenetic studies, yet only superficial efforts have been made to study dengue disease to date. DF and DHF can be caused by both primary and secondary infections by any of the four serotypes of the dengue virus. Differences in host susceptibility to infectious disease and disease severity cannot be attributed solely to the virus virulence. In this context, host factors are of utmost importance, determining the final outcome of the infection (Guzman et al., 2000).

B.1. HLA and DF:

Increased expression of HLA class I and II molecules on infected cells has been reported for flaviviral infections, including dengue viral infection. It is possible that the level of the immune response generated against virus peptides presented by HLA molecules may be responsible for the immunopathology of dengue viral infection (King and Kesson, 2003). CD8+ cytotoxic T lymphocytes (CTLs) play an important role in controlling virus infected cells. The HLA class I antigens loaded with viral antigen-derived peptides along with costimulatory receptor/ligand stimuli mediate interactions between CD8+T cells and target cells. To escape recognition and destruction by CD8+T lymphocytes, viruses have developed strategies to inhibit the expression and/or function of HLA class I antigens. Thus, HLA class I molecules restrict CD8+CTL function and mediate immune responses against ‘endogenous’ antigens and virally infected targets. HLA class I alleles consist of HLA-A, -B and -C. Its products have a wide distribution and are present on the surface of all nucleated cells and platelets. HLA-A*0203 and HLA*B*52 were associated with less severe DF in patients with secondary infections. Moreover, HLA-B44, -B62, -B76 and -B77 also appear to be protective against developing clinical disease after secondary DV infection (Stephens et al., 2002).

HLA class II molecules are involved in the presentation of ‘exogenous’ antigens to T helper cells. Class II HLA products consist of HLA-D, -DR, -DP and -DQ. They are distributed on B cells, macrophages, dendritic cells, Langerhan’s cells and activated T
cells. LaFleur et al. (2002) suggested that among Mexicans, HLA-DR4 may be a genetic factor that is protective against DHF.

**B.2. HLA and DHF:**

Polymorphisms in the HLA class I region gene are associated with DHF disease susceptibility. Chiewsilp et al. (1981) were the first to report an association between HLA class I and the severity of DV infection, followed by Paradoa Perez et al. (1987), who showed a significant difference in HLA-A1, HLA-B blank, HLA-Cw1 and HLA-A29 antigens in DHF when compared with normal control group. Recent studies confirm that classical HLA class I alleles are associated with the clinical outcome of exposure to DV, in previously exposed and immunologically primed individuals (Stephens et al., 2002). A study by Sierra et al. (2007) found a significant association between HLA class I polymorphism and DHF disease susceptibility, but polymorphism in the HLA-DRB1 was associated with protection.

T-lymphocyte activation during dengue is thought to contribute to the pathogenesis of DHF. Gagnon et al. (2001) examined T-cell receptor Vb gene use by a reverse transcriptase-PCR assay in DF and DHF and found that there exists a borderline significance between them. These data suggest that the differences in T-cell activation in DHF and DF are quantitative rather than qualitative and that T-cells are activated by conventional antigen(s) and not a viral superantigen.

**B.3. HLA class III and DHF:**

Genes in the class III region encode a number of proteins, including complement proteins (C4A, C4B and C2), TNF-α and TNF-β and heat shock proteins (Cooke and Hill, 2001). Loke et al. (2001) studied promotor polymorphisms in the TNF-α gene but did not find an association with DHF. However, Fernandez-Mestre et al. (2004) studied a single-nucleotide polymorphism and reported a significant increase of the TNF-308A allele in patients with DHF.
B.4. Non-HLA genetic factors and DHF:

A few studies have investigated the association between susceptibility to DHF and polymorphic non-HLA alleles, for example vitamin D receptors (VDR), Fcγ receptor II (FcγRII), IL-4, IL-1 repeat alleles (IL-1RA) and mannose binding lectin (MBL). All subclasses of IgG have a widely distributed Fcγ receptor to mediate antibody-dependent enhancement. A few infections are associated with an arginine to histidine substitution at position 131 of the FcγRIIA (Fijen et al., 1993; Sanders et al., 1994), whereas less susceptibility to DHF has been reported with the homozygotes for the arginine variant at position 131 of the FcγRIIA gene (Loke et al., 2002).

