Studies on biomolecules associated with vascular dysfunction in Dengue Haemorrhagic Fever / Dengue Shock Syndrome

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By

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DECLARATION

The research work embodied in this thesis entitled **"Studies on biomolecules associated with vascular dysfunction in Dengue Haemorrhagic Fever / Dengue Shock Syndrome"** being submitted to Jadavpur University, Kolkata has been carried out at ICMR-National Institute of Cholera and Enteric Diseases, Beleghata, Kolkata under the supervision of Dr. Provash Chandra Sadhukhan, Virus Laboratory, ICMR- National Institute of Cholera and Enteric Diseases. This work is original and has not been submitted in part or in full, for any degree or diploma to this or any other university.

Priya Kumari Verma
Index No.: 114/19/Life Sc./26

Dedicated to
my family

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Priya Kumari Verma

~ Priya Kr. Verma

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Thesis Title: Studies on biomolecules associated with vascular dysfunction in Dengue Haemorrhagic Fever / Dengue Shock Syndrome

Abstract

Dengue fever is a rapidly emerging tropical disease and an important cause of morbidity in its severe form worldwide. This fever remains a global health concern, causing >500,000 hospitalisations annually due to severe dengue; only in Southeast Asia, the Pacific and the Americas. This self-limiting, acute febrile illness is caused by the dengue virus and is transmitted through Aedes mosquitoes. Most dengue infections result in mild symptoms but a subset of cases progresses to severe forms, known as severe dengue [Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS)]. A wide spectrum of the pathophysiology is associated with the transition of dengue fever to severe dengue, which is driven by the host immune response.

In this doctoral study, a total of 620 samples were included. Among them 380 samples were from dengue-suspected hospital-admitted patients and 240 samples were from the early phase of dengue fever for molecular serotyping. Dengue samples were screened for the presence of dengue NS1 antigen and IgM/IgG antibodies by enzyme-linked immunosorbent assay for dengue early phase and late phase identification. From early-phase samples, viral RNA was extracted from NS1 seropositive samples and subjected to molecular serotyping, genotyping and phylogenetic analysis. Co-circulation of all four dengue serotypes with DENV-2 as the prevalent serotype in the year 2018 (76%) and 2019 (47%), whereas DENV-4 in the year 2020 (47%), DENV-3 in 2021 (72%) and 2022 (55%) were observed. Phylogenetic analysis of prevalent serotypes showed Genotype-I of DENV-4 and Genotype-III of DENV-3 whereas Genotype II of DENV-2 was the major circulating DENV strain during the study period. Dengue fever-associated clinical manifestations, biochemical parameters and liver functional profile observed among hospital-admitted dengue patients. A multivariate logistic regression approach was used for making a regression model including dengue-associated clinical symptoms. 70% of patients showed thrombocytopenia, with petechia being the most common bleeding manifestation. A significant change in trends of dengue-associated clinical manifestations and differential expression of liver functional profile with different phases of transition of dengue fever was observed in this study population.

A mass-spectrometry-based proteomic approach was used to find the candidate proteins biomolecules associated with the pathogenesis of a severe form of dengue fever. We uniquely performed this prospective study among hospital-admitted dengue-infected patients from different phases (acute and critical) of dengue fever at two time points. Pairwise patient samples were subjected to high-throughput qualitative, quantitative proteomic, protein array and bioinformatics analysis to find the novel biomolecules and elucidate their intricate molecular networks underlying the pathophysiology of severe dengue. Pathway analysis was performed using PANTHER, Reactome and KEGG databases to find significantly enriched pathways and proteins. Protein-Protein Interaction (PPI) network between the dengue virus and host proteins was depicted in the search for

proteins associated with severe dengue pathophysiology. The set of elucidated proteins was validated via Western blot, Real-Time PCR and ELISA techniques. This study has validated expression patterns of Apo AI, AII, AIV, ApoB and ApoE among the apolipoproteins and E-Cadherin (epithelial cadherin), VEGF (Vascular endothelial growth factor), FGFR1 (fibroblast growth factor receptor 1), VCAM1 (Vascular cell adhesion molecule 1), IRF3 (Interferon regulatory factor 3), IFN-γ (Interferon-gamma) and ANGPT1 (Angiopoietin 1) among the endothelial proteins and cytokines from the significant proteins list via Western blot. Ten candidate genes were selected for Real-Time PCR-based validation. After narrowing down the Real-Time PCR result, group of top six biomolecules i.e. PTX3 (Pentraxin 3), LBP (Lipopolysaccharide Binding Protein), Fibronectin, IGFBP-2 (Insulin-like Growth Factor Binding Protein-2), POST (periostin) and Serpin were validated as top-notch proteins associated with severe dengue pathophysiology via ELISA-based mass validation from hospital-admitted patient's samples. The ROC curve's logistic regression analysis of signature proteins identified via ELISA has an average empirical AUC of above 0.7 with 95% CI. Protein docking results reveal a significant interaction between viral and host proteins, which is linked to severe dengue pathophysiology. STRING analysis depicted the protein-protein interactions of candidate proteins.

In conclusion, a comprehensive understanding of severe dengue pathogenesis requires a multidimensional approach that encompasses dengue virus changing patterns in a population, viral-host interactions, immune response dynamics and vascular dysfunction mediated by protein biomolecules. The identified eminent biomolecules panel and clinical markers from the liver have the potential to enhance predictive and diagnostic accuracy, risk stratification, patient management and therapeutic interventions among severe dengue patients.

Signature of Candidate

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List of Abbreviations

	Abbreviations
DF	Dengue Fever
DHF/DSS	Dengue Hemorrhagic Fever/ Dengue Shock Syndrome
DENV	Dengue virus
NVBDCP	National Center for Vector Born Diseases
WHO	World Health Organization
^{O}N	Degree North
^{O}S	Degree South
^{O}C	Degree Celsius
E protein	Envelope protein
C protein	Capsid protein
pr-M	Pre-membrane protein
RNA	Ribonucleic Aid
ELISA	Enzyme-Linked Immunosorbent Assay
EIA	Enzyme Immuno-Assay
IFA	Immunofluorescence Assay
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
cDNA	Complimentary Deoxyribonucleic acid
NS	Non-Structural
UTR	Untranslated Region
ORF	Open Reading Frame
ER	Endoplasmic Reticulum
BSA	Bovine Serum Albumin
mM	Mili Molar
DTT	Dithiothreitol
TOF	Time of Flight
CID	Collision-Induced Dissociation
PSM	Peptide-spectrum matches
XIC	Extracted Ion Chromatogram
FDR	False Discovery Rate
ddCT	Delta-Delta Cycle Threshold
HRP	Horseradish peroxidase

CCU	Critical Care Unit
ADE	Antibody-dependent enhancement
TP	Total Protein
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CNC	Constitutional Complications
GIC	Gastrointestinal Complications
MSC	Musculoskeletal Complications
URC	Upper Respiratory Tract Complications
g/dL	gram/decilitre
U/L	unit per litre
KD	Kilodalton
Kb	Kilobase
mg	Milligrams
µg	Micrograms
µl	Microlitres
min	Minutes
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC/Orbitrap MS	Liquid Chromatography/ Orbitrap Mass Spectrometry
CID	Collision-Induced Dissociation
DDA	Data Dependent Acquisition
SWATH-MS	Sequential Window Acquisition of All Theoretical- Mass Spectra
PANTHER GO	Protein Analysis Through Evolutionary Relationships Gene Ontology
KEEG	Kyoto Encyclopedia of Genes and Genomes
NASBA	Nucleic Acid Sequence-Based Amplification
FC	Fold Change
PPI	Protein-Protein Interactions
PDGFAA	Platelet-Derived Growth Factor
TIMP 1	Tissue inhibitors of metalloproteinases
MMP	Matrix Metalloproteinase
DPP4	Dipeptidyl peptidase 4
IP 10	Interferon-Gamma Inducible Protein 10

PA1	Plasminogen Activator Inhibitor 1
LDLr	Low Density Lipoprotein Receptors
SR-B1	Scavenger Receptor B1
Apo AI	Apolipoproteins AI
AII	Apolipoproteins AII
AIV	Apolipoproteins IV
ApoB	Apolipoproteins B
ApoE	Apolipoproteins E
E-Cadherin	Epithelial Cadherin
VEGF	Vascular Endothelial Growth Factor
FGFR1	Fibroblast Growth Factor Receptor 1
VCAM1	Vascular Cell Adhesion Protein 1
IRF3	Interferon Regulatory Factor 3
IFN-γ	Interferon Gamma
ANGPT1	Angiopoietin 1
UTRs	Untranslated Regions
ADE	Antibody-Dependent Enhancement
DHHI	Dengue virus and Human Host Interactome
CD	Cluster of Differentiation
Th1	T Helper Cell 1
Th2	T Helper Cell 2
IgM	Immunoglobulin M
IgG	Immunoglobulin G
HLA	Human Leukocyte Antigen
PPRs	Pattern Recognition Receptors
TLR	Toll Like Receptors
MDA5	Melanoma Differentiate on Associated Gene 5
RIG-I	Retinoic-acid Inducible Gene 1
RDTs	Rapid diagnostic tests
IFA	Immunofluorescence assays
NTDs	Neglected tropical diseases
NO	Nitric Oxide
STAT1	Signal Transducer and Activator of Transcription

B-cell	B Lymphocytes
T cell	T Lymphocytes
dNTPs	Deoxynucleotide triphosphate
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
qRT PCR	Quantitative Real Time Polymerase Chain Reaction
PTX3	Pentraxin 3
LBP	Lipopolysaccharide Binding Protein
Fb	Fibronectin
IGFBP	Insulin-like Growth Factor Binding Protein
POST	Periostin
ROC	Receiver Operating Curve
AUC	Area Under Curve
RMSD	Root Mean Square Deviation
HADDOCK	High Ambiguity Driven protein-protein Docking
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins

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Chapter 1: Introduction

Key facts:

- *Dengue fever is the most common arboviral disease affecting humans (WHO), caused by the dengue virus, a single-stranded RNA virus belonging to the Flaviviridae family*
- *Dengue fever is endemic in tropical and subtropical regions worldwide, especially in urban and semi-urban areas. Regions with a higher incidence include Southeast Asia, the Pacific Islands, the Americas, Africa and the Caribbean.*
- *40% of the worldwide population lives in endemic areas resulting in 390 million new cases per year with 2,50,000 to 5,00,000 severe complicated forms and annually 25,000 deaths occur due to severe dengue*
- *There are four distinct serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4). Infection with one serotype does not provide immunity against the others, and subsequent infections with different serotypes can lead to more severe forms of the disease. Severe forms can be life-threatening and require prompt medical attention.*
- *Early detection of warning signs of severe dengue is crucial for timely clinical intervention. These warning signs may include severe abdominal pain, persistent vomiting, rapid breathing, bleeding and lethargy.*
- *There is no specific antiviral treatment for dengue. Management involves supportive care, including fluid replacement to address dehydration and pain relievers for fever and pain*
- *No effective vaccine available against DENV, "Dengvaxia" is available in some countries with its limitations but not approved in India*
- *Host factors play a major role in the transition from dengue fever to severe dengue; hence, intricate insight is required*
- *The genomic diversity of DENV among the circulating strains needs to be observed as they might be co-related with dengue disease severity*
- *Public health strategies to prevent severe dengue include community education, vector control programs and early detection and management of dengue case*

1. Epidemiology and burden of disease

Dengue fever is an acute systemic arboviral disease; transmitted between mosquitos and their human host globally with both endemic and epidemic transmission cycles (1). This is a major cause of morbidity and mortality in the tropical and subtropical regions of the world, making it a more serious public health concern.

1.1.a. Global Prevalence of dengue fever

Over the previous 50 years, there has been an alarming 30-fold increase in dengue cases (1). Dengue is endemic in around 129 countries such as the regions of Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific. The region's most severely impacted include the Americas, Southeast Asia and the Western Pacific, with Asia accounting for around 70% of the world's disease burden (1). Forty percent of the world's population or over 2.5 billion people, live in tropical and sub-tropical regions and are thus susceptible to dengue infection (1), (2). According to one modelling estimate, there are 390 million dengue virus infections annually, of which 96 million result in clinical manifestations (3). Explosive dengue outbreaks are happening and the disease is expanding to other regions, including Europe. In 2010, imported cases were found in three more European countries, while local transmission was first documented in France and Croatia (1). 2019 had the highest number of dengue cases ever documented worldwide, while Afghanistan saw the first known incidence of dengue transmission in 2019 (1). 753 deaths and 171,991 cases of dengue fever were reported in Africa in 2023. From 42 countries and territories in the Americas, there were 2049 fatalities and 4.1 million suspected dengue infections, with 6710 severe cases (0.16% of suspected cases) and 15 countries reporting an active outbreak. In 2023, the most confirmed cases have been recorded from Pakistan, Saudi Arabia and Oman. Even though dengue fever is not endemic in the WHO European

Region and travel is the primary cause of cases, reports of autochthonous illnesses have been made in several nations since 2010 (1), including Croatia, France, Israel, Italy, Portugal, and Spain. With the most comprehensive data available, 2500 dengue cases were reported to WHO in 2018 using the regional annual surveillance data collection technique; the majority of cases were from Germany, France, and the United Kingdom. Rare autochthonous cases and outbreaks were recorded in Italy (n = 82), France (n = 43) and Spain (n = 3) in 2023. As a result, the real number of dengue cases in 2023 is probably understated. Routine dengue testing is uncommon in Member States of the WHO European Region unless there is a travel history and clinical suspicion. There has only been one recorded death in Italy (1) connected to an imported, travel-associated case; no additional deaths tied to 2023 have yet to be reported in European countries. It is acknowledged that dengue is prevalent in ten of the eleven WHO member states in Southeast Asia. Many countries, most notably Bangladesh and Thailand, reported a considerable rise in dengue incidence in 2023 when compared to previous years (1), (4). Among the top 30 most endemic countries are Thailand, Sri Lanka, Myanmar, Indonesia, India, and Sri Lanka. In 2023 there were more cases of dengue in Thailand and Bangladesh than in 2022. Bangladesh had a significant increase in instances by November 2023, total 308,167, as opposed to 62,382 cases recorded for the whole of 2022 (1), (4). Dengue cases increased by more than 300% in Thailand, from 46,678 in 2022 to 136,655 in 2023 (1), (4). As of 2023, (1), (4), dengue disease is still present in Brazil, Colombia, the Cook Islands, Fiji, India, Kenya, Paraguay, Peru, the Philippines, the Reunion Islands, and Vietnam. Dengue was thought to be a non-fatal fever illness that had periodic, self-limiting outbreaks prior to World War II (4). Massive dengue haemorrhagic fever outbreaks have coincided with a marked change in epidemiology and disease severity after World War II. This was first observed in Asia, where the post-war period was characterised by economic boom and

urbanisation. Before the 1980s, severe dengue was a relatively rare disease (5), where the observed significant expansion of dengue has increased disease severity and is linked to the advent of several serotypes. The international burden of dengue fever in the last 5 years (**Figure 1a**) and high-risk zones of southeast Asia (**Figure 1b**) depict a global alarming risk of dengue fever.

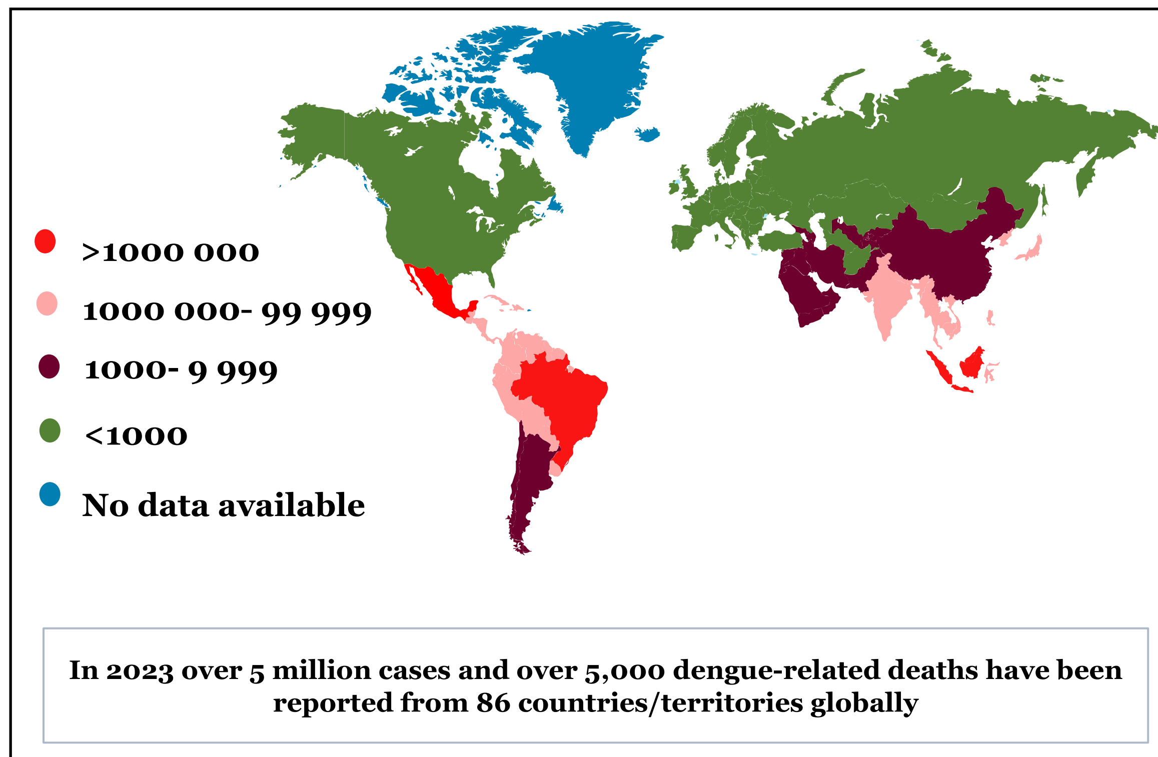

Fig.1.a: International burden of dengue fever, from year 2018 to 2023, showing southeast Asia as the 2nd most affected regions (Created as per data available on WHO website https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue)

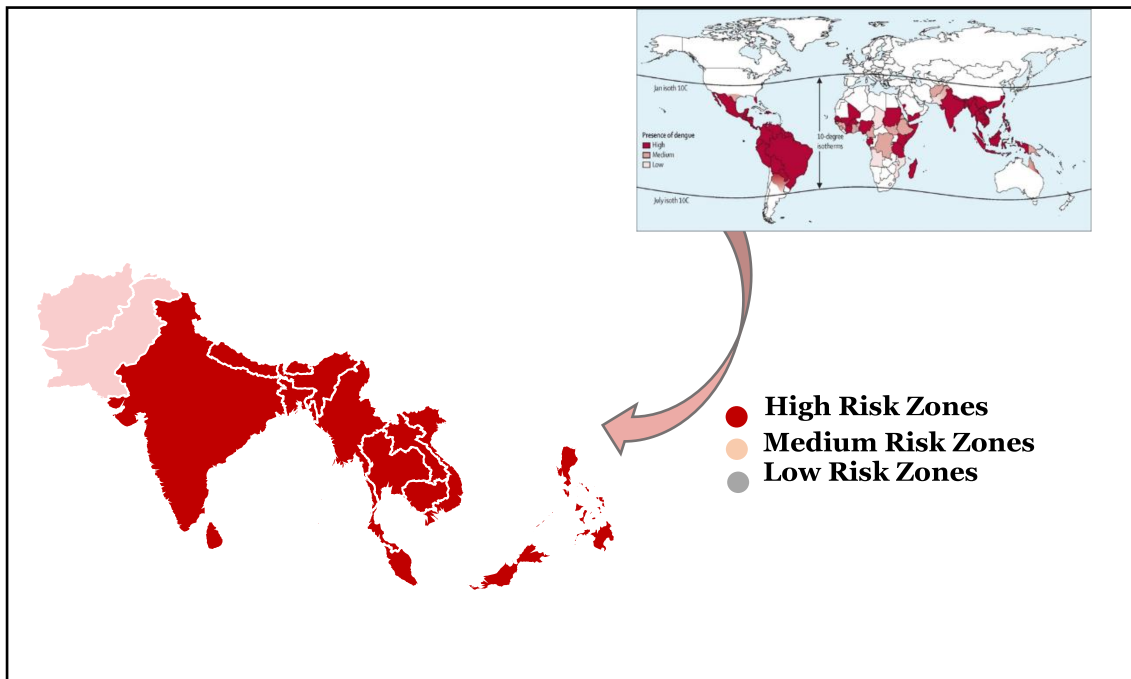

Fig. 1.b: High risk Zones in Southeast Asia, contributing maximum number of clinical dengue infections worldwide (Created as per data available on WHO website https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue)

Dengue serotype distribution can also vary greatly on a worldwide basis (6). Different serotype outbreaks may occur at different periods in different nations and locations (1). International travel and trade can help dengue and its serotypes spread. Individuals who are infected and migrate from one place to another can transfer new serotypes to areas where they were previously uncommon. Dengue vaccinations, when available, may be tailored to protect against many serotypes, as serotype co-circulation is frequent in many places. It is crucial to highlight that the distribution of dengue serotypes and their epidemiological data can provide insight into the frequency of certain serotypes in different locations.

1.1.b. Dengue situation in India

Southeast Asia (SEA) and the Western Pacific regions among all the WHO regions, contribute approximately 75% of the global burden of dengue. These are the hyperendemic hotspots for DENV infections (6), (7), (8). India is the global epicentre for Dengue, contributing approximately 34% of the clinical infections worldwide. The number of cases dramatically increased after the year 1990 and almost all the states are now endemic for the disease (8). As per the National Vector Borne Diseases Control Programme, the active Indian cases of Dengue increased strikingly by 23.21% within a mere period of 5 years (2019-2023) (9) **(Figure 2)**. Dengue is highly seasonal in India and the majority of the cases are reported during the Indian summer monsoon period, i.e. June to October (9), (10). During winter the temperature and humidity decrease, with a decline in several active cases. The reasons underlying is that temperature greatly influences the rate of mosquito hatching at different stages of their life cycle. Biting rate, mosquito survival and mortality rates are also influenced by temperature. Replication of the Dengue virus inside the vector is known to be accelerated at higher temperatures (11), (12) and humidity influences the longevity of mosquitoes (13). As a result, due to climate change, the expansion of two more months of dengue transmission risk occurs in wide geographical regions of India (14). The high population density also accelerates the secondary transmission of dengue in India. In the present decade, the expansion of dengue fever from urban to rural settings has been observed which inflicts an alarming health concern and socio-economic burden in endemic areas. Converging our focus from all over India to West Bengal, same scenario of the disease eruption was observed. The wide Gangetic districts of West Bengal, including Kolkata, Howrah, North and South 24 Parganas, Murshidabad, Nadia and Medinipur are the endemic foci of this viral disease during summer to monsoon and post-monsoon season (15). Household containers without lids, uncovered water tanks, improper sanitation and

drainage system as well as large population density contribute to the disease outbreak. The gradual climate change in this geographical location makes it far more suitable for the virus to replicate and propagate within its vector and human host (14). In an interesting study, it was also reported that co-infection of Dengue and chikungunya viruses is also causing fever (16). Dengue virus has four serotypes, DENV-1, DENV-2, DENV-3, and DENV-4. These serotype's distribution can change over time and throughout different geographies (1). Dengue serotype distribution varies from year to year and area to region. Outbreaks can be triggered by a single dominant serotype or a mix of many. Some places may have a cyclical pattern of serotype dominance, in which one serotype becomes more prevalent in some years before falling and being replaced by another. Serotype distribution is monitored by national health authorities and organisations to help guide vaccination plans and public health actions. North, West-Central, East and South India have shown periodic increases in dengue cases and deaths during the last 2-4 years (17). A comparative report of dengue sequences from India till 2018, observed that all four dengue serotypes have been co-circulating in the nation since 2000. Even though dengue sequence reporting from diverse regions of the nation is infrequent and concentrated in urban areas, the number of yearly cases and fatalities over the last two decades have correlated well with the numeral of available sequences every year. We found an upsurge in DENV-2 and DENV-4 serotypes in particular after 2011 and 2014, respectively (18), (19).

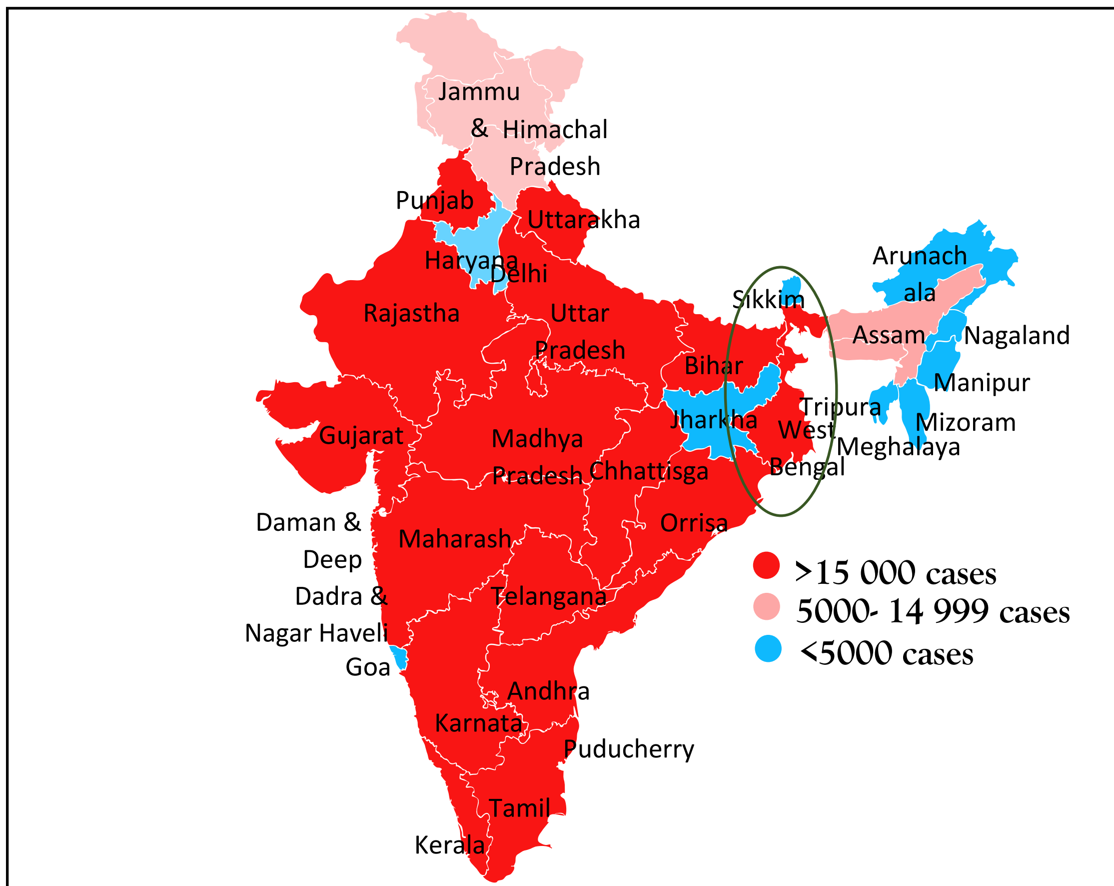

Fig. 2: National burden of dengue fever depicted categorizing the number of cases into three classes (High to low) from last five-year NVBDCP data showing India as global epicenter of dengue fever (Created as per data available at NVBDCP website https://ncvbdc.mohfw.gov.in/index1.php?lang=1&level=1&sublinkid=5776&lid=3690)

The number of sequences reported from North and South India peaked periodically at the same time as the reported cases (20). Sequential peaks in the genomes of North Indians show a pattern of serotype replacement. Due to frequent outbreaks, Delhi provided the majority of the sequences from North India (80.5%) and outbreaks in Delhi are strongly correlated with the spikes in dengue sequences in 2006, 2010, and 2013. In line with previous research, DENV-3 was the predominant strain during the epidemic in 2006 (20), whereas DENV-1 and DENV-2 serotypes were responsible for the outbreaks in 2010 and 2013 in India, respectively (20). While dengue outbreaks have been reported in East and West Central India, there are fewer sequences available from these regions, most likely as a result of insufficient genomic surveillance. However, from 2016 to 2018, all serotypes

were detected in West-Central India, whereas DENV-2 became the most common serotype in East India (22). The number of sequences in South India peaked in 2009, 2013, and 2016, and there were related peaks in the number of cases and deaths in 2009–10, 2012–13, and 2017. In conclusion, DENV-1 and DENV-3 were the most common serotypes in India up till 2012. Since then, DENV-2 has spread over much of India, with DENV-4 becoming established in South India (18). However, during the past few years, West Bengal has seen a sharp shift in the pattern of circulating predominant serotypes. The detailed trail of circulating serotypes in West Bengal is deliberated in the discussion section.

1.2.Dengue in Travellers

Travellers have a significant impact on worldwide dengue epidemiology because they introduce a variety of dengue strains and serotypes into areas where mosquitoes may spread the virus (23). Additionally, travellers do a valuable service by alerting others in advance about events occurring in other parts of the world. Travellers often transfer the dengue virus from developing tropical countries with inadequate laboratory facilities to developed nations with laboratories equipped to detect different serotypes of the virus (24). Access to research facilities can yield more detailed information about a virus, such as its serotype and even its genotypes. The systematic collecting of clinical specimens and the banking of serum or isolates may be advantageous in the future when new technologies become accessible.

Researchers looked into at month-by-month morbidity using data from the Geo Sentinel monitoring Network (www.geosentinel.org), which was gathered longitudinally over a ten-year period. Of the 24,920 sick return travellers, 33 monitoring locations saw (25). The international community can be promptly informed about the onset of epidemics in

endemic areas where there is no dengue surveillance or reporting, as well as the geographic spread of virus serotypes and genotypes to new areas, which increases the risk of severe dengue, by using sentinel surveillance to share information about dengue in travellers. The information can also assist doctors in temperate nations, many of whom lack training in clinical tropical diseases, in keeping an eye out for cases of dengue fever among recently returned tourists.

Since dengue may manifest differently in travellers than in the endemic population which is mostly composed of children with pre-existing immunity, its clinical symptoms and aftereffects can also be investigated in travellers, the majority of whom are adults and non-immune. The drawback of this kind of sentinel surveillance is that it lacks a denominator, making it impossible to assess the occurrence of actual danger. An increase in travel to dengue-endemic regions, for instance, maybe the cause of the surge in incidence among travellers. The global pattern, wherein travellers are more frequently infected in Asia, is consistent with the condition documented in European studies. Dengue is mostly a disease of active travellers, as opposed to the situation in Asia, where it primarily affects young children (26), (27), (28).

1.3. Factors associated with infestation of Dengue Virus

Adaptations in both virus and vector support the urban DENV life cycle. Vector specialization to feed on humans and to breed in artificial aqueous environments provided in urban settings are directly associated with the spread of dengue virus. Also, virus adaptation to the urban vectors and human adaptation of DENV serotypes to one another caused the escape of the cross-neutralization by heterologous antibodies and enhanced the risk of secondary dengue infection. Increased human population growth and urbanization

in cities affect vector and host densities (1), (4). Also, changes in the environment such as urban environments, especially slums provide more vector breeding habitats. Traveling of infected humans, transport of materials containing infected mosquito eggs and larvae are directly associated with geographic spread of vectors and viruses. The global incursion of Aedes aegypti and Aedes albopictus mosquitos caused a global infestation of the dengue virus.

1.4. Dengue Pathology: Clinical Diagnosis of Dengue and Severe Dengue

Dengue fever in humans can vary from extremely minor symptoms that go unnoticed known as asymptomatic dengue fever to dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS), which can be fatal. Dengue fever is also known as ***Break-bone fever or Saddleback (biphasic) fever*** (29)**.** Infection with any of the four DENV serotypes developed a wide range of clinical symptoms. The severity of the sickness is determined by the virus's serotype and strain, as well as the age, genetic background and immunological condition of the specific human host (29). Undifferentiated fever, dengue fever (DF), and dengue haemorrhagic fever (DHF) were the three types of symptomatic dengue virus infections **(Figure 3)**. Dengue fever with warning signs is further subdivided into four severity levels. Patients with grade I and II grades severity are categorized as having dengue haemorrhagic fever (DHF) and patients with grade III and IV severity defining dengue shock syndrome (DSS) (30). Many reports of challenges in using this categorization (31), which were summarised in a comprehensive literature review by Chen et al in 2018 (32), have been published. A detailed characterization of different types of dengue fever is described below in **Figure 3**.

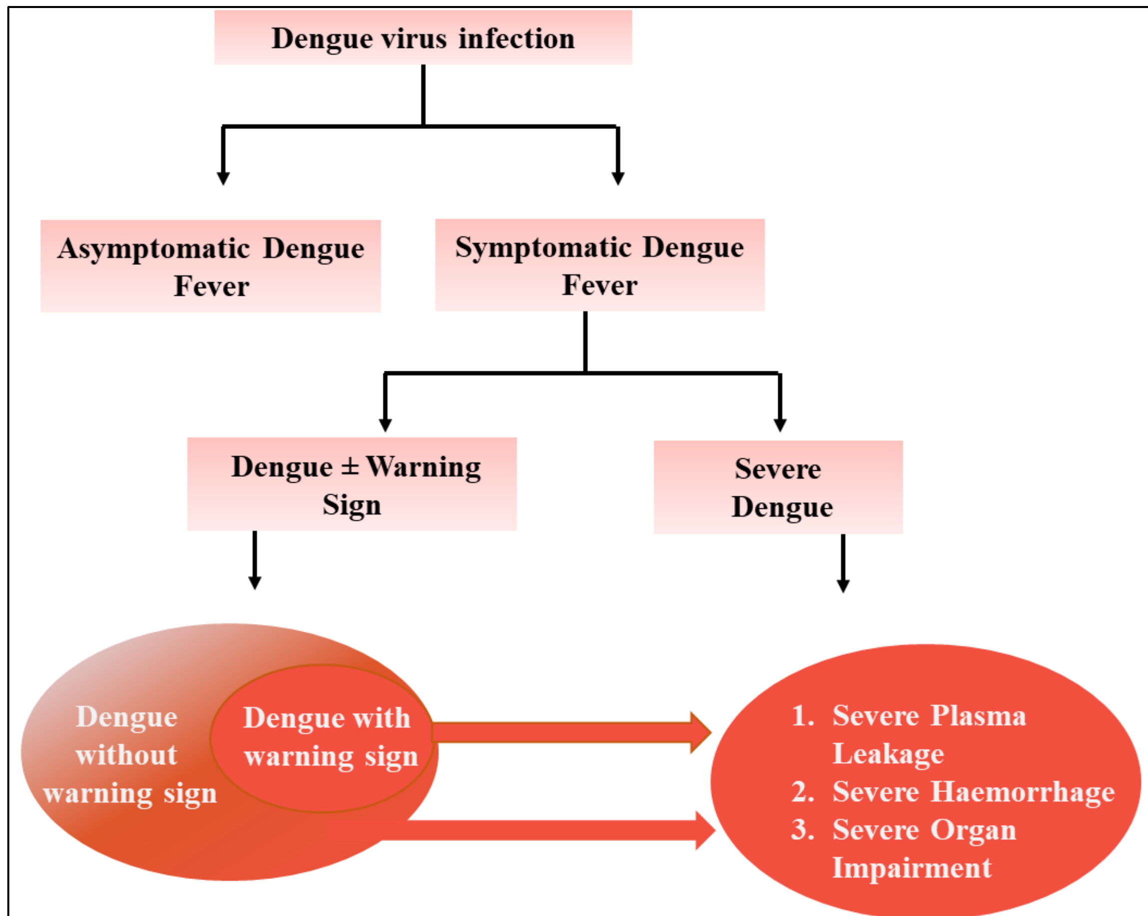

Fig. 3: Classification of Dengue fever

1.4.a. Dengue Fever

Dengue fever is distinguished by a fast onset of fever; however, the severity of the symptoms varies depending on the patient (33). A variety of aches and pains are typical; frequently, the headache is retro-orbital and is accompanied by symptoms such as rash, myalgia, lack of appetite, nausea, vomiting, and stomach discomfort. Symptoms may also include changes in taste (metallic flavour) and flushing of the face. The clinical definition of DF involves two or more of the following symptoms, in addition to fever: headache, vomiting, retro-orbital discomfort, muscle or joint pain, rash, haemorrhagic manifestation, or leucopenia. Mild haemorrhagic skin signs such as petechiae may be noticed. Other

symptoms include spontaneous bleeding, including gum bleeding, increased menorrhagia haemorrhage, gastrointestinal bleeding, and haematuria (33).

Also, DENV infection can cause symptoms such as loss of appetite, constipation, diarrhoea, respiratory discomfort, a moderate sore throat, and an altered perception of taste. A fever of 102°F to 105°F may develop and remain for two to seven days, accompanied by pharyngitis, conjunctivitis (pink eye), and lymphadenopathy (swollen lymph nodes). Up to 50% of infected people get a rash, and a second rash may arise later on the body and extend to the extremities. Petechiae (broken capillary blood vessels) visible as red or purple patches and acute pruritus (itching) may develop when body temperatures return to normal. Mild to severe hemorrhagic manifestations, including petechiae and purpura (bleeding under the skin seen as red or purple spots) may occur, along with gastrointestinal haemorrhages/bleeding, bleeding gums, menorrhagia (heavy menstrual bleeding), epistaxis (nosebleed), hematuria (bloody urine), and jaundice (yellow pigmentation of the skin) (29), (32). Dengue fever is seldom lethal, with an acute phase lasting three to seven days and a lengthy convalescent (recovery) phase. The convalescent phase, which includes feelings of weakness and despair, can extend for weeks (29), (34).

1.4.b Severe Dengue

A severe dengue infection is distinguished by plasma leakage, shock, fluid build-up, respiratory difficulty, severe bleeding, and organ damage (1), (33). Hypovolaemia intensifies as dengue vascular permeability rises, culminating in shock around defervescence. The compensatory mechanism keeps blood pressure normal while generating tachycardia and peripheral vasoconstriction, which results in chilly extremities and delayed capillary refill time. Prolonged hypotensive shock and hypoxia can result in multi-organ failure and a challenging clinical outcome. A pulse pressure of 20 mm Hg or

inadequate capillary perfusion in children may signal a more serious shock. Hypertension is commonly accompanied by protracted shock, which is sometimes aggravated by significant bleeding. Major bleeding is nearly invariably accompanied by deep shock, which can result in multiple organ failure and progressive disseminated intravascular coagulation (35). Even in the absence of significant plasma leakage or shock, unusual symptoms such as acute liver failure and encephalopathy may occur. The majority of dengue deaths occur in individuals who are in deep shock, especially if the situation is worsened by fluid overload (36).

Severe dengue should be considered if the patient is from a dengue-risk location and has a fever lasting 2-7 days along with any of the following symptoms:

- Tachycardia, clammy, chilly extremities, capillary refill time longer than three seconds, weak or undetectable pulse, narrow pulse pressure, or, in late shock, unrecordable blood pressure are all signs of plasma leakage.
- A large amount of blood
- A shift in awareness (convulsions, coma, lethargy or restlessness)
- Severe organ malfunction (acute liver failure, acute renal failure, encephalopathy or encephalitis, or other atypical symptoms, cardiomyopathy) or other odd signs, including persistent vomiting, increasing or severe stomach pain, and jaundice.

Approximately 1 to 2% of DF progresses to DHF, distinguished from DF by an increase in vascular permeability (1). Severe dengue patient percentage among the population may change based on vector infestations and dengue outbreak rates (35). In haematological tests, this is shown as thrombocytopenia, increased haematocrit, and hypoproteinemia. High temperature (up to 39 degrees Celsius), which can be biphasic, haemorrhages, thrombocytopenia and hemo-concentration, hepatomegaly, and symptoms of circulatory

failure are among the clinical findings. The WHO defines DHF as fever, thrombocytopenia, and an increased haematocrit.

Vascular abnormalities, thrombocytopenia, and coagulation problems are all symptoms of DHF and DSS (36). DSS symptoms include narrow pulse pressure or hypotension, in addition to the symptoms indicated for DHF. Shock affects around one-third of DHF patients. Dengue fever or dengue hemorrhagic fever can progress to a more serious state after 3-7 days of the sickness after the fever has subsided (35). Patients have considerable stomach discomfort before the onset of shock. The skin becomes chilly and the pulse becomes quick and narrow, which are common indicators of circulatory failure (1). Patients in shock are in danger and plasma volume replacement treatment is required to keep them alive. Death rates from the severe types of dengue sickness, DHF and DSS, can be dramatically decreased with timely supportive care. Correctly timed supportive care can considerably lower the death rates of the severe types of dengue sickness, DHF and DSS (35). The criteria for dengue ±warning signs and severe dengue are explained in **Figure 4.** The case fatality rate in DHF and DSS is substantial (40% - 50%); however, with excellent physiologic fluid replacement treatment, rates should be around 3-5% (1), (35), (36).

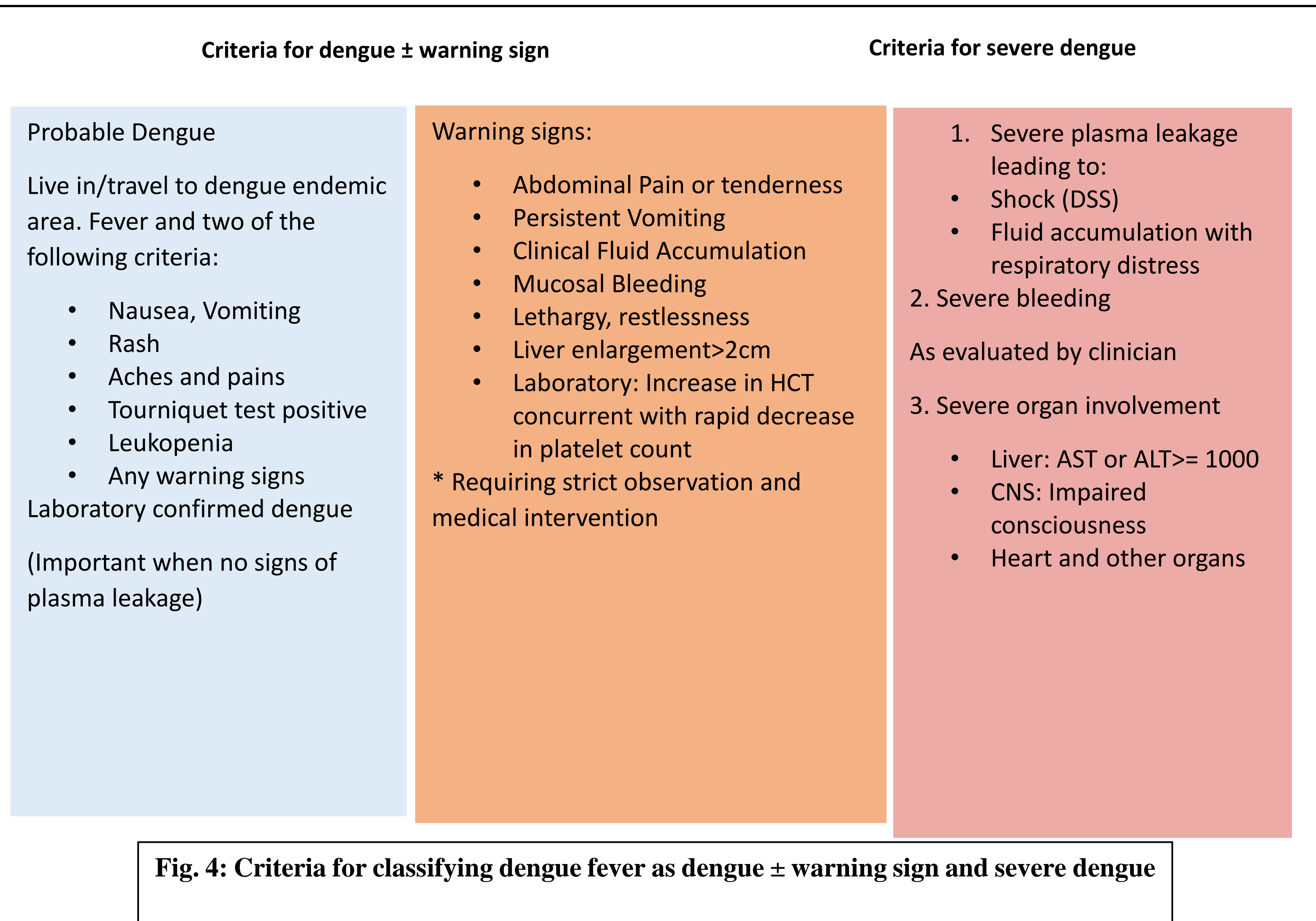

Fig. 4: Criteria for classifying dengue fever as dengue ± warning sign and severe dengue

1.5. Types of Severe Dengue: Clinical diagnosis and risk factors

1.5.a. Dengue Haemorrhagic Fever (DHF)

Dengue Haemorrhagic Fever (DHF) is an illness that predominantly affects children but now due to the global infestation of this virus, adults are also equally prone to this disease (29). A more virulent or infective isotype of DENV has a higher risk of causing significant illness, with high levels of viremia associated with the development of DHF (1). The most serious illness risk is ADE caused by subsequent DENV infection with a heterologous serotype. Host factors that increase the risk of progression to severe disease include being female, type AB blood, several human leukocyte antigen class I alleles, a single-nucleotide polymorphism in the tumour necrosis factor gene and a promoter variant of the DC-SIGN receptor genes (37), (38).

Race and polymorphisms in the vitamin D receptor and Fc receptor genes are host characteristics that minimise the likelihood of developing severe illness following a subsequent DENV infection (39). DHF initially mimics a primary DENV infection and is characterised by a rapid onset of fever lasting two to seven days with no DHF-specific symptoms. However, when the fever has subsided, plasma leakage is a clinical sign of DHF (29). When the fever declines, there are indicators of circulatory failure, hemorrhagic manifestations, thrombocytopenia (a reduction in platelets in the blood), and haemoconcentration (an increase in the quantity of red blood cells). Symptoms of haemorrhagic manifestations include purpuric lesions at the site of intravenous access, petechiae and purpura on the torso and limbs, epistaxis, gastrointestinal haemorrhage, bleeding gums, and haematuria with ground-coffee looking vomit and melena (black tarry faeces). The melena is linked to iron oxidation in the gastrointestinal system due to elevated haemoglobin levels. Some individuals have mild to severe shock as a result of blood loss and reduced blood flow if not diagnosed early (40).

1.5.b. Dengue shock syndrome (DSS)

Fever and other nonspecific signs and symptoms are followed by a fast deterioration of the patient's state in dengue shock syndrome (DSS). When these individuals' fevers diminish, their skin may become cold, blotchy, and congested. Circumoral cyanosis, a quick and weak pulse, restlessness, stomach discomfort, and rapid advancement into a critical stage of shock owing to plasma leakage are additional symptoms. Other DSS symptoms are comparable to DHF symptoms and can lead to death. Antishock therapy can aid recovery if started as soon as symptoms of DSS appear (33), (36).

1.6. Phases of dengue fever

Dengue fever is a complex and ever-changing illness. It has a broad clinical range with both severe and mild clinical symptoms (1). The sickness occurs rapidly after the incubation period and is divided into three stages: febrile, critical, and recovery. The management of a disease with multiple presentations is generally easy, affordable, and extremely successful in saving lives as long as precise and appropriate measures are undertaken. Early detection and comprehension of clinical difficulties during the various stages of the disease is critical, leading to a reasonable approach to case management and a positive clinical result.

Primary and secondary care activities, triage and treatment choices; where patients are first seen and evaluated are crucial in deciding the clinical outcome of dengue. A well-managed front-line approach not only eliminates needless hospital admissions but also saves dengue patients' lives. Early detection and notification of dengue patients observed in primary and secondary care is critical for recognising outbreaks and establishing an early response (33). Different phases of dengue fever are described below in **Figure 5**.

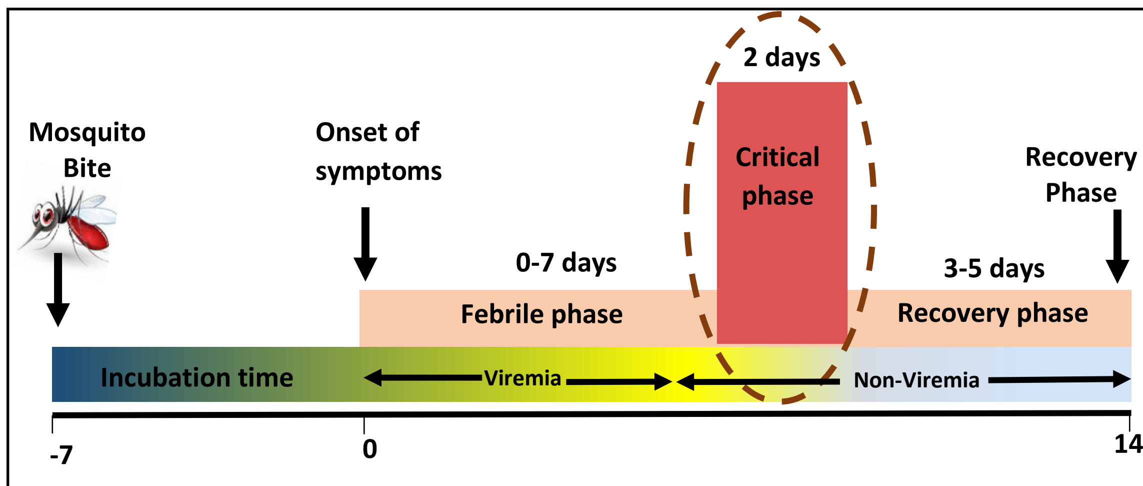

Fig 5a: Different phases of dengue fever starting from febrile to critical to recovery with viremia and non-viremia phases

1.6.a. Febrile phase

Patients usually get a high-grade fever all of a sudden. This acute febrile phase generally lasts 2-7 days and is characterised by face flushing, erythema of the skin, generalised body discomfort, myalgia, arthralgia, and headache (1). Sore throat, injected pharynx, and conjunctival injection may occur in certain people. Anorexia, nausea, and vomiting are all frequent symptoms. In the early fever phase, clinically distinguishing dengue from non-dengue febrile disorders might be challenging. A positive tourniquet test during this stage raises the likelihood of dengue. Furthermore, these clinical characteristics are identical across severe and non-severe dengue patients. Monitoring for warning signals and other clinical measures is therefore vital for detecting advancement to the critical phase. Mild hemorrhagic symptoms such as petechiae and mucosal membrane bleeding (nose and gums) are possible. Massive vaginal bleeding (in women of reproductive age) and gastrointestinal bleeding are possible but uncommon at this stage. After a few days of fever, the liver is frequently enlarged and painful. The first irregularity in a complete blood count is a gradual reduction in total white cell count, which should alert the doctor to the possibility of dengue (1), (3), (30).

1.6.b. Critical phase

After acute phase of sickness, a rise in capillary permeability may occur in tandem with increasing haematocrit levels. This is the start of the critical period. Clinically significant plasma leakage typically lasts 24-48 hours. Plasma leakage is frequently preceded by progressive leukopenia and a fast fall in platelet count. Patients with no increase in capillary permeability will improve at this time, whereas those with increased capillary permeability may worsen as a result of reduced plasma volume (1).

Plasma leaking varies in severity. Depending on the degree of plasma leakage and the volume of fluid treatment, pleural effusion and ascites may be clinically apparent. As a result, chest X-rays and abdomen ultrasonography can be important diagnostic techniques. The extent of the rise over the baseline haematocrit frequently indicates the severity of plasma leakage. When a critical amount of plasma is lost due to leakage, shock develops. It is frequently preceded by warning symptoms. During shock the body temperature drop below average. The subsequent organ hypoperfusion caused by prolonged shock culminates in increasing organ damage, metabolic acidosis, and disseminated intravascular coagulation. This, in turn, causes significant bleeding, lowering the haematocrit in severe shock. Instead of the typical leukopenia found during this stage of dengue, individuals with significant bleeding may have an increase in total white cell count. Furthermore, serious organ damage such as severe hepatitis, encephalitis, or myocarditis, as well as severe bleeding, may occur in the absence of evident plasma leakage or shock.

Non-severe dengue is defined as those who improve after defervescence. Some individuals reach the crucial phase of plasma leakage without defervescence. Clinical care and clinical service delivery changes in the whole blood count should be utilised to guide the commencement of the critical phase and plasma leakage in these individuals. Those who are deteriorating will exhibit warning indications, which is referred to as dengue with

warning signals. Dengue cases with warning symptoms will most likely recover with early intravenous rehydration. Whereas, some cases progress to severe dengue (1), (33), (34).

1.6.c. Recovery Phase

Among patients who survive the critical 24-48 hours reabsorption of extravascular compartment fluid occurs gradually over the next 48-72 hours. General health improves, hunger returns, gastrointestinal problems subside, the haemodynamic state stabilises, and diuresis occurs. Some individuals may develop a rash with "islands of white in a sea of red" (33). Some people may develop generalised pruritus. During this period, bradycardia and electrocardiographic changes are prevalent (33). Because of the delusional action of reabsorbed fluid, the haematocrit stabilises or falls. White blood cell count normally begins to climb immediately after defervescence, however platelet count usually recovers later than white blood cell count. If excessive intravenous fluid is used, respiratory distress from significant pleural effusion and ascites can occur at any time (36). Excessive fluid treatment during the critical and/or recovery periods is related to pulmonary oedema or congestive heart failure (33).

1.7. Primary vs Secondary Dengue infection: Understanding the Key Differences

One of the critical aspects of dengue infection is the distinction between primary and secondary infections, which has important implications for disease severity and management.

1.7.a. Primary Dengue Infection

A primary dengue infection occurs when an individual is exposed to the dengue virus for the first time. The virus has four serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. When a person is infected with one serotype, they develop lifelong immunity to that specific serotype but remain susceptible to infection by the other serotypes.

During a primary infection, the virus enters the body through the bite of an infected mosquito. It then replicates in various cells, including immune cells such as dendritic cells and macrophages. The immune system responds by producing antibodies, primarily Immunoglobulin M (IgM), to fight the virus. Symptoms of primary dengue infection typically include high fever, severe headache, joint and muscle pain, rash, and sometimes mild bleeding.

Most primary dengue infections are mild, and many individuals may not even realize they have been infected. However, in some cases, especially in children and older adults, the infection can progress to severe dengue or dengue hemorrhagic fever (DHF), characterized by plasma leakage, severe bleeding, and organ impairment. Severe dengue requires prompt medical attention and intensive care.

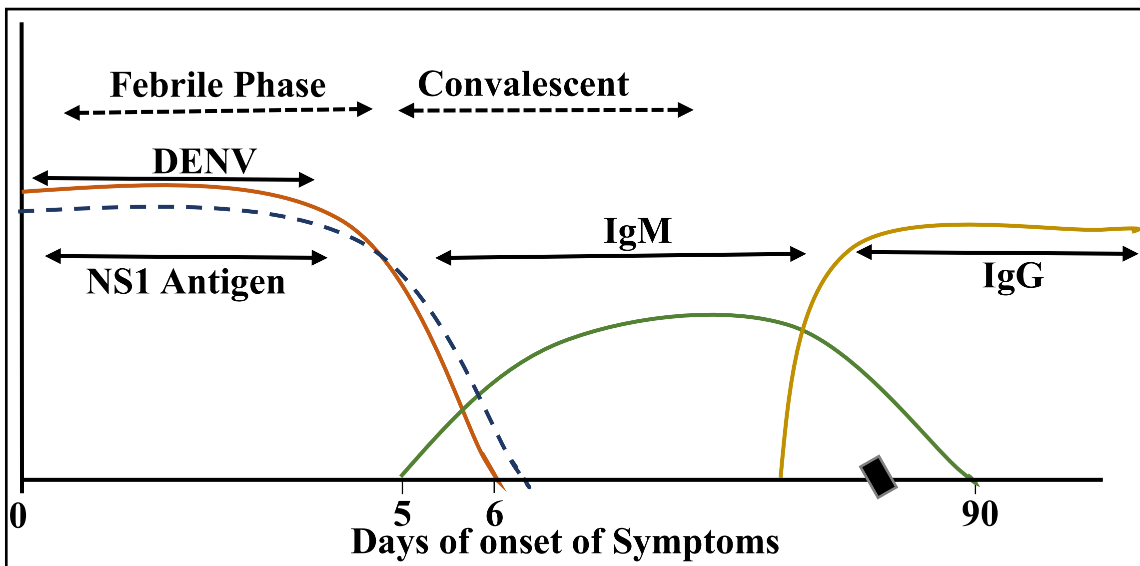

Fig. 5b.i: Immune responses during primary dengue infection

1.7.b. Secondary Dengue Infection

A secondary dengue infection occurs when a person who has previously been infected with one serotype is exposed to a different serotype of the virus. In this case, the immune system recognizes the new serotype but may mount an exaggerated immune response due to the presence of antibodies from the previous infection. This phenomenon is known as antibody-dependent enhancement (ADE).

ADE can lead to more severe symptoms compared to a primary infection. The antibodies from the first infection can facilitate the entry of the new serotype into immune cells, leading to increased viral replication and a heightened inflammatory response. As a result, secondary dengue infections are more likely to progress to severe dengue or DHF, especially if the immune system's response is not effectively regulated. Also, Cytokine storms in secondary Dengue infection can be a significant concern. In secondary Dengue infections, the immune system may overreact, leading to a cytokine storm. This is characterized by an excessive release of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-γ), etc. These cytokines can cause systemic inflammation and contribute to the severity of the disease, including the development of Dengue hemorrhagic fever (DHF) or Dengue shock syndrome (DSS) **(Figure 5b)**.

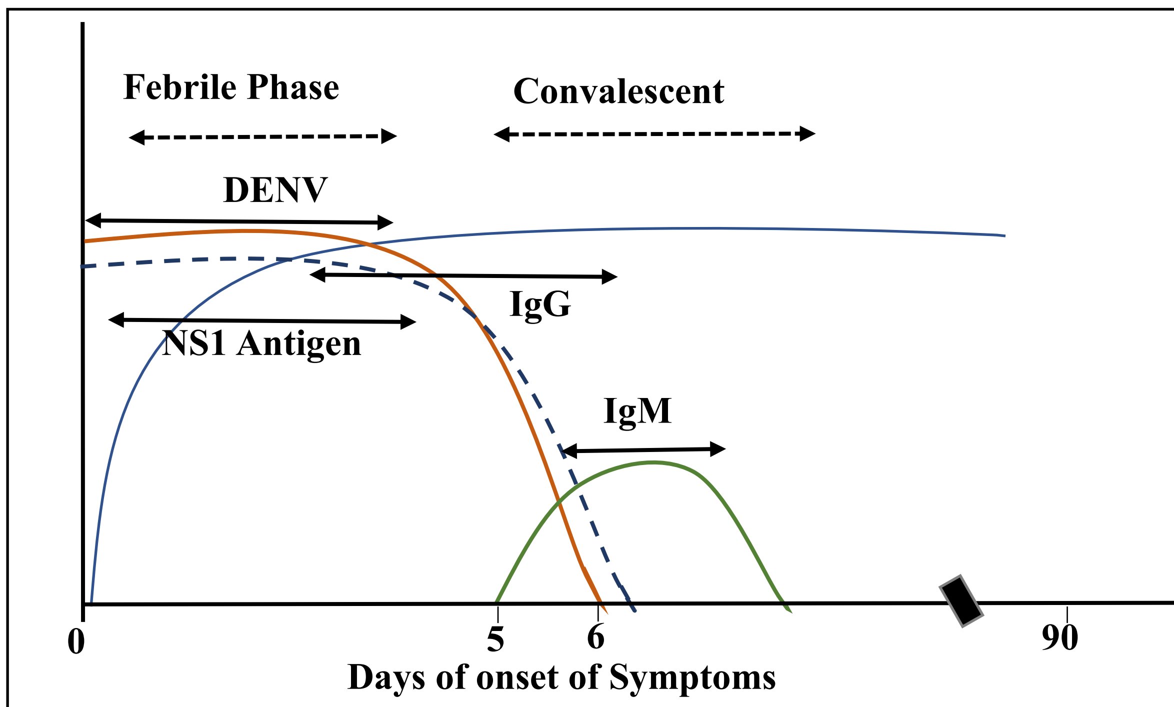

Fig. 5b.ii: Immune responses during secondary dengue infection

Table. 1.a: Key differences between primary vs secondary dengue

Factors	Primary dengue infection	Secondary dengue infection
Immune Response	In primary infection, the immune response is primarily directed at the infecting serotype	In secondary infection, ADE and cytokine Strom can lead to an exaggerated immune response due to cross-reactive antibodies
Severity	Primary infections are less severe compared to secondary infections	Secondary infections are generally more severe, with a higher risk of developing severe dengue i.e. DHF or DSS compared to primary infections
Risk Factors	Risk factors for primary infections include age, adults are more susceptible to dengue infection	Risk factors for severe dengue in secondary infections include age (children and older adults are more vulnerable), genetic factors, and viral factors such as the specific serotype and viral load

1.8. Dengue virus Transmission

1.8.a Spread and infestation of dengue virus

DENV's only known vectors are mosquitos of the *Aedes* genus. *Aedes aegypti, Aedes albopictus*, and *Aedes polyniensis* of the subgenus *Stegomyia* are known to be capable of

becoming infected with DENV, replicating it, and transmitting it (42). These are at least partially domesticated and anthropophilic creatures. A mosquito's life cycle is divided into four stages: egg, larva, pupa, and adult, with the first three requiring an aquatic environment. The duration of the developmental stages is determined by the temperature of the environment and the availability of food during the larval stage. At normal temperatures, *Aedes aegypti* takes approximately 8-10 days to mature (36), (42), (43). *Aedes aegypti* and *Aedes albopictus* mosquitos infected with DENV transmit the virus to a human host. The primary vector, *Aedes aegypti*, is a tiny black and white mosquito with a tropical and subtropical geographic range **(Figure 6a**). Domesticated Aedes aegypti like to deposit eggs in different artificial containers found in or near homes, resulting in increased mosquito output in these places. Adult *Aedes aegypti* prefers to feed on human hosts throughout the day. Peak feeding hours are just after sunrise and shortly before sunset, however, feeding has been seen throughout the day. The same mosquito can feed on many humans during the same blood meal, allowing infections to spread to multiple hosts. Dengue virus particles may withstand the stomach digestive fluids of mosquitos and pass through the stomach wall barrier, infecting midgut epithelial cells. DENV quickly multiplies and disseminates into the hemocoel before travelling to, infecting, and concentrating in the salivary glands, completing the five-to-seven-day cycle. DENV is then transferred to a new human host during a blood feed by the mosquito's proboscis and infected salivary glands (42), (44). After a human host is bitten by an infected mosquito, the virus incubates for three to fourteen days before causing dengue illness symptoms (43).