The immunoregulatory effects of 1,25-dihydroxyvitamin D3 (1,25D3), including activation of monocytes, stimulation of cellular immune responses, and suppression of immunoglobulin production and lymphocyte proliferation, are mediated by the VDR gene (MacDonald et al., 1994). Expression of VDR may affect susceptibility to DHF as activated B and T lymphocytes express VDR and 1,25D3 affects monocytes, the main sites of DV infection and replication (Halstead & Rourke, 1977). The t allele at position 352 of the VDR gene was associated with resistance to severe dengue, although the exact mechanism needs to be explored (Loke et al., 2002). While several mutations in the mannose binding protein gene have been associated with viral infections (Ji et al., 2005; Thio et al., 2005), they did not have any effect on susceptibility to DHF (Loke et al., 2002).

C. Transporter associated with Antigen Processing gene and immunity

The transporter associated with antigen processing (TAP) is a protein that delivers cytosolic peptides to the lumen of the endoplasmic reticulum (ER) where they associate with MHC class I molecules (McCluskey et al., 2004). The TAP heterodimer comprises TAP1 and TAP2 proteins, members of the ATP-binding cassette (ABC) family of transporter molecules, which contain multiple transmembrane (TM) spanning segments and a cytoplasmic nucleotide-binding domain. Peptides bind to the cytosolic loops between TM segments 4–6, involving both TAP1 and TAP2 (Bauer and Tampe., 2002).
C.1. TAP polymorphism and peptide selection:

The TAP loci are linked and lie adjacent to the genes encoding the two low molecular weight polypeptide (LMP) immunoproteasome subunits, LMP2 and LMP7 in the class II region of the MHC. Tapasin is also encoded in the MHC near HLA-DP. This observation suggests the co-evolution of the linked genes controlling the creation of peptide antigens in the cytoplasm (immunoproteasomes LMP2 and 7), the capture and transportation of these peptides into the ER (TAP1 and TAP2) and then their presentation to T cells (MHC class I molecules) (Abarca et al., 2002). One consequence of the linkage of these genes might be their coordinated regulation by cytokines, which might benefit from open chromatin or shared promoter elements, such as in the intergenic region between TAP1 and LMP2 (Dovhey et al., 2000; Seliger et al., 2002).

Another explanation for MHC linkage of the TAP loci could involve the selection of favorable combinations of TAP alleles with alleles of the immunoproteasome to customize peptide specificities for polymorphic MHC class I molecules. Human TAP selects peptides that are generally well suited to the binding preferences of polymorphic HLA class I molecules, suggesting the co-evolution of these genes independent of their linkage (Obst et al., 1995).

Sequence polymorphism has also been reported in genes of rats, mice, and humans (Fig. 5). Recent studies of rat TAP proteins have provided clear evidence that TAP polymorphisms can have pronounced functional effects, which arise during the induction of allospecific major histocompatibility complex class I restricted CTL responses (Schumacher, 1994; Ford, 2004). Furthermore, mutation analysis of the rat TAP2 gene has shown that even a single substitution of an amino acid can result in dramatic change in substrate selectivity (Daniel et al., 1997; Momburg, 1994). There are six human TAP1 and four humanTAP2 alleles formally recognized but other rare alleles were also reported (Powis et al., 1993). Polymorphism in human TAP genes generally results in differences in only one or two amino acids, and these are scattered throughout the protein (Fig. 5). The location of TAP polymorphisms does not rule out functional
differences in peptide selection and this might explain the association of TAP polymorphism with immune disorders (Obst et al., 1995).

![Diagram of TAP molecules]

Fig. 5: A topological model of human TAP molecules (McCluskey et al., 2004). Naturally occurring allelic polymorphism is highlighted on the topological model by red stars. The large red star at the carboxyl terminus of TAP2 denotes a deletion of 18 residues in TAP2*0101.

C.3. TAP as target of viral subversion:

The TAP, and associated components of the peptide loading complex (PLC), are not only essential for direct antigen presentation to CD8+ T cells, but are also required for the cross-presentation of exogenous antigen delivered to the cytoplasm via phagosomes (Lehner et al., 2004; Houde et al., 2003). The large dsDNA herpes viruses are particularly adept at immune evasion through subversion of antigen presentation (Boname et al., 2004). Impaired TAP expression can be achieved transcriptionally or by specific interference of TAP function by viral proteins. For example, during lytic pseudo-rabies virus infection, the product of the virion host shut-off (vhs) gene, UL41, induces degradation of cellular mRNAs, including those encoding MHC class I and TAP (Ambagala et al., 2003). During flavivirus infection, transient increase in peptide transport through TAP has been reported and its biological significance in viral evasion of natural killer cell has also been recognized (Momburg et al., 2001). However no studies are available pertaining to dengue viral infection and TAP protein mediated peptide selection.
studies are available pertaining to dengue viral infection and TAP protein mediated peptide selection.

C.4. TAP gene polymorphism and viral infections:

In hepatitis C virus infection, TAP2*0103 was found to be closely associated with low serum ALT activity and viral load among Japanese patients (Kuzushita et al., 2001). The potential role of TAP gene for susceptibility to hepatitis B viral (HBV) infection revealed that TAP1^637 and TAP2^651 sites were associated with the risk of HBV infection (Xu et al., 2007). Isoleucine at TAP2^379 was found to carry an increased risk for esophageal carcinoma development in HPV infected individuals (Cao et al., 2005).

C.5. TAP gene polymorphism and dengue viral infection:

Role of HLA gene polymorphisms in different clinical spectrum of dengue infection leads to consideration that the TAP gene polymorphisms could contribute additional information on genetic susceptibility to DHF/DSS (Chaturvedi et al., 2006). However to best of our knowledge no studies are available on association between TAP gene polymorphisms and dengue viral infection.

2.9.3. Human platelet antigens and their importance in thrombocytopenia and abnormal coagulatory profile:

A. Platelet specific glycoprotein complexes and its polymorphisms

The adhesion of platelets is mediated through a superfamily of membrane, usually grouped into glycoprotein (GP) complexes (Kapalan et al., 1991). The glycoprotein complex, GPIIb/IIa is the most abundant receptor in the platelet membrane and is known as the fibrinogen receptor. One of the most frequent alloantigen that is expressed on GPIIIa is known as Human Platelet Antigen-1 (HPA-1). HPA-1a/b polymorphism was identified as a single nucleotide change (T/C) causing an amino acid substitution (Leucine33Proline). The most frequent antigen is termed as ‘a’ and the other is designated as ‘b’. Similarly GPIbα is the largest polypeptide in the GPIb-IX-V glycoprotein complex and contains the binding region for von Willibrand Factor (vWF).
It has amino acid dimorphism (Threonine\textsubscript{45}Methionine) known as human platelet antigen-2 (HPA-2) that lies adjacent to the vWF-binding site.

**B. Clinical implications on HPA polymorphisms**

**B.1. Role of autoantibody-mediated platelet destruction in thrombocytopenia:**

Platelet GPs are suitable targets of immune recognition and immune attack. Individuals missing a certain HPA alloepitopes are immunized against them following the transfusion of platelets or during pregnancy with the antigen-positive fetus (Brussel et al., 1997, Porcelijn et al., 1998, Kroll et al., 1998). Usually the platelet-specific antibody produced during the above said clinical settings binds to the corresponding antigen and enables the removal of affected platelets (Stratton et al., 1989). HPA-1 is responsible for neonatal alloimmune thrombocytopenia (NAIT) in approximately 80% of the cases (Smith et al., 1999). The second commonest platelet antigen involved in NAIT is HPA-5b followed by HPA-3 (Smith et al., 1999, Kapalan et al., 1991). Alloantibody specific for HPA-1a was found to be associated with established Post-transfusion purpura (PTP) cases (Shulman et al., 1961). Certain alloepitopes, such as HPA-2a, can also become a preferential target of the autoimmune attack compared to their allelic pair HPA-2b (Thude et al., 1999).