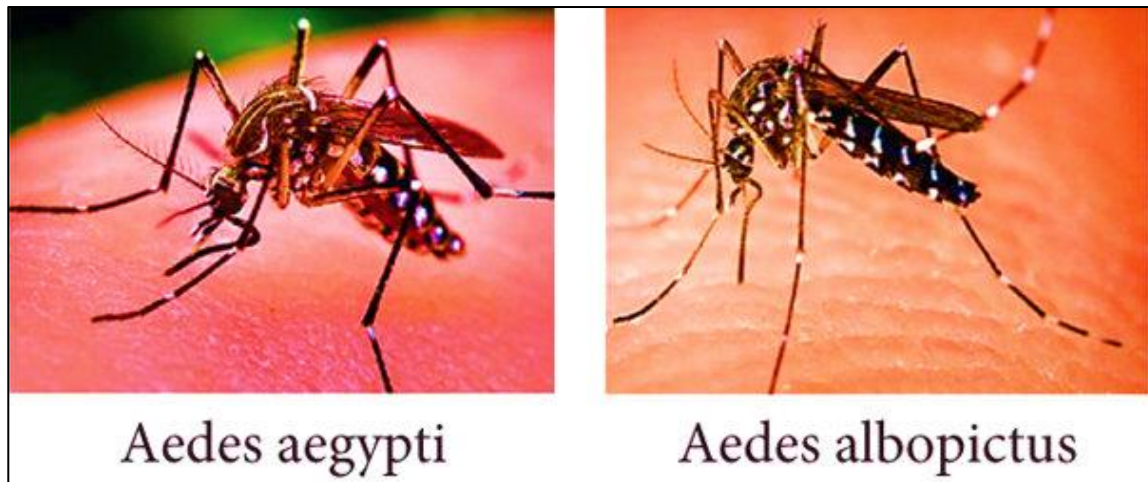

Fig. 6a: Dengue virus vectors in the wild and urban environments (Ref: Huang YJ et. al, Flavivirus-mosquito interactions. Viruses. 2014 Nov 24;6(11):4703-30).

The epidemiological trio of dengue includes the pathogen, mosquito vector and human host. The life cycle of the major vector *Aedes aegypti* is directly influenced via ambient temperature and rainfall (45). Higher temperatures might increase dengue risk by increasing the rate of mosquito development and reducing the virus incubation period in endemic regions (8)**.** Rainfall, on the other hand, can have strikingly contrasting non-linear effects on disease transmission. Where heavy rainfall might flush away eggs, larvae and pupae but water logging due to excessive rainfall might also create new breeding habitats in the longer term (46), (47). Water storage in dry climate areas might also aggravate the situation. A dry climate can lead to human behaviour of saving water in water storage containers, which might become future breeding sites for *Aedes aegypti.* Thus, climatic conditions may affect the virus, the vector and/or human behaviour both directly and indirectly increasing the disease prevalence (7). A wide range of factors are associated with this rapid increase in dengue prevalence, including globalization, urbanization, travel, demographic change, inadequate domestic water supplies, lack of sanitation and global warming. In addition. These are directly influencing the spread of the main vectors *Aedes*

aegypti and *Aedes albopictus* (10). Both these mosquito species rapidly spread from their continent of origin either via the infected host or via containers where these lay eggs (9). Climate change may lead to changes in these determinants of dengue transmission by multiple, inter-related mechanisms. As a consequence of global warming, a 0.75°C rise in temperature has been observed in the past 100 years. This alarming rise in temperature is directly proportional to the dengue outbreak in a non-linear manner (45).

1.8.b. Dengue virus transmission cycles in the wild and urban environments

The sylvatic cycle, which involves non-human primates (monkeys) and jungle mosquitoes, and the human-to-human epidemic transmission cycle, which occurs in urban environments, are the two known transmission cycles for dengue viruses. DENV's life cycle includes a replication stage in both mosquito and primate hosts. After an infected blood meal from a viremic host, the mosquito can infect new primate hosts after 8 hours to 1 day. In both cycles, the virus is passed from an infected mosquito female to the progeny mosquitoes by transovarial transmission and may potentially be sexually transmitted (43), (48). Dengue virus transmission cycle in wild and urban environments is shown in **Figure 6.b**.

Fig. 6b: Dengue virus transmission cycles in the wild and urban environments

1.9. The Vector

This mosquito is a tropical and subtropical species that is found all over the world, primarily between latitudes 35^0 N and 35^0 S. These geographical limitations roughly correspond to a winter isotherm of 10^0 C. Aedes aegypti has been reported as far north as 45^0 N, although these incursions happened during warmer months (explained earlier), and the mosquitoes did not survive the winter. Aedes aegypti is also uncommon above 1000 metres due to cooler temperatures. Immature stages are found in water-filled environments, mainly in artificial containers close to human houses and frequently indoors. According to research, the majority of female Aedes aegypti may spend their entire lives in the wild. This means that people, not mosquitos, spread the virus quickly within and between groups. Aedes albopictus, Aedes polynesiensis, and numerous species of the Aedes scutellaris complex have also been linked to dengue outbreaks (43). Each of these species has its ecology, behaviour, and geographic range. Aedes albopictus has spread from Asia to Africa, the Americas, and Europe in recent decades, encouraged in part by the worldwide trade in worn tyres, where eggs are deposited when they contain rainwater. In the lack of water, the eggs can survive for months (43), (45).

1.10. The Host

The primary host for the dengue virus is humans. When a mosquito bites a person infected with the dengue virus, it acquires the virus along with the blood meal. The virus then replicates in the mosquito's body, and the mosquito can transmit the virus to another human when it takes its next blood meal. This transmission cycle perpetuates the spread of the virus in human populations.

It's important to note that while humans are the primary host for the dengue virus, the virus can also infect and replicate in other primates, including non-human primates like monkeys. However, these animals are not considered major contributors to the transmission cycle of dengue in human populations. Dengue virus is an RNA virus, that requires contact, binding, and penetration to get access to the host's cellular machinery and multiply. It is emitted from the salivary gland of the pathogenic female Aedes aegypti mosquito, where it begins replication in secondary tissues such as salivary glands before passing to the human host. DENV infects various cell groups, including epithelial, fibroblast, monocytes, macrophages, dendritic, endothelial, and hepatocytes due to its diverse binding to various molecules (9), (43), (49).

1.11.Laboratory Diagnosis of dengue

The current laboratory diagnosis of dengue is based on the identification of DENV infection indicators in patient serum. These include viral components and antibodies seen in patient blood at various stages of infection. This complicates the identification of acute dengue infection, and many test types or matched samples are frequently required for an accurate diagnosis. Furthermore, information about the date of the sample illness start is required for selecting an appropriate diagnostic approach. Diagnostic tests differ by nation, and in addition to commercial test kits, in-house diagnostic procedures are extensively utilised. According to WHO, laboratory criteria for confirming dengue virus infection include the presence of at least one of the following inpatient sample(s):

a. Detection of DENV-NS1 antigen
b. Detection of anti-DENV antibodies
c. Detection of Dengue virus genome
d. Isolation of live dengue virus

1.11.a. Detection of DENV-NS1 antigen

Dengue NS1 antigen-based detection is widely used for early detection of viral infection. NS1 antigen detection is a DENV-specific approach for dengue diagnosis that, unlike RT-PCR-based methods, it does not require specialised laboratory equipment and is simple to use. The non-structural protein 1 (NS1) antigen, present in the dengue virus genome is an indication of the replicative phase of the virus and hence used for early detection. Early diagnosis, usually within five days of symptoms onset and fever is achieved using this procedure. Rapid diagnostic tests (RDTs) or ELISA assays on blood samples are frequently used in their execution. Because of their high sensitivity and specificity, NS1 tests are useful for timely diagnosis and patient treatment.

1.11.b. Detection of anti-DENV antibodies

Dengue is detected via IgM and IgG-based methods also, which includes detecting certain antibodies that the immune system produces in reaction to the dengue virus. IgM antibodies can last for around two to three months after infection and are usually detected during the first week. For the initial phase after 5 days of fever, they are sensed. Conversely, IgG antibodies develop later in the course of illness and typically last for years. IgG detection is useful for diagnosing later stages of illness by serological means, verifying prior exposure to the virus, and identifying prior dengue infections. These antibodies are frequently found in blood samples using techniques like ELISA (Enzyme-Linked Immunosorbent Assay) or rapid diagnostic assays (RDTs), which help with the diagnosis and epidemiology surveillance of dengue fever. Reliable serodiagnosis is based on paired samples in which a diagnostic increase in antibody titres can be seen between acute (five days after onset) and convalescent (five to seven days after onset). IgM antibody titres in initial infections rise to detectable levels in 80% of patients by day 5 following fever start

(50), (51). IgG antibodies become detected immediately after IgM antibodies, and by day 7, the majority of individuals have measurable levels. Commercial kits for detecting E-protein antigens are also available, however they are less sensitive.

Traditional dengue diagnostic approaches rely on identifying antibodies against the dengue virus in the patient's serum. The envelope proteins of all species of the genus flavivirus exhibit structural similarities, resulting in serological cross-reactions in serological assays. Cross-reactive antibodies from prior flavivirus infections or immunisations might result in false positive test findings, particularly for IgG detection. Neutralisation tests are the only serological procedures that are reliable for classifying flavivirus infections and dengue virus infections based on the infection-producing serotype. These procedures are not employed in routine diagnostics since they are time-intensive and necessitate the introduction of infectious viruses.

The capture IgM-enzyme immuno-assay (Mac-EIA) based on the inactivated viral antigen is the most extensively used serological approach for anti-DENV antibodies (33).

Immunofluorescence assays (IFA), lateral flow/immunochromatography-based fast tests, and immunoblot approaches (52), (53) have been employed in addition to EIA-based methods to detect anti-dengue IgM. IgG detection strategies are similar to those used for IgM detection; additional approaches include dot-blot and IFA.

1.11.c. Detection of Dengue virus genome

The detection of the DENV genome in patient serum samples allows for precise dengue diagnosis at an early stage of the disease when serological approaches are ineffective. The viremic phase of the sickness corresponds with the fever, and its length ranges from one to one hundred days (54). A positive test result allows for diagnosis; however, a negative test result does not rule out the potential of dengue since viremia levels and timing vary across

individuals. Although the time of the sampling is critical for RNA detection, the storage and handling of the samples are as vital for maintaining viral RNA in the samples.

DENV genome detection, in comparison to other diagnostic procedures, necessitates numerous handling stages, including sample RNA extraction and the test itself. Unlike serological approaches, viral RNA detection is susceptible to contamination and necessitates specialised laboratory facilities and equipment. Unlike serological approaches, DENV genome detection allows for specific quick DENV diagnosis from a single early phase sample and allows for the typing of the infection-causing serotype, which is critical for epidemiological follow-up.

Conventional RT-PCR

To identify DENV RNA in patient samples, several reverse-transcription polymerase chain reaction (RT-PCR)-based techniques have been utilised. In general, viral RNA is isolated from blood samples, reverse transcribed to cDNA in a separate procedure or a one-step method, and then amplified. Amplification targets may include highly conserved parts of the DENV genome, such as the NS5 and 3' UTR, or locations with greater variability, such as the C-prM and E-gene regions. Using ethidium bromide staining, the amplification products of traditional RT-PCR are visualised on agarose gels. Amplicons are often hundreds of base pairs long and can be used to investigate the sequence of the amplified PCR product using sequencing the PCR product (55). Performing many rounds of amplification can improve the sensitivity of the RT-PCR experiment. The second amplicon in nested amplification is positioned within the first. Traditional RT-PCR procedures require a separate detection phase in addition to the amplification stage, making them slower than newer real-time approaches that include both processes. However, because traditional methods are resilient, real-time applications have not completely supplanted them.

Real-time RT-PCR

In viral nucleic acid detection, two major real-time PCR methods have been widely employed. These include sequence-specific detection of amplified PCR products employing fluorogenic labelled probes and approaches based on the accumulation of a fluorescent dye attached to the double-stranded DNA amplicon in a sequence-unspecific way (56). In both cases, the fluorescence generated is proportional to the amplified target sequence and may be measured. Real-time PCR amplicons are often shorter than those utilised in traditional PCR. Furthermore, the PCR apparatus and chemicals employed allow for very fast PCR steps, detecting the findings concurrently and thereby reducing the time necessary for the experiment. Different manufacturers have created a variety of equipment that differ in the technology utilised to do the thermal analysis (57). Sequence-specific real-time PCR relies on probes that bind to the target sequence and are amplified in PCR. TaqMan-based methods, which employ Taq DNA polymerase and dual-labelled probes, are commonly employed in the detection of DENV RNA. These probes have a fluorescent dye on one end and a quencher dye on the other, which prevents the fluorescent reporter dye from generating fluorescence. The probe is constructed downstream of the PCR primer site in the target sequence, and Taq DNA polymerase cleaves it during the primer extension phase, separating the quencher and reporter molecules. This extremely specific approach enables the quantification of fluorescence intensity throughout each PCR cycle. To identify and type DENV RNA, several TaqMan-based RT-PCR techniques were applied (56). Methods based on SYBR Green I dye, which binds to double-stranded DNA, detect the fluorescence produced by the dye. The specificity of these approaches is determined by the primers employed, however, melting curve analysis of amplification products allows them to discover varied sequences. The melting temperature is determined by the amplified

product sequence, and this approach has been utilised to identify DENV genomes in real time PCR (56).

1.11.d. Virus isolation

Although virus isolation is not often employed in routine diagnostics, it provides conclusive confirmation of DENV infection. The sampling period and optimum sample preservation are critical in viral genome identification (60), (61). Currently, cultivated mosquito cells (Aedes albopictus C6/36) are the most often utilised technique of DENV culture from patient sera, although numerous other mosquito-cell lines and mammalian cell lines typically employed in viral cultures, such as monkey kidney cells, are also appropriate. Inoculation with live mosquitoes is the most sensitive but impractical approach known. Dengue virus's potential to generate cytopathic effects (CPE) on infected cells varies and is most likely based on viral strain features. DENV may be identified and serotype determined in viral isolation cells utilizing dengue virus-specific RT-PCR techniques or monoclonal antibodies in an immunofluorescence format.

Other Detection methods

RT-PCR-based DNA microarray detection (58), as well as amplification by nucleic acid sequence-based amplification (NASBA), have been employed in the detection of DENV RNA (59).

1.12. Prevention & Treatment

1.12.a. Vector Control

Effective mosquito control requires a multifaceted approach, integrating various methods and engaging communities to sustainably reduce the risk of dengue transmission. Environmental management and chemical control measures, such as larvicides and

adulticide space sprays, have been utilised with some success to decrease or eliminate DENV transmission. Cleaning stagnant water in containers near houses and public spaces, as well as the usage of mosquito nets, are other measures of control (62). Controlling mosquito populations is a critical strategy in reducing the incidence of dengue fever, as the disease is primarily transmitted by Aedes mosquitoes, particularly Aedes aegypti and Aedes albopictus. Various methods are employed to control these mosquito populations such as

Environmental Management and Source Reduction: This involves eliminating or managing mosquito breeding sites, primarily through reducing standing water where mosquitoes lay their eggs. Emptying containers such as flower pots, buckets, and discarded tires that can collect water. Ensuring that garbage and waste are properly disposed of and not left to accumulate and collect water. Covering water storage containers to prevent mosquitoes from accessing them.

Biological Control: Utilizing natural predators or biological agents to control mosquito populations, Fish such as guppies or mosquito fish (Gambusia affinis) can be introduced into water bodies to feed on mosquito larvae. Bacillus thuringiensis israelensis (Bti) is a bacterium that produces toxins lethal to mosquito larvae but is safe for humans and other animals. Releasing mosquitoes infected with the Wolbachia bacterium, which reduces the ability of mosquitoes to transmit dengue virus.

Chemical Control: Chemicals like temephos, methoprene, and pyriproxyfen that are applied to water to kill mosquito larvae. Insecticides like pyrethroids used to kill adult mosquitoes. These can be applied through outdoor fogging or indoor spraying. Spraying insecticides in areas with high mosquito activity, especially during outbreaks.

Genetic Control: Using genetic modification techniques to reduce mosquito populations or their ability to transmit dengue. Releasing sterilized male mosquitoes into the wild to

mate with females, resulting in no offspring. Releasing genetically modified mosquitoes that produce offspring that die before reaching adulthood or that are less capable of transmitting dengue.

Personal Protection and Community Participation: Reducing mosquito bites through individual and community efforts. Applying DEET, picaridin, or oil of lemon eucalyptus-based repellents to uncovered skin. Using bed nets, particularly treated with insecticides, to protect against mosquito bites while sleeping. Wearing long-sleeved shirts and long pants, particularly during peak mosquito activity times which is early morning and late afternoon.

Public Health Education and Community Engagement: Educating communities about the importance of mosquito control and engaging them in proactive measures. Conducting campaigns to educate people about dengue transmission, symptoms, and mosquito control methods. Organizing community efforts to clean up potential mosquito breeding sites. Engaging local leaders and community organizations to promote and sustain mosquito control activities.

Integrated Vector Management (IVM): Combining multiple control methods in a coordinated and sustainable manner. Conducting surveillance to identify mosquito breeding sites and population density. Using a combination of environmental management, biological control, chemical control, and community engagement. Continuously monitoring the effectiveness of control measures and making adjustments as needed.

Furthermore, more effective vector control strategies may reduce virus transmission even further. These include the use of long-lasting insecticide-treated window curtains and water container covers, as well as control-released larvicides that give several months of treatment (1). Currently, no DENV vaccine is easily accessible, and techniques to prevent DENV infection, including mosquito control, are restricted (62). Insecticides and bed nets

can assist to minimise mosquito-mediated transmission. With the number of individuals at risk of infection increasing and no cures for active disease, vaccine development or preventative techniques are critical.

1.12.b. Treatment

There is no specific antiviral treatment approved for dengue fever. Treatment for dengue primarily involves symptomatic care to manage symptoms and complications. Anti-pyrogenic medicine like Aspirin and Bufen is prohibited in dengue infections as they increase bleeding manifestations and stomach pain in DENV-infected patients. On medical guidance, paracetamol can be administered. If one or more indications of DHF are observed, the patient should be rushed to the hospital. During the transport to the hospital, the patient is given fluid to drink or injected intravenously (3), (30). In most situations, early and effective replenishment of plasma losses using a plasma expander or fluid and electrolyte solution leads to a good outcome. DSS is quickly reversible with enough and proper fluid supplementation. Resuscitation from shock as soon as possible, as well as repair of metabolic and electrolytic abnormalities, will avoid disseminated intravascular coagulation. Because high fever, anorexia, and vomiting cause thirst and dehydration in DHF patients, fluid intake by mouth should be adequate. Plain water is superior to an electrolyte replacement solution or fruit fluid is preferable to combat diarrhoeal symptoms. Convulsions are possible during the acute febrile period. Patients with hyperpyrexia, particularly those with a history of feverish convulsions, may benefit from antipyretics. Salicylates must be avoided since they might result in bleeding and acidity. Although paracetamol is recommended for fever reduction, it should be taken with care. In an outpatient rehydration centre, parental fluid treatment can be administered to patients who are dehydrated due to fever, vomiting, or anorexia. Shock is considered a medical

emergency. It is critical to administer intravenous fluid immediately to increase plasma volume. Ringer's lactate or Ringer's acetate or 5% glucose dissolved in physiological saline should be given as a fast intravenous bolus (10-20 ml/kg) within 20 minutes. If necessary, another bolus can be given to increase the fluid dosage to 20-30 ml/kg (30).

1.12.c. Vaccine development

The increasing dissemination and outbreaks of dengue virus infection and sickness highlight the critical need for the development of a DENV vaccine. The development of vaccine against dengue virus has been ongoing for several decades, but because of the complex pathophysiology of dengue and the need to simultaneously target all four virus serotypes, progress has been slow. The development of the disease to DHF and DSS owing to subsequent infection emphasises the necessity for a vaccine to protect not only naive patients but also those who have previously had dengue (62). The ideal DENV vaccine would not be reactogenic, would provide life-long immunity against infection with all four dengue virus serotypes and would be inexpensive (63). Due to heterogeneity in DENV transmission intensity and circulation, vaccine trials should be conducted in a variety of risk groups. Although no commercially accessible DENV vaccine is presently available, numerous forms of marginally effective vaccinations have been produced. Among them, clinical studies for live attenuated viruses and chimeric viruses using yellow fever vaccination and attenuated DENV as backbones have been approved (63). Furthermore, genetically modified DENV vaccines targeting the prM, E, or NS1 genes have been tried with encouraging outcomes (64).

There are only two licenced dengue vaccines: Dengvaxia (CYD-TDV, Sanofi) and QDENGA (TAK-003, Takeda).

One of the notable vaccine candidates is Dengvaxia, developed by Sanofi Pasteur. Dengvaxia is a tetravalent vaccine designed to protect against altogether four serotypes of DENV. It received regulatory approval in several countries, but its implementation faced challenges and controversies. Some concerns were raised about the vaccine's efficacy and its potential to increase the risk of severe dengue in individuals who had not been previously exposed to the virus. As a result, recommendations for its use varied among different regions and populations.

Dengue Tetravalent Vaccine (Live and Attenuated) known as QDENGA (TAK-003) is a dengue vaccine based on a live-attenuated dengue virus serotype 2. This vaccine serves as the genetic "backbone" for all four dengue virus serotypes and is meant to protect against all of them. The Group of Experts of WHO's Strategic Advisory on Immunisation (SAGE) confirmed QDENGA's efficacy against all four serotypes. This vaccine is recommended for use in individuals > 4 years of age as the recommendations mentioned below. Recommendations propose an initial cautionary approach and tactics due to lack of broad-spectrum information in dengue-naive adults and especially elderly people. Vaccination is recommended before travelling to an endemic country for travellers with a history of self-reported (hospitalised or a policlinic tested) dengue fever. Also, dengue-naive travellers aged 4-16 years old, are recommended to be vaccinated regardless of travel duration. We suggest immunisation only for lengthier travels that are relevant to the travel location for travellers aged 17-60 years old. To serve as a reference, they propose a vacation of more than six weeks to Southeast Asia, an area with one of the highest global incidences of dengue disease. QDENGA has not been investigated in people over 60 years of age, yet. Therefore, it's advised to avoid vaccination in this age group, until more data is available (65).

Chapter 2: Review of literature

2.1. Neglected Tropical Diseases

Neglected tropical diseases (NTDs) are a group of parasitic, viral, bacterial, fungal, and non-communicable illnesses that affect over one billion people worldwide (66). They include diseases like dengue fever, malaria, lymphatic filariasis, schistosomiasis, and Chagas disease, etc. which thrive in poverty, lack of access to clean water and sanitation, and limited healthcare infrastructure. NTDs are interconnected with social determinants of health, such as inadequate housing, poor sanitation, and lack of education, contributing to the persistence and spread of these diseases. The debilitating effects of NTDs compromise physical well-being, economic productivity, education, and social cohesion. Efforts to address NTDs have gained momentum, with initiatives like the World Health Organization's Neglected Tropical Diseases Roadmap aiming to control, eliminate, or eradicate these diseases by 2030. However, challenges persist, hindering progress towards achieving these ambitious goals. NTDs receive a disproportionately small share of global health funding, which hampers the development and implementation of effective prevention and treatment strategies. They often operate silently, leading to a lack of awareness and understanding, leading to delayed diagnosis and treatment (66), (67). The global distribution of neglected tropical diseases is shown in **Figure 7** below.

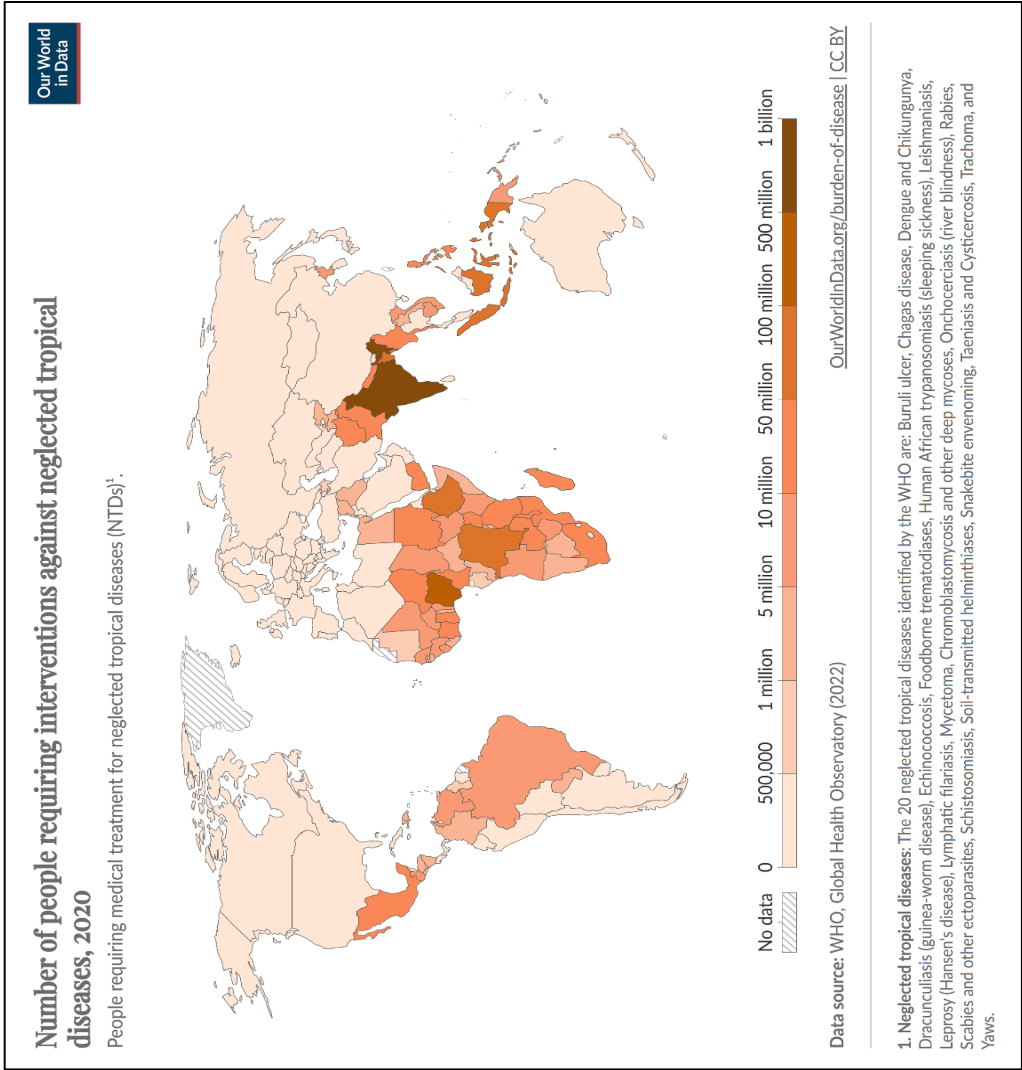

Fig. 7: Global distribution of neglected tropical disease (Ref: Our World in Data: https://ourworldindata.org)

2.2. Viruses

Viruses are microscopic infectious agents, incapable of independent life and can only replicate within the cells of a living host organism. They are composed of genetic material, either DNA or RNA, surrounded by a protein coat called a capsid (68). Some viruses also have an outer envelope derived from the host cell membrane. This obligatory intra-cellular parasite uses the host's cell machinery, components and energies for the production of their

progeny virions. Viruses can infect various types of organisms, including animals, plants, fungi, bacteria (bacteriophages), and archaea. They cause a wide range of diseases in humans, animals, and plants, ranging from mild illnesses to severe and potentially fatal conditions. The life cycle of a virus typically involves the following stages: Attachment and Entry, Replication and Transcription, Assembly and release. Virus genomes undergo evolution which enables them to adapt according to environmental changes. Viruses can cause a variety of diseases, such as the common cold, influenza, HIV/AIDS, COVID-19, hepatitis, and many others. All three domains of life Eukarya, Prokarya and Archaea are parasitized by their specific virus and they co-evolved with their host organisms (68), (69).

2.2.a. Flavivirus

The name Flavivirus (also known as flaviviruses) is derived from flavus, the Latin word for yellow. Flavivirus is a genus of viruses belonging to the Flaviviridae family, which includes several significant human pathogens (70). Flaviviruses *impose a constant threat on global public health* as infections due to them are the most prevalent viral infections worldwide. Currently, Flaviviruses infect more than 400 *million people annually* (71). *Flaviviruses* are classified according to their vector associations: viruses transmitted by mosquitos (39 species), viruses transmitted by ticks (13 species), and viruses for which no arthropod vector has been identified, known vector viruses (16 species) (72). Furthermore, many speculative species have been identified with the genus, including genetically different lineages recovered from bats, dubbed Tamana bat virus (TABV) and mosquito cells, dubbed Cell fusing Agent virus (CFAV) (72). In the past 70 years, this epidemiological pattern has often appeared, notably during outbreaks of the West Nile and dengue viruses as well as the most recent, explosive Zika pandemic. Dengue fever is the most prevalent arboviral disease worldwide. The primary mode of transmission for

flaviviruses is through arthropod vectors, particularly mosquitoes and ticks (42). Human infections usually occur when an infected vector bites a person, introducing the virus into the bloodstream. Flaviviruses have a widespread global distribution, with different species prevalent in various geographical regions. Some well-known flaviviruses include Dengue virus, Zika virus, West Nile virus, Japanese encephalitis virus and Yellow fever virus. Flaviviruses can cause a spectrum of clinical manifestations. While some infections may result in mild flu-like symptoms, others can lead to severe diseases such as hemorrhagic fever, encephalitis, or congenital malformations in newborns (as seen with Zika virus). Certain flaviviruses are responsible for significant outbreaks and epidemics, placing a considerable burden on public health systems. For example, Dengue virus is a leading cause of mosquito-borne viral illnesses globally, with millions of cases reported annually (73). Vaccines have been developed for some flaviviruses to prevent infections and reduce the severity of diseases. The emergence of new flaviviruses or the re-emergence of known ones in different regions is a constant concern. Zika virus, for instance, gained international attention due to its association with microcephaly and other neurological disorders. Flaviviruses represent a diverse group of viruses with significant implications for global public health and their genetic evolution is explained in **Figure 8**. The TBEV complex is represented in green, the DENV complex in blue, and the WNV complex in red. The two tick-borne viruses depicted are TBEV and Powassan virus (POWV); all others are mosquito-borne. Yellow fever virus (YFV); West Nile virus (WNV); Japanese encephalitis virus (JEV). Phylogenetic tree rooted to Tamana bat virus (TABV) as the outgroup to demonstrate flavivirus diversity. The scale bar denotes a 6% change in amino acid (74). Understanding their biology, transmission dynamics, and the development of effective preventive measures are essential components in the ongoing efforts to control and mitigate the impact of flavivirus-associated diseases on human populations.

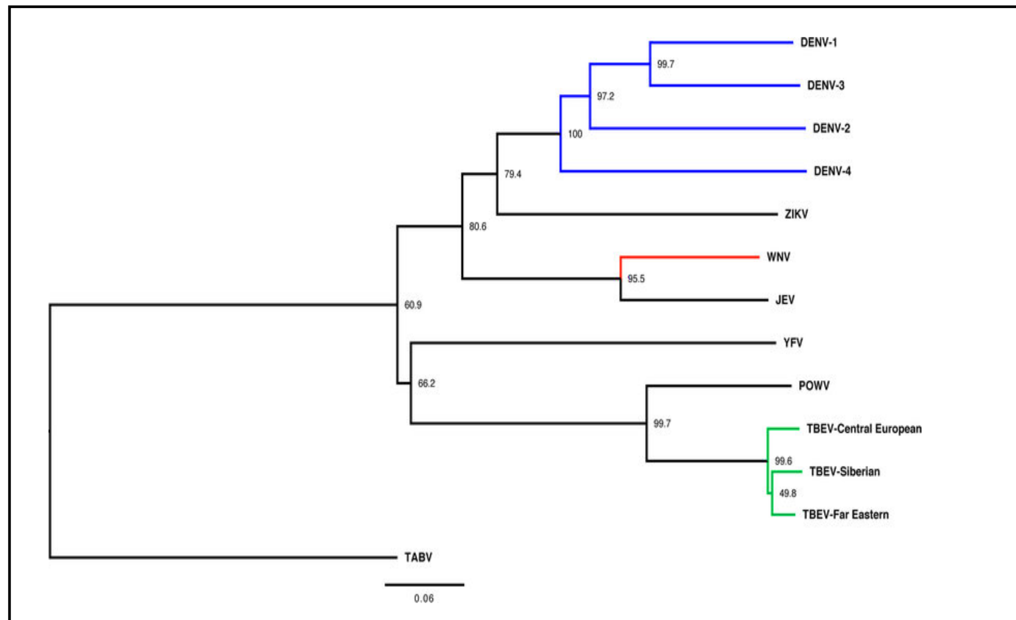

Fig. 8: Phylogenetic tree of therapeutically significant flaviviruses based on Envelope protein diversity (Ref: Saeed O et.al, Dengue Virus Disease: From Origin to Outbreak. Elsevier Inc.; 2019. 9–16 p.)

2.3. History of dengue virus

Dengue virus is a rampant arthropod-borne virus and a major global health concern, causing dengue fever, a flu-like illness, and in severe cases, potentially life-threatening complications, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (1). The history of the dengue virus can be traced back centuries **(Figure 9)**, with evidence suggesting that the virus has been infecting humans for at least several hundred years. The earliest known recorded outbreak resembling dengue-like symptoms occurred in 1779-1780 in Asia, Africa, and North America (75). In the 19th century, several large outbreaks of dengue-like illness were documented in various parts of the world, including sia, the Caribbean, and the Americas. In 1901, during an outbreak in the Philippines, the U.S. Army physician Dr. Albert Sabin discovered that dengue fever was caused by a virus (75).

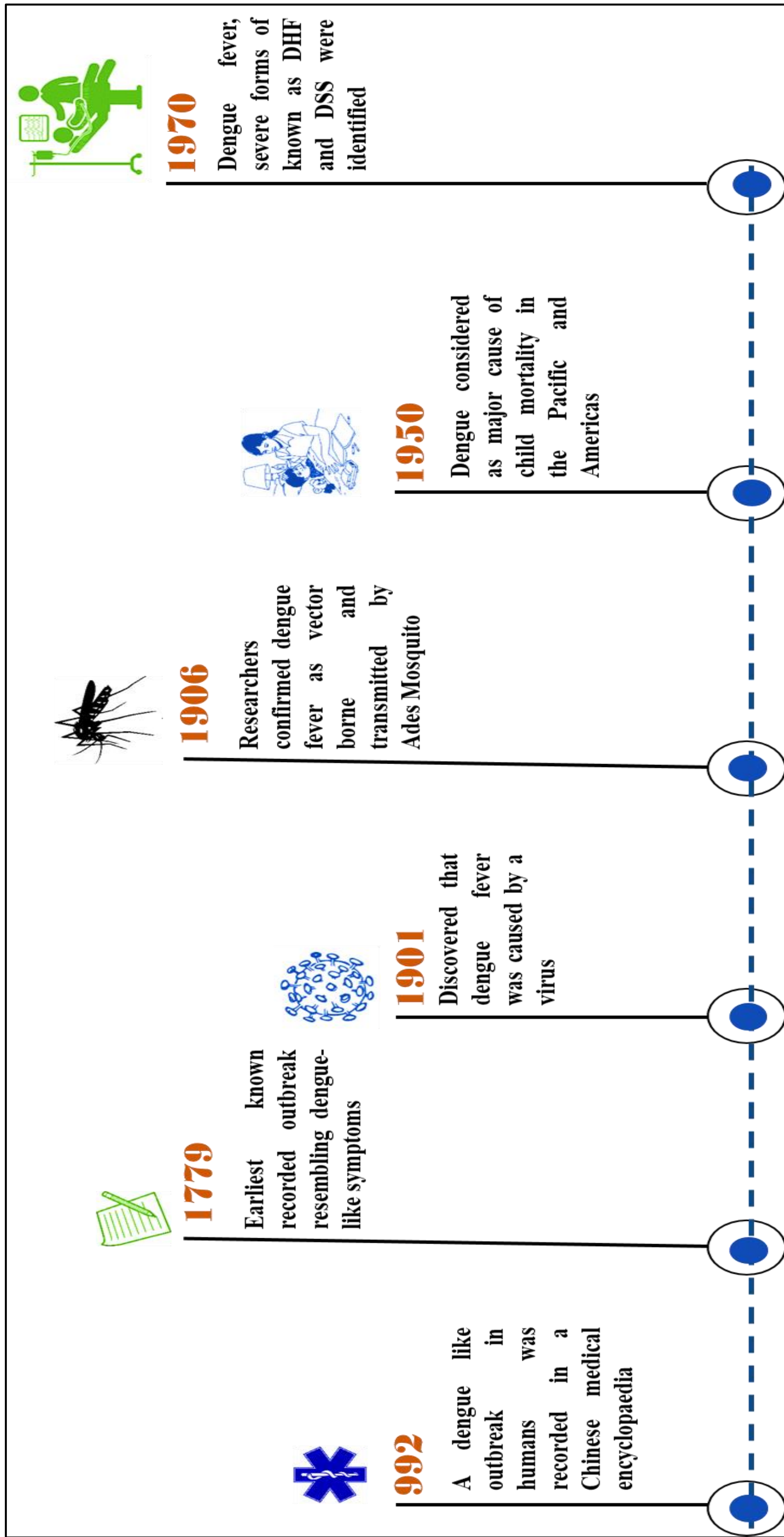

Fig. 9: History of dengue virus

In 1906, the transmission of dengue fever through mosquitoes was confirmed by the identification of Aedes aegypti as the primary vector (75). In 1944, the dengue virus was isolated and classified into four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) by

Dr. Albert Sabin and his colleagues (76).The virus was named "dengue" after the Swahili phrase "ki-denga pepo," which means "cramp-like seizure caused by an evil spirit" (5). Throughout the 20th century, dengue fever continued to spread globally, particularly in tropical and subtropical regions. The epidemics from India include those from Calcutta in year 1963, Vishakhapatnam in 1964, again in West Bengal in 1968, Ajmir in 1969, Kanpur in 1969, Delhi in 1970, Rajasthan in 1985 and Delhi in 1996 (5), (77).

Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS): In the 1950s, severe forms of dengue infection known as DHF and DSS were identified, characterized by increased vascular permeability, bleeding tendencies, and organ involvement. DHF and DSS can lead to life-threatening complications and are more commonly associated with secondary infections with a different dengue serotype. In the late 20th and early 21st centuries, dengue fever has seen a resurgence and has become a major public health concern worldwide (5), (36). Increased international travel, urbanization, population growth and climate change have contributed to the spread and severity of dengue outbreaks. The history of the dengue virus reflects its long-standing presence and its impact on human populations across different continents.

2.4.Genomic organization of dengue virus

2.4.a. Introduction of DENV genome

The dengue virus (DENV) is an enveloped RNA virus belonging to the *Flaviviridae* family and the genus *Flavivirus* (57). It is the etiological agent of dengue fever, a mosquito-borne viral illness that poses a significant global public health burden. Understanding the genome and proteins of the dengue virus is crucial for comprehending its pathogenesis, replication cycle, and the development of potential therapeutic interventions.

The genome of the dengue virus is a single-stranded, positive-sense, RNA molecule approximately 10.7 kilobases in length. It consists of a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). The genome encodes a polyprotein precursor that is subsequently cleaved into three structural proteins (C, M/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) via post translation and co-translational mechanism (57) (**Figure 10**).

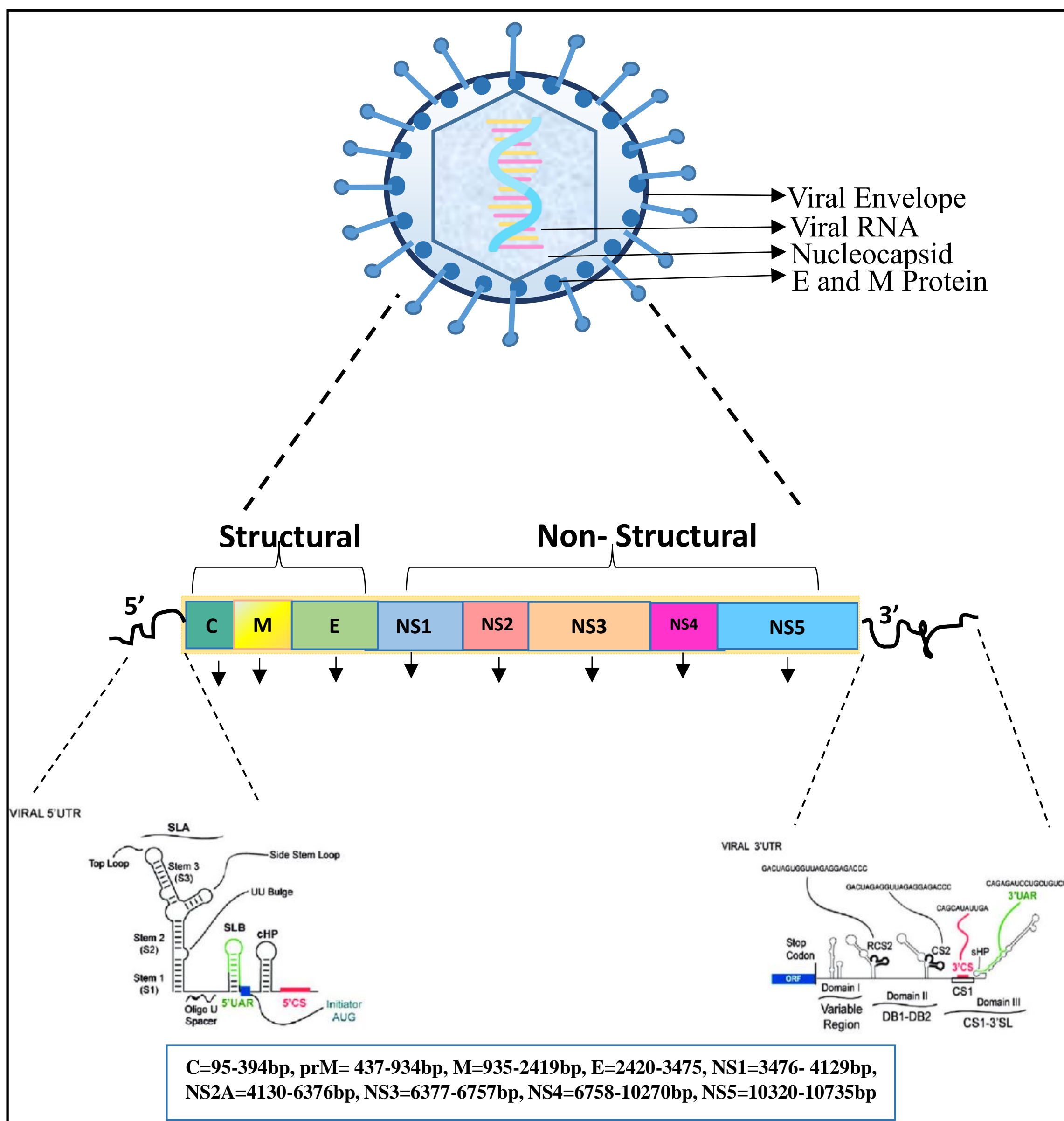

Fig. 10: Genomic organization of dengue virus showing three structural genes and seven non-structural genes

2.4.b. Structural Proteins

The capsid protein is a homodimer with 100 amino acid residues that includes an inherently disordered N-terminal domain and four α-helical sections. Genome encapsidation involves C. The C protein is essential for the assembly of viral particles and is involved in the packaging of viral RNA. The recruitment of viral RNA during assembly and the release of the genome during infection are two mechanisms that are linked to this function. Capsids are present in lipid droplets and nucleoli, despite the fact that particle formation takes place on endoplasmic reticulum membranes (78).

M has 75 aa residues, whereas prM has 166 aa residues. In order to prevent the fusion peptide E from undergoing premature fusion prior to viral release, prM/M serves as a cap-like structure. During viral assembly, the prM/M protein chaperones the E protein. Through receptor binding and fusion, the E protein enables viral attachment and entrance into host cells. It is essential for triggering defensive immune responses and a primary target for neutralizing antibodies (57). Surface proteins on the virion envelope called structural proteins E and M are used to distinguish between immature and mature viruses.

The juvenile virus is known as "spiky" because spikes appear on its surfaces when M proteins connected to a precursor membrane protein (pr) form heterodimers with E proteins. In mature virions, furin cleaves soluble pr from M protein, adhering the M proteins and resulting in the absence of pr protein from the mature viral membrane (79). The envelope of the virus contains the viral surface proteins E and M. The E glycoprotein is the main target of neutralizing antibodies and is involved in crucial processes such as viral attachment to cells and membrane fusion. It contains the main epitopes (virus-specific and cross-reactive epitopes) that are recognized by neutralizing antibodies (80, 81).

The flavivirus fuses to target cells by an internal fusion peptide found in domain II of this protein, while cellular receptor-binding motifs (81, 82) are found in domain III. This protein

is composed of three structural and functional domains. Subcomplex- and type-specific epitopes dominate domains I and III, while major flavivirus groups and subgroup cross-reactive epitopes dominate domain II (83, 84).

The M protein comes in two varieties. Cell-associated (immature) virions include prM, the precursor of the M protein, which combines with the E protein to produce a prM-E heterodimer. In acidic post-Golgi vesicles, prM functions as a chaperone for the E protein, preventing irreversible inactivation during viral transport to the cell surface (79, 85). Through this contact, prM helps in viral assembly and budding into the lumen of the endoplasmic reticulum. Before being released, intracellular virions are not contagious; this is due to a host-cell-derived furin-like protease that cleaves prM into the soluble pr peptide and the particle-associated M protein (86). The E protein cannot undergo the structural alterations required for DENV membrane fusion at low pH levels when prM is uncleaved. Thus, immature DENV is essentially non-infectious (31). Depending on the degree of prM cleavage, different amounts of prM and M may be present in the extracellular particles. The envelope protein is a class II N-glycosylated dimeric membrane fusion protein with 493–495 amino acid residues (53 kDa). E is present in mature DENV as 90 homodimers that create a "smooth" protein shell by lying flat against the DENV surface. The three different domains that make up each monomer subunit are I, II, and III. E is in charge of determining the host range, tropism, and pathogenicity of the virus and facilitates its binding and fusing to the host cell membrane (domain III). Roughly 30% of prM carrying immature particles have been found in DENV-infected cells (74, 87). Some of the differences in cleavage efficiency may be explained by the charged residues around the furin consensus sequence at the prM cleavage junction; moreover, structural variations amongst flaviviruses at the prM junction modify prM cleavability.

2.4.c. Non-structural Proteins

NS1 is a 46 kDa protein that is anchored in glycosyl-phosphatidylinositol (GPI) and may be found both within and outside of cells. By preventing complement activation, NS1 contributes to both viral defences and the viral RNA replication complex. A versatile protein, NS1 is involved in immunological evasion, pathogenicity, and viral replication. It is secreted as a soluble hexamer and is employed as a dengue infection diagnostic marker (32, 57). Later in this book, NS1 protein's precise mechanism and role in vascular dysfunction are discussed. In addition to influencing host immune responses, NS2A takes involvement in the assembly and replication of viral RNA. NS2A A 22 kDa protein with 218 aa residues.

NS2A is involved in the coordination of changes related to RNA packaging, replication, and interferon (IFN) antagonistic activity.

Processing of viral polyproteins requires NS2B, which is a cofactor for the NS3 protease. NS2B is a membrane-associated protein with 130 amino acid residues (14 kDa). As a cofactor in the structural activation of NS3's DENV serine protease, NS2B joins forces with NS3 to produce the DENV protease complex. RNA helicase, RNA-dependent RNA polymerase and serine protease are just a few of the several enzymatic functions that NS3 exhibits. It participates in the processing of polyproteins, the reproduction of viruses, and the avoidance of host immunological reactions. a 70 kDa (618 aa) protein. The helicase domain is found between residues 180 and 618, whereas the protease domain is N-terminal, spanning residues 1 to 180. With RNA helicase, RNA triphosphatase (RTP/NTPase), and chymotrypsin-like serine protease activity, NS3 is a multifunctional protein.

NS3 is involved in both RNA replication and the cleavage of the DENV polyprotein. Important functions in viral replication and host cell regulation, including as immune evasion and membrane rearrangements, are played by NS4A and NS4B. The tiny

hydrophobic proteins NS4A and NS4B have respective molecular weights of 150 aa (16 kDa) and 245–249 aa (27 kDa). They are both essential membrane proteins. Membrane changes brought on by NS4A are crucial for viral propagation. By directly interacting with NS3, NS4B promotes viral RNA replication while inhibiting IFN-induced signal transduction. The most conserved DENV protein is NS5, a 900 aa residue (104 kDa) protein. The RNA-dependent RNA polymerase is found at residues 270–900, whereas the methyltransferase domain is found at residues 1–269. NS5 is a bifunctional enzyme that exhibits both RNA-dependent RNA polymerase activity and methyltransferase activity. The dengue virus's biggest and most conserved protein is called NS5. It is essential for viral replication and immune evasion and has methyltransferase and RNA-dependent RNA polymerase functions. The development of antiviral drugs is thought to have NS5 as a major target (49, 73).

2.5. Genetic diversity and evolution of DENV

The DENV RNA-dependent RNA polymerase, like all RNA viruses, generates mistakes during replication and causes variability in the virus genome. As a result, dengue viruses persist as communities of genetic variants known as quasispecies (31). Recombination contributes to further heterogeneity in DENV genomes (89), (90). Because of genetic drift and selective pressures, the relative proportions of variations in viral population shift throughout time. The virus phenotype is selected based on its interactions with the environment. DENV phenotypic features affecting host interactions in primates and mosquitoes, such as infectivity, replication and transmission efficiency are critical. It has been shown that the majority of DENV genome changes are detrimental, emphasising the relevance of selection in DENV evolution (90).

Dengue viruses are highly adapted to their mosquito hosts and are thought to have evolved from mosquito viruses that initially adapted to sylvatic life cycles shared by rainforest mosquitos and non-human primates (90). According to evolutionary research, the DENVs engaged in human epidemics are derived from sylvatic DENV strains. Because sylvatic transmission cycles have been proven for all four serotypes, it is thought that the four serotypes evolved independently from their sylvatic progenitors. The transition from sylvatic to human-to-human urban cycles was made feasible by mosquitoes feeding on humans in rural areas. The sylvatic life cycles of dengue virus are only found in Africa, whereas they are seen for all dengue virus kinds in the Malay peninsula, implying an association with an Asiatic origin for dengue viruses (1). DENV evolutionary rates are 7.6×10^{-4} substitutions/site/year (48). Based on these rates, it has been predicted that dengue viruses diverged from their progenitors roughly thousand years ago and that zoonotic transfer from sylvatic to urban epidemic cycles happened between 15 and 40 years ago (91), (92). The virus's adaptability to human-to-human transmission in various geographical zones was most likely responsible for the diversity. This evolutionary adaptation not only allowed the four serotypes to co-circulate, but it also had a significant impact on their pathogenicity to humans.

2.6. DENV Serotype and genotype

A serotype is a classification within a species based on the variations in the antigens on the surface of the organism. In the case of viruses, such as the dengue virus, serotyping is often done based on the specific proteins or antigens present on the viral surface. In the context of the dengue virus, there are four serotypes: Dengue virus serotype 1 (DENV-1), Dengue virus serotype 2 (DENV-2), Dengue virus serotype 3 (DENV-3) and Dengue virus serotype 4 (DENV-4) (49). Each serotype represents a distinct strain of the virus with slightly

different surface antigens. This means that infection with one serotype does not provide immunity to the other serotypes. The genotypes of different serotypes of the dengue virus are depicted in **Table 1.b** and **Figure 11.**

Table. 1.b: Classification of DENV genotypes of different serotypes and their distributions (Ref: Jagtap S, Pattabiraman C, Sankaradoss A, Krishna S, Roy R. Evolutionary dynamics of dengue virus in India. PLoS Pathog. 2023)

Serotype	Genotype and their distribution
DENV-1	I Strains from East Africa, Southeast Asia and China
	II Strains from Thailand (between 1950s and 1960s)
	III Sylvatic strain collected in Malaysia and Malaysian strain
	IV Strains from the West Pacific islands and Australia
	V Strains collected in the Americas, West Africa, and some strains from Asia
DENV-2	Asian 1 Strains from Malaysia and Thailand
	Asian 2 Strains from Vietnam, China, Taiwan, Sri Lanka and the Philippines
	Cosmopolitan Strains from Australia, East and West Africa, the Pacific and Indian ocean islands, the Indian subcontinent and the Middle East
	American Strains from Latin America and older strains collected from the Caribbean, the Indian subcontinent and Pacific Islands in the 1950s and 1960s
	Asian/American Strains from Thailand and Vietnam and strains collected in the Americas
	Sylvatic Strains collected from humans, forest mosquitoes or sentinel monkeys in West Africa and Southeast Asia
DENV-3	I Strains from Indonesia, Malaysia, the Philippines and recent isolates from the South Pacific islands
	II Strains from Thailand, Vietnam and Bangladesh
	III Strains from Sri Lanka, India, Africa and Samoa and 1962 strain from Thailand
	IV Strains from Puerto Rico, Latin and Central America and the 1965 Tahiti strain DENV-4
DENV-4	I Strains from Thailand, the Philippines, Sri Lanka, and Japan
	II Strains from Indonesia, Malaysia, Tahiti, the Caribbean and the Americas
	III Thai strains that are distinct from other Thai isolates
	IV Sylvatic strains from Malaysia

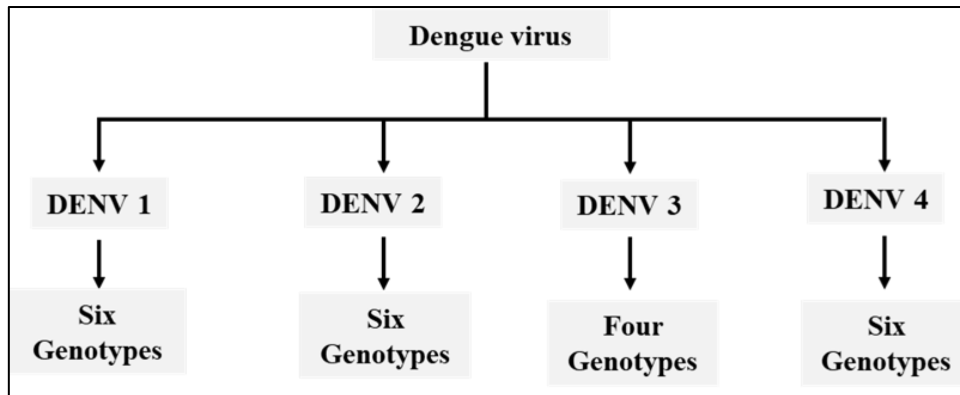

Fig. 11: Classification of dengue serotypes and their genotypes

The presence of multiple serotypes is a significant factor in the epidemiology and clinical manifestation of dengue fever. Individuals who have been infected with one serotype are at risk of more severe disease if subsequently infected with a different serotype. This phenomenon is known as antibody-dependent enhancement (ADE) and it underscores the importance of considering all four serotypes in vaccine development and public health strategies to combat dengue fever. The study found a strong link between DENV-1 and DENV-3, with DENV-2 forming a single cluster with these serotypes and DENV-4 forming a individual group with a single serotype. Dengue viruses are varied; the nucleotide sequences of the four DENV serotypes differ by 5-35%. DENV-4 looks to be the most divergent of the four DENV serotypes, followed by DENV-2, with DENV-1 and DENV-3 being the most closely related to one another (93), (94). Dengue viruses of the same serotype can be divided into various genotypes (or subtypes) based on phylogenetic study. The envelope gene is the most commonly used genomic area in phylogenetic research; however, sequences from other genes have been used to determine the DENV genotype.

The phylogenetic study also supports the biological split of dengue viruses based on transmission cycle characteristics. Dengue virus strains arising from nonhuman primates and rainforest mosquitoes belong to the sylvatic genotype and are distinct from epidemic variants linked with urban human-to-human transmission. Depending on the author,

multiple different approaches and styles of numbering or naming the pandemic DENVs have been proposed, resulting in different designations for a given genotype. DENV-4 is divided further into three primary genotypes which include one sylvatic genotype and two epidemic genotypes first discovered in South East Asia (genotype I) and Indonesia (genotype II) (18), (95), (96). DENV-2 viruses have been divided into six genotypes. The sylvatic genotype comprises strains from South-East Asia and Africa. The five most prevalent genotypes of DENV-2 include the Cosmopolitan genotype (IV), which has a global distribution and one genotype originally described from Central and South America, American genotype (IV). Furthermore, two lineages of Asian origin (Asian genotypes I and II) (97) are separated and one genotype from the Americas genetically connected with Asiatic viruses is referred to as the American-Asian genotype (III), which has been associated with severe disease (98).

DENV3 viruses have been divided into five lineages: the Indian subcontinent (III), Thailand (II), the Americas (IV), Southeast Asia (V) and one lineage found in Southeast Asia and the South Pacific (I) (48). Although no sylvatic strains of DENV-3 have been identified or sequenced, serological studies have provided evidence for their presence. DENV-1 viruses have been determined to have five distinct genotypes, one of which is sylvatic (III). Four etiological genotypes originated from Thailand (II), other regions of Asia (I), the Americas and Africa (V) and the South Pacific region (IV) are the origins of DENV (91), (99).

Genotyping is particularly useful in tracking the spread of the virus and understanding its evolution over time. Different genotypes of each dengue virus serotype can be found in various geographical regions and shifts in predominant genotypes can occur. Researchers use genetic information to study the molecular epidemiology of dengue and to monitor changes in the virus that might affect disease patterns and severity. It's important to note

that while serotypes refer to variations in the surface antigens, genotypes refer to genetic variations within the virus's entire genome. Both serotyping and genotyping are crucial for a comprehensive understanding of the dengue virus and for informing public health strategies, including vaccine development and outbreak management (73), (100).

2.7. DENV life cycle in cell and replication mechanism

The risk of DENV infection is determined by the host's age, virus serotype, genotype and genetic background. DENV infects a wide range of cells, including macrophages, monocytes, and dendritic cells. DENV enters the cell by receptor-mediated endocytosis.

2.7.a. DENV life cycle in cell

The virus is constructed and budded off when the viral RNA is digested and reproduced (101), (102). Dengue viremia occurs three to fourteen days after infection and peaks around the onset of symptoms (103). DC-SIGN on macrophages and human mannose-binding receptor (MR) are primary receptors used by DENV and CLEC5A as signalling receptors for sensing the invasion of dengue virus and then sending signals to stimulate macrophages to secrete cytokines. Heparin sulphate, a mannose receptor on CD14+ monocytes and GRP78/Bip on hepatic cells are also known as cell surface receptors (104), (105).

Dengue virus interacts with the cellular receptor via E-protein, which enables receptor-mediated virion uptake by endocytosis. The low pH of the endosome after internalisation causes a conformational shift in the E-protein, revealing a fusion peptide mediating the fusion of the virion and endosome membranes (106). As a result, the viral envelope ruptures and the capsid is released into the cell. The capsid dissociates, releasing the viral DNA into the cytoplasm of the host cell, where it is translated into a single big polyprotein **(Figure 12).**

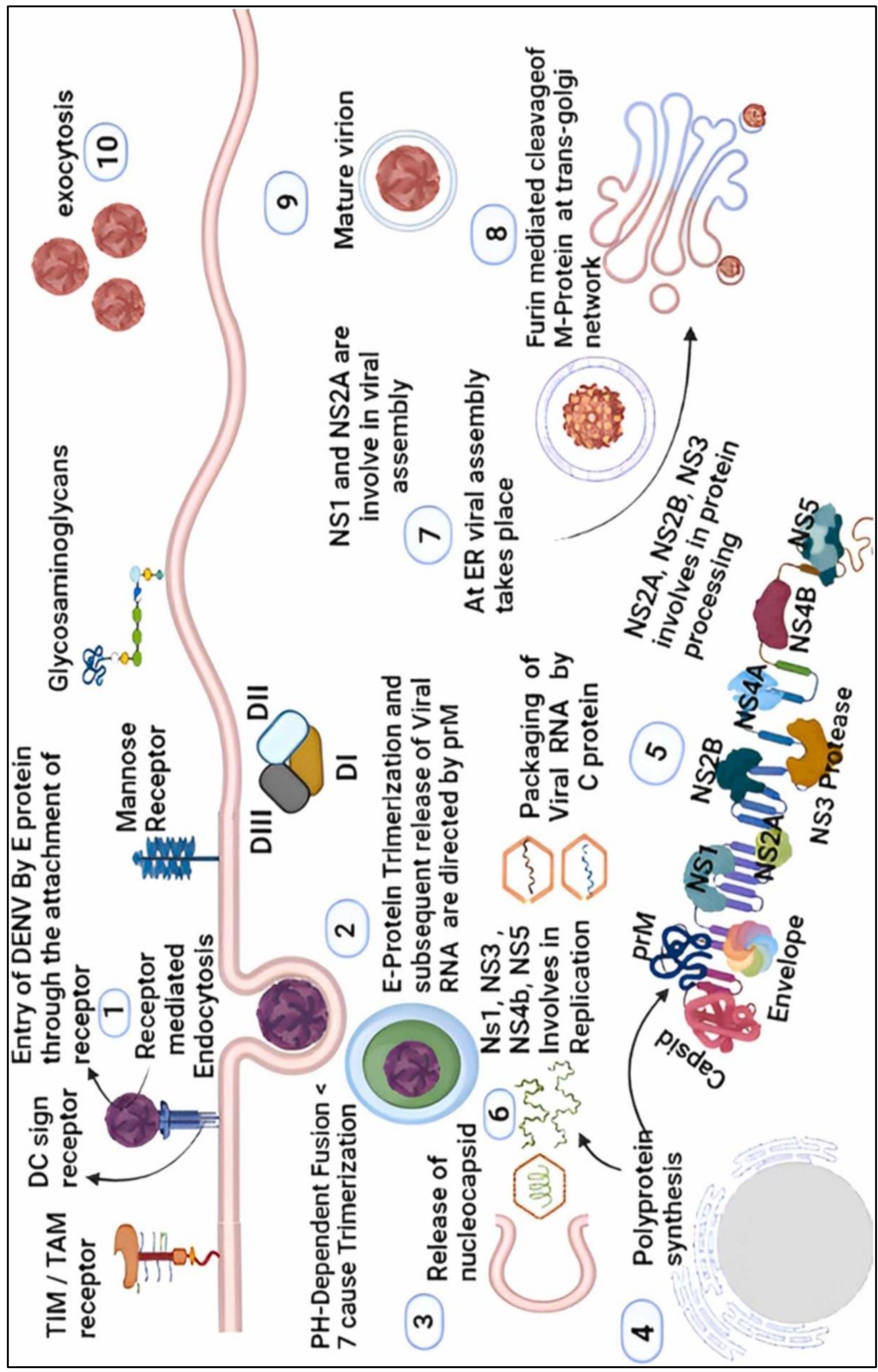

Fig. 12: Dengue virus life cycle. DENV infects and replicates involving several steps, including virus attachment, receptor-mediated endocytosis, virus uncoating, viral protein generation, vRNA replication, virus assembly and release (Cruz-Oliveira C, et. al, Receptors and routes of dengue virus entry into the host cells. (Ref: FEMS Microbio Rev. 2015;39(2):155–70)

The polyprotein is directed to the endoplasmic reticulum (ER), where it is orientated by signal sequences and membrane anchor domains, leaving it on the cytosolic and ER luminal sides. Individual viral proteins are formed as a result of processing by virus and host-encoded proteases: three structural proteins: capsid (C), a precursor for the membrane

protein (prM), envelope protein (E) and seven non-structural proteins that have functions mediating replication, polyprotein processing and virion assembly (106), (107), (108). The replication occurs in virus-induced vesicular membrane structures linked to the ER. The viral positive sense RNA genome is replicated via a negative-sense intermediary, which serves as a template for the formation of more genomic positive strands. The genomes are packaged into capsids via an unknown method. The virions enter the ER lumen as immature forms with surface prM and E proteins. Immature virions are transported through the trans-Golgi network, where they are matured by cleavage of pr/M, resulting in infectious, mature virions that are exocytosis out of the cell (109).