**B.2. Influence of HPA-2 polymorphism on thrombosis:**

The HPA-2 system is of clinical interest, because platelet-specific alloantibodies have been implicated in the pathogenesis of NAIT, PTP and refractoriness to HLA-matched platelet transfusion (Kiefel et al., 2001). Several other clinical studies were performed to determine whether the HPA-2 system is associated with an increased risk of thrombosis due to influence of the HPA-2 polymorphisms on the vWF-GPIb\(\alpha\) interaction. Although earlier study failed to demonstrate the HPA-2a/2b or HPA-2b/2b genotype as a risk factor (Carlsson et al., 1997), Baker et al. (2001) found an association of the HPA-2a/2b or HPA-2b/2b genotype with ischemic stroke.
C. Etiopathogenesis of Thrombocytopenia in dengue infection:

Concomitant with endothelial permeability in dengue infection is a marked thrombocytopenia. However, the exact cause for thrombocytopenia, seen often in DF and always in DHF, is unclear. It has been proposed that dengue infection results in the transient suppression of haematopoiesis, presumably to limit the damage to progenitor cells during the elimination of infected cells (Russa and Innis, 1995). This is supported by the finding that growth and division of cord blood mononuclear cells was inhibited by dengue infection, via the effects of macrophage inflammatory protein-1α (MIP-1 α) (Murgue et al., 1998). It has been demonstrated that dengue virus binds to platelets in the presence of virus specific-antibody, suggesting that immune-mediated clearance may account for the observed thrombocytopenia (Wang et al., 1995). A similar mechanism of immune-mediated clearance initiated by anti-virus antibody, which cross-reacts with platelets, has also been proposed by Lin et al. (2001). An analysis of dengue infected patient sera revealed the presence of anti-NS1 antibodies, IgM but not IgG, which cross-reacted with human platelets. The antibodies were shown to not only be able to induce complement mediated lysis but also to inhibit platelet aggregation. The levels of these autoantibodies were higher in DHF patients than in DF patients and persisted for several months after illness (Lin et al., 2001).

Other studies have demonstrated that anti-platelet antibodies were also produced in a mouse model of dengue infection and thrombocytopenia (Huang et al., 2000). Anti-dengue antibodies which cross-react with other components of the coagulation system including plasminogen (Markoff et al., 1991; Chungue et al., 1994) and fibrinogen (Falconar, 1997) and also endothelium (Lin et al., 2002) have also been described. Immune-mediated clearance, and other mechanisms, could result in the disruption of the coagulation system as suggested by the higher tissue-type plasminogen activator to plasminogen activator inhibitor ratio seen in DHF patients (Lei et al., 2001; Huang et al., 2001).

Together, these studies suggest an immune mediated clearance of platelets similar to that in the autoimmune thrombocytopenic purpura might be associated with dengue
infection. Interestingly, chronic autoimmune thrombocytopenic purpura is associated with elevated concentrations of IL-2, IL-10 and IFN-γ in the serum (Semple et al., 1996). While anti-platelet antibodies in dengue patients appear to persist beyond illness, it is perhaps only in the presence of high concentrations of pro-inflammatory cytokines and mediators and activated complement, as in DF and DHF, that they lead to platelet clearance. The clearance of platelets may then contribute to vascular leakage following cytokine and mediator-induced endothelial permeability. Hence these observations lead to the contemplation that HPA 1 and 2 gene polymorphism might be one of the factors associated with the etiopathogenesis of thrombocytopenia and aberrant coagulation phenomenon seen in dengue viral infection. To best of our knowledge no studies are available on association between HPA gene polymorphisms and dengue infection.

2.9.4 Production of reactive oxygen species and inflammatory mediators during dengue infection:

A. Generation of Free radicals and its physiological role

Free radicals are compounds / molecules / atoms which are having one or more unpaired electrons. They are highly reactive chemical species, trying to combine with any compound they come across, to satisfy their valency. They have very short half life with high damaging activity toward macromolecules like lipids, proteins and nucleic acids (Berlett and Stadtman, 1997). The free radicals may be either derivatives of oxygen or derivatives of other molecules like reactive nitrogen species (RNS). Most important free radicals in biological system are derivatives of oxygen species which are called as reactive oxygen species (ROS).

Phagocytic cells such as macrophages and neutrophils, on activation, undergo an oxidative burst that produces highly toxic ROS that are designed to kill the invading pathogens. This oxidative burst is mediated by the NADPH oxidase system, and results in a marked increase in oxygen consumption and the production of superoxide (O2−). This allows the concentrated release of oxidants formed subsequently. Superoxide is converted to hydrogen peroxide (H2O2) either spontaneously or more rapidly when catalyzed by
superoxide dismutase, an enzyme that occurs in two isoforms, one of which is inducible by inflammatory cytokines such as TNF-α (Berlett and Stadtman, 1997).