2.7.b. Viral Genome Replication

DENV genomes have a 100-nucleotide long 5'-UTR region with a hairpin stem-loop shape and a 7-methyl guanylate cap (101). The 3'-UTR region is 450 nucleotides long, has a conserved 3' stem-loop required for efficient replication, a conserved sequence CS1 required for genome cyclization and lacks a poly(A) tail (110). DENV RNA is translated into the cytoplasm by host cell machinery to produce viral polymerase. RNA-dependent RNA polymerase attaches to the 5' stem-loop structure and interacts with the 3'-end to generate the negative or template strand of the genome by cyclizing the complete genome. The template strand is employed in the Golgi apparatus to transcribe the positive strand genome (111). Invaginations of the ER membrane create a membrane-bound milieu essential for RNA production and viral assembly. Many positive viral RNA stands are associated with ER membrane invaginations that enfold around active replication complexes (RCs) and establish membrane-bound viral replication compartments. Membrane compartments keep viral RNAs and proteins distinct from cellular components, allowing for more effective RNA synthesis and viral assembly (112).

2.7.c. Viral Assembly and Budding

DENV is quickly packed and organised into the virion on the ER of infected cells. The ER aids in the orientation of the capsid, allowing the viral RNA and the C protein to form nucleocapsid complexes (113). The nucleocapsid complex forms the heterodimer complex with the addition of prM and E proteins from the ER. With the addition of sugar residues, this complex is transported to the Golgi apparatus for post-translational modification. With a prM-E-lipid sheath, the complex buds through the ER lumen. While the virus is being exported via exocytic vesicles, furin-mediated cleavage of prM results in the production of mature M proteins, which leads E proteins to a conformational shift to a homodimer form (112).

Chapter 3: Immune responses and pathogenesis of dengue and severe dengue

3.1. Immune Response to Dengue Infection

DENV infects human Langerhans and dermal/interstitial dendritic cells, which move to lymph nodes and deliver viral antigens to T-cells, triggering cellular and humoral immune responses (114). DENV also infects macrophages and monocytes, causing T-cell activation of DENV-specific CD4+ memory T cells and CD8+ T lymphocytes (115). To further engage the immune system, active T cells produce cytokines and chemical mediators. Tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-7 (IL-7), interleukin-6 (IL-6) and platelet-activating factor are all produced by infected monocytes and macrophages. These variables, as well as complement activation products (C3a and C5a) and histamine, are related to the severity of sickness because they contribute to increased vascular permeability, plasma leakage, and shock (116). Antibodies predominantly directed against the E and M glycoproteins are generated to develop acquired immunity to the infecting serotype of DENV. The primary infection stage of DENV infection is characterised by a sluggish and low-titer antibody response. The first kind of antibody to arise is immunoglobulin M (IgM). IgM antibodies rise between three to ten days of fever onset, peak approximately two weeks later, and then drop to undetectable levels over the next few months. Immunoglobulin G (IgG) antibodies are detected at a low titre one week after the fever starts and gradually rise over time (36), (51). Each dengue serotype provides particular immunity to that serotype. As a result, infection with all four dengue serotypes can occur in a single person (29). A secondary antibody response arises after a second DENV exposure. During the acute phase of this infection, IgG antibodies are generated and titer rise significantly to high levels in the subsequent two weeks. During a subsequent DENV infection, IgM antibody levels are much lower IgM and IgG levels are crucial for

identifying primary and secondary dengue infections, as they increase after the first infection and decrease with secondary infections (50). Individuals who have a subsequent DENV infection with a heterologous serotype are at a high risk of developing severe illness due to antibody-dependent enhancement (ADE). In ADE, an existing heterologous dengue antibody specific for the virus serotype from the main infection recognises the new DENV serotype after secondary infection, generating a non-neutralizing antigen-antibody complex. Fc receptors on the cell surface attach to the Fc portion of the antigen-antibody combination, allowing the virus to bind. The virus is then internalised by the cell, which allows it to proliferate (117).

3.2. Cell tropism

Dengue virus can be found in peripheral blood for a brief length of time during the early stages of the disease, with levels presumably matching the number of infected tissues. DENV cell tropism includes the immune system, liver, and endothelial cells of blood arteries, according to postmortem and in vivo investigations of DENV-infected patients. Dengue virus has been demonstrated to infect several cell types after being injected into the human circulation by a mosquito. The first targets might be epidermal dendritic cells (skin Langerhans cells) (114) and keratinocytes (97). When the virus infects the lymph nodes, monocytes and macrophages become the primary targets of the infection, disseminating the virus through the lymphatic system to cells of the mononuclear lineage in the liver and spleen, including monocytes, myeloid dendritic cells, and macrophages (118).

3.3. Pathogenic mechanisms of dengue virus

Several viral and host-specific variables have been linked to dengue illness severity and pathogenesis. Because identical clinical signs resembling severe dengue in humans are not present in other species, the study of dengue pathogenesis currently lacks a viable animal model. Murine models produced by experimental infection with DENV frequently develop a neurological illness observed relatively infrequently in infected humans (119), (120). Non-human primates infected with DENV produce viremia, although replication is less efficient than in humans, resulting in lower levels of viremia. However, when a large dosage of the virus is employed, Rhesus macaques show sub-cutanic haemorrhagic symptoms (121).

Several hypotheses and the participation of diverse components have been postulated to explain the pathogenesis of dengue; nevertheless, the required or sufficient elements are unknown and the understanding of dengue pathogenesis in humans is primarily based on fatal cases analysed. Host genetic variables, age, immunological condition, chronic illnesses, and viral strain features are all connected with severe disease outcomes (122). The observed disparities in illness patterns among locations show the participation of many causes, such as the fact that in Southeast Asia, DHF primarily affects children, whereas it primarily affects adults in the Americas. It has been proposed that distinct DENV serotypes and lineages may have biological features that are related to pathogenicity (2). However, these are unlikely to fully explain the reported pathogenicity because all DENV strains may induce DHF/DSS.

Because viral load in humans correlates with illness severity (123),(124) the lack of immune responses to restrict DENV replication and the impact of infected cells on other cells in the body are likely to play a significant role. The elevated viral load is thought to cause apoptosis and necrosis in numerous tissues, as well as an imbalance in the profiles of

soluble mediators and cytokines, which results in endothelial cell dysfunction and blood coagulation abnormalities seen in DHF and DSS. Based on epidemiological research linking DHF to secondary infections (123), the role of immunological enhancement in dengue sickness was suggested. According to the antibody-enhancement (ADE) theory, cross-reactive antibodies bind to the illness-causing virus but are unable to neutralise it. These antibodies can be maternal in early infants or antibodies from an earlier infection with a heterologous DENV serotype. These antibody-virus complexes promote monocyte-macrophage infection by attaching to their Fc-receptors. In vitro experiments revealed that the presence of cross-reactive non-neutralizing antibodies boosted DENV infection in macrophage lineage cells (117).

According to ADE theory, increased infection activates a cascade of events that leads to immunopathology via T-cell activation, interferons, complement cascade and platelet activation, cytokines and altered endothelial and epithelial cell activities. It is hypothesised that the increased infection incidence causes greater viremia in individuals with secondary infections, which finally leads to plasma leakage. The commencement of plasma leakage, however, happens after the peak of viremia, implying an immune-mediated process. Cross-reactive antibodies known to exist against E-protein were considered to be responsible for the enhancement; however, a subsequent investigation revealed that they were mostly directed against the prM protein of DENV (117). Whereas the ADE theory emphasises the relevance of pre-existing antibodies in dengue development, it does not explain DHF in primary infections. Dengue-virus-specific T-cell responses have also been linked to severe illness and have been proposed to have a role in infection clearance and immunopathogenesis. The recovered activated T-cells from individuals with secondary infections were shown to have a poor affinity for the infective serotype. Instead, they demonstrated a strong affinity for the heterologous serotype, which was most likely the one

seen earlier (125). It has also been postulated that molecular mimicry plays a role in dengue pathogenesis by inducing an autoimmune response. An amino acid residue region in the DENV-4 envelope protein was discovered to mimic a protein family of blood clotting proteins. The patient antibodies against E-protein were also shown to cross-react with plasminogen (126). Antibodies to the NS1 protein have also been demonstrated to bind blood clotting factors, integrins, and endothelial cells (41). The mechanisms of the complement system in dengue pathogenesis are poorly understood, although they are thought to play an essential role. Complement activation is related to plasma leakage, which corresponds to high quantities of C3a and C5a activation products in patient plasma. Furthermore, patients with DSS have lower levels of complement components. The NS1 protein of the Dengue virus has been shown to interact with the complement inhibitory factor clusterin and the soluble NS1 protein has also been demonstrated to activate complement, which is aided by anti-NS1 protein antibodies (127).

3.4. Secondary Dengue Infections Risk Factors

A single serotype dengue infection offers lifelong immunity against heterotypic serotypes, but also transient cross-protection. Controlling infections and the propagation of viruses depend heavily on the humoral response. An individual can be infected by more than one serotype at the same time. Cross-reactive neutralizing activity ages whereas specific neutralizing IgG antibodies remain active for decades. According to preliminary evidence, there may be a continuous selection process for human dengue-virus-neutralizing antibodies, with homologous reactivity rising and heterotypic cross-responses falling. Early research conducted in Thailand revealed that DHF/DSS was most prevalent in two groups: children who were born to mothers who were resistant to dengue and children who experienced a mild or silent dengue illness before contracting a different dengue serotype.

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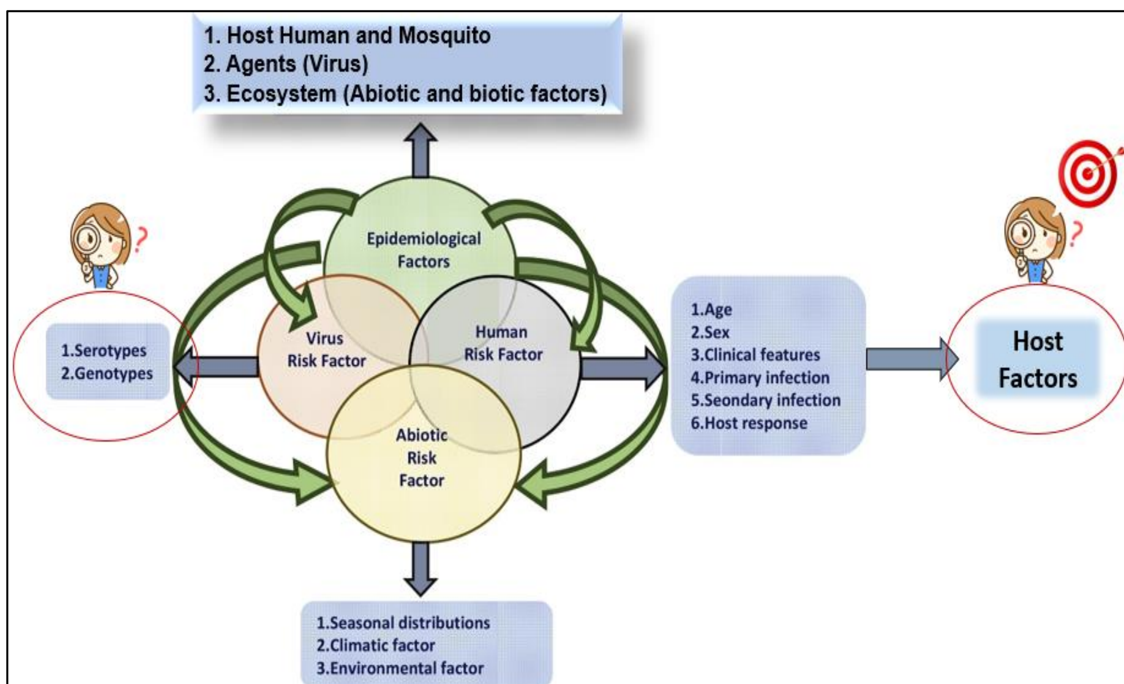


Fig. 13: Various risk factors associated with severe dengue

1. Prior Dengue Infection: People who have previously contracted one dengue virus serotype will be more likely to become very sick from future infections (1).

2. Age: Severe dengue is more likely to affect children, teenagers, and older persons. Severe illness symptoms are more likely to occur in younger people (73).

3. Immune Response: When a person has antibodies against one serotype of dengue and contracts an infection with a different serotype, a condition known as antibody-dependent enhancement (ADE) may occur. This may result in an advanced stage of the illness (34).

4. Viral Load: A higher risk of severe dengue has been linked to higher viral loads. The severity of the illness may vary depending on the quantity of virus in the circulation (130).

5. Host Genetics: A person's vulnerability to severe dengue may be influenced by certain genetic variables. Both the severity of the illness and the immunological response can be impacted by genetic differences. One genome-wide association study (GWAS) on dengue, for instance, found a correlation between variations in phospholipase C, epsilon 1 and DSS, phosphopeptide-related sequence B (MICB), major histocompatibility complex (MHC) class I, and phospholipase C (37).

6. Chronic Health illnesses: People who already have underlying medical illnesses, such as diabetes, hypertension, or other chronic diseases, may be more susceptible to severe dengue.

7. Secondary Infections: Severe dengue is more likely when secondary infections occur from a different dengue virus serotype. This has to do with the phenomena of enhancement that is dependent on antibodies.

8. Immune Status: The degree of a dengue infection may vary depending on the immune system's capacity to build a successful defence.

9. Delay in Medical Care: There is a higher chance of complications and unfavourable results when medical attention and appropriate care are delayed. To reduce the chance of developing severe dengue, early detection and appropriate medical management are essential.

10. Mosquito Exposure: The risk of infection is increased by residing in or visiting regions with a high concentration of Aedes mosquitoes, which are the carriers of the dengue virus. Living in or visiting dengue-endemic areas requires people to take precautions against mosquito bites, seek medical assistance as soon as symptoms appear, and adhere to hospital protocols for dengue fever treatment. Furthermore, future study may shed light on the risk factors connected to severe dengue. These all factors play a crucial role in pathogenesis of dengue fever and severe dengue.

3.5. Severe dengue hypothesis: Cytokine Strom and Antibody-Dependent Enhancement

Cytokine Strom-Immuno-modulating proteins and their role in dengue and severe dengue with vascular endothelial dysfunction

Cytokines and chemokines are small proteins ranging from 8 to 40 kDa, also known as immune-modulating proteins. Secreted proteins cytokines intricate with cell signalling, play a role in induction, inhibition and the effector phases of the immune and inflammatory pathway. The small subset of cytokines known as chemokines recruit and exert chemotactic passage of other cells to a localized zone for exerting different types of biological effects such as inflammation and homeostasis (131). There are two main predominant pathways or hypotheses for DHF/DSS pathogenesis; the first one is antibody-dependent enhancement

(ADE) and the second is T cell immunopathology (cytokine storm) causing an imbalanced immune system (134),(135) (**Figure 14.a**).

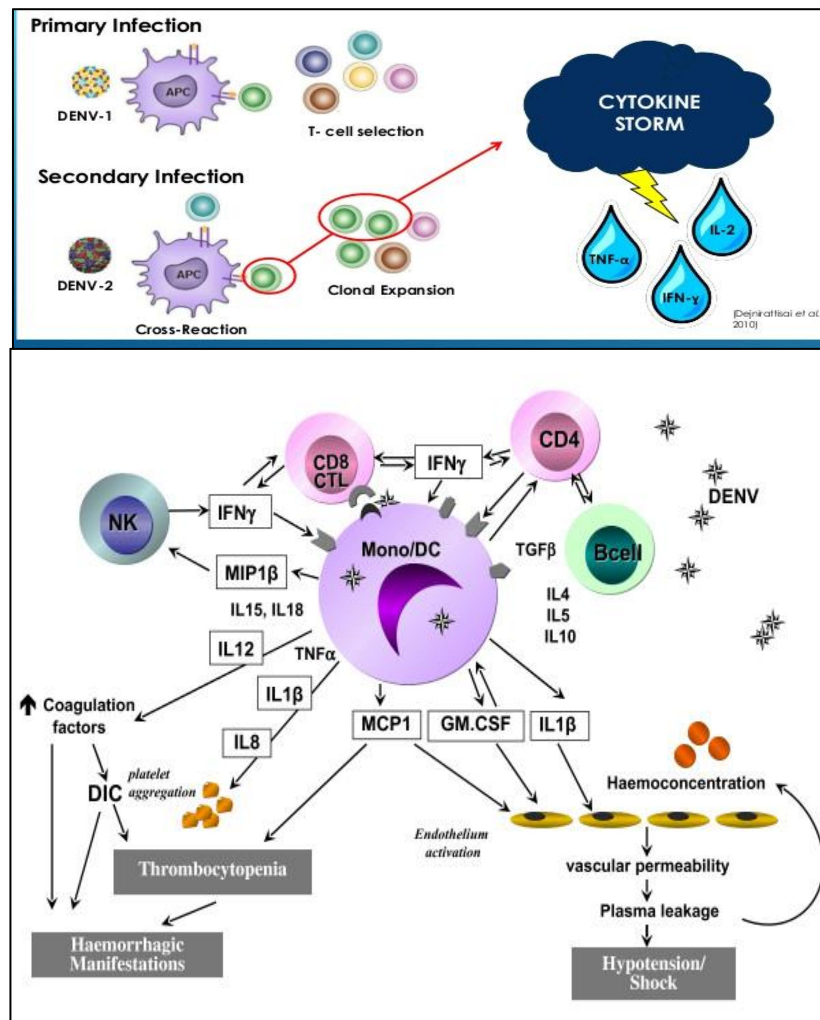


Fig. 14.a: Detailed mechanism of cytokine Strom (Ref: Patro ARK, Mohanty S, Prusty BK, Singh DK, Gaikwad S, Saswat T, Chattopadhyay S, Das BK, Tripathy R, Ravindran B. Cytokine Signature Associated with Disease Severity in Dengue. Viruses. 2019 Jan 8;11(1):34.

Severe dengue in neonates and children is caused by maternally generated DENV IgG antibodies and/or altered cytokine profiles (30). Severe dengue also results from secondary dengue infection. The immuno-modulating protein profiles of dengue and severe dengue vary, and these variations are linked to the development of plasma leakage, enhanced microvascular permeability, and thrombocytopenia as well as alterations in the clinical course of dengue virus (113). It has been noted that there is a change in the TH1 (Type 1

Helper) type response in DF to the TH2 (Type 2 Helper) response in DHF/DSS. This change is thought to be brought about by modified immune regulating proteins. Our dengue interactome representation demonstrated the direct involvement of biological pathways such IF-γ, IL-27, IL-35, IL-12, IL-21, IL-6, IL-23, IL-9, IL-4, type-1 interferon and TNF related signaling pathways in vascular dysfunction.

Thus, immune-modulating proteins are always desirable and should be pursued as possible early prognostic indicators for dengue severity prediction. The primary characteristic of severe dengue is elevated vascular permeability, which leads to widespread plasma leakage in the body's cavities and tissue spaces, including the pleural, pericardial, and peritoneal cavities in DHF patients. This can cause profound shock (136). The basic anatomical blood-tissue barrier is made up of the vascular endothelial cells that line the inner lumen of blood vessels and capillaries. These endothelial cells play a crucial role in controlling the flow of nutrients into the tissue, the dynamics of the microcirculation, and the permeability of macromolecules. They are highly systematized and physiologically defined. They are crucial to the immune system's operations and the body's homeostasis.

Thus, immune-modulating proteins are always desirable and should be pursued as possible early prognostic indicators for dengue severity prediction. The primary characteristic of severe dengue is elevated vascular permeability, which leads to widespread plasma leakage in the body's cavities and tissue spaces, including the pleural, pericardial, and peritoneal cavities in DHF patients. This can cause profound shock (136). The basic anatomical blood-tissue barrier is made up of the vascular endothelial cells that line the inner lumen of blood vessels and capillaries. These endothelial cells play a crucial role in controlling the flow of nutrients into the tissue, the dynamics of the microcirculation, and the permeability of macromolecules. They are highly systematized and physiologically defined.

They are crucial to the body's hemostasis and immune system processes. However, endothelial cell activities in microcirculatory beds and the integrity of this barrier are not constant and are physiologically influenced by a variety of stimuli (131, 136). When endothelial cells respond to immunological responses, their permeability dramatically changes, particularly when it comes to proinflammatory cytokines like TNF-α, IFN-γ, IL-6, and IL-8 (8), (133). Patients with a heterologous dengue serotype who have a subsequent dengue infection are more likely to get dengue hemorrhagic fever. The mechanism behind it is enhanced viral replication caused by macrophages absorbing more of the dengue virus-enhancing antibody complex.

The dengue virus peptides were given to memory T cells after being complexed with human leucocyte antigen (HLA) molecules. This resulted in the proliferation and generation of proinflammatory cytokines, which in turn had an impact on vascular endothelial cells and ultimately led to plasma leakage. Therefore, the higher incidence of DHF/DSS would be compatible with the quick induction of cross-reactive memory T-cells after a recurrent dengue infection. Vascular permeability is eventually increased by a "tsunami" of inflammatory cytokines, the cascade of memory T-cell activation, and other chemical mediators produced mainly by T cells, monocytes/macrophages, and endothelial cells. Increased disease severity during dengue infection is mostly due to the interaction of several inflammatory cytokines' actions on vascular endothelial cells (131, 137).

The pathophysiology of the vascular endothelial cell, which serves as the dengue virus's battleground, has been the subject of substantial research for many years; nonetheless, the etiology of DHF remains poorly understood, mostly because suitable animal models are rarely used. A thorough review of all the existing literature was conducted to determine the role of immune-modulation chemicals and possibly important molecules in dengue and severe dengue with increased vascular dysfunction.

Antibody-dependent enhancement (ADE)

This is a phenomena where pre-existing antibodies facilitate a virus's entrance into host cells rather than shielding it from attack. There are four different serotypes of dengue virus (DENV-1 to DENV-4), and having been infected with one serotype before does not provide protection against subsequent infections (138). When a person who has already contracted one serotype is exposed to another, ADE becomes a worry (**Figure 14.b**). Here's how antibody-dependent enhancement works in the context of dengue fever:

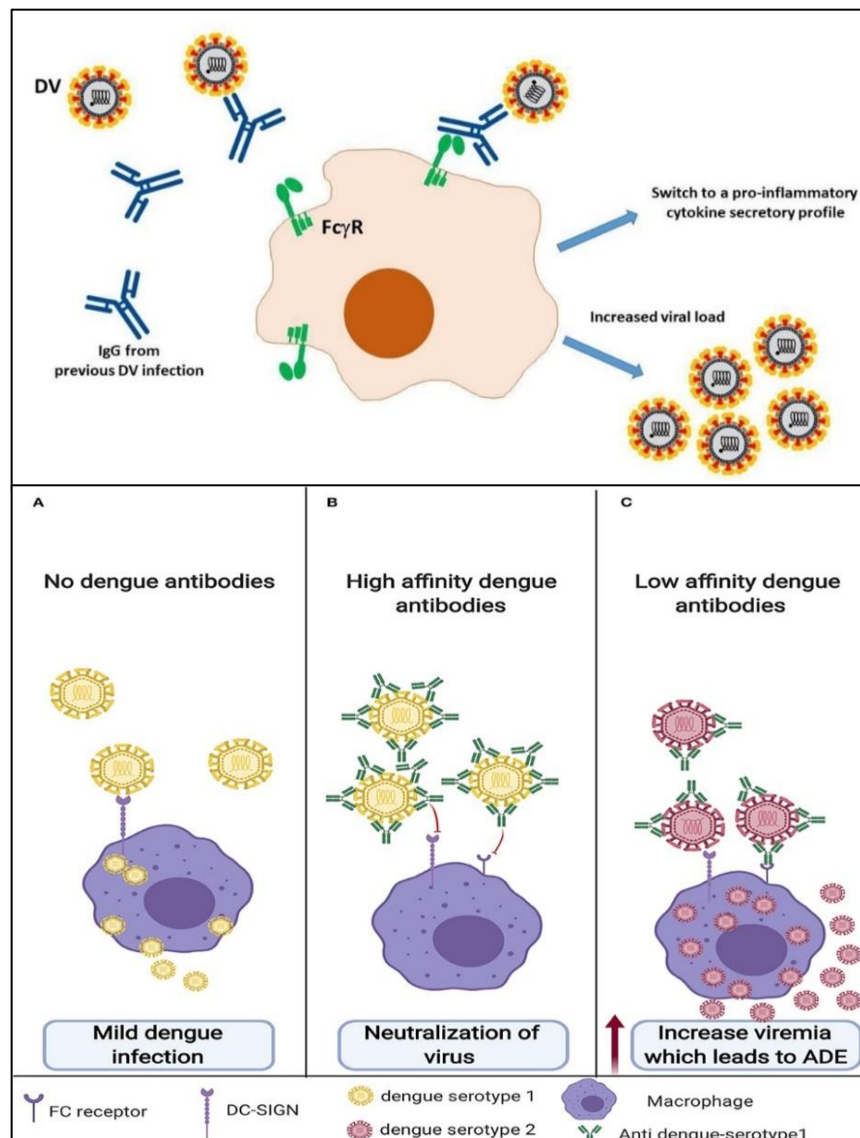


Fig. 14.b: Detailed mechanism of antibody-dependent enhancement (Ref: Guzman MG, et. al, The complexity of antibody-dependent enhancement of dengue virus infection. Viruses. 2010 Dec;2(12):2649–62)

1. Initial Infection: When a person is first infected with one serotype of the dengue virus, the immune system generates antibodies specific to that serotype (primary infection). These antibodies help clear the virus from the bloodstream and provide immunity against that particular serotype.

2. Subsequent Infection with a Different Serotype: If the person is later exposed to a different dengue serotype, the pre-existing antibodies may recognize and bind to the new virus (secondary infection). When antibody-virus complexes are internalised into cells via FcRs, a greater proportion of target cells are infected, potentially leading to increased viral output. During an initial dengue infection, cross-reactive antibodies with little neutralising action are produced. These antibodies attach to the second infectious virus during secondary infection. Increased viral generation has traditionally been regarded as the result of an increase in the number of infected Fc-R-bearing cells, as well as an accelerated rate of internalisation and cell infection by immune complexes. It has been proposed that infected individuals have higher viremia and so suffer from greater severity. However, these antibodies may not effectively neutralize the virus. Instead, the virus-antibody complex may facilitate the entry of the virus into certain immune cells, such as macrophages, through a process known as ADE (139), (140).

3. Enhanced Viral Replication: Once inside the immune cells, the dengue virus can replicate more efficiently, leading to higher viral loads and potentially more severe disease. This phenomenon is associated with the development of severe dengue, including dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (141).

4. Cytokine Storm: ADE can trigger an exaggerated immune response, often referred to as a cytokine storm. This uncontrolled release of pro-inflammatory molecules can lead to increased vascular permeability, bleeding, and organ damage (137).

Extrinsic Vs. Intrinsic ADE

Extrinsic and intrinsic ADE are the two types of ADE. The term "extrinsic ADE" describes mechanisms like higher rates of receptor engagement and internalization of virus-immune complexes that are not inherent to mononuclear phagocytes. In fact, extrinsic factors were thought to be mostly in charge of the detrimental effects of dengue ADE-related pathogenesis until research on the Ross River Virus (RRV) revealed otherwise. Innate immune suppression resulting from the prolonged incubation of virus-infected cells with anti-virus IgG resulted to ADE-mediated chronic productive infection of macrophages (142). Intrinsic ADE is characterized by internalized virus-immune complexes that influence innate immune effectors to enhance replication and release (143). Stated differently, extrinsic ADE increases the bulk of infected cells whereas intrinsic ADE increases the "burst size" of infected cells, or the quantity of virus released from an infected cell. This work focuses on intrinsic ADE during dengue infections and the several ways in which this phenomenon facilitates the production and proliferation of DENV (142).

3.6. Primary dengue vs Secondary dengue infection immune responses

The main source of the extreme pathogenicity linked to intrinsic ADE-induced secondary dengue infection is the evasion of the host's innate immune responses. An in-depth knowledge of the immune response that arises during canonical/primary dengue infection is necessary in order to completely comprehend the immune evasion pathways associated with intrinsic ADE. Pathogen recognition receptors (PRRs) are the first to recognize the invasive pathogen during canonical DENV entry, which takes place by receptor-mediated endocytosis. While MDA5 (melanoma differentiate on associated gene 5) and RIG-I (retinoic-acid inducible gene 1) recognize viral RNA escape from these vesicles, TLR3 (Tool Like Receptors 3) and TLR7 identify the virus in endosomes (144). Proinflammatory

cytokines including IL-12, IL-8, IFN-γ and IFN-α are produced in response to the activation of TLR-dependent and independent pathways (145).

This, together with STAT1 activation by IFNs, leads to the formation of nitric oxide (NO) radicals, which aids in the limitation of DENV replication and dissemination (143).

Effects on Adaptive Immune Response

Pathogen clearance requires a balanced Th-1 (T cell helper type 1) and Th-2 type immune response to every infection. Th-2 reactions lead to increased production of anti-inflammatory cytokines, which are characterized by type-2 or antibody-mediated immunity, whereas Th-1 responses result in the production of pro-inflammatory cytokines and greater phagocytic activity. Th-2 cytokines, including IL-1, IL-10, and IL-13, promote the growth of B cells, which in turn increases the generation of antibodies (146). A skewed Th-2 immune response in dengue-ADE only makes matters worse by promoting the production of sub-neutralizing antibodies, which aid in immune complex-mediated DENV entry into vulnerable cells (140).

3.7. Knowledge gap and rationale of the study

The pathogenesis of severe dengue is complex and involves intricate interactions between the virus, host immune responses and various biomolecules. Severe dengue imposes a substantial socioeconomic burden, necessitating a comprehensive understanding of the biological factors that contribute to the transition of dengue fever to severe dengue (1) (147). Understanding the molecular mechanisms underlying severe dengue pathophysiology is crucial for developing effective therapeutic strategies for patient management **(Figure 15)**. Dengue virus serotypes and genotypes also play a crucial role in severe dengue development. Hence, in search of severe dengue patients, an epidemiological study is needed to observe the serotypic shift and their relation to severe dengue. Parallel

clinicopathological factors also play a crucial role in severe dengue (WHO) therefore continuous monitoring is required for risk assessment. In recent years, extensive research has focused on identifying and characterizing the bio-molecules associated with severe dengue, shedding light on their roles in disease progression and potential diagnostic and prognostic applications (148). Biomolecules associated with severe dengue encompass various aspects of the host-pathogen interaction, including viral replication and immune responses (149). They are involved in processes such as immune cell activation, cytokine signalling, endothelial dysfunction, coagulation abnormalities, and vascular leakage, all of which contribute to the pathogenesis of severe dengue (150). Notably, certain biomolecules are differentially expressed or exhibit distinct patterns in severe dengue cases compared to mild dengue or healthy controls, highlighting their potential as biomolecules for disease severity and prognosis (3). The dysregulated immune response plays a pivotal role in severe dengue pathogenesis and vascular endothelium is a key target in severe dengue (6). But the actual mechanism by which only a few dengue-infected patients develop severe dengue and not all is still unknown. To address this critical knowledge gap, this extensive study has focused on identifying and characterizing biomolecules associated with severe dengue. In this study, we have facilitated the high-throughput molecular biology, clinical assessment, qualitative and quantitative proteomic, protein array bioinformatics etc. analysis to identify the novel biomolecules and elucidate their intricate molecular networks underlying the pathophysiology of severe dengue. We aim to explore the intriguing domain of severe dengue-associated biomolecules, highlighting their potential as prognostic and diagnostic markers as well as therapeutic targets and hence validating the elucidated proteins via Real-Time PCR, Western blot, and ELISA techniques.

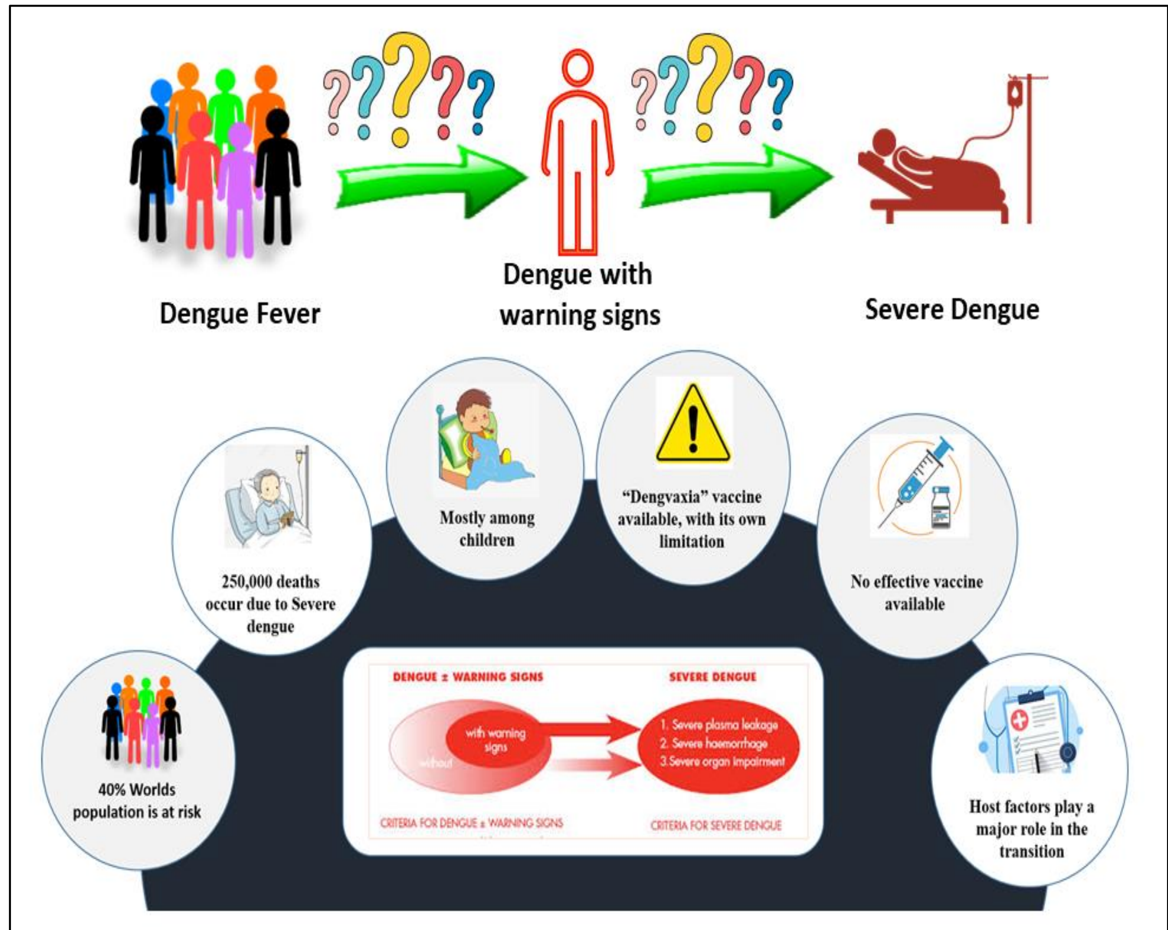


Fig. 15: Rational and knowledge gap towards the search for severe dengue biomolecules

Chapter 4: Objectives of the study

1. To study the serological and molecular conformation of DENV infection in clinically dengue-suspected patients and search for severe dengue patients among the study population
2. To study the clinicopathological association of dengue infection with different dengue virus serotypes and potential parameters associated with severe dengue
3. To find potential biomolecule(s) in serum associated with vascular dysfunction in the study population
4. To study the correlation between dengue virus and expression of serum biomarkers, and to find any specific marker(s) that can be used as surrogate marker(s) for early detection of DHF and or/ DSS

Chapter 5: Materials and Methodology

5.1. Study Population

Clinically suspected dengue fever patients as per WHO guidelines, were admitted to the ID & BG Hospital, Beliaghata; Dr B. C. Roy Post Graduate Institute and R. G. Kar Medical College and Hospital, three tertiary care hospitals in Kolkata were enrolled in this study. Blood samples were collected from different phases of dengue fever patients. The definition of phases is acute phase A = 2-7 days of fever, acute phase B = 48 to 72 hours after collection of acute phase A samples, critical phase A = 24 to 48 hours after defervescence, critical phase B= 48 hours after collection of critical phase A samples. Patients' samples from the acute phase were categorized as dengue fever (DF) i.e. dengue±warning signs whereas samples from the critical phase were categorized as severe dengue (DHF/DSS) population. In this investigation, samples were collected from the general ward (acute to recovery) and critical ward (critical to recovery/death phase) pairwise i.e. at two-time points with the help of our clinical collaborators. Apart from paired samples, unpaired samples from acute-phase patients were also collected from hospitals, to observe the circulating pattern of dengue serotypes. Blood samples were collected without having any age and gender bar.

A total of 380 dengue suspected hospital-admitted patients were recruited in this study and an additional 240 patients' samples from the dengue early phase were only collected for the dengue serotyping study to observe the prevalent serotype in each year. Among hospital-admitted patients, 32 acute phase samples were paired and among critical phase 19 paired samples were obtained. Plasma samples from six healthy individuals were collected and used as control samples. Samples were transported to ICMR-NICED, Virus

Laboratory in mini coolers to maintain the cold chain, divided into aliquots, and stored at -80°C for further analysis.

In a protein marker validation study, the sample size is a crucial consideration as it directly impacts the statistical power and reliability of the study results. Severe Dengue belongs to the rare disease of the Phenomena group and hence it is challenging to gather a large number of samples. Conducting protein marker validation studies is resource-intensive, both in terms of time and funding. This study is exploratory into the potential association of the protein marker with severe dengue conditions. Such studies are designed to generate hypotheses and guide future research. Also, the target population is highly homogeneous, meaning that the individuals within the study share similar characteristics and traits relevant to the protein marker, therefor limited sample numbers are justified.

5.2. Patient Selection Criteria

Dengue patients' classification was done based on dengue-associated clinical manifestation according to WHO guidelines (18). Patients were admitted with fever and any dengue symptoms such as nausea, vomiting, rash, headache, retro-orbital pain, myalgia, arthralgia, leukopenia, recurrent vomiting, abdominal pain, tenderness, lethargy/ restlessness, mild pleural effusion/ ascites, minor bleeding from any site, downturn platelet count was considered as dengue warning signs cases. Patients with severe plasma leakage leading to shock (DSS), fluid accumulation with respiratory distress, severe bleeding as evaluated by a clinician and severe organ involvement were considered severe dengue cases.

At the time of sample collection, written informed consent and pertinent data including demographics (patient address, age, sex, etc.) obtained from the patients or their legal guardians. Also, patient follow-up up done to collect phase-specific biochemical data.

5.3. Ethical Deceleration

This study protocol complied with the Helsinki Declaration of 1975 (151), amended in 2013 and was approved by the Institutional Ethical Committee, Indian Council of Medical Research-National Institute of Cholera and Enteric Diseases (ICMR-NICED), Kolkata. Subjects willing to participate in the study were enrolled and informed written consent was obtained for host and viral genetic analysis. Clinical collaborators assisted in collecting samples to gain access to DENV seropositive blood samples and clinical data from patients. Children (12 years old) were recruited with their parents' permission. Adolescents (12 to 17 years old) were recruited with both the patient's and their parent's permission. Before blood collection, all subjects provided informed written consent at the time of enrolment before taking blood samples. Those who refused to provide blood samples were not included in this study. Co-infected patients with other viruses/diseases were excluded.

5.4. Sample collection

2mL of blood was drawn by venipuncture by qualified phlebotomists and collected in EDTA vials. Collected samples were transported to the ICMR-NICED Virus Laboratory under the cold chain within 1 hour of collection.

5.5. Sample separation and storage

Collected samples were kept for 1-2 hrs in a 4° C freezer for the separation of blood and plasma. No centrifugation technique was used for sample separation to protect blood cells from shock. Plasma and serum were separated using clean pipetting procedures and aliquoted into multiple 1.5mL micro-centrifuge tubes labelled with Lab Code No. and

promptly frozen at -80°C freezer (**Figure 16**). The plasma was used for DENV serology assay, molecular serotyping, proteomics analysis, protein arrays, Western blot and ELISA while whole blood was used for total RNA extraction from whole blood. To prevent protein and RNA degradation, multiple freeze-thaw cycles were avoided.

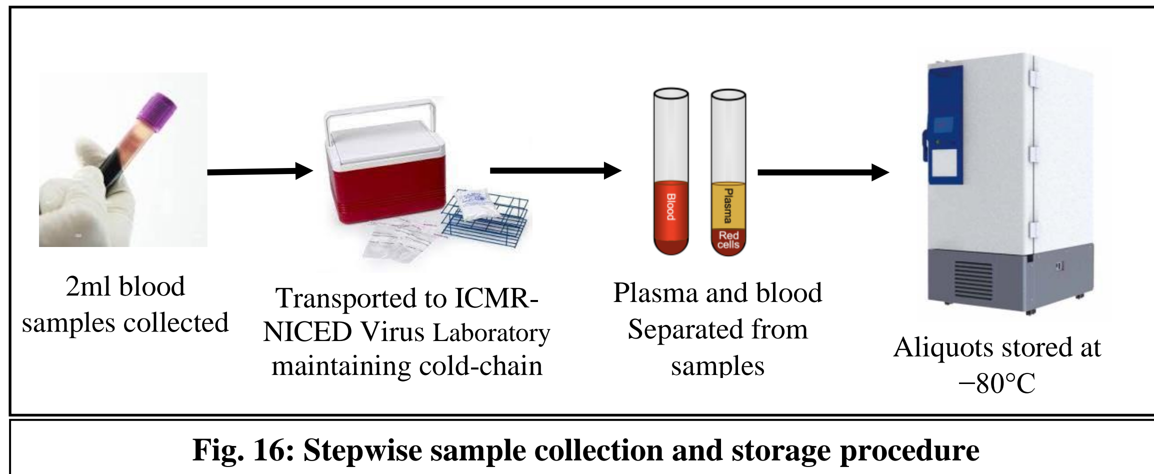


Fig. 16: Stepwise sample collection and storage procedure

5.6. Demographic and clinical data collection

Demographic data (age, sex, etc.) were obtained from the Hospital records section. All signs, symptoms and biochemical parameters of each hospital-admitted dengue patient were monitored until they were discharged as per WHO guidelines (2011). DF-associated manifestations were categorized as constitutional, gastrointestinal, respiratory, neurological, musculoskeletal and bleeding manifestations as per their physiological association and recorded after the patient examination. Fluid accumulation in body cavities was assessed by a sonogram of the abdomen (ascites) or chest X-rays for pleural effusion. Liver profile examination includes estimation of total protein (TP) using the Biuret method, albumin using the Bromo Cresol Method, alanine transaminase (ALT) and aspartate aminotransferase (AST) using the Reitman and Frankel method were analyzed and noted accordingly. Intravenous fluids and platelet transfusion were given to severe patients as per the WHO Handbook of Dengue Patient Management (152). Dengue cases were grouped as

DF and severe dengue (DHF with Grades I and II complications, DSS with Grades III and IV complications).

5.7. NS1 or IgM/IgG serology

Patient's samples were subjected to dengue NS1 for dengue detection in the early phase whereas, IgM/IgG ELISA (Pan Bio, Brisbane, Australia) was for dengue detection in the late phase of dengue fever. IgM and IgG levels are crucial for identifying primary and secondary dengue infections, as they increase after the first infection and decrease with secondary infections (153). Different phases of dengue fever have been categorized based on their ELISA profiles and associated clinical manifestations as per WHO guidelines, 2011 (8). 100 µl of standard or diluted samples were added to the wells of a micro-ELISA plate, pre-coated with gene-specific antibody then incubated for 90 minutes at 37°C. The liquid was then discarded and immediately 100 µl of Biotinylated Detection Ab working solution was added to each well and incubated for 60 minutes at 37°C. The plate was aspirated and washed 3 times and 100 µl of HRP conjugate working solution was added and incubated for 30 minutes at 37°C. The plate was then aspirated and washed 5 times to remove any excess and unbound antigen-antibody complex. The complex was then incubated in micro-wells with newly prepared substrate solution, Substrate solution of 90 µl was added and incubated for 15 minutes at 37°C. After which the reaction was terminated by adding 50 µl of stop solution. The absorbance was then measured at 450 nm on a spectrophotometer. The enzyme-substrate reaction results in the formation of a blue colour, which indicates that the Ag-Ab reaction has happened in the micro-well. The stop solution (2N H_2SO_4) was added in the last stage and the optical density of the produced colour was measured at 450nm on a spectrophotometer.

5.8. Detection of DENV Virus RNA

Nucleic acid-based detection of DENV was offered to measure an active viremia in DENV-infected individuals. This RNA-based detection of DENV was adapted from the method of Lanciotti et al., 1992 (154).

DENV Molecular Serotyping

Viral RNA Extraction

QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) was used to extract viral RNA from DENV seropositive plasma samples according to the manufacturer's protocol. The extraction method coupled a silica-based membrane's selective binding characteristics with a spin technique. To assure the separation of intact viral RNA, the samples were first lysed under extremely denaturing conditions to inactivate RNases Briefly, 140µL plasma was pipetted to 1.5mL microcentrifuge tube, 560µL of freshly prepared Buffer AVL containing 5.6µL carrier RNA was then added in the microcentrifuge tube and mixed by pulse-vortexing for 15s. The tube was briefly centrifuged to remove drops from inside of the lid and incubated at room temperature (15–25°C) for 10 minutes. 560µL of ethanol (96–100%) was then added and mixed by pulse-vortexing for 15s. After mixing, the tube was briefly centrifuged to remove drops from inside the lid. 630µL of the solution from the previous step was added to the QIAamp mini-column without wetting the rim. Then it was centrifuged at 6000 x g (8000 rpm) and the tube containing the filtrate was discarded. 500µL of Wash Buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for 1min and the tube containing the filtrate was discarded. 500µL of Wash Buffer AW2 was added next and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3min. To remove surplus ethanol, the QIAamp mini column was centrifuged at maximum speed for 1 minute. The column was then put in a 1.5mL DNase-RNase-free microcentrifuge tube. 50µL of elution

buffer AVE was added and incubated for 2 minutes at room temperature and then centrifuged at 6000 x g (8000 rpm) for 1 minute. The eluted viral RNA was aliquoted in small batches of 10 µl each **(Figure 17)**. Aliquots were either utilised for additional downstream processing or kept at -80°C until needed.

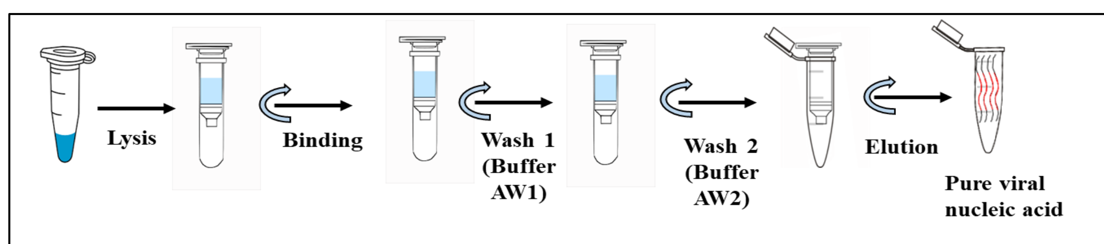


Fig. 17: Stepwise Viral RNA Extraction procedure

Qualitative-Polymerase Chain Reaction (PCR) for dengue Virus RNA detection by Semi Nested RT-PCR

Amplification of the DENV genome was performed by multiplex semi-nested RT-PCR according to the method of Lanciotti et al., 1992 which can differentiate 4 dengue serotypes. The stepwise protocol of dengue serotyping is explained in **Figure 18**. This includes first-round RT-PCR using highly conserved D1 (forward) and D2 (reverse) primers which are homologous for all 4 serotypes. The 1st step RT-PCR was carried out in a 20 µl reaction volume consisting of 2 µl of 10X PCR buffer-II, 1.6 µl of 10mM dNTPs, 1 µl each of 10 µM of forward and reverse primers (D1 and D2), 1.2 µl of 25mM of $MgCl_2$, 0.4 U of AMV RT (Promega, USA), 0.5U of Taq Polymerase (Invitrogen) and 3 µl of extracted RNA. The reaction conditions were 1hr at 42ºC, initial denaturation at 94ºC for 5 min followed by 35 cycles, 94ºC for 30 secs, 55ºC for 1 min, 72ºC for 2 min followed by a final extension at 72ºC for 5 min.

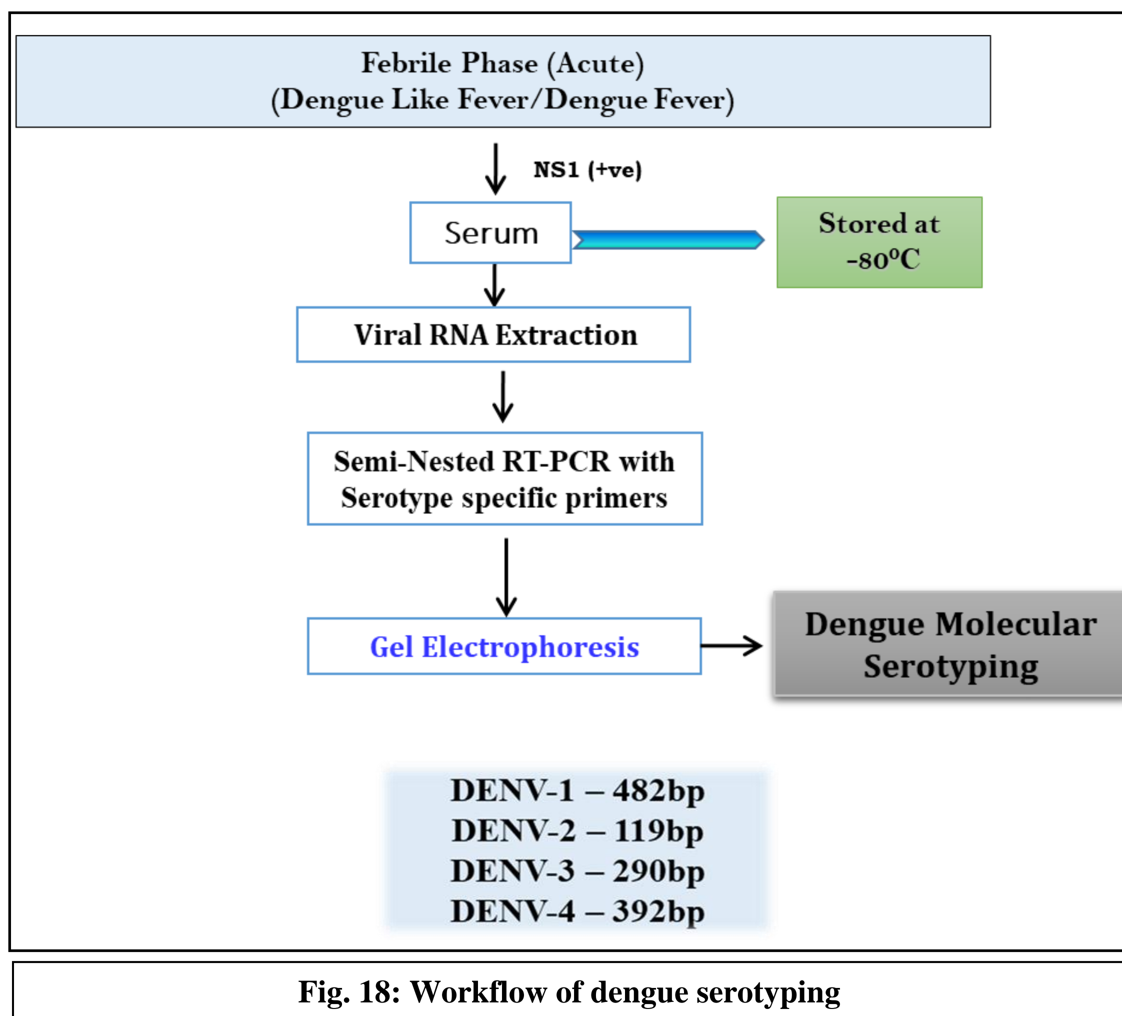


Fig. 18: Workflow of dengue serotyping

Serotype-specific amplification of DENV was performed by nested PCR by using 1:100 times dilution of the amplicon of first-round PCR as the template in second-round nested PCR. The second round was performed in a total volume of 25 µl using 2.5 µl of 10X PCR buffer-II, 2.0 µl of 10 mM dNTPs, 2.5 µl of 25 mM $MgCl_2$, 1 µl each of 10 µM of forward and reverse primers (D1, TS1, TS2, TS3 and TS4), 0.625U of Taq polymerase (Invitrogen, USA) and 2.5 µl of first-round diluted PCR product. The PCR was performed at initial denaturation of 94ºC for 5 minutes followed by 94ºC for 30 secs, 55ºC for 1 min, and 72ºC for 2 min for 30 cycles with a final extension at 72ºC for 5 min. (**Table 2a and Table 2b**). A set of four serotypes of positive RNA was used in each multiplex nested RT-PCR as a positive control. The expected size of PCR products is 482bp for DENV-1, 119bp for DENV-2, 290bp for DENV-3 and 392bp for DENV-4. The obtained nested PCR products

were run on 1.5% agarose gel stained with ethidium bromide and visualized under the Gel Documentation System (Bio-Rad, USA).

Table 2a: First-round RT-PCR Master-mix protocol and thermal cycler conditions

Reagents	1X
10X PCR buffer-II	2.0 µl
25mM $MgCl_2$	1.2 µl
10mM dNTPs	1.6 µl
D1F (10µM)	1.0 µl
D2R (10µM)	1.0 µl
AMV RT (Promega, USA)	0.04 U
Taq Polymerase (Invitrogen)	0.1.0 U
Extracted RNA	3.0 µl

	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	
42ºC	95°C	94°C	55°C	72°C	72°C	4°C
60 min	5 min	30 secs	1 min	2 min	7 min	∞
1x cycle		35 x cycles				

Table 2b: Second-round semi-nested PCR Master-mix protocol and thermal cycler conditions

Reagents	1X
10X PCR buffer-II	2.5 µl
10mM dNTPs	2.0 µl
25mM $MgCl_2$	2.5 µl
D1F(10µM)	1.0 µl
TS1 (10µM)	1.0 µl
TS2 (10µM)	1.0 µl
TS3 (10µM)	1.0 µl
TS4 (10µM)	1.0 µl
Taq polymerase (Invitrogen, USA)	0.12 U
RT-PCR amplicon diluted to 1:100 times	2.5 µl

<table>
<tr><th>Initial Denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final Extension</th><th></th></tr>
<tr><td>94°C</td><td>94°C</td><td>55°C</td><td>72°C</td><td>72°C</td><td>4°C</td></tr>
<tr><td rowspan="2">5 min</td><td>30 secs</td><td>1 min</td><td>2 min</td><td rowspan="2">7 min</td><td rowspan="2">∞</td></tr>
<tr><td colspan="3">30 x cycles</td></tr>
</table>

Table 2.c: List of dengue serotyping primers

Serotyping primers	Primer Sequence (5' to 3')
D1	TCAATATGCTGAAACGCGCGAGAAACCG
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC
TS1	CGTCTCAGTGATCCGGGGG
TS2	CGCCACAAGGGCCATGAACAG
TS3	TAACATCATCATGAGACAGAGC
TS4	CTCTGTTGTCTTAAACAAGAGAGG

5.9. Dengue Genotyping

cDNA synthesis

Complementary DNA was synthesized for a region encompassing the partial structural region from extracted viral RNA and subjected to a polymerase chain reaction. Complementary DNA was synthesized for a region encompassing the partial C-prM gene region of the DENV genome from extracted viral RNA. For each sample, 12 µl of the master mix was prepared by adding 2.4 µl FBS, 1.2 µl of 10µM dNTPs, 0.6 µl of reverse primer, 0.6 µl DTT, 0.3U of Maxima RT, 0.06U AMV RT and 5 µl isolated RNA. Thermal conditions for cDNA synthesis were at 50ºC for 75 minutes continuing to 70ºC for 15 minutes.

Reverse Transcriptase-Polymerase chain reaction

RT-PCR was performed using an expanded long templet PCR system (Roche® Life Science Products) using primers given in **Table 3.c**. PCR was performed in 25 µl of reaction volume containing 11.25 µl of Roch buffer-III, 2.5 µl of 10mM dNTPs, 3 µl each

of 10 µM of forward and reverse primers, 0.2 U of expand long templet PCR system (Roche® Life Science Products) and 2.5 µl of isolated RNA. Thermal cycler conditions were 94°C for 2 minutes followed by 35 cycles of 94°C for 10s, 60°C for 30s and 68°C for 2min each, the final extension step was carried out at 68°C for 7 minutes in an ABI 9700 thermo-cycler (**Table 3.a and 3.b**). The PCR amplicons of DENV-1, DENV-2, DENV-3 and DENV-4 were electrophoresed in 1% agarose gel (Sigma-Aldrich, St. Louis, USA) stained with ethidium bromide and documented using a gel documentation system (Bio-Rad, Hercules, USA).

Table 3.a: cDNA synthesis PCR Master-mix protocol and thermal cycler conditions

Reagents	1X
5X Fast buffer	2.4 µl
10mM dNTPs	1.2 µl
DTT	0.6 µl
$D^{I}R$ (10µM)	0.6 µl
DH_2O	6.84 µl
AMV RT (Promega, USA)	0.04 U
SIII RT (Invitrogen)	0.2 U
Extracted RNA	5.0 µl

STAGE 1	STAGE 2	STAGE 3
50°C	75°C	4°C
75 mins	15 mins	∞
1x cycle	1x cycle	

Table 3.b: Expand™ Long Template PCR Master-mix protocol and thermal cycler conditions

Reagents	1X
Roch buffer-II	2.5 µl
dNTPs (10mM)	3.0 µl
$D^{1}F$(10µM)	2.5 µl
$D^{1}R$ (10µM)	2.5 µl
DH_2O	11.25 µl
Expand™ Long Template PCR System (Roch-Sigma Aldrich)	0.12 U
cDNA	2.5 µl

STAGE 1	STAGE 2			STAGE 3			STAGE 4	STAGE 5
94°C	94°C	60°C	68°C	94°C	60°C	68°C	68°C	4°C
2 mins	10s	30s	2 mins	15s	30s	2 mins	7 mins	∞
1x cycle	10x cycles			25x cycles			1x cycle	

Table 3.c: List of dengue primers used for dengue genotyping for PCR -amplification

Serotypes	Primer Sequence (5' to 3')	Amplicon Size
DENV-2	GAGCCTTCTGGATAGCTGAAGC(F)	520bp
	CCATTCGGAGACAGCTACATC(R)	
DENV-3	TCAATATGCTGAAACGCGTGAGAAACCG (F)	990bp
	CTCCTCAGGCAAAACCGCT (R)	
DENV-4	TCAATATGCTGAAACGCGAGAGAACCG (F)	962bp
	CCACTTCCTTGGCTGTTGTCTTGATC (R)	

Purification of PCR amplified product for Sequencing via gel elution

The positive amplicons were gel purified for sequencing using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purification system used a simple bind-wash-elute procedure. The DNA fragment was excised from agarose gel with a clean, sharp scalpel. The gel slice was first weighed in an Eppendorf tube. Three volumes of Buffer QG to 1 volume gel were added. (100mg gel ~100 µl). The mixture was incubated at 50°C for 10min (or until the gel slice had completely dissolved). The tube was vortexed every 2 to 3min to help dissolve the gel. After the gel slice had been dissolved completely, the colour of the mixture was checked to be yellow (similar to Buffer QG without dissolved agarose). If the colour of the mixture was orange or violet, 10 µl 3 M sodium acetate, pH 5.0 was added and mixed. 1 gel volume of isopropanol to the sample was added and mixed. QIAquick spin column was placed in the provided 2mL collection tube. The sample was added to the QIAquick column and centrifuged for 1 minute at 13000 x g. The flow-through was discarded and the QIAquick column was placed back into the same tube. 500 µl of Buffer QG was added to the QIAquick column and centrifuge for 1min at 13000 x g. 750 µl Buffer PE was added to the QIAquick column and centrifuged for 1min. The column was allowed to stand for 2 to 5 minutes after the addition of Buffer PE and the clean 1.5ml microcentrifuge tube. To elute DNA, 30 µl Buffer EB was added (10mM Tris-Cl, pH 8.5)

to the centre of the QIAquick membrane and centrifuged. The purified DNA was analyzed on a gel to check the purity of the extraction (**Figure 19**).

Sequencing Reaction

Sequencing Reaction and product purification:

The purified products from each serotype were subjected to a SANGER sequencing reaction. In all cases, both inner sense primer and antisense inner primer were used for sequencing and directly used for DNA sequencing analysis in an automated DNA Sequencer, model 3130XL (ABI, USA) using Big Dye terminator 3.1 kit (ABI, USA). Cycle Sequencing PCR was performed. After the BigDye Terminator sequencing reaction, excess labelled nucleotides (excess nucleotides obscure data in the early part of the sequence and can interfere with base calling) were removed with the Ethanol/EDTA/sodium acetate precipitation method. Briefly, two master mixes were prepared: Master Mix-1 was prepared of 10 µl of miliQ water and 2 µl of 125 mM EDTA per reaction. 12 µl of master mix1 was added to each reaction containing 10 µl of sequence reaction. The contents were mixed gently. Master Mix-2 of 2 µl of 3M NaOAc (sodium acetate) pH-4.6 and 50 µl of ethanol per reaction was prepared. 52 µL of Master Mix 2 was added to each reaction (**Table 3.d**). The whole mixture was incubated at room temperature for 15 minutes. Centrifugation at 12,000g for 20mins was done, and the supernatant was aspirated. The pellet was washed by adding 500 µl of 75% ethanol at room temperature and centrifuged at 12000g for 5 minutes. The supernatant was aspirated and the 75% ethanol wash step was repeated once more. Then the pellet was air dried and re-suspended in 15µL of formamide. The contents were briefly vortexed and heated at 95ºC for 5 minutes. After snap chilling in ice for 5 minutes, and then it was vortexed again and a short spin in a microfuge. The concentration of 25ng of the eluted purified product was used for the

sequencing reaction. The re-suspended pellet mixture was then loaded directly into 96 well plates and loaded into an automated DNA sequencer 3130 XL (ABI, USA).