ROS are produced during normal aerobic cell metabolism, have important physiological roles in maintaining cell redox status, and are required for normal cellular metabolism including intracellular signaling pathways and the activity of transcription factors such as NF-κB, activator protein 1 and hypoxia-inducible factor-1α (HIF-1α) (Baldwin, 1996). NF-κB is a dimeric transcription factor that is present in the cytoplasm of most resting cells but that upon activation can undergo rapid nuclear translocation and induce gene expression. Some of these agents that are capable of inducing NF-κB activity include viral genes such as (Human immunodeficiency virus (HIV)-1, Cytomegalovirus), immuno- receptors (IL-2 receptor α-chain, T cell receptor β2), cell adhesion molecules, cytokines and growth factors, chemokines, acute phase proteins, oxidative stress- related enzymes and anti-apoptotic proteins (Baeuerle and Baichwal, 1997). In addition, ROS produced by phagocytes also seem to have important physiological roles in priming the immune system. The physiological production of ROS by phagocytes in response to antigen affects T cell–antigen interactions and possibly induces apoptosis of autoreactive antigenic T cells, thereby preventing autoimmune responses (Larbi et al., 2007).

B. Oxidative stress and Macromolecular damage

B.1. Oxidative stress and lipid peroxidation:

The major consequences of oxidative stress are DNA damage, lipid peroxidation and oxidative protein modification, which may alter cellular integrity and affect cell signaling. Reactive oxygen species, when generated close to cell membranes, can induce lipid peroxidation (oxidation of membrane phospholipids) and the accumulation of their products including malondialdehyde, 4-hydroxy-2-nonenal, acrolein and F2-isoprostanes. Some of the chemically and metabolically stable lipid oxidation products have been used as in vivo biomarkers (Esterbauer et al., 1991). The unsaturated hydroperoxides generated from peroxidation of polyunsaturated lipids can break down, usually in the presence of reduced metals or ascorbate, to a host of mono- and bi-functional reactive aldehydes.
Evidence is accumulating for these aldehydes are being involved in many pathophysiological effects associated with oxidative stress by NFkB activation (Fig. 6).

![A schematic diagram showing NFkB activation and regulation by lipid peroxides (Suematsu et al., 1981).](image)

**Fig. 6:** A schematic diagram showing NFkB activation and regulation by lipid peroxides (Suematsu et al., 1981). LP = lipid peroxides; CYTO = proinflammatory cytokines; ROS = reactive oxygen species; NFkB = nuclear factor kB; NIK = NFkB inducible kinase; IkB = inhibitory protein IkB; TNF-α = tumour necrosis factor α; IL-1β = interleukin-1β; IL-10 = interleukin-10.

***B 2. Oxidative stress and protein damage:***

Free Radicals have high affinity towards molecules with nucleophilic character. They covalently bind to these compounds and this may lead to the loss of function of the molecules attacked, typical examples are enzymes. Enzymes may lose their catalytic activity and the receptors may be inactivated after being attacked by free radicals. Covalent binding to a protein may initiate allergic reactions, since a protein that has been structurally modified by a foreign metabolite might exhibit antigenic properties (Younes, 1999).

Direct damage to proteins or chemical modification of amino acids in proteins during oxidative stress and glyco-oxidation can give rise to protein carbonyls, which may
serve as biomarkers for general oxidative stress (Stadtman & Berlett, 1998). Oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains leads to formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative. Direct oxidation of lysine, arginine, proline, and threonine residues may also yield carbonyl derivatives. In addition, carbonyl groups may be introduced into proteins by reactions with aldehydes (4-hydroxy-2-nonenal, malondialdehyde) produced during lipid peroxidation or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins (Serdar et al., 2002). Carbonyl groups as the evidence of protein oxidation have been associated with oxidative stress in many diseases (Berlett and Stadtman, 1997). Thus the presence of carbonyl groups in proteins has therefore been used as a marker of ROS-mediated protein oxidation.

B.3. Oxidative stress and Sialic acid:

Sialic acid (N-acetylneuraminic acid) is a negatively charged nine carbon monosaccharide with a molecular weight of 309 and a pKa value of 2.6. It is commonly attached by an α-glycosidic linkage to the non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids (Schauer, 1995). Bound sialic acid is of major importance in cell biology because of its external position on glycoproteins and glycolipids, and on the outer cell membranes. Sialic acid participates in the stabilization of the conformation of glycoproteins and cellular membranes. The structural diversity of sialic acids can also determine or modify recognition by antibodies, by a variety of endogenous sialic acid-binding lectins, as well as by microbial agglutinins, toxins, and adhesins (Varki, 1997; Karlsson, 1998). Sialic acid is involved in many other vertebrate functions, including cell-cell interactions in processes such as the trafficking of blood cells during inflammation, the control of neuronal plasticity, and the interactions of tumour cells during the metastatic process (McEver, 1997; Rutishauser and Landmesser, 1996). Furthermore sialic acid contributes to the regulation of the permeability of the vascular endothelium (Schauer, 2000).
It has been suggested that ROS are capable of degrading glycoproteins, which are important components of extracellular matrices, thus leading to an impairment of proteoglycan functions (Moseley et al., 1995). It was also reported that ROS are capable of modifying glycoprotein *in vitro* (Mccord, 1974; Halliwell, 1978). It was recently reported that oxidative damage by copper ion and H$_2$O$_2$ causes the site-specific degradation of N-linked oligosaccharide at N-acetylglucosamine residues (Eguchi et al., 2002). Hence there is accumulating evidence that sialic acid, as well as proteins, lipids, and DNA, may be a target molecule of ROS.

The relation between inflammation and sialic acids is that the most of the inflammatory proteins are sialoglycoproteins which are likely to lose the sialic acid residue in conditions of oxidative stress, cancer, arteriosclerosis, diabetes, and inflammation. (Tanaka et al., 1998; Yamamoto et al., 1995; Crook et al., 2000; Painbeni et al., 1997). ROS and peroxylipids are also related to inflammation as major exacerbation factors (Nagata, 2005). It is considered that therefore, the opportunity for free sialic acid to be produced and to encounter H$_2$O$_2$ will be increased by inflammation. Iijima et al., (2007) demonstrated that the sialic acid counteracts the cytotoxicity of H$_2$O$_2$, and this antioxidant activity was a result of a direct chemical reaction, whereby sialic acid reduces H$_2$O$_2$ in the culture media.

C. Oxidative stress and viral infections

Peterhans (1979) was among the first workers to demonstrate that a Sendai virus could generate ROS from phagocytes. Later, influenza viruses and paramyxoviruses were shown to activate monocytes and polymorphonuclear leukocytes *in vitro* to generate ROS (Peterhans et al., 1987). Activated phagocytes release not only ROS but also the pro-oxidant cytokine, TNF-α. In influenza virus infection, children with Reye's syndrome frequently have release of TNF-α from macrophages (Gloenbock et al., 1991; Cooperstock et al., 1975).

The observation that infection of mice with influenza A resulted in a decrease in the total concentration of lung glutathione and the antioxidant vitamins C and E provided evidence that viral infection was associated with oxidant stress *in vivo* as well as *in vitro*.
(Hennet et al., 1992). It is reported that children with Reye's syndrome exhibit increased serum lipid peroxides and lipofuscin-like substances in their livers (Brown et al., 1982). Human hepatocytes infected with influenza B virus showed depression of mitochondrial respiration when incubated with supernatant media from activated macrophages. (Schwarz et al., 1994). Because the depressant effects were inhibited by anti-TNF-α monoclonal antibody and by the antioxidant, vitamin E, it was concluded that infection had rendered the cells more susceptible to the pro-oxidant effects of TNF-α (Jacoby and Choi, 1994).

Observations about interactions between ROS and HIV illustrate the complex nature of these interactions. HIV-seropositive humans exhibit decreased concentrations of naturally occurring antioxidant reductants such as total acid-soluble thiols, cysteine, and glutathione in plasma, peripheral blood monocytes, and lung epithelial-lining fluids (Buhl et al., 1989; Staal et al., 1992; Eck et al., 1989; Roederer et al., 1992). Roederer et al. (1991) have demonstrated that T cells with high intracellular glutathione levels are selectively lost as the HIV infection progresses. Plasma malondialdehyde is increased in patients with HIV, suggesting the infection results in oxidative stress for the host lipids (Srnnerborg et al., 1988). Advanced HIV infection is associated with increased serum catalase, an activity correlating with increased scavenging of $\text{H}_2\text{O}_2$, which may serve to compensate for the deficiencies of glutathione and other antioxidants reported above (Left et al., 1992).