Table 3.d: Big Dye reaction master-mix protocol and thermal cycler conditions

Master Mix-1	
Reagents	**1X**
DH_2O	10 µl
125 mM EDTA	2.0 µl

Master Mix-2	
Reagents	**1X**
Ethanol	50 µl
3M NaOAc (sodium acetate)	2.0 µl

Initial Denaturation	Denaturation	Annealing	Extension	
96°C	96°C	50°C	60°C	4°C
1 min	10 secs	5 secs	4 min	∞
	25 x cycles			

Table 3.e: List of dengue primers used for sequencing reaction

Serotypes	**Primer Sequence (5' to 3')**
DENV-2	GAGCCTTCTGGATAGCTGAAGC(F)
	CCATTCGGAGACAGCTACATC(R)
DENV-3	TCAATATGCTGAAACGCGTGAGAAACCG (F)
	CTCCTCAGGCAAAACCGCT (R)
DENV-4	TCAATATGCTGAAACGCGAGAGAACCG (F)
	CCACTTCCTTGGCTGTTGTCTTGATC (R)

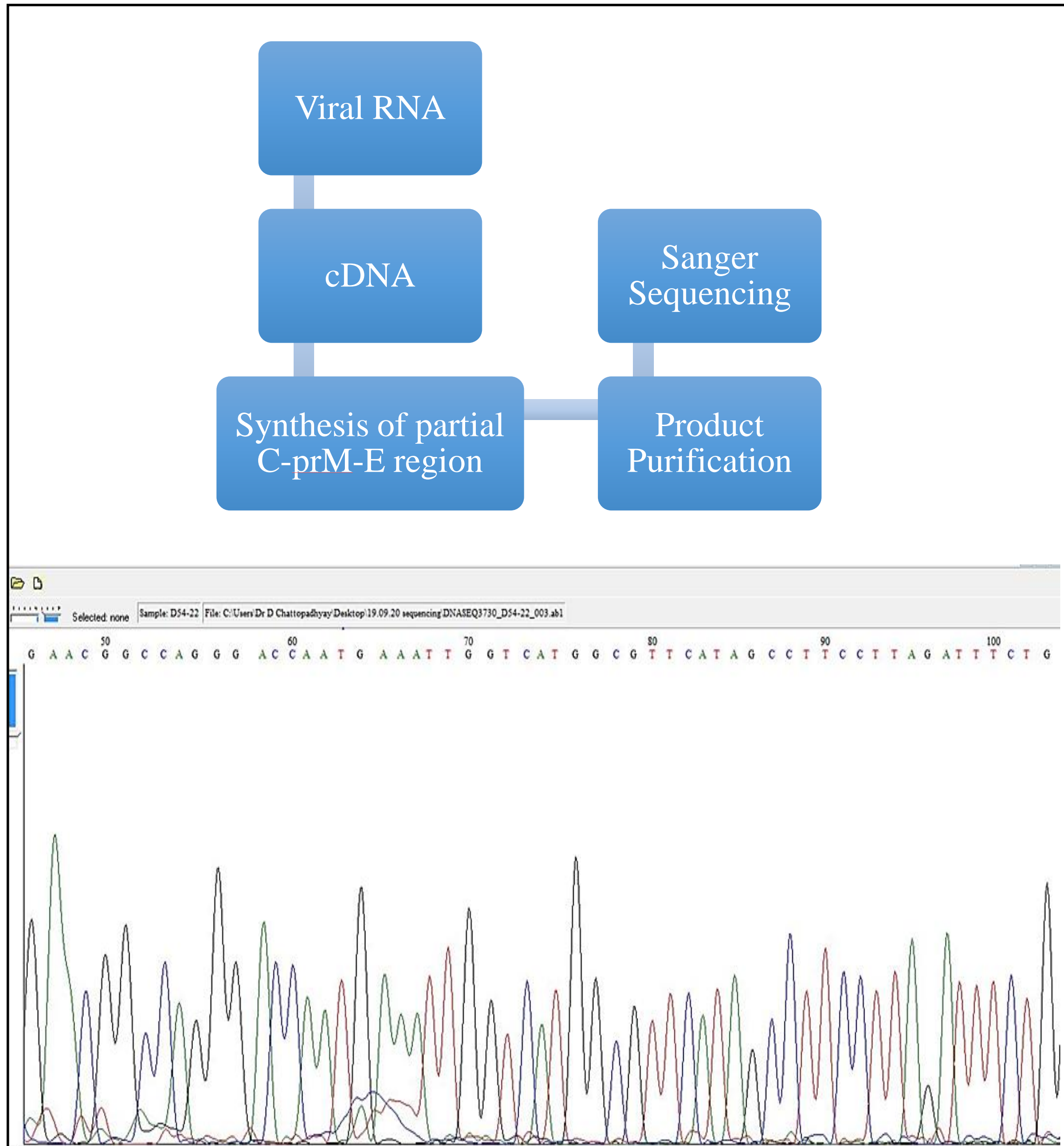


Fig. 19: Dengue Genotyping Workflow and Chromatogram obtained from Sanger sequencing from DENV isolates

Sequencing reaction and Phylogenetic analysis

Obtained sequences were edited using the Bio-Edit tool and a BLAST search was done using the NCBI website (155) to confirm sequence identities. Phylogenetic analysis was performed using 40 representative sequences of different geographical regions retrieved from Gene Bank for DENV 2, DENV 3 and DENV 4 serotypes in each phylogenetic tree.

To investigate the evolutionary linkage among laboratory isolates and reference strains, partial sequences of the representative DENV laboratory obtained sequence were aligned with DENV reference strains using the Molecular Evolutionary Genetics Analysis (MEGA-10) tool. The evolutionary history was concluded using the Neighbor-Joining (NJ) method and the Tamura-Nei model (156).

The variation rates among various sites were modelled with a gamma distribution pattern (shape parameter = 1). The differences in the composition bias among all sequences were considered in the evolutionary comparison. The evolutionary distances were calculated using the Maximum Composite Likelihood method (-7113.82) and are in the units of the number of base substitutions per site (157). Codon positions 1st+2nd+3rd+Noncoding were included in the settings. All positions containing gaps and missing data were eliminated for this purpose.

5.10. Biomolecule identification

5.10.a. Qualitative proteomic Analysis

Proteomics analysis was performed using phase-specific samples collected at two-time points from hospital-admitted patients. Various steps followed in the protein identification process are explained below starting from plasma quality check by protein estimation using the Bradford technique (158).

Protein estimation using the Bradford technique

To estimate protein in our clinical plasma samples, firstly a standard BSA solution of 1mg/ml is prepared. Then pipette 0, 2, 4, 6,10,15 and 20 µl of Bovine Serum Albumin (BSA) into the assigned well of 96 well plates. Then pipette 20 µl of diluted unknown

samples into wells individually. Then 40 µl of Bradford reagent (Cat. No: B6916, Sigma) was added to all wells. The final volume of each well was adjusted to 200 µl by pipetting distilled water into it. After 5 minutes and before 1 hour, compare the absorbance at 595nm (OD595) to a reagent blank (100 µl of distilled water). Calculate the protein conc. In diluted samples by comparing, it to the BSA standard curve. If the OD595 of the diluted extract is too high or too low, make a more appropriate dilution. Diverse proteins have significant variations in their dye-binding capabilities, resulting in diverse test results. Proteins were estimated twice i.e. pre depletion and post-depletion. Post-depletion protein samples of 650 ng to 1 mg are ideal for proteomics reactions.

In-solution tryptic digestion and qualitative mass spectrometry

To delineate the plasma proteome profiles, a nano-LC- Orbitrap mass spectrometer (LTQ Orbitrap XL, Thermo Scientific, USA) was employed. The mass spectrometry work was carried out at the central facility of CSIR-Indian Institute Chemical Biology, Kolkata.

Human plasma samples contain albumin, IgG and other abundant proteins as high as 70% of the total plasma proteome. Therefore, we needed to deplete the most abundant proteins to identify the less abundant proteins in plasma for biomolecule(s). We took 10 µl raw plasma samples initially to deplete albumin and IgG using the Merck Millipore Pure Proteome Albumin/IgG depletion kit (LSKMAGD12-1). In brief, 10 µl plasma was diluted in 100 µl 1X PBS. 400 µl of Pure Proteome Albumin/IgG magnetic bead slurry were washed off their storage buffer, equilibrated with 1X PBS thrice and added to 10 µl of the samples. This plasma and bead mixture was incubated for 1.5 h at room temperature on an end-to-end rotating mixer. The depleted plasma was estimated using a DC estimation reagent and 50 µg of depleted plasma was reduced using 10mM DTT at 60ºC for 30 min. Post reduction, the sample was alkylated with 20 mM iodoacetamide for 40 min at RT in

the dark. The excess iodoacetamide was quenched with 5mM DTT for 10 min and the sample was digested with 1 µg/µl Trypsin gold (Promega, cat# V5280) O/N at 37ºC in a shaker incubator. The digestion reaction was stopped the next morning with 0.1% (v/v) formic acid and the digested samples were dried using a speed vac. Dried peptides were reconstituted in 0.1% (v/v) formic acid and run in the Orbitrap-TOF. Pre-analytical steps were kept constant for all the samples including healthy control. This experiment for the difference between proteomic profiling designed as continuous variable data, based on H1 for superiority, interference about absolute difference, type I error rate .5, power (1-β) = 80% 0 (0.8), sampling ratio (group A/ group B/ group C) = 1, Mean under H0 (arithmetic mean of sample value) = 1.5, std deviation of samples =1.2, min. detectable effect=1, superiority margin =1. For this study sample size was calculated as, group A=3, group B=3, and group c=3 (159).

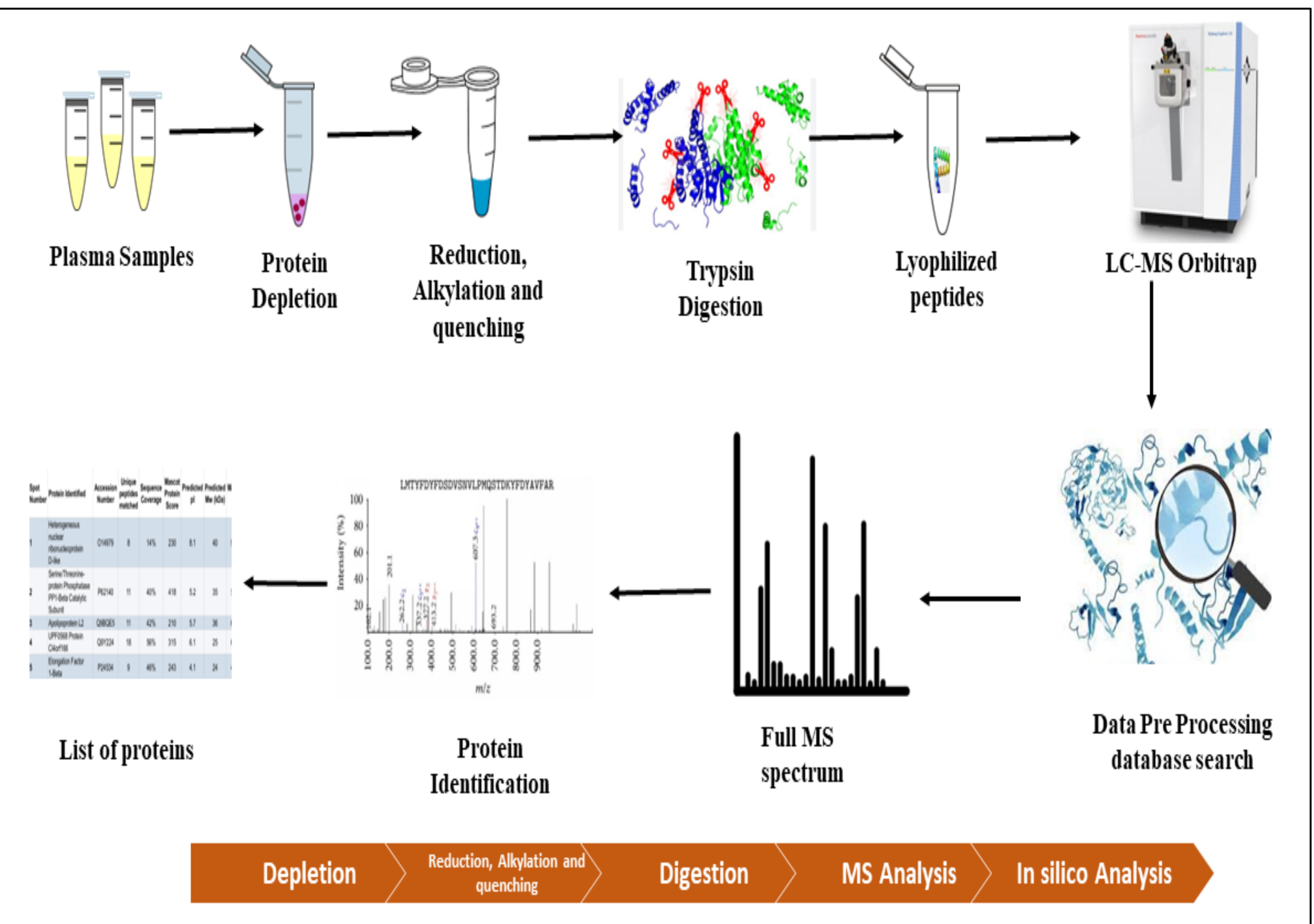


Fig. 20: Schematic diagram showing the procedure of LC-MS

Data Acquisition from Orbitrap Liquid Chromatography-Mass Spectrometry (LC-MS)

The acquisition was data-dependent. The total MS run time was 145 min. The scan range for Orbitrap was from 350 to 2000(m/z) with a minimum of 3 peaks and a resolution of 60,000. Ionic fragmentation was performed by the collision-induced dissociation (CID) method. The normalized collision energy for each run was 35. Detection and digitization of peaks were performed by Fourier transformation and data acquisition was conducted in positive ion mode. Protein identification was performed by Thermo Proteome Discoverer v1.4.0 (160). MS/MS spectra were matched against SEQUEST with the assistance of a fixed-value PSM validator. The static modification was set for N-terminal acetylation and carbamidomethylation of Cysteine. The dynamic modification was set for Methionine (oxidation), Tyrosine (phosphorylation) and Lysine (acetyl). The maximum missed cleavage number was set to two. Swissprot reviewed database (updated with Uniprot release 2015 and 46,000 protein entries) (161) was used as a reference software for protein identification. The minimum number of peptides for assigning a protein was set to two and mass tolerance for precursor ions was set to 10 ppm. The schematic workflow of the LC-MS procedure is shown in **Figure 20.**

5.10.b. Quantitative proteomics Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS)

SWATH-MS from paired plasma samples of different groups of dengue-infected patients along with healthy controls were performed at the National Facility for Biochemical and Genomic Resources (NFBGR), CSIR-IGIB, New Delhi, India.

Sample preparation

Ten (10) µl of serum samples were used for protein precipitation. To 90µl of 1X Phosphate Buffer Saline (PBS), 10 µl serum was added and vortex mixed. Protein precipitation was performed using pre-chilled acetone. Briefly, to 100 µl diluted protein extract, four times the volume of pre-chilled acetone was added, vortex mixed and centrifuged at 15000 g for 10 minutes at 4°C. The supernatant was discarded, and the protein pellets were air-dried at room temperature and suspended in 0.1 M Tris-HCl with 8M urea, pH 8.5. Protein quantitation was performed using the Bradford assay.

Reduction, alkylation, and trypsin digestion

Twenty (20) µg of protein from each sample was reduced with 2 mM of Dithiothreitol (DTT) for 30 minutes at 60°C, followed by alkylation using 2.2 mM of Iodoacetamide (IAA) at room temperature (in the dark) for 30 minutes. These samples were then subjected to trypsin digestion in an enzyme-to-substrate ratio of 1:20 (trypsin: protein) for 16 hours at 37°C. Finally, the tryptic peptides were vacuum-dried in a vacuum concentrator.

Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) Data acquisition

Peptides from each sample were cleaned up using an Oasis HLB cartridge (Waters, Milford, MA) as per the manufacturer's protocol. SWATH-MS analysis for the samples was performed on a quadrupole-TOF hybrid mass spectrometer (TripleTOF 6600, SCIEX) coupled to an Eksigent NanoLC-425 system. Optimized source parameters were used, and curtain gas and nebulizer gas were maintained at 25 psi and 30 psi respectively. The ion spray voltage was set to 5.5 kV and the temperature was set to 250°C. About 4 µg of peptides were loaded on a trap column (ChromXP C18CL 5 µm 120 Å, Eksigent, SCIEX) and online desalting was performed with a flow rate of 10 µl per minute for 10 minutes.

Next, peptides were separated on a reverse-phase C18 analytical column (ChromXP C18, 3 µm 120 Å, Eksigent, SCIEX) in a 57-minute gradient with a flow rate of 5 µl/minute using water with 0.1% formic acid and acetonitrile with 0.1% formic acid.

SWATH method was created with 100 precursor isolation windows, defined based on precursor m/z frequencies in Data Dependent Acquisition (DDA) run using the SWATH Variable Window Calculator (SCIEX), with a minimum window of 5 m/z. Data was acquired using Analyst TF 1.7.1 Software (SCIEX). Accumulation time was set to 250 msec for the MS scan (400–1250 m/z) and 25 msec for the MS/MS scans (100–1500 m/z). Rolling collision energies were applied for each window based on the m/z range of each SWATH and a charge 2+ ion, with a collision energy spread of 5. The total cycle time was 2.8 seconds.

Data acquisition from SWATH-MS

For identification of the proteins using SWATH analysis, a high-pH fractionated peptide library for human serum proteins (obtained from SCIEX) comprising 465 proteins was used. SWATH peaks were extracted using this library in SWATH 2.0 microapp in PeakView 2.2 software (SCIEX), excluding shared peptides. The processing settings for peak extraction were: a maximum of 10 peptides per protein, 5 transitions per peptide, >95% peptide confidence threshold and 1% peptide false discovery rate (FDR). XIC extraction window was set to 55 minutes with 75 ppm XIC Width. All information was exported in the form of MarkerView (.mrkw) files. Data were normalized using total area sum normalization in MarkerView 1.2.1 and further analysis was performed in MS Excel and R Studio 4.3.2.

5.11. Pathway analysis of proteomic data

Pathway and network analysis approaches are used for interpreting proteomics results. Pathway analysis refers to data analysis that seeks to find active pathways or pathway modules in functional proteomics data. Biological pathways are classified into signalling routes, gene regulatory pathways, metabolic pathways, etc. all of which are carefully curated in credible databases. Qualitative proteomics data subjected to pathway analyses using PANTHER (protein analysis through evolutionary relationships) (http://www.pantherdb.org/) database (162). The list of proteins analysed for their categorization in GO (Gene Ontology)- Slim Biological process, GO- Slim Molecular Function, PANTHER pathways and protein class. Whereas two different pathway analyses were performed from Quantitative proteomic data using PANTHER (http://www.pantherdb.org/) and the reactome database (https://reactome.org/PathwayBrowser/#/) (163). A reactome is a database of reactions, pathways and biological processes. The list of the proteins was searched for significant enrichment of the molecular functions and KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathways. KEGG pathway is a collection of manually drawn pathway maps representing our knowledge of molecular interaction, reaction and relation networks.

5.12. Dengue viral protein-host protein interactome

The Interactome refers to all molecular interactions in cells, particularly protein-protein interactions. In this study, we have analysed the protein-protein interaction network of the dengue virus genome and human host proteins. The functions of a protein can be roughly determined from its interacting partners. Interactions of 10 DENV proteins with their host interactors were analysed. Out of these 10 proteins, 3 were structural (C, E and prM) and 7

were non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). These 10 viral proteins were already reported to interact with 535 host proteins (164). This protein-protein interaction (PPI) network was depicted with the help of Cytoscape v3.8.2 where the host proteins were represented as blue rectangular edges and the DENV proteins were marked as different coloured rectangular hubs. Protein-protein interactions (PPIs) depict connections between two or more proteins, representing complicated biological activities.

5.13. Proteome profiler array analysis

Total of three (N=3) critical patient samples (obtained on the day of transition to severe dengue) along with a healthy control were subjected to proteome profiler analysis as per manufacturer protocol. Two different types of proteome profiler analyses were performed as mentioned below

I. Human angiogenesis array kit (Cat. No.- ARY007, R&D system) for the parallel determination of the relative level of human angiogenesis-related proteins

II. Human cytokine array kit (Cat. No.- ARY005B, R&D system) for the parallel determination of the relative level of selected human cytokine and chemokine.

Protein Array Protocol

All the reagents and samples were prepared as directed in the Array kit manufacturer protocol. 2 mL of Array Buffer 4 was given through a pipette into each well of the 4-well Multi-Dish. Array Buffer 4 serves as a block buffer. Using flat-tip tweezers each membrane to be used from between the protective sheets was removed and placed in a well of the 4-Well Multi-Dish with the array number facing upward. The tray was then incubated for one

hour on a rocking platform shaker in such a way that each array rocks end to end in its well. While the membranes were blocked, samples were prepared by adding up to 1 mL of each sample to 0.5 mL of Array Buffer 4 in separate tubes. The final volume of 1.5 mL was adjusted with Array buffer 5. 15 micro-litres of reconstituted Human Cytokine Array Detection Antibody Cocktail were added to each prepared sample. Then it was mixed and incubated for one hour at room temperature. Array Buffer 4 was aspirated from wells of the 4-Well Multi-dish and then samples/antibody mixtures were added as prepared in steps 5 and 6. Then the lid was placed on the 4-well multi-dish. The multi-dish was incubated overnight at 2-8 degrees C on a rocking platform. Each membrane was removed carefully and placed into individual plastic containers with 20 ml of 1X Wash Buffer. 4 Well multi-dish were rinsed with deionized and distilled water and dried thoroughly. Each membrane was washed with 1X Wash Buffer for 10 minutes on a rocking platform shaker. A total of three washes were done with 2 times repetitions. Sreptavidin-HRP was diluted in Array Buffer 5 using the dilution factor on the vial label. 2.0 ml of Streptavidin-HRP was pipetted into each well of the 4-Well Multi-dish. Then each membrane was carefully removed from its wash container, excess buffer was drained from the membrane. The membrane was returned to the 4-Well Multi-dish containing the diluted Sreptavidin-HRP and then the wells were covered with the lid and incubated for 30 minutes at room temperature on a rocking platform shaker. Each array was then washed as described previously. Each membrane is then removed carefully from its wash container. Excess Wash Buffer was allowed to drain from the membrane by blotting the lower edge onto paper towels. Each membrane was placed on the bottom sheet of the plastic sheet protector with the identification number facing up. 1 ml of prepared Chemi reagent was pipetted and mixed evenly onto each membrane and carefully covered with the top sheet of the plastic sheet protector. The air bubble was gently smoothened out Chemi reagent Mix was spread evenly

to all corners of each membrane and incubated for 1 minute. Paper towels were positioned on top and sides of plastic sheet protectors containing the membranes and carefully squeezed out excess Chemi reagent Mix. The plastic sheet protector was removed and carefully laid an absorbent lab wipe on top of the membrane to blot off any remaining Chemi reagent Mix. Leaving the membranes on the bottom plastic sheet protector, the membranes were covered with plastic wrap and gently smoothed out any air bubbles. The excess plastic wrap was wrapped around the back of the sheet protector so that the membranes and the sheet protector were completely wrapped. The membranes were placed with the identification numbers facing up in an autoradiography film cassette. Membranes were exposed to X-ray film for 1-10 mins. Multiple exposure times were required.

Data acquisition from proteome array

The positive signals developed on the array film were identified by placing the transparency overlay template provided with the kit. The pair of reference spots in the three corners of the array was adjusted with film for proper alignment. The location of controls, angiogenesis protein and cytokine proteins captured on the membrane is listed in the appendix provided with each kit, respectively. Pixel density is calculated based on the signal observed from each protein representing the spot. Relative change in protein expression of identified proteins calculated with healthy control for relative quantification of individual proteins.

This doctoral study performed a multi-approach analysis to find severe dengue pathogenesis and its biomolecules. Starting from a clinicopathological approach to find clinical markers. The proteomics approach includes qualitative and quantitative proteomics, in vivo host and viral protein interactions via Cytoscape and then protein array.

From all these aspects we have identified a panel of biochemical markers and also a panel of candidate protein biomolecules. These protein biomolecules upon identification subjected to Western bolt, Real-time time and ELISA-based validations among different groups of dengue patients.

5.14. Biomolecule validation experiments

5.14.a. Western blot analysis

A comparative study for the validation of differentially expressed proteins in plasma between hospital-admitted dengue-infected patients and healthy control was done. Human plasma samples were diluted 50 times in 1X PBS before Western blotting. Protein concentrations were measured using Bradford's method and equal amounts (~30µg) of plasma samples were run. Samples were separated on 1mm 8% and 10% sodium dodecyl sulphate (SDS) polyacrylamide gels and transferred onto PVDF membranes (Merck-Millipore). Post transfer, membranes were blocked with 5% or 3% skimmed milk (HiMedia, Cat. No. TC194) and probed using the antibodies tabulated in **Table 4**.

The PVDF membranes were washed 3 times with 1X TBST and incubated with Alkaline phosphatase-conjugated secondary antibodies for 2 hours at room temperature. Washing was done first by Tris buffered saline (TBS) followed by 3X TBST. {For 1L TBS: 24g Tris-HCl (formula weight: 157.6 g), 5.6g Tris base (formula weight: 121.1 g), 88g NaCl (formula weight: 58.4g), 900mL distilled water was added and dissolved), TBST (TRIS - buffered saline, 0.1% Tween20 (For 1L): 100 ml of TBS 10X, 900mL distilled water, 1ml Tween 20. Membranes were washed 3 times with TBST and developed using NBT-BCIP (Fermentas R0841, R821) in 1X alkaline phosphatase buffer. After the development of coloured products on the membranes, they were washed with water, dried and images were

acquired using a scanner. Images were quantified using Image J software with Transferrin as loading control for plasma samples.

Table 4: List of antibodies used for validation via Western blot and their manufacturer's details

Description & Catalog no.	Description & Catalog no.	Molecular weight (kDa)
E-Cadherin Rabbit pAb (A16811)	ABclonal Technology, USA	125
IRF3 Rabbit pAb (A2172)	ABclonal Technology, USA	50
FGFR1 Rabbit pAb (A0082)	ABclonal Technology, USA	100-120
VEGF Rabbit pAb (A12303)	ABclonal Technology, USA	16/34/45
ANGPT1 Rabbit pAb (A7877)	ABclonal Technology, USA	51
ICAM-1 Rabbit pAb (A5597)	ABclonal Technology, USA	89/92
VCAM1 Rabbit pAb (A0279)	ABclonal Technology, USA	100-120
IFNG Rabbit pAb (A12450)	ABclonal Technology, USA	17/23
Transferrin Rabbit pAb (A1448)	ABclonal Technology, USA	77
Apo AII Rabbit pAb (A14690)	ABclonal Technology, USA	35
ApoB Rabbit mAb (A4184)	ABclonal Technology, USA	270/520
Apo AI mouse mAb (sc-376818)	Santa Cruz Biotechnology, USA	30
Apo AIV mouse mAb (sc-374543)	Santa Cruz Biotechnology, USA	45
Goat Anti-rabbit ALP conjugate (G21079)	Invitrogen	

5.14.b. Quantitative Real-time PCR

Samples for Real-Time-PCR-based validation were selected as per WHO, 2011 clinical patients' management guidelines (165). Real-time-PCR-based biomolecule validation was performed among selected 12 adult paired samples of the acute phase and critical phase i.e. 24 samples from each phase group along with 6 healthy controls.

Isolation of RNA from Whole Blood

The differentially expressed proteins identified by the proteomic analysis (quantitative proteomic and proteomic array) were validated using real-time PCR. Total RNA was isolated from plasma samples using Qiagen RNA from a blood kit (Cat. No. 52304) according to the manufacturer's protocol (**Figure 21**).

Procedure

1. 100 µl of human whole blood mixed with 500 µl of buffer EL in an appropriately 1.5ml Eppendorf tube.
2. Then incubate for 10–15 min on ice. Mixed by vortexing briefly 2 times during incubation. The cloudy suspension became translucent during incubation, indicating lysis of erythrocytes. Incubation time can be extended to 20 min. as per requirement.
3. The blood and EL mix were centrifuged at 400 x g for 10 minutes at 4°C, and the supernatant was collected and discarded. After centrifugation, leukocytes will form a pellet. Ensure that all supernatant has been removed. The subsequent wash procedure will remove trace numbers of erythrocytes, which give the pellet a red tinge.
4. 4. To the cell pellet, add 200 µl of buffer EL added. By short vortexing, cells were re-suspended.
5. Supernatant removed and discarded after centrifuging at 400 x g for 10 minutes at 4°C.
6. Buffer RLT added to pelleted leukocytes according to the Table given below. By vortex or pipet mix (**Table 5**).
7. Pipet lysate directly into a QIA shredder spin column in a 2 ml collection tube (provided) and centrifuge for 2 min at maximum speed to homogenize. Discard the QIA shredder spin column and save homogenized lysate. To avoid aerosol

Table 5: Volume of buffer RLT used for lysis

Buffer RLT (µl)	**Healthy whole blood (ml)**	**No. of leukocytes**
350	Up to 0.5	Up to 2×10^6
600	0.5 to 1.5	Up to 2×10^6 to 10^7

formation, adjust the pipet to ≥750 µl to ensure that the lysate can be added to the QIA shredder spin column in a single step.

8. Add 1 volume (350 µl or 600 µl) of 70% ethanol to the homogenized lysate and mix by pipetting. Do not centrifuge. A precipitate may form after the addition of ethanol.

9. Carefully pipet the sample, including any precipitate which may have formed, into a new QIA amp spin column in a 2 ml collection tube (provided) without moistening the rim. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). The maximum loading volume is 700 µl. If the volume of the sample exceeds 700 µl, successively load aliquots onto the QIAamp spin column and centrifuge twice by dividing it into half volume. Discard flow-through and collection tube.

10. Transfer the QIA amp spin column into a new 2 ml collection tube. Apply 700 µl Buffer RW1 to the QIA amp spin column and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash. Discard flow-through and collection tube.

11. Place the QIA amp spin column in a new 2 ml collection tube. Pipet 500 µl of Buffer RPE into the QIA amp spin column and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard flow-through and collection tube.

12. Carefully open the QIA amp spin column and add 500 µl of Buffer RPE. Close the cap and centrifuge at full speed (20,000 x g, 14,000 rpm) for 3 min.

13. Recommended: Place the QIA amp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer RPE carryover.

14. Transfer the QIA amp spin column into a 1.5 ml microcentrifuge tube (provided) and pipet 30–50 µl of RNase-free water directly onto the QIA amp membrane.

Centrifuge for 1 minute at ≥8000 x g (≥10,000 rpm) to elute. Repeat if >0.5 ml whole blood (or >2 x 106 leukocytes) has been processed. The purity of RNA was checked by spectrophotometric analysis of the A260/280 ratio and concentration was determined from the A260 value.

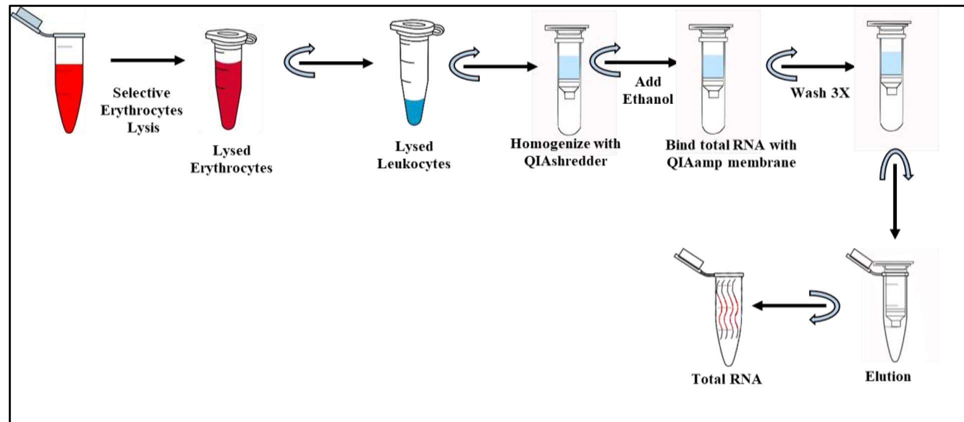


Fig. 21: Procedure of RNA extraction from Whole Blood

cDNA synthesis

Complementary DNA was synthesized from RNA isolated from whole blood using targeted gene-specific primers. For each sample, 12 µl of the master mix was prepared by adding 2.4 µl FBS, 1.2 µl of 10 µM dNTPs, 0.6 µl of reverse primer, 0.6 µl DTT, 0.3U of SIII RT (Invitrogen, Carlsbad, CA), 0.06U AMV RT and 5 µl isolated RNA. Thermal conditions for cDNA synthesis were at 50ºC for 75 minutes continuing to 70ºC for 15 minutes.

Quantitative Real-time PCR-SYBR Green

SYBR Green is a dsDNA binding dye used to estimate amplicon quantity during the PCR by monitoring total fluorescence emission. The dye binds to the minor groove of dsDNA, but not to ssDNA. A real-time PCR Master mix was prepared by adding 10 µl SYBR green mix with 3 µl nuclease-free water, 1 µl of forward, 1 µl reverse gene-specific primers and

5 μl of synthesized cDNA. Real-time quantification of the candidate genes was performed using a power SYBR-green assay (Applied Biosystems, USA) with β actin as the housekeeping gene. Primer list of targeted gene given in **Table 6**. Each sample was loaded in duplicate and run at 40 cycles of the ddCT programme on an ABI prism 7500 machine (Applied Biosystem, USA). After each run, melting curves were generated to confirm the amplification of specific transcripts. To determine the relative level of gene expression, the comparative threshold cycle (ddCT) method was employed after the normalization of a respective gene of interest expression level against that of β actin (dCT). In this same manner, the gene expression of a total of 10 sets of prognostic genes was measured in dengue-infected patients. The relative expression of the genes in terms of fold change (2−ddCT) was calculated and plotted as violin plots.

Table 6: Primer list for Real-Time based quantitative validation

Sl. No.	Target Protein	Primer Sequence (5' to 3')	Amplicon size
1.	LBP-F	CTACAGGGCTCCTTTGATGTCA	126bp
	LBP-R	CACGTCAGCGATGTCACTG	
2.	Fb-F	TCTGTGCCTCCTATCTATGTGC	85bp
	Fb-R	GAGGGACCACGACAACTCTTC	
3.	Vt-F	TGACCAAGAGTCATGCAAGGG	116bp
	Vt-R	ACTCAGCCGTATAGTCTGTGC	
4.	PTX 3 F	CATCTCCTTGCGATTCTGTTTTG	156bp
	PTX 3 R	CCATTCCGAGTGCTCCTGA	
5.	Serpin-F	AGCCCAACGATGACTACTTACT	172bp
	Serpin-R	ACCCAAGAGTTGATGTCCTTTCT	
6.	PDGFA-F	GCAAGACCAGGACGGTCATTT	135bp
	PDGFA-R	GGCACTTGACACTGCTCGT	
7.	Ang F	GAGCTGAGCGACGCTAGATG	144bp
	Ang R	GGGTTGACTCGAAGTGCAGAG	
8.	IGFBP2-F	GACAATGGCGATGACCACTCA	123bp
	IGFBP2-R	CAGCTCCTTCATACCCGACTT	
9.	SYNE2-F	ACCTCCAATGGTGGTGGAC	150bp
	SYNE2-R	CGTGCCAATGTTAGCCACA	
10.	POSTN-F	CTCATAGTCGTATCAGGGGTCG	138bp
	POSTN-R	ACACAGTCGTTTTCTGTCCAC	
11.	Beta actin-F	CTCGACACCAGGGCGTTATG	230bp
	Beta actin-R	CCACTCCATGCTCGATAGGAT	

5.14.c. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA-based biomolecule validation was performed in a group of 92 samples, which included 24 paired samples from the acute dengue phase, 19 paired samples from the critical dengue phase and 6 healthy controls (**Table 6**).

Assay Protocol

ELISA-based validation of topmost candidate protein biomolecules performed using paired samples from each phase as per manufacturer protocol (Cat. No.: E-EL-H2104, E-EL-H6081, E-EL-H6160, E-EL-H0179, E-EL-H6037, E-EL-H6108). Dilutions of standard and samples prepared as per provided protocol. 100 µl of standard or diluted samples were added to the wells of a micro-ELISA plate, pre-coated with gene-specific antibody then incubated for 90 minutes at 37°C. The solution was then discarded and immediately 100 µl of Biotinylated Detection Ab working solution was added to each well and incubated for 60 minutes at 37°C. The plate was aspirated and washed 3 times and 100 µl of HRP conjugate working solution was added and incubated for 30 minutes at 37°C. The plate was then aspirated and washed 5 times to remove any excess and unbound antigen-antibody complex. The complex was then incubated in micro-wells with newly prepared substrate solution, Substrate solution of 90 µl was added and incubated for 15 minutes at 37°C. After which the reaction was terminated by adding 50 µl of stop solution. The absorbance was then measured at 450 nm on a spectrophotometer. The enzyme-substrate reaction results in the formation of a blue colour, which indicates that the Ag-Ab reaction has happened in the micro-well. The stop solution (2N H_2SO_4) was added in the last stage, and the optical density of the produced colour was measured at 450nm on a spectrophotometer. The ELISA must be optimized for high sensitivity and specificity, ensuring low biomolecule concentration detection and preventing cross-reactivity with other proteins. A standard

curve is generated using known concentrations of the target protein. This standard curve serves as a reference to quantify the biomolecules levels in samples. The topmost biomolecule levels in these samples are quantified based on the standard curve.

5.15. Protein Docking

To find the interactions between viral NS1 proteins and topmost candidate proteins, protein-protein docking was performed in the HADDOCK 2.4 server (166). To perform these interactions structures of DENV NS1 protein and targeted host were downloaded from the Protein Data Bank and visualized, edited and protein active sites were selected using Discovery Studio 2021. Active sites of viral proteins and host proteins were mapped and identified. Then each viral host protein docked with DENV NS1 protein to find the interacting sites. These interactions showing viral NS1 proteins are actively associated with host factors related to vascular pathogenesis.

5.16. STRING: Functional proteins association network

STRING Protein-Protein Interaction Networks Functional Enrichment Analysis performed using STRING 12.0 database (167). Validated target genes are observed to have a functional association with nitric oxide, which is the major protein behind vascular endothelial dysfunction. We depicted the functional network of NO (Nitric Oxide) with other candidate proteins of this study such as E Cadherin, Vascular endothelial growth factor, Fibroblast growth factor receptor, Vascular cell adhesion protein, Interferon-gamma, Fibronectin, Insulin-Like Growth Factor Binding Protein 2, Serpin, Periostin, Pentraxin 3, Angiopoietin, Lipopolysaccharide binding protein. K mean interaction modelling was also performed to find the significant functional clustering among our target

proteins with nitric oxide synthase endothelial. Predicting underlying interaction between NO and validated biomolecules of this study can provide insights into potential therapeutic targets to mitigate the severity of dengue and improve patient outcomes.

5.17. Statistics Analysis

Collected data were organized with necessary modifications. The pattern of dengue circulating serotypes, its associated clinical manifestations and thrombocytopenia were shown as a percentage variable. Dengue-associated clinical manifestations were analysed using GraphPad Prism 9.3.1 software for multivariate linear logistic modelling by receiver operating curve (ROC). Serotype-specific clinical manifestations were analysed using the Pearson $\chi 2$ test. We used mean ± SD for the day-wise assessment of Biochemical parameters and did Tukey's test box plot of the same. Continuous variables were presented as mean ± standard deviation or median (Range) while categorical variables are expressed as frequencies percentage. The existence of differences in groups was assessed using the chi-square test for linear trend when appropriate and calculating the odds ratio (OR) within a 95% confidence interval (CI). A p-value of <0.05 was used as the criterion for statistical significance. Pathway enrichment P values analysed in PANTHER database. Whereas Heatmap, Real-Time and ELISA data statistics were performed via GraphPad Prism 9.2. ELISA Statistical analysis is performed to compare target protein levels between the two groups (acute vs. critical) and ROC curves (receiver operating characteristic) generated. This analysis determines if the protein is differentially expressed and whether it can effectively distinguish between the acute and critical groups. Several parameters are considered for validation, including sensitivity, specificity, positive and negative predictive values, and area under the ROC curve (AUC). These metrics help to assess the

biomolecule's prognostic and diagnostic performance (168). All the statistical analyses were performed using the Statistical Package of GraphPad PRISM.

In the study of dengue biomolecules, statistical analysis is essential because it offers a methodical, quantitative way to examine large, complicated data sets. Statistical tools are useful in this research because they help interpret patterns, trends and connections in data, which is important when it comes to understanding biomolecular interactions and dynamics. We have used an array of statistical methodologies, including differential expression analysis, clustering approaches and ROC analysis to evaluate the relevance of biomolecular alterations linked to dengue infection. The identification of important biomolecules, pathways, and regulatory mechanisms implicated in the disease's course is facilitated by these investigations.

A brief methodology workflow of this doctoral study encompassing an approach to reach all four objectives and A brief workflow of identification and validation of severe dengue biomolecules is depicted in **Figure 22. a** and **Figure 22. b**.

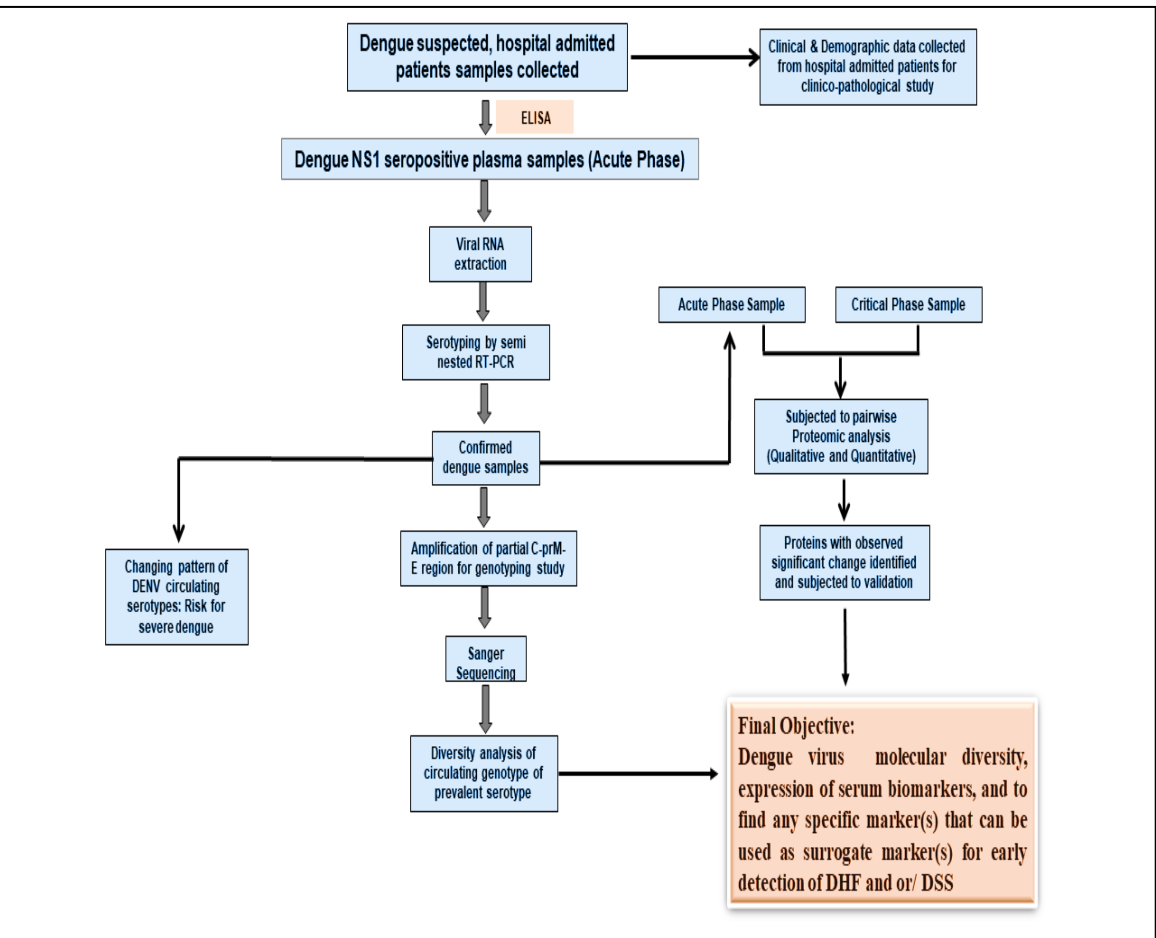


Fig. 22. a: Methodology Workflow of this doctoral study encompassing approach to reach all four objectives

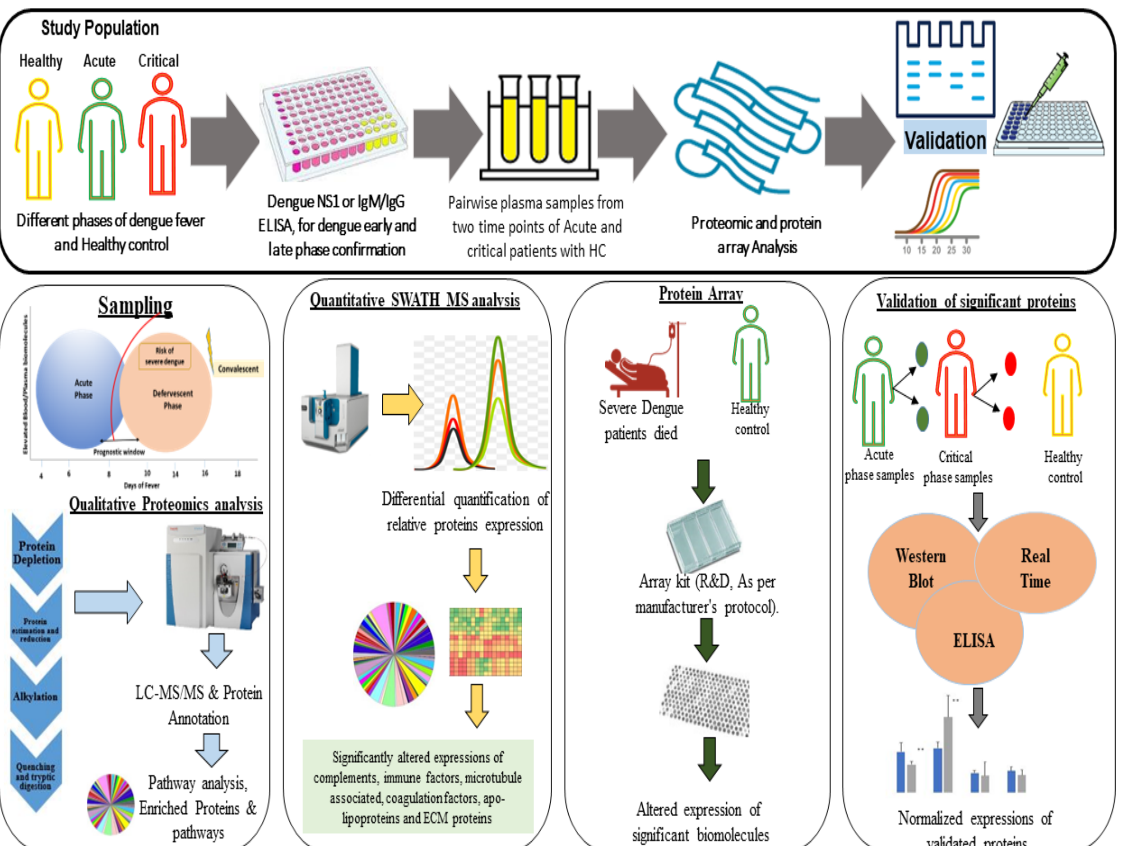


Fig 22.b: Workflow of identification and validation of severe dengue biomolecules

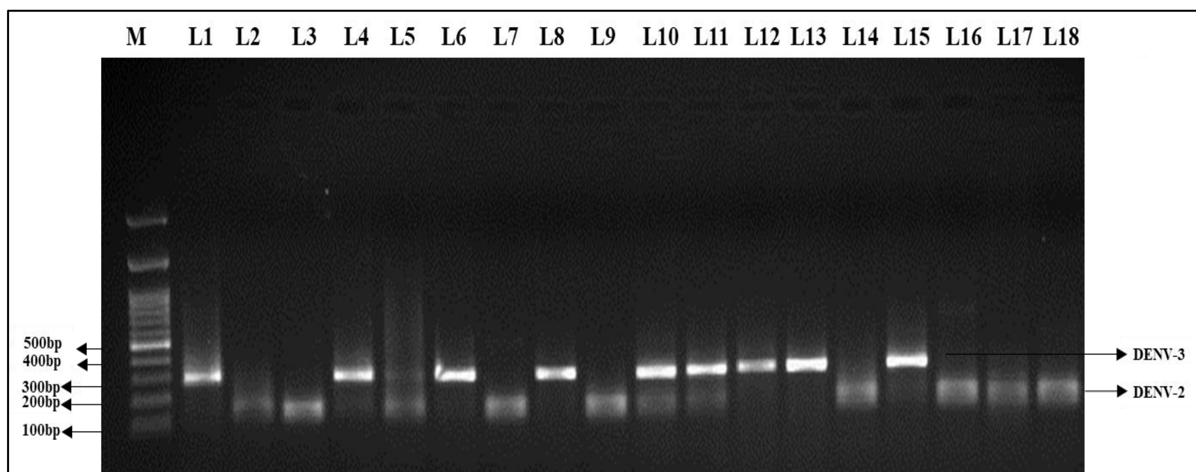
Chapter 4: Results

6.1. Molecular serotyping, genotyping and diversity analysis of DENV

Among hospital-admitted patients only (N= 380 dengue suspected), 320 dengue patients tested dengue positive. Among them 220 patients tested positive for dengue NS1 antigen, 84 patients tested positive for dengue IgM/IgG and 16 patients tested negative. Positive patients were classified according to the WHO guidelines and the highest number of dengue cases without warning signs (93%, n = 284) followed by severe dengue in 7% (n=20) of patients. Severe dengue patients developed Grades III and IV complications and four patients died due to those complications. In this study we have primarily recruited the severe dengue patients for finding biomolecules associated with severe dengue and hence the percentage of severe dengue patients is much higher than the actual scenarios.

To identify the circulating dengue serotype and genotype an additional 240 NS1 positive samples were recruited from different district hospitals of West Bengal to achieve an average of 100 samples from each year to observe the actual scenario of circulating dengue serotype. Although those samples were not suitable for proteomics and biomolecules study. Analysis of dengue serotyping data showed co-circulation of all four serotypes among our study samples. Among the RNA-positive patients in the study population, DENV-2 was observed as the major circulating serotype in 2018 with a high prevalence of 76% along with other co-circulating serotypes DENV-3 (16%), DENV-1 (4%) and DENV-4 (4%). In 2019, the scenario remained unchanged noticeably as infection with other serotypes increased but the major circulating strain remained DENV-2 (47%) followed by DENV-3, DENV-1 and DENV-4 (28%, 18% and 7%). In 2020, DENV-4 (47%) was the prevalent circulating serotype followed by DENV-2 (34 %), DENV-3 (12%) and DENV-1 (7%). Whereas in 2021, DENV-3 (72%) was the prevalent circulating serotype followed by

DENV-2 (18%), DENV-4 (6%) and DENV-1 (4%). The prevalence pattern of DENV-3 was found to be continued in 2022, with DENV-3 (55%) as the major circulating serotype again with co-circulation of DENV-2 (40%) as the second major serotype followed by DENV-1 (4%) and DENV-4 (2%). Among the dengue NS1-positive patients 56% were males and 44% were female patients. Age-based distribution of dengue infections indicated that adults belonging to the age group 21-30 were the most susceptible group followed by adolescents belonging to the age group 11-20 years (p-value <0.001). Year-wise genotyping of prevalent serotypes is shown in **Table 7**, showing Genotype II of DENV-2, Genotype III of DENV-3 and Genotype I of DENV-4 as major circulating strains. Sequencing data analysis of DENV-2 (n = 12), the prevalent serotype of the year 2018-19, DENV-4 (n = 12) the prevalent serotype of the year 2020, showed genotype II of serotype 2 and genotype I of serotype 4 was the prevalent strain of that year. However, genotype III of serotype 3 (n = 12) was observed as the prevalent strain of the year 2021. Gel Electrophoresis picture of each serotype genotyping is depicted in **Figure 25.a.i, Figure 25.a.ii and Figure 25.a.iii**. Phylogenetic analysis of all three genotypes is shown in **Figure 25.b.i, Figure 25.b.ii** and **Figure 25.b.iii**. As DENV-1 was never observed as a prevalent serotype during this study period, no genotyping was performed from this serotype.



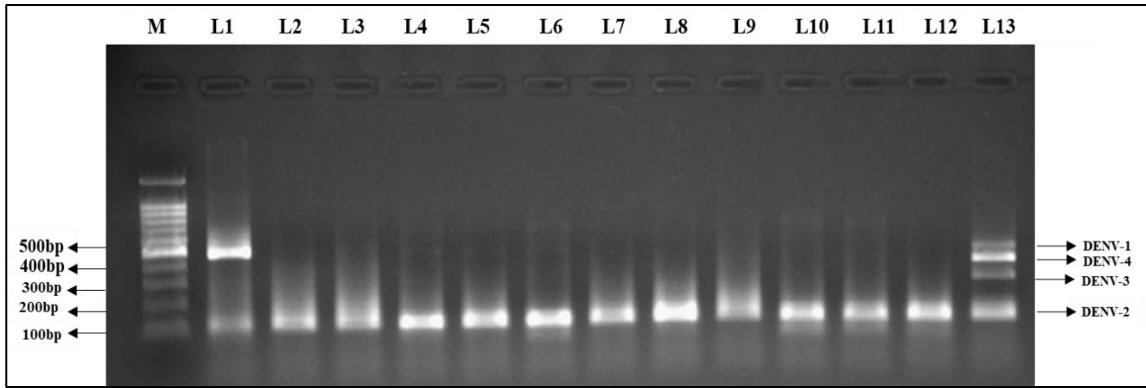


Fig. 23.a: Gel Electrophoresis picture of different dengue serotypes, DENV-1, DENV-2, DENV-3 and DENV-4, M: Marker (100bp), (a) L1 to L18: DENV positive samples (b) L1 to L12: DENV RT-PCR (Reverse Transcriptase-PCR) positive samples, L13: positive control (DENV-1 =482bp, DENV-2=119bp, DENV-3= 290bp, DENV-4= 392bp)

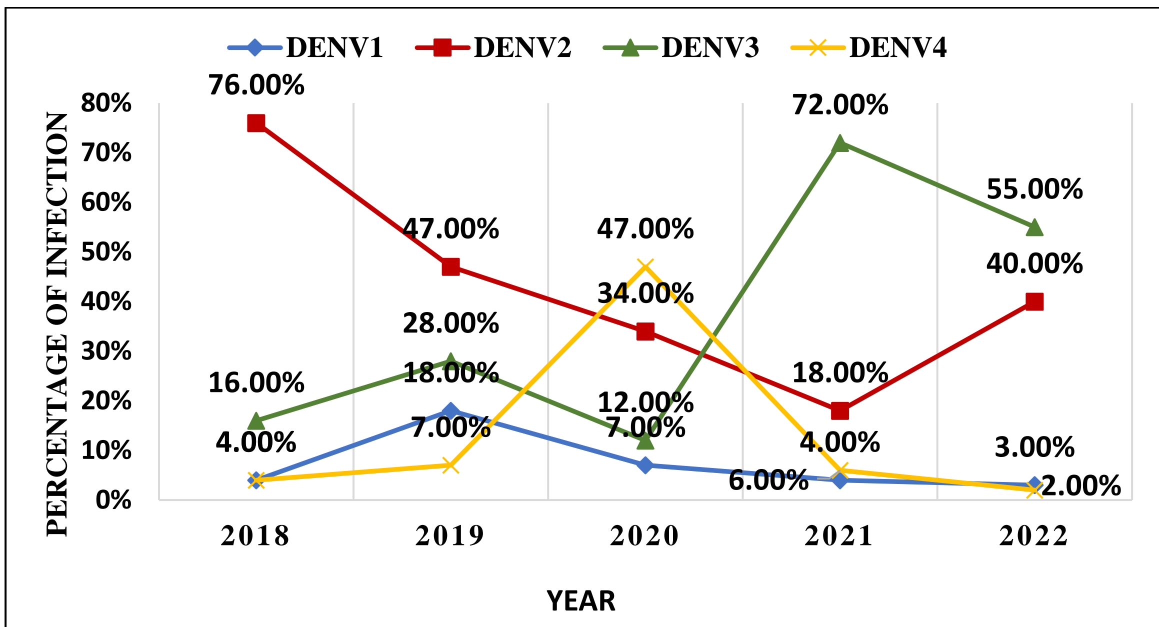


Fig. 23.b.: Year-wise distribution of dengue serotype in West Bengal. showing a rapid change in the pattern of circulating DENV serotypes

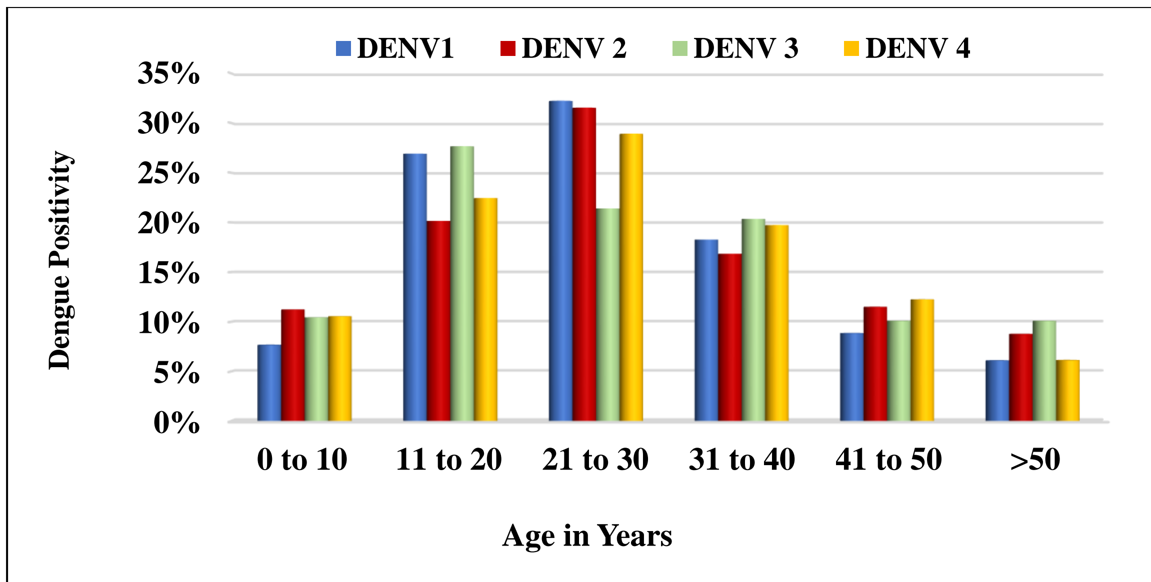


Fig. 24.a: Age -based distribution of DENV among study population

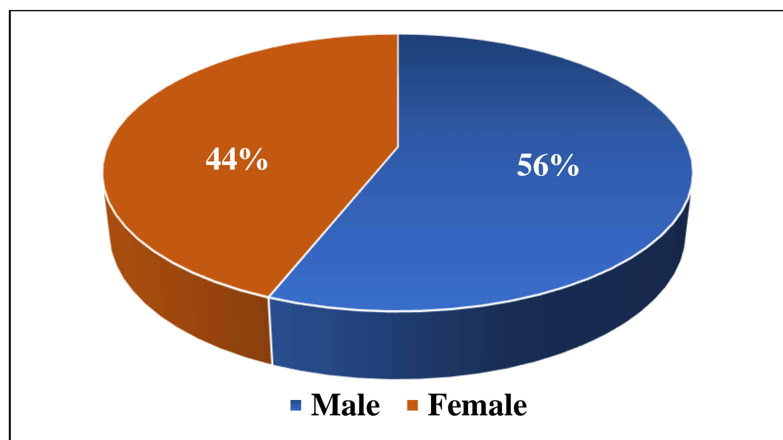


Fig. 24.b: Gender-based distribution of DENV among study population

YEAR	DENV GENOTYPE
2018	DENV-2, Genotype II
2019	DENV-2, Genotype II
2020	DENV-4, Genotype I
2021	DENV-3, Genotype III
2022	DENV-3, Genotype III

Table 7: Year-wise genotyping of prevalent DENV serotypes

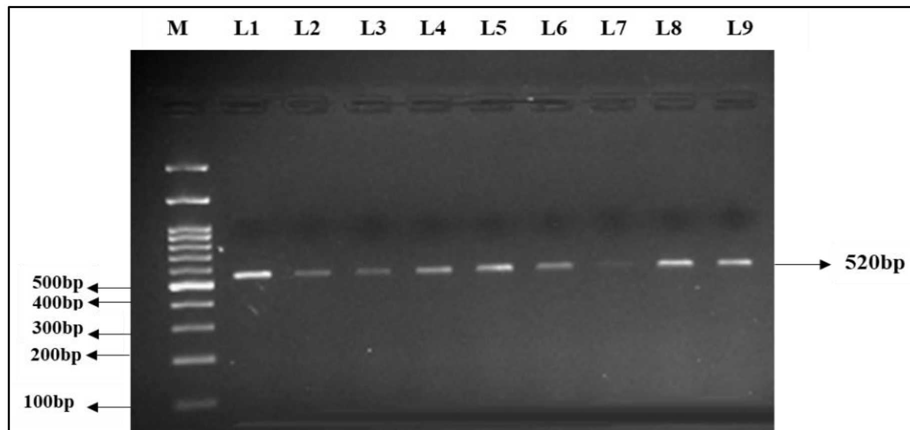


Fig. 25.a.i: Gel electrophoresis picture of DENV 2-Genotype II in 2018 and 2019 during the study period, M: Marker (100bp), L1 to L10: DENV-2 positive samples

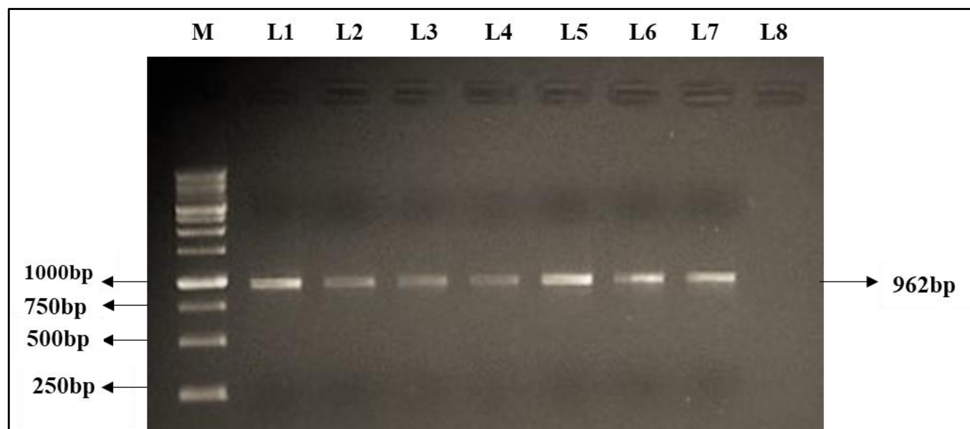


Fig. 25.a.ii: Gel electrophoresis picture of DENV 4-Genotype I in 2020 during the study period, M: Marker (1000bp), L1 to L8: DENV-4 positive samples

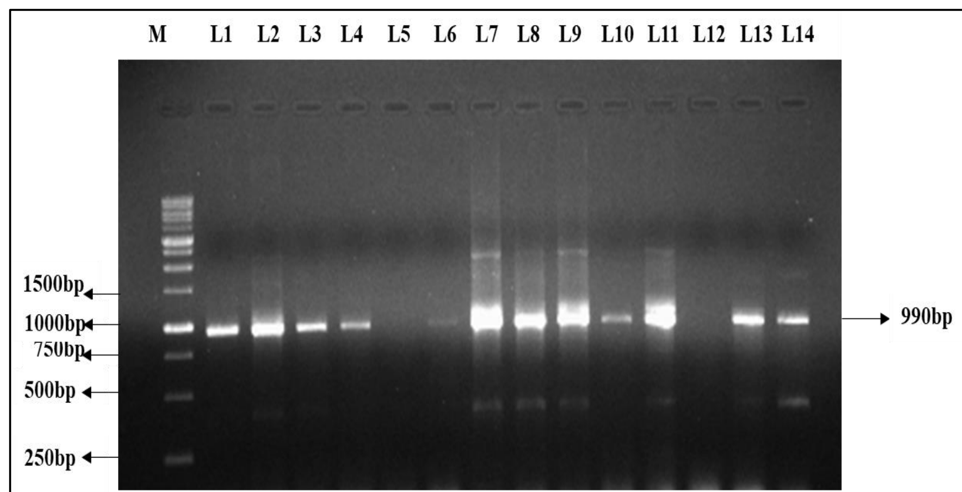


Fig. 25.a.iii: Gel electrophoresis picture of DENV 3-Genotype III in 2021 during the study period, M: Marker (1000bp), L1 to L14: DENV-3 positive samples

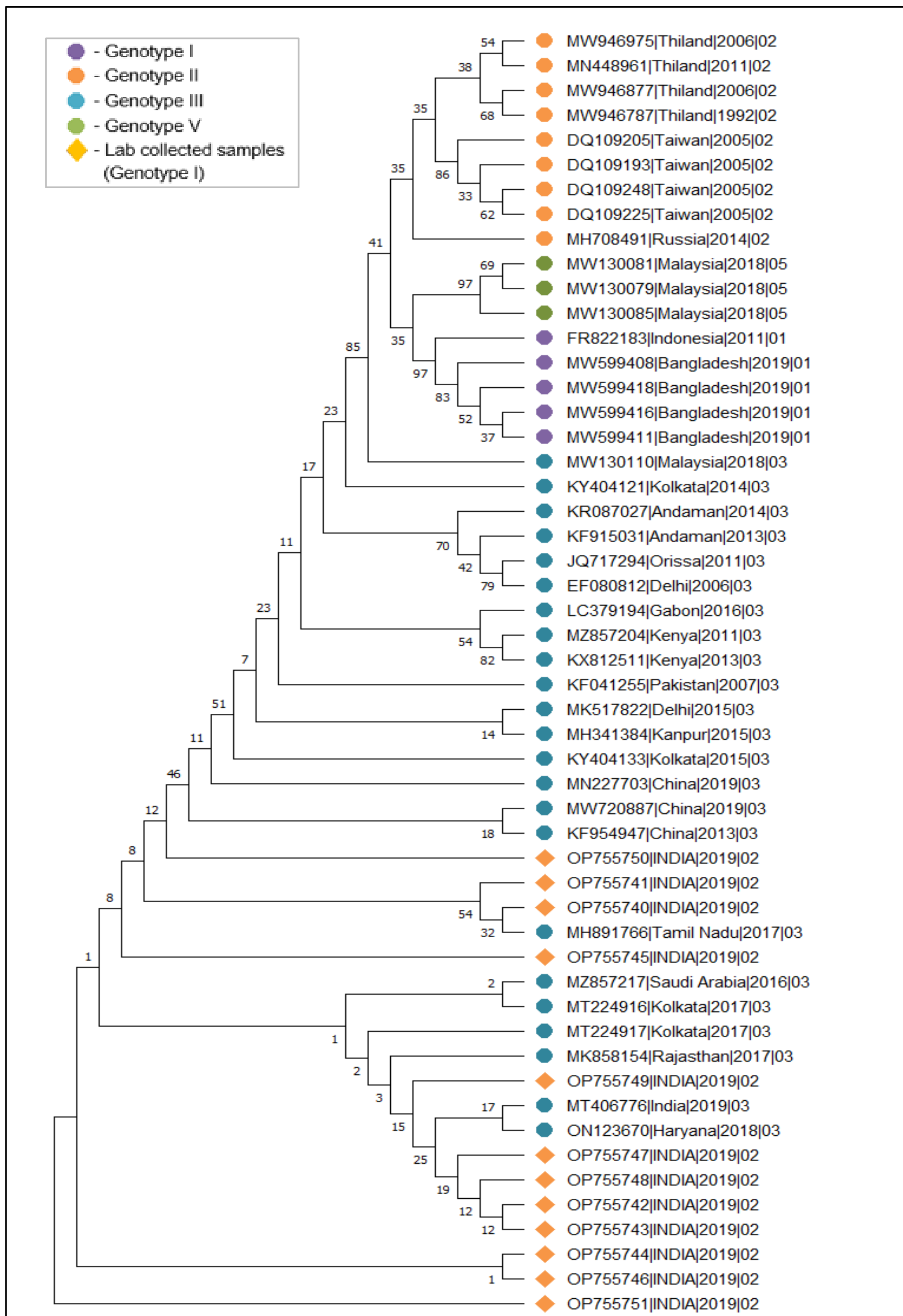


Fig. 25.b.i: Phylogenetic analysis of DENV-2 Genotype II collected during this study (n=12), with other genotypes of DENV-2 collected from NCBI website via Maximum like hood method, Bootstrap set to 500

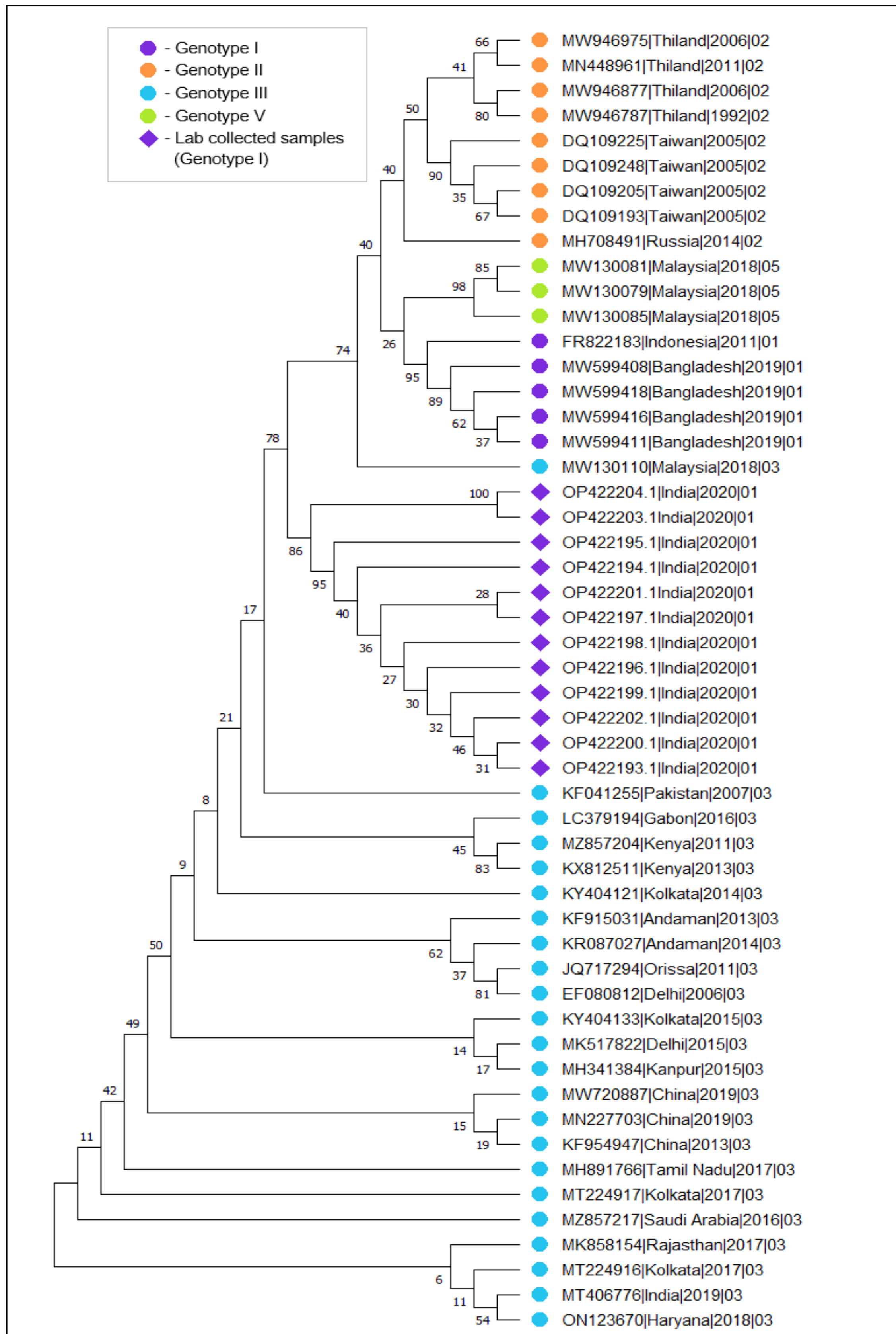


Fig. 25.b.ii: Phylogenetic analysis of DENV4 Genotype I (n=12), with other genotypes of DENV-4 collected from NCBI website via Maximum like hood method, Bootstrap set to 500 via Maximum like hood method, Bootstrap set to 500

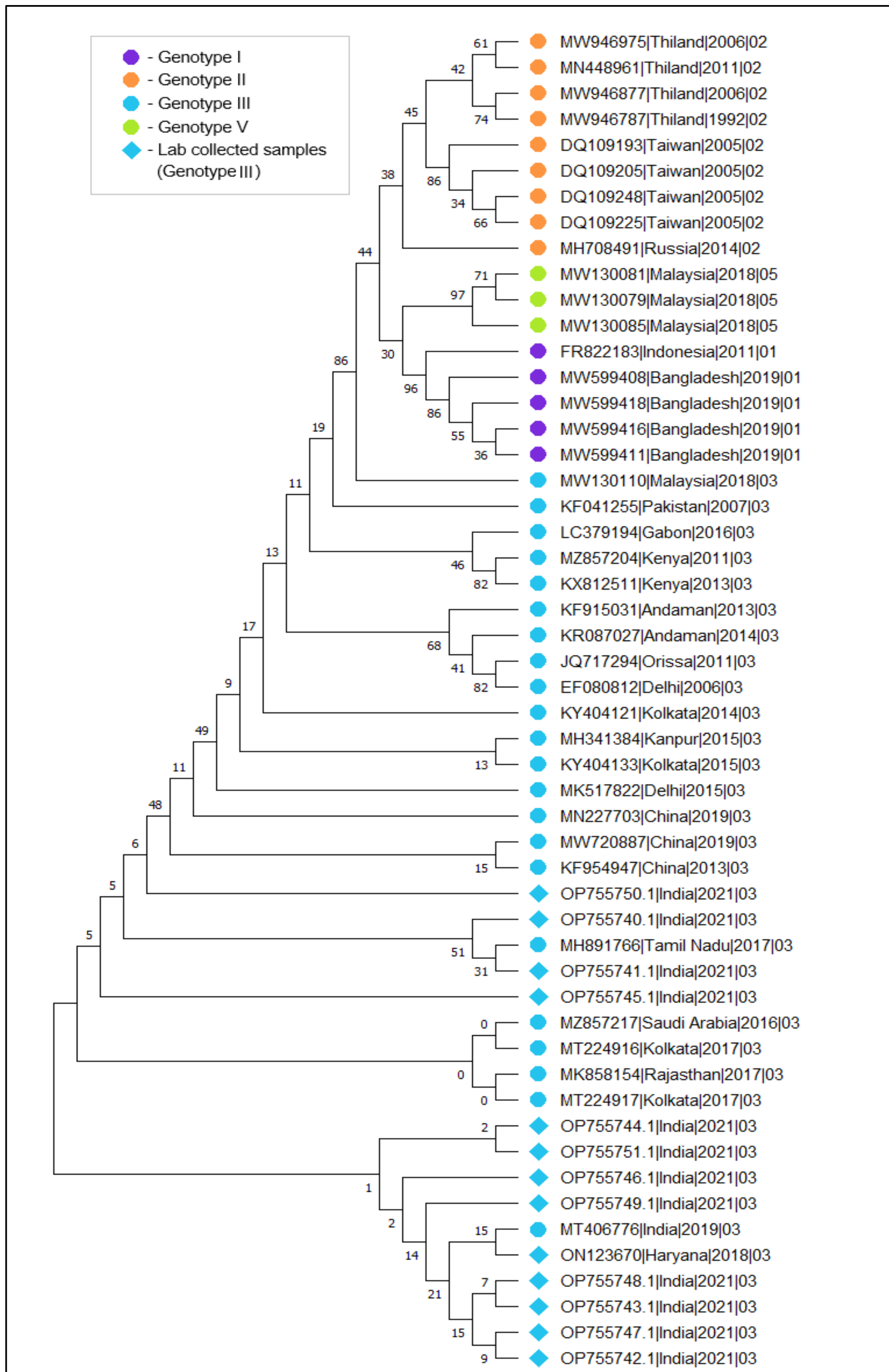


Fig. 25.b.iii: Phylogenetic analysis of DENV3 Genotype III (n=12), with other genotypes of DENV-3 collected from NCBI website via Maximum like hood method, Bootstrap set to 500 collected during this study, via Maximum like hood method, Bootstrap set to 500

6.2.a. Clinicopathological corelation

To correlate disease severity in patients, WHO suggested case classification and level of severity identification manifestations were taken into consideration. This part of a study was conducted on hospital-admitted 170 dengue-infected patients. Dengue-associated clinical symptoms were observed and depicted in **Figure 26**. Common clinical symptoms related to dengue infection were pyrexia, nausea, abdominal pain, vomiting and anorexia (observed in >40% of patients). Most of the dengue-infected patients suffered from acute atypical symptoms of fever at the time of admission. Some of the classical dengue symptoms such as joint pain, retro-orbital pain and body pain were absent or much less among the dengue-positive patients, whereas fluid accumulation and ascites were significantly high. Bleeding manifestations were observed in 53 patients, among them, petechiae were present in 87% of patients followed by melena in 75% and hematemesis in 25% of patients (**Figure 27**). Some specific correlation between dengue clinical manifestations and their serotype was also observed and shown in **Table 8**. A majority of individuals infected with DENV-2 mainly suffered from constitutional (91.04%) and gastrointestinal complications (88.05%).

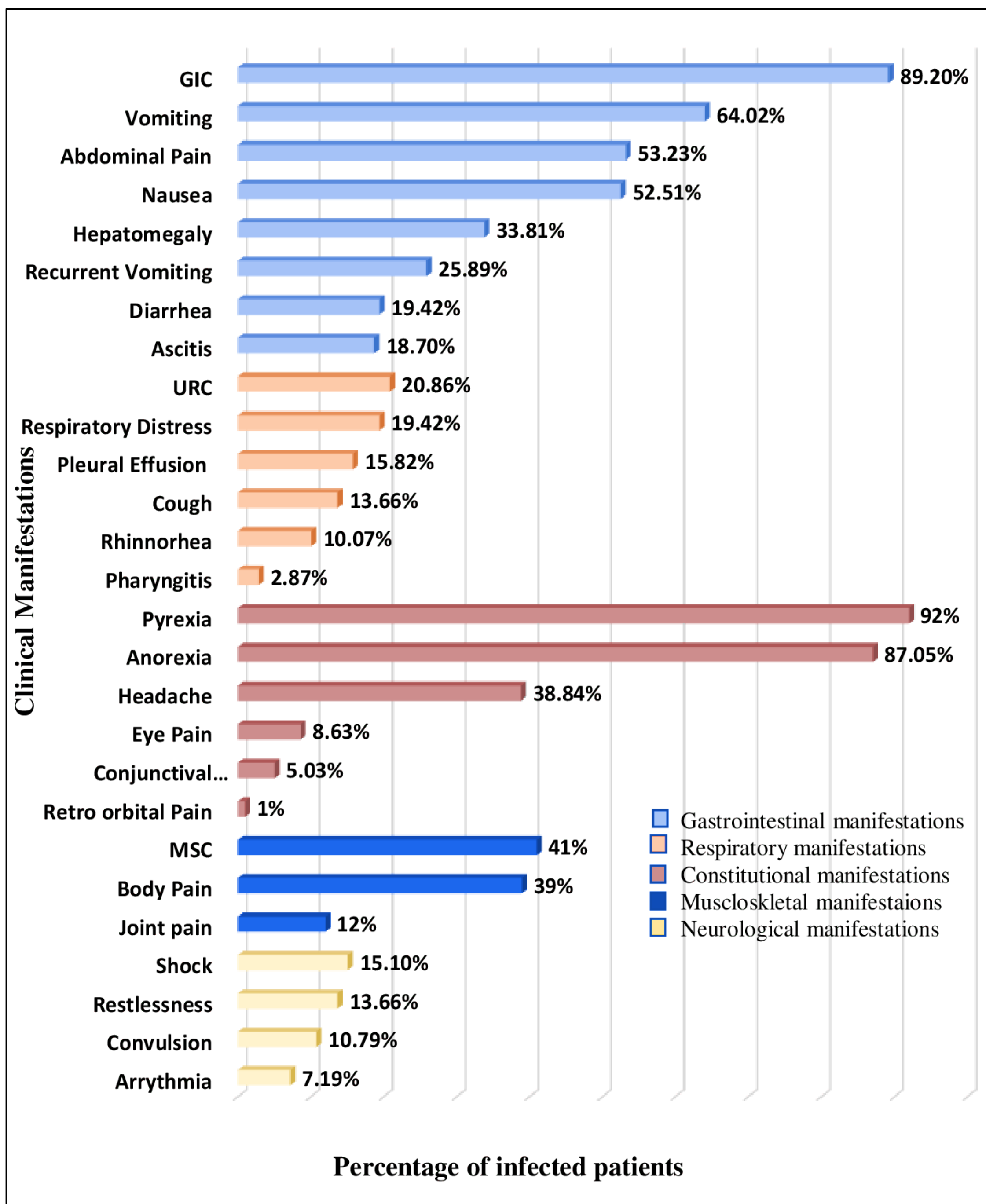


Fig. 26: Categorization and Graphical representation of different symptoms and physiological manifestations associated with dengue patients

Table 8: Different sign and clinical symptoms associated with various dengue serotypes in dengue patients with their statistical significance

Dengue fever-associated physiological manifestations	DENV-1 (6.31%, n=6)	DENV-2 (70.52%, n=67)	DENV-3 (18.94%, n=18)	DENV-4 (4.21%, n=4)	*P -value*
CNC	6(100%)	61(91.04%)	14(77.77%)	4(100%)	<0.001
GIC	6(100%)	59(88.05%)	18(100%)	4(100%)	<0.001
MSC	2(33.33%)	26(38.8%)	6(33.33%)	2(50%)	<0.001
URC	1(16.66%)	8(11.94%)	9(50%)	3(75%)	<0.001
Thrombocytopenia	2(33.33%)	46(68.65)	13(72.22%)	4(100%)	<0.001
Rash	3(50%)	23(34.32%)	11(61.11%)	3(75%)	<0.001
Fluid Leak	3(50%)	17(25.37%)	6(33.33%)	4(100%)	<0.001
Shock	1(16.66%)	11(16.41%)	7(38.88%)	4(100%)	0.299
Bleeding	5(90%)	10(14.92%)	5(27.77%)	4(100%)	0.023

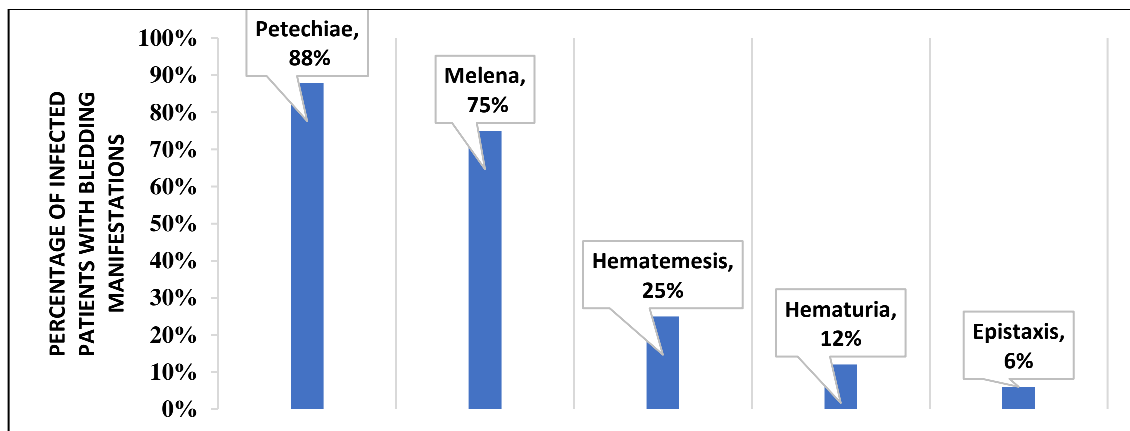


Fig. 27.a: Distribution of different bleeding manifestations among hospital admitted dengue patients (n=53)

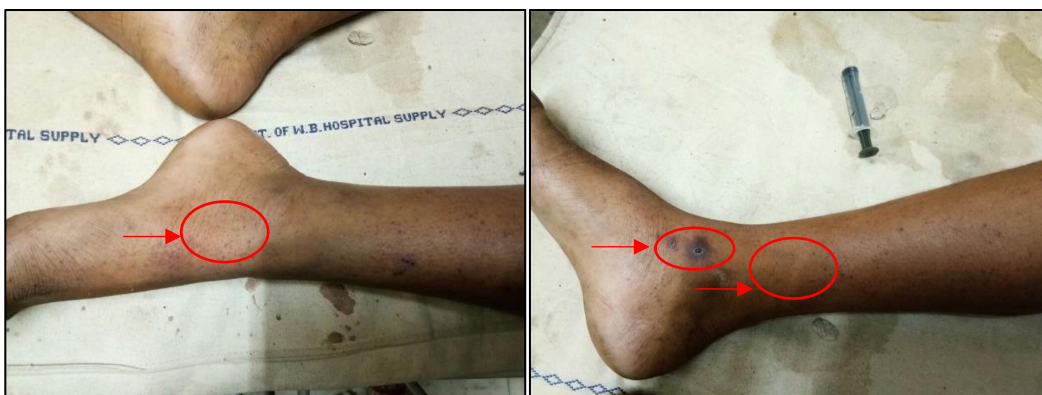


Fig. 27.b: Images of Petechial Hemorrhages in dengue-infected patients obtained from RG Kar Medical College and Hospital, Kolkata

6.2.b. Platelet transfusion and its outcome

Haematological analysis showed thrombocytopenia in 70% of patients, as per the patient's classification platelet count and transfusion pattern recorded day-wise to observe close proximal relation of dengue severity with decreased platelet count. Platelet transfusion was done in severe patients as per WHO guidelines (152) and observed transfusion outcomes are shown in **Table 9.**

Table 9: Platelet transfusion and its clinical outcome in dengue-infected severe patients

	Grade of severity among severe patients	%age of severe patients who received a transfusion	Lowest Platelet count+ day	Average Platelet count at the time of transfusion	Unit of platelet transfusion+ day	Clinical outcome
Thrombocytopenia observed in 70% of all dengue-infected patients	**DHF (Moderate-14%)**	75%	20,000/µl+4	29,750/µl	2 to 4 units + 4 to 8 days	With different bleeding manifestations, patients recovered
	DSS (Severe-3%)	100%	10,000/µl+6	21,250/µl	4 to 6 units + 4 to 10 days	With different bleeding manifestations, patients died

6.2.c. Liver functional profile of DENV-infected patients

Serum TP (Total Protein) was measured in Groups I and II dengue patients with a mean of 6.44 versus 6.30 g/dl in DF patients, 5.06 versus 4.96 g/dl in DHF (with Grades I and II complications), and 4.93 versus 3.62 g/dl in DSS (with III and IV complications), respectively (**Figure 28.a**). Depletion in albumin was also observed with a mean of 4.30 g/dl in Group I 4.23 g/dl in Group II of DF, 3.72 g/dl in Group I and 3.49 g/dl in Group II of DHF and 3.01 g/dl Group I and 2.3 g/dl in Group II of DSS patients, respectively (**Figure 28.b**). Elevation in liver enzymes was observed among patients with a mean AST (Aspartate aminotransferase) value of 66.42 U/L in Group I and 84.59 U/L in Group II of DF, 102.76U/L in Group I and 167.68 U/L in Group II of DHF, 140.73 U/L in Group I, and

729.54 U/Lin Group II of DSS patients (**Figure 28.c**). Similarly, ALT (Alanine aminotransferase) values were 63.90 U/L in Group I and 91.83 U/L in Group II of DF, 164 U/L in Group I, and 287 U/L in Group II of DHF, 193.6 U/L in Group I, and 791.30 U/L in Group II of DSS were observed (**Figure 28.d**).

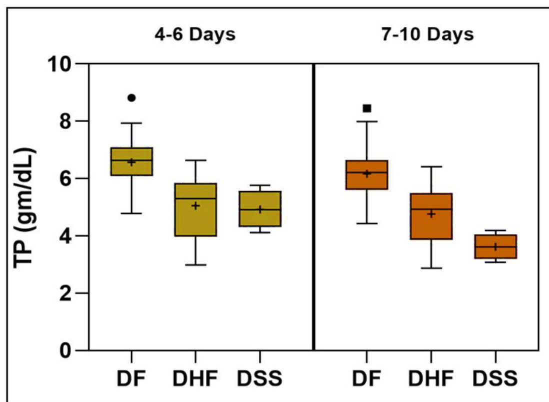


Fig. 28.a

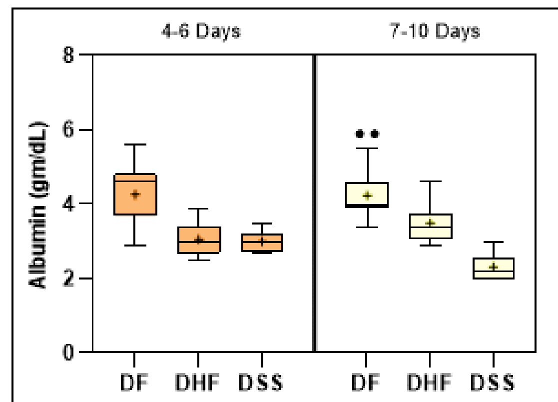


Fig. 28.b

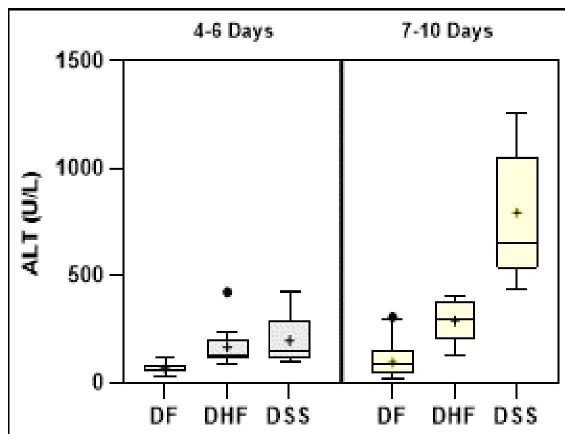


Fig. 28.c

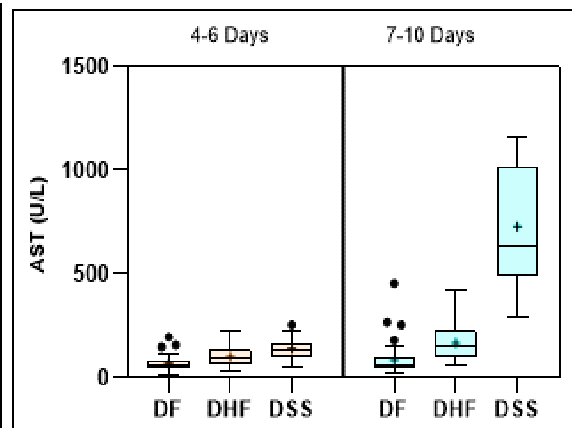


Fig. 28.d

Fig. 28.a to 28.d: Observational representation of day-wise Total protein, Albumin and liver enzymes (ALT, AST) association with dengue fever and severe dengue patients

6.2.d. Regression logistic analysis

Receiver Operating Curve (ROC) is used for determining the best cut-off values and to model the relationship between binary response variables. A total of 170 patients were analyzed in ROC 1 based on the dengue positive and negative outcomes as a dependent variable associated with observed clinical manifestations shown in **Figure 29.a**. We have evaluated an alternative regression model shown in ROC 2. In the alternative model, we have analyzed 139 dengue seropositive patients based on dengue serotypes and IgM positivity as a dependent variable shown in **Figure 29.b**. A multivariate logistic regression approach was used for making the regression model including parameters such as gastrointestinal complications, nausea, vomiting, abdominal pain, recurrent vomiting, ascites, loose stool, liver enlargement, upper respiratory tract complications, rhinorrhea, cough, sore throat, pleural effusion, respiratory distress, different types of rashes, rash with itchiness, musculoskeletal complications, body pain, joint pain, constitutional complications, eye pain, headache, anorexia, tourniquet test, bleeding, fluid accumulation, convulsion, arrhythmia, puffy eyes, thrombocytopenia. For multivariate analysis, dengue-associated clinical symptoms were classified as a categorical variable and their observations transformed into binary forms of “0” and “1”, where “1” denotes “yes” and “0” denotes “no” and analysed with dengue positivity or negativity as a dependent outcome in ROC model 1 (n=170), whereas as RNA positivity or IgM positivity in ROC model 2 (n = 139). Both descriptive and inferential statistics were used for analysing the data.

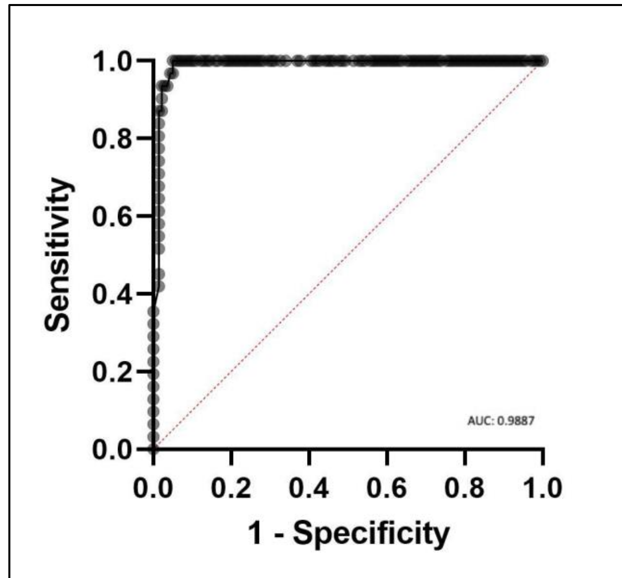


Fig. 29.a: Receiver operating curve of dengue associated clinical manifestation with dengue positivity and negativity

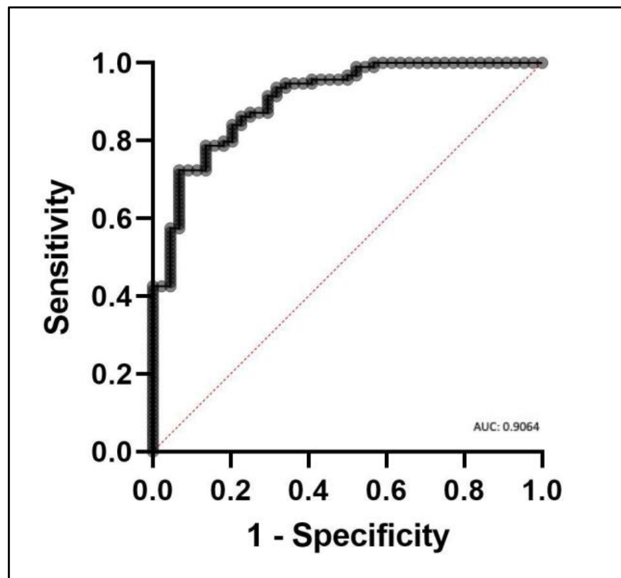


Fig. 29.b: Receiver operating curve of dengue associated clinical manifestation with dengue serotype positivity and IgM (serotype negativity)

6.3. Proteomics study in search for biomolecules and their validation

6.3.a. Protein annotation using Orbitrap Mass spectrometry and pathway analysis

Using the nano-LC/Orbitrap mass spectrometer run and subsequent analyses, we have annotated around 6000 proteins from acute phase patient's samples, 5000 proteins from critical phase patient's samples and 4300 proteins from healthy controls individuals respectively. The annotated proteins from the MS run were analyzed for their enrichment in Gene Ontology-based Biological Processes, Molecular Functions, Protein Classes and PANTHER pathways in the PANTHER (v 16.0) database. Using the above database it was evident that the annotated proteins belong to a wide range of protein classes (membrane traffic protein, viral or transposable element protein, extracellular matrix protein, structural proteins, defence/immunity protein, scaffolding proteins, etc.), involved in an array of pathways (B cell activation, apoptosis signalling pathway, Ubiquitin-proteasomal pathway, autophagy pathway, VEGF signalling pathway, Wnt-signaling pathway, Heme biosynthesis, Toll-receptor signalling pathway, TGF-β signalling pathway, T-cell activation, Notch signalling pathway, Transcription activation, Synaptic vesicle trafficking and many more). Pathway analyses of the significantly enriched proteins annotated from Orbitrap MS are represented in **Figure 31. (i-iv)**.

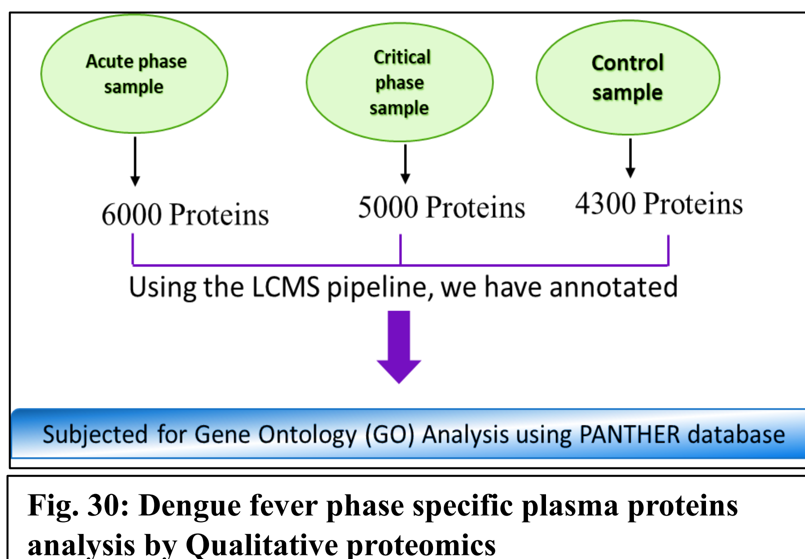
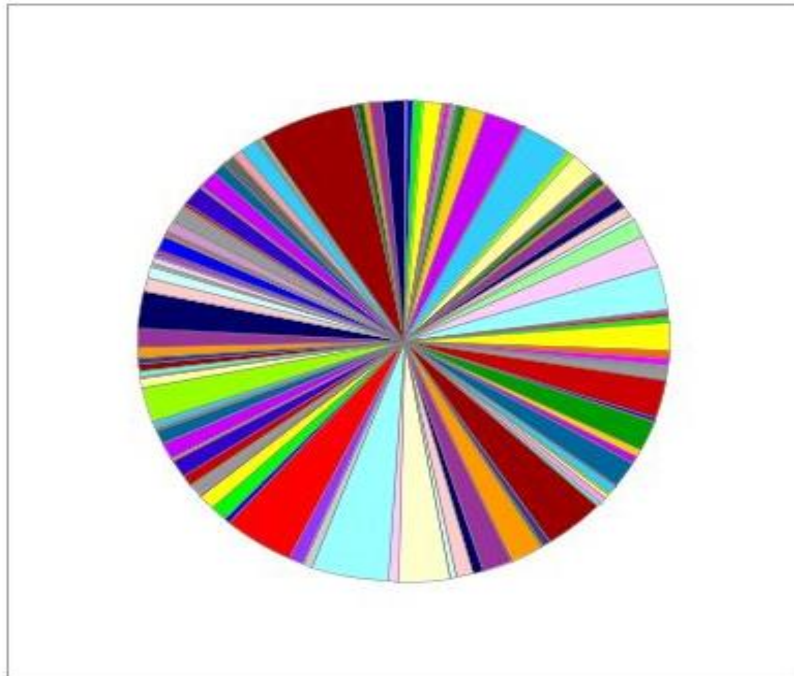


Fig. 30: Dengue fever phase specific plasma proteins analysis by Qualitative proteomics

(i)

PANTHER Pathway

Total # Genes: 6339 Total # pathway hits: 2133



Click to get gene list for a category:

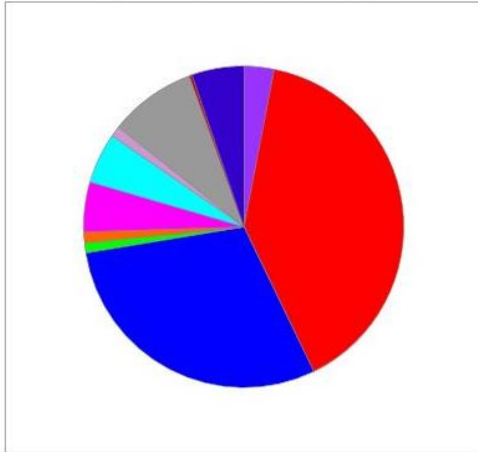
- 2-arachidonoylglycerol biosynthesis (P05726)
- 5-Hydroxytryptamine biosynthesis (P04371)
- 5-Hydroxytryptamine degredation (P04372)
- 5HT1 type receptor mediated signaling pathway (P04373)
- 5HT2 type receptor mediated signaling pathway (P04374)
- 5HT3 type receptor mediated signaling pathway (P04375)
- 5HT4 type receptor mediated signaling pathway (P04376)
- ATP synthesis (P02721)
- Adenine and hypoxanthine salvage pathway (P02723)
- Adrenaline and noradrenaline biosynthesis (P00001)
- Alanine biosynthesis (P02724)
- Allantoin degradation (P02725)
- Alpha adrenergic receptor signaling pathway (P00002)
- Alzheimer disease-amyloid secretase pathway (P00003)
- Alzheimer disease-presenilin pathway (P00004)
- Aminobutyrate degradation (P02726)
- Anandamide degradation (P05728)
- Androgen/estrogene/progesterone biosynthesis (P02727)
- Angiogenesis (P00005)
- Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)
- Apoptosis signaling pathway (P00006)
- Arginine biosynthesis (P02728)
- Ascorbate degradation (P02729)
- Asparagine and aspartate biosynthesis (P02730)
- Axon guidance mediated by Slit/Robo (P00008)
- Axon guidance mediated by netrin (P00009)
- Axon guidance mediated by semaphorins (P00007)
- B cell activation (P00010)
- Beta1 adrenergic receptor signaling pathway (P04377)
- Beta2 adrenergic receptor signaling pathway (P04378)
- Beta3 adrenergic receptor signaling pathway (P04379)
- Blood coagulation (P00011)
- Bupropion degradation (P05729)
- CCKR signaling map (P06959)
- Cadherin signaling pathway (P00012)
- Cell cycle (P00013)
- Cholesterol biosynthesis (P00014)
- Circadian clock system (P00015)
- Coenzyme A biosynthesis (P02736)
- Cortocotropin releasing factor receptor signaling pathway (P04380)
- Cytoskeletal regulation by Rho GTPase (P00016)
- DNA replication (P00017)
- De novo purine biosynthesis (P02738)
- De novo pyrimidine deoxyribonucleotide biosynthesis (P02739)
- De novo pyrimidine ribonucleotides biosythesis (P02740)
- Dopamine receptor mediated signaling pathway (P05912)
- EGF receptor signaling pathway (P00018)
- Endogenous cannabinoid signaling (P05730)
- Endothelin signaling pathway (P00019)
- Enkephalin release (P05913)
- FAS signaling pathway (P00020)
- FGF signaling pathway (P00021)
- Formyltetrahydrofolate biosynthesis (P02743)
- Fructose galactose metabolism (P02744)
- GABA-B receptor II signaling (P05731)
- Gamma-aminobutyric acid synthesis (P04384)
- General transcription by RNA polymerase I (P00022)
- General transcription regulation (P00023)
- Glycolysis (P00024)
- Gonadotropin-releasing hormone receptor pathway (P06664)
- Hedgehog signaling pathway (P00025)

- Heme biosynthesis (P02746)
- Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)
- Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)
- Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction (P00028)
- Histamine H1 receptor mediated signaling pathway (P04385)
- Histamine H2 receptor mediated signaling pathway (P04386)
- Histamine synthesis (P04387)
- Huntington disease (P00029)
- Hypoxia response via HIF activation (P00030)
- Inflammation mediated by chemokine and cytokine signaling pathway (P00031)
- Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)
- Insulin/IGF pathway-protein kinase B signaling cascade (P00033)
- Integrin signalling pathway (P00034)
- Interferon-gamma signaling pathway (P00035)
- Interleukin signaling pathway (P00036)
- Ionotropic glutamate receptor pathway (P00037)
- Isoleucine biosynthesis (P02748)
- JAK/STAT signaling pathway (P00038)
- Leucine biosynthesis (P02749)
- Mannose metabolism (P02752)
- Metabotropic glutamate receptor group I pathway (P00041)
- Metabotropic glutamate receptor group II pathway (P00040)
- Metabotropic glutamate receptor group III pathway (P00039)
- Methionine biosynthesis (P02753)
- Methylmalonyl pathway (P02755)
- Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)
- Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)
- N-acetylglucosamine metabolism (P02756)
- Nicotine degradation (P05914)
- Nicotine pharmacodynamics pathway (P06587)
- Nicotinic acetylcholine receptor signaling pathway (P00044)
- Notch signaling pathway (P00045)
- O-antigen biosynthesis (P02757)
- Opioid prodynorphin pathway (P05916)
- Opioid proenkephalin pathway (P05915)
- Opioid proopiomelanocortin pathway (P05917)
- Ornithine degradation (P02758)
- Oxidative stress response (P00046)
- Oxytocin receptor mediated signaling pathway (P04391)
- PDGF signaling pathway (P00047)
- PI3 kinase pathway (P00048)
- Parkinson disease (P00049)
- Pentose phosphate pathway (P02762)
- Phenylethylamine degradation (P02766)
- Plasminogen activating cascade (P00050)
- Proline biosynthesis (P02768)
- Purine metabolism (P02769)
- Pyrimidine Metabolism (P02771)
- Pyruvate metabolism (P02772)
- Ras Pathway (P04393)
- S-adenosylmethionine biosynthesis (P02773)
- Salvage pyrimidine deoxyribonucleotides (P02774)
- Salvage pyrimidine ribonucleotides (P02775)
- Succinate to proprionate conversion (P02777)
- Sulfate assimilation (P02778)
- Synaptic vesicle trafficking (P05734)
- T cell activation (P00053)
- TCA cycle (P00051)
- TGF-beta signaling pathway (P00052)
- Tetrahydrofolate biosynthesis (P02742)
- Threonine biosynthesis (P02781)
- Thyrotropin-releasing hormone receptor signaling pathway (P04394)
- Toll receptor signaling pathway (P00054)
- Transcription regulation by bZIP transcription factor (P00055)

- Transcription regulation by bZIP transcription factor (P00055)
- Ubiquitin proteasome pathway (P00060)
- VEGF signaling pathway (P00056)
- Valine biosynthesis (P02785)
- Vasopressin synthesis (P04395)
- Vitamin B6 metabolism (P02787)
- Vitamin D metabolism and pathway (P04396)
- Wnt signaling pathway (P00057)
- mRNA splicing (P00058)
- p38 MAPK pathway (P05918)
- p53 pathway by glucose deprivation (P04397)
- p53 pathway feedback loops 2 (P04398)
- p53 pathway (P00059)

(ii)

PANTHER GO-Slim Molecular Function
Total # Genes: 6339 Total # function hits: 4837

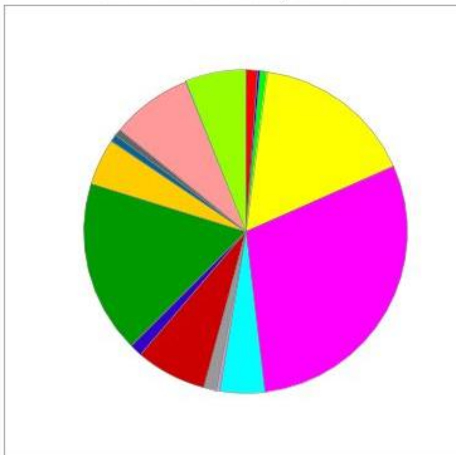


Click to get gene list for a category:

- ATP-dependent activity (GO:0140657)
- binding (GO:0005488)
- catalytic activity (GO:0003824)
- cytoskeletal motor activity (GO:0003774)
- low-density lipoprotein particle receptor activity (GO:0005041)
- molecular adaptor activity (GO:0060090)
- molecular function regulator (GO:0098772)
- molecular transducer activity (GO:0060089)
- structural molecule activity (GO:0005198)
- transcription regulator activity (GO:0140110)
- translation regulator activity (GO:0045182)
- transporter activity (GO:0005215)

(iii)

PANTHER GO-Slim Biological Process
Total # Genes: 6339 Total # process hits: 11127



Click to get gene list for a category:

- behavior (GO:0007610)
- biological adhesion (GO:0022610)
- biological phase (GO:0044848)
- biological process involved in interspecies interaction between organisms (GO:0044419)
- biological regulation (GO:0065007)
- biomineralization (GO:0110148)
- cellular process (GO:0009987)
- developmental process (GO:0032502)
- growth (GO:0040007)
- immune system process (GO:0002376)
- localization (GO:0051179)
- locomotion (GO:0040011)
- metabolic process (GO:0008152)
- multicellular organismal process (GO:0032501)
- pigmentation (GO:0043473)
- reproduction (GO:0000003)
- reproductive process (GO:0022414)
- response to stimulus (GO:0050896)
- rhythmic process (GO:0048511)
- signaling (GO:0023052)

(iv)

Fig. 31: Pathway analyses of the proteins annotated from Nano LC/Orbitrap MS depicting significantly enriched proteins via (i) PANTHER Pathway, (ii) PANTHER GO slim Molecular Function, (iii) PANTHER GO slim Biological Process, (iv) PANTHER Protein class

6.3.b. Identification and Quantification of pathway enriched and patient-specific proteins

In SWATH-MS1 run was obtained from a paired sample of acute phase patients with equivalent healthy control and the SWATH-MS2 run was obtained from a paired sample of critical phase patients with equivalent healthy control subjected to pathway analysis individually. Quantitative proteomics phase-specific analysis is depicted in **Figure 32** and pathway analysis of each phase is shown in **Figures 33 and 34**. Using SWATH-MS, we were able to identify and quantify 290 proteins from the paired acute phase plasma samples

and 253 from paired critical phase plasma samples. Differential expression of 239 common plasma proteins was analysed after a comparative analysis of acute phase paired and critical phase paired samples that had already been analyzed qualitatively using Orbitrap-mass spectrometry. Equivalent numbers of healthy control used in each SWATH-MS analysis; list of proteins submitted to ProteomeX server**.** The significantly altered expressions of proteins obtained from SWATH-MS fall under the categories of complements, immune activating, microtubule-associated, coagulation factors, apolipoproteins and endothelial proteins. Various groups of proteins such as angiogenesis (P00005), blood coagulation (P00011), cadherin signalling pathway (P00012), interleukin signalling pathway (P00036), inflammation-mediated cytokine and chemokine signalling pathway (P00031), etc. found to be significantly enriched in PANTHER pathway analysis (p-value: $P < 0.05$), **Figure 33** and **Figure 34.**

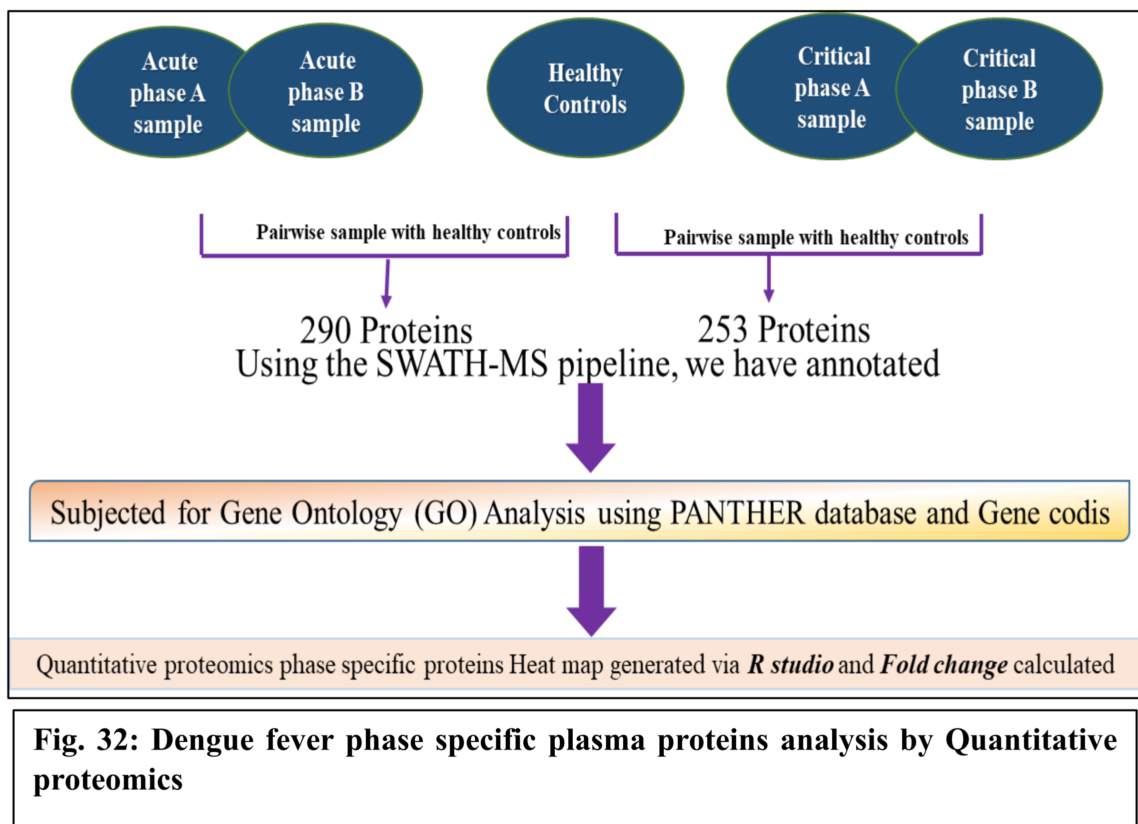
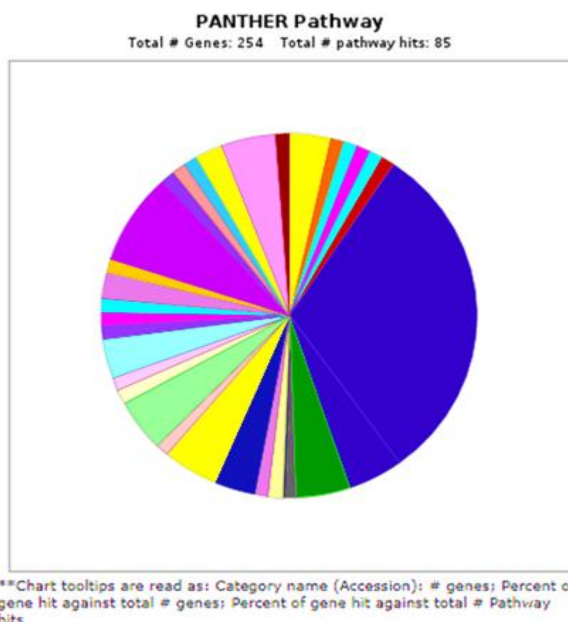


Fig. 32: Dengue fever phase specific plasma proteins analysis by Quantitative proteomics



Click to get gene list for a category:

- Alzheimer disease-presenilin pathway (P00004)
- Angiogenesis (P00005)
- Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)
- Apoptosis signaling pathway (P00006)
- Axon guidance mediated by semaphorins (P00007)
- B cell activation (P00010)
- Blood coagulation (P00011)
- CCKR signaling map (P06959)
- Cadherin signaling pathway (P00012)
- Cytoskeletal regulation by Rho GTPase (P00016)
- FAS signaling pathway (P00020)
- Fructose galactose metabolism (P02744)
- Glycolysis (P00024)
- Gonadotropin-releasing hormone receptor pathway (P06664)
- Huntington disease (P00029)
- Inflammation mediated by chemokine and cytokine signaling pathway (P00031)
- Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)
- Insulin/IGF pathway-protein kinase B signaling cascade (P00033)
- Integrin signalling pathway (P00034)
- Interleukin signaling pathway (P00036)
- Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)
- Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)
- Nicotinic acetylcholine receptor signaling pathway (P00044)
- Parkinson disease (P00049)
- Plasminogen activating cascade (P00050)
- Pyruvate metabolism (P02772)
- T cell activation (P00053)
- Toll receptor signaling pathway (P00054)
- Vitamin D metabolism and pathway (P04396)
- Wnt signaling pathway (P00057)
- p53 pathway (P00059)

Fig. 33: Pathway enrichment of the proteins identified using SWATH-MS from acute dengue patients, PANTHER analysis

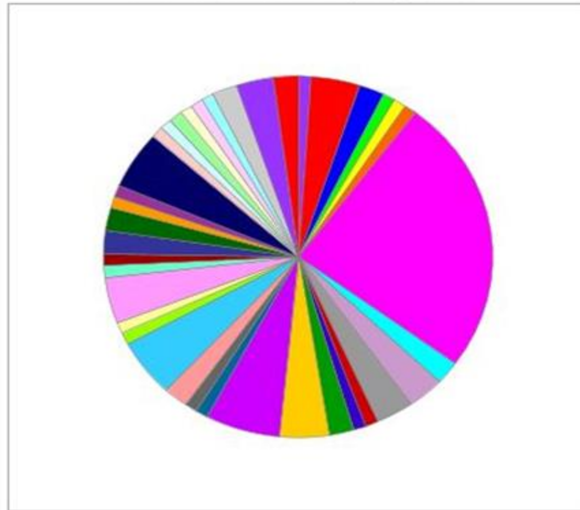


Fig. 34: Pathway enrichment of the proteins identified using SWATH-MS from critical dengue patients, PANTHER analysis

6.3.c. KEGG (Kyoto Encyclopaedia of Genes and Genomes) Pathway analysis

KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway analysis is a bioinformatics tool used to interpret high-throughput genomic and transcriptomic data. KEGG pathway analysis of 239 differentially expressed (after SWATH-MS comparative analysis of paired acute and paired critical phase samples) proteins was performed to understand the biological functions associated with a set of genes or proteins. This analysis provided insights into the pathways these genes or proteins are involved in, shedding light on their roles in different cellular processes. By identifying the pathways that are significantly enriched with differentially expressed genes or proteins, we can discern the biological relevance of their experimental findings. This is crucial for understanding the underlying mechanisms of diseases or biological phenomena. The analysis can generate hypotheses about the relationships between genes, proteins and cellular processes. This information can guide further experimental studies to validate the predictions made by the pathway analysis. The significant pathways after the KEGG analysis of the SWATH-MS gene are shown in **Figure 35.a-** Compliment and coagulation pathways, **Figure 35.b-** Viral protein interactions with Chemokine and Cytokine Receptors, **Figure 35.c-** Cholesterol Metabolism and Figure, **35.d-** ECM (Extra Cellular Matrix) interaction Receptors.

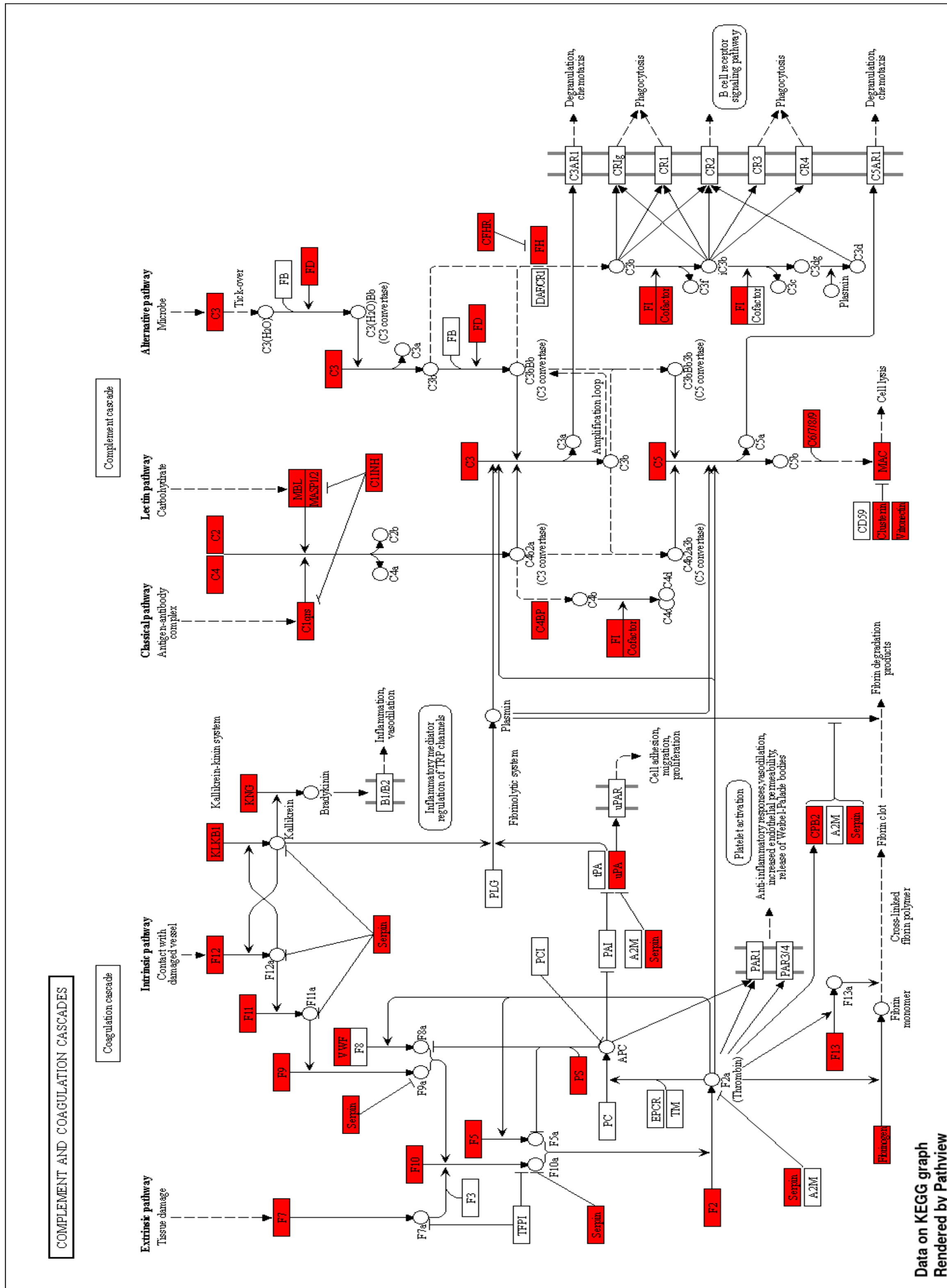


Fig. 35.a

Fig. 35.b

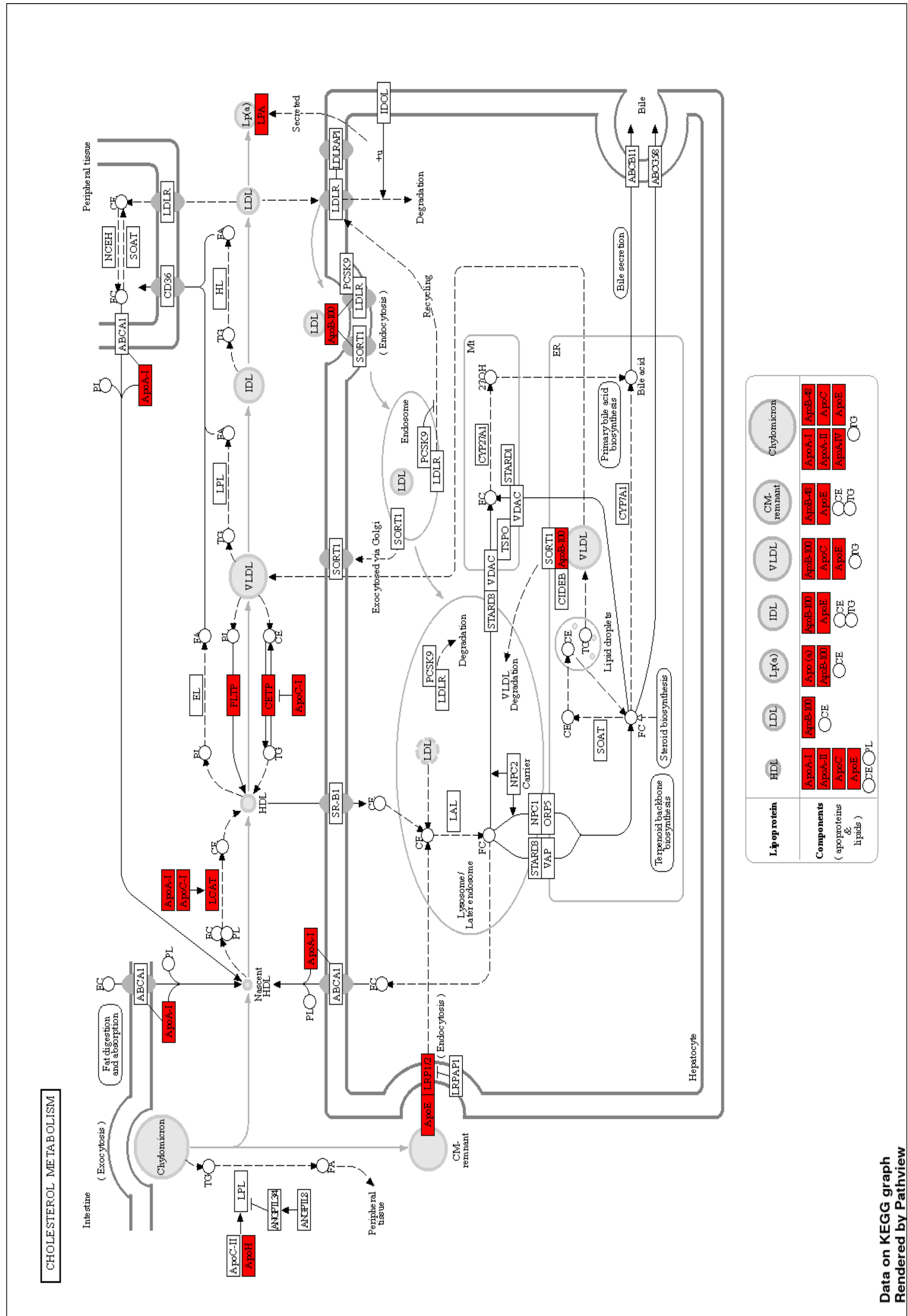


Fig. 35.c

Fig. 35.d

Fig. 35.a to 35.d: KEGG pathway diagrams highlighting SWATH-MS genes associated with complement and coagulation pathways, viral protein interactions with cytokine and cytokine receptors, ECM-receptor interactions and cholesterol metabolism

6.3.d. Heat map of significantly changed proteins

Heat map analysis is a visualization technique used to explore patterns of protein/gene expression across different experimental conditions, samples or time points. Heat maps provide a visual representation of protein/gene expression patterns, making it easier to identify trends and clusters within large datasets. Patterns may reveal relationships between genes or distinct expression profiles associated with different clinical complications/ diseases. We have also maps performed hierarchical clustering on samples based on gene expression profiles. This clustering can reveal similarities or differences between samples, helping to group them based on their molecular characteristics. Heat map analysis aids in the identification of potential biomolecules by highlighting protein/genes that show consistent and significant expression changes across different conditions. These biomolecules can be crucial for diagnostic or prognostic purposes in dengue diseases. We have visualized the differential expression of genes between different phases of dengue fever. Which helped in identifying genes that are upregulated or downregulated in response to severe dengue. Among differentially expressed SWATH-MS proteins, the top 50 proteins were selected based on fold change cut-off value (Upregulation=1.5 and above, Downregulation= 0.3 and below) from the severe dengue group and depicted via Heat map and cluster analysis, **Figure 36**. Fold change expression of the differentially expressed protein of interest shows that Apo CII, Apo CIII, Apo E upregulated, Apo CI, Apo M, Apo a, and Apo AI downregulated among apolipoproteins; coagulation factor VIII, XI, XIIIB, V upregulated, Heparin cofactor II, Plasma kallikrein OS, Thrombospondin I OS, Platelet factor IV OS, Coagulation factor XIII, IX, XII, antithrombin III, prothrombin OS downregulated, complement factor C4a, C1q subunit A, B and C, Complement component C9, Complement C1s subcomponent, C1r like protein, complement component C7, Complement C4b, upregulated and Complement component C6 downregulated, vascular

cell adhesion protein 1, N cam L1 like protein, EGF containing tubulin like EMP 1, periostin, integrin β6, L selectin, COMP upregulated whereas desmogling II, NCAM 1, contactin 1 downregulated.

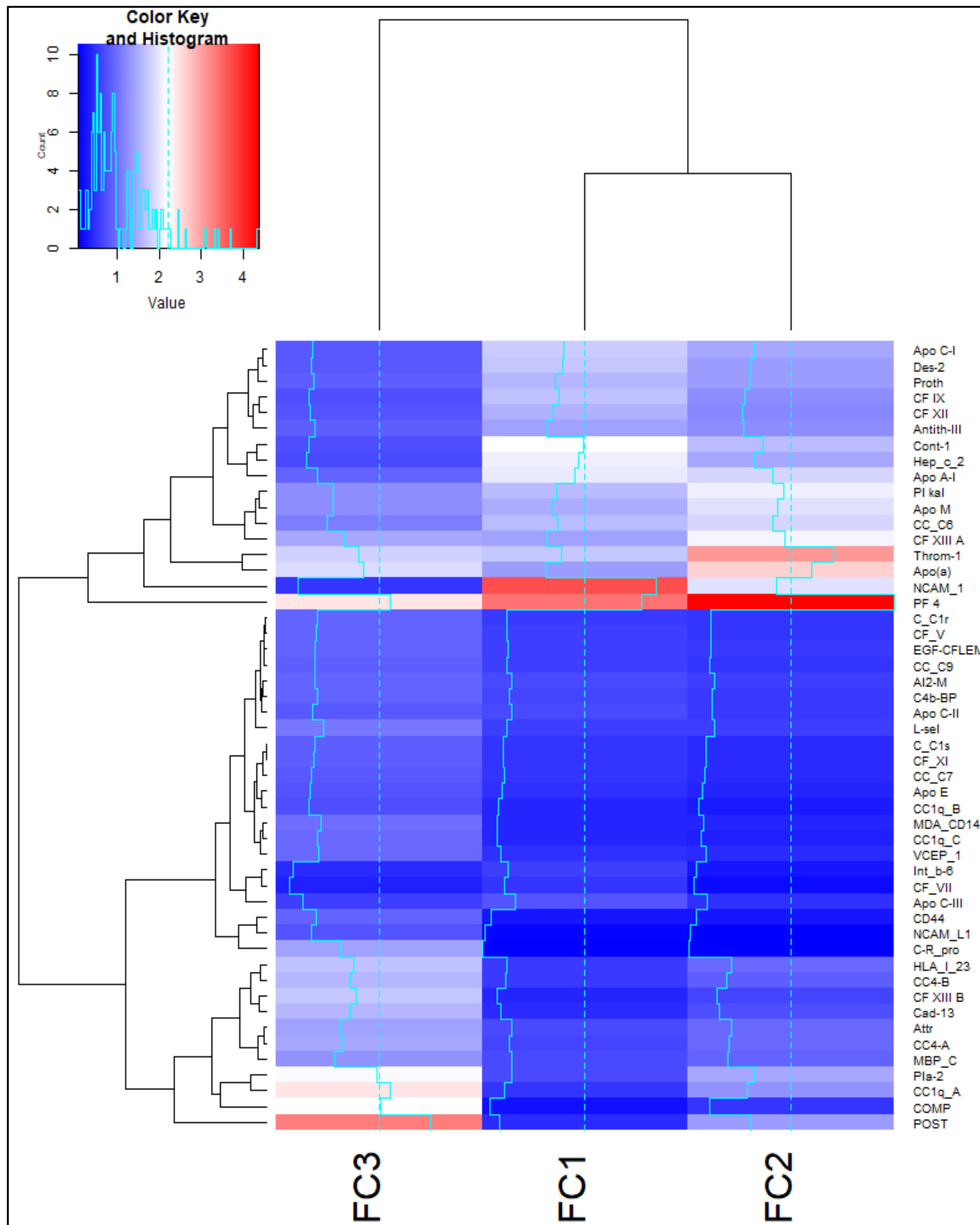


Fig. 36: Heat map of differentially expressed significant proteins obtained by SWATH-MS analysis based on fold change of protein with cluster analysis and histogram using R studio. FC1= Fold changes acute phase vs control, FC2= Fold changes critical phase vs control, FC3= Fold change critical phase vs acute phase of dengue-infected patients

6.4. REACTOME Analysis

The REACTOME database provides curated pathways of biological reactions, molecular events, and functional annotations. REACTOME pathway analysis helps researchers interpret the functional significance of gene sets by associating them with specific biological pathways. This aids in understanding how genes work together to carry out cellular processes. This places individual genes in a broader biological context, revealing their involvement in specific pathways and cellular functions. This context is crucial for understanding the role of genes in normal cellular processes and disease mechanisms. Analysing SWATH-MS annotated genes in the context of REACTOME pathways can provide insights into the molecular mechanisms underlying severe dengue. This is particularly valuable for identifying dysregulated pathways and potential therapeutic targets. This integrated approach provides a more comprehensive understanding of the molecular events in the pathophysiology of severe dengue. Also, a crosstalk between different pathways was observed via this analysis. This is important for understanding how signalling cascades and cellular processes are interconnected, providing a more holistic view of biological regulation. We looked for the enrichment of the Reactome pathways identified from SWATH-MS (**Figure 37**). It appeared that various pathways including programmed cell death, signal transduction, protein localization, extracellular matrix organization, metabolism etc. pathways are enriched with the SWATH-MS proteins. The enriched pathways are depicted in. A similar PANTHER pathway analysis was performed with these sets of proteins as depicted in **Figure 39.**

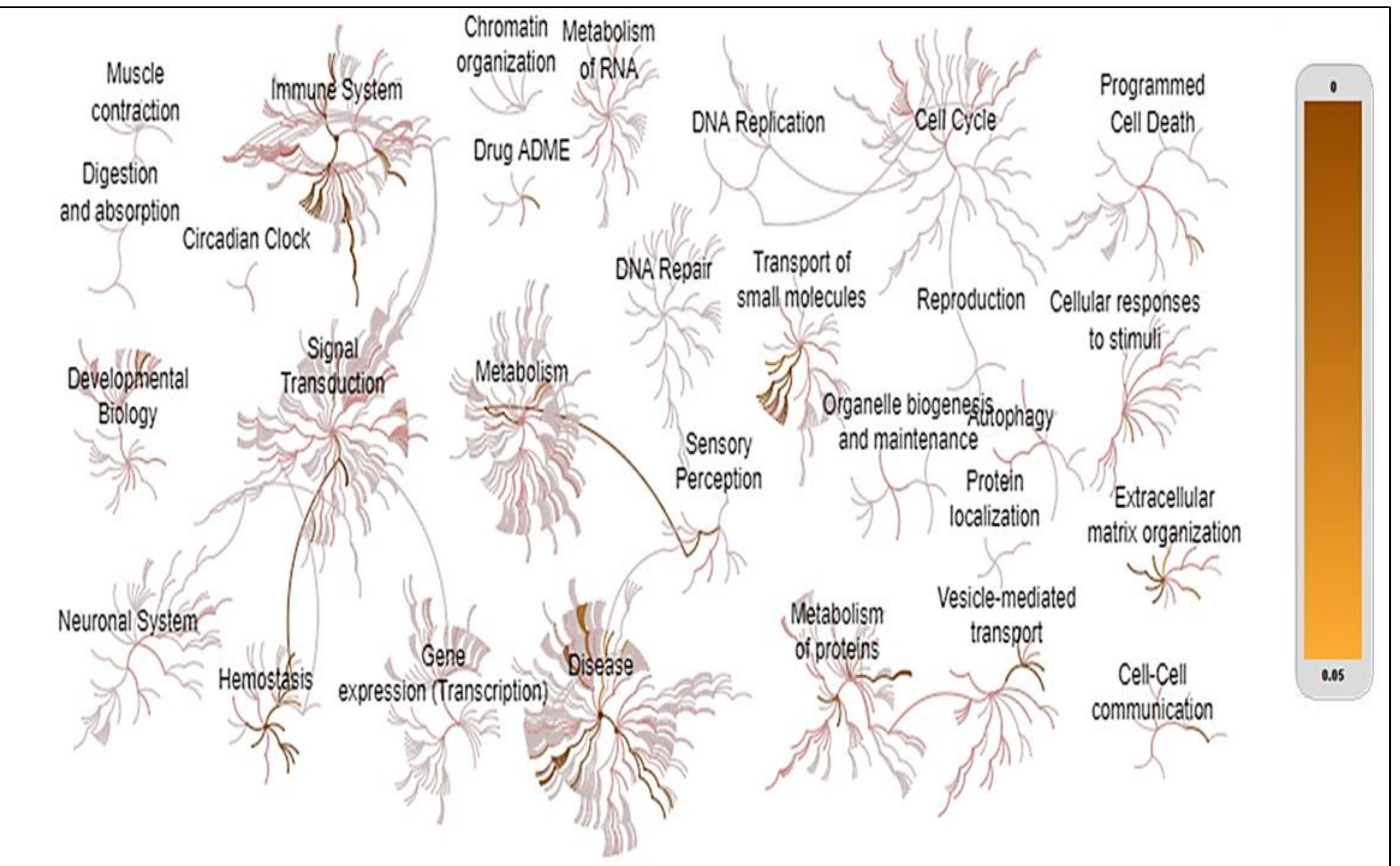


Fig. 37: REACTOME analysis of differentially expressed SWATH -MS proteins, annotating significant biological processes with protein

6.5.The DENV-Human host protein INTERACTOME

We have analyzed the interactions of 10 DENV proteins with their host interactors. These 10 viral proteins were already reported to be interacting with 535 host proteins (164). This protein-protein interaction (PPI) network was depicted with the help of Cytoscape v3.8.2 where the host proteins were represented as blue rectangular edges and the DENV proteins were marked as different coloured rectangular hubs in **Figure 38.**

We have analyzed this PPI network using Gene Ontology software (geneontology.org). In total, 471 Biological Processes (BP) and 58 Molecular Functions (MF) were significantly enriched with these host-interacting partners. In addition, 11 PANTHER and 233 Reactome pathways were enriched significantly with these DENV interacting host proteins. The enriched KEGG pathways include the JAK/STAT signalling pathway (P00038), Interferon-gamma signalling pathway (P00035), Inflammation mediated by chemokine and cytokine signalling pathway (P00031), Apoptosis signalling pathway (P00006), etc. As expected, we observed that the term interferon-gamma signalling pathway is enriched significantly with seven host proteins, viz. PIAS3, PIAS1, STAT1, JAK2, JAK1, IFNGR2, IFNGR1. Upon analysis, 11 GO terms were observed to be significantly enriched with apoptosis-related pathways (GO:0042981, GO:0006915, GO:0008625, GO:0043524, GO:0036462, GO:2001244, GO:1902042, GO:2001243, GO:2000427, GO:0051402, GO:1901216). Six Reactome pathways related to unfolded protein response (UPR) (R-HSA-381119) are enriched with the interacting proteins. Interestingly, we observed enrichment of Autophagy (R-HSA-9612973), chaperone-mediated autophagy (R-HSA-9613829), Late endosomal microautophagy (R-HSA-9615710) and Selective autophagy (R-HSA-9663891) to be significantly coming up with the host interactors. We also observed that IRE1alpha activates chaperones (R-HSA-381070) and HSP90 chaperone cycle for steroid hormone receptors (SHR) (R-HSA-3371497) pathways to be significantly enriched with the host

interactors. Quite unexpectedly, plasma lipoprotein assembly (R-HSA-8963898) and Plasma lipoprotein remodelling (R-HSA-8963899) popped up as the two highly enriched Reactome terms with the host proteins. Interestingly, Angiogenesis (P00005), and Integrin signalling pathway (P00034) were also observed as enriched hubs. Based on the DENV-host PPI network and subsequent enrichment analyses, we tried to focus their contribution on some of the crucial molecular pathophysiology of dengue fever. Analysed pathways enriched with the DENV-host interactome using the PANTHER database(http://www.pantherdb.org/). The enriched pathways include blood coagulation, angiogenesis, apoptosis, cadherin signalling, EGF-receptor signalling, endothelin signalling pathways etc. among others. The representative pie charts of PPI PANTHER pathways are provided in **Figure 39.**

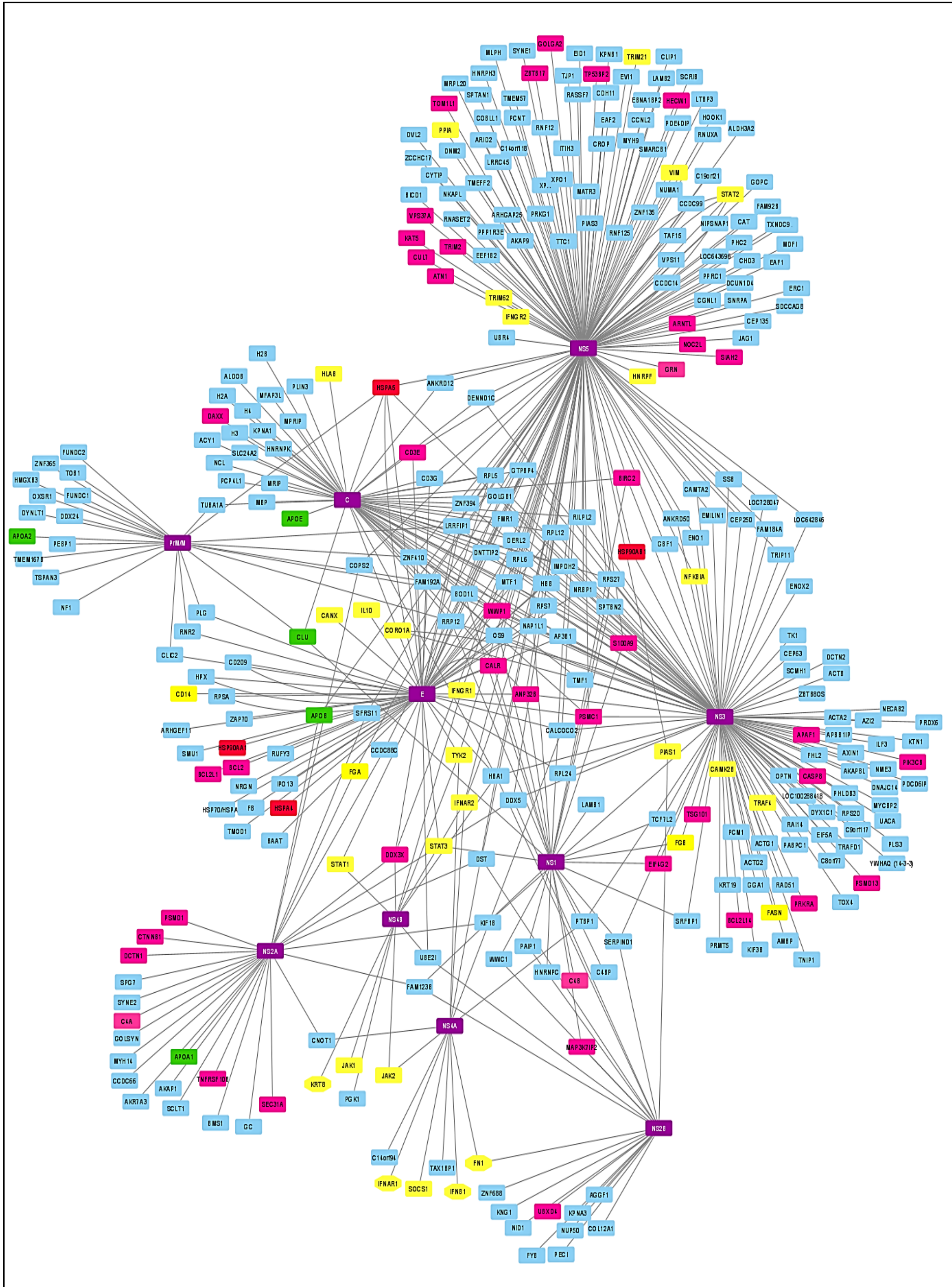


Fig. 38: DENV-Human host interactome Protein-Protein-Interaction (PPI) analysis, Host proteins were represented as blue rectangular edges and the DENV proteins were marked as different coloured rectangular hubs

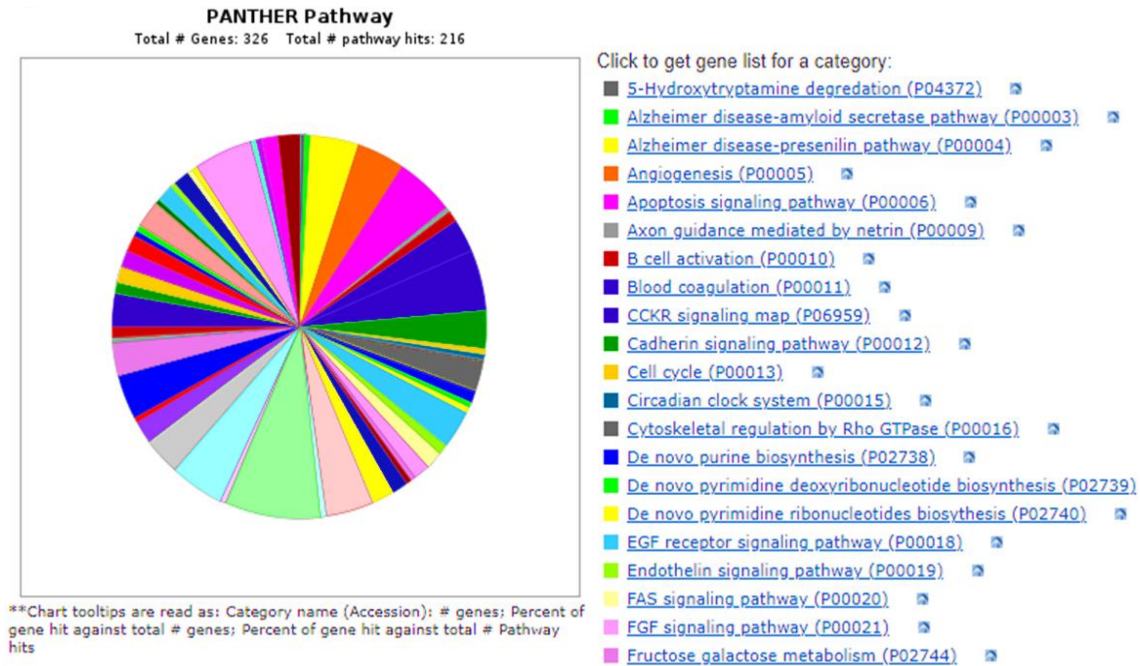


Fig. 39: Pathway analysis of dengue virus and host proteins interactome common proteins

6.6. Overlap between dengue virus-host protein interactome with the differentially expressed proteins as obtained from SWATH-MS

Among the proteins identified using SWATH-MS and the 338 unique host proteins interacting with the DENV proteins, ten proteins were observed to be common as depicted in **Figure 40**. Interestingly, 25 PANTHER pathways were found to be common between the pathways enriched significantly with SWATH proteins and the host protein interactors **Figure 40**. The common proteins and pathways were tabulated in **Tables 10 and 11**. This analysis was performed online (http://www.listdiff.com/) and the result gives us confidence that the differentially expressed proteins obtained from SWATH-MS might have functional relevance toward the dengue disease pathogenesis.

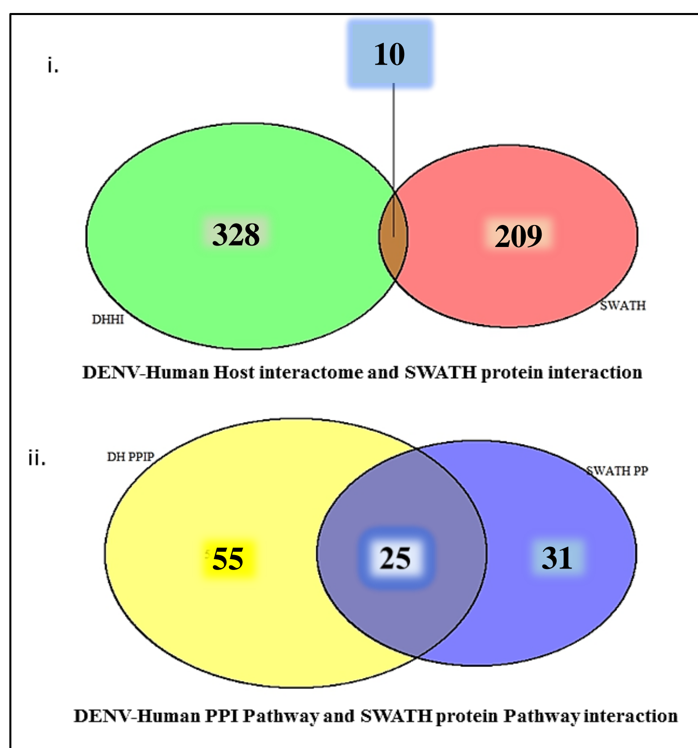


Fig. 40: Protein and pathway overlap interaction via Venn Diagram in R studio, denoting ten common proteins among SWATH enriched proteins and DENV-Human Host interactome whereas 25 common pathways among SWATH protein pathways and DENV-Human Host Protein-Protein Interaction pathways (refer to Tables 10 and 11)

Table 10: Common proteins between SWATH and PPI

PANTHER Pathway ID	Name of Pathway
P00044	Nicotinic acetylcholine receptor signaling pathway
P06959	CCKR signaling map
P00011	Blood coagulation
P00024	Glycolysis
P00054	Toll receptor signaling pathway
P00029	Huntington disease
P00053	T cell activation
P00016	Cytoskeletal regulation by Rho GTPase
P00049	Parkinson disease
P00031	Inflammation mediated by chemokine and cytokine signaling pathway
P06664	Gonadotropin-releasing hormone receptor pathway
P00050	Plasminogen activating cascade
P00005	Angiogenesis
P00012	Cadherin signaling pathway
P00036	Interleukin signaling pathway
P00033	Insulin/IGF pathway-protein kinase B signaling cascade

P00010	B cell activation
P00057	Wnt signaling pathway
P04396	Vitamin D metabolism and pathway
P00020	FAS signaling pathway
P00004	Alzheimer's disease-presenilin pathway
P00059	p53 pathway
P00006	Apoptosis signaling pathway
P00034	Integrin signalling pathway
P02744	Fructose galactose metabolism

Table 11: Common proteins between pathways enriched significantly via. PANTHER analysis and SWATH proteins and the host protein interactions

Common Protein	Associated Pathway & mechanisms
ALDOB	Glycolysis (BioCyc) and Fructose metabolism, Gluconeogenesis
AMBP	Binding and Uptake of Ligands by Scavenger Receptors and Vesicle-mediated transport, Scavenging of heme from plasma
APOA1	Platelet degranulation, ABC transport as lipid homeostasis, PPARA activates gene expression, scavenging of heme from plasma, scavenging by class b receptors
APOA2	PPARA activates gene expression, regulation of insulin-like GF, transport and uptake by insulin-like GF binding protein, post-translational protein phosphorylation, chylomicron assembly, chylomicron remodelling, retinoid metabolism and transport
APOB	Cell surface interactions, vascular wall, scavenging by class B, A, F and H receptors
APOE	Nuclear signalling by ERBB 4, Scavenging by class A receptors, regulation of insulin-like GF, transport and uptake by Insulin like BP, Transcriptional regulation by AP 2 family of transcription factor, PTM protein phosphorylation
CD14	Erythrocyte phagosome pathway, caspase activation via death receptor in the presence of ligand, TLR 4 cascade, transfer of LPS from LBB carrier CD14
HBB	Erythrocytes take up O2 and release CO2, Scavenging heme from plasma, neutrophil degranulation, chaperon-mediated autophagy,
ITIH3	Platelet degranulation and pre-apoptosis pathways
KNG1	Platelet degranulation, Extrinsic pathway of fibrin clot formation, peptide ligand-binding receptors, G- alpha (q) signalling events, regulation of insulin like GF transport and uptake by insulin like GF binding proteins

6.7. Protein Array

Several proteins were found to be upregulated in severe dengue patients on the day of the transition from dengue fever to severe dengue. Cytokines such as CCL5 (CC-chemokine ligand 5), RANTES (regulated upon activation, normal T cell expressed and secreted), ICAM1 (intercellular adhesion molecule1), CD54 (Cluster of Differentiation 54), SERPIN E1, PA1 (plasminogen activator inhibitor 1), CXCL 10 (CXC chemokine ligand 10) and IP 10 (interferon-gamma inducible protein 10) were found to be significantly upregulated **(Figure 41.a**). Whereas among angiogenesis proteins angiogenin, DPP4 (Dipeptidyl peptidase 4), endostatin, collagen 18, IGFBP1 (insulin like growth factor binding protein 1), IGFBP2, IGFBP3, MMP8 (matrix metalloproteinase), MMP9, PTX 3 (Pentraxin 3), PDGFAA (Platelet-derived growth factor), TIMP 1 (Tissue inhibitors of metalloproteinases), TIMP 4 and thrombospondin were found to be significantly upregulated **(Figure 41.b**). A 20 to 80-fold overexpression of the number of proteins mediating angiogenesis and cytokine pathways was observed among critical patients. From this multiapproach analyses starting from qualitative proteomic to quantitative proteomic to protein array to dengue virus and host protein INTERACTOME analysis, we have narrowed down and identified a panel of proteins associated with severe dengue pathophysiology **(Figure 42**).

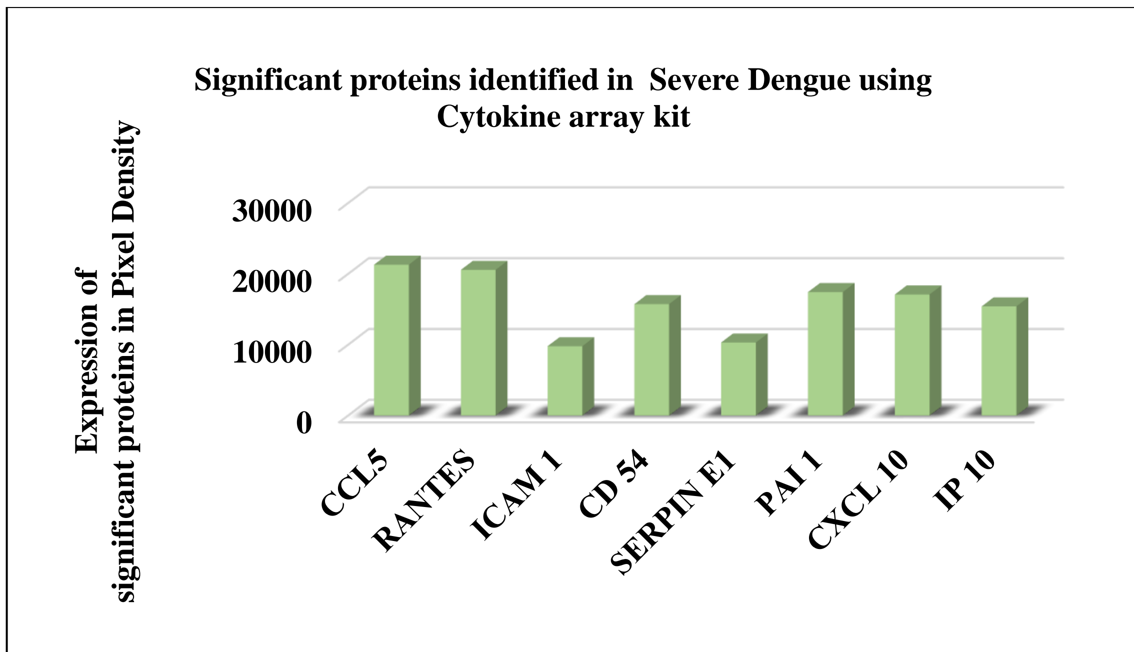


Fig. 41.a: Differentially expressed array of proteins among critical dengue death patients using cytokine proteome array kit, showing significant surge of cytokine proteins during their transition period (critical phase A)

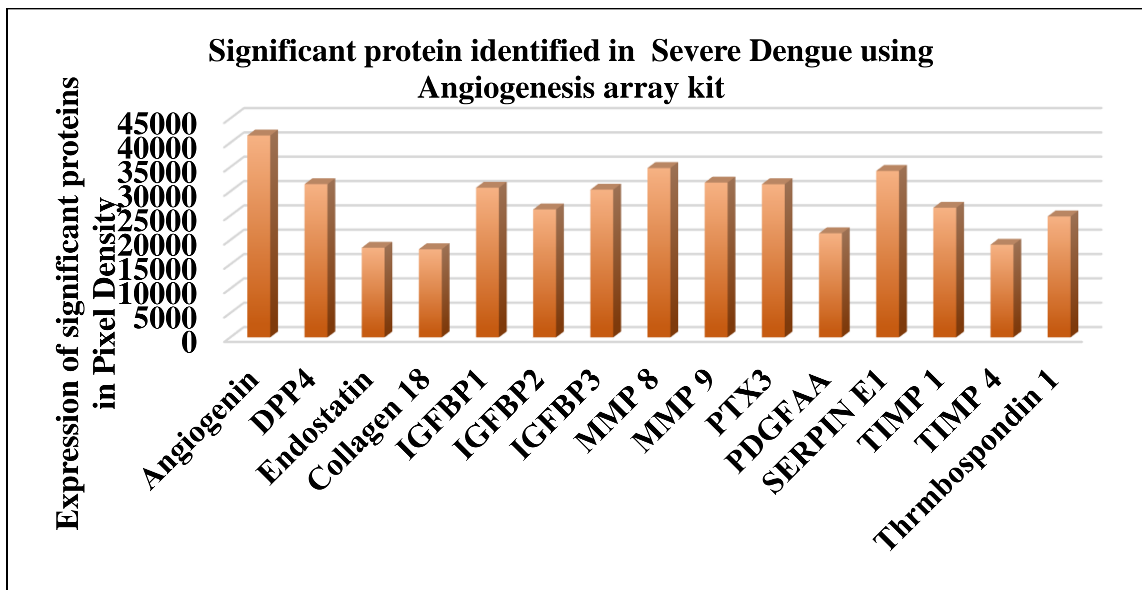


Fig. 41.b: Differentially expressed array of proteins among critical dengue death patients using angiogenesis proteome array kit, showing significant surge of angiogenesis proteins during their transition period (critical phase A)

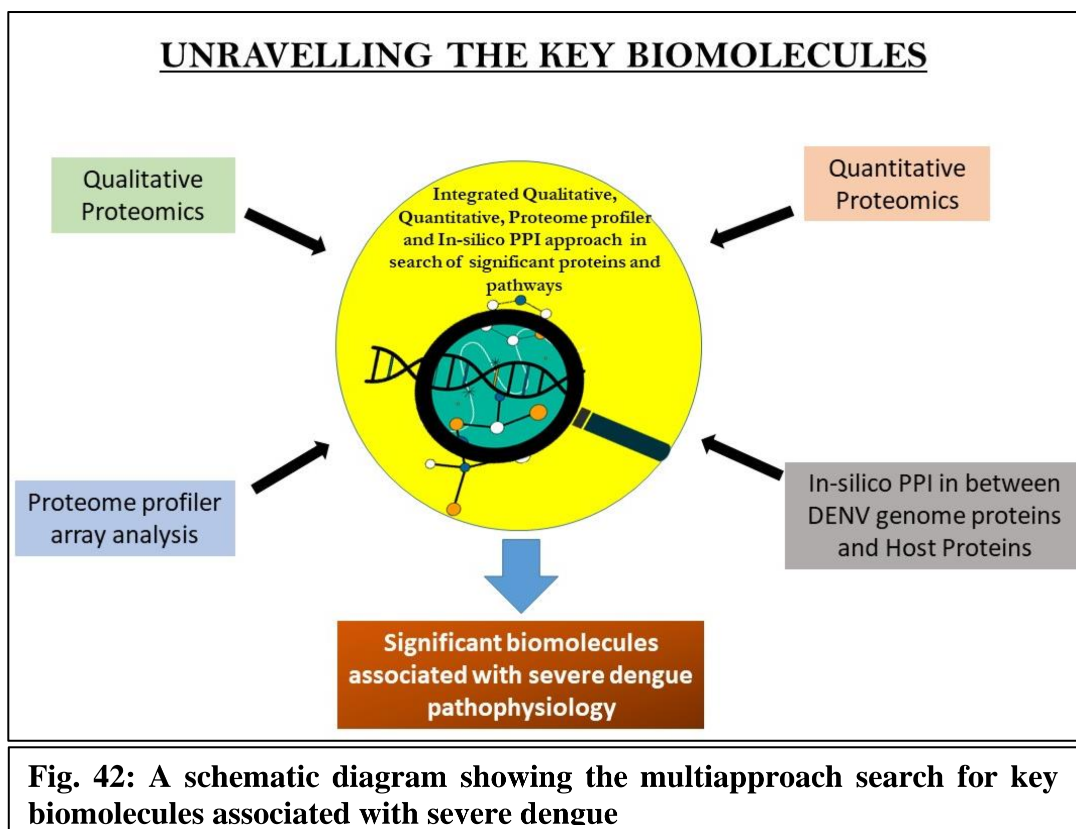


Fig. 42: A schematic diagram showing the multiapproach search for key biomolecules associated with severe dengue

6.8. Validation of protein expressions identified from SWATH-MS and Orbitrap

Identified proteins/genes were subjected to validation in two parts, initially, we did Western blot validation of some significant proteins and in another part, a different set of genes were validated using Real-Time PCR and ELISA-based techniques. We validated some of the protein expressions as obtained from SWATH-MS using a Western blot. As evident from proteomics data, an array of apolipoproteins, cytokines and endothelial proteins revealed differential expressions using SWATH-MS in Dengue patients as compared to their healthy counterparts. We validated expression patterns of Apo AI, AII, AIV, ApoB and ApoE among the apolipoproteins and E-Cadherin (epithelial cadherin), VEGF (Vascular endothelial growth factor), FGFR1 (fibroblast growth factor receptor 1), VCAM1 (Vascular cell adhesion molecule 1), IRF3 (Interferon regulatory factor 3), IFN-γ (Interferon-gamma) and ANGPT1 (Angiopoietin 1) among the endothelial proteins and cytokines from the list. The representative Western blot images and the corresponding bar

diagrams of the Apolipoproteins, endothelial proteins and cytokines protein expressions are provided in **Figure 43.a** and **Figure 43.b**. A detailed role of validated Apolipoproteins endothelial proteins and cytokines protein in severe dengue is described in **Table 12** and **Table 13**.

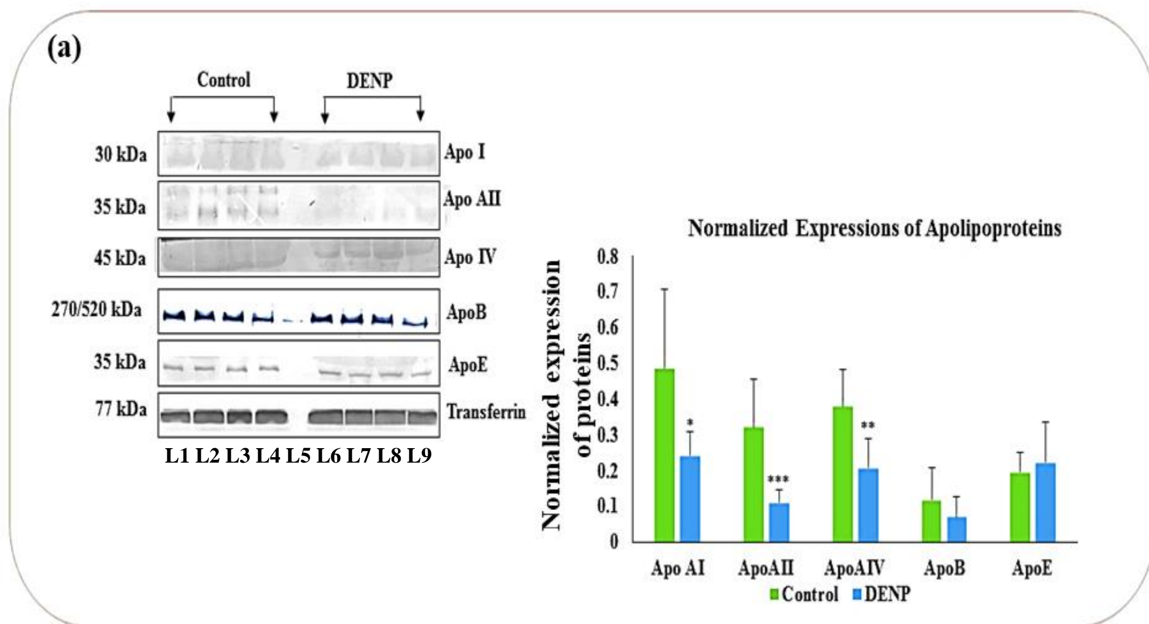


Fig. 43.a: Validated expression patterns of Apo AI, AII, AIV, ApoB and ApoE among the apolipoproteins identified from the SWATH-MS and Orbitrap-MS data. n ≥ 3 for all data sets and significance was calculated using Student's unpaired t-test. *p ≤ .05p ≤ .01, ***p ≤ .001. Lane L1 to L4: Healthy control samples, L6 to L9: Dengue patient samples from critical phase**

Table 12: Role of Apolipoprotein in severe dengue

Sl. No.	Apo lipoproteins Name	Role in severe dengue
1.	**Apo I**	Play a multifaceted role in severe dengue by exerting anti-inflammatory, antioxidant, influence on the entry of the dengue virus into host cells and its subsequent replication and endothelial-protective effects
2.	**Apo II**	Play with roles in lipid metabolism, endothelial protection, and immune modulation. Its protective effects on the endothelium and its ability to modulate inflammation are particularly relevant in severe dengue

3.	Apo IV	Play a multifaceted role in severe dengue, involving anti-inflammatory, influence the entry of the dengue virus into host cells, antioxidative, vascular protective, and immune-modulating functions
4.	Apo B	Role in severe dengue through its involvement in lipid metabolism, endothelial dysfunction, immune response modulation, and coagulation pathways. Alterations in Apo B levels and lipid profiles during dengue infection can contribute to disease pathogenesis and severity
5.	Apo E	ApoE plays a role in modulating the immune response, influence the entry of the dengue virus into host cells and its subsequent replication, Lipid Metabolism and Inflammation, Genetic Polymorphisms and maintain endothelial cell integrity

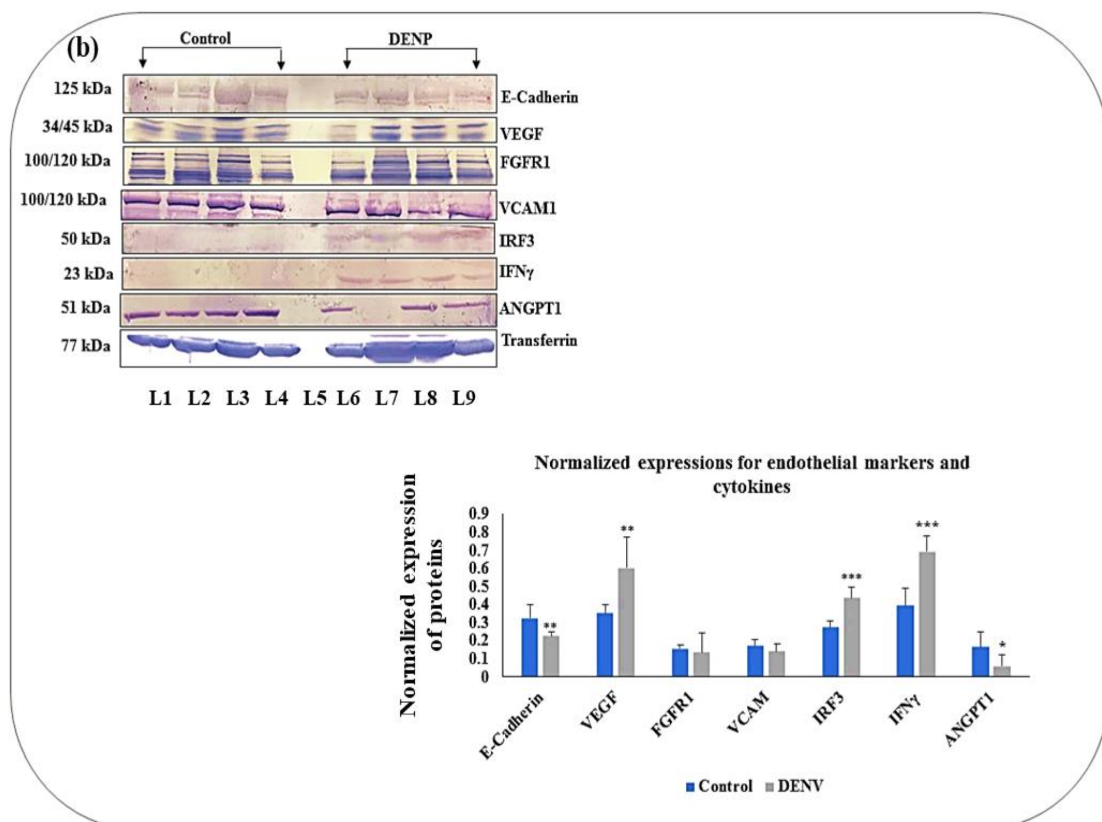


Fig. 43.b: Validated expression patterns of E-Cadherin, VEGF, FGFR1, VCAM1, IRF3, IFN-γ and ANGPT1 among the endothelial proteins and cytokines identified from the SWATH-MS and Orbitrap-MS data. n ≥ 3 for all data sets and significance was calculated using Student's unpaired t-test. *p ≤ .05, **p ≤ .01, ***p ≤ .001. Lane L1 to L4: Healthy control samples, L6 to L9: Dengue patient samples from critical phase

Table 13: Role of selected proteins in severe dengue

Sl. No.	Protein Name	Role in severe dengue
1.	**E Cad**	Disruption of E-Cadherin-mediated cell-cell adhesion contributes to endothelial dysfunction and increased vascular permeability, leading to plasma leakage and organ dysfunction
2.	**VEGF**	A key regulator of angiogenesis and vascular permeability. In severe dengue, elevated levels of VEGF contribute to increased vascular permeability
3.	**FGFR1**	Involved in endothelial cell proliferation and angiogenesis. In severe dengue, FGFR1 signaling may contribute to vascular remodeling and endothelial dysfunction
4.	**V cam**	An adhesion molecule expressed on endothelial cells that facilitates leukocyte adhesion and migration. Increased expression of VCAM-1 during severe dengue contributes to immune cell infiltration into tissues
5.	**IRF 3**	A transcription factor involved in the innate immune response. Activation of IRF3 signaling in response to dengue virus infection leads to the production of type I interferons (IFNs), which play a crucial role in antiviral defense
6.	**IFN-γ**	IFN-γ is a pro-inflammatory cytokine produced by activated T cells and natural killer (NK) cells. Elevated levels of IFN-γ have been observed in severe dengue and are associated with the cytokine storm phenomenon
7.	**Angiopoietin**	Angiogenic growth factors that regulate vascular stability and permeability. Dysregulation of angiopoietin-Tie2 signaling during severe dengue can lead to endothelial dysfunction

The integrated analysis of SWATH MS and Proteome array also leads us to a panel of proteins, associated with severe dengue pathophysiology. The sampling procedure for real-time-based validation is depicted in **Figure 44**. Real-time PCR results from paired Group I (pairwise acute) and Group II (pairwise critical), of selected genes are shown in **Figure 46**, with their upregulation and downregulation. After narrowing down candidate biomolecules (**Figure 46**) based on the Real-Time result group of six proteins i.e. PTX3 (Pentraxin 3), LBP (Lipopolysaccharide Binding Protein), Fibronectin, IGFBP2 (Insulin-like Growth Factor Binding Protein-2), POST (periostin), and Serpin were selected for validation via ELISA in search of our top-notch proteins. Patient sampling for validation purposes is depicted in **Table 14** and their detailed clinical parameters which includes

platelet pattern, ALT, AST, Albumin and Total Protein are provided in **Figure 45.a to 45.e.** Phase-specific change in expression (upregulation) of the targeted biomolecules along with Logistic Regression Analysis ROC curves are shown in **Figure 47.a to 47.f.** Also, the role of selected candidate proteins for Real-Time PCR in severe dengue is listed in **Table 15**.

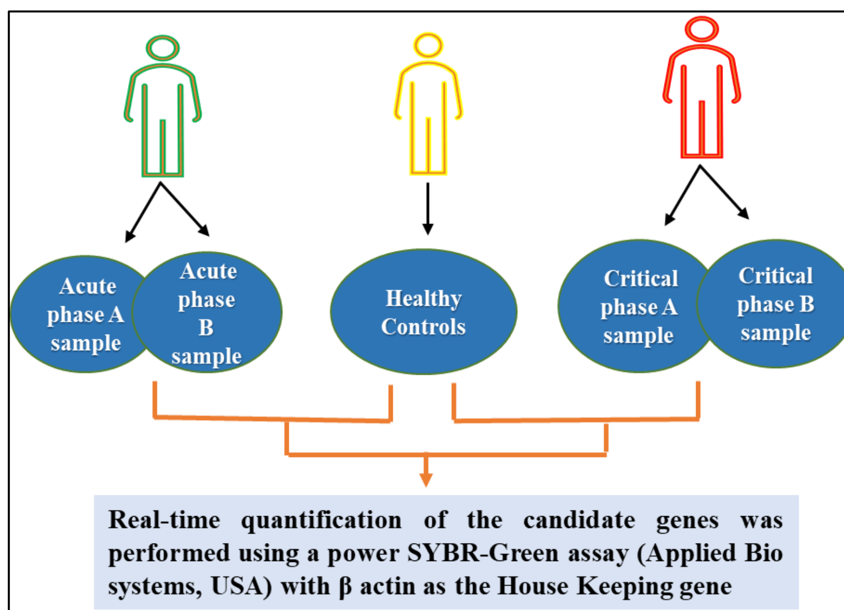


Fig. 44: Visual representation of sampling procedure for Real Time PCR quantification

Table 14: Study population details for sample validation

	ACUTE PATIENTS	**CCU PATIENTS**
Study Population	24 Paired (24×2=48)	19 Paired (19×2=38)
No. of males (number, percentage)	11(46%)	8(42%)
No. of Female (number, percentage)	13(54%)	11(58%)
Age (year, median value)	30.5	45
Days of Fever (median value)	5	10.5
Healthy Control (N)=6		
Total Population (N)=92		

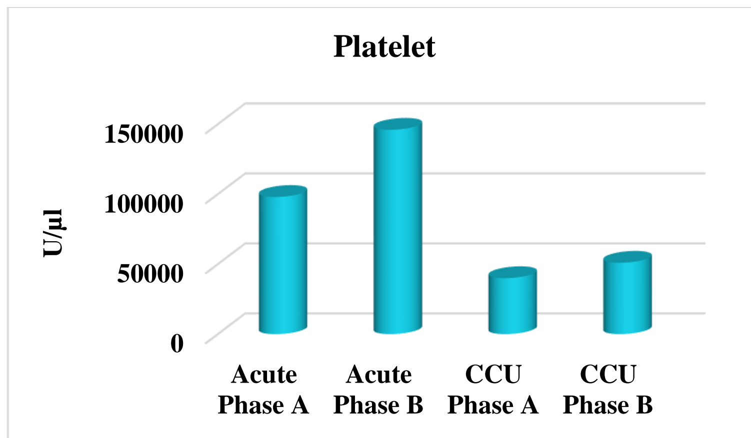


Fig. 45.a

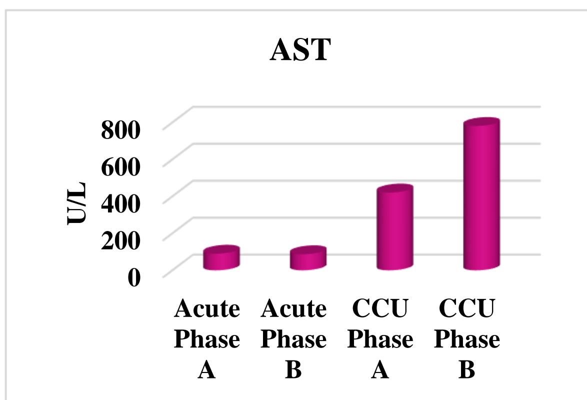


Fig. 45.b

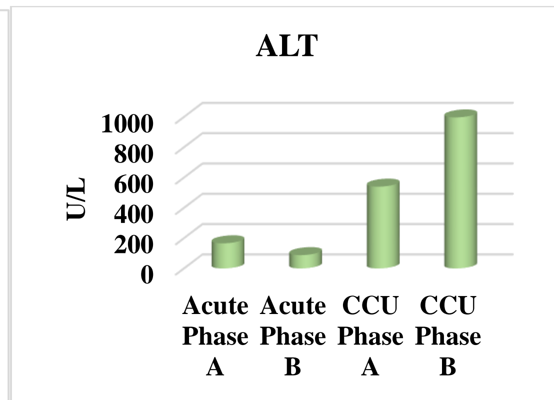


Fig. 45.c

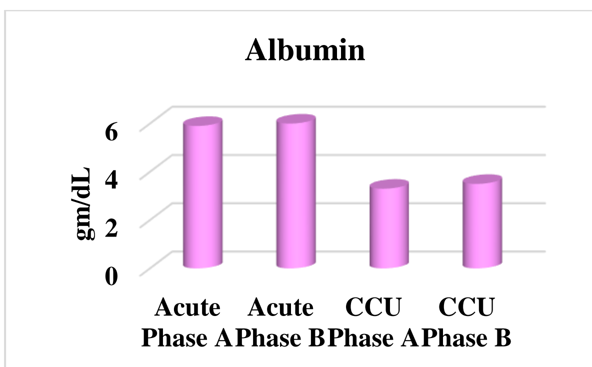


Fig. 45.d

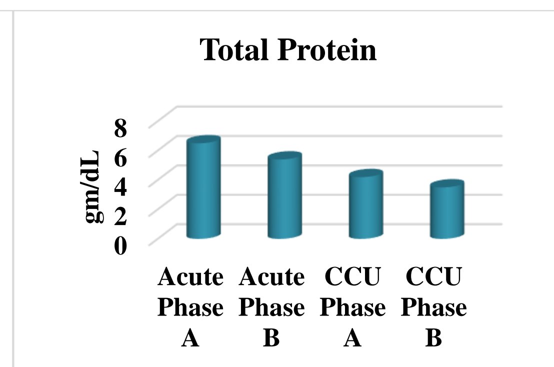


Fig. 45.e

Fig. 45.a to 45.e: Platelet and Liver profile of hospital admitted patients in acute and critical phases at two time points, selected for biomolecules validation

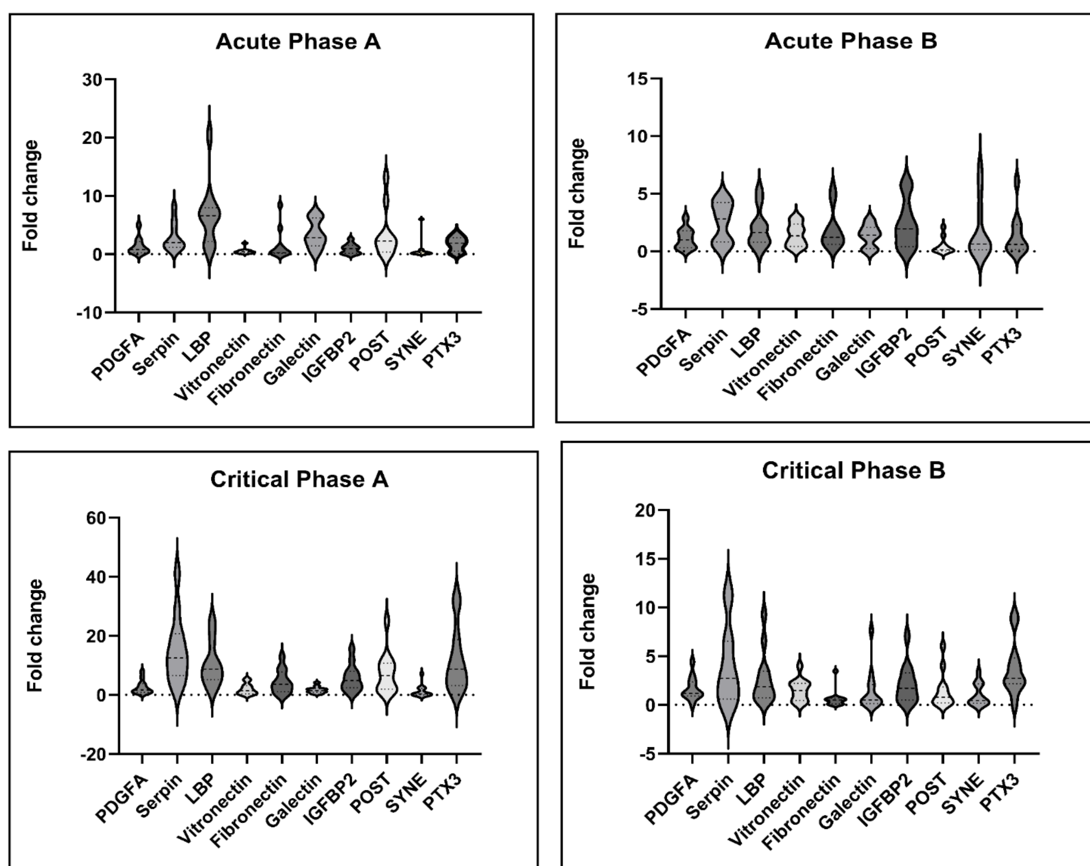
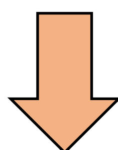


Fig. 46: Real-Time PCR-based quantitative analysis of a selected panel of genes showing phase-specific changes in acute and critical phases at two time points

Table 15: Role of selected candidated proteins in severe dengue

Sl. No.	Protein Name	Role in severe dengue
1.	**Fibronectin**	Fibronectin is involved in cell adhesion and migration, and its increased levels have been associated with endothelial cell dysfunction
2.	**Angiogenin**	Angiogenin is a potent angiogenic factor that promotes blood vessel formation. In severe dengue, dysregulated angiogenesis may contribute to vascular permeability and endothelial dysfunction
3.	**Periostin**	Periostin is involved in tissue repair and remodelling. Its expression has been linked to fibrosis, a process that can occur in severe dengue, particularly in organs such as the liver
4.	**Serpin E1**	Serpin E1 is a regulator of fibrinolysis and can promote thrombosis when dysregulated. Elevated levels of Serpin E1 have been associated with coagulopathy and increased risk of bleeding in severe dengue cases
5.	**PTX3**	Pentraxin 3 (PTX3) is an acute-phase protein involved in the innate immune response. It plays a role in inflammation and tissue remodeling and may

		contribute to the immune dysregulation seen in severe dengue.
6.	**IGFBP 2**	Growth factor involved in cell proliferation and survival. Its dysregulation may contribute to tissue damage and organ dysfunction in severe dengue.
7.	**SYNE2**	Protein is involved in cell structure and function. Its role in severe dengue is not well-defined but may be related to cellular responses to infection and inflammation
8.	**PDGFA**	Growth factor involved in cell proliferation and migration. Its dysregulation may contribute to vascular dysfunction and tissue damage in severe dengue.
9.	**LBP**	Involved in the immune response to bacterial infections. Its role in severe dengue may relate to immune activation and inflammatory processes.
10.	**Vitronectin**	Involved in cell adhesion and complement regulation. Its dysregulation may contribute to endothelial dysfunction and immune activation in severe dengue.



Sl. No.	Target Protein Selected for ELISA-based validation
1.	Fibronectin
2.	Periostin
3.	Serpin E1
4.	PTX3
5.	IGFBP2
6.	LBP

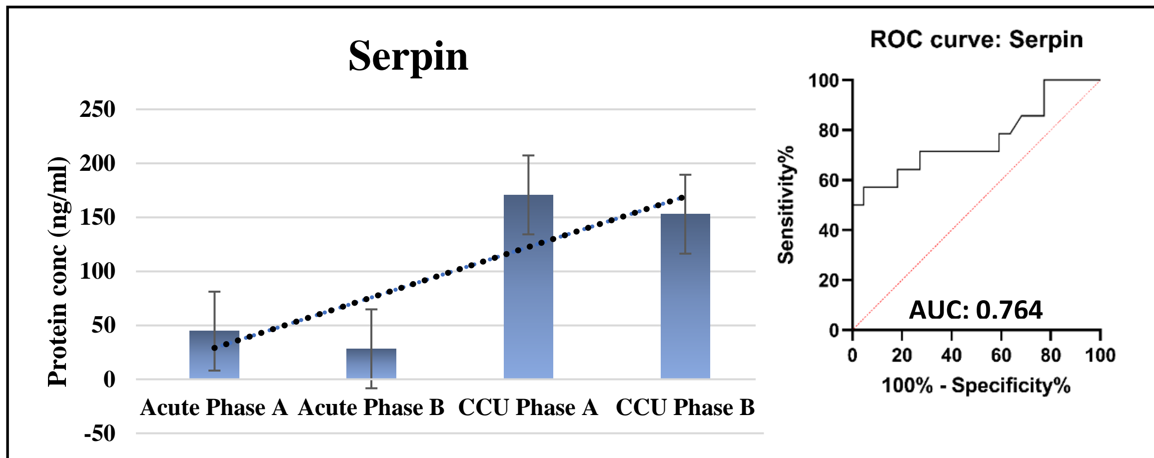


Fig. 47.a: Phase-specific upregulation of targeted proteins Serpin in early and late critical phases with their ROC curve (comparing critical vs acute values) AUC values showing the clinical utility of selected biomolecules as a candidate protein for severe dengue identification

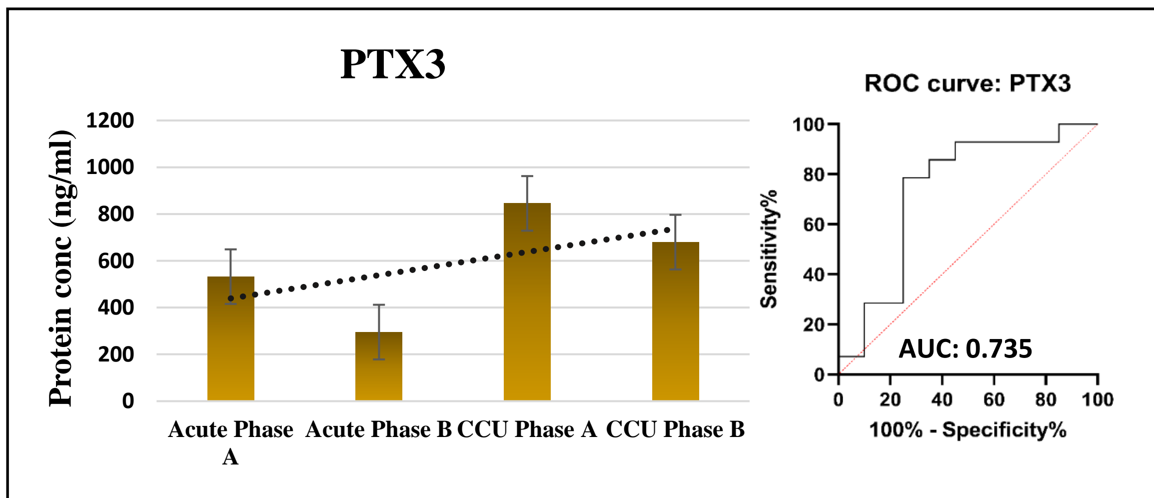


Fig. 47.b: Phase-specific upregulation of targeted proteins PTX3 in early and late critical phases with their ROC curve (comparing critical vs acute values) AUC values showing the clinical utility of selected biomolecules as a candidate protein for severe dengue identification

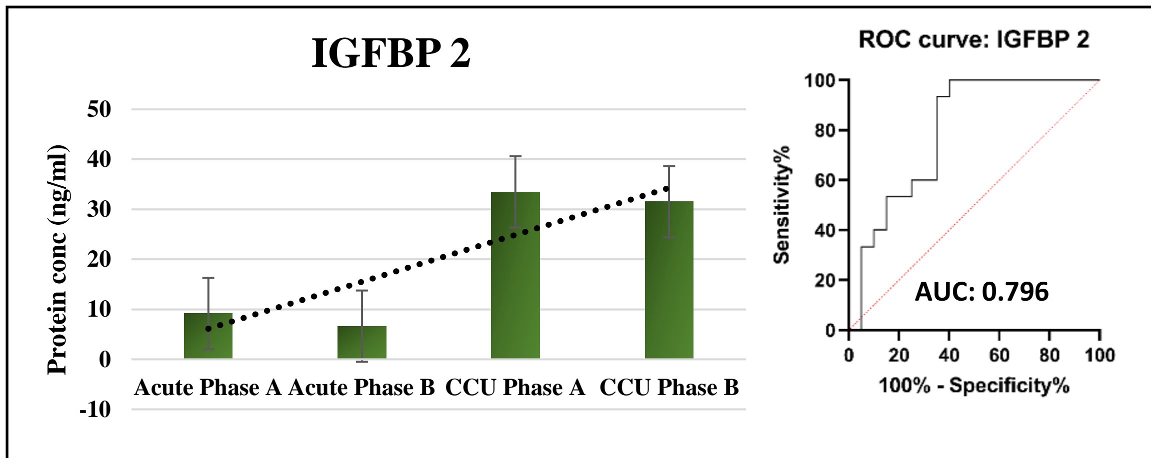


Fig. 47.c: Phase-specific upregulation of targeted proteins in early and late critical phases with their ROC curve (comparing critical vs acute values) AUC values showing the clinical utility of selected biomolecules as a candidate protein for severe dengue identification

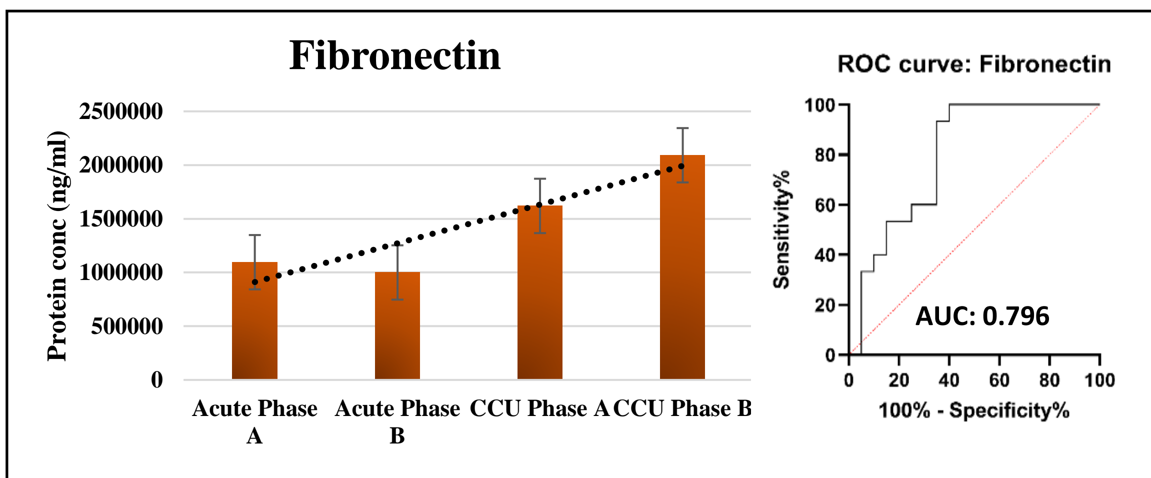


Fig. 47.d: Phase-specific upregulation of targeted proteins in early and late critical phases with their ROC curve (comparing critical vs acute values) AUC values showing the clinical utility of selected biomolecules as a candidate protein for severe dengue identification

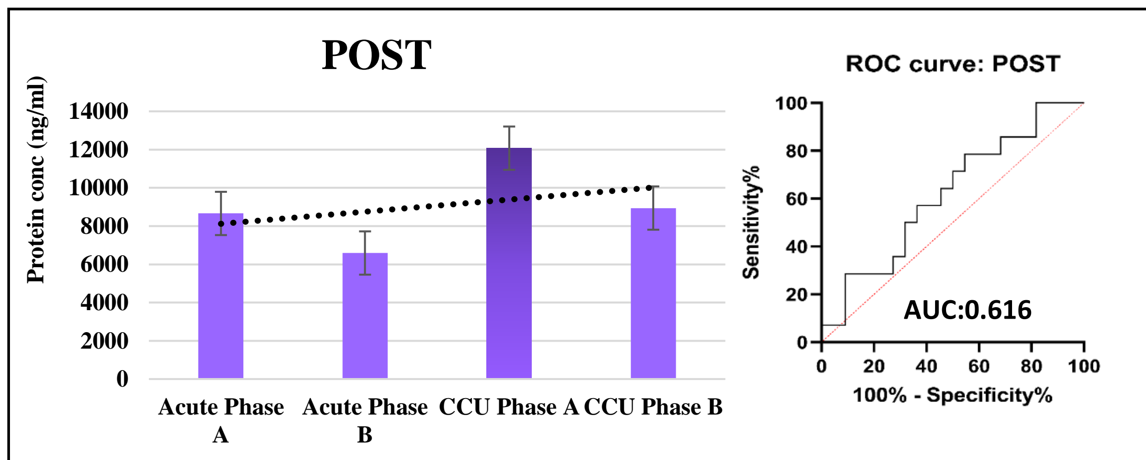


Fig. 47.e: Phase-specific upregulation of targeted proteins in early and late critical phases with their ROC curve (comparing critical vs acute values) AUC values showing the clinical utility of selected biomolecules as a candidate protein for severe dengue identification

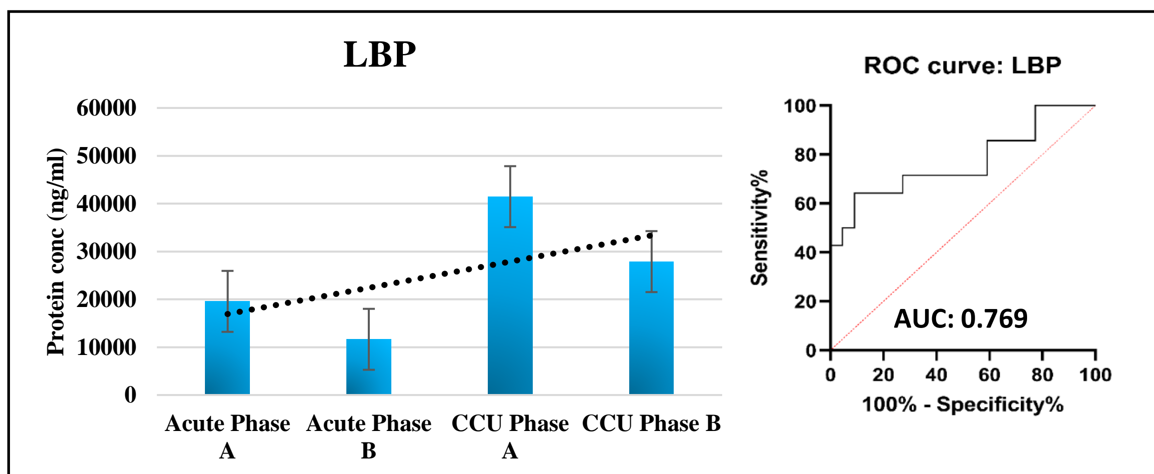


Fig. 47.f: Phase-specific upregulation of targeted proteins in early and late critical phases with their ROC curve (comparing critical vs acute values) AUC values showing the clinical utility of selected biomolecules as a candidate protein for severe dengue identification

6.9. Protein - Protein Docking

Dengue virus NS1 protein is found to be interacting with our candidate ELISA proteins. Considering the protein-specific role of candidate proteins in biological cascades of severe dengue pathophysiology, they docked with viral NS1 protein and sorted out based on their

RMSD (Root Mean Square Deviation) values. Low RMSD values represent great models and hence interaction between NS1 with proteins PTX3 (0.7+/-0.5), Serpin (2.2+/-1.3), LBP (0.4+/-0.2) and IGFBP2 (6.7+/-0.1) is of great importance **Figure 48**.

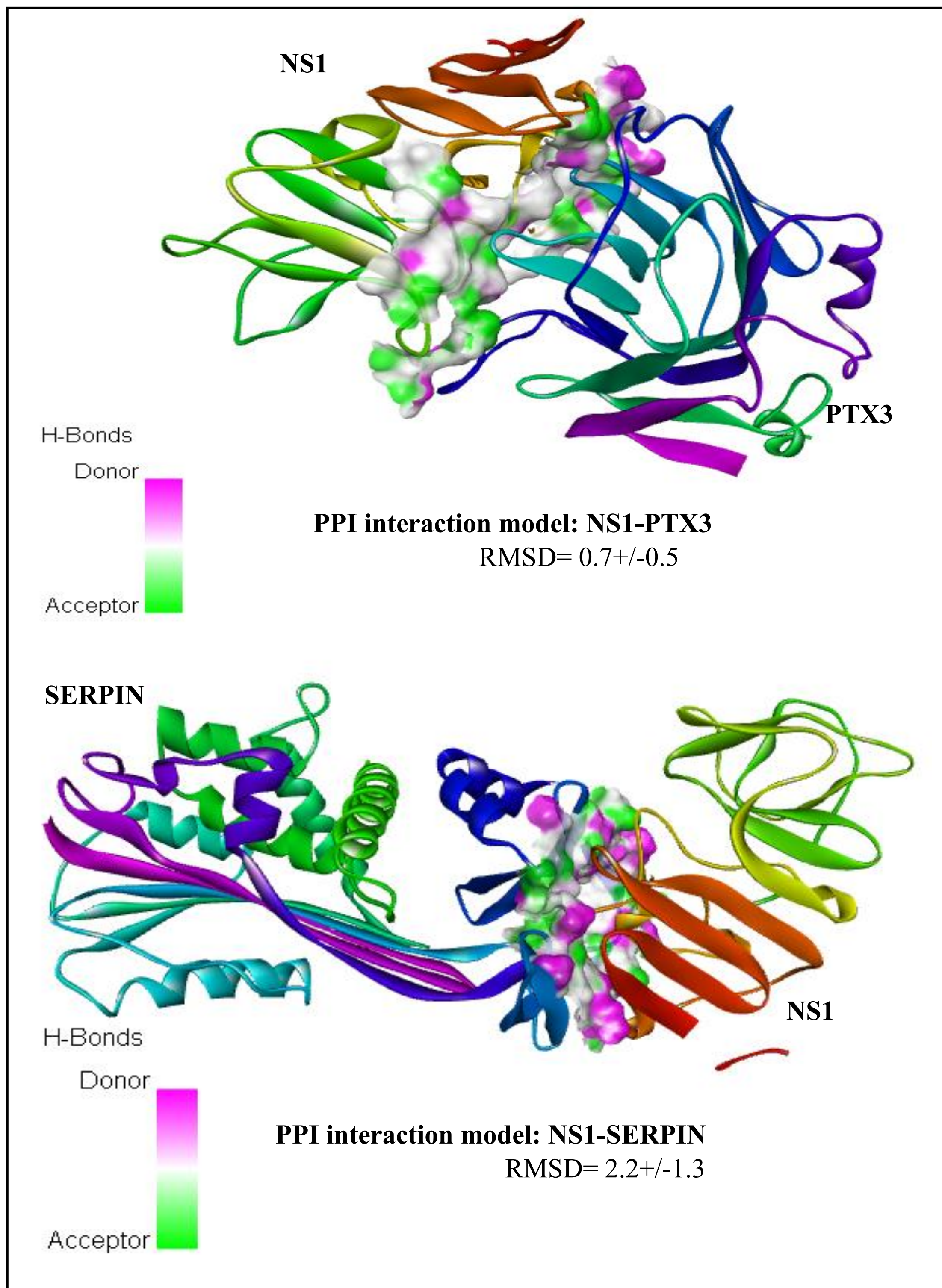


Fig. 48 Cont.

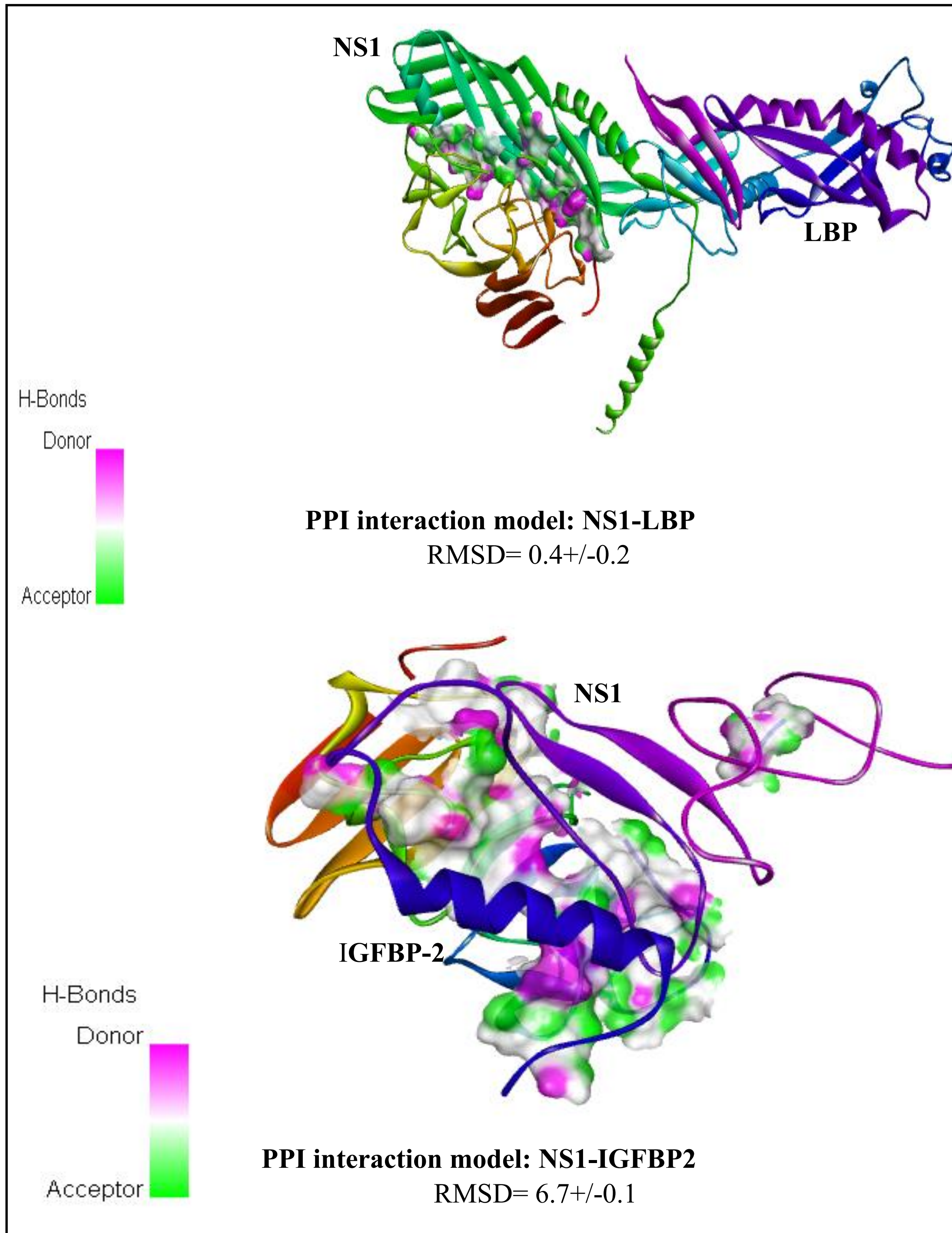
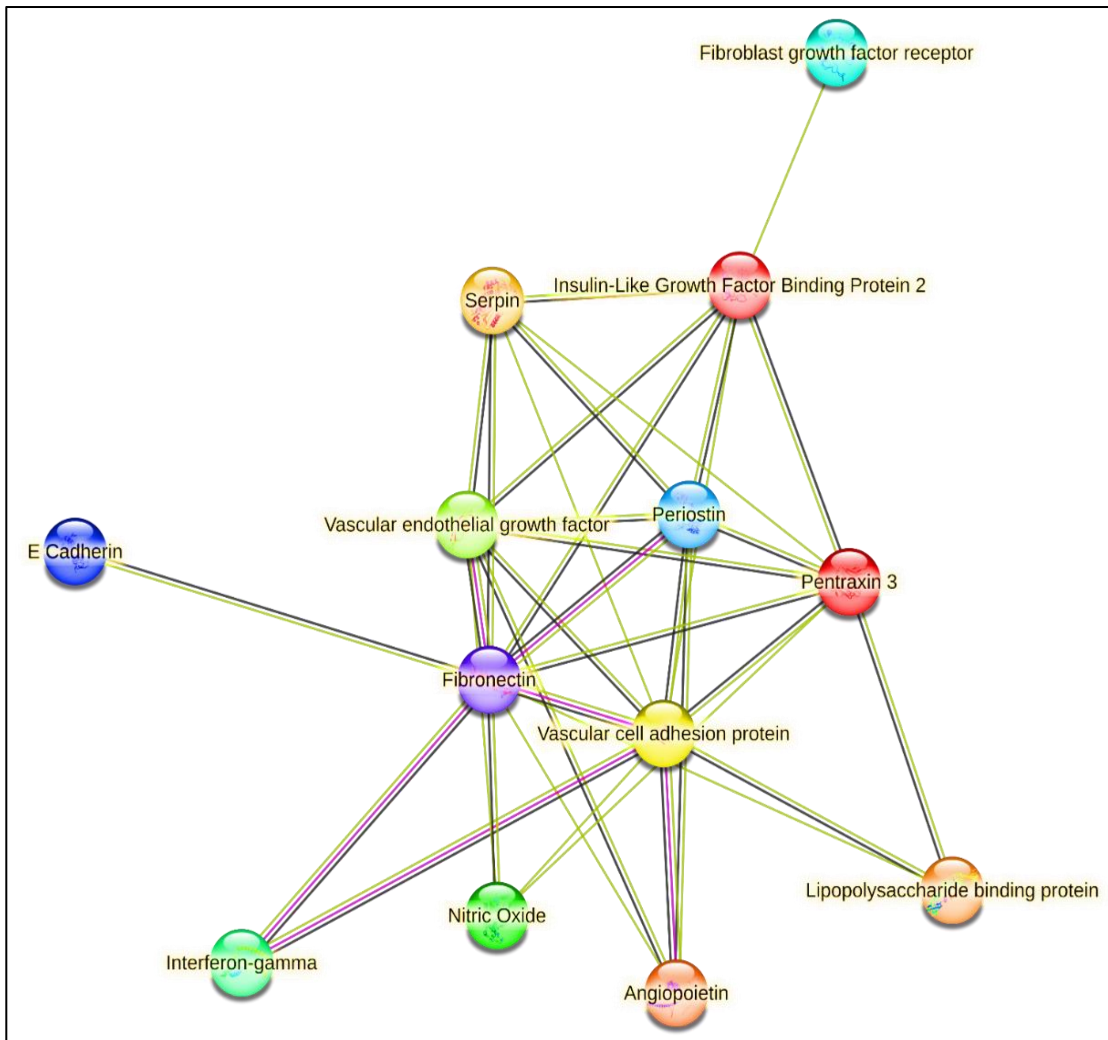


Fig. 48: Protein-Protein Interaction (PPI) model performed among viral NS1 protein and host PTX3, Serpin, LBP and IGFBP-2 proteins via docking with their significant RMSD values

6.10. Functional proteins association network

Validated genes in this study such as E Cadherin, Vascular endothelial growth factor, Fibroblast growth factor receptor, Vascular cell adhesion protein, Interferon-gamma, Fibronectin, Insulin-Like Growth Factor Binding Protein 2, Serpin, Periostin, Pentraxin 3, Angiopoietin, Lipopolysaccharide binding protein are found to be significantly interacting with nitric oxide synthase endothelial (NO) in functional modelling and also in K mean modelling. Various colours of edges show different types of interaction among these proteins as explained in **Figure 49.a**. Also, various colour of clusters showing the clustering pattern in K mean model where the minimum clustering number was set to 3 (**Figure 49.b**). In order to maintain vascular homeostasis, nitric oxide (NO) is essential for controlling blood vessel tone, suppressing platelet aggregation, and preventing leukocyte adhesion. Endothelial dysfunction and NO depletion are important pathogenic factors that contribute to the severity of dengue. Star protein of this doctoral study in direct association with severe dengue pathophysiology listed in **Figure 50** with their upregulation and downregulation pattern.



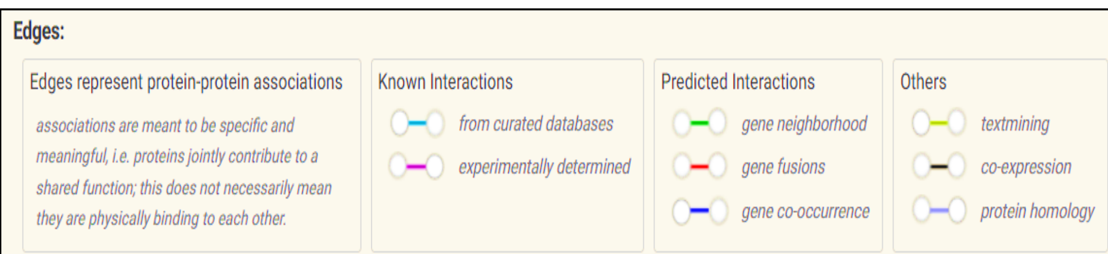
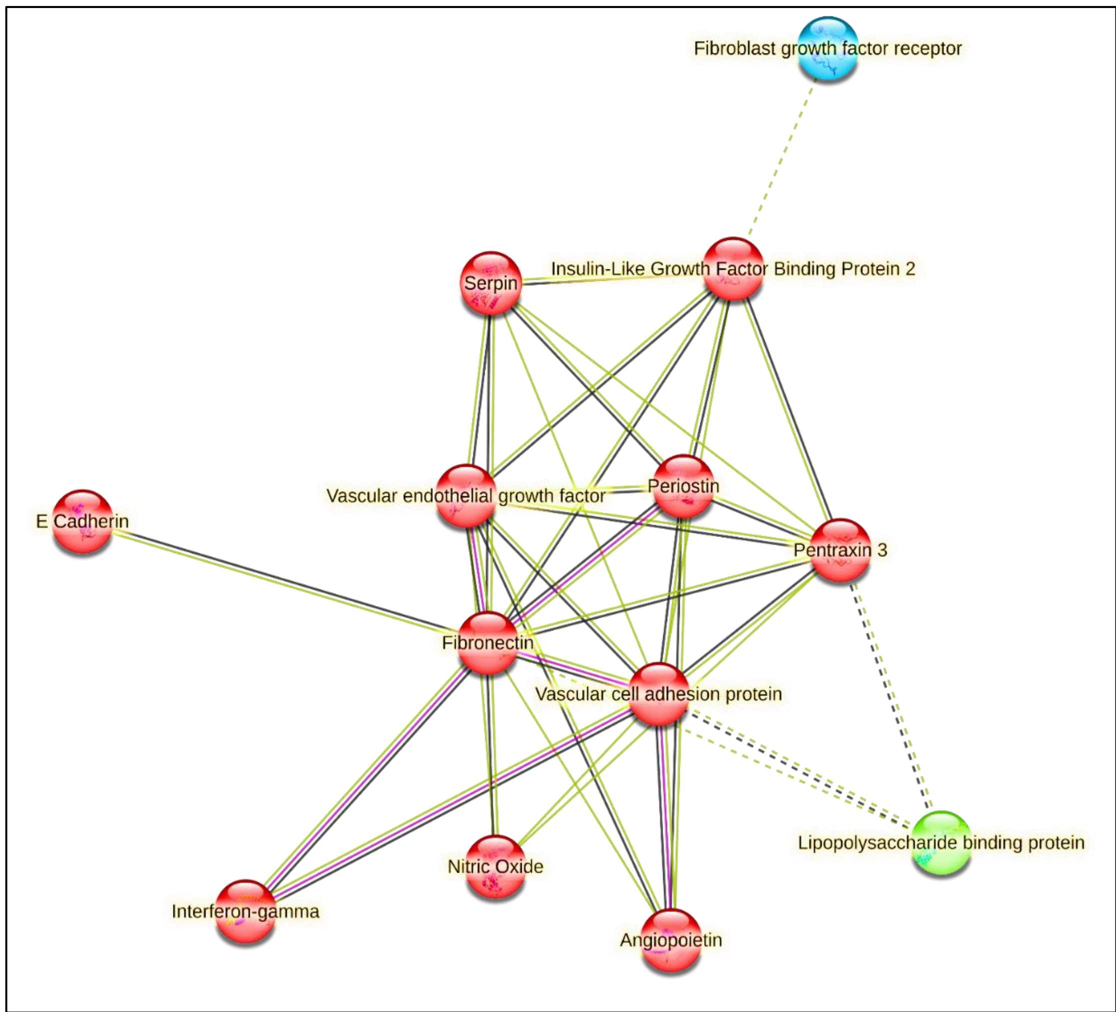


Fig. 49.a: STRING Functional proteins association network analysis via functional annotations



Clusters

bubble	cluster Id	gene count	protein names
🔴	Cluster 1	9	E Cadherin, Fibronectin, Insulin-Like Growth Factor Binding Protein 2, Interferon-gamma, Nitric Oxide, Pentraxin 3, Periostin, Serpin, Vascular cell adhesion protein
🟢	Cluster 2	1	Lipopolysaccharide binding protein
🔵	Cluster 3	1	Fibroblast growth factor receptor

Fig. 49.b: STRING Functional proteins association network analysis via K mean clustering

STAR PROTEINS OF OUR STUDY

Sl. No.	Apo lipoproteins involved in cascade of virus replication and entry
1.	Apo I ↓
2.	Apo II ↓
3.	Apo IV ↓
4.	Apo B ↓
5.	Apo E ↑

↑ Protein Upregulated

↓ Protein Downregulated

Sl. No.	Proteins involved in cascade vascular dysfunction
1.	E Cad ↓
2.	Angiopoitein ↓
3.	FGFR1 ↓
4.	V cam ↓
5.	IRF 3 ↑
6.	IFN γ ↑
7.	Fibronectin ↑
8.	IGFBP 2 ↑
9.	Serpin ↑
10.	Periostin ↑
11.	PTX3 ↑
12.	VEGF ↑
13.	LBP ↑

Sl. No.	Top protein-protein docking interactions
1.	NS1-PTX3
2.	NS1-SERPIN
3.	NS1- LBP

Based on Root Mean Square Deviation (RMSD) value of model

Fig. 50: List of star proteins validated in this study with their upregulation and downregulation pattern

Chapter 7: Discussion

Dengue virus molecular serotyping and genotyping

Dengue fever, the emerging and neglected tropical disease has become a major public health concern due to the rapidly progressing geographical spread of vector infestation. Monitoring of dengue molecular epidemiology is crucial, especially considering the consequences of severe dengue infection. India being a tropical country, contributed 34% of global dengue infection (1). The first Dengue Haemorrhagic Fever (DHF) outbreak occurred in Kolkata (then Calcutta) in 1963 with 30% of cases showing haemorrhagic manifestations (169), since then, West Bengal has witnessed several dengue outbreaks. West Bengal being the 4th populous state of India, is severely affected by dengue each year during monsoon and post-monsoon season. In this study, co-circulation of all the four dengue serotypes with a rapid change in the pattern of prevalent serotypes. An earlier dengue serotyping study from this lab reported a dengue outbreak in Kolkata in the year 2012 with more severe dengue cases due to DENV-3 serotype (170). A rapid change in circulating pattern of dengue serotyping observed as DENV-2 in 2018 and 2019, DENV-4 in 2020 whereas DENV-3 in 2021 and 2022 (**Figure 23**). The changing pattern of dengue serotypes has become a significant concern in recent years. The dynamic shift and circulation of different serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) have contributed to an increase in the incidence and severity of dengue outbreaks. This serotype variability affects immunity and increases the risk of severe dengue, as individuals previously infected with one serotype are at higher risk of severe disease upon infection with a different serotype. Understanding these patterns is crucial for developing effective vaccination strategies and public health interventions to mitigate the impact of dengue. The prevalence variability can be explained by the studies by Nisalak et. al., which show an

increase in serotype diversity can subsequently increase disease prevalence, which can cause a reduction in the circulating serotypes (171). A shift of dominance from DENV-2 to DENV-1 and DENV-3 was also reported in 2012 in Kolkata, which caused the 2012 dengue outbreak (172). Studies by Balmaseda et al., have shown that the predominance of clinical symptoms could also be influenced by the majorly circulating serotype (173) but the risk of severe dengue could not completely be explained by a prior infecting serotype (174). The re-emergence of DENV-2 after a gap of a few years in West Bengal caused an utmost outbreak of dengue fever. As postulated in the literature, DENV-2 is known as the most prevalent serotype worldwide (175). In this study, among the DENV-infected population, there were multiple severe dengue patients and four patients died due to grade III and IV dengue complications (152). Primary dengue infection with one serotype usually gives lifelong homotypic immunity to that serotype, but only short-term immunity to the other serotypes. Subsequent infection with a different serotype increases the risk of severe complications. Alterations in circulating serotypes in the same geographical region, especially in an endemic region, increase the risk of more secondary dengue infections in the population (176). Clinical and pathological manifestations of both dengue fever and severe dengue are similar during the febrile phase, but in the case of severe dengue, the patient's condition deteriorates suddenly after this phase leading to severe dengue (DHF-grade I and II complications, DSS-grade III and IV complications). On the other hand, severe dengue also exhibits some pathophysiological changes, such as capillary leakage, thrombocytopenia, and coagulopathy (152).

Generally, dengue infection is considered a pediatric disease, but it is currently becoming a growing problem in adults throughout tropical countries. In this study, age-specific dengue fever distribution among the different age groups, adults between the age of 21–30 showed the highest DENV infection followed by the age group of 11–20 years (**Figure 24**).

This data significantly (p-value < 0.05) corroborates with the previous study from Thailand in which 30–40% of dengue infected population were adults (177). Analysis of demographic data showed the incidence of DENV infection was higher in males in comparison to females, co-relating with the WHO's earlier interpretations of higher dengue preponderance in males (178). Hence, these findings suggest that there may be age bias and gender-related alterations in dengue occurrence, which might be due to exposure differences among male and female adolescents. As dengue virus life cycle involves both mosquito and human host, which might be a contributing factor behind the low rate of variation among DENV in comparison to other RNA viruses. The genomic region selected for dengue virus serotyping and genotyping is from a conserved region and most of the mutations reported in this region are of silent type (179). Also, several reports corroborated that the DENV genotype classification is based on less than 6 % of nucleotide deviation within a selected genomic region (180). Results showed that prevalent DENV circulating serotypes observed during this study period were also prevalent in their subtypes i.e. same genotype of prevalent serotype was observed. DENV-2, genotype II; DENV-3, genotype III and DENV-4 genotype-I were the prevalent subtypes in circulation (**Figure 25**). Indian strains from Madurai, Pune and Bangalore showed > 90 % similarity with genotype I of DENV-4 whereas strains from Delhi, Madhya Pradesh and Tamil Nadu showed > 90 % similarity with genotype III of DENV-3 isolated in this study. Previous dengue serotyping and genotyping study in the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV) in 2018 encompassing 21 Virus Research and Diagnostic Laboratory (VRDLs) from across India also reported DENV-3 genotype III and DENV-4 genotype I as prevalent circulating strain in India (181). Optimal virulence is higher in epidemiological contexts where only one serotype is circulating. Dengue is hyperendemic (4 serotypes circulating) in nature and therefore, the transition from two circulating

serotypes to hyper-endemism selects for more virulent viral strains. This may be relevant for understanding observed genotype replacement dynamics in areas with increasing dengue serotype circulation. The recurring dengue occurrences in West Bengal emphasize the necessity of dengue serotyping and genotyping studies. These studies are crucial for understanding serotype distribution, epidemiological insights and public health interventions. Furthermore, serotype and genotype-based molecular surveillance of DENV is required to explore and understand pathogenicity and clinical manifestations associated with dengue. The level of serotype and genotype diversity indicates the sustainability and adaptability of the virus in the population. A change in the pattern of serotype and genotype is being noticed in West Bengal, specifically, the presidency province with significant pathogenic diversities. This indicates the increased fitness of circulating viral strains due to changing environmental conditions and the inhabitant's immunity. Hence, the diversity of different dengue serotypes and their subtype, the competence of the dengue vectors and their anthropophilic physiognomies, coupled with the muddled and hazardous urbanization and suburbanization pattern of developing countries, mostly located in tropical and subtropical zones, make the management of this arbovirus infection very challenging and complex.

Clinicopathological alterations of symptoms and severe dengue clinical biomolecules

The global incidence of dengue has grown dramatically in recent decades with no specific available treatment, but early detection and access to proper clinical management lower the fatality rates below 1% (35). Identification of the circulating serotype is added value in managing the consequences of secondary infection and correlating the disease severity with

the dengue serotype as observed by Kalyanarooj S. et. al. in 2011 (182). In this study, DENV-2 was observed as a major circulating serotype (**Figure 24**). The main deceptive illness in this study population was gastrointestinal complications such as vomiting observed in 64.02%, abdominal pain in 53.23%, and nausea in 52.51% of patients. Patients with discerned hepatomegaly (33.81%), were observed with an enlargement of liver >2 cm which may have occurred due to inflammation of liver cells by DENV infection. Recurrent vomiting, diarrhoea, and ascites were observed in 25.89%, 19.42%, and 18.70%, respectively (**Figure 26**), stating these symptoms are significantly increased than those observed in the previous study (183). Upper respiratory tract complications were observed in 20.86% of patients with respiratory distress in 19.42%, and pleural effusion in 15.82% (**Figure 26**) which leads to the development of severe respiratory problems mainly in severe patients DHF (with Grades I and II complications) and DSS (with Grades III and IV complications), which correlates the previous finding that ultrasonography for pleural effusion leads to determine disease severity and beneficial to patient management (184). Constitutional complications with pyrexia in 92%, anorexia in 87.50%, headache in 38.84%, pain in the eyes in 8.63%, conjunctival congestion in 5.03% of patients, and no retro-orbital pain (**Figure 26**) were observed in denial of the previous study where 60% and 42% of patients showed headache and retro -orbital pain respectively (183). This study also contradicts a previous study where 85% (182) and 33% (185) of patients presented with fever during dengue infection. Musculoskeletal complications were observed in 41% of patients in this study population mainly as body pain (39%) and joint pain (12%), which contradicts previous studies where 81% and 84.5% of patients suffered from myalgia (185), (186). Neurological complications were observed as a shock in 15.10%, restlessness in 13.66%, convulsion in 10.79% and arrhythmia in 7.19% (**Figure 26**). This study proposes that changes in classical dengue symptoms may occur due to changes in circulating

serotypes with genetic diversity. Various bleeding manifestations were observed in 38% (n = 53) of dengue infected patients as shown in **Figure 27**. In DSS, the majority of patients developed petechiae and hematemesis as major bleeding manifestations accompanied by others; one patient developed petechiae with GI bleeding, one hematemesis with melena, and the last two developed petechiae with hematemesis and melena but all of them represented petechiae rash type. Petechiae being the major rash type, 88% of all bleeding manifestations correlated with the findings of studies conducted by G.N. Malavige, 2006, signifying it as a salient manifestation of DHF and DSS (186). Dengue infection is well known to be associated with constitutional manifestations (187) but is also present in many other infections. This study uniquely denotes a significantly higher frequency of constitutional manifestations, the general feature of dengue infection with different serotype infections (**Table 8**). Various serotypes of the dengue virus (DENV) have common constitutional symptoms, including high fever, intense headache, retro-orbital discomfort, myalgia, arthralgia, and rash. With varying serotype infections, however, these signs might fluctuate greatly in terms of frequency and severity. Also, a notable increase in gastrointestinal complications was observed with different serotypes. Among our clinicopathological co-relation study population 71% of patients was infected with DENV-2 (n= 67). Also, among DENV-2 infected patient's population 88% of patients observed with major Gastrointestinal Complications (GIC). Patients infected with other dengue serotypes also showed GIC but the percentage is significantly high in DENV-2 infected patients in comparison to other serotypes. On the other hand, individuals with DENV-2 infection had a higher prevalence of gastrointestinal complications also corroborates with a previous study from India by Kumaria et al. (188) which demonstrated that more GIC mainly abdominal pain is associated with DENV-2 compared to **Table 8** other serotypes. Muscle cells are known to be involved in the production of proinflammatory cytokines

(189), (190). Also, there are studies indicating a link between dengue infection and musculoskeletal dysfunction by Salgado et al. (190) It was observed that MSC was present in approximately 40% of individuals of all serotypes in this study (**Table 8**). A few respiratory complications were also observed to be associated with dengue, mainly among severe patients. In this study population, no particular serotype was observed to be associated with bleeding manifestation. Hence, secondary dengue infection with any serotype is a major factor behind the development of DHF/DSS. Thrombocytopenia (platelet count <1,50,000/µl) is another persistent finding with dengue, most of the dengue-infected patients admitted to the hospital had thrombocytopenia. The patient situation is alarming when the platelet count is <1 00 000/µl, but it's considered to be serious below 40,000/µl. In this study, platelet count was significantly low in severe dengue patients. Platelet transfusion and patient treatment outcomes are diverse because all platelet transfused patients were not recovered or improvised (**Table 9**). This supports the previous study of 106 pediatric patients in the severe dengue phase with thrombocytopenia and coagulopathy, where no significant difference in the incidence of haemorrhage has been observed between patients who received preventive platelet transfusions compared to those who did not (191). Similar observations were made in other studies in various parts of India by Ratageri et al. (192) and Jakribettu et al. (193). Hence, the correlation of platelet counts with bleeding manifestation or with disease severity observed in patients was also observed previously in different studies (194), (195) The major factor behind depleted platelets are DENV-induced bone marrow hypoplasia. Significant platelet destruction occurs due to platelet disseminated intravascular coagulation, functional disruption of these cells is also associated with increased apoptosis by the complement system and through the involvement of antiplatelet antibodies (196). The regression models of dengue patients obtained from this study have high sensitivity. The area under the curve (AUC) of the ROC

evaluates the model's ability to classify the status of the model in the association of disease. The best-case ROC would look like a 90° angle and the AUC value of the best-fitting model of the ROC curve is 1 (197). The AUC value for our regression models was estimated as 0.9888 for ROC 1 and 0.9064 for ROC 2 (**Figures 29.a and 29.b**). In the acute and febrile phases, dengue patients suffered from more clinical manifestation as shown in relevance to serotype positivity. A combination of clinicopathological manifestations was more informative than the individual symptoms. More work is needed on the algorithm of dengue-associated laboratory results as the independent variable, which will improve the sensitivity of the model. A model with high accuracy will help in the initial step of dengue evaluation and will also reduce the misdiagnosis of DF. This type of predictive and regression model will help in making a down-turn decision tree to analyze the need to perform an additional examination or laboratory tests for patient management. As per the WHO definition, a significant decrease of serum albumin >0.5 g/dl from baseline or <3.5 g/dl is indirect evidence of plasma leakage (198). Low TP and albumin were noticed in most of the severe dengue cases and approached their alarming lower limit with progression of disease severity. Albumin and TP depleted severely in dengue patients with Grades III and IV complications (**Figure 28**). DF patients also showed hypoalbuminemia but their value was not depleted beyond the lower limit. However, there are reports of low albumin levels (<3.5 g/dl) in patients with DF and DHF (199). The hypothesis behind the mechanism of increased protein excretion in urine is that during DENV infection the endothelial cells of glycocalyx are disrupted either by the direct action of the virus or by the NS1 antigen causing plasma leakage (196), (199). Usually, high values of albumin reflect the integrity of the vascular endothelium; however, albumin levels less than 3 g/dl may be an early indicator of vascular permeability alteration. Therefore, the degree of hypoalbuminemia and proteinuria can act as a predictor to detect plasma leakage as well as

the degree of disease severity from DHF (complications Grades I, II) to DSS (complications Grades III, IV). The liver is one of the main organs involved in dengue infection (200). The range of liver dysfunction in dengue may vary from mild damage with an elevation of liver enzymes to severe damage like jaundice. In children, the aminotransferase levels of more than 10 times indicated that the children are at higher risk of hepatic involvement during infection, with the possibility of developing hepatic encephalopathy (200). In this study, AST and ALT values were high in classical dengue infection and more than 10 times higher in severe dengue cases, with the highest value of 1056 U/L of ALT in DSS patients on the 10th day after the onset of fever (at the time of death) (**Figure 28**). Hepatic dysfunction during dengue infection in children was reported in various studies (201). This type of abnormal liver profile parameters may give rise to jaundice and fulminant hepatitis-like situations during or post-dengue infection. Liver involvement happened through an inflammatory process in the parenchyma and was provoked directly or indirectly by the DENV, reducing the diameter of the lumen biliary canaliculi and causing an obstruction. ALT and AST may act as important markers to evaluate and monitor the severity of this infection because the elevation of transaminases is common in severe dengue patients along with liver enlargement.

Proteomic-based approach for identification of protein biomolecules and validation of panel of biomolecules

Dengue virus infection pathophysiology involves a complex molecular network between the host and the dengue viral protein. Due to these interactions, the clinical symptoms of dengue fever vary, from mild dengue fever to severe complications to even death with grade III and IV complications (152). Therefore, the identification and validation of new

biomolecules in search of diagnostic and predictive indicators of severe dengue are essential. In this study, we investigated alterations in serum proteome during different phases of dengue fever and identified changes in the expressions of multiple proteins. As only 1 to 2% of dengue-infected patients develop its severe form and hence, the search for phase-specific severe dengue patients is very challenging (128). There are several earlier reports on plasma and serum profiling of dengue fever patients from different parts of the world (202), (203), (204). To the best of our knowledge, this study reports the first dengue phase-specific comprehensive analysis of plasma protein alterations in dengue-infected patients. In comparison to previously reported proteomic studies, we have also reported a greater number of differentially expressed plasma proteins in dengue patients and performed their functional pathway analysis for a better understanding of their biological context with dengue pathogenicity. Dengue fever cases have surged by 30-fold in the last 50 years (13). Only 1-2% of dengue-infected patients develop severe dengue (205), which is associated with a high risk of mortality and the critical phase develops after defervesence, the chase for critical dengue patients is a real tussle. The search for reliable biomolecules associated with severe dengue is crucial for understanding and managing the life-threatening disease, as they have emerged as valuable tools for predicting and monitoring its progression.

DENV-2 is a prevalent serotype in India, co-circulating with the other three serotypes (206), prompting a proteomics study on patients infected with this serotype. Nano LC/Orbitrap MS-based protein annotation approach was used initially to have a holistic idea about the protein composition at different stages of dengue fever. Patients in acute phase category were suffered from the acute stage of dengue fever, which is marked by a high body temperature, intense headache, discomfort behind the eyes, aches in the joints and muscles, rash, and light bleeding. Various proteins implicated in the inflammatory

response, immunological activation, and viral replication are expected to be enhanced during this phase when the immune system is actively reacting to the viral infection. Patients in critical phase were at more advanced stage of dengue fever known as the critical phase. Major clinical complications such as platelet drop, plasma leakage, haemorrhage organ dysfunction, etc. were observed during this phase by our clinical collaborators. Healthy individuals without any current or past dengue infection were used as a control group for baseline comparison of protein expression. The proteomic profile of healthy controls is essential for identifying changes specific to dengue infection and its phases. By comparing the protein expression levels between healthy controls and dengue-infected patients we can discern disease-specific proteomic alterations, enhancing the understanding of dengue pathogenesis and aiding in the development of diagnostic and therapeutic strategies. In qualitative MS analysis, 6000 proteins from acute phase patient's samples, 5000 proteins from critical phase patient's samples and 4300 proteins from healthy control individuals were identified respectively. Various biological processes and pathways were enriched with these proteins (**Figure 31**). Most of these proteins were common throughout all the categories, but a subsection of the identified unique proteins might be helpful to understand the transition from dengue fever (DF) to its severe, life-threatening form. To address this query, SWATH-MS was utilized, which revealed differential expressions of unique proteins in different phases of dengue fever. The differentially expressed proteins belong to a category of Apoliproteins, clotting factors, immune activating, microtubule-associated, Coagulation factors, Complement, and extra-cellular matrix proteins found to be upregulated and downregulated as mentioned earlier in the results. The pathway analysis of these proteins might determine the fate of a particular DF towards a more severe form. In **Figures 33, 34 and 35**, PANTHER and KEGG databases highlighted multiple proteins found to be associated with the pathophysiology of severe dengue.

The approach of this study is unique starting from patient recruitments i.e. dengue phase-specific and at two time points from each phase. Also, this study has combined various protein identification and validation techniques in search of potential biomolecules for predicting severe dengue and observed viral protein interaction with top validated biomolecules. Quantitative proteomics is a powerful approach used in the field of biomedical research to study changes in protein expression levels and identify potential biomolecules associated with various diseases (207), (208) including severe dengue. These differentially expressed groups of proteins may be predicting factors for dengue severity. A recent group of studies suggested the role of an array of apolipoproteins in Dengue pathophysiology. We too, observed a group of apolipoproteins in this study to be deregulated during dengue pathogenesis. Li et. al, reported the association of Apo AI with dengue viral particles and cell receptor SR-BI to promote entry of the dengue virus inside host cells (209). Our SWATH-MS data demonstrated the differential expressions of Apo AI, AII, AIV, B, CI, CII, CIII, D, E, M and L1. Using Western blot analysis, we have validated significantly downregulated expressions of Apo AI, AII and AIV in the dengue-infected patients recruited in our experiment (**Figure 43**). Transferrin protein was used as a housekeeping control gene for the Western blot experiment and target protein expressions were normalized with Transferrin. In Western blotting, gene normalization using transferrin is an essential method for precise protein quantification. The glycoprotein transferrin, which is involved in the transport of iron, is frequently employed as a housekeeping protein because of its consistent expression across a range of cell types and experimental settings. To compare the relative intensities of the target protein and transferrin, both have been detected on the same membrane throughout this normalization step. Since the interaction of SR-BI to Apo AI is known to be crucial for facilitating the entry of the DENV particles inside host cells (210), these downregulated expressions of

Apo AI, AII and AIV might be a compensatory effect exerted by the host cells upon DENV infection (209). Apolipoproteins play a vital role in the dengue virus entry and replication process. Dengue virus manipulates lipid raft integrity and utilizes cholesterol component and apolipoprotein for virus internalization LDLr (Low Density Lipoprotein Receptors) and SR-B1 (Scavenger Receptor B1) receptors (209), (210). We believe that the synergistic effects of all the apolipoproteins are an important avenue in dengue pathogenesis. Increased vascular permeability, resulting in plasma leakage and hemorrhagic tendency, is a hallmark of dengue hemorrhagic fever (DHF) and subsequent shock (211). Interestingly, direct infection of endothelial cells with DENV particles that might result in the death of these cells has not been well documented. Rather, elevated levels of capillary filtration (resulting in higher extracellular water) were observed in DHF patients as compared to healthy controls or DF patients (212), (213). Although infection of endothelial cells and death of patients is not reported in severe dengue but many reports on the activation of endothelial cells during DENV infection are available (214), (215). These are manifested by the elevation of several biomolecules, including soluble vascular cell adhesion molecules (sVCAM-1) and soluble intercellular adhesion molecule 1 (sICAM-1), especially in severe cases (216). As observed from SWATH-MS data, both Cadherin-13 and E-Cadherin were downregulated and VCAM-1 was upregulated in the later phase of dengue as compared to the control. We validated a similar pattern of expression of E-Cadherin (**Figure 43**) in dengue samples using Western blot analysis. Also, elevated levels of VEGF and FGFR were observed in critical dengue patients in comparison to control samples (**Figure 43**). The increased production of VEGF during DHF leads to a transcellular gap and fenestration formation of vesicles (216). These morphological changes in endothelium might lead to increased vascular permeability. FGFR reciprocally affects viral replication and infectivity, Inhibition of FGFR4 decreases replication and increases specific infectivity of virions, an

increase in specific infectivity is linked to enhanced proteolytic cleavage of prM and hence associated with severe dengue. IRF3 and IFN-γ were observed as significantly high in critical dengue patients in SWATH-MS analysis and validated in **Figure 43**. In another study using loss-of-function approaches for various molecules, it was demonstrated that the IRF-3-associated pathway predominantly utilizes IFN-γ and, to a lesser degree type I IFNs to stimulate interleukin production (217). Dengue-induced thrombocytopenia and endothelial dysfunction are associated with an imbalance in angiopoietin-2: angiopoietin-1 plasma levels. Ang1 is required for protecting against endothelial hyper-permeability. In this study, Ang1 was found to be down-regulated during dengue infection (218), (219).

Endothelial cell activation might also lead to changes in the coagulation state of patients (220). In corroboration to this, we have observed differential expression patterns of an array of proteins involved in the blood coagulation cascade, viz., Factor VII, IX, XI, XII, XIII, Heparin cofactor, Fibrinogen α and β, from our SWATH-MS data. Effects of DENV on the host coagulation cascade are highly complex. Alterations in the coagulation system, specifically clotting factors and fibrinolytic system, have been observed to contribute to the pathogenesis of DHF previously (221). Studies suggest that dengue infection may trigger the activation of the complement system, potentially enhancing the inflammatory response (222). The extracellular matrix (ECM) is crucial for tissue and organ structural integrity, and alterations in its composition can lead to severe dengue (221). Endothelial dysfunction and vascular permeability are significant processes in dengue virus pathogenesis that contribute to the successful dissemination of DENV, immune cell migration, and the release of soluble compounds from the blood to the tissue stroma, hence increasing disease severity (141). In this doctoral study, we have focused on proteins associated with vascular endothelium dysfunction. Therefore, angiogenesis and cytokine protein arrays have been selected to cover the broad range of proteins belonging to these groups **(Figure 41)**. Protein

arrays allow the simultaneous analysis of multiple proteins in a single experiment, making it a powerful tool for high-throughput screening. Elevated levels of angiogenesis proteins in severe dengue cases observed in this protein array, suggest their potential role in the increased vascular permeability which is associated with severe manifestations. Whereas, cytokines are essential in dengue immune response, leading to a "cytokine storm" in severe cases. This dysregulated immune response contributes to severe dengue symptoms like dengue haemorrhagic fever and dengue shock syndrome. The cytokine storm in severe dengue is characterized by an imbalance in pro-inflammatory and anti-inflammatory responses, leading to vascular leakage, plasma loss, and, in some cases, shock (137). From both experiments, a panel of significant proteins was selected for validation purposes as mentioned earlier. While quantitative proteomic and protein arrays can identify potential biomolecules, RT-PCR and ELISA serve as validation methods in this study. Real-time PCR quantified mRNA levels, compares across samples, and confirms that protein expression changes correlates with gene expression of selected proteins (**Figure 46**). However, it’s beneficial to combine RT-PCR with other validation methods, due to its limitation such as potential variations in RNA quality, and the requirement for careful assay design. Hence for large-scale robust validation of our best six proteins, we have chosen the ELISA method. Six sets of proteins chosen for validation by this method include Fibronectin, Periostin, Serpin E1, PTX3, IGF2, and LBP (**Figure 47.a to 47.f**). As predicted from Real Time PCR results, phase-specific changes in these proteins observed in ELISA results and directly associated with severe dengue pathogenesis.

Starting from PTX3 (Pentraxin 3), a gene associated with the immune response, particularly in the context of inflammation and infections. PTX3 is an essential component of the innate immune system and may be involved in recognizing and responding to dengue virus infections (223). It is part of the body's defence mechanism against the virus. This study

aims to determine, pentraxin 3 level with different phases of dengue fever to assess its potential usefulness as a prognostic tool. Our data show that pentraxin 3 is persistently elevated with disease severity in comparison to non-severe dengue patients. The magnitude of the increase in pentraxin 3 levels varied from more than 10-fold in patients suffering from dengue fever± with/without warning signs (Acute Phase) to as much as 80-fold in dengue fever± with warning signs (critical phase). This is hypothesised that this protein plays an important role in the early phases of inflammation by recognising microbial moieties, activating the classical complement system, and improving detection by macrophages and dendritic cells (224). PTX3 modulates the innate immune response by helping to opsonize and remove apoptotic or necrotic cells (223). Hence, PTX3 correlate with the severity of dengue fever and can act as a prognostic marker during the onset of vascular inflammation to track the transition of dengue fever to severe dengue. Fibronectin is a glycoprotein involved in cell adhesion and migration. It plays a role in the extracellular matrix and wound healing (225). In the context of dengue, fibronectin doesn't directly relate to the virus itself but might be involved in the host's response to infection or tissue repair due to the inflammatory nature of dengue.

POST (Periostin) protein plays a crucial role in tissue development and repair, particularly in connective tissues (226). While the specific relationship between the POSTN gene and dengue virus is not as extensively studied as other genes, this study observed that periostin could be involved in the host's response to severe dengue infection. In many viral infections, various genes related to tissue repair and inflammation are activated as part of the host's defence mechanisms. This gene encodes a secreted extracellular matrix protein that functions in tissue development and regeneration, including wound healing, and ventricular remodelling following myocardial infarction (226).

LBP (Lipopolysaccharide binding protein) is a part of the innate immune system that plays a role in recognizing and responding to infections. Lipopolysaccharide is a crucial immune stimulant that activates the innate immune system through a membrane-bound CD14/Toll-like receptor 4 complex. LBP and sCD14 influence the systemic response to LPS release in circulation, with LBP synthesized by the liver to accelerate LPS transfer (227). However, during severe dengue infections, the virus can lead to an extreme immune response, leading to multiple-fold overexpression of this protein.

Among critical dengue patients, the Serpin gene was highly upregulated. Serpins, or serine protease inhibitors, are a family of proteins that regulate the activity of proteases, enzymes involved in various biological processes such as fibrinolysis and cell migration inhibition (228). Serpins can modulate inflammation and influence the immune response to the virus affecting aspects like inflammation, tissue damage and activation of immune cells. As reported earlier the increased level of serpine1 is associated with dengue hemorrhagic fever which leads to thrombocytopenia, plasma leakage and coagulation abnormalities in children (161), (229).

IGFBP2 (Insulin-Like Growth Factors Binding Protein 2) is involved in regulating the activity of insulin-like growth factors (IGFs) in the body. Dengue primarily affects the immune system, and the focus of the study has been on various immune-related genes and proteins. IGFBP2 could indirectly influence the response to dengue infection through its role in regulating cell growth and differentiation, more research is needed to understand its precise involvement in the context of dengue. The ROC curve, and AUC value of candidate proteins was more than 0.500, suggesting a great ROC model of selected proteins(168), (230).

Biomolecules validated in this study were observed to be significantly interacting with dengue virus NS1 protein in protein-protein docking (**Figure 48**). Thus, the structural

analogue of biomolecules validated in this study might have the potential to halt pathogenesis caused by dengue virus NS1 protein. The NS1 protein plays a crucial role in the pathogenesis of the dengue virus, contributing to various aspects of severe dengue transition (231), (232). Till now, no molecule has been identified or validated as a dengue biomolecule among the many substances produced and released into the host blood during DENV infection. The exact mechanisms of dengue virus interaction with ECM (extracellular matrix) proteins and their contribution to dengue pathogenesis are still under active research. Given the importance of knowing aetiology, this study is the need of an hour.

The functional interaction between proteins plays a crucial role in understanding cellular processes and functions. K-means clustering is a widely used method in bioinformatics to analyse protein-protein interactions, and the STRING database serves as a valuable resource for this purpose (233). In the context of protein interactions, K-means clustering in the STRING database enables the grouping of proteins based on similarities in their interaction patterns (**Figure 49**). This clustering method helps to identify functional modules and potential protein complexes within the cellular network (234). By applying K-means clustering we can uncover meaningful insights into the organization and regulation of cellular processes. This approach aids in the identification of key proteins that may serve as hubs or connectors within biological networks, shedding light on the intricate relationships and signalling pathways that underlie various physiological and pathological conditions. Ultimately, the integration of K-means clustering with the extensive protein interaction data available in the STRING database enhances our understanding of the complex web of interactions that govern cellular function. Among these hub genes based on all results a gene trifecta of Serpin, PTX3 and IGFBP2 combination might have great importance towards predicting severe dengue and can act as a point of care biomolecules

with further research and clinical validations. NO is synthesized by endothelial cells through the action of endothelial nitric oxide synthase (eNOS). In severe dengue, several factors contribute to the depletion of NO (235), (236). Severe dengue is associated with heightened oxidative stress, which can inactivate NO. Reactive oxygen species (ROS) produced during the inflammatory response react with NO to form peroxynitrite, a reactive nitrogen species that further damages endothelial cells and depletes NO levels. The elevated levels of pro-inflammatory cytokines can downregulate eNOS expression and activity, leading to reduced NO production. Additionally, these cytokines can promote the expression of inducible nitric oxide synthase (iNOS) in immune cells, leading to the production of large amounts of NO, which can paradoxically contribute to oxidative stress and endothelial damage (235), (236). During severe dengue activated platelets release substances that can impair endothelial function and NO production. As the NO is a potent vasodilator and its depletion leads to unopposed vasoconstriction, increasing vascular resistance and contributing to impaired tissue perfusion and organ dysfunction. NO helps maintain the integrity of the endothelial barrier. Its depletion exacerbates endothelial dysfunction and promotes plasma leakage, leading to edema, ascites, and pleural effusion. Reduced NO levels can lead to increased platelet aggregation, thrombosis, and disseminated intravascular coagulation (DIC), complicating severe dengue, further damaging endothelial cells and perpetuating a cycle of inflammation and endothelial dysfunction (235), (236). Thus, the direct interaction of validated biomolecules of this study with NO depicting an underlying role of them in NO depletion during severe dengue.

The study validated biomolecules in severe dengue, revealing their diagnostic and prognostic importance. These biomolecules can differentiate severe dengue patients from milder ones, aiding in risk stratification and guiding treatment interventions.

Conclusion and future prospects

This five-year doctoral study encompasses the multifactorial approach to the pathophysiology of severe dengue to combat the battle against it. Among the first two objectives, the circulatory pattern of dengue serotypes, subtypes and clinicopathological changes has been established. Whereas in next two objective protein biomolecules associated with severe dengue were identified and validated. This study uniquely converged the virus and host factors in the etiologic mechanism of severe dengue. The epidemiological part of this study showed DENV-2 as a predominant etiological serotype followed by DENV-3. The dengue-associated spectrum of clinical manifestations seems to be changing over the period. Different serotypes have been linked to various clinical outcomes. Knowledge of circulating strains can help with patient management and treatment techniques. Dengue serotypes play a key role in the disease spectrum and dengue resurgence. Therefore, serotype-based studies are needed for better reflection of dengue virus virulence. The goal of developing a regression logistic model is to determine the sensitivity and specificity of dengue-associated clinical symptoms to provide preliminary data in the approach towards making a decision tree in the clinical management of dengue fever. A predictive and sensitive model at the initial step of patient evaluation can help in the clinical management of dengue cases and DF-associated morbidity and mortality. In inference, serotyping and genotyping of dengue virus are indispensable tools for understanding the epidemiology, improving clinical management, guiding vaccine development and informing public health strategies. Future advancements in diagnostic technologies, data integration and global collaboration will enhance our ability to combat dengue and reduce its global burden.

Next, the proteomics-based part of this study identifies changes in protein expression levels during different phases of dengue fever. This study reveals apo-lipoproteins associated with severe dengue pathogenesis via enhancing viral entry and replication, while cytokines and endothelial proteins are associated with vascular dysfunction and viral pathogenesis. These biomarkers have provided insights into the mechanisms underlying severe dengue, including immune dysregulation, endothelial dysfunction and coagulopathy. Identification and monitoring of biomolecules in severe dengue cases are of paramount importance for early diagnosis and patient management. A combination of clinical parameters and significant biomolecules can aid in risk stratification and guide appropriate interventions. Our study highlights the potential of quantitative proteomics in unravelling biomolecules associated with severe dengue. The identified biomolecules panel holds promise for improving diagnostic accuracy, risk stratification and therapeutic interventions in severe dengue patients. This phase-specific (acute, intermediate/critical and recovery/death) study uniquely helps in developing signature molecular profiles for early prognosis of severe dengue enabling the creation of personalized therapies and interventions. Further research is needed to validate the utility of these biomolecules and to fully exploit the clinical utility of these candidate biomolecules, to translate them into practical applications that benefit patients and public health efforts. Also, the findings suggest the development of a signature molecular profile for early detection of severe dengue could lead to design drugs to halt the transition from dengue fever to severe dengue. Identified protein markers will improve patient outcomes and inform strategies to control and prevent severe dengue morbidity and mortality. Developing and validating biomarker-based diagnostic kits and prognostic tools for use in clinical settings, ensuring they are accessible, especially in resource-limited regions where dengue is prevalent, a major future prospect of this proteomic based biomolecule study. Also, to explore the potential of biomarker-guided therapies, including

targeted treatments and immunomodulatory approaches, to improve patient outcomes. By encouraging national and international collaborations to standardize biomarker research protocols, share data and enhance the overall impact of research efforts on a global scale, we can fight against dengue and severe dengue in the near future.

Bibliography

1. Dengue and severe dengue [Internet]. [cited 2024 Feb 6]. Available from: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue
2. Gulber DJ. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 2002;10(2):100–3.
3. National Guidelines for Clinical Management of Dengue Fever 2023.pdf.
4. Dengue Situation Update 685.
5. Saeed O, Asif A. Dengue virus disease; the origins [Internet]. Dengue Virus Disease: From Origin to Outbreak. Elsevier Inc.; 2019. 9–16 p. Available from: http://dx.doi.org/10.1016/B978-0-12-818270-3.00002-3
6. Shepard DS, Halasa YA, Tyagi BK, Adhish SV, Nandan D, Karthiga KS, et al. Economic and disease burden of dengue Illness in India. Am J Trop Med Hyg. 2014;91(6):1235–42.
7. Ebi KL, Nealon J. Dengue in a changing climate. Environ Res. 2016;151:115–23.
8. Mutheneni SR, Morse AP, Caminade C, Upadhyayula SM. Dengue burden in India: Recent trends and importance of climatic parameters. Emerg Microbes Infect [Internet]. 2017;6(8). Available from: http://dx.doi.org/10.1038/emi.2017.57
9. Shuman EK. Global climate change and infectious diseases. Int J Occup Environ Med. 2011;2(1):11–9.
10. Murray NEA, Quam MB, Wilder-Smith A. Epidemiology of dengue: Past, present and future prospects. Clin Epidemiol. 2013;5(1):299–309.
11. Gratz NG. Emerging and resurging vector-borne diseases. Annu Rev Entomol. 1999;44:51–75.
12. Carrington LB, Armijos MV, Lambrechts L, Scott TW. Fluctuations at a Low Mean Temperature Accelerate Dengue Virus Transmission by Aedes aegypti. PLoS Negl Trop Dis. 2013;7(4).
13. Messina JP, Brady OJ, Golding N, Kraemer MUG, Wint GRW, Ray SE, et al. The current and future global distribution and population at risk of dengue. Nat Microbiol. 2019;4(9):1508–15.
14. Kakarla SG, Caminade C, Mutheneni SR, Morse AP, Upadhyayula SM, Kadiri MR, et al. Lag effect of climatic variables on dengue burden in India. Epidemiol Infect. 2019;147:1–10.
15. Majhi J, Singh R, Yadav V, Garg V, Sengupta P, Atul P, et al. Dynamics of dengue outbreaks in gangetic West Bengal: A trend and time series analysis. J Fam Med Prim Care. 2020;9(11):5622.

16. Debnath F, Provash C, Chakraborty A, Dutta S. Dengue fever outbreak by more than one serotype in a municipal area of Kolkata, Eastern India. J Vector Borne Dis. 2019;56(4):380–2.
17. Dengue: National Center for Vector Borne Diseases Control (NCVBDC) [Internet]. [cited 2024 Feb 6]. Available from: https://ncvbdc.mohfw.gov.in/index1.php?lang=1&level=1&sublinkid=5776&lid=3690
18. Jagtap S, Pattabiraman C, Sankaradoss A, Krishna S, Roy R. Evolutionary dynamics of dengue virus in India. PLoS Pathog. 2023;19(4):1–25.
19. Ángel R del, Murray N, Quam M, Wilder-Smith A, Tripathi SK, Gupta P, et al. Emergence of new lineage of dengue virus 3 (genotype III) in Lucknow, India. Iran J Microbiol. 2013;5(7):68–75.
20. Bharaj P, Chahar HS, Pandey A, Diddi K, Dar L, Guleria R, et al. Concurrent infections by all four dengue virus serotypes during an outbreak of dengue in 2006 in Delhi, India. Virol J. 2008;5:1–5.
21. Pandey A, Diddi K, Dar L, Bharaj P, Chahar HS, Guleria R, et al. The evolution of dengue over a decade in Delhi, India. J Clin Virol. 2007;40(1):87–8.
22. Guzmán MG, Kourí G. Dengue: an update. Lancet Infect Dis. 2002;2(1):33–42.
23. Lau CL, Weinstein P, Slaney D. Dengue surveillance by proxy: Travellers as sentinels for outbreaks in the Pacific Islands. Epidemiol Infect. 2013;141(11):2328–34.
24. Semenza JC, Sudre B, Miniota J, Rossi M, Hu W, Kossowsky D, et al. International Dispersal of Dengue through Air Travel: Importation Risk for Europe. PLoS Negl Trop Dis. 2014;8(12).
25. Wilson ME. The traveller and emerging infections: Sentinel, courier, transmitter. J Appl Microbiol Symp Suppl. 2003;94(32):1–11.
26. Jelinek T, Mühlberger N, Harms G, Corachàn M, Grobusch MP, Knobloch J, et al. Epidemiology and clinical features of imported dengue fever in Europe: Sentinel surveillance data from TropNetEurop. Clin Infect Dis. 2002;35(9):1047–52.
27. Schwartz E, Weld LH, Wilder-Smith A, Von Sonnenburg F, Keystone JS, Kain KC, et al. Seasonality, annual trends, and characteristics of dengue among Ill returned travelers, 1997-2006. Emerg Infect Dis. 2008;14(7):1081–8.

28. Wichmann O, Gascon J, Schunk M, Puente S, Siikamaki H, Gjørup I, et al. Severe dengue virus infection in travelers: Risk factors and laboratory indicators. J Infect Dis. 2007;195(8):1089–96.
29. Gubler DJ. Dengue and Dengue Hemorrhagic Fever. Trop Infect Dis Princ Pathog Pract 2-Vol Set CD-ROM. 2005;11(3):813–22.
30. Dengue guidelines for diagnosis, treatment, prevention and control treatment, prevention and control treatment, prevention and control [Internet]. Available from: www.who.int/tdr
31. Zybert IA, van der Ende-Metselaar H, Wilschut J, Smit JM. Functional importance of dengue virus maturation: Infectious properties of immature virions. J Gen Virol. 2008;89(12):3047–51.
32. Chen HR, Lai YC, Yeh TM. Dengue virus non-structural protein 1: A pathogenic factor, therapeutic target, and vaccine candidate. J Biomed Sci. 2018 Jul 24;25(1).
33. World Health Organization & UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Handbook for clinical management of dengue [Internet]. World Health Organization. 2012. 111 p. Available from: https://apps.who.int/iris/handle/10665/76887
34. Halstead SM. Dengue and dengue hemorrhagic fever. Handb Zoonoses Second Ed Sect B Viral Zoonoses. 2017;11(3):89–99.
35. Dengue and severe dengue [Internet]. Available from: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue
36. Wang WH, Urbina AN, Chang MR, Assavalapsakul W, Lu PL, Chen YH, et al. Dengue hemorrhagic fever – A systemic literature review of current perspectives on pathogenesis, prevention and control. J Microbiol Immunol Infect. 2020 Dec 1;53(6):963–78.
37. Stephens HAF, Klaythong R, Sirikong M, Vaughn DW, Green S, Kalayanarooj S, et al. HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. Tissue Antigens. 2002;60(4):309–18.
38. Fernández-Mestre, Mercedes & Gendzekhadze, Ketevan & Rivas-Vetencourt, P & Layrisse, Zulay. (2004). TNF-α-308A allele, a possible severity risk factor of hemorrhagic manifestation in dengue fever patients. Tissue antigens. 64. 469-72. 10.1111/j.1399-0039.2004.00304.x.

39. Guzman MG, Kouri G. Dengue haemorrhagic fever integral hypothesis: confirming observations, 1987-2007. Trans R Soc Trop Med Hyg. 2008;102(6):522–3.
40. Sumarmo, Wulur H, Jahja E, Gubler DJ, Suharyono W, Sorensen K. Clinical observations on virologically confirmed fatal dengue infections in Jakarta, Indonesia. Bull World Health Organ. 1983;61(4):693–701.
41. Roy SK, Bhattacharjee S. Dengue virus: Epidemiology, biology, and disease aetiology. Can J Microbiol. 2021;67(10):687–702.
42. Huang YJS, Higgs S, Horne KME, Vanlandingham DL. Flavivirus-Mosquito interactions. Viruses. 2014;6(11):4703–30.
43. Transmission of DENV [Internet]. [cited 2024 Feb 7]. Available from: https://www.cdc.gov/dengue/training/cme/ccm/page45901.html
44. Halstead SB. Dengue virus-mosquito interactions. Annu Rev Entomol. 2008;53:273–91.
45. Shuman EK. Global climate change and infectious diseases. Int J Occup Environ Med. 2011;2(1):11–9.
46. Githeko AK. Advances in developing a climate based dengue outbreak models in Dhaka, Bangladesh: Challenges & opportunities. Indian J Med Res. 2012;136(1):7–9.
47. Sarfraz MS, Tripathi NK, Tipdecho T, Thongbu T, Kerdthong P, Souris M. Analyzing the spatio-temporal relationship between dengue vector larval density and land-use using factor analysis and spatial ring mapping. BMC Public Health. 2012;12(1):1–19.
48. Vasilakis N, Holmes EC, Fokam EB, Faye O, Diallo M, Sall AA, et al. Evolutionary Processes among Sylvatic Dengue Type 2 Viruses. J Virol. 2007;81(17):9591–5.
49. Denguefever.pdf. https://www.mayoclinic.org/diseases-conditions/dengue-fever/symptoms-causes/syc-20353078
50. Changal KH, Raina AH, Raina A, Raina M, Bashir R, Latief M, et al. Differentiating secondary from primary dengue using IgG to IgM ratio in early dengue: An observational hospital based clinico-serological study from North India. BMC Infect Dis. 2016;16(1):1–7.
51. Guzman MG y KG. MINIREVIEW Advances in Dengue Diagnosis. Clin Diagn Lab Inmunol. 1996;3(6):621–7.

52. Kuno G, Cropp CB, Wong-Lee J, Gubler DJ. Evaluation of an IgM immunoblot kit for dengue diagnosis. Am J Trop Med Hyg. 1998;59(5):757–62.
53. Wu SJL, Paxton H, Hanson B, Kung CG, Chen TB, Rossi C, et al. Comparison of two rapid diagnostic assays for detection of immunoglobulin M antibodies to dengue virus. Clin Diagn Lab Immunol. 2000;7(1):106–10.
54. O'Grady P, DeSalle R. Phylogeny of the Genus . Genetics. 2018;209(1):1–25.
55. Bakonyi T, Hubálek Z, Rudolf I, Nowotny N. Novel flavivirus or new lineage of West Nile virus, Central Europe. Emerg Infect Dis. 2005;11(2):225–31.
56. Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. Nucleic Acids Res. 2002;30(6):1292–305.
57. Murugesan A, Manoharan M. Dengue virus [Internet]. Emerging and Reemerging Viral Pathogens: Volume 1: Fundamental and Basic Virology Aspects of Human, Animal and Plant Pathogens. Elsevier Inc.; 2019. 281–359 p. Available from: http://dx.doi.org/10.1016/B978-0-12-819400-3.00016-8
58. Nordström H, Falk KI, Lindegren G, Mouzavi-Jazi M, Waldén A, Elgh F, et al. DNA microarray technique for detection and identification of seven flaviviruses pathogenic for man. J Med Virol. 2005;77(4):528–40.
59. Wu SJL, Eun Mi Lee, Putvatana R, Shurtliff RN, Porter KR, Suharyono W, et al. Detection of dengue viral RNA using a nucleic acid sequence-based amplification assay. J Clin Microbiol. 2001;39(8):2794–8.
60. Teichmann D, Göbels K, Niedrig M, Sim-Brandenburg JW, Làge-Stehr J, Grobusch MP. Virus Isolation for Diagnosing Dengue Virus Infections in Returning Travelers. Eur J Clin Microbiol Infect Dis. 2003;22(11):697–700.
61. Yamada KI, Takasaki T, Nawa M, Kurane I. Virus isolation as one of the diagnostic methods for dengue virus infection. J Clin Virol. 2002;24(3):203–9.
62. Hombach J, Jane Cardosa M, Sabchareon A, Vaughn DW, Barrett ADT. Scientific consultation on immunological correlates of protection induced by dengue vaccines. Report from a meeting held at the World Health Organization 17-18 November 2005. Vaccine. 2007;25(21):4130–9.
63. Torres-Flores JM, Reyes-Sandoval A, Salazar MI. Dengue Vaccines: An Update. BioDrugs. 2022;36(3):325–36.
64. Apt D, Raviprakash K, Brinkman A, Semyonov A, Yang S, Skinner C, et al. Tetravalent neutralizing antibody response against four dengue serotypes by a single chimeric dengue envelope antigen. Vaccine. 2006;24(3):335–44.

65. Angelin M, Sjölin J, Kahn F, Ljunghill Hedberg A, Rosdahl A, Skorup P, et al. Qdenga® - A promising dengue fever vaccine; can it be recommended to non-immune travelers? Travel Med Infect Dis. 2023;54(June):10–2.
66. Centers for Disease Control and Prevention [Internet]. [cited 2024 Feb 7]. Available from: https://www.cdc.gov/index.htm
67. Our World in Data [Internet]. [cited 2024 Feb 7]. Available from: https://ourworldindata.org/
68. Virus Structures - Biology LibreTexts [Internet]. [cited 2024 Feb 7]. Available from:https://bio.libretexts.org/Bookshelves/Introductory_and_General_Biology/Introductory_Biology_(CK12)/07%3A_Prokaryotes_and_Viruses/7.08%3A_Virus_Structures
69. Fauquet CM, Fargette D. International Committee on Taxonomy of Viruses and the 3,142 unassigned species. Virol J. 2005;2:1–10.
70. Chen T, He X, Zhang P, Yuan Y, Lang X, Yu J, et al. Research advancements in the neurological presentation of flaviviruses. Rev Med Virol. 2019;29(1):1–12.
71. Qian X, Qi Z. Mosquito-Borne Flaviviruses and Current Therapeutic Advances. Viruses. 2022;14(6).
72. de Lamballerie X, Crochu S, Billoir F, Neyts J, de Micco P, Holmes EC, et al. Genome sequence analysis of Tamana bat virus and its relationship with the genus Flavivirus. J Gen Virol. 2002;83(10):2443–54.
73. About Dengue: What You Need to Know | Dengue | CDC [Internet]. [cited 2024 Feb 7]. Available from: https://www.cdc.gov/dengue/about/index.html
74. Duehr J, Lee S, Singh G, Foster GA, Krysztof D, Stramer SL, et al. Tick-Borne Encephalitis Virus Vaccine-Induced Human Antibodies Mediate Negligible Enhancement of Zika Virus Infection In Vitro and in a Mouse Model. mSphere. 2018;3(1):e00011-18.
75. Pepper OHP. A Note on David Bylon and Dengue. Ann Med Hist. 1941;3(5):363–8.
76. Dick OB, San Martín JL, Montoya RH, Del Diego J, Zambrano B, Dayan GH. Review: The history of dengue outbreaks in the Americas. Am J Trop Med Hyg. 2012;87(4):584–93.
77. Tuiskunen Bäck A, Lundkvist Å. Dengue viruses – an overview. Infect Ecol Epidemiol. 2013;3(1):19839.

78. Byk LA, Gamarnik AV. Properties and Functions of the Dengue Virus Capsid Protein. Annu Rev Virol. 2016;3(July):263–81.
79. Yap SSL, Nguyen-Khuong T, Rudd PM, Alonso S. Dengue virus glycosylation: What do we know? Front Microbiol. 2017;8(JUL):1–16.
80. Guirakhoo F, Heinz FX, Mandl CW, Holzmann H, Kunz C. Fusion activity of flaviviruses: Comparison of mature and immature (prM-containing) tick-borne encephalitis virions. J Gen Virol. 1991;72(6):1323–9.
81. Modis Y, Ogata S, Clements D, Harrison SC. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc Natl Acad Sci U S A. 2003;100(12):6986–91.
82. Crill WD, Roehrig JT. Monoclonal Antibodies That Bind to Domain III of Dengue Virus E Glycoprotein Are the Most Efficient Blockers of Virus Adsorption to Vero Cells. J Virol. 2001;75(16):7769–73.
83. Mandl CW, Guirakhoo F, Holzmann H, Heinz FX, Kunz C. Antigenic structure of the flavivirus envelope protein E at the molecular level, using tick-borne encephalitis virus as a model. J Virol. 1989;63(2):564–71.
84. Roehrig JT, Bolin RA, Kelly RG. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology. 1998;246(2):317–28.
85. Guirakhoo, F et al. "The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein." Virology vol. 191,2 (1992): 921-31. doi:10.1016/0042-6822(92)90. 1992;(January).
86. Gary T. Furin at the cutting edge: from protein traffic to embryogenesis and disease. Mol Cell. 2007;3(10):753–66.
87. Elshuber S, Mandl CW. Resuscitating Mutations in a Furin Cleavage-Deficient Mutant of the Flavivirus Tick-Borne Encephalitis Virus. J Virol. 2005;79(18):11813–23.
88. Kurosu T. Quasispecies of dengue virus. Trop Med Health. 2011;39(4 SUPPL.):29–36.
89. Worobey M, Rambaut A, Holmes EC. Widespread intra-serotype recombination in natural populations of dengue virus. Proc Natl Acad Sci U S A. 1999;96(13):7352–7.

90. Waman VP, Kolekar P, Ramtirthkar MR, Kale MM, Kulkarni-Kale U. Analysis of genotype diversity and evolution of Dengue virus serotype 2 using complete genomes. PeerJ. 2016;2016(8):1–23.
91. Pollett S, Melendrez MC, Maljkovic Berry I, Duchêne S, Salje H, Cummings DAT, et al. Understanding dengue virus evolution to support epidemic surveillance and counter-measure development. Infect Genet Evol. 2018;62(September 2017):279–95.
92. Rexliene J, Sridhar J. Genetic diversity and evolutionary dynamics of dengue isolates from India. Virusdisease. 2019 Sep;30(3):354-359. doi: 10.1007/s13337-019-00538-1. Epub 2019 Jul 24.
93. Venkatachalam R, Subramaniyan V. Homology and conservation of amino acids in E-protein sequences of dengue serotypes. Asian Pac J Trop Dis. 2014;4(S2):S573–7.
94. Dwivedi VD, Tripathi IP, Tripathi RC, Bharadwaj S, Mishra SK. Genomics, proteomics and evolution of dengue virus. Brief Funct Genomics. 2017;16(4):217–27.
95. Lanciotti RS, Gubler DJ, Trent DW. Printed in Great Britain Molecular evolution and phylogeny of dengue-4 viruses. J Gen Virol. 1997;78:2279–86.
96. Twiddy SS, Woelk CH, Holmes EC. Phylogenetic evidence for adaptive evolution of dengue viruses in nature. J Gen Virol. 2002;83(7):1679–89.
97. Lewis JA, Chang GJ, Lanciotti RS, Kinney RM, Mayer LW, Trent DW. Phylogenetic Relationships of Dengue-2 Viruses. Virology. 1993;197(1):216–24.
98. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, et al. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology. 1997;230(2):244–51.
99. Lanciotti RS, Lewis JG, Gubler DJ, Trent DW. Molecular evolution and epidemiology of dengue-3 viruses. J Gen Virol. 1994;75(1):65–75.
100. Malik S, Ahsan O, Mumtaz H, Tahir Khan M, Sah R, Waheed Y. Tracing down the Updates on Dengue Virus—Molecular Biology, Antivirals, and Vaccine Strategies. Vaccines. 2023;11(8):1–15.
101. Clyde K, Kyle JL, Harris E. Recent Advances in Deciphering Viral and Host Determinants of Dengue Virus Replication and Pathogenesis. J Virol. 2006;80(23):11418–31.

102. Gubler DJ, Suharyono W, Tan R, Abidin M, Sie A. Viraemia in patients with naturally acquired dengue infection. Bull World Health Organ. 1981;59(4):623–30.
103. Lee E, Nestorowicz A, Marshall ID, Weir RC, Dalgarno L. Direct sequence analysis of amplified dengue virus genomic RNA from cultured cells, mosquitoes and mouse brain. J Virol Methods. 1992;37(3):275–88.
104. Cruz-Oliveira C, Freire JM, Conceição TM, Higa LM, Castanho MARB, Da Poian AT. Receptors and routes of dengue virus entry into the host cells. FEMS Microbiol Rev. 2015;39(2):155–70.
105. Miller JL, DeWet BJM, Martinez-Pomares L, Radcliffe CM, Dwek RA, Rudd PM, et al. The mannose receptor mediates dengue virus infection of macrophages. PLoS Pathog. 2008;4(2).
106. Nayak V, Dessau M, Kucera K, Anthony K, Ledizet M, Modis Y. Crystal Structure of Dengue Virus Type 1 Envelope Protein in the Postfusion Conformation and Its Implications for Membrane Fusion. J Virol. 2009;83(9):4338–44.
107. Allison SL, Schalich J, Stiasny K, Mandl CW, Heinz FX. Mutational Evidence for an Internal Fusion Peptide in Flavivirus Envelope Protein E. J Virol. 2001;75(9):4268–75.
108. Bartenschlager R, Miller S. Molecular aspects of Dengue virus replication r P ut ho ro of. Rev Lit Arts Am. 2008;3:1–11.
109. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the Flavivirus life cycle. Nat Rev Microbiol. 2005;3(1):13–22.
110. Brinton MA, Basu M. Functions of the 3' and 5' genome RNA regions of members of the genus Flavivirus. Virus Res. 2015;206:108–19.
111. Filomatori CV, Lodeiro MF, Alvarez DE, Samsa MM, Pietrasanta L, Gamarnik AV. A 5′ RNA element promotes dengue virus RNA synthesis on a circular genome. Genes Dev. 2006;20(16):2238–49.
112. Seema SJ. Molecular Mechanism of Pathogenesis of Dengue Virus. Indian J ClinBiochem. 2005;20(2):92–103.
113. Fernandez-Garcia MD, Mazzon M, Jacobs M, Amara A. Pathogenesis of Flavivirus Infections: Using and Abusing the Host Cell. Cell Host Microbe. 2009;5(4):318–28.

114. Wu SJL, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, et al. Human skin Langerhans cells are targets of dengue virus infection. Nat Med. 2000;6(7):816–20.
115. Kelley JF, Kaufusi PH, Nerurkar VR. Dengue hemorrhagic fever-associated immunomediators induced via maturation of dengue virus nonstructural 4B protein in monocytes modulate endothelial cell adhesion molecules and human microvascular endothelial cells permeability. Virology. 2012;422(2):326–37.
116. Srikiatkhachorn A, Kelley JF. Endothelial cells in dengue hemorrhagic fever. Antiviral Res. 2014;109(1):160–70.
117. Dejnirattisai, Wanwisa Jumnainsong, Amonrat Onsirisakul N, Fitton P, Vasanawathana, Sirijitt Limpitikul W, Puttikhunt C, Edwards C, Duangchinda, Thaneeya Supasa, Sunpetchuda Chawansuntati, Kriangkrai Malasit P, et al. Cross-Reacting Antibodies Enhance. Science. 2010;328(May):745–8.
118. Martina BEE, Koraka P, Osterhaus ADME. Dengue virus pathogenesis: An integrated view. Clin Microbiol Rev. 2009;22(4):564–81.
119. Shresta S, Kyle JL, Beatty PR, Harris E. Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology. 2004;319(2):262–73.
120. Bente DA, Melkus MW, Garcia JV, Rico-Hesse R. Dengue Fever in Humanized NOD/SCID Mice. J Virol. 2005;79(21):13797–9.
121. Onlamoon N, Noisakran S, Hsiao HM, Duncan A, Villinger F, Ansari AA, et al. Dengue virus - Induced hemorrhage in a nonhuman primate model. Blood. 2010;115(9):1823–34.
122. Guzmán MG, Kourí G. Dengue: An update. Lancet Infect Dis. 2002;2(1):33–42.
123. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis. 2000;181(1):2–9.
124. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, Green S, et al. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis. 2002;186(8):1165–8.
125. Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, et al. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med. 2003;9(7):921–7.

126. Markoff LJ, Innis BL, Houghten R, Henchal LS. Development of cross-reactive antibodies to plasminogen during the immune response to dengue virus infection. J Infect Dis. 1991;164(2):294–301.
127. Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmeca S, Auethavornanan K, et al. Vascular leakage in severe dengue virus infections: A potential role for the nonstructural viral protein NS1 and complement. J Infect Dis. 2006;193(8):1078–88.
128. Rosenberger KD, Alexander N, Martinez E, Lum LCS, Dempfle CE, Junghanss T, et al. Severe dengue categories as research endpoints—results from a prospective observational study in hospitalised dengue patients. PLoS Negl Trop Dis. 2020 Mar 1;14(3).
129. Rathore AP, Farouk FS, St. John AL. Risk factors and biomarkers of severe dengue. Curr Opin Virol. 2020 Aug 1;43:1–8.
130. Alcalá AC, Ludert JE. The dengue virus NS1 protein; new roles in pathogenesis due to similarities with and affinity for the high-density lipoprotein (HDL)? PLoS Pathog. 2023 Aug 1;19(8).
131. Patro ARK, Mohanty S, Prusty BK, Singh DK, Gaikwad S, Saswat T, Chattopadhyay S, Das BK, Tripathy R, Ravindran B. Cytokine Signature Associated with Disease Severity in Dengue. Viruses. 2019 Jan 8;11(1):34. doi: 10.3390/v11010034.
132. Guzman MG, Vazquez S. The complexity of antibody-dependent enhancement of dengue virus infection. Viruses. 2010 Dec;2(12):2649–62.
133. Srikiatkhachorn A, Mathew A, Rothman AL. Immune-mediated cytokine storm and its role in severe dengue. Semin Immunopathol. 2017 Jul 1;39(5):563–74.
134. Guzman MG, Vazquez S. The complexity of antibody-dependent enhancement of dengue virus infection. Viruses. 2010 Dec;2(12):2649-62. doi: 10.3390/v2122649. Epub 2010 Dec 8.
135. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. Semin Immunopathol. 2017 Jul;39(5):517-528. doi: 10.1007/s00281-017-0639-8. Epub 2017 May 29. PMID: 28555385.
136. Teo A, Chua CLL, Chia PY, Yeo TW. Insights into potential causes of vascular hyperpermeability in dengue. PLoS Pathog. 2021 Dec 9;17(12):e1010065. doi: 10.1371/journal.ppat.1010065. PMID: 34882753; PMCID: PMC8659665.

137. Lee YH, Leong WY, Wilder-Smith A. Markers of dengue severity: a systematic review of cytokines and chemokines. J Gen Virol. 2016 Dec;97(12):3103-3119. doi: 10.1099/jgv.0.000637. Epub 2016 Oct 21. PMID: 27902364.
138. Teo A, Tan HD, Loy T, Chia PY, Chua CLL. Understanding antibody-dependent enhancement in dengue: Are afucosylated IgG1s a concern? PLoS Pathog. 2023 Mar 30;19(3):e1011223. doi: 10.1371/journal.ppat.1011223. Erratum in: PLoS Pathog. 2023 Oct 18;19(10):e1011736.
139. Katzelnick LC, Gresh L, Halloran ME, Mercado JC, Kuan G, Gordon A, Balmaseda A, Harris E. Antibody-dependent enhancement of severe dengue disease in humans. Science. 2017 Nov 17;358(6365):929-932. doi: 10.1126/science.aan6836. Epub 2017 Nov 2.
140. Ubol S, Halstead SB. How Innate Immune Mechanisms Contribute to Antibody-Enhanced Viral Infections. Clin Vaccine Immunol. 2010 Dec;17(12):1829–35.
141. Vervaeke P, Vermeire K, Liekens S. Endothelial dysfunction in dengue virus pathology. Rev Med Virol. 2015 Jan;25(1):50–67.
142. Halstead S. Dengue Antibody-Dependent Enhancement: Knowns and Unknowns. In 2015. p. 249–71.
143. Narayan R, Tripathi S. Intrinsic ADE: The Dark Side of Antibody Dependent Enhancement During Dengue Infection. Front Cell Infect Microbiol. 2020 Oct 2;10:580096.
144. Bhatt P, Sabeena SP, Varma M, Arunkumar G. Current Understanding of the Pathogenesis of Dengue Virus Infection. Curr Microbiol. 2021;78(1):17–32.
145. Ubol S, Halstead SB. How Innate Immune Mechanisms Contribute to Antibody-Enhanced Viral Infections. Clin Vaccine Immunol. 2010 Dec;17(12):1829–35.
146. Brad Spellberg, John E. Edwards, Type 1/Type 2 Immunity in Infectious Diseases, Clinical Infectious Diseases, Volume 32, Issue 1, 1 January 2001, Pages 76–102.
147. Shepard DS, Halasa YA, Tyagi BK, Adhish SV, Nandan D, Karthiga KS, et al. Economic and disease burden of dengue Illness in India. Am J Trop Med Hyg. 2014;91(6):1235–42.
148. John DV, Lin YS, Perng GC. Biomarkers of severe dengue disease - A review. J Biomed Sci. 2015;22(1):1–7.
149. Waqar AB. Dengue Virus: Host-Pathogen Interactions and Emerging Role of DNA Vaccines. J Hum Virol Retrovirology. 2016;3(3).

150. Calderón-Peláez MA, Coronel-Ruiz C, Castellanos JE, Velandia-Romero ML. Endothelial Dysfunction, HMGB1, and Dengue: An Enigma to Solve. Viruses. 2022;14(8):1–22.
151. World Medical Association- Declaration of Helsinki—Ethical Principles for Medical Research Involving Human Subjects—WMA—The World Medical Association [Internet]. Available from: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-formedical-research-involving-human-subjects/
152. Handbook for Clinical Management of Dengue [Internet]. title: Handbook%2Bfor%2BCliniCal%2BManageMent%2Bof%2Bden gue#1%5Cnhttp://scholar.google.com/scholar?hl=en%26btnG=Search %26q=intitle; 2012. Available from: https://www.who.int/about/licensing/copyright_form/en/index.html%5Cn
153. Changal KH, Raina AH, Raina A, Raina M, Bashir R, Latief M, et al. Differentiating secondary from primary dengue using IgG to IgM ratio in early dengue: an observational hospital based clinico-serological study from North India. BMC Infect Dis. 2016 Nov 28;16(1):715.
154. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol. 1992;30(3):545–51.
155. Nucleotide BLAST: Search nucleotide databases using a nucleotide query [Internet]. [cited 2024 Mar 2]. Available from: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome
156. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci. 2004 Jul 27;101(30):11030–5.
157. Maximum Composite Likelihood Method [Internet]. [cited 2024 Mar 2]. Available from: https://www.megasoftware.net/web_help_7/hc_mcl.htm
158. Bradford protein assay. In: Wikipedia [Internet]. 2023 [cited 2024 Mar 2]. Available from: https://en.wikipedia.org/w/index.php?title=Bradford_protein_assay&oldid=1183720874

159. Bujang MA. A Step-by-Step Process on Sample Size Determination for Medical Research. Malays J Med Sci. 2021 Apr;28(2):15-27.
160. Proteome Discoverer Software - IN [Internet]. [cited 2024 Mar 2]. Available from: https://www.thermofisher.com/in/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/proteome-discoverer-software.html
161. UniProt [Internet]. [cited 2024 Mar 2]. Available from: https://www.uniprot.org/
162. pantherdb.org [Internet]. [cited 2024 Mar 2]. Available from: https://pantherdb.org/
163. Home - Reactome Pathway Database [Internet]. [cited 2024 Mar 2]. Available from: https://reactome.org/?ref=blog.opentargets.org
164. Dey L, Mukhopadhyay A. DenvInt: A database of protein–protein interactions between dengue virus and its hosts. PLoS Negl Trop Dis. 2017 Oct 19;11(10):e0005879.
165. Handbook for Clinical Management of Dengue [Internet]. 2012. Available from: https://www.who.int/about/licensing/copyright_form/en/index.html%5Cnhttp://scholar.google.com/scholar?hl=en%26btnG=Search%26q=in
166. Van Zundert GCP, Rodrigues JPGLM, Trellet M, Schmitz C, Kastritis PL, Karaca E, et al. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. J Mol Biol. 2016 Feb;428(4):720–5.
167. STRING: functional protein association networks [Internet]. [cited 2024 Mar 2]. Available from: https://string-db.org/
168. Sonego P, Kocsor A, Pongor S. ROC analysis: applications to the classification of biological sequences and 3D structures. Brief Bioinform. 2008 May;9(ue 3):198-209,.
169. Gupta N, Srivastava S, Jain A, Chaturvedi UC. Dengue in India. Indian J Med Res. 2012 Sep;136(3):373–90.
170. Saha K, Ghosh M, Firdaus R, Biswas A, Seth B, Bhattacharya D, et al. Changing pattern of dengue virus serotypes circulating during 2008-2012 and reappearance of dengue serotype 3 may cause outbreak in Kolkata, India. J Med Virol. 2016 Oct;88(10):1697–702.
171. Nisalak A, Endy TP, Nimmannitya S, Kalayanarooj S, Thisayakorn U, Scott RM, et al. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am J Trop Med Hyg. 2003 Feb;68(2):191–202.

172. Goswami S, Rahman M, Chakraborti R, Karmakar J, Bhattacharya N, Bandyopadhyay B. Shift of Dominance of Circulating Dengue Virus Serotypes During the Period 2014-17 in Kolkata. In 2021 [cited 2024 Mar 8]. Available from: https://www.semanticscholar.org/paper/Shift-of-Dominance-of-Circulating-Dengue-Virus-the-Goswami-Rahman/e46aba950c9a3f628edb061fda347f55ba3d7236
173. Balmaseda A, Hammond SN, Pérez L, Tellez Y, Saborío SI, Mercado JC, Cuadra R, Rocha J, Pérez MA, Silva S, Rocha C, Harris E. Serotype-specific differences in clinical manifestations of dengue. Am J Trop Med Hyg. 2006 Mar;74(3):449-56. PMID: 16525106.
174. Alvarez M, Rodriguez-Roche R, Bernardo L, Vázquez S, Morier L, Gonzalez D, et al. Dengue hemorrhagic Fever caused by sequential dengue 1-3 virus infections over a long time interval: Havana epidemic, 2001-2002. Am J Trop Med Hyg. 2006 Dec;75(6):1113–7.
175. Waman VP, Kolekar P, Ramtirthkar MR, Kale MM, Kulkarni-Kale U. Analysis of genotype diversity and evolution of Dengue virus serotype 2 using complete genomes. PeerJ. 2016 Aug 24;4:e2326.
176. Gupta A, Rijhwani P, Pahadia MR, Kalia A, Choudhary S, Bansal DP, et al. Prevalence of Dengue Serotypes and Its Correlation With the Laboratory Profile at a Tertiary Care Hospital in Northwestern India. Cureus. 13(5):e15029.
177. Patumanond J, Tawichasri C, Nopparat S. Dengue Hemorrhagic Fever, Uttaradit, Thailand. Emerg Infect Dis. 2003 Oct;9(10):1348–50.
178. Comprehensive Guideline for Prevention and Control of Dengue and Dengue Haemorrhagic Fever [Internet]. Revised and expanded. Available from: https://apps.who.int/iris/handle/10665/204894
179. Dash PK, Parida MM, Saxena P, Kumar M, Rai A, Pasha ST, et al. Emergence and continued circulation of dengue-2 (genotype IV) virus strains in northern India. J Med Virol. 2004 Oct;74(2):314–22.
180. Fatima Z, Idrees M, Bajwa MA, Tahir Z, Ullah O, Zia MQ, et al. Serotype and genotype analysis of dengue virus by sequencing followed by phylogenetic analysis using samples from three mini outbreaks-2007-2009 in Pakistan. BMC Microbiol. 2011 Sep 10;11:200.
181. Alagarasu K, Patil JA, Kakade MB, More AM, Yogesh B, Newase P, et al. Serotype and genotype diversity of dengue viruses circulating in India: a multi-

centre retrospective study involving the Virus Research Diagnostic Laboratory Network in 2018. Int J Infect Dis IJID Off Publ Int Soc Infect Dis. 2021 Oct;111:242–52.

182. Kalayanarooj S. Clinical manifestations and management of Dengue/ DHF/DSS. Trop Med Health. 2011;39(4):83–7.
183. Rai S, Bhatia A, Bharmal T, Samant P, Rai M. Dengue: a clinicopathological correlation. Int J Sci Res. 2016;5(10):2013–6.
184. Communication S. Nature and clinical course of pleural effusion in dengue fever. Int J Intern Emer Med. 2018;1(1):1006.
185. Pervin M, Ali TS, M. Clinical and laboratory observations associated with the 2000 dengue outbreak in Dhaka. Bangladesh. 2000;28.
186. Banerjee M, Chatterjee T, Choudhary GS, Srinivas V, Kataria VK. Dengue: a clinicohaematological profile. Med J Armed Forces India. 2008;64(4):333–6.
187. Hasan MJ, Tabassum T, Sharif M. Comparison of clinical manifestation of dengue fever in Bangladesh: an observation over a decade. BMC Infect Dis. 2021;21(1):1–10.
188. Kumaria R. Correlation of disease spectrum among four Dengue serotypes: a five years hospital-based study from India. Braz J Infect Dis. 2010;14(2):141–6.
189. Warke RV, Becerra A, Zawadzka A. Efficient dengue virus (DENV) infection of human muscle satellite cells upregulates type I interferon response genes and differentially modulates MHC I expression on bystander and DENV-infected cells. J Gen Virol. 2008;89(Pt 7):1605–15.
190. De Rossi M, Bernasconi P, Baggi F, de Waal Malefyt R, Mantegazza R. Cytokines and chemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation. Int Immunol. 2000 Sep;12(9):1329-35. doi: 10.1093/intimm/12.9.1329. PMID: 10967028.
191. Lumpur K. Preventive transfusion in dengue shock syndrome—is it necessary? J Pediatr. 2003;3476(03):682–4.
192. Ratageri VH, Shepur TA, Wari PK, Chavan SC, Mujahid IB, Yergolkar PN. Clinical profile and outcome of dengue fever cases. Indian J Pediatr. 2005;72:705–6.
193. Jakribettu RP, Boloor R, Thaliath A. Correlation of clinicohaematological parameters in paediatric dengue: a retrospective study. J Trop Med. 2015;647162.

194. Ojha A, Nandi D, Batra H. Platelet activation determines the severity of thrombocytopenia in dengue infection. Sci Rep. 2017;7:41697.
195. de Almeida RR, Paim B, de Oliveira SA, Souza AS Jr, Gomes ACP, Escuissato DL, Zanetti G, Marchiori E. Dengue Hemorrhagic Fever: A State-of-the-Art Review Focused in Pulmonary Involvement. Lung. 2017 Aug;195(4):389-395. doi: 10.1007/s00408-017-0021-6. Epub 2017 Jun 13.
196. de Azeredo EL, Monteiro RQ, de-Oliveira Pinto LM. Thrombocytopenia in Dengue: Interrelationship between Virus and the Imbalance between Coagulation and Fibrinolysis and Inflammatory Mediators. Mediators Inflamm. 2015;2015:313842. doi: 10.1155/2015/313842. Epub 2015 Apr 27.
197. GraphPad Prism 9 Curve Fitting Guide—Interpreting Logistic ROC Curves [Internet]. Available from: https://www.graphpad.com/guides/prism/latest/curve-fitting/reg_logistic_roc_curves.htm
198. Comprehensive Guideline for Prevention and Control of Dengue and Dengue Haemorrhagic Fever. Revised and expanded edition [Internet]. WHO Regional Office for South-East Asia; 2011 [cited 2024 Mar 2]. Available from: https://iris.who.int/handle/10665/204894
199. Ravishankar K, Vinoth PN, Venkatramanan P. Biochemical and radiological markers as predictors of dengue severity in children admitted in a tertiary care hospital. Int J Contemp Pediatr. 2015;2(4):311–6.
200. Trung DT, Thi L, Thao T, Hien TT, Hung NT, Vinh NN. Liver involvement associated with dengue infection in adults in Vietnam. Am J Trop Med Hyg. 2010;83(4):774–80.
201. Verma P, Banerjee S, Baskey U. View of Prevalence of hepatic dysfunction in children with dengue fever | Pediatric Review: International Journal of Pediatric Research. J Med. 2022;94:4348–58.
202. Ray S, Srivastava R, Tripathi K, Vaibhav V, Patankar S, Srivastava S. Serum Proteome Changes in Dengue Virus-Infected Patients from a Dengue-Endemic Area of India: Towards New Molecular Targets? OMICS J Integr Biol. 2012 Oct;16(10):527–36.
203. Yadav A, Shamim U, Ravi V, Devi P, Kumari P, Maurya R, Das P, Somani M, Budhiraja S, Tarai B, Pandey R. Early transcriptomic host response signatures in the serum of dengue patients provides insights into clinical pathogenesis and

disease severity. Sci Rep. 2023 Aug 29;13(1):14170. doi: 10.1038/s41598-023-41205-2.

204. Cui L, Pang J, Lee YH, Ooi EE, Ong CN, Leo YS, et al. Serum metabolome changes in adult patients with severe dengue in the critical and recovery phases of dengue infection. PLoS Negl Trop Dis. 2018 Jan 24;12(1):e0006217.
205. Centers for Disease Control and Prevention. 2023 [cited 2024 Mar 8]. Dengue Clinical Presentation | CDC. Available from: https://www.cdc.gov/dengue/healthcare-providers/clinical-presentation.html
206. Alagarasu K, Patil JA, Kakade MB, More AM, Yogesh B, Newase P, et al. Serotype and genotype diversity of dengue viruses circulating in India: a multi-centre retrospective study involving the Virus Research Diagnostic Laboratory Network in 2018. Int J Infect Dis. 2021 Oct 1;111:242–52.
207. Pando-Robles V, Oses-Prieto JA, Rodríguez-Gandarilla M, Meneses-Romero E, Burlingame AL, Batista CVF, et al. Quantitative proteomic analysis of Huh-7 cells infected with Dengue virus by label-free LC-MS. J Proteomics. 2014;111(11):16–29.
208. Martínez-Betancur V, Martínez-Gutierrez M. Proteomic profile of human monocytic cells infected with Dengue Virus. Asian Pac J Trop Biomed. 2016 Jan 8;6.
209. Li Y, Kakinami C, Li Q, Yang B, Li H. Human Apolipoprotein A-I Is Associated with Dengue Virus and Enhances Virus Infection through SR-BI. PLOS ONE. 2013 Jul 24;8(7):e70390.
210. Ahmad RI, Nor FM, Mui WS, Rahman THA. The Role of Apolipoproteins in Dengue Infection: A Review. IIUM Med J Malays [Internet]. 2021 Apr 1 [cited 2024 Mar 8];20(2). Available from: https://journals.iium.edu.my/kom/index.php/imjm/article/view/130
211. Lee YH, Leong WY, Wilder-Smith A. Markers of dengue severity: a systematic review of cytokines and chemokines. J Gen Virol. 2016 Dec;97(12):3103–19.
212. Ferreira RAX, de Oliveira SA, Gandini M, Ferreira L da C, Correa G, Abiraude FM, et al. Circulating cytokines and chemokines associated with plasma leakage and hepatic dysfunction in Brazilian children with dengue fever. Acta Trop. 2015 Sep;149:138–47.
213. Dejnirattisai W, Duangchinda T, Lin CLS, Vasanawathana S, Jones M, Jacobs M, et al. A complex interplay among virus, dendritic cells, T cells, and cytokines in

dengue virus infections. J Immunol Baltim Md 1950. 2008 Nov 1;181(9):5865–74.

214. Manchala NR, Dungdung R, Pilankatta R. Proteomic analysis reveals the enhancement of human serum apolipoprotein A-1(APO A-1) in individuals infected with multiple dengue virus serotypes. Trop Med Int Health TM IH. 2017 Oct;22(10):1334–42.

215. Methods in Enzymology | Proteomics in Biology, Part A | ScienceDirect.com by Elsevier [Internet]. [cited 2024 Mar 8]. Available from: https://www.sciencedirect.com/bookseries/methods-in-enzymology/vol/585/suppl/C

216. Murgue, Bernadette & Cassar, Olivier & Deparis, Xavier. (2001). Plasma concentrations of sVCAM-1 and severity of dengue infections. Journal of Medical Virology. 65. 97 - 104. 10.1002/jmv.2007.

217. An IRF-3-, IRF-5-, and IRF-7-Independent Pathway of Dengue Viral Resistance Utilizes IRF-1 to Stimulate Type I and II Interferon Responses - PubMed [Internet]. [cited 2024 Mar 8]. Available from: https://pubmed.ncbi.nlm.nih.gov/29117564/

218. Michels M, van der Ven AJAM, Djamiatun K, Fijnheer R, de Groot PG, Griffioen AW, et al. Imbalance of angiopoietin-1 and angiopoetin-2 in severe dengue and relationship with thrombocytopenia, endothelial activation, and vascular stability. Am J Trop Med Hyg. 2012 Nov;87(5):943–6.

219. Mapalagamage M, Handunnetti SM, Wickremasinghe AR, Premawansa G, Thillainathan S, Fernando T, et al. High Levels of Serum Angiopoietin 2 and Angiopoietin 2/1 Ratio at the Critical Stage of Dengue Hemorrhagic Fever in Patients and Association with Clinical and Biochemical Parameters. J Clin Microbiol. 2020 Mar 25;58(4):e00436-19.

220. Srikiatkhachorn A, Kelley JF. Endothelial cells in dengue hemorrhagic fever. Antiviral Res. 2014 Sep;109:160–70.

221. Chuang Y, Linn Y, Liu C, Liu H, Liao S. Factors contributing to the disturbance of coagulation and fibrinolysis in dengue virus infection. J Formos Med Assoc. 2013 Jan;112(1):12–7.

222. Charles A Janeway J, Travers P, Walport M, Shlomchik MJ. The complement system and innate immunity. In: Immunobiology: The Immune System in Health

and Disease 5th edition [Internet]. Garland Science; 2001 [cited 2024 Mar 8]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27100/

223. Deban L, Jaillon S, Garlanda C. Pentraxins in innate immunity: lessons from PTX3 Cell Tissue Res. Vol. 343. 2011. p. 237–49.
224. Garlanda C, Bottazzi B, Sironi M, Mantovani A. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annu Rev Immunol. 2005;23(4):337-366.
225. Hsiao C, Cheng H, Huang C, Ou M, Huang J, Khoo K. Fibronectin in cell adhesion and migration via N-glycosylation. Oncotarget. 2017 Aug 7;8(41):70653–68.
226. National Library of medicine [Internet]. cell%20attachment%20and%20fibroblast%20proliferation. Available from: https://www.ncbi.nlm.nih.gov/gene/10631#:~:text=PERIOSTIN%20plays%20a%20key%20role
227. van de Weg CA, Koraka P, van Gorp EC, Mairuhu AT, Supriatna M, Soemantri A, van de Vijver DA, Osterhaus AD, Martina BE. Lipopolysaccharide levels are elevated in dengue virus infected patients and correlate with disease severity. J Clin Virol. 2012 Jan;53(1) 38-42. doi:10.1016/j.jcv.2011.09.028. PMID: 22014848.
228. Soares T, Gonzalez B, Torquato R, Lemos F, Silva A. Functional characterization of a serine protease inhibitor modulated in the infection of the Aedes aegypti with dengue virus. Biochemie. 2018 Jan;144:160–8.
229. Djamiatun K, Faradz SM, Setiati TE, Netea MG, Ven AJ, Dolmans WM. Increase of plasminogen activator inhibitor-1 and decrease of transforming growth factor-b1 in children with dengue haemorrhagic fever in Indonesia. J Trop Pediatr. 2011;57(6):424–32.
230. Mansanguan C, Hanboonkunupakarn B, Muangnoicharoen S, Huntrup A, Poolcharoen A, Mansanguan S. Cardiac evaluation in adults with dengue virus infection by serial echocardiography [Internet]. Vol. 21. 2021. p. 1–13. Available from: https://doi.org/10.1186/s12879-021-06639-x
231. Tadkalkar N, Prasad S, Gangodkar S, Ghosh K, Basu A. Dengue Virus NS1 Exposure Affects von Willebrand Factor Profile and Platelet Adhesion Properties of Cultured Vascular Endothelial Cells. Indian J Hematol Blood Transfus. 2019;35(3):502–6.

232. Bhatt P, Sabeena SP, Varma M, Arunkumar G. Current Understanding of the Pathogenesis of Dengue Virus Infection. Curr Microbiol. 2021;78(1):17–32.
233. STRING: functional protein association networks [Internet]. [cited 2024 Mar 8]. Available from: https://string-db.org/
234. How to implement k-means algorithm on string data [Internet]. Stack Overflow. 2012 [cited 2024 Mar 8]. Available from: https://stackoverflow.com/q/13518701
235. Chaturvedi UC, Nagar R. Nitric oxide in dengue and dengue haemorrhagic fever: necessity or nuisance? FEMS Immunol Med Microbiol. 2009 Jun;56(1):9–24.
236. Yacoub S, Lam PK, Huynh TT, Nguyen Ho HH, Dong Thi HT, Van NT, et al. Endothelial Nitric Oxide Pathways in the Pathophysiology of Dengue: A Prospective Observational Study. Clin Infect Dis. 2017 Oct 16;65(9):1453–61.

Data availability statement

The data that support the findings of this study are uploaded to the proteomics public repositories. Qualitative and quantitative proteomics data are available via ProteomeXchange with identifiers **PXD050773** and **PXD050774**. All DENV sequence data are publically available on the Gene bank (Accession no. **OP422204.1, OP422203.1, OP422202.1, OP422201.1, OP422200.1, OP422199.1, OP422198.1, OP422197.1, OP422196.1, OP422195.1, OP422194.1, OP422193.1; OP755740.1, OP755751.1, OP755741.1, OP755742.1, OP755743.1, OP755746.1, OP755744.1, OP755745.1, OP755747.1, OP755749.1, OP755750.1, OP755748.1, PP237825, PP237826, PP237827, PP237828, PP237829, PP237830, PP237831, PP237832, PP237833, PP237834, PP237835, PP237836).** Any additional data in association with this study are available on request from the corresponding author.

List of Published Original Articles

1. **Priya Verma**, Upasana Baskey, Kamalika Roy Choudhury, Supradip Dutta, Sagnik Bakshi, Raina Das, Palas Mondal, Sudipta Bhaduri, Dipankar Majhi, Shanta Dutta and Provash Chandra Sadhukhan. "Changing pattern of circulating dengue serotypes in the endemic regions: An alarming risk to the healthcare system during the pandemic" Journal of Infection and Public Health. 11 October 2023 **https://doi.org/10.1016/j.jiph.2023.10.014, Impact Factor: 6.7**

2. **Priya Verma,** Sayan Banerjee, Upasana Baskey, Supradip Dutta, Sagnik Bakshi, Raina Das, Sandip Samanta, Shanta Dutta, Provash C Sadhukhan. "Clinicopathological alteration of symptoms with serotype among dengue infected pediatric patients" Journal of Medical Virology. 2022 Sep;94(9):4348-4358. **https://doi.org/10.1002/jmv.27862, Impact Factor: 20.63**

3. Sandip Kumar De, Sarmistha Ray, Yogita Rawat, Subrata Mondal, Arpita Nandy, **Priya Verma**, Anuradha Roy, Provash Sadhukhan, Chandrima Das, Sankar Bhattacharyya, Dulal Senapati. "Porous Au-seeded Ag nanorod networks conjugated with DNA aptamers for impedimetric sensing of DENV-2" Sensors and Actuators B: Chemical, Volume 348, 1 December 2021, 130709, **https://doi.org/10.1016/j.snb.2021.130709, Impact Factor: 9**

4. Upasana Baskey, **Priya Verma**, Debottam Pal, Palas Mandal, Supradip Dutta, Arita Biswas, Sagnik Bakshi, Raina Das, Dipankar Majhi, Sudipta Bhaduri, Shanta Dutta and Provash Chandra Sadhukhan, Geographical Information System (GIS)-Aided Evaluation of Changing Trends of Dengue in Identifying Secondary-Infection Prone Areas in West Bengal, India: A Five-Year Epidemiological Study, **Journal Name: Indian Journal of Medical Research, Impact Factor: 5.2**

5. Supradip Dutta, Shreyasi Nath, Dr Sk. Mahiuddin Ahammed, Raina Das, Sagnik Bakshi, Moumita Majumdar, Anwesha Ghosh, **Priya Verma**, Upasana Baskey, Shanta Dutta and Provash Chandra Sadhukhan, Assessment of direct-acting antiviral therapy in different HCV genotype-infected chronic liver disease, patients of West Bengal, India, **Journal Name: Indian Journal of Medical Research, Impact Factor: 5.2**

List of Manuscripts communicated and under review

1. Kamalika Roy Choudhury, **Priya Verma**, Aleepta Guha Ray, Sandip Samanta, Asish Manna, Arun Bandyopadhyay, Shanta Dutta & Provash C. Sadhukhan, Differential proteomic profiling at different phases of dengue infection: an intricate insight from proteins to pathogenesis, **Journal Name: Journal of Proteome Research, Impact Factor: 5.3**

2. **Priya Verma**, Anwesha Ghosh, Upasana Baskey, Asis Manna, Shanta Dutta and Provash Chandra Sadhukhan. "Signature proteins as prognostic biomolecules for predicting severe dengue: Unravelling the path to enhanced diagnosis and treatment" **Journal Name: Journal of Medical Virology, Impact Factor: 12.7**

3. Sagnik Bakshi, Supradip Dutta, Aritra Biswas, Raina Das, Promisree Choudhury, Upasana Baskey, **Priya Verma**, Shanta Dutta, and Provash Chandra Sadhukhan Unique HCV distribution among chronic kidney disease (CKD) patients in West Bengal, India. Author: **Journal Name: Haemodialysis, Impact Factor: 1.3**

4. Sagnik Bakshi, Mahiuddin Ahammed, Raina Das, Supradip Dutta, Shreyasi Nath, Moumita Majumdar, Anwesha Ghosh, **Priya Verma,** Upasana Baskey, Prosanto Chowdhury3 Shanta Dutta1 and Provash Chandra Sadhukhan, Efficacy of various combinations of Direct-Acting Antiviral (DAA) against HCV in West Bengal, India. **Journal Name: Journal of Virus Eradication, Impact Factor: 5.1**

5. Raina Das, Supradip Dutta, Sagnik Bakshi, Aritra Biswas, Shreyasi Nath, Moumita Majumdar, **Priya Verma,** Upasana Baskey, Shanta Dutta and Provash Chandra Sadhukhan. "Emergence of HCV genotype 6 and its new variants among intravenous drug users in Manipur, a North Eastern state of India" **Journal Name: Therapeutic Advances in Infectious Diseases, Impact Factor: 3.6**

6. Upasana Baskey, **Priya Verma,** Palas Mandal, Supradip Dutta, Sagnik Bakshi, Raina Das, Shanta Dutta and Provash Chandra Sadhukhan, Emergence of Dengue Virus Serotype 3, genotype 1 in India. **Journal Name: Emerging Infectious Diseases, Impact Factor: 11.8**

List of Published abstracts of papers presented at different scientific meetings

1. **Priya Verma,** Anwesha Ghosh, Upasana Baskey, Shanta Dutta and Provash Chandra Sadhukhan. An intricate insight from proteins to pathogenesis: Unravelling key biomolecules associated with severe dengue. 7th Molecular Virology Meeting, 2023, IISC-Bangalore

2. Raina Das, Moumita Majumdar, Sagnik Bakshi, Supradip Dutta, **Priya Verma**, Upasana Baskey, Anwesha Ghosh, Shanta Dutta, Provash Chandra Sadhukhan. Identifying mutations conferring resistance to Sofosbuvir in DAA naive Genotype 3a isolates from HCV-infected patients in Kolkata, West Bengal, 31st Annual Meeting of Indian National Association for Study of the Liver (INASL) –3rd to 6th 2023

3. **Priya Verma**, Anwesha Ghosh, Upasana Baskey, Shanta Dutta and Provash C. Sadhukhan. "An octennial study on changing paradigm of dengue serotypes and their genotypes: A GIS aided mapping of dengue hotspots in search of risk zones for secondary dengue infection- (3rd prize in Turbo Talk oral presentation), iSOVECON2023- The Second International Conference of the Society for Vector Ecology (SOVE), India from 13-16 March 2023 at ICMR-VCRC, Puducherry.

4. Upasana Baskey, **Priya Verma**, Anwesha Ghosh, Supradip Dutta, Sagnik Bakshi, Raina Das, Shreyashi Nath, Shanta Dutta and Provash Chandra Sadhukhan. "Trends in serotypes shift of Dengue virus and its outbreak in West Bengal, India" 15th International Conference of Medical Anthropology - Control of Malaria and other Vector-borne & Zoonotic Diseases: -Challenges and Opportunities in Implementation Research 12-14 December 2022, Osmania University

5. **Priya Verma,** Kamalika Roy Choudhury, Anwesha Ghosh, Upasana Baskey, Supradip Dutta, Sagnik Bakshi, Raina Das, Shreyshi Nath, Shanta Dutta and Provash Chandra Sadhukhan. "Diarrhoea and Gastrointestinal complications are associated with dengue virus infection: A proteomics approach to understanding their patterns and causes" 16th Asian Conference on Diarrhoeal Disease and Nutrition (ASCODD-2022) November 11-13, 2022, Kolkata, India

6. **Priya Verma**, Upasana Baskey, Supradip Dutta, Sagnik Bakshi, Raina Das, Promisree Chowdhury, Aritra Biswas, Shanta Dutta and Provash Chandra Sadhukhan. Rapid Change of Dengue Virus Serotypes in West Bengal over Last 4 Years (2015-2018). 6th Molecular Virology Meeting 2019 (IIT-KGP)

7. **Priya Verma**, Upasana Baskey, Supradip Dutta, Sagnik Bakshi, Raina Das, Promisree Chowdhury, Ronita Dey, Shanta Dutta and Provash Chandra Sadhukhan. Changing trends of clinical manifestation of dengue have a co-relation with dengue serotype. 8th CME Society of tropical medicine and infectious disease in India, Kolkata

8. Sagnik Bakshi, Supradip Dutta, Raina Das, **Priya Verma**, Upasana Baskey, Promisree Chowdhury, Aritra Biswas, Shanta Dutta and Provash Chandra Sadhukhan Hepatitis C Virus Drug Resistance is not Uncommon in Eastern India. 6th Molecular Virology Meeting 2019 (IIT-KGP)

9. Upasana Baskey, Supradip Dutta, Promisree Chowdhury, **Priya Verma**, Sagnik Bakshi, Raina Das, Aritra Biswas, Shanta Dutta and Provash Chandra Sadhukhan. "Comparative Genomic Analyses of Hepatitis C Virus Infection in Multi-Transfused Thalassemia Patients in West Bengal". 6th Molecular Virology Meeting 2019 (IIT-KGP)

10. Sagnik Bakshi, Supradip Dutta, Raina Das, Shreyashi Nath, **Priya Verma**, Upasana Baskey, Shanta Dutta and Provash Chandra Sadhukhan. Unique HCV genotype distribution pattern among two different high-risk groups (HRGs) populations in West Bengal, India. 8th CME Society of tropical medicine and infectious disease in India, Kolkata

11. Upasana Baskey, Supradip Dutta, **Priya Verma**, Aritra Biswas, Shanta Dutta and Provash Chandra Sadhukhan. "Rapid Change of Dengue Virus Serotypes in West Bengal over Last 3 Years (2015-2017)". 7th CME Society of tropical medicine and infectious disease in India, Kolkata

12. **Priya Verma**, Upasana Baskey, Kamalika Roy Choudhury, Raina Das, Shanta Dutta, Provash Chandra Sadhukhan. "Liver Pays Dengue Toll in Body Along with Wide Range of Gastrointestinal Complications Strongly Associated with DENV Serotype 2 Infection in Children" DOI: https://doi.org/10.1016/j.jceh.2021.10.123, Impact Factor: 1

13. Shreyasi Nath, Supradip Dutta, Raina Das, Sagnik Bakshi, Moumita Majumdar, **Priya Verma**, Upasana Baskey, Anwesha Ghosh, Shanta Dutta, Provash Chandra Sadhukhan, Molecular Epidemiology of Hepatitis C Virus Infection and Evaluation of the Outcome of Direct Acting Antivirals Treatment in Patients with Chronic Liver Disease in West Bengal, India, January 2023, Journal of Clinical and Experimental Hepatology 13:S182, DOI:10.1016/j.jceh.2023.07.040, Impact Factor: 1

14. Raina Das, Supradip Dutta, Sagnik Bakshi, **Priya Verma**, Upasana Baskey, Shanta Dutta, Provash Chandra Sadhukhan. "Comparative Study on Genomic Diversity of Hepatitis C Virus among High Risk Group (HRG) Individuals in the North-Eastern State of Manipur, India" DOI:https://doi.org/10.1016/j.jceh.2021.10.132, Impact Factor: 1

15. Raina Das, Shreyasi Nath, Supradip Dutta, Sagnik Bakshi, Moumita Majumdar, **Priya Verma**, Upasana Baskey, Anwesha Ghosh, Shanta Dutta, Provash Chandra Sadhukhan. Identifying mutations conferring resistance to Sofosbuvir in DAA naive Genotype 3a isolates from HCV-infected patients in Kolkata, West Bengal, January 2023Journal of Clinical and Experimental Hepatology 13:S182, DOI:10.1016/j.jceh.2023.07.040, Impact Factor: 1

List of conferences, symposia, seminars, meetings, workshop attended

1. An intricate insight from proteins to pathogenesis: Unravelling key biomolecules associated with severe dengue. 7th Molecular Virology Meeting, 2023, IISC-Bangalore
2. An octennial study on changing paradigm of dengue serotypes and their genotypes: A GIS aided mapping of dengue hotspots in search of risk zones for secondary dengue infection- Certificate of participation and 3rd prize in Turbo Talk oral presentation
3. 16th ASCODD, 2022 (ICMR-NICED): Certificate of participation and poster presentation
4. 28th Annual Scientific meeting of Indian National Association for Study of the Liver (INASL): Certificate of participation and poster presentation
5. 6th Molecular virology meeting 2019 (IIT-KGP): Certificate of participation and poster presentation
6. 8th CME Society of tropical medicine and infectious disease in India: Certificate of participation and poster presentation
7. Monsoon Advanced Proteomics School (MAPS-hybrid mode) 2023, Novel sample strategies, OMICS and Big data analysis towards Bio-Marker Discovery, July 25-26, 2023, IIT- Bombay: Certificate of participation
8. Vector control and vaccines for mosquito-borne diseases: Dengue, Chikungunya and Zika, on Thursday, April 12, 2023: Certificate of participation
9. Workshop on MALDI-ToF/TOF and imaging: Certificate for participation in hands on training on MALDI-ToF, 29th December, 2022, IIT- Khragpur: Certificate of participation
10. Path breaking contribution of ICMR - NIV in fighting COVID-19”. Webinar at 1st April 2021 at 4 PM: Certificate of participation
11. National Doctor’s Day celebration at ICMR-NICED. Virtual seminar on “COVID-19: Changing scenario”. 1st July, 2021: Certificate of participation
12. Open Science: a multifaceted framework to improve science and health outcomes. (ICMR-Elsevier: Publishing Workshop series. 2021). Webinar at October 29th, 2021.

13. Observation of World Antimicrobial Awareness Week. November 18th-24th, 2021 at ICMR-NICED.
14. Climate Change and Assessment of Dengue Geography in India". Aug 12, 2021
15. ICMR-Elsevier: Trends in Global Virology. Webinar on November 19th, 2021.
16. 11th annual meeting on Proteomics Society of India (PSI- 2019): Certificate of participation
17. Molbio- True lab hands-on training on real-time quantitative micro-PCR system: Certificate of participation
18. Cross talk in biological sciences- From the concept of application (RMVC – Rahara, Kolkata): Certificate of participation
19. 7th CME Society of tropical medicine and infectious disease in India: Certificate of participation