D. Oxidative stress and Dengue viral infection

While pathogenesis of DHF is not clear, oxidative stress appears to play a role. Gil et al. (2004) analyzed serum levels of total antioxidant status (TAS), peroxidation potential (PP), glutathione (GSH), lipid peroxidation measured as hydroperoxides, and MDA and 4-hydroxyalkenals (MDA + 4-HDA), as well as antioxidant enzymatic activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx), in 22 patients with dengue fever. High levels of PP, MDA + 4-HDA, SOD and low levels of GPx and total hydroperoxides were found in patients in comparison with controls. In patients with dengue fever, increases in plasma concentrations of retinol and beta-carotene were seen,
whereas decreases were observed for glutathione and total antioxidant status (Klassen et al., 2004). In dengue infection, high levels of glutathione peroxidase correlated with spontaneous bleeding and intensity of oxidative stress was found to influence the clinical presentation of dengue (Rojas et al., 2007). However, previous reports available on this aspect, although describe the changes in DF cases, lack details in DHF and DSS (Gil et al., 2004; Klassen et al., 2004; Rojas et al., 2007; Ray et al., 1999).

E. Cytokines and Oxidative stress

E.1. Role of cytokines in the immunopathogenesis of DHF:

Evidences suggest a massive T-cell activation during DHF, which could explain partly if not totally the mechanism of plasma leakage through cytokine production and infected cell lysis by CD4+ and CD8+ dengue-specific T lymphocyte (Rothman and Ennis, 1999; Hober et al., 1998). Cytokines could be released either directly from monocytes/macrophages as a result of infection or after interactions between infected and immune cells, or both. Cytokines that may induce plasma leakage such as IFN-γ, IL-2, and TNF-α are increased in DHF cases (Hober et al., 1998; Green et al., 1999). Also, IFN-γ enhances uptake of dengue particles by target cells through increasing Fc cell receptors (Kontny et al., 1988). Mukerjee et al. (1995) detected a cytotoxic factor of 22–25 kDa in sera of DHF patients. The factor was able to increase capillary permeability in mice and was capable of reproducing in mice all the pathological lesions that are seen in human beings.

The role of TNF-α in the pathogenesis of the disease is critical, and it probably initiates several processes relating to plasma leakage and haemorrhage (Anderson et al., 1997). Avirutnan et al. (1998) have shown that infection of human endothelial cells with dengue virus induces the secretion of IL-8 and formation of nonlytic complement complexes. Release of higher amount of IL-6 and IL-10 was also observed. Both cytokines could activate endothelial cells modulating the expression of the adhesion molecules as well as altering endothelial cell morphology (King et al., 2000). Complement activation as a result of immune complexes (virus-antibody) or immune
activation and cytokine production could be also involved in the mechanism of plasma leakage.

E.2. TNF-α and Oxidative stress:

TNF-α can either be released from activated phagocytes into the circulation, or in some infections it can be synthesized in infected host cells. In either case, TNF-α can act on host cell mitochondria, producing a pro-oxidant effect as it has been shown to inhibit mitochondrial respiration at Site II, the site of superoxide production (Schulze-Osthoff et al., 1992). TNF-α also acts to release NF-κB from the cytoplasmic inhibitor protein IκB and translocates to the nucleus where the transcription factor binds to DNA, inducing the transcription of cellular and/or viral genes (Schreck et al., 1991). During infection, ROS increases the TNF-α secretion by NF-κB induction (Fig. 6). The likelihood of oxidative stress in dengue viral infection might up regulate the production of certain proinflammatory cytokines which might result in an exacerbation of dengue disease. However, no in vivo studies are available in context of relationship between oxidative stress and inflammatory response in dengue.
Role of Transporter Associated with antigen Processing (TAP) and Human Platelet Antigen (HPA) gene polymorphism in diverse pathogenesis of dengue viral infection